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Agrobacterium-mediated transformation system for the drought and excess light stress-tolerant wild watermelon (*Citrullus lanatus*)

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Abstract Wild watermelon plants are drought and excess light stress-tolerant despite carrying out normal C_3-type photosynthesis. In this study, a system was established for the genetic transformation of wild watermelons. Adventitious shoots were induced efficiently from cotyledon explants on medium containing $4\,mg\,l^{-1}$ of benzyladenine. The explants were infected with *Agrobacterium* carrying a plasmid containing selectable marker genes, *npt*II (neomycin phosphotransferase), *hpt* (hygromycin phosphotransferase), and a reporter gene *gus*A (β-glucuronidase). A β-Glucuronidase (GUS) histochemical assay showed that kanamycin was more effective than hygromycin as the selective agent for transformation. The presence of T-DNA in the regenerated shoots was confirmed by amplification of the transgene using polymerase chain reaction. Southern blot analysis revealed stable integration of the transgene in the T_1 progeny. The system presented here will provide an experimental basis for molecular studies of wild watermelon genes, and thus facilitate an understanding of their contribution to stress tolerance in this plant.

Key words: Wild watermelon, *Citrullus lanatus*, *Agrobacterium*, adventitious regeneration.

Water deficits are one of the most important environmental factors restricting plant growth and productivity, and the genetic improvement of stress tolerance in plants is an urgent challenge for the future of agriculture (Khush 1999). During the last decade, the molecular responses of plants to stress have been mainly studied using model plant species such as *Arabidopsis* (Seki et al. 2003). However, these plants are not, by nature, particularly tolerant to stress, and application of such knowledge to the engineering of stress-tolerant plants has been met with only limited success. One promising approach to overcome this limitation is to study drought-tolerant mechanisms in specific plant species that can withstand severe environmental stresses.

Wild watermelons (*Citrullus lanatus* sp.) from the Kalahari Desert exhibit exceedingly high tolerance to drought and excess light stresses, and have been used as an excellent model system for studying how C_3 plants survive severe environmental stresses (Yokota et al. 2002). The uniqueness of this plant is exemplified by its accumulation of a novel compatible solute, citrulline (Kawasaki et al. 2000), which is one of the most potent scavengers of hydroxyl radicals (Akashi et al. 2001). Moreover, a number of unique genes are up-regulated in wild watermelon leaves during stress (Akashi et al. 2004), making this plant an attractive source of useful genetic traits for molecular approaches to the breeding of crop plants. However, in order to analyze these genes further using advanced techniques such as gene-knockout by RNAi and measurement of gene expression with chimeric promoter-reporter systems, development of an efficient transformation system is needed.

One of the requirements for *Agrobacterium*-mediated transformation is availability of a protocol for plant regeneration from explants. Although a few reports have been published with regard to regeneration of mature plants from explants in domesticated watermelons (Tabei et al. 1993; Compton and Gray 1993), cucurbitaceous plants including watermelons are known to be recalcitrant with respect to these processes. In addition, there have been only a few reports on the genetic transformation of domesticated watermelons (Choi et al. 1994; Ellul et al. 2003). For wild watermelons, no system for successful plant regeneration and genetic transformation has been reported so far.

In this paper, we report an efficient protocol for *Agrobacterium*-mediated stable transformation of wild watermelon plants. Factors affecting transformation and regeneration are also discussed.

Materials and methods

Bacterial strains and vectors

Agrobacterium strains C58C1rif^R and EHA101 (Hood et al. 1986) carrying the plasmid pIG121-Hm (Akama et al. 1992) were used for transformation. The *Agrobacterium* strains were grown in 20 ml of Luria broth (LB) liquid medium supplemented with 50 mg l^{-1} kanamycin, 30 mg l^{-1} hygromycin, 50 mg l^{-1} rifampicin and 10 mg l^{-1} acetosyringone at 28°C until an optical density of 0.5–0.7 at 600 nm was reached. The cells were collected by centrifugation and resuspended in 500 ml of liquid medium containing Murashige and Skoog (MS) salt and 4 mg l^{-1} of N^6-benzyladenine (BA).

Plant materials, tissue culture and transformation

Seeds of wild watermelon, *Citrullus lanatus* sp. No. 101117-1 (Kawasaki et al. 2000), were decoated using a blade and forceps. The peeled seeds were sterilized in 5% sodium hypochlorite and 0.05% Tween-20 for 5 min, and rinsed five times with sterile water. They were then soaked in sterilized water in the dark at 28°C overnight, placed on basal medium (BM) containing Murashige and Skoog (MS) salts, 10 mg l^{-1} thiamine-HCl, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose and 0.8% agar, and cultured in the dark at 28°C for 4 days. The expanded cotyledons were cut into disks 5 mm in diameter. Infection and cocultivation of explants with *Agrobacterium* were performed by soaking the explants in *Agrobacterium* suspension for 10 min, followed by incubation for 3 days in the dark on BM supplemented with 4 mg l^{-1} BA and 10 mg l^{-1} acetosyringone. After cocultivation, the explants were rinsed with 500 mg l^{-1} carbenicillin and 200 mg l^{-1} cefotaxime, transferred to BM supplemented with 4 mg l^{-1} BA, 100 mg l^{-1} kanamycin, 200 mg l^{-1} carbenicillin and 100 mg l^{-1} cefotaxime, and maintained at 28°C under a 16-hour photoperiod. After 3–4 weeks, regenerating shoots were excised from the explants, and the roots were induced on BM containing 100 mg l^{-1} kanamycin, 200 mg l^{-1} carbenicillin and 100 mg l^{-1} cefotaxime. Plants that were still green after this selection period were then subcultured every 2 weeks on BM supplemented with 100 mg l^{-1} kanamycin and 3 g l^{-1} gellan gum (Wako Chemical, Osaka, Japan). When roots were at least 4-cm long, plants were transferred to a mixture of standard soil for horticulture : vermiculite (1 : 1) in pots and the humidity was gradually decreased to greenhouse conditions.

β-glucuronidase (GUS) assay

Histochemical GUS analysis was performed essentially as described previously (Topping et al. 1991) with some modifications. Tissues were incubated overnight at 37°C in 100 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton X-100 and 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indoyl glucuronide (X-gluc). The tissues were then soaked in 70% ethanol for several hours to remove chlorophyll. Quantification of GUS-expressing units was made by counting the number of blue spots on the tissues.

PCR analysis

Genomic DNA was isolated from leaves of wild watermelons as described previously (Akashi et al. 2004). The primers used for amplification of a 1-kbp fragment of the *gus*A gene were 5′-AGTGAAGGG-CGAACAGTTCCT-3′ and 5′-TCATTGTTTGCCTCCC-TGCT-3′. The conditions for PCR were 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min. The products were separated by electrophoresis on a 0.8% agarose gel, stained by ethidium bromide and visualized under ultraviolet light.

Southern blot analysis

Genomic DNA (10 μg) from transgenic and wild-type plants, and pIG121-Hm plasmid DNA (0.5 ng) were digested overnight at 37°C with 100 units of either *Bam*HI or *Xba*I restriction enzymes. DNA digests were separated by electrophoresis on a 0.8% agarose gel and blotted onto a nylon membrane (Hybond N+, Amersham) according to the standard procedures (Sambrook et al. 1989). For probe preparation, a 1-kbp *gus*A fragment was generated by PCR using pIG121-Hm plasmid DNA and *gus*A-specific primers (above). The amplified DNA fragment was purified by MinElute Gel Extraction Kit (QIAGEN, Valencia, CA), and used as template for a radioactive probe by StripAble Probe Synthesis Kit (Ambion, Austin, TX). Hybridization was performed as described previously (Sambrook et al. 1989).

Results and Discussion

Effect of plant growth hormones on shoot regeneration

To establish an explant regeneration system for wild watermelons, the efficiency of shoot induction from immature cotyledons was examined by incubating the tissues for four weeks on BM containing various levels of auxin (naphthaleneacetic acid: NAA) and cytokinin (N^6-benzyladenine: BA) (Figure 1). Immature cotyledons were used as the explant source, as this tissue has been shown to be an excellent source of plant regeneration in domesticated watermelons (Tabei et al. 1993). The percentage of explants that produced shoots was greatest in BM containing 4 mg l^{-1} BA (Figure 1). Under these conditions, shoots were generated from 91% of the

explants. Although addition of 0.2 mg l^{-1} NAA stimulated shoot induction when BA concentration was 1 mg l^{-1}, supplementation by NAA impaired shoot regeneration when BA concentration was in the range of 2–4 mg l^{-1}. This contrasts with the optimal conditions reported for domesticated watermelons, where relatively high concentrations of both auxin and cytokinin were essential for regeneration (Tabei et al. 1993). In all subsequent experiments, BM supplemented with 4 mg l^{-1} BA was used for shoot induction of the wild watermelons.

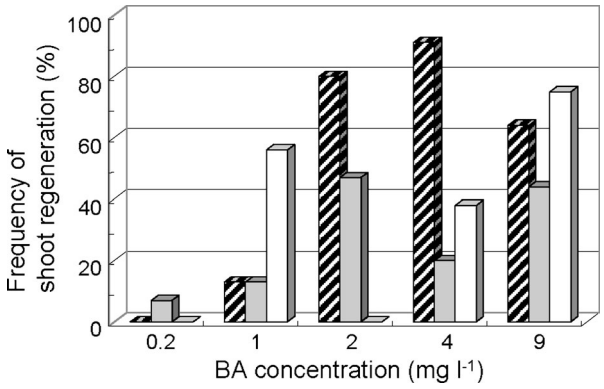

Figure 1. Effects of plant growth hormones on the frequency of shoot regeneration from cotyledon explants of wild watermelon. Explants were cultured on BM containing various concentrations of BA supplemented with 0.1 mg l^{-1} NAA (gray boxes), 0.2 mg l^{-1} NAA (white boxes) or without NAA supplementation (hatched boxes). The values represent the number of explants regenerating shoots as a percentage of the total number of explants cultured. At least 30 explants were used for each experiment.

Effects of Agrobacterium strains and antibiotics on the transient expression of the gusA gene

To optimize conditions for *Agrobacterium*-mediated DNA transfer to wild watermelon explants, the effects of different *Agrobacterium* strains and concentrations of selective antibiotic were examined. Two *Agrobacterium* strains, C58C1 and EHA101, were transformed with the binary vector pIG121-Hm harboring neomycin phosphotransferase (*npt*II) and hygromycin phosphotransferase (*hpt*) genes as selection markers. A reporter β-glucuronidase (*gus*A) gene containing a plant intron sequence to suppress GUS expression in *Agrobacterium* was also included in the construct. These strains were cocultivated with wild watermelon explants and incubated for 10 days on BM supplemented with various concentrations of kanamycin or hygromycin. The presence of the transgene in emerging callus of the explants was detected by transient expression of the *gus*A gene (Figures 2A, B).

Cocultivation with the *Agrobacterium* strain EHA101 led to a much higher percentage of GUS-positive explants and a higher average number of GUS-expressing zones (blue spots in the explants) compared with the C58C1 strain (Table 1), suggesting that EHA101 is more suitable for genetic transformation of wild watermelons. We also found that selection by kanamycin gave a higher frequency of GUS-positive explants than hygromycin. The greatest frequency of GUS-positive explants was achieved when the explants were infected with *Agrobacterium* strain EHA101 and selected on BM supplemented with 75 mg l^{-1} of kanamycin.

It has been reported that preculture of explants on shoot induction medium prior to *Agrobacterium*

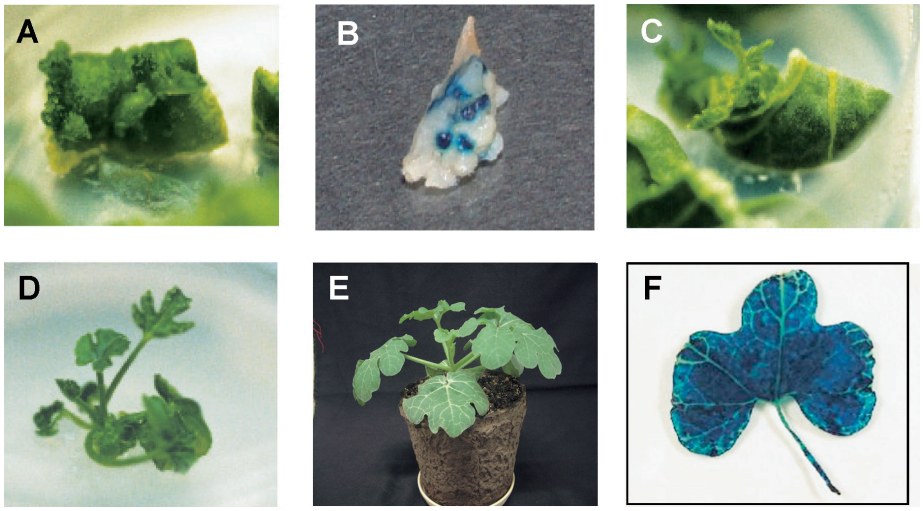

Figure 2. *Agrobacterium*-mediated transformation in wild watermelons. (A) Induction of transformed callus on the cut edge of a cotyledon explant ten days after inoculation with *Agrobacterium*. (B) Histochemical GUS assay of a cotyledon explant ten days after inoculation. (C) Shoot formation on a cut edge of a cotyledon explant cultured on BM containing 100 mg l^{-1} kanamycin four weeks after inoculation. (D) Transgenic shoot (T_0 plant) six weeks after inoculation. (E) T_1 progeny grown in soil for three weeks. (F) GUS expression in a leaf of T_1 progeny.

infection significantly improves efficiency of transformation in domesticated watermelons (Choi et al. 1994). In wild watermelons, however, two days of preculture did not increase the percentage of GUS-positive explants nor the mean number of blue spots per explant (data not shown). Therefore, we decided not to include the preculture treatment in our protocol.

Selection of transgenic plants

To establish a method for selection of transgenic plants, the inoculated explants were cultivated on various concentrations of either hygromycin or kanamycin, and the efficiency of shoot induction was examined four weeks after inoculation (Table 2). When hygromycin was used as the selective antibiotic, the efficiency of transgenic shoot induction was very low. On medium containing 15 to 50 mg l^{-1} hygromycin, nearly all the explants gradually turned brown and died without forming shoots (data not shown). Only one shoot was regenerated from one explant cultured on medium containing 30 mg l^{-1} hygromycin, but after subsequent transplantation to fresh medium, this shoot did not develop further and eventually died two weeks after transplantation. Although shoots were induced at a higher frequency on medium containing lower concentrations (3–5 mg l^{-1}) of hygromycin, these shoots appeared to be 'escapes' because no GUS expression was observed in their tissues.

In contrast, shoots were induced at high efficiency on medium containing kanamycin as the selective agent (Table 2). Most of these shoots grew vigorously after subsequent transplantation to fresh medium containing kanamycin at the same concentration (Figure 2D). GUS histochemical assay of the leaves harvested from these shoots showed that 100 mg l^{-1} kanamycin was suitable for transgenic shoot regeneration, since the frequency of regenerated shoots that exhibited GUS-staining throughout their entire tissues was highest (13 out of 81) at this concentration (Table 2). Some shoots exhibited only partial GUS-staining, i.e., some regions of the leaf tissues were stained blue while other parts of the same tissues were unstained (data not shown). These results indicate that some sectors of these tissues failed to integrate the foreign gene and became chimeric with respect to transgene integration.

Table 1. Effect of *Agrobacterium* strains and antibiotic concentrations on transient GUS expression.

Agrobacterium strain	Antibiotic conc. (mg l^{-1})	Frequency of GUS+explants (%)[a]	Average number of blue spots per explant
C58C1	hygromycin:		
	20	0	0
	50	9	0.2
	kanamycin:		
	20	31	3.2
	50	18	0.9
	75	13	4.1
	100	50	1.5
	125	14	5.0
EHA101	hygromycin:		
	20	38	9.0
	50	33	6.4
	kanamycin:		
	20	49	7.9
	50	25	9.1
	75	88	5.8
	100	73	6.0
	125	20	2.1

[a] Values represent the number of explants with blue spots as a percentage of the total number of explants in the histological GUS assay. For each experiment 7–20 explants were tested.

Table 2. Efficiency of transgenic shoot regeneration with different levels of selective antibiotics.

Antibiotic (mg l^{-1})	Regenerated shoots/ total explants[a]	Shoots after transplantation[b]	GUS-negative shoots[c]	Partially GUS-stained shoots[c]	Shoots GUS-stained throughout[c]
Hygromycin:					
3	36/88	20	20	0	0
5	9/78	2	2	0	0
15	0/84	—	—	—	—
30	1/126	0	—	—	—
50	0/126	—	—	—	—
Kanamycin:					
50	64/224	64	58	3	3
75	107/616	90	48	40	2
100	99/644	81	29	39	13
150	1/168	1	0	1	0

[a] The number of regenerated shoots was recorded four weeks after *Agrobacterium* inoculation.

[b] The regenerated shoots were transplanted to fresh medium, and the number of vigorously growing shoots was counted two weeks after transplantation.

[c] Leaves from the transplanted shoots were subjected to GUS histochemical assays two weeks after transplantation, and classified as GUS-negative, partially GUS-stained or GUS-stained throughout as judged by their spatial staining patterns.

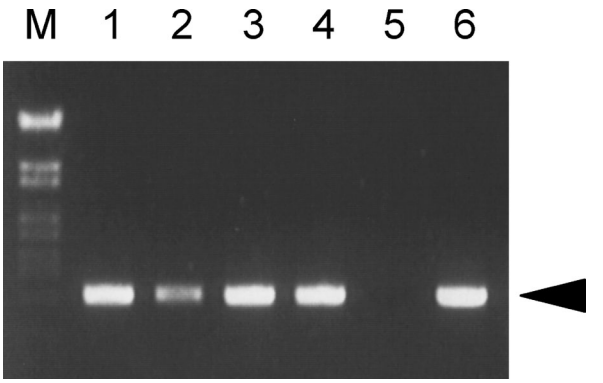

Figure 3. PCR analysis of transgenic shoots (T_0 plants) using *gus*A gene-specific primers. M, *λ-sty*I size marker; lanes 1–4, transgenic shoot candidates; lane 5, untransformed control plant; lane 6, pIG121-Hm plasmid as a positive control. The arrowhead points to amplification of the 1-kbp fragment for the *gus*A gene.

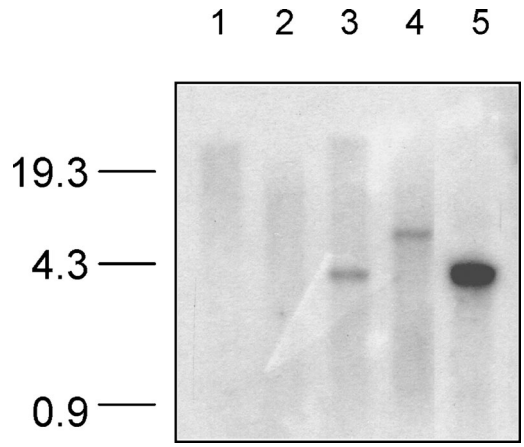

Figure 4. Southern blot analysis of T_1 progeny. The 1-kbp PCR fragment amplified from the *gus*A gene was used as a probe. Total genomic DNA (5 μg) from an untransformed control plant (lanes 1, 2), a transformed plant (lanes 3, 4), and pIG121-Hm plasmid DNA (0.5 ng) as the positive control (lane 5), was digested with *Bam*HI (lanes 1, 3, 5) and *Xba*I (lanes 2, 4). Marker sizes are shown in kbp.

The shoots that exhibited GUS-staining throughout were cultured for two more weeks. Transmission of the transgene in these shoots was examined by PCR using primers for the *gus*A gene. All four shoots tested in this analysis showed amplification of the predicted 1-kbp fragment (Figure 3, lanes 1–4), confirming successful transmission of the foreign gene during culturing.

Analysis of the T_1 progeny

Two transgenic shoots were cultivated further and acclimatized to soil culture under greenhouse conditions. In subsequent steps, small sections of leaves were randomly chosen, amputated and used in histochemical GUS analysis. The results were always positive for GUS expression (data not shown). The plants produced normal male and female flowers, and T_1 seeds were obtained from these two lines through manual self-pollination. A germination assay of T_1 seeds on BM containing 100 mg l^{-1} kanamycin revealed that the ratios of resistant to sensitive T_1 seedlings in these two lines were both approximately 3 : 1, indicating transmission of the single marker gene copy in the expected Mendelian ratio. The T_1 plants were phenotypically normal (Figure 2E), and GUS staining was clearly visible in their leaves (Figure 2F).

One of the T_1 plants was then used for Southern blot analysis to confirm stable integration of the transgene and to estimate the number of transgenes inserted. Using a fragment of the *gus*A gene as a probe, the T_1 plant gave hybridization bands of 4.0-kbp in *Bam*HI- and 6.7-kbp in *Xba*I-digested DNA (Figure 4, lanes 3, 4). Since the pIG121-Hm T-DNA contains only one recognition site for *Xba*I, digestion of genomic DNA with this enzyme should detect hybridization fragments composed of both the T-DNA and flanking genome sequences. Because the number of T-DNA inserts is expected to equal the number of bands detected in this manner, this transgenic plant appeared to have a single copy of the transgene. In the case of *Bam*HI digestion, a 4.0-kbp band was detected that corresponded to the expected size for an internal T-DNA fragment consisting of the *gus*A and *hpt* genes.

The results of GUS expression assays, PCR and Southern blot analyses have provided proof for successful transgene integration in the wild watermelon genome via *Agrobacterium*. This is the first report to present transformation of wild watermelon plants. The methods described here should create new opportunities for investigating the functions and regulatory mechanisms of specific genes in wild watermelons, and provide a useful experimental system for elucidating the mechanisms of drought and high light stress tolerance in this xerophyte.

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