BIOLOGIA PLANTARUM 53 (3): 578-582, 2009

BRIEF COMMUNICATION

# Regeneration of transgenic citrus plants from the trimmed shoot/root region of etiolated seedlings

D.L. LI, B. TAN, Y.X. DUAN and W.W. GUO*

*National Key Laboratory of Crop Genetic Improvement, National Center of Crop Molecular Breeding, Huazhong Agricultural University, Wuhan 430070, P.R. China*

## Abstract

Transformation and high efficient regeneration of transgenic plants from the trimmed etiolated shoot/root region (TESRR) of Anliucheng sweet orange [*Citrus sinensis* (L.) Osb.] seedling was reported. A visual green fluorescent protein (GFP) marker gene was introduced to evaluate transformation efficiency by using the explants from TESRR and epicotyls. The transformation protocol was: infection 20 min, co-culture 3 d, selection culture 30 d, and rooting 15 d. Out of a total of 288 sprouted shoots obtained from TESRR, 34 shoots (11.8 %) yielded GFP expression. In contrast, only 2 (3.0 %) of the 67 sprouted shoots from epicotyl transformation yielded GFP expression. In all plants showing the green fluorescence an expected 500 bp GFP fragment was proved by PCR analysis. Southern blot analysis further confirmed the integration of GFP gene into citrus genome. Transgenic plantlets were obtained within 80 d using the TESRR, compared within 150 d by using epicotyls.

*Additional key words*: *Agrobacterium tumefaciens*, sweet orange, GFP, plantlet regeneration, transformation

Since first citrus transgenic research was reported by Kobayashi and Uchimyia (1989), many transgenic citrus were obtained using different explants. Epicotyl and internode are often used in citrus transformation for their low cost and ease of regeneration (Moore *et al*. 1992, Peña *et al.* 1995, Dominguez *et al.* 2002, Orbovic and Grosser 2006, Ballester *et al.* 2007, Duan *et al.* 2007b). Cotyledon (Khawale *et al.* 2006), protoplast (Guo *et al.* 2005), and embryogenic callus (Li *et al.* 2002, 2003; Duan *et al.* 2007a) have also been used in citrus transformation. For early evaluation of genetically modified characteristics, the mature tissue explants can also be used (Cervera *et al.* 1998).

The green fluorescent protein (GFP) is a good marker gene. Direct visualization of gene expression in individual cells is possible without cell lysis and subsequent biochemical analysis, and tissue distortion caused by fixation, staining and sectioning could be avoided (Chiu *et al.* 1996). The use of GFP could significantly reduce labor, cost and time in citrus transformation (Ghorbel *et al.* 1999, Duan *et al.* 2007b). By spatial visualization and revealing temporal patterns of gene expression *in vivo*, GFP facilitated citrus transformation (Ghorbel *et al.* 1999). Transgenic plants with the gene GFP could also be used in further studies by biotechnological approaches. For instance, GFP cell lines were used as a visual marker in citrus somatic fusion (Olivares-Fuster *et al.* 2002, Guo and Grosser 2005, Cai *et al.* 2006).

In this paper we describe a method to obtain transgenic citrus plants in a short time through the *Agrobacterium*-mediated transformation by using GFP as a reporter gene.

Seeds were extracted from mature fruits of *Citrus sinensis* (L.) Osbeck cv. Anliucheng and treated with 1 M NaOH for 10 min, followed by 2 % NaClO solution treatment for 15 min. The sterilized citrus seeds were then rinsed with sterile distilled water for three times and the inner seed coats of the resulting seeds were stripped off. Embryos were placed on Murashige and Tucker

*Received* 28 September 2007, *accepted* 26 February 2008.

*Abbreviations*: TESRR - trimmed etiolated shoot/root region; AS - acetosyringone; BA - 6-benzyladenine; GFP - green fluorescent protein; GUS - β-glucuronidase; IBA - indole butyric acid; KIN - kinetin; MT medium - Murashige and Tucker (1969) medium; NAA - α-napthaleneacetic acid; NPT II - neomycin phosphotransferase II.

*Acknowledgements*: This research was financially supported by the national NSF of China, the Ministry of Education (20060504014), the National 863 project of China (2007AA10Z182), and the NSF of Hubei province of China (2008CDA069).

* Author for correspondence; fax: (+86) 27 8728 0016, e-mail: guoww@mail.hzau.edu.cn

(1969; MT) solid based medium containing 25 g $dm^{-3}$ sucrose + 7.5 g $dm^{-3}$ agar, and germinated at 26 °C in the dark. The trimmed etiolated seedlings (TESRR), including partial shoot and root regions (1.5 - 2.0 cm), were used as explants for transformation after 4 weeks cultivation in the dark and 1 week at a 16-h photoperiod. The epicotyls (1.5 cm) were also used for transformation to compare its transformation efficiency with that of the TESRR.

*Agrobacterium tumefaciens* strain EHA-105 carrying the plasmid pBIN m-*gfp5*-ER (Haseloff *et al.* 1997) was used. A fresh single colony was cultivated on solid Luria-Bertani (LB) medium (10 g $dm^{-3}$ peptone, 5 g $dm^{-3}$ yeast extract, 10 g $dm^{-3}$ sodium chloride, 15 g $dm^{-3}$ agar) containing kanamycin (50 mg $dm^{-3}$) and rifampicin (25 mg $dm^{-3}$), for 48 h at 28 °C. The bacteria were collected, transferred to liquid MT medium in an orbital shaker at 28 °C and 250 rpm for 1 h, and then adjusted to an absorbance $A_{600} = 0.5$.

TESRR and epicotyls were infected with A. *tumefaciens* for 20 min, and then dried with sterile filter paper and co-cultivated on SMT [MT medium plus 0.5 g $dm^{-3}$ benzyladenine (BA), 0.5 g $dm^{-3}$ kinetin (KIN) and 0.1 g $dm^{-3}$ naphthaleneacetic acid (NAA); Guo *et al.* 2002] medium containing 100 mM acetosyringone (AS) for 3 d in the dark at 23 °C. After co-culture, TESRR were blotted dry with sterile filter paper and transferred to SMT medium, supplemented with kanamycin (50 mg $dm^{-3}$) and cefotaxime (400 mg $dm^{-3}$). The cultures were maintained in the dark for 7 d at 28 °C and then transferred to a 16-h photoperiod (irradiance of 33 μmol $m^{-2}$ $s^{-1}$) at 28 °C. When the shoots grew up to 0.3 - 0.5 mm (about 30 d), the explants with GFP expression were transferred to RMT (half strength MT basal medium plus 0.5 mg $dm^{-3}$ NAA, 0.1 mg $dm^{-3}$ IBA, and 0.5 mg $dm^{-3}$ activated charcoal)


Fig. 1. Regeneration of TESRR explants: *A* - TESRR after trimming; *B* - regenerated shoots after being screened on selective medium for 30 d; *C*, *D* - regeneration of resistant shoots from transformed TESRR; *E* - transgenic shoot self-rooted in the RMT medium; *F* - strong root system of the transgenic shoot; *G* - transgenic plant in greenhouse; *H*, *I* - transgenic shoots with bright green fluorescence. *Bar* = 1 mm.

without antibiotics to induce new roots. The complete plantlets were then transferred to pots and grown in greenhouse.

After co-cultivation, the explants were screened for GFP transient fluorescence and periodical examination was conducted with a *Leica* fluorescence stereomicroscope (*MZFLIII,* Wetzlar, Germany) 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 and used in the following analysis.

Standard PCR technique was employed to detect the presence of the GFP gene in leaf samples from the regenerated putative transgenic plantlets. The GFP primers used were GPL: 5'-AGGACCATGTGGTCTCTCTT-3' and GPR: 5'-TGGCCAACACTTGTCACTAC-3', which produced a 500 bp fragment. For PCR analysis, DNA was denatured at 94 °C for 3 min followed by 30 amplification cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) and 5 min at 72 °C . Amplified DNA was detected by UV radiation after electrophoresis of each sample on 2 % (m/v) agarose-ethidium bromide gels. For Southern blot analyses, DNA was extracted from PCR positive and non-transformed plants using the CTAB method according to Cheng *et al.* (2003). A total of 15 μg genomic DNA digested with *Hind* III (which has only one restriction enzyme site in the plasmid) was separated on a 0.8 % agarose gel and transferred onto *Hybond*-$N^+$ blotting membrane under alkaline conditions. The membrane was probed to specific PCR fragment of GFP labeled with $P^{32}$.

The etiolated seedlings were obtained after 30 d of culture. As detected with a fluorescence microscope, GFP expression was maximal after 3 d co-culture, subsequently both the level of expression and the number of foci expressing the GFP decreased (data not shown). After 30 d selection culture with kanamycin, the TESRR that exhibited green fluorescence (Fig. 1*H*) were regenerated from wounding surface (shoot, root and cotyledon region), and transferred to RMT medium to induce new roots. Most adventitious buds were produced from shoot and cotyledon region, only few came from root region (Fig. 1*B,C,D*). The regenerated shoots with GFP expression from epicotyls were placed on RMT medium for rooting, or shoot-tip grafted. Roots had a better growing ability when regenerated from TESRR (Fig. 1*F*). If there were more than two sprouted buds with GFP expression, only one bud was kept, and the remaining ones were regenerated by rooting as for the epicotyl explants. Shoots that had no green fluorescence were removed. The shoots with GFP expression indicated that they contained stably transformed cells. Then transgenic plants with strong root system were transferred to pots in greenhouse (Fig 1*G*). Transgenic plants were obtained after 78 d. For the transgenic plants regenerated from epicotyls, about 140 - 170 d were required (elongating culture 30 d, rooted culture 30 d and 30 d seedling acclimation). Totally, ten independent transgenic plant lines were obtained. The transgenic shoots from epicotyls were also regenerated.

In the presence of kanamycin, adventitious shoots on TESRR were delayed by about 1-week (data not shown), and the number and quality of shoots was significantly higher than that of the controls (epicotyls). Adventitious shoots on epicotyls were delayed by about two weeks. The TESRR, that had green fluorescence buds, were transferred to RMT medium without antibiotics directly for root regeneration. Without antibiotics, the root regenerated better than that with antibiotics. After transferring the transgenic plants to greenhouse, the plants exhibited normal growth compared to non-transformed controls. With about 300 TESRRs and 900 epicotyls inoculated in this experiment, a total of 34 green fluorescent shoots were obtained from 288 sprouted buds (11.8 %) by using the TESRR explants, compared with only 2 green fluorescent shoots from 67 sprouted buds (3.0 %) by using epicotyl explants. All the transgenic citrus obtained with TESRRs were pure GFP expression shoots. These results showed that using the TESRR explants had higher transformation efficiency.

Genetic transformation is a laborious task. It is possible to overcome the difficulties by using GFP as a

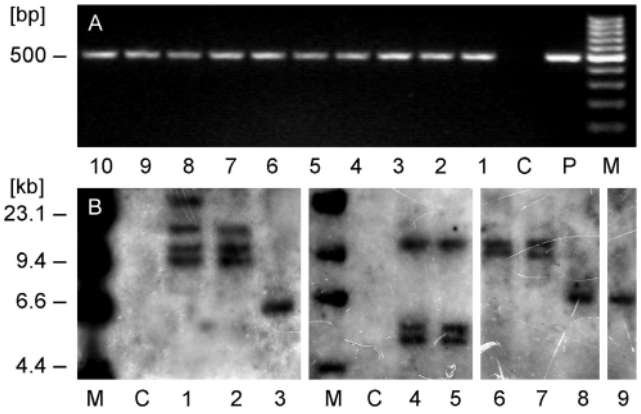


Fig. 2. PCR (*A*) and Southern blot (*B*) analysis of the GFP gene from GFP-expressing plants. *A* - 500 bp GFP gene fragment in transgenic plants. *Lane M* is 100 bp DNA ladder, *lane C* is non-transformed control, *lane P* is plasmid, *lanes 1 - 10* are transformed plants. *B* - Genomic DNA digested with *Hind*III, which has only one enzyme site in the plasmid. *Lanes* 1 - 9 is GFP expressing plants, *lane C* is non-transformed plant, *lane M* is λ DNA/*Hind* III molecular mass marker. Molecular masses are indicated *on the left*.

marker gene (Moore *et al.* 1992, EI-Shemy *et al.* 2007, Uzelac *et al.* 2007, Suwanaketchanatit *et al.* 2007). GFP expression could be detected transiently during co-cultivation. The cutting end of transformed TESRR and epicotyls exhibited green fluorescence, while the non-transformed controls showed red autofluorescence (Fig. 1*H, I*). 30 d later, the whole TESRR or the sprouted buds that had GFP activity were transferred to RMT medium without antibiotics (Fig. 1*E*). GFP expression could be detected *in vivo* and thus reduce the toxicity of antibiotics. PCR analysis revealed that 100 % of the buds with green fluorescence were positive transgenic events. This demonstrates that the GFP is a good marker gene to identify the transgenic events at early stage in *Agrobacterium*-mediated transformation. Because GFP can be detected *in vivo*, escapes and chimeric shoots could be easily identified. Though some silenced transgenic shoots were lost, it is still a great assistance for citrus breeding and gene function analysis using GFP as a marker gene.

PCR analysis was performed on all transgenic plants to confirm the presence of GFP transgenes. The expected band of 500 bp fragment was obtained from the plasmid in all transgenic plants while no band was detected in non-transformed control plant (Fig. 2*A*). The GFP gene was used as a probe to confirm the integration pattern of the transgene. Three events of single-copy insertion were identified by the presence of single band when *Hind*III which cuts once within the T-DNA region was used. The other six lines showed multiple-copy insertion with two, three and four bands. No hybridization signal was detected in the non-transformed control plant (Fig. 2*B*). These results further confirmed the integration of GFP gene into citrus genome.

Explant type is crucial for *Agrobacterium*-mediated transformation. Citrus genetic transformation has been tried by using various kinds of explants. Embryogenic calluses are convenient and available at any time for transformation especially of seedless cultivars. Protoplasts isolated from callus lines as explants are also easy to obtain pure clones, but protoplast isolation and culture system is only well established in a few laboratories in the world. Compared with other explants, the callus had high transformation efficiency and low chimeric events (Li *et al.* 2002). But regeneration difficulties limited the use of these two types of cultures. Cotyledon, epicotyl and internode, mature tissue come from seeds, their utilization as explants is hampered by seed availability and limited season. There have some reports to improve the transformation efficiency (Duan *et al.* 2007b), but the procedure is tedious. In all transformation methods, transgenic shoots from the explants need a rootstock to graft or a time period to root. Rooting of transgenic citrus plants has been a problem in most reports. Normally, transgenic shoots were micrografted on a non-transgenic rootstock because of the difficulty in rooting (Peña *et al.* 1995, Duan *et al.* 2007b). As a new explant for citrus transformation, the TESRR is also from seeds and it is superior to overcome the difficulties in rooting. In regeneration stages, the grafting and rooting periods were saved. Because the root system comes from seed germination, it has strong root system for further growth. Thus the advantages of TESRR explant, is to obtain transgenic citrus plants in a shorter time and to ensure better further growth. As with the epicotyls and internodes, the TESRR development is also limited by seed availability, and explant number is less than that of epicotyls.

## References


Ballester, A., Cervera, M., Peña, L.: Efficient production of transgenic citrus plants using isopentenyl transferase positive selection and removal of the marker gene by site-specific recombination. - Plant Cell Rep. **26**: 39-45, 2007.

Cai, X.D., Liu, X., Guo W.W.: GFP expression as an indicator of somatic hybrids between transgenic Satsuma mandarin and calamondin at embryoid stage. - Plant Cell Tissue Organ Cult. **87**: 245-253, 2006.

Cervera, M., Juárez, J., Navarro, A., Pina, J.A., Durán-Vila, N., Navarro, L., Peña, L., 1998. Genetic transformation and regeneration of mature tissues of woody fruit plants bypassing the juvenile stage. - Transgenic Res. **7**: 51-59, 1998.

Cheng, Y.J., Guo, W.W., Yi, H.L., Pang, X.M., Deng, X.X.: An efficient protocol for genomic DNA extraction from citrus species. - Plant mol. Biol. Rep. **21**: 117a-117g, 2003.

Chiu, W.L., Niwa, Y., Zeng, W.K., Hirano, T., Kobayashi, H., Sheen, J.: Engineered GFP as a vital reporter in plants. - Curr. Biol. **6**: 325-330, 1996.

Dominguez, A., Fagoaga, C., Navarro, L., Moreno, P., Peña, L.: Regeneration of transgenic citrus plants under non selective conditions results in high-frequency recovery of plants with silenced transgenes. - Mol. Genet. Genomics **267**: 544-556, 2002.

Duan, Y.X., Guo, W.W., Meng, H.J., Tao, N.G., Li, D.D., Deng, X.X.: High efficient transgenic plant regeneration from embryogenic calluses of *Citrus sinensis*. - Biol. Plant. **51**: 212-216, 2007a.

Duan, Y.X., Liu, X., Fan, J., Li, D.L., Wu, R.C., Guo, W.W.: Multiple shoot induction from seedling epicotyls and transgenic citrus plant regeneration containing the green fluorescent protein gene. - Bot. Stud. **48**: 165-171, 2007b.

EI-Shemy, H., Khalafalla, M., Fujita, K., Ishimoto, M.: Improvement of protein quality in transgenic soybean plants. - Biol. Plant. **51**: 277-284, 2007.

Ghorbel, R., Juarez, J., Navarro, L., Peña, L.: Green fluorescent protein as a screenable marker to increase the efficiency of generating transgenic woody fruit plants. - Theor. appl. Genet. **99**: 350-358, 1999.

Guo, W.W., Cheng, Y.J., Deng, X.X.: Regeneration and molecular characterization of intergeneric somatic hybrids between *Citrus reticulata* and *Poncirus trifoliata*. - Plant Cell Rep. **20**: 829-834, 2002.

Guo, W.W., Duan, Y.X., Olivares-Fuster, O., Wu, Z.C., Arias, C.R., Burns, J.K., Grosser, J.W.: Protoplast transformation and regeneration of transgenic Valencia sweet orange plants

containing the juice quality related pectin methylesterase gene. - Plant Cell Rep. **24**: 482-486, 2005.

Guo, W.W., Grosser, J.W.: Somatic hybrid vigor in *Citrus*: direct evidence from protoplast fusion of an embryogenic callus line with a transgenic mesophyll parent expression the GFP gene. - Plant Sci. **168**: 1541-1545, 2005.

Haseloff, J., Siemering, K.R., Prasher, D.C., Sarah, H.: Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. - Proc. nat. Acad. Sci. USA **94**: 2122-2127, 1997.

Khawale, R.N., Singh, S.K., Garg, G., Baranwal, V.K., Ajirlo, S.A.: *Agrobacterium*-mediated genetic transformation of Nagpur mandarin (*Citrus reticulata* Blanco). - Curr. Sci. **91**: 1700-1705, 2006.

Kobayashi, S., Uchimiya, H.: Expression and integration of a foreign gene in orange (*Citrus sinensis* Osb.) protoplasts by direct DNA transfer. - Jap. J. Genet. **64**, 91-97: 1989.

Li, D.D., Shi, W., Deng, X.X.: *Agrobacterium*-mediated transformation of embryogenic calluses of Ponkan mandarin and the regeneration of plants containing the chimeric ribonuclease gene. - Plant Cell Rep. **21**: 153-156, 2002.

Li, D.D., Shi, W., Deng, X.X.: Factors influencing *Agrobacterium*-mediated embryogenic callus transformation of Valencia sweet orange (*Citrus sinensis*) containing the *pTA29-barnase* gene. - Tree Physiol. **23**: 1209-1215, 2003.

Moore, G.A., Jacono, C.C., Neidigh, J., Lawrence, S.D., Cline, K.: *Agrobacterium*-mediated transformation of *Citrus* stem segments and regeneration of transgenic plants. - Plant Cell Rep. **11**: 238-242, 1992.

Murashige, T., Tucker, D.P.H.: Growth factor requirements of citrus tissue culture. - In: Chapman, H.D. (ed.): Proceedings of the 1st Citrus Symposium. Pp. 1155-1161. Riverside 1969.

Olivares-Fuster, O., Duran-Vila, N., Navarro, L.: Green fluorescent protein as a visual marker in somatic hybridization. - Ann. Bot. **89**: 491-497, 2002.

Orbovic, V., Grosser, J.W.: Citrus. - Methods mol. Biol. **344**: 177-189, 2006.

Peña, L., Cervera, M., Juarez, J., Navarro, A., Pina, J.A., Duran-Vila, N.: *Agrobacterium*-mediated transformation of sweet orange and regeneration of transgenic plants. - Plant Cell Rep. **14**: 616-619, 1995.

Suwanaketchanatit, C., Piluek, J., Peyachoknagul, S., Huehne, P.S.: High efficiency of stable genetic transformation in *Dendrobium via* microprojectile bombardment. - Biol. Plant. **51**: 720-727, 2007.

Uzelac, B., Ninkovic, S., Smigocki, A., Budimir. S.: Origin and development of secondary somatic embryos in transformed embryogenic cultures of *Medicago sativa*. - Biol. Plant. **51**: 1-6, 2007.