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BRIEF COMMUNICATION

Agrobacterium-mediated transformation in *Citrullus lanatus*

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Abstract

Agrobacterium tumefaciens-mediated transformation was used to produce transgenic watermelon. Cotyledonary explants of *Citrullus lanatus* Thumb (cv. Daesan) were co-cultivated with *Agrobacterium* strains (LBA4404, GV3101, EHA101) containing pPTN289 carrying with *bar* gene and pPTN290 carrying with *nptII* gene, respectively. There was a significant difference in the transformation frequency between bacteria strains and selective markers. The EHA101/pPTN289 showed higher transformation frequency (1.16 %) than GV3101/pPTN289 (0.33 %) and LBA4404/pPTN289 or /pPTN290 (0 %). The shoots obtained (633 and 57 lines) showed some resistance to glufosinate and paromomycin, respectively. Of them, the β-glucuronidase positive response and PCR products amplified by *bar* and *nptII* specific primers showed at least 21 plants resistant to glufosinate and at least 6 plants to paromomycin. Southern blot analysis revealed that the *bar* gene integrated into genome of transgenic watermelon. Acclimated transgenic watermelons were successfully transplanted in the greenhouse and showed no phenotypic variation.

Additional key words: *Agrobacterium* strains, β-glucuronidase, glufosinate, paromomycin, transgenic watermelon.

Watermelon, one of the most important vegetable crops, is eaten chiefly as a fresh fruit, because of its sweetness and flavor. It originated from tropical and subtropical Africa, and now is widely distributed throughout the tropics, South Asia, and East Asia including China. Fresh watermelon is also rich in lycopene, a potent antioxidant that has been shown to reduce human risk to cancer (Gaster 1997). By using pathogen-resistant or abiotic stress-resistant cultivars watermelon yields can be increased. The cultivars have been developed by traditional breeding to have resistance to watermelon fruit blotch (Rane and Latin 1992), to watermelon mosaic virus (Gillaspie and Wright 1993), and to zucchini yellows mosaic virus (Boyhan *et al*. 1992). Recent advances in insertion of bacterial, fungal and virus resistance genes through *Agrobacterium*-mediated transformation would facilitate the development of new disease resistant genotypes without significantly altering the genetic composition, and have made it possible to improve their productivity and quality beyond the limit of traditional breeding. *Agrobacterium*-mediated transformation of watermelon has been very difficult. Of these difficulties, selection of transformants was a problem as the cotyledons are moderately resistant to kanamycin (Gaba *et al*. 2004). Actually, selection on glufosinate using the *bar* gene for *Basta* herbicide resistance was more efficient than selection with the *nptII* gene in the production of transgenic cucumber and melon (Cho *et al*. 2005a, b). The *bar* gene, encoding for phosphinothricin acetyltransferase (PAT) which detoxifies glufosinate, has been widely used as a selectable marker in the soybean transformation system (Zhang *et al*. 1999). Since numerous studies have been conducted for plant regeneration *via* organogenesis or somatic embryogenesis from cotyledons or immature embryos of watermelons (Compton and Gray 1993), only a few reports for the watermelon transformation system have been achieved (Choi *et al*. 1994, Ellul *et al*. 2003, Compton *et al*. 2004). Tricoli *et al*. (2002) used this system to introduce the virus resistance gene into the watermelons. In these studies, kanamycin has been only used as a selective agent to produce transgenic watermelons, and the

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Abbreviations: BA - benzyladenine; GUS - β-glucuronidase; IBA - indolebutyric acid; MS - Murashige and Skoog's medium; PCR - polymerase chain reaction.
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phosphomannose isomerase has been rarely used (Reed *et al*. 2001). To our knowledge, no work has been reported to date evaluating this marker in a watermelon transformation system. This paper describes a transformation system for watermelons by co-culturing cotyledon explants with *Agrobacterium* harboring the pPTN289 binary vector carrying *bar* gene as a selectable marker.

Zygotic embryos of F_1 hybrid watermelon (*Citrullus lanatus* Thumb*,* cv. Daesan) were dissected out of the mature seeds and the surface disinfected with 70 % ethanol for 1 min and 1 % sodium hypochlorite for 15 min, and then rinsed three times with sterile deionized-distilled water. These seeds were germinated in the dark on MS medium (Murashige and Skoog 1962). The pH of all media was adjusted to 5.8 before autoclaving. Medium was dispensed into plastic Petri dishes. After 7 - 10 d of incubation, cotyledons of seedlings 2 to 3 cm long were excised, avoiding the shoot apex. The transformation of watermelon was performed with the binary vectors pPTN289 (Tom Clemente, unpublished data) and pPTN290 (Howe *et al*. 2005), which contained the herbicide resistance gene (*bar*) and the neomycin phosphotransferase gene (*nptII*) as selective marker, respectively. Disarmed *Agrobacterium tumefaciens* strains (LBA4404, EHA101, GV3101) were used as helper strains in a binary vector system. Each binary vector was introduced into *A. tumefaciens* strains LBA4404 (Ooms *et al*. 1981), EHA101 (Hood *et al*. 1993), GV3101 (Koncz and Schell 1986) by the freeze-thaw method, respectively (An *et al*. 1987). The *Agrobacteria* were grown in YEP medium amended with the appropriate antibiotics to an absorbance (A_{650}) 0.6 to 0.8 at 28 °C. The pellets after centrifuged at 890 *g* for 10 min resuspended to a final A_{650} 0.6 to 0.8 in $^1/_{10}$ MS basal medium amended with 3.2 mg dm^{-3} benzyladenine (BA), 0.5 mg dm^{-3} indolebutyric acid (IBA), 39 mg dm^{-3} acetosyringone (AS) and 3 % sucrose. The medium was buffered with 3.9 g dm^{-3} MES, pH 5.4. Cotyledonary explants inoculated with *Agrobacterium* suspensions were incubated for 30 min on co-cultivation media (pH 5.4) supplemented with 3.9 g dm^{-3} MES, 100 mg dm^{-3} cysteine, 3.2 mg dm^{-3} BA, 0.5 mg dm^{-3} IBA, 0.039 mg dm^{-3} acetosyringone and 3 % sucrose. Six explants were cultured per 90 × 15 mm Petri dish and the explants were positioned with the adaxial side on a filter paper laid over the media. After co-cultivation the explants were washed with three times by a sterilized distilled water and then were cultured on shoot induction medium (MS salt, MS vitamin, 3 % sucrose, 3.2 mg dm^{-3} BA, 0.5 mg dm^{-3} IBA, 5 mg dm^{-3} glufosinate, 50 mg dm^{-3} ticarcillin, 50 mg dm^{-3} cefotaxime, 50 mg dm^{-3} vancomycin, 0.58 g dm^{-3} MES, pH 5.6) for 4 weeks. Explants were subcultured to fresh medium after 2 weeks, and primary shoots developed from the explants were cut and discarded. Following 4 weeks of culture on shoot induction medium, explants were transferred to shoot elongation medium (MS salt, 0.1 mg dm^{-3} IBA, 0.5 mg dm^{-3} GA_3, 3 mg dm^{-3} glufosinate, 50 mg dm^{-3} ticarcillin, 50 mg dm^{-3} cefotaxime, 50 mg dm^{-3} vancomycin, 0.58 g dm^{-3} MES, pH 5.6) solidified with 0.8 % agar. Subculture to fresh medium was done every two weeks, and culture conditions during co-cultivation, shoot induction and elongation were 16-h photoperiod at irradiance of 46 μmol $m^{-2}s^{-1}$ and temperature of 24 °C. In the case of pPTN290 carrying with the *nptII* gene, selection agent was used a paromomycin at concentration of 100 mg dm^{-3} in place of glufosinate. Elongated shoots were transferred to rooting medium comprised of $^1/_2$ MS salts, 3 % sucrose, 0.58 g dm^{-3} MES, 50 mg dm^{-3} cefotaxime, 0.8 % agar, pH 5.6. The rooted plants were transferred to soil. Five replicates were prepared for each *Agrobacterium* co-cultivation with about 100 cotyledon explants. A histochemical β-glucuronidase assay was done on flower of plants grown in soil to verify T-DNA transfer (Jefferson *et al*. 1987) by immersing them with a 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution overnight at 37 °C. The flowers were subsequently cleared in 70 % ethanol prior to visualization. Plants (T_0) acclimated in soil were screened for tolerance to the herbicide *Liberty*® by application of a 0.1 % solution of the herbicide with a cotton swab to the upper leaf of the plant at maturity. A 200 mg dm^{-3} stock of *Liberty*® was diluted in water for the leaf painting assay. Leaf tissue was observed for herbicide tolerance 5 d post application. Genomic DNA was extracted from young leaf by method with sodiumdodecylsulphate according to Dellaporta *et al*. (1985) with some modifications and was used as template in amplification. Presence of the *nptII* and *bar* coding region in the genomic DNA was analysed by PCR amplification using forward primer sequence 5'-ATG AGC CCA GAA CGA CGC CCG GCC-3' and reverse primer sequence 5'-TGC CAG AAA CCC ACG TCA TGC CAG TT-3' for a *bar* fragment of 500 bp size, and forward primer 5'-GAG GCT ATT CGG CTA TGA CTG-3' and reverse primer 5'-ATC GGG AGC GGC GAT ACC GTA-3' for a *nptII* fragment of 650 bp size, respectively. A genomic DNA extracted from the 4 putative transgenic watermelon leaf with GUS positive response, polymerase chain reaction (PCR) product and herbicide tolerance as described by Dellaporta *et al*. (1985). Ten μg of the genomic DNA was digested with *Eco*RI, separated by electrophoresis in 0.8 % agarose gel and transferred to *Zeta-Probe® GT* nylon membrane (*Bio-Rad*, Hercules, USA). The DNA was fixed to the membrane by UV cross linking. A DNA fragment containing the *bar* gene consisted of the entire 600 bp open reading frame (ORF) and obtained from the pPTN289 vector by *Bg1*II digestion and then was used to generate a ^{32}P-labeled probe. The probe was prepared by random primer synthesis incorporating ^{32}P-dCTP utilizing *Prime-It*® II kit (*Stratagene*). Hybridization and washing conditions for Southern blot analysis followed the *Zeta-Probe® GT* manufacturer's instructions.

To determine the optimum cultivar for shoot organogenesis, we examined the frequency of cotyledon with

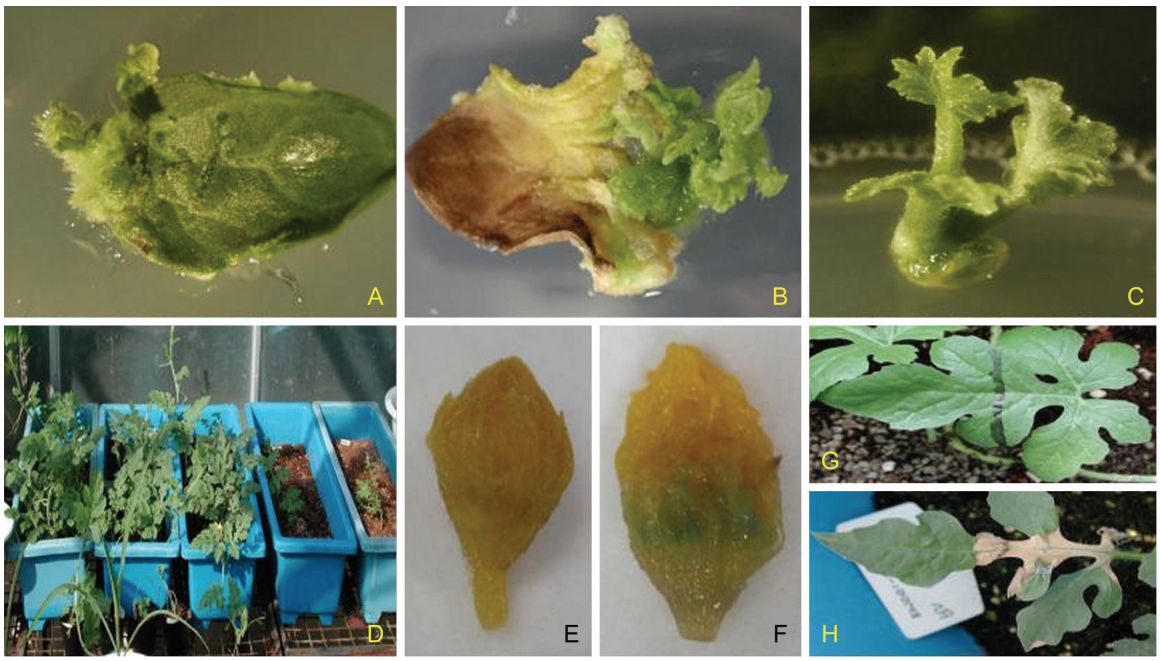

Fig. 1. Plant regeneration from cotyledonary explants of watermelon transformed with pPTN289 or pPTN290 vectors and molecular analysis for the transgenic watermelon. *A*, *B*, *C* - Adventitious shoots formation on shoot induction medium with 5 mg dm^{-3} glufosinate. *D* - Transgenic watermelon grown in soil. *E* - GUS negative response in flower of non-transgenic plant. *F* - GUS positive response in flower of transgenic plant. *G* - Herbicide-resistance response in the leaf of transgenic leaf. *H* - Necrosis in leaf of non-transgenic plant with *Basta* treatment.

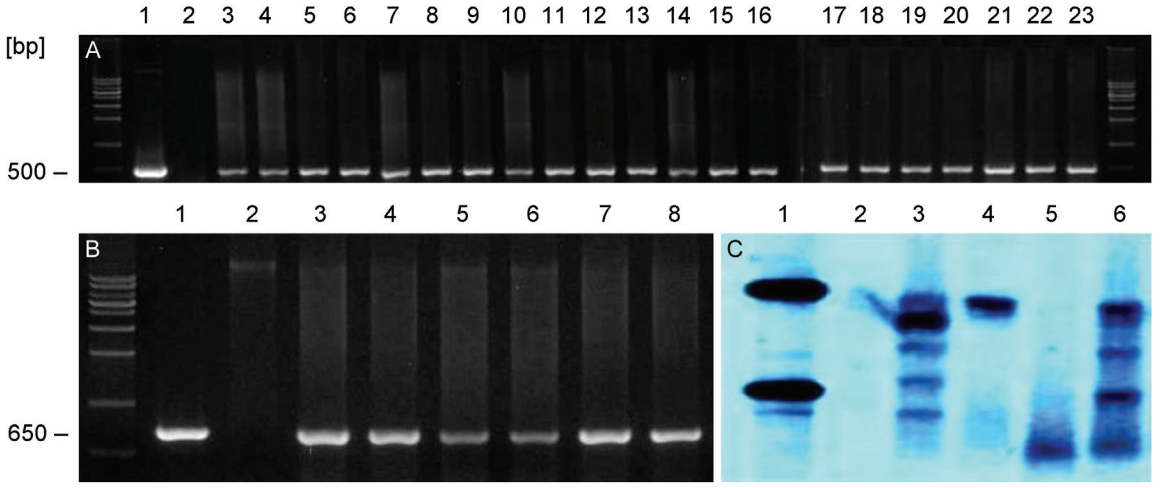

Fig. 2. Polymerase chain reaction (PCR) and Southern blot analysis of transgenic watermelon. *A* - PCR products (500 bp) of positive control (*lane* 1), negative control (*lane* 2) and putative transgenic events (*lanes* 3 - 23) using the specific *bar* primers. *B* - PCR products of (650 bp) of positive control (*lane* 1), negative control (*lane* 2) and putative transgenic events (*lanes* 3 - 8) using the specific *nptII* primers. *C* - Total genomic DNA was digested with *EcoR*I. The 600 bp *bar* probe obtained from the pPTN289 vector by *Bg1*II digestion and then labeled with ^{32}P-dCTP, was hybridized with the Plasmid vector DNA (pPTN289) digested with *EcoR*I (*lane* 1), and genomic DNA (10 μg) of negative control (*lane* 2) and T_0 plants (*lanes* 3 - 6).

adventitious shoots on shoot induction medium in twenty cultivars of domestic watermelon. The cv. Daesan gave the maximum frequency (> 95 %) for shoot organogenesis. The cotyledon explants of cv. Daesan were inoculated with *Agrobacterium* suspension and then incubated on shoot induction medium supplemented with a glufosinate (pPTN289) or paromomycin (pPTN290) as selective agents. After 3 weeks of culture, many cotyledon explants were turned brown and necrosis was observed. After 6 weeks of culture, a few of these cotyledon explants showed adventitious shoot formation (Fig. 1*A,B*). To remove any non-transformed shoots, we

transferred only those shoots having expanded leaves to shoot elogation medium containing 3 mg dm^{-3} glufosinate or 50 mg dm^{-3} paromomycin. The elongated shoots were then separated, transferred to rooting medium (Fig. 1*C*). A total of 633 (19.7 %) and 57 (1.8 %) shoots on selection medium containing glufosinate and paromomycin, respectively, elongated and rooted, and 21 (0.7 %) and 6 (0.2 %) of these plantlets showed GUS positive response in flowers (Fig. 1*F*), and the 21 transformants with GUS positive response were also resistance to glufosinate, and grew normally to maturity without symptoms of *Basta* herbicide damage (Fig. 1*G*). Whereas, the leaves of control seedlings became necrotic and fell off (Fig. 1*H*). In the transgenic watermelon with GUS positive response, PCR products were amplified the expected 500 bp fragment (Fig. 2*A*) of *bar* gene and 650 bp fragment (Fig. 2*B*) of *nptII* gene, respectively. All GUS-positive shoots rooted on the rooting medium and then were transplanted to soil and grown to maturity in a greenhouse (Fig. 1*D*). The frequency of GUS positive response was shown in GV3101/pPTN289 (0.33 %), EHA101/pPTN289 (1.16 %), and EHA101/pPTN290 (0.86 %), but no GUS positive response in other strains (LBA4404/pPTN289, GV3101/pPTN290, LBA4404/ pPTN290) (data not shown). Like these, the highest frequency (1.16 %) was with the strain EHA101/ pPTN289. The herbicide resistance gene, *bar* derived from *Streptomyces hygroscopicus* (Thompson *et al*. 1987) has been an effective selectable marker gene in the production of transgenic crops such as cotton (Keller *et al*. 1997), lettuce (Mohapatra *et al*. 1999), soybean (Zhang *et al*. 1999) and bottle gourd (Han *et al*. 2005). Actually, the cotyledons and *bar* gene have often been used as explants and selective marker genes for *Agrobacterium* infection in our *Cucurbitacea* transformation studies, including cucumber (Cho *et al*. 2005a) and melon (Cho *et al*. 2005b). The *nptII* gene encodes an enzyme, neomycin phosphotransferase II which detoxifies paromomycin or kanamycin, has been used as an effective selectable marker in cucumber, melon, and watermelon (Gaba *et al.* 2004). The *nptII* gene in our experiment was a less effective than *bar* gene as selective marker gene for watermelon transformation. When the genomic DNA of four randomly selected plants (Fig. 2*C*, *lanes* 3 - 6) with resistance to *bar* gene was digested with *Eco*RI and subjected to Southern blot analysis, 4 plants tested possessed the *bar* gene as single (Fig. 2*C*, *lanes* 4,5) or multiple copies (Fig. 2*C*, *lanes* 3,6).

In conclusion, we have developed a stable protocol for the genetic transformation of watermelon on selection medium with glufosinate as selective agents after *Agrobacterium* co-cultivation. The process is simple, and many plants including melon and cucumber can be used in a transformation experiment to introduce useful genes.

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