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DEVELOPMENT OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION PROTOCOL FOR MATURE SEED-DERIVED CALLUS TISSUES OF *CITRUS* CULTIVAR 'GAILIANGCHENG ORANGE X WEIZHANG SATSUMA MANDARIN'

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ABSTRACT

The current study was conducted to develop *Agrobacterium*-mediated transformation protocol for mature seed-derived callus tissues of *Citrus* cultivar 'GWZ' (Gailiangcheng orange x Weizhang Satsuma mandarin) using *gfp* as reporter. Transformation experiments were performed with *Agrobacterium tumefaciens* strain 'EHA-105', harboring a binary vector 'pBin19'. Some important transformation factors including Kanamycin, and Acetosyringone concentrations, optical density of Agrobacterium culture, pre-culturing period, immersing time, and co-culturing period were investigated based on gfp fluorescence. The *gfp* fluorescence enabled us in early detection and recovery of transformants. Explants pre-cultured for three days before immersing in bacterial suspension for 20 min at OD^{600}: 0.6 significantly improved the transformation efficiency. By combining the best combinations of the transformation factors, we achieved highest transformation efficiency (43.56%). The integration of *gfp* gene in transgenic plantlets was confirmed by PCR analysis. The protocol reported herein would help in transformation of useful genes in tree fruit species particularly in the Citrus cultivar under study.

INTRODUCTION

Citrus is one of the most economically important fruit trees grown in tropical and sub tropical regions in the world. China is leading among *Citrus* producing countries in the world. *Citrus* ranks second among all fruits grown in China with respect to area and production. Southern China is one of the most important centers for *Citrus* diversity and related genera. The important cultivars grown in this part of China are loose skinned mandarins and many commercially important hybrid *Citrus* cultivars including Gailiangcheng orange x Weizhang Satsuma mandarin. This cultivar is very popular for its excellent taste and appealing colour in China and other countries. The demands from domestic as well as foreign consumers for this hybrid cultivar are very high. To meet the increasing demands and further improve its quality and yield, it is very essential to transfer some useful genes in *Citrus* cultivar 'Gailiangcheng orange x Weizhang Satsuma mandarin'.

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Citrus breeding through conventional techniques is very difficult due to its complex reproductive biology such as low genetic diversity, apomixes, polyembryony, high heterozygosity, long juvenility, and auto-incompatibility (Boscariol *et al*., 2003). Biotechnological techniques including genetic transformation have made it possible to overcome some of these limitations (Hammerschlag and Litz, 1992). Among various transformation techniques, *Agrobacterium*-mediated transformation has been extensively used in the genetic improvement of *Citrus* species from the last two decades. This technique has made it possible to introduce a particular trait of interest from one cultivar to another elite *Citrus* cultivar without altering its original traits (Zanek *et al*., 2008). The currently available *in vitro* and *Agrobacterium*-mediated transformation protocols are species or even cultivar dependent (Pena *et al.,* 2007; Zhao *et al*., 2010; Bachchu *et al*., 2011; Donmez *et al.,* 2013; Orbovic *et al*., 2013). It is very necessary to develop an *Agrobacterium* - mediated transformation protocol for *Citrus* species particularly for the cultivar under study. Varying transformation efficiencies had been reported in previous *Agrobacterium*- mediated-transformation studies on *Citrus*. Li *et al*. (2002, 2003)

obtained highest transformation efficiencies (20 and 30%) in *Agrobacterium* mediated transformation of Ponkan mandarin and Valencia sweet orange respectively. Dutt and Grosser (2009) have reported 47, 40, 25 and 8% transformation efficiencies in Carrizo, Duncan, Hamlin, and Mexican lime respectively. Various factors such as explants types, pre-culture and co-cultured periods, immersion time, optical density of *Agrobacterium* culture, Acetosyringone and Kanamycin concentrations had beneficial effects on transformation efficiency in earlier studies (Dutt and Grosser, 2009; Zhao *et al*., 2010; Bachchu *et al.,* 2011; Fu *et al*., 2011; Khan *et al.,* 2011, 2012; Orbovic *et al.,* 2013; Liu *et al.,* 2013). Among different kinds of explants used in *Agrobacterium* mediated transformation of *Citrus* species, embryogenic callus is favored over other explants, because it can be easily used as explant all over the year (Ribas *et al*., 2011).

In transformation studies, sometimes only a small portion of plant cells become transformed, while a large number of cells remain untransformed (escaped). So it is necessary to include a selection marker together with the desired gene, which can distinguish transformed cells from non transformed ones. Green fluorescent protein (*gfp*) is a very useful reporter (Shimomura *et al*., 1962) which is widely used in the development of *Citrus* transformation protocols. Although a large number of citrus species specific transformation protocols have been developed, according to our knowledge no reports are available to date on transformation of the *Citrus* cultivar 'Gailiangcheng orange x Weizhang Satsuma mandarin'. Hence the current study was initiated to develop a highly efficient *Agrobacterium*-mediated transformation and regeneration protocol for *Citrus* species particularly for the cultivar under study.

MATERIALS AND METHODS

Plant Materials

Green, healthy, and friable embryogenic calluses (explants) induced from mature seeds of the *Citrus* cultivar (Gailiangcheng orange x Weizhang Satsuma mandarin) were collected from National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China. The maintenance and regeneration of embryogenic callus cultures were performed by following Khan *et al*. (2011) with minor modifications. Briefly calluses were cultured onto MT (Murashige and Tucker 1969) basal medium supplemented with 3 % sucrose and incubated at 25 ± 1ºC under 16/8-hour light/dark cycle (Cool white fluorescent lamps, 40 µ mol m^{-2} s^{-1}). The pH of the medium used in the current study was adjusted at 5.8 – 6.0 by using 1N HCl or 1N NaOH before autoclaving for 15 min at 105 KPa and 121°C. The cultures were sub-cultured at two weeks intervals on same medium at least three times. The media compositions used in the current study have been listed in Table 1.

Agrobacterium tumefaciens strain and binary vector

Agrobacterium tumefaciens strain 'EHA-105' harboring a binary vector pBIN-m*gfp*5-ER (Haseloff *et al.,* 1997) was used in the current study. The T-DNA of the vector containing *nptII* as a selectable marker and green fluorescence protein (*gfp*) as a reporter under the control of cauliflower mosaic virus 35S promoter were used (Duan *et al*., 2007).

Evaluation of factors affecting transformation efficiency

Before actual transformation of the *Citrus* cultivar 'Gailiangcheng orange x Weizhang Satsuma mandarin', some important factors influencing transformation efficiency were optimized. These are: pre-culturing period of explants (0, 1, 2, 3, 4, 5, 6 days), optical density of *Agrobacterium* culture (OD_{600}: 0.2, 0.3, 0.4, 0.6, 0.8, 1.0), immersion time (5, 10, 15, 20 min), co-culturing period of explants (0, 1, 2, 3, 4, 5, 6 days), Kanamycin (Kan) concentrations (0, 50, 100, 150, 200, 250 mg/L) and Acetosyringone (AS) concentrations (0, 50, 100, 150, 200, 250 mg/L). Each experiment included three replicates of 30 explants per treatment and was repeated three times. All factors were optimized based on transient *gfp* expression. A particular factor optimized in one experiment was followed in the subsequent transformation experiments.

Preparation of *Agrobacterium* culture

Agrobacterium tumefaciens strain 'EHA-105' was grown on semi solid LB medium (5 g/L Tryptone, 10 g/L NaCl, 5 g/L Malt Extract supplemented with 50 mg/L Kan and incubated under dark at 28°C. After 2 days, *Agrobacterium* culture was selected and multiplied on LB medium. The bacterial cultures were suspended in liquid MT medium containing 10 mg/L AS. The suspension was continuously shaken in an orbital shaker (200 rpm) at 28 °C for 2h until OD_{600} was measured at 0.6 using UV-Spectrophotometer (Shimadzu Model UV-1206, Japan).

Transformation, Selection and regeneration of transformants

The embryogenic calli (0.5–1.0 mm in diameter) were cultured for 3 days on callus proliferation medium (CPM) containing MT salts supplemented with 3% sucrose before immersing in *Agrobacterium* suspension. After immersing for 20 min in bacterial suspension, explants were washed in double distilled (DD) water and blot dried on sterile filter paper before transferred to petri- plates containing 25 mL semi-solid co-cultivation medium (CCM) which consisted of MT salts and 10 mg/L AS and incubated in the dark for 3 days. The cultures were then transferred on selection medium (SM) containing MT salts, 50 mg/L Kan, 400 mg/L Cefotaxime and 30 g/L sucrose. They were sub-cultured at 3 weeks intervals until resistant callus lines appeared. The resistant lines were multiplied by sub-culturing on SM at 2 weeks intervals and then transferred on embryogenesis medium (EM) containing MT salts, 2 % Glycerol, 50 mg/L Kan and 200 mg/L Cefotaxime. The regenerates showing *gfp* fluorescence clearly were sub-cultured at 2 weeks intervals. The regenerated shoots were excised from embryos and transferred to shoot elongation medium (SEM) which consisted of MT salts, 0.2 mg/L BA, 0.2 mg/L IAA, 0.2 mg/L GA_3 and 30g/L sucrose. The elongated shoots were rooted by culturing on root inducing medium (RIM) composed of 1/2 MT salts, 0.5 mg/L NAA, 0.1 mg/L IBA, 0.5 g/L activated charcoal and 30 g/L sucrose. Well developed plantlets with good root system were transferred into small plastic pots filled with soil, perlite and

vermiculite (3:1:1) and acclimatized in greenhouse for about two months. Fully acclimatized plants were transferred into field for further studies.

Detection of *gfp* fluorescence

Fluorescence of *gfp* gene in the resistant embryogenic calli and regenerated embryos was detected by using Leica-fluorescence stereomicroscope (MZF III, Chroma-Technology Corp., USA) attached with digital camera. The transformation efficiency of putative transgenic explants was calculated as the number of *gfp* positive explants out of total number of explants cultured (Cervera *et al*., 1998).

PCR analysis

For confirmation of *nptII* and *gfp* genes integration through PCR, genomic DNA from fresh leaves of six randomly selected transgenic plantlets and one non-transgenic plantlet was isolated according to Khan *et al*. (2012). The primer sequences used for *nptII* gene were 5'-TGCGCTGCGAATCGGGAGCG-3' (reverse) and 5' -GAGGCTATTCGGCTATGACT- 3' (sense) and that for *gfp* gene were 5'-TGGCCAACACTTGTCACTAC-3' (forward) and 5'- AGGACCATGTGGTCTCTCTT-3' (reverse). PCR reaction mixture (20 µ L) containing 50 ng genomic DNA, 0.1 m M of each gene specific primer, 0.2 m M of dNTPs, 1.5 m M Mg Cl_2 and 1.0 U *Taq* DNA polymerase was used. PCR analyses were carried out in a Thermocycler (PTC-200, MJ Research, USA). For amplification of *nptII* gene products, reactions were subjected to 35 cycles of 30s at 94°C, 30s at 60°C and 42s at 72°C. The amplification of *gfp gene* products, reactions were carried out at 35 cycles of 1 min at 94 °C, 1 min at 55°C and 1.5 min at 74°C. The expected amplified product sizes were 710bp and 500bp for *nptII* and *gfp* genes respectively. The amplified products were electrophoresed on 1 % (w/v) agarose gel and visualized by UV transillumination after staining with ethidium bromide.

Statistical analysis

Each experiment included three replicates of 30 explants per treatment and was repeated three times. The data was statistically analyzed using SAS ver. 6.12 (SAS Institute, 1995, Cary, NC) statistical software packages and the differences among means were calculated by Duncan's Multiple Range Test (DMRT) at $\alpha = 0.05$ level of significance.

RESULTS AND DISCUSSION

Optimization of factors influencing transformation efficiency

In the current study, we optimized some important factors influencing transformation efficiency before actual transformation including Kanamycin, and Acetosyringone concentrations, optical density of *Agrobacterium* culture, pre-culturing period, immersing time, and co-culturing period on the basis of *gfp* fluorescence.

Kanamycin (Kan) concentration

Optimization of Kanamycin concentrations in selection medium is one of the most important steps in the development of *Citrus* transformation protocol. Among various Kan concentrations tested in the current study, 50 mg/L Kan resulted into formation of resistant callus lines at highest rate (50.67 %) compared to other treatments. Increased Kan concentrations (100, 150, 200 and 250 mg/L) caused necrosis and reduction in the calli survival rate i.e. 35.35, 21.33, 8.33 and 2.67 % respectively (Fig. 1). Non transformed callus lines become necrotic and dead in the selection treatments. To minimize escapes and to prevent necrosis, we selected 50 mg/L Kan concentrations in subsequent transformation studies. Similar results had been reported in previous studies (Khan *et al.,* 2011; Iantcheva *et al.,* 2014). Contrary to our findings, Kan in higher concentrations in selection medium resulted into higher transformation efficiency (Bezirganoglu *et al*., 2013; Wang *et al.,* 2013).

Table 1. Media composition

Medium	Composition
CPM (Callus proliferation medium)	MT + 30 g/L Sucrose* + 7 g/L Agar*
PCM (Pre-culture medium)	MT + 0.5 g/L Malt Extract + 1.5 g/L Glutamine + 30 g/L Sucrose
CCM (Co-culture medium)	MT + 10 mg/L Acetosyringone + 30 g/L Sucrose
SM (Selection medium)	MT + 50 mg/L Kanamycin + 400 mg/L Cefotaxime + 30 g/L Sucrose
EM (Embryogenesis medium)	MT + 2% Glycerol + 50 mg/L Kanamycin + 200 mg/L Cefotaxime
EMM (Embryo maturation medium)	MT + 0.5 mg/L Malt Extract + 2% Glycerol + 50 mg/L Kanamycin
SEM (Shoot elongation medium)	MT + 0.2 mg/L BA + 0.2 mg/L IAA + 0.2 mg/L GA_3 + 30 g/L Sucrose
RIM (Root inducing medium)	1/2 MT + 0.5 mg/L NAA + 0.1 mg/L IBA+ 0.5 g/L Activated Charcoal + 30 g/L Sucrose

* Solidified all the media with 0.8 % Bacto-Agar and autoclaved at 105 KPa (121°C for 15 min)

Table 2 Transformation efficiencies in *Agrobacterium* mediated transformation of *Citrus* cultivar Gailiangcheng orange x Weizhang Satsuma mandarin'

No. of experiments	No. of inoculated calli	No. of transgenic events [a] (plants)[1]	Transformation efficiency (%)[2]
03	280	127	45.36

1) Transgenic events represent transformed calli which are producing Kanamycin resistant plants

2) Transformation efficiency was calculated using the number of transgenic events from all inoculated calli

a) An independent transformation event is defined as an explant or callus which regenerated after 8 wk culture on the selection medium.

Pre-culturing period of explants

Pre-culturing period of explants is one of the most critical factors affecting transformation efficiency.

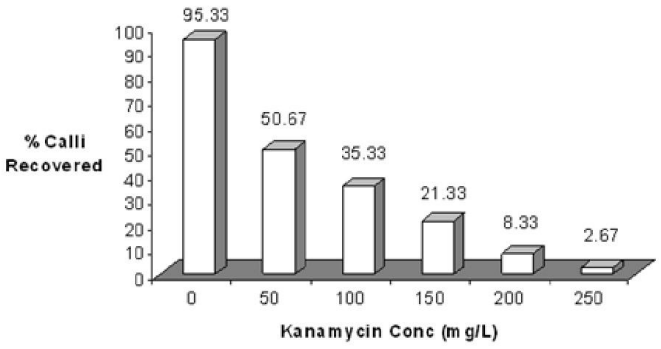

Figure 1. Effect of Kanamycin concentrations on survival of callus lines. Each experiment was repeated three times. Means were significantly different at $P \leq 0.05$ according to Duncan's Multiple Range Test (DMRT). Data represents mean values of three replicates

During this period, explants become more vulnerable for bacterial infection causing increase in transformation efficiency. In the current study, explants were cultured on MT medium supplemented with 0.5 g/L Malt Extract, 1.5 g/L Glutamine and 30 g/L sucrose for 0 to 5 days before immersing in bacterial suspension. We observed that explants pre-cultured for 3 days had higher transformation efficiency (56.67%) compared with control and other treatments (Fig. 2a). The transformation efficiency progressively decreased in lower and longer than the 3 days pre-culturing period. Similarly, explants not subjected to pre-culture period prior to immersion in bacterial suspension, exhibited lowest transformation efficiency (< 10%). The beneficial effects of pre-culturing period on transformation efficiency had been reported in previous studies (Li *et al*., 2002 and 2003; Duan *et al*., 2007). In contrast to our findings, 5 days pre-culture period was found optimum for *C. sativus* by Vengadesan *et al.* (2005) and Selvaraj *et al*. (2010). Therefore, 3 days pre-culturing period was found optimum for transformation in the current study.

Acetosyringone concentration

Acetosyringone is playing a significant role in enhancing transformation efficiency. In the current study, transformation efficiency differed at varying concentration of AS added in co-culture medium. Figure (2b) shows that addition of 10 mg/L AS in co-culture medium had significantly increased transformation efficiency (51.33%) compared to other treatment. However, transformation efficiency decreased with the increasing AS concentrations in co-culture medium. It was noted in this study that addition of over 10 mg/L AS in co-culture medium had negative effects on transformation efficiency. Therefore we selected 10 mg/L AS for including in co-culture medium in subsequent experiments. Contrary to our findings, Dutt and Grosser (2009) obtained highest transformation efficiencies at 100 µM AS in transformation of Carrizo and Hamlin Sweet oranges.

Optical density of *Agrobacterium* culture (O.D.)

Selection of an appropriate optical density of *Agrobacterium* culture is one of the important steps in the development of transformation protocol. In the current study, explants were immersed in bacterial suspension at O.D. levels (0, 0.2, 0.4, 0.6, 0.8 and 1.0).

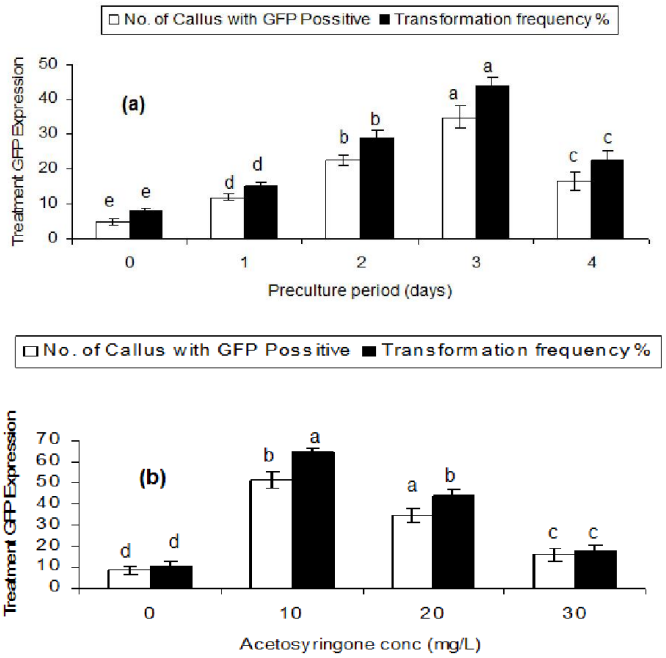

Figure 2. Factors influencing *gfp* gene expression and transformation efficiency; 2a) Effect of pre-culture period on transformation efficiency and *gfp* gene expression; 2b) Effect of Acetosyringone (AS) concentrations on transformation efficiency and *gfp* gene expression. Data represents mean values ± standard error (SE) of three replicates; each experiment was repeated three times. Means with common letters were not significantly different at $P \leq 0.05$ according to Duncan's Multiple Range Test (DMRT)

We achieved highest transformation efficiency (46.67%) when explants were infected in bacterial suspension at OD_{600}: 0.6 (Fig. 3a). Bacterial suspension below $OD_{600:}$ 0.6 had no effects, whereas higher O.D. caused gradual reduction in transformation efficiencies. Our results are in line with the findings of Mondal *et al*. (2001) and Yong *et al.* (2014). Miao *et al.* (2009) obtained higher transformation efficiencies at an OD_{600}: 0.8, whereas Nanasato *et al.* (2013) at an $OD_{600:}$ 0.5. Hence bacterial concentrations of OD_{600}: 0.6 were selected for subsequent transformation studies.

Immersion of explants in bacterial suspension

As depicted in Fig. (3b), immersion of explants in bacterial suspension for different time periods (5, 10, 15, 20 min) had variable effects on transformation efficiency. We obtained highest transformation efficiency (80%), when explants were immersed in bacterial suspension for 20 min. Immersion for longer times negatively affected the transformation efficiency, where most of the explants died due to *Agrobacterium* overgrowth. Our findings are in agreement with some previous reports (Sahoo and Tuteia, 2012; Wittaya *et al.,* 2009). Bunnag and Tangpong (2012) achieved higher transformation efficiency by infecting explants for 10 min. Therefore, 20 min immersion in bacterial suspension was considered appropriate for use in actual transformation studies.

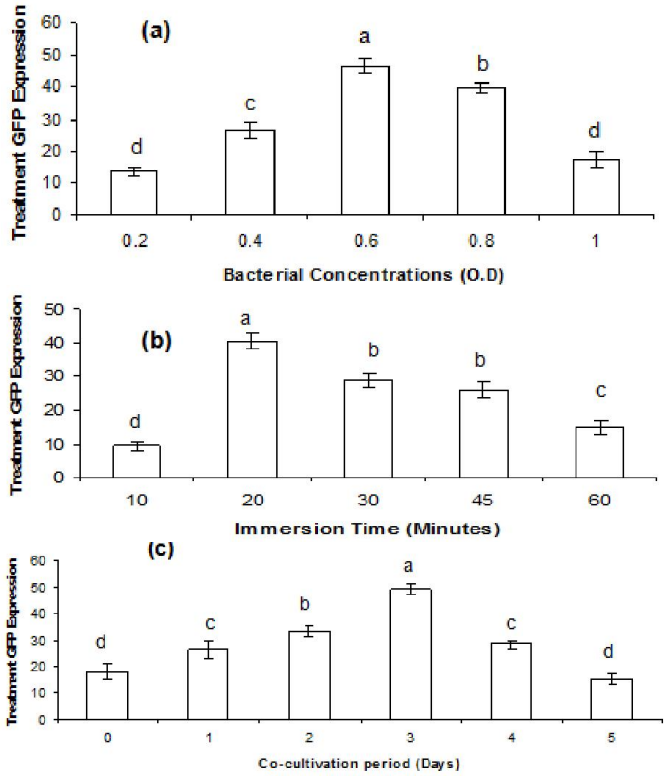

Figure 3. Factors influencing *gfp* expression and transformation efficiency 3a) Effect of optical density of *Agrobacterium* culture ($O.D_{600}$) on *gfp* expression and transformation efficiency 3b) Effect of immersion time on *gfp* expression and transformation efficiency 3c) Effect of co-culture period on *gfp* expression and transformation efficiency. Data represents mean values ± standard error (SE) of three replicates; each experiment was repeated three times. Means with common letters were not significantly different at $P \leq 0.05$ according to Duncan's Multiple Range Test (DMRT)

Co- culturing period of explants

The genetic elements encoded by '*vir* regulon' are induced during co- culturing period of explants, which results in integration of T-DNA into plant genome (Han *et al*., 2005). Bacterial overgrowth and tissues necrosis occur during unfavorable co-culture periods. In the current study, significantly higher transformation efficiency (49%) was recorded when explants were cultured on co-culture medium for 3 days and lowest (26.33%) at day 1 (Fig. 3c). Our results are consistent with the findings of previous studies (Wang *et al*. 2013; Yong *et al*. 2014). More than 3 days of co-cultivation was declared favorable by Mourgues *et al*. (1996) and Song *et al*. (2007). Co-cultivation of explants for 3 days was found optimum in this study, which was followed in subsequent studies.

Transformation and regeneration of transformants

An efficient transformation and regeneration protocol was successfully developed for the *Citrus* cultivar 'Gailiangcheng orange x Weizhang Satsuma mandarin' by optimizing several transformation factors. As can be seen in Figure (4a), embryogenic calli induced from mature seeds were cultured on callus proliferation medium (CPM, Table 1) for 3 days prior to immersion in bacterial suspension. The pre-cultured calli were immersed in bacterial suspension for 20 min and blot dried on sterile filter paper after washing three times with sterile double distilled (DD) water. The infected calli were cultured on co-culture medium (CCM, Table 1) and incubated at 23°C under dark conditions for 3 days. Following co-cultivation, explants were inoculated on selection medium (SM) and incubated at 25 ± 1°C under dark for 6 weeks. The explants were sub-cultured at 2 weeks intervals. Under these selective conditions, putatively transformed calli appeared in variable number as well as in size (Fig. 4b).

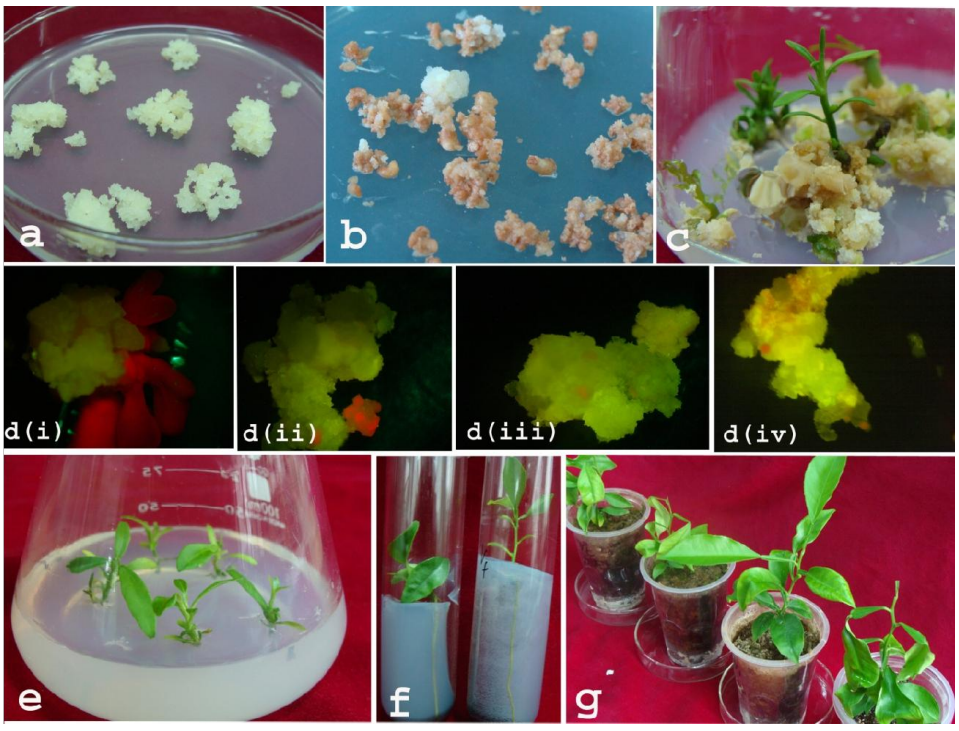

Figure 4. Transformation and regeneration of transgenic plants using embryogenic calluses as explants. a) Pre-cultured embryogenic callus lines; b) Selection of Kanamycin resistant callus lines; c) Regeneration of shoots from resistant callus lines and adventitious embryos; d- i, ii, iii, iv) *gfp* fluorescence in the resistant callus lines and regenerated embryos; e) Elongation of regenerated transgenic shoots; f) Rooting in transgenic shoots; g) Acclimatization of transgenic plantlets in soil conditions

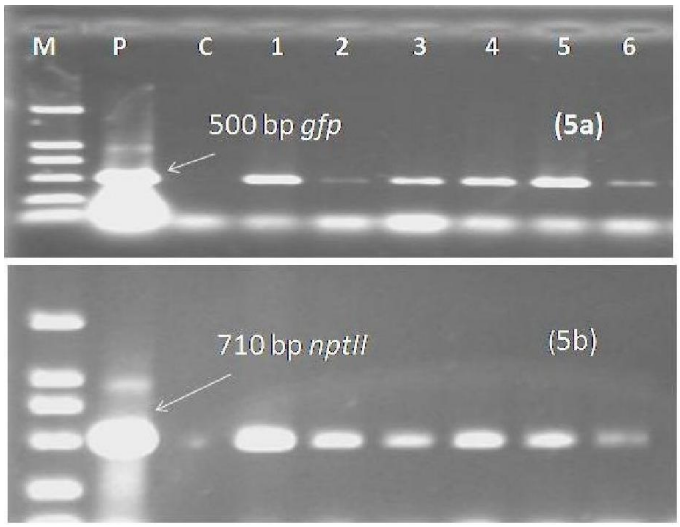

Figure 5. PCR analysis of the transgenic plants with primers specific to: 5a) *gfp* gene and 5b) *nptII* gene. M: Molecular size marker (100 bp ladder). C: Control (Untransformed plant). P: Positive control (plasmid DNA). Lanes: 1-6 are independent transgenic plants overexpressing *gfp* and *nptII* genes respectively. Arrows: Pointing the bands size of the *gfp* and *nptII* genes

After about 6-8 weeks of culturing on Kanamycin-enriched embryogenesis medium (EM), each resistant callus line regenerated several putatively transformed somatic embryos. These embryos were transferred to embryos maturation and shoot induction medium (EMM) (Fig. 4c). As shown in Fig. 4 d (i, ii, iii, andiv), embryos and shoots exhibiting strong *gfp* fluorescence under stereomicroscope were separated and transferred to shoot elongation medium (SEM) and incubated at 25 ± 1^{o}C for 3 weeks under 16/8 light/dark cycle (Fig.4e). Elongated shoots were cultured on root inducing medium (RIM) to develop into whole plantlets (Fig. 4 f). We produced 127 putatively transformed plantlets in the current study (Table 2). Transgenic plantlets at the height of 5 cm were planted into plastic pots containing mixture of soil, perlite and vermiculite (3:1:1) and acclimatized under greenhouse conditions for about 2 months (Fig. 4 g). Well developed transgenic plants with good root system were transferred into field for further studies.

Agrobacterium tumefaciens mediated genetic transformation systems using embryogenic callus cultures had been successfully reported for various *Citrus* species by optimizing transformation factors (Li *et al*., 2002, 2003; Duan *et al.,* 2007; Dutt and Grosser, 2010; Zhao *et al*., 2010; Bachchu *et al.,* 2011; Khan *et al*., 2011; Donmez *et al*., 2013). The *gfp* fluorescence enabled us in early detection and recovery of transformed cell lines, embryos and shoots. By optimizing some critical transformation factors, we achieved highest transformation efficiency (45.35%) (Table 2). Thist transformation efficiency is higher than several previous reports (Duan *et al*., 2007, Dutt and Grosser, 2009, Boscariol *et al*., 2003).

Dutt and Grosser (2009) obtained 40, 25 and 8% transformation efficiencies in Duncan, Hamlin, and Mexican lime respectively, 10% in Bingtangcheng sweet orange by Duan *et al*. (2007) and 23.8% in Valencia sweet orange by Boscariol *et al*. (2003). However, transformation efficiency achieved in the current study is lower than those of Dutt and Grosser (2009) who reported 47% in Carrizo citrange. The differences in transformation efficiencies might be due to transformation factors and cultivar used. Use of *gfp* gene as a reporter has increased the transformation and selection efficiencies in previous studies (Cervera *et al.,* 1998; Ghorbel *et al*., 1999; Grosser *et al.,* 2000; Dutt and Grosser, 2009; Khan *et al*., 2011; Orbovic and Grosser, 2015) which are consistent with our current findings. This study describes the first successful *Agrobacterium* mediated transformation and regeneration protocol for the cultivar 'Gailiangcheng orange x Weizhang Satsuma mandarin, which will be very useful for transformation of other cultivars of *Citrus*.

Molecular analysis of transgenic plantlets

PCR analysis was conducted in order to confirm integration of *nptII* and *gfp* genes in randomly selected six transgenic plantlets. About 127 putatively transgenic plants were produced and highest transformation efficiency (43.56%) was obtained in the current study. Fig. (5 a, b) shows PCR confirmation of randomly selected six transgenic plantlets and one non transgenic plant. Transgenic plants yielded the expected DNA fragments i.e 500 bp and 710 bp corresponding to *gfp* gene (Fig. 5 a, Lanes: 1-6) and *nptII* gene (Fig. 5b, Lanes: 1-6) respectively as did by the plasmid. No amplification products were detected in the non-transgenic plant (Fig. 5 a, b; lane: C).

Conclusion

We developed an efficient and simple *Agrobacterium*-mediated transformation and regeneration protocol for the *Citrus* cultivar 'Gailiangcheng orange x Weizhang Satsuma mandarin' by optimizing some critical transformation factors including Kanamycin, and Acetosyringone concentrations, optical density of *Agrobacterium* culture, pre-culturing period, bacterial immersing time, and co-culturing period. To our knowledge, this is the first report on *Agrobacterium*-mediated transformation of the above *Citrus* cultivar. The protocol opens up new avenue for genetic improvement of the current as well as other *Citrus* cultivars with valuable genes to attain sustainable and higher production.

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