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D. D. Li · W. Shi · X. X. Deng

Agrobacterium-mediated transformation of embryogenic calluses of Ponkan mandarin and the regeneration of plants containing the chimeric ribonuclease gene

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Abstract Ponkan (*Citrus reticulata* Blanco), one of the most important commercial cultivars of mandarin, is very seedy. In this study, the chimeric ribonuclease gene (*barnase*) driven by an anther tapetum-specific promoter (*pTA29*) was introduced into embryogenic callus of Ponkan by *Agrobacterium*-mediated transformation using the *bar* gene as a selectable marker. In contrast to previous reports, embryogenic calluses were used as the explant for *Agrobacterium* infection and transgenic plant regeneration. Selection of transformed callus was accomplished using basta. After 3 days of co-culture, calluses were transferred to MT medium with 50 mg/l basta and 400 mg/l cefotaxime. Resistant calluses were recovered and proliferated after three to four subcultures and then regenerated plantlets. A total of 52 resistant plants were recovered, of which 43 were verified to be transformants by polymerase chain reaction amplification of a fragment of the transgene. Southern hybridization of seven randomly selected transformed plants further confirmed their transgenic nature. The potential of this strategy for breeding citrus seedless types is discussed.

Keywords Ponkan · *pTA29-barnase* · Male sterility · Genetic transformation · *Agrobacterium tumefaciens*

Abbreviations *BA*: 6-Benzyladenine · *CaMV*: Cauliflower mosaic virus · *gfp*: Green fluorescent protein · *gus:* β-Glucuronidase · *IBA*: 3-Indole butyric acid · *KT*: Kinetin · *NAA*: α-Naphthaleneacetic acid · *NPTII*: Neomycin phosphotransferase · *PCR*: Polymerase chain reaction · *SDS*: Sodium dodecyl sulfate

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D.D. Li · W. Shi · X.X. Deng (✉)
National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, P.R. China
e-mail: dxxwwlj@public.wh.hb.cn
Tel.: +86-27-87281300, Fax: +86-27-87280016

Introduction

Ponkan (*Citrus reticulata* Blanco), a prolific, very tasty and easy peeling mandarin, is one of the most important commercial cultivars in China, but its seedy trait is a key demerit. From the point of view of consumers, seedlessness is an attractive trait for fresh citrus fruit. Many economically important commercial citrus varieties worldwide are seedless. In conventional citrus seedless breeding, selection from bud mutation and seedling variation has been commonly used. Other strategies include the induction of seedless mutation by irradiation treatment (Hensz 1977) and the creation of triploids via interploidy hybridization using autotetraploid or allotetraploid somatic hybrids as one of the parents (Chandler et al. 2000; Deng et al. 1996b; Guo et al. 2000). In fact, a number of different mechanisms will lead to seedless citrus fruit, including pollen sterility, embryo-sac abortion, chromosome ploidy variation and self-incompatibility (Deng et al. 1996a). Of these factors, pollen sterility is the most common one applied, as seen in most satsuma mandarin and navel orange cultivars. Male sterility will lead to complete pollen sterility, and the induction of male sterility in seedy cultivars with a desirable genetic background and the parthenocarpic trait by genetic transformation is a possible and promising strategy by which to obtain seedless varieties.

The induction of male sterility in plants by the chimeric *barnase* gene, derived from *Bacillus amyloliquefaciens* (Hartley 1988), was first reported by Mariana et al. (1990) and has since been applied in maize (Liu et al. 2000), alfalfa (Rosellini et al. 2001), rapeseed (Zhou et al. 1997), rice (Lin et al. 1998) and many other plant species. Since most citrus cultivars, including Ponkan, have the parthenocarpic character and since male sterility is a useful approach to develop seedless citrus fruit, we introduced a chimeric ribonuclease gene (*pTA29-barnase*) into embryogenic callus of cv. Ponkan in an effort to obtain male-sterile plants and, subsequently, a seedless clone of Ponkan.

Considerable progress in citrus has been made in the time from the first report on genetic transformation

(Kobayashi and Uchimiya 1989) to the establishment of efficient and reliable protocols to obtain transgenic plants. The use of epicotyl segments and protoplasts as explants for genetic transformation has been applied widely in most reports using *Agrobacterium* and electroporation, respectively (Cervera et al. 2000; Olivares-Fuster et al. 2000; Pena and Navarro 1999; Serres and Stang 1992). Different kinds of genes, mostly reporter genes such as *gus* (Gutierrez-E et al. 1997; Moore et al. 1992; Pena et al. 1995, 1997), *NPTII* (Moore et al. 1992; Pena et al. 1995), *gfp* (Ghorbel et al. 1999; Olivares-Fuster et al. 2000) and the coat protein gene of citrus tristeza closterovirus (Dominguez et al. 2000; Ghorbel et al. 2000; Yang et al. 2000), have been introduced into different citrus cultivars. In addition to epicotyls and protoplasts, internode segments from greenhouse-grown plants have also been used as explants for transformation (Cervera et al. 1998). In the investigation reported here, we used embryogenic calluses as explants for *Agrobacterium*-mediated transformation and plant regeneration, which is in contrast to previous reports. The efficient transformation of this system indicates that it will be a good alternative to obtain transgenic citrus plants in the future.

Materials and methods

Plant material

Embryogenic callus of mandarin cv. Ponkan (*Citrus reticulata* Blanco) was induced from nucellar tissue (Huo et al. 1999) and subcultured on solid MT (Murashige and Tucker 1969) basal medium containing 50 g/l sucrose. Prior to infection with *Agrobacterium tumefaciens*, the calluses were cultured in liquid MT medium supplemented with 0.5 g/l malt extract, 1.5 g/l glutamine and 50 g/l sucrose for 4 days.

Bacterial strain and vector

A. tumefaciens strain EHA105 harboring the binary plasmid pBI-TBSBAR, kindly provided by Dr. W.B. Li (Institute of Genetics, Chinese Academy of Science, Beijing), was used as the vector system for transformation. The phosphinothricin acetytransferase gene (*bar*) driven by the CaMV 35S promoter and terminator sequence was used as the selectable marker gene. The ribonuclease gene (*barnase*) was driven by the anther tapetum-specific promoter *pTA29* (Fig. 1). Bacteria were cultured overnight at 28°C in YEB medium containing 50 mg/l kanamycin and then collected by centrifugation at 2,500 *g* for 10 min and adjusted to an OD_{600}=0.5 in MT liquid medium.

Determination of basta concentration in selection medium

To determine the optimum selection conditions, we carried out a preliminary experiment. The control calluses were cultured on antibiotic-free medium and on the same medium containing basta at different concentrations (10–60 mg/l). After 30 days of culture, most calluses on medium containing 10–30 mg/l basta became brown, but there were a few that still grew slowly. Those on medium containing 40–50 mg/l basta became necrotic and died. Therefore, a selection procedure of culture on MT medium containing 50 mg/l basta was used for subsequent transformation experiments.

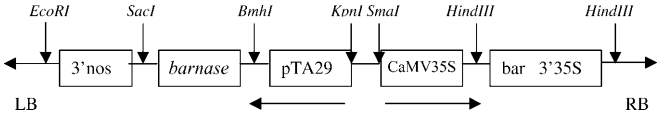

Fig. 1 A linear map of the T-DNA region of pBI-TBSBAR. *LB* Left T-DNA border, *3′nos* NOS terminator, *barnase* ribonuclease gene, *pTA29* anther tapetum-specific promoter, *CaMV 35S* cauliflower mosaic virus 35S promoter, *3′35S* CaMV 35S terminator, *RB* right T-DNA border

Transformation and regeneration

Embryogenic calluses were immersed in an *Agrobacterium* suspension for 25 min, blotted dry on sterile filter paper and transferred to solidified MT medium with 100 μ*M* acetosyringone. After 3 days of co-culture in darkness, the calluses were transferred to MT medium containing 50 mg/l basta and 400 mg/l cefotaxime. Five weeks later, most of the calluses had became necrotic and died. The calluses that did proliferate on the selection medium were subcultured onto fresh MT medium containing 50 mg/l basta and 400 mg/l cefotaxime three or four times at 4-week intervals.

The resistant calluses were transferred to MT medium supplemented with 2% glycerol (Vardi et al. 1989) for the induction of somatic embryos. Media for shoot and root development were prepared as in previous reports (Deng et al. 1993; Guo and Deng 1999). For shoot induction, embryoids were transferred to MT medium supplemented with 0.5 mg/l BA, 0.5 mg/l KT and 0.1 mg/l NAA. The resultant shoots were then excised and transferred to root induction medium containing half-strength MT medium supplemented with 0.5 mg/l NAA, 0.1 mg/l IBA, 25 g/l sucrose and 0.5 g/l activated charcoal.

DNA extraction and molecular analysis

DNA was extracted from leaves of individual resistant plants and non-transformed control calluses using the SDS method (Shi et al. 1998). PCR amplification was carried out using the following conditions: 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C. The primers used for amplification of the *barnase* sequence were 5′-ACTGCAGGATCCATGGCACAGGTTATCAACACGT-3′ and 5′-CCCCTCGAGCTCGTTATCTGATCTTTG-3′. For Southern hybridization, DNA samples (10 μg) were digested with *Eco*RI, separated on an 0.8% agarose gel and transferred onto Hybond-N^+ blotting membrane under alkaline conditions. The *barnase* gene probe DNA was prepared from 420 bp of PCR products amplified from pBI-TBSBAR with the primer described above and labeled with [^{32}P]. Hybridization and autoradiography were according to the manufacturer's instructions (Clark 1998).

Results and discussion

After 5 weeks of selective culture, resistant callus lines kept growing while the others became necrotic and died (Fig. 2). The resistant calluses from ten plates were recovered and subcultured three times in fresh selective medium for multiplication and then transferred to MT medium containing 2% glycerol for somatic embryo induction. Four weeks later, somatic embryos developed (Fig. 3).Of these, 210 somatic embryos were selected and transferred to medium for shoot induction; 5 weeks later, 86 shoots had elongated (Fig. 4). Entire plantlets were recovered on root induction medium. A total of 52 resistant plants were recovered from different somatic embryos.

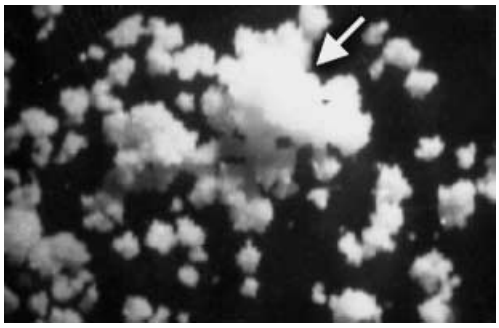

Fig. 2 The basta-resistant cv. Ponkan calluses (*arrow*) growing on selective medium

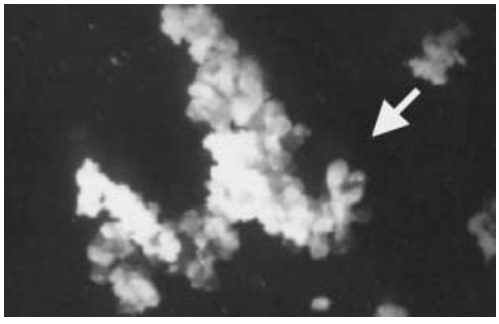

Fig. 3 Ponkan somatic embryos (*arrow*) regenerated on MT medium containing 2% glycerol

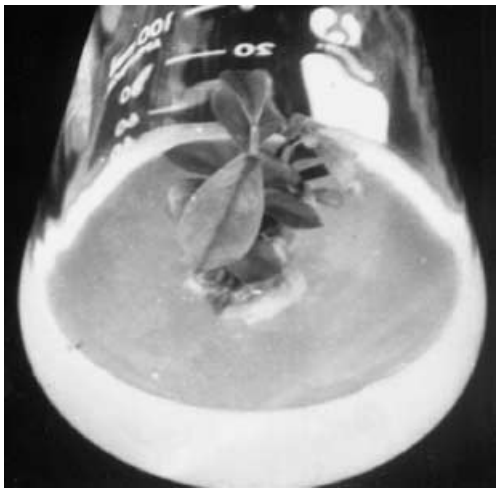

Fig. 4 Shoots induced from the Ponkan somatic embryos

Fig. 5 PCR amplification of the regenerated Ponkan plants. *Lane M* Marker, *lane 1* plasmid DNA, *lane 2* non-transformed control, *lanes 3–10* transgenic plants

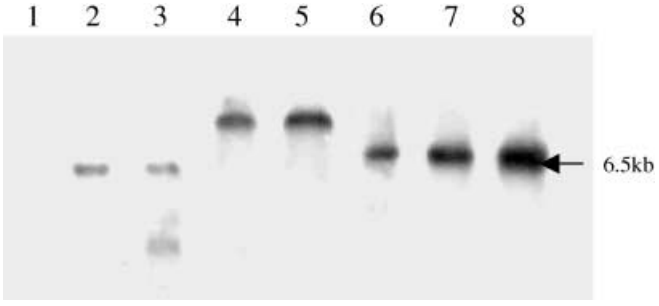

Fig. 6 Southern hybridization analysis of the transgenic plants. DNA was cut with *Eco*RI. *Lane 1* Non-transformed control, *lane 2–8* transgenic plants

DNA was extracted from each of the regenerated resistant plants. PCR amplification revealed that 43 of the 52 regenerated plants had the expected 420-bp band, as did the plasmid control, while no such band was present in the non-transformed control calluses (Fig.5). The positive results suggested that the *barnase* gene was present in these plants. The negative results of the remaining nine plants could be due to plant regeneration of non-transformed calluses or because the *barnase* gene was not stably integrated into the plant genome and subsequently lost during the following subcultures.

The [^{32}P]-labeled probe was used to hybridize with digested genomic DNA of seven randomly selected PCR-positive plants and non-transformed control calluses. Different banding patterns were found in the transgenic plants, while no bands were found in the non-transformed control calluses (Fig. 6). These results confirm the integration of the *barnase* gene in the genome of these transgenic plants. On the basis of the banding patterns, at least three independent transformants are represented among the tested lines, and it appears that single-copy inserts predominated (4, 5, 6, 7, 8). This result is consistent with *Agrobacterium*-mediated transformation reported previously in citrus and other crops (Cervera et al. 2000).

Compared with previous investigations in which various kinds of explants were used, citrus callus as the material for infection and regeneration is a different *Agrobacterium*-mediated genetic transformation system. In this system, we determined a rate of resistant calluses (weight of resistant calluses/infected calluses after the first 45 days of selective cultivation) higher than 20%. Compared to the 0.1% internodal stem-segment transformation efficiency achieved by Gutierrez-E et al. (1997) and the 20% co-transformation rate of protoplasts observed by Olivares-Fuster et al. (2000), we have proven the transformation system described here to be efficient. In addition, the genetically identical background of the material, large numbers of transgenic plants, easy proliferation and rare occurrence of chimeras during regenera-

tion are a positive feature of this transformation system. Moreover, transgenic calluses can also be preserved over the long-term and induced to regenerate transgenic plants when necessary. Obvious disadvantages of this system are that resulting plants are juvenile and that an evaluation of traits related to fruit characters requires a longer time. This technique is also not suitable for varieties with no embryogenic potential.

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