In Vitro Regeneration and Genetic Transformation of *Cucumis metuliferus* through Cotyledon Organogenesis

Yu-Tsung Lin, Chia-Wei Lin, Chien-Hung Chung, and Mei-Hsiu Su
Department of Agronomy, National Chung Hsing University, Taichung 40227, Taiwan

Hsiu-Yin Ho, Shi-Dong Yeh, and Fuh-Jyh Jan¹
Department of Plant Pathology, National Chung Hsing University, Taichung 40227, Taiwan

Hsin-Mei Ku
Department of Agronomy, National Chung Hsing University, Taichung 40227, Taiwan

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Abstract. This study was undertaken to establish for the first time an efficient regeneration and transformation system for *Cucumis metuliferus* line PI292190, which is the source of a well-defined resistant gene, *Wmv*, that provides resistance against *Papaya ringspot virus* type P (PRSV-P) and PRSV-W (formerly known as Watermelon mosaic virus 1, WMV-1). Different combinations of growth regulators were evaluated for the regeneration of cotyledon explants. Adventitious buds or shoot primordia were obtained within 3 to 4 weeks on regeneration medium. After shoot development, adventitious buds or shoot primordia were transferred to elongation medium for 3 to 4 weeks and these shoots were subcultured onto rooting medium for another 1 to 2 weeks. Under optimal culture conditions, a total of 7 to 10 weeks was necessary to obtain *C. metuliferus* plantlets from cotyledons. Furthermore, transgenic plants were successfully obtained using an *Agrobacterium tumefaciens*-mediated transformation method as shown by polymerase chain reaction analysis and histochemical β -glucuronidase (GUS) assay. A total of nine transgenic plants were developed from 360 cotyledon explants, giving a transformation frequency of 2.5%.

Cucumis metuliferus (Naud.) Mey, one of the traditional vegetables originated from South Africa, provides high-quality nutrition sources and is consumed daily as a vegetable by many rural communities in Africa (Kristkova et al., 2003). Its other names include jelly melon, horned melon, or hedgehog gourd, and it is generally marketed as Kiwano. It is an important supplement of fiber and mineral elements, namely potassium, calcium and magnesium (Odhav et al., 2007; Romero-Rodriguez et al., 1992). The novel flavor of *C. metuliferus* fruit may be useful in an innovative tea or beverage. The fruits contain high antioxidant com-

pounds (Breithaupt et al., 2002) and medicinal properties when applied to laboratory rats (Motlhanka, 2008; Wannang et al., 2007). Moreover, *C. metuliferus* was reportedly able to provide competence for heat and salt tolerance (Benzioni et al., 1991), prolong fruit shelf life (Mendlinger et al., 1992), and increase resistance against *Cucurbit yellowing stunting disorder virus* (Lopez-Sese and Gomez-Guillamon, 2000), PRSV-P, PRSV-W (formerly known as WMV-1) and *Squash mosaic virus* (Provvidenti and Gonsalves, 1982; Provvidenti and Robinson, 1974, 1977), whitefly (Lopez-Sese and Gomez-Guillamon, 2000), fusarium wilt caused by *Fusarium oxysporum* f. sp. melonis (Nisini et al., 2002), and root-knot nematode (Chen and Adelberg, 2000; Nugent and Dukes, 1997; Siguenza et al., 2005; Wehner et al., 1991).

The previously described regeneration procedures for *C. metuliferus* vary greatly with respect to the genotype of plants, the explant sources, and growth regulators used (Adelberg, 1998; Beharav and Cohen, 1994; McCarthy et al., 2001; Punja et al., 1990; Raharjo and Punja, 1993; Tang and Punja, 1989). The frequency of shoot formation in *C. metuliferus* PI 292190 from petiole explants was 14.6% (Raharjo and Punja, 1993); however, 0% to 30% from leaf explants and 0% to 10% from

cotyledon explants were observed (Punja et al., 1990).

In addition, no information on transformation of *C. metuliferus* is available so far, although ample transformation systems for melon, watermelon, squash, muskmelon, and cucumber have been developed by *Agrobacterium tumefaciens*-mediated transformation (Choi et al., 1994; Fang and Grumet, 1990; Gonsalves et al., 1994; Pang et al., 2000; Vasudevan et al., 2007) or microprojectiles protocols (Chee and Slightom, 1992). It was reported that genotype and growth regulators are determining factors influencing regeneration efficiency in the Cucurbitaceae family (Oridate et al., 1992). 2, 4-dichlorophenoxy-acetic acid (2,4-D) is more frequently used in genotype of *Cucurbita* species for somatic embryogenesis, yet naphthaleneacetic acid (NAA) is used in *Cucumis* species. In addition, benzyladenine (BA) was shown to give the best response on shoot production derived from cotyledon explants in *Cucumis* species (Abrie and van Staden, 2001; Chee, 1991; Niedz et al., 1989) and shoot primordia formation through organogenesis on NAA/BA or NAA/zeatin combinations (Punja et al., 1990).

In this study, a successful organogenic regeneration procedure (with greater than 58% efficiency) for *C. metuliferus* is described. Meanwhile, two binary vectors, pBI121 (Chen et al., 2003) and TWBI, were used in *A. tumefaciens*-mediated transformation. *A. tumefaciens*-mediated delivery of the β -glucuronidase gene (*gus*) in *C. metuliferus* was obtained at a frequency of 2.5%. This is the first report on an efficient regeneration and *A. tumefaciens*-mediated transformation in *C. metuliferus*.

Materials and Methods

Plant material and regeneration system.

The seedcoats of *C. metuliferus* line PI 292190 were removed and the naked seeds were surface-disinfected by dipping in 0.5% sodium hypochlorite for 10 min followed by three rinses in sterile distilled water. The sterilized seeds were placed on Murashige and Skoog (MS) medium (pH 5.7) including Gamborg B5 vitamins (Duchefa, The Netherlands) for 1 to 2 d. Embryos were removed from sterilized seed and the residual cotyledons were used as explants. Each enlarged cotyledon was longitudinally and transversally dissected to eight equal parts for regeneration. After 3 to 4 weeks on regeneration medium (using various combinations of NAA, BA, and 2,4-D as shown in Table 1), callus, adventitious buds, or shoot primordia derived from cotyledon explants were excised and transferred to elongation medium (0.1 mg·L⁻¹ BA, 0.02 mg·L⁻¹ NAA). After further incubation for 3 to 4 weeks, the elongated shoots were transferred to rooting medium (MS medium containing 0.5 mg·L⁻¹ indole-3-butyric acid) and maintained in a tissue culture room under 16-h light/8-h dark cycles with a photon flux rate of 55 to 65 $\mu\text{Em}^{-2}\cdot\text{s}^{-1}$ from cool white fluorescent lamps. The rooted shoots

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¹To whom reprint requests should be addressed; e-mail fjjan@nchu.edu.tw, hmku@nchu.edu.tw.

were acclimatized in a greenhouse under controlled conditions of $26 \pm 2^\circ\text{C}$. All plant growth regulators were filter-sterilized and added into the medium, which had been autoclaved (15 min at 121°C and ≈ 1 kPa) after pH adjustment (pH 5.7). Approximately 15 mL of medium was dispensed into disposable petri dishes (90×20 mm) for regeneration and elongation and 30 mL of rooting medium was dispensed into Magenta GA7 vessels for rooting.

Selection of kanamycin and DL-phosphinothricin. To determine the appropriate concentration of kanamycin and DL-phosphinothricin (PPT) for selection of transgenic explants, *Agrobacterium*-infected cotyledon explants were placed on S1 medium (MS medium supplemented with $1.0\text{ mg}\cdot\text{L}^{-1}$ BA and $0.02\text{ mg}\cdot\text{L}^{-1}$ NAA) containing different concentration of kanamycin or PPT (Table 2). Surviving explants with adventitious buds or shoot primordia were excised and transferred to S2 medium (MS medium supplemented with $0.1\text{ mg}\cdot\text{L}^{-1}$ BA, $0.02\text{ mg}\cdot\text{L}^{-1}$ NAA) under the same selection conditions. The number of surviving shoots was recorded after 2 weeks. The concentration of kanamycin or PPT that gave the highest frequency of shoot development was chosen as the criterion for the transformation of *C. metuliferus*.

Transformation of *Cucumis metuliferus*. *A. tumefaciens* strain LBA4404 harboring the binary vector pBI121 (Clontech Laboratories, Palo Alto, CA) carrying the neomycin phosphotransferase II (*nptII*) and *gus* genes was used for plant transformation under kanamycin selection. The *nptII* and *gus* genes of binary vector pBI121 were driven by the nopaline synthase (*nos*) promoter and *Cauliflower mosaic virus* (CaMV) 35S promoter, respectively (Fig. 1A). Another binary vector, TWBI, containing the chimeric *bar* (phosphinothricin resistance gene) driven by the CaMV 35S promoter (Fig. 1B) was also transformed into *Agrobacterium* strain LBA4404 for *C. metuliferus* transformation under PPT selection. Cotyledon explants were inoculated with freshly grown-overnight bacterial suspension at 100-fold dilution in MS liquid medium supplemented with $20\text{ }\mu\text{M}$ acetosyringone. After 10 min, the cotyledons were directly transferred into TR medium (the same as S1 medium without any selective component) and placed in the dark for 4 d and then under a 16-h photoperiod for another 3 d. The transformants were then transferred to TS1 (S1 medium supplemented with $150\text{ mg}\cdot\text{L}^{-1}$ kanamycin and $250\text{ mg}\cdot\text{L}^{-1}$ carbenicillin) and subcultured weekly for 3 to 4 weeks. The newly generated buds or shoot primordia were transferred to TS2 (S2 medium supplemented

with $150\text{ mg}\cdot\text{L}^{-1}$ kanamycin and $250\text{ mg}\cdot\text{L}^{-1}$ carbenicillin) media and subcultured weekly for another 3 to 4 weeks. The number of adventitious buds and shoot primordia formed at the apical site of cotyledon explants were recorded in TS2 medium for a period of 3 to 4 weeks. The rooting procedure (on rooting medium supplemented with $250\text{ mg}\cdot\text{L}^{-1}$ carbenicillin) and acclimation of transformants were the same as described for regeneration experiments. Acclimatized plants were analyzed for transgene by polymerase chain reaction (PCR) and GUS assays.

Detection of transgenes. Plant genomic DNA was extracted from leaves of transgenic plants by a modified CTAB method (Fulton et al., 1995). Forty nanograms of plant genomic DNA was used for PCR analysis performed with denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min for 30 cycles and a final extension at 72°C for 5 min. PCR analysis for the *nptII* gene was performed using primers FJJ2002-14 (5'-CCCCTCGGTATCCAATTAGAG-3') and FJJ2002-15 (5'-CGGGGGGTGGCGAAGAACTCCAG-3') (Fig. 1A). PCR analysis of the *gus* gene was performed using primers FJJ1999-12 (5'-ATATGGA TATCTCCACTGACGTAAGGGATG-3') located in the CaMV 35S promoter region and FJJ2001-15 (5'-TGATAATCATCGC AAGAC-3') covering the *nos* terminator region (Fig. 1A). Southern blot analysis was performed to confirm the integration of the *gus* gene in the transformants that gave positive PCR results for both transgenes (*nptII* and *gus* genes). Vector DNA (as positive control) and genomic DNA of transformed and non-transformed plants were digested by *ScaI* and *XhoI*, electrophoresed in 1% agarose gels, and blotted onto nylon membranes. The membranes were hybridized with a P^{32} -labeled probe of a 2.0 kb *gus* gene fragment, which was PCR amplified from plasmid template with primers FJJ1999-12 and FJJ2001-15. In addition, a histochemical assay to detect GUS activity was performed as described by Jefferson (1987). Leaf tissues were immersed in the reaction buffer (100 mM Tris, 50 mM NaCl, and 0.1% Triton-X 100, pH 7.0) containing 2 mM potassium ferricyanide and 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as substrates. The reaction was performed under a mild vacuum for 1 min after incubation at 37°C for 1 to 2 weeks. The tissues were washed with 70% ethanol before observation. The efficiency of transformation was calculated by dividing the number of PCR-positive plants for both *nptII* and *gus* genes by the total number of explants used in three independent experiments.

Results

Regeneration of cotyledon explants. Some friable, white or pale yellow calli were observed on the medium containing auxin only (NAA and 2,4-D), whereas buds or shoot primordia were observed after adding BA at a concentration of 0.5 or $1.0\text{ mg}\cdot\text{L}^{-1}$ (Table 1). Generally, cotyledon explants enlarged

Table 1. The effect of auxins (NAA, 2,4-D) and cytokinin (BA) concentration on regeneration of buds or shoot primordia derived from *Cucumis metuliferus* cotyledon explants.

Concn of growth regulator ($\text{mg}\cdot\text{L}^{-1}$)			Number of cotyledon explants	Number of calli (-C), buds (-B), or shoot primordia (-SP) (frequency) ^a	
BA	NAA	2,4-D			
0.5	0.02		48	27-C (27%), 20-B (20%), 15-SP (31.3%)	
1.0	0.02		48	40-C (40%), 29-B (29%), 20-SP (41.7%)	
1.0			48	23-C (23%), 19-B (39.6%), 8-SP (16.7%)	
	0.02	0.2	160	160-C (100%), 7-B (4.4%)	
	0.02	0.5	160	160-C (100%)	
	0.02	1.0	160	160-C (100%)	
	0.2		50	36-B (72%)	
	0.5		50	37-B (74%)	

^aThe frequency was calculated by using the number of calli (-C), buds (-B), or shoot primordia (-SP) divided by the number of cotyledon explants in each experiment.

NAA = naphthaleneacetic acid; 2,4-D = 2, 4-dichlorophenoxy-acetic acid; BA = benzyladenine.

Table 2. Effect of kanamycin and DL-phosphinothricin (PPT) concentration on regeneration of buds or shoot primordia from *Agrobacterium*-inoculated *Cucumis metuliferus* cotyledon explants.

Concn of selective agent		Number of cotyledon explants	Number (frequency) of surviving cotyledon explants ^a	Number of calli (-C), buds (-B), or shoot primordia (-SP) (frequency) ^a
Kanamycin ($\text{mg}\cdot\text{L}^{-1}$)	PPT ($\text{mg}\cdot\text{L}^{-1}$)			
50		333	177 (62.1%)	1-C (0.6%), 40-B (22.6%), 13-SP (7.3%)
150		379	341 (89.9%)	52-B (15.2%), 52-SP (15.2%)
200		433	301 (78.1%)	30-B (10%), 6-SP (2%)
	33.8	200	63 (31.5%)	0
	22.5	171	68 (39.7%)	1-B (1.5%)
	16.9	267	223 (83.5%)	19-B (8.5%)
	13.5	545	410 (75.2%)	44-B (10.7%), 12-SP (2.9%)
	11.3	170	101 (59.4%)	0
	2.7	261	93 (35.6%)	21-B (22.6%), 21-SP (22.6%)
	1.0	268	155 (57.8%)	31-B (24%), 24-SP (15.5%)

^aCotyledon explants surviving on regeneration medium containing kanamycin or PPT at different concentrations after 2 weeks.

^bThe frequency was calculated with the number of calli (-C), buds (-B), or shoot primordia (-SP) divided by the number of surviving cotyledon explants, respectively.

considerably and developed into mature callus, buds, or shoot primordia on the margins within 3 weeks after being transferred into regeneration medium (Fig. 2A). When the

mature globular calli were transferred to elongation medium, the regenerated calli failed to form shoots. However, most adventitious buds or shoot primordia were observed

preferentially at the proximal cotyledons (Fig. 2B). When transferred to elongation medium, these buds and shoot primordia continued to grow and formed mature shoots after 3 to 4 weeks. In some cases, cotyledon explants proliferated profusely and formed clusters of shoot apices, which did not develop into normal shoots on elongation medium (Fig. 2C). Some explants directly developed into leaf-like structures without forming buds or shoot primordia (Fig. 2D). In terms of bud or shoot formation, the medium containing 0.02 mg·L⁻¹ NAA and 1.0 mg·L⁻¹ BA provided a suitable development. In most cases, buds or shoot primordia developed into normal shoots and leaves (Fig. 2E–F). All elongated shoots were transferred to rooting medium for 1 to 2 weeks (Fig. 2G–H). The plantlets were acclimatized in the greenhouse (Fig. 2I) with the survival rate greater than 95%.

Sensitivity of cotyledons to kanamycin and DL-phosphinothricin. To test the optimal concentrations of kanamycin and PPT for transformation, *Agrobacterium*-infected cotyledon explants were placed on the selection medium supplemented with various concentrations of kanamycin or PPT (Table 2). After 3 weeks on selection medium, the survival rate of cotyledon explants were 62.1%, 89.9%, and 78.1% when kanamycin was supplemented at 50, 150, and 200 mg·L⁻¹, respectively. Fifty-two buds and 52 shoot primordia were obtained from 341 surviving explants when kanamycin was supplemented at 150 mg·L⁻¹, whereas fewer buds or shoot primordia were obtained at 50 or 200 mg·L⁻¹ (Table 2). For PPT selection, most cotyledon explants became pale yellowish or developed partial necrosis 3 weeks after being transferred to media supplemented with various concentrations of PPT from 1.0 to 33.8 mg·L⁻¹. The survival rates varied from 31.5% at 33.8 mg·L⁻¹ of PPT to 83.5% at 16.9 mg·L⁻¹ of PPT (Table 2). Although it is cheaper and easier to screen out nontransformants by using PPT than kanamycin, more restriction on buds and shoot primordia development was noticed on PPT selective medium. Therefore, kanamycin at a concentration of 150 mg·L⁻¹ was used for further establishment of *C. metuliferus* transformation.

Transformation of *Cucumis metuliferus*. The frequency of buds or shoot differentiation of inoculated explants varied from 43.3% to 68.3% in three independent experiments (Table 3). Explants with buds or shoot primordia were dissected and subcultured weekly in TS2 medium for 3 to 4 weeks. Most buds or shoots elongated but some did not develop into normal shoots. These abnormal shoots were hyperhydric and formed unexpanded leaf structures. In contrast, the elongated shoots showed various developments, including no root formation, short root formation, and formation of a strong and branched root system in rooting medium. From these experiments, a total of 53 putative transgenic plantlets was successfully developed with healthy root systems. All of them were acclimatized in the greenhouse (Fig. 2I).

Fig. 1. Schematic diagram of the T-DNA region of the binary plasmids pBI121 (A) and TWBI (B). (A) The Ti plasmid contains neomycin phosphotransferase II (*nptII*) and β -glucuronidase (*GUS*) reporter genes. These two genes were separately fused under the control of the nopaline synthase (*NOS-pro*) and *CaMV* 35S promoters (*CaMV* 35S-pro), respectively. (B) The *bar* gene conferring resistance to the herbicide Basta is driven by the *CaMV* 35S promoter and the *NOS* terminator (*NOS-ter*). RB represents the right border and LB is the left border of the T-DNA.

Fig. 2. Plant regeneration and transformation of *Cucumis metuliferus* line PI 292190. (A) Formation of callus at the cut surface of the cotyledon explants on medium containing 0.02 mg·L⁻¹ NAA and 0.2 mg·L⁻¹ 2,4-D after 3 weeks of culture (C = callus). (B) Adventitious shoots formation at the proximal end of the cotyledon in regeneration medium (0.02 mg·L⁻¹ NAA and 1.0 mg·L⁻¹ BA) after 3 weeks of culture (P = protuberance). (C) Adventitious buds showing shoot clustering and severe hyperhydricity after several subcultures. (D–E) Explants showing differentiation into a leaf without the formation of buds or shoot primordia. Formation of abnormal leaves that did not elongate (L = leaf-like structure). (F) Healthy plantlets growing in elongation medium. (G–H) Healthy roots generated in transgenic *C. metuliferus* plantlet obtained after 1 to 2 weeks culturing in the rooting medium. (I) Acclimatized putative transgenic plants. NAA = naphthaleneacetic acid; 2,4-D = 2, 4-dichlorophenoxy-acetic acid; BA = benzyladenine.

Detection of the presence of transgenes. DNA isolated from the upper leaves of transformants, untransformed *C. metuliferus* or the pBI121 plasmid was used as template DNA for PCR analysis of transgenes. The presence of a 1.2-kb fragment corresponding to the expected size of the *nptII* gene was detected in transformants and pBI121 plasmid but not in the untransformed *C. metuliferus* (Fig. 3A). A fragment of 2.0 kb for the *gus* gene was found in transformants and pBI121 but not in untransformed *C. metuliferus* (Fig. 3B). A total of 21 plants were positive in PCR analysis of the *nptII* gene but only nine of those plants were also positive for the *gus* gene. Furthermore, the results of Southern blot analysis showed the presence of the *gus* gene in the transformants and positive control but not in the untransformed control (Fig. 4). Finally, a GUS expression assay was conducted in transformants showing positive PCR reaction for the *gus* gene and non-transgenic *C. metuliferus* as a negative control. GUS activity was detected in transformed shoots (Fig. 5A) along the margins of leaves in transgenic plant (Fig. 5B) but not in the non-transgenic control (Fig. 5C) after incubation at 37 °C for 2 d. Based on the results of PCR analysis and GUS assay, the transformation frequency of *C. metuliferus* was measured to 2.5% (Table 3).

Discussion

An efficient regeneration system (greater than 58%) was obtained for *C. metuliferus* after *A. tumefaciens* infection and the adventitious buds and shoot primordia developed within 3 to 4 weeks. The buds and shoot primordia were cultured on elongation medium for 3 to 4 weeks and finally on rooting medium for another 1 to 2 weeks. A total of 7 to 10 weeks were needed to obtain transformants. An average transformation rate of 2.5% was achieved; however, a rate as high as 24.2% was obtained in one of the experiments (Table 3).

In this study, cotyledons were chosen as the explant source to establish an efficient regeneration and transformation system for *C. metuliferus* line PI 292190. Direct organogenesis from cotyledons offers the following advantages: the easier availability of cotyledon, the avoidance of somatic mutations that may be associated with callus, and it is technically easy and rapid (Vasudevan et al., 2007). It has been widely applied for in vitro regeneration of different genotypes of *C. melo* (Adelberg et al., 1994; Dong et al., 1991; Ezura et al., 2000; Ficcadenti and Rotino, 1995; Gaba et al., 1999; Galperin et al., 2003; Gonsalves et al., 1994; Molina and Nuez, 1995), *C. sativus* (Vasudevan et al., 2007; Vengadesan et al., 2005), *Cucurbita pepo* (Kathiravan et al., 2006), *Citrullus lanatus* (Chaturvedi and Bhatnagar, 2001), and *C. metuliferus* line PI 482439 (Adelberg, 1998). It was reported that the proximal edges of cotyledon explants conferred the highest regenerability in *C. melo* (Gaba et al., 1999; Gonsalves et al., 1994). In this study, it was

Table 3. Transformation frequency of *Cucumis metuliferus*.^z

Expt.	Number of cotyledon explants inoculated	Number (frequency) of adventitious buds or shoot primordia ^y	Number (frequency) of shoot differentiation ^x	Number of rooted plantlets	Number (frequency) of TP ^w
1	120	52 (43.3%)	36 (30.0%)	2	0 ^v
2	120	82 (68.3%)	43 (35.8%)	14	10 (11.6%)
3	120	75 (62.5%)	55 (45.8%)	37	17 (24.2%)
Total	360	209 (58.0%)	134 (37.2%)	53	9 (2.5%)
SE		12.8	7.8	14.5	

^zPrecultured cotyledon explants were inoculated with *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pBI121.

^yBuds or shoot primordia differentiation was recorded after 2 to 3 weeks of culture on TS1 medium. The frequency was calculated by using the number of adventitious buds or shoot primordia divided by the number of cotyledon explants inoculated in each experiment.

^xOnly elongated adventitious buds or shoot primordia were counted. The frequency was calculated by using the number of shoot differentiation divided by the number of cotyledon explants inoculated in each experiment.

^wTP = transgenic plants that were polymerase chain reaction (PCR)-positive in the detection of *nptII* and *gus* gene. Frequency was calculated by using total number of PCR-positive plantlets divided by the total number of cotyledon explants.

^vThe putative transgenic plants died after acclimatizing in the greenhouse and were not subject to PCR analysis.

Fig. 3. Polymerase chain reaction (PCR) analysis of *nptII* and β -glucuronidase genes. PCR amplified fragments of (A) *nptII* gene and (B) β -glucuronidase (*gus*) gene from putative transgenic *Cucumis metuliferus* (Lanes 1 to 18), non-transgenic *Cucumis metuliferus* PI 292190 (Lane 19), and plasmid pBI121 (Lane 20). The expected fragment sizes of 1.2 kb for the *nptII* gene and 2.0 kb for the *gus* gene were obtained. Two sets of primers were used for *nptII* and *gus* PCR detection. The primers FJJ2002-14 and FJJ2002-15 were designed for *nptII* detection and a 1.2-kb fragment was amplified. A 2.0-kb fragment was amplified for *gus* detection using primers FJJ1999-12 and FJJ2001-15.

also observed that proximal edges of cotyledon explants produced more buds and shoot primordia (Fig. 2B). Normal adventitious buds or shoot primordia were successfully regenerated at a frequency of 58% from cotyledon explants and from which the frequency of shoot formation reached 37.2% (Table 3). It was suggested that a higher concentration of NAA (0.37 mg·L⁻¹) or a lower concentration of 2,4-D (0.11 mg·L⁻¹) in combination with 0.22 mg·L⁻¹ BA can hinder the formation of callus from explants in *C. metuliferus* line PI 292190 (Raharjo and Punja, 1993). In this study, we observed callus regeneration in medium containing 0.02 mg·L⁻¹ NAA and 0.2 to 0.5 mg·L⁻¹ 2,4-D but this callus failed to regenerate shoots (Fig. 2A). The enhancement of shoot development using higher concentrations of BA in regeneration medium has been reported for five different selected cucurbit cultivars (Abrie and van Staden, 2001). In a study conducted by Gaba et al. (1999), the regeneration of *Cucumis* species from epidermis and the subepidermal layer was principally derived

Fig. 4. Southern blot analysis using α -³²P labeled probe derived from a fragment of the *gus* gene. Lane 1, positive control (vector pBI121 digested by *ScaI*); Lanes 2 and 5, transformants 960822-5; Lanes 3 and 6, transformants 960822-17; Lanes 4 and 7, untransformed negative control (*Cucumis metuliferus* line PI 292190). Genomic DNA of Lanes 2 to 4 and 5 to 7 plants was digested by *ScaI* and *XhoI*, respectively.

from the adaxial surface of cotyledon explants when BA concentrations ranging from 0.22 to 2.2 mg·L⁻¹ were used in the culture medium. In this study, 0.02 mg·L⁻¹ NAA

Fig. 5. Histochemical localization of β -glucuronidase gene (GUS) expression. The transformed explants (A), leaves of transgenic plants (B) and non-transgenic control, *Cucumis metuliferus* line PI 292190 (C) were analyzed for 2,4-dichlorophenoxyacetic acid (GUS) staining.

combined with $1.0 \text{ mg}\cdot\text{L}^{-1}$ BA were used in regeneration medium for cotyledon explants and from which adequate buds or shoot primordia were regenerated and later developed into normal shoots. However, some explants in this study were found to produce many buds but only few shoots (Fig. 2C). A similar phenomenon has also been reported in regeneration studies of other cucurbits (Colijn-Hooymans et al., 1994; Compton and Gray, 1993). Gonsalves et al. (1994) reported that buds developing from the epidermis or sub-epidermal layer, especially those located at the cut part of the cotyledon proximal to the seed apex, and the first organogenic meristems formed in vitro inhibited the development of further buds. It was suggested that excising the larger shoots permits more buds to develop further (Gaba et al., 1999).

The phenomenon of “escape” from antibiotic selection in the transformation of *C. melo* was reported by Akasaka-Kennedy et al. (2004). In previous work, cotyledon explants under 75 to $100 \text{ mg}\cdot\text{L}^{-1}$ kanamycin selection regenerated at frequencies of 15% for *C. sativus* (Vengadesan et al., 2005), 30% for muskmelon (Fang and Grumet, 1990), and 75% to 90% for melon (Dong et al., 1991). A high proportion of escapes was encountered in cucumber when embryo suspension culture was used under kanamycin selection (Schulze et al., 1995). The occurrence of the escapes may be the result of inadequate selective pressure or cross-protection by secreted products of contaminating microbial cells (Dong et al., 1991). Therefore, it is important to establish an efficient selection for the transformation system, especially for systems using regeneration through direct organogenesis from cotyledon explants (Gaba et al., 1999). In this study, although a high concentration of kanamycin ($150 \text{ mg}\cdot\text{L}^{-1}$) was used, the occurrence of escape was difficult to avoid completely. As shown in Table 3, an average of 58% of the *Agrobacterium*-infected cotyledon explants formed multiple shoots on selective media. However, only nine of 53 putative transgenic lines were detected to contain both *nptII* and *gus* genes in PCR analysis, indicating a certain degree of escape or chimeric plants regenerated from *C. metuliferus*

PI 292190. It has been postulated that the phenomenon of cross-protection against kanamycin occurred in melon or other *Cucumis* species and that transformation rate of inoculated melon on selection media is higher than on non-selective media (Dong et al., 1991). Therefore, increasing the selection pressure enables the transformed cells to compete with the non-transformed cells more effectively resulting in a slight decrease in the level of escapes and chimeric plants. Other selection agents such as methotrexate or PPT were also used for efficient selection of transgenic plants in melon and cucumber transformation (Dong et al., 1991; Vengadesan et al., 2005). However, we observed a lower surviving frequency of cotyledon explants on PPT than on kanamycin. Very few shoots regenerated under the various concentrations of PPT, although buds or shoot primordia developed at a low concentration. We speculated that *C. metuliferus* is very sensitive to PPT. When kanamycin was used for selection at concentrations of 50, 150, and $200 \text{ mg}\cdot\text{L}^{-1}$, the survival rates reached 62.1%, 89.9%, and 78.1%, respectively (Table 2). Based on the high survival rate of 89.9%, kanamycin at $150 \text{ mg}\cdot\text{L}^{-1}$ was subsequently used for *C. metuliferus* PI 292190 transformation. In the case of the GUS histochemical assay, the expression of the *gus* gene was detected only along the margins of leaves and leaf petioles in transformants, indicating that the CaMV 35S promoter was preferentially expressed only in certain tissues (Fig. 5). GUS activity driven by the CaMV 35S promoter was reported to be very active in vascular bundles and epidermal and parenchyma cells of young leaf petioles and petal and gradually decreases as the tissue matured (Dong et al., 1991).

In conclusion, an efficient (greater than 58%) regeneration procedure that only required 3 to 4 weeks to complete was developed and with that transgenic plants of *C. metuliferus* PI 292190 could be obtained in less than 2 months. The transformation rate of *C. metuliferus* line PI 292190 was reached at 2.5%. This is the first successful attempt for the transformation of any wild *Cucumis* species.

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