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BRIEF COMMUNICATION

Establishment of an *Agrobacterium*-mediated transformation system for *Fortunella crassifolia*

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Abstract

Epicotyl segments of kumquat (*Fortunella crassifolia* Swingle cv. Jindan) were transformed with *Agrobacterium tumefaciens* GV3101 harboring neomycin phosphotransferase gene (*npt* II) containing plant expression vectors. Firstly, the explants were cultured in darkness at 25 °C on kanamycin free shoot regeneration medium (SRM) for 3 d, and then on SRM supplemented with 25 mg dm^{-3} kanamycin and 300 mg dm^{-3} cefotaxime for 20 d. Finally, they were subcultured to fresh SRM containing 50 mg dm^{-3} kanamycin monthly and grown under 16-h photoperiod. Sixty five kanamycin resistant shoots were regenerated from 500 epicotyl explants after four-month selection. Shoot tips of 20 strong shoots were grafted to 50-day-old kumquat seedlings and survival rate was 55 %. Among the 11 whole plants, 3 were transgenic as confirmed by Southern blotting. This is the first report on transgenic kumquat plants, and a transformation efficiency of 3.6 % was achieved.

Additional key words: epicotyl, kanamycin, kumquat, shoot tip grafting, PCR, Southern blotting.

As a modern alternative way for creating new genetic resources, genetic transformation was successfully applied to citrus since 1990 (Vardi *et al.* 1990). Up to now, transgenic plants have been obtained in *Poncirus trifoliata* Rafin., *Citrus sinensis* Osbeck, *C. aurantium* L., *C. reticulata* Blanco, *C. limon* Burm, *C. aurantifolia* Swing., *C. grandis* Osbeck, and *C. paradisi* Macf. (reviewed in Peña and Navarro 1999, Yang *et al.* 2003, Petri and Burgos 2005). However, reports on transgenic kumquats (*Fortunella* sp.) are not available.

Though very close to *Citrus* plants, kumquats have a very notable advantage, short juvenile phase, over *Citrus* plants. The juvenile phase of kumquats is only about 2 years, while that of *Citrus* is 5 - 12 years. Therefore, kumquats are most suitable among various citrus for evaluating functions of genes related to fruit characteristics or studying expression patterns of fruit specific promoters by genetic transformation since fruits can be obtained in a much shorter time. Moreover, kumquats are plants of high economic and ornamental value. Therefore, establishment of a genetic transformation system for kumquats has dual significances for research as well as commercial production.

The media used in this study were based on Murashige and Skoog (1962; MS) medium or 1/2 MS medium supplemented with 3 % (m/v) sucrose and 0.7 % (m/v) *Bacto* agar at a pH of 5.8. Routine culture conditions were 26 ± 1 ºC, 16-h photoperiod with an irradiance of approximately 45 μmol m^{-2} s^{-1} and a relative humidity of about 60 % unless otherwise stated.

Seeds were collected from ripe fruits of kumquat (*Fortunella crassifolia* Swingle cv. Jindan), surface sterilized and sowed to 1/2 MS medium as described in our former study (Yang *et al.* 2006). Seedlings were obtained after two-week seed germination in darkness and additional two-week culture under light.

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Abbreviations: BA - 6-benzyladenine; KT - kinetin; MS - Murashige and Skoog; NAA - 1-naphthaleneacetic acid; *npt* II - neomycin phosphotransferase II gene; SRM - shoot regeneration medium.
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Shoot regeneration medium (SRM) applied in this study was MS medium supplemented with 8.88 μM 6-benzyladenine (BA), 5.37 μM 1-naphthaleneacetic acid (NAA), and 4.65 μM kinetin (KT) (Yang *et al*. 2006). To establish the optimal kanamycin concentration for selection, kanamycin was added to SRM to reach final concentrations of 0, 25, 50, 75, 100, 125 and 150 mg dm^{-3}, respectively. Epicotyls were excised from the four-week-old seedlings, cut into 1.0 cm segments with a vertical incision of 1 to 2 mm deep at adaxial end, and placed horizontally or vertically on media with various concentrations of kanamycin. Number of regenerated shoots was recorded after 30 d culture under light.

Agrobacterium tumefaciens GV3101 (Koncz and Schell 1986) harboring a pBI121 (Jefferson *et al.* 1987, Chen *et al.* 2003) derived, neomycin phosphotransferase II gene (*npt* II)-containing plant expression vector, either pBI-aCSBCH (β-glucuronidase gene of pBI121 was replaced by an antisense *C. sinensis* carotene β-ring hydroxylase gene) or pBI-pCSCL (35S CaMV promoter of pBI121 was replaced by a *C. sinensis* chlorophyllase promoter). The agrobacteria were cultured overnight in YEP liquid medium supplemented with 75 mg dm^{-3} kanamycin, 25 mg dm^{-3} gentamicin and 50 mg dm^{-3} rifampicin in a shaker at 180 rpm and 28 °C. The cells were pelleted at 3500 rpm and 4 °C for 10 min, resuspended and diluted to absorbance (A_{600}) 0.5 with MS liquid medium supplemented with 30 g dm^{-3} sucrose. Epicotyl explants were prepared as described above, and incubated with the agrobacteria for 30 min at room temperature, then blotted dry with sterilized filter paper and placed horizontally on SRM. After co-cultivation for 3 d in darkness at 25 °C, the explants were blotted with sterilized filter paper and transferred to SRM supplemented with 25 mg dm^{-3} kanamycin and 300 mg dm^{-3} cefotaxime, cultured in darkness at 25 °C for 20 d, and then the explants were subcultured monthly to SRM supplemented with 50 mg dm^{-3} kanamycin. After 4 subcultures, strong adventitious shoots were excised for shoot tip grafting.

Aseptically raised 50-d-old kumquat seedlings were decapitated to around 5 cm as rootstocks for grafting, and a vertical incision of around 5 mm deep was made to each rootstock. Adventitious kanamycin resistant shoots were V-shaped at base and inserted into the incision of the rootstocks, then the grafting sites were wrapped with *Parafilm M* (*Pechiney Plastic Packaging*, Menasha, USA). All manipulations were conducted under sterile conditions and the grafted plants were transferred to sterilized culture vessels on 1/2 MS medium for further growth of 4 weeks. The recovered plantlets were hardened for 1 week and transferred to soil as described in Yang *et al.* (2006). Young leaves from 10-month-old plants were sampled for DNA extraction.

Genomic DNA from putative transgenic and non-tranformed control leaves was isolated according to Kim *et al.* (1997) except that the temperature for extraction was 65 °C rather than room temperature. 0.5 g leaf tissues from each plant was applied. *npt* II gene primers NPTUP (5' GTTCTTTTTGTCAAGACCGACC 3') and NPTDP (5' CAAGCTCTTCAGCAATATCACG 3') were commercially synthesized (*Shanghai Sangon*, Shanghai, China) and used to amplify a fragment of 562 bp. A 0.025 cm^3 PCR reaction mixture contained the 1× PCR buffer (*New England Biolabs*, Boston, USA), 40 ng of genomic DNA, 2.0 mM $MgCl_2$, 0.4 mM dNTPs, 0.2 μM NPTUP, 0.2 μM NPTDP, and 0.3 U *Taq* DNA polymerase (*New England Biolabs*). Amplification was performed in thermal cycler (*Eppendorf Mastercycler*, Madison, USA) with following conditions: initial denaturation at 94 ºC for 5 min, 30 cycles of 30 s denaturation at 94 ºC, 40 s annealing at 55 ºC and 1 min extension at 72 ºC followed by a final 10 min extension step at 72 ºC. The reaction products were analyzed on 1.0 % agarose gel containing 5×10^{-4} g dm^{-3} ethidium bromide.

Southern blotting was performed with 9 μg of genomic DNA. The DNA was digested by *Eco*R I overnight, separated on 0.8 % agarose gel, and then transferred to positively charged nylon membrane (*Roche Diagnostics*, Mannheim, Germany) according to Sambrook *et al.* (2001). Preparation of DIG-labelled *npt* II gene probe DNA, hybridization and autoradiography were performed according to *DIG* application manual for filter hybridization (*Roche Diagnostics*).

Concentrations of kanamycin from 0 to 150 mg dm^{-3} were evaluated to determine the lowest effective threshold. Every 100 explants were applied for each kanamycin concentration and explant placement. The lowest effective concentration was tremendously affected by explant placement, being 25 and 100 mg dm^{-3}, respectively for explants placed horizontally and vertically. Less than 5 % of horizontally placed explants regenerated shoots when the concentration of kanamycin was kept over 25 mg dm^{-3}, and the shoots were found to be obviously weaker when the concentration was over 50 mg dm^{-3}.

Npt II gene in transformed shoots can detoxify kanamycin. However, too high a concentration of kanamycin might still be toxic to transformed cells. To keep a balance between alleviating growth inhibition of transformed shoots and eliminating escapes, selection was carried out using kanamycin at 25 mg dm^{-3} for initial 20 d and 50 mg dm^{-3} for the following stages. In addition, for those explants placed vertically, the regeneration of non-transformed shoots was not effectively inhibited unless the concentration of kanamycin was as high as 100 mg dm^{-3}. Therefore, the explants were only placed horizontally in further experiments.

Every 250 epicotyl segments were infected with *A. tumefaciens* GV3101 harbouring a recombinant binary plasmid either pBI-aCSBCH or pBI-pCSCL, respectively. Earliest resistant buds were observed after 20 d selection but new adventitious buds kept forming until 2 to 3 month after selection. Bleach and then browning of some explants was observed after one-month selection, and about half of explants completely died after four-

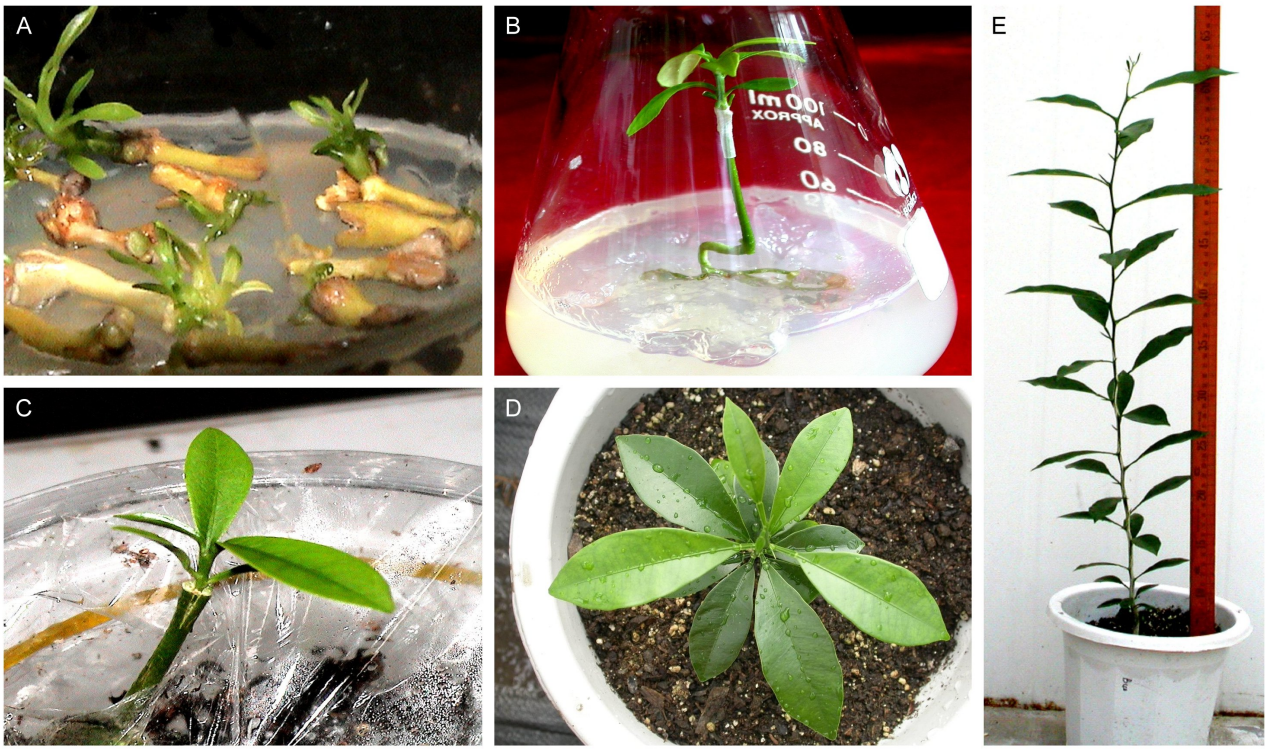

Fig. 1. Production of putative transformed *Fortunella crassifolia*: *A* - regenerated shoots after four-month selection, *B* - shoot tip grafted plantlets kept growing in culture vessel for 3 weeks, *C* - hardening of putative transformants, *D* - 10-month-old plant, *E* - 15-month-old plant.

month selection (Fig. 1*A*). Most shoots were regenerated from the cut surface of the segments and all shoots exclusively located at the morphologically upper end of explants (Fig. 1*A*). After four-month selection, 45 and 20 resistant shoots (shoots shorter than 2 mm were not included) were regenerated from pBI-aCSBCH and pBI-pCSCL transformation, respectively. The average regeneration frequency, defined as the number of regenerated resistant shoots against number of explants applied, for kumquat in this study was 13 %. This regeneration frequency was higher than 5.6 % for *Poncirus trifoliata* (Iwanami *et al*. 2004) but much lower than 37.1 % and 162.5 % reported for *Citrus sinensis* and citrange, respectively (Yu *et al*. 2002).

In our previous study (Yang *et al*. 2006), 74 % of regenerated shoots from tissue culture rooted in rooting medium. However, in preliminary experiments, none of the shoots from kanamycin selection in this study successfully rooted even in kanamycin free rooting medium. Similar results were frequently reported on other citrus (Peña and Navarro 1999) though rooting was successful for *C. paradisi* Macf. (Costa *et al*. 2002). The results suggested that kanamycin might result in loss of rooting ability of shoots. As an alternative, shoot tip grafting is commonly used to produce whole plants of citrus (Peña and Navarro 1999).

Every 10 strong resistant shoots from pBI-aCSBCH or pBI-pCSCL transformation were excised for shoot tip grafting. The grafted plants were transferred to sterilized culture vessels on 1/2 MS medium for further growth. After 4 weeks, 11 plantlets survived as shown by vigorous shoot growth especially leaf expanding (Fig. 1*B*). The survival rate of shoot tip grafting was 55 %, while for citrange and *C. sinensis* Osbeck, a rate of 100 % or near 100 % was reported (Peña and Navarro 1999, Yu *et al*. 2002). Improvement in grafting techniques might be necessary to increase the survival rate. However, when shoots from tissue culture were applied, the survival rate was over 90 %. Therefore, the low survival rate at least partially resulted from adverse effect of kanamycin selection and the kumquat might be quite sensitive to kanamycin, which is in line with a low concentration threshold for selection for this plant.

The recovered whole plantlets were subjected to hardening (Fig. 1*C*) for 1 week, and then the plantlets were transferred to soil in greenhouse. After ten-month growth, the plants grew over 10 leaves (Fig. 1*D*), and at 15 months since planting, the plants grew to a height of 35 to 85 cm (Fig. 1*E*).

DNA was extracted from young leaf of 10-month-old plants to identify transformation by PCR and Southern blotting. Five out of 11 plants were identified to be putative transformants by PCR (Fig. 2*A*) and 3 of them were true transgenic as confirmed by Southern blotting (Fig. 2*B*). Transformation positive percentage, the ratio of the number of Southern blotting positive shoots against number of total kanamycin resistant shoots, was 27.3 % in this study, which is lower than those reported for other

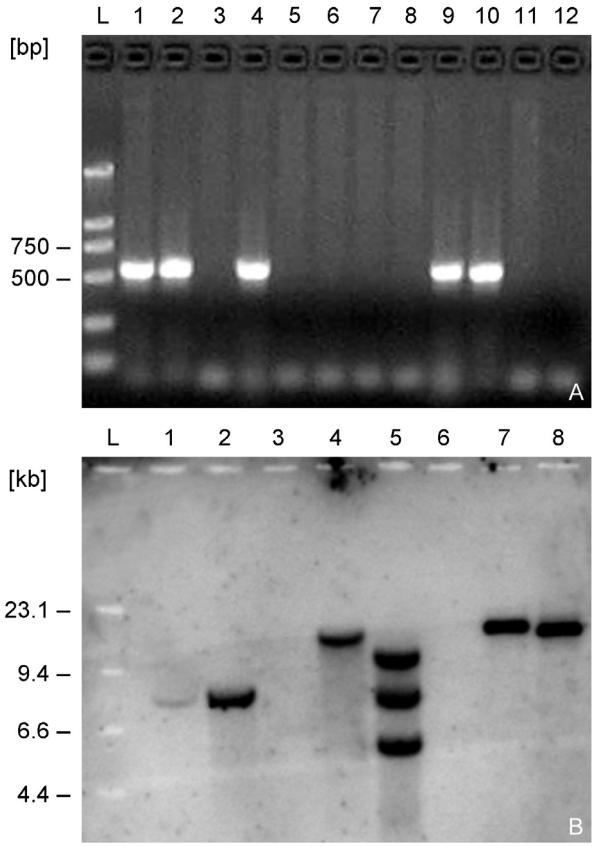

Fig. 2. PCR (*A*) and Southern blotting (*B*) analysis of putative transformants. *A*: *lanes L* - DL2000 DNA ladder (Takara); *lane 1 - 11* - putative transformants B1, B2, B3, B4, B13, C1, C2, C3, C6, C9, C11; *lane 12* - non-transformed control plant. *B*: *lanes L* - λDNA / H*ind* III DNA ladder; *lane 1-5* - putative transformants B1, B2, B4, C6, C9; *lane 6* - non-transformed control plant; *lane 7* - pBI-aCSBCH plasmid; *lane 8* - pBI-pCSCL plasmid.

citrus species, such as 90 % (Cervera *et al*. 1998) and 88 % (Wong *et al*. 2001) for citrange, or 87 % for *P*. *trifoliata* (Wong *et al*. 2001). Low kanamycin concentration applied in this study might partially explain the high percentage of escape shoots. However, for kumquat, it was not suitable to increase the kanamycin concentration, as in our preliminary studies, over 90 % explants bleached and died after one-month selection when the concentration of kanamycin applied in initial 20 d selection was raised to 50 mg dm^{-3}, and the regeneration frequency was less than 1 % after four-month selection.

The transformation efficiency, calculated by multiplying the regeneration frequency with transformation positive percentage, was 3.6 % for kumquat in this study, which is similar to 3.5 % for *C*. *aurantifolia* Swing. (Domínguez *et al*. 2000), 2.1 - 4.6 % for *P*. *trifoliata* (Iwanami *et al*. 2004), 3.6 - 6.6 % for *C*. *aurantium* (Ghorbel *et al*. 2000), and 4.3 % for *C*. *sinensis* (Yu *et al*. 2002) but lower than 41.3 % (Cervera *et al*. 1998) and 40 % (Yu *et al*. 2002) for citrange (Wong *et al*. 2001). Genotype is a determinant factor for woody fruit tree transformation (Petri and Burgos 2005), and kumquat might represent one of recalcitrant species to be transformed among citrus. However, it is possible that the transformation efficiency can be improved by applying some supervirulent *A. tumefaciens* strains such as EHA105 (Cervera *et al*. 1998, Domínguez *et al*. 2000, Švábová *et al.* 2005) or co-cultivating in liquid medium (Uraney *et al.* 2005).

The putative transformant B4 was PCR positive but non-transformed as shown by Southern analysis. Such false-positive result was also reported in *P*. *trifoliata* (Iwanami *et al*. 2004) but no explanation was given. In the present study, we suggest that this might result from contamination of trace survived *Agrobacterium* on leaf surface, since *Chv* A, a gene located in chromosome of *A. tumefaciens* (Hooykaas and Beijersbergen 1994), was detected in this DNA sample by PCR (data not shown). In addition, multicopy integration was also observed in this study, which is frequently reported in the studies of citrus transformtion (Cervera *et al*. 1998, Costa *et al*. 2002, Domínguez *et al*. 2000, Ghorbel *et al*. 2000, Iwanami *et al*. 2004, Wong *et al.* 2001).

The study was the first report on transformation of kumquat. A final transformation frequency of 3.6 % was achieved with epicotyls as explants and mediated by *A. tumefaciens*. Owing to shorter juvenile phase of kumquat, the established transformation protocol is especially useful for further work on evaluating functions of genes related to fruit characteristics or studying expression patterns of fruit specific promoters by genetic transformation.

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