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Genetic Transformation of Water Spinach (*Ipomoea aquatica*)

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Abstract

Water spinach (*Ipomoea aquatica*) has high nutritional value and is considered one of the most important vegetables in Southeast Asia. Because of its quick growth and efficient absorption of various substances, it has been suggested to be useful for sequestration of environmental pollutants as well as offering a source of medical materials. We have developed and established a system for stable genetic transformation by infecting cut cotyledons with *Agrobacterium* harboring the *GUS* gene as a model case after evaluating conditions of bacterial cell density, growth phase and concentrations of acetosyringone. The resulting transgenic plants grew normally to maturity, and exhibited stable GUS activity. Thus, genetic modification of *I. aquatica* can be readily achieved, thereby improving its quality for whatever traits desired.

Key words: Genetic transformation, *Ipomoea aquatica*, Water spinach.

Abbreviations

GUS, β-Glucuronidase; *HPT*, Hygromycin phosphotransferase.

Ipomoea aquatica (water spinach or pakbung) is a fast growing aquatic vegetable in Southeast Asia. It requires only two weeks of cultivation at an appropriate temperature after sowing. After decapitation of main stems, new shoots grow from each stem nodule, making continuous harvesting is possible. Nitrogen and phosphorus absorption rates of this plant are as high as with the water hyacinth (Furukawa and Fujita, 1993). Due to these properties, *I. aquatica* has become one of the major vegetables in Thailand and nearby countries. In addition, because of its capacity to grow in polluted water, it is now recognized as a plant which can potentially be utilized for purification purposes (Furukawa and Fujita, 1993). It was reported that *I. aquatica* accumulates significant amounts of toxic metals such as Fe, Cu, Cr, Mn and Pb in its leaves (Rai and Sinha, 2001). In addition, it has been suggested to be a useful medicinal plant because of its hypoglycaemic activity (Malalavidhane *et al.*, 2000). An aqueous extract of *I. aquatica* was in fact found to be effective as the drug tolbutamide in reducing the blood glucose levels of glucose-challenged Wistar rats (Malalavidhane *et al.*, 2001).

These properties have stimulated attempts to construct genetically modified *I. aquatica*, which may be able to rapidly absorb and metabolize pollutants from environments, or to produce high amounts of medicinal substances. For example, it has been suggested that this plant can be utilized for sulfur remediation because of its rapid uptake and metabolism of exogenous sulfuric compounds (Ernst, 1998). Transformation of *Ipomoea* species by *Agrobacterium* has so far been reported for cotyledon-derived hairy roots of *I. trichocarpa* (Otani *et al.*, 1996) and embryogenic callus-derived apical meristem of *I. batatas* (Otani *et al.*, 1998), but not for *I. aquatica*. In this paper, we report the transformation system using adventitious shoots of this species with *Agrobacterium tumefaciens*.

Seeds of *I. aquatica* purchased from Takii Co. (Kyoto, Japan) were surface sterilized with 70% ethanol for 2 min, 1% NaOCl for 30 min and then rinsed five times with sterile distilled water. After final immersion in sterile distilled water for 30 min, the seeds were transferred to MS solid medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose and 2.5 g l^{-1} Gellan Gum, pH 5.8 in a 300 ml plastic bottle containing 50 ml of medium

for germination. The incubation conditions were 25 ℃ under 16-h photo period and light intensity of 24 μmol $m^{-2}s^{-1}$ (white fluorescent tube). Cotyledon segments including petiole-like cotyledon bases, excised from one week-old seedlings, were used as explant sources based on our previous finding that those preparations efficiently regenerated (Akaracharanya *et al.*, 2001).

Agrobacterium tumefaciens EHA 101 (Hiei *et al.*, 1994; Yokoi *et al.*, 1996), harboring a plasmid pIG121-Hm containing β-glucuronidase (GUS), hygromycin phosphotransferase (HTP) and neomycin phosphotransferase II (NPT II) genes in the T-DNA region of the plasmid, was used. A single colony was inoculated into 10 ml YEP medium (10 g l^{-1} bacto-peptone, 10 g l^{-1} yeast extract, 5 g l^{-1} sodium chloride) containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} hygromycin in 125 ml flask, and incubated at 25 ℃ with aeration by shaking at 160 rpm for 3 days. A 1-ml culture aliquot was then transferred to fresh 10 ml YEP medium containing the same agents and incubated under the same conditions for 24 h (late log phase; $2.7x10^8$ cells ml^{-1} as counted by bacterial cell counting chamber slide), and used for transformation.

Hundred cotyledon segments with petiole-like cotyledon bases were suspended in 30 ml of MS medium containing $4.5x10^6$ cells ml^{-1} of *A. tumefaciens* and 50 μM acetosyringone in a 50 ml plastic tube for 0.5 h with shaking at 80 rpm. Infected cotyledon segments were filtered through sterilized stainless mesh and blotted dry on sterilized filter paper and cultured on MS solid medium containing 50 μM acetosyringone (James *et al.*, 1993) at 25 ℃ in dark for 4 days. In order to determine efficient conditions for *Agrobacterium*-mediated transformation, the followings were examined: *A. tumefaciens* culture in growth phases after culture for 18 h (mid log phase), 24 h (late log phase) or 36 h (stationary phase); *A. tumefaciens* cell density of $4.5x10^6$, $9x10^6$ or $1.8x10^7$ cells ml^{-1}; inoculation periods of 0.5, 1.0 or 2.0 h; and acetosyringone concentrations of 0, 50, 100 and 200 μM. One hundred cotyledon segments were used in each experiment. The assay was repeated three times.

The infected cotyledon segments were washed with 100 ml of sterilized water containing 300 mg l^{-1} cefotaxime twice and then blotted dry on sterilized filter paper. Samples were cultured on a modified MS solid medium (Mori *et al.*, 1999; Akaracharanya *et al.*, 2001) containing 10 μM TDZ (thidiazuron) and 300 mg l^{-1} cefotaxime at 25 ℃ under a 16 h-photo period and light intensity of 24 μmol $m^{-2}s^{-1}$ (white fluorescent tubes) for one month (transferred to new medium every two-weeks). The adventitious shoots formed were transferred to a modified MS solid medium containing 10 μM TDZ and 25 mg l^{-1} kanamycin and 25 mg l^{-1} hygromycin and 300 mg l^{-1} cefotaxime and further incubated for one month. Kanamycin and hygromycin were used to select transformants containing full length *GUS* gene, because *GUS* gene was placed between *HTP* and *NPTII* genes. The survived shoots were then transferred to hormone-free MS solid medium containing 300 mg l^{-1} cefotaxime and incubated under the same conditions with continuous light. Plantlets with well developed shoots and roots were transferred to diluted MS solid medium without supplementation with any organic compound.

Histochemical GUS assays were performed for cotyledon segment, transient assay, and for kanamycin and hygromycin resistant adventitious shoots according to the method of Gallagher (1992). For staining, the materials were incubated in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) solution with a composition modified to 50 mM Na_2HPO_4, 0.5 mM $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$. After 16 h at 37 ℃, these explants were immersed in 99.5% ethanol for chlorophyll bleaching and then observed under a dissecting microscope.

Extraction of DNA from leaves of plantlets having positive GUS activity was carried out by a modified method of Edwards *et al.* (1991), supplemented with a phenol/chloroform extraction step. The primers used for amplifying the *GUS* gene were 5'-GACGTTCCAACCACGTCTTC-3' and 5'-TCACGGGTTGGGGTTTCTAC-3' and those for the *HPT* gene were 5'-GCGTGACCTATTGCATCTCC-3' and 5'-TTCTACACAGCCATCGGTCC-3'. The reaction mixture for PCR was incubated in a DNA thermal cycler (Perkin Elmer Cetus 2400) under the following conditions: 96 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 2 min with a final 5 min extension at 72 ℃.

In order to estimate the effects of various factors on *Agrobactreium*-mediated transformation, the number of cotyledon segments, which transiently expressed the GUS gene, were counted after cocultivation with *A. tumefaciens* EHA101 harboring pIG121-Hm. Infected cotyledon segments were transferred to MS solid medium containing acetosyringone, incubated in the dark for 4 days, and GUS activity was estimated histochemically (**Fig. 1A**). We defined the GUS positive to indicate all segments with blue color at any strength, because they were clearly different from those without infection. The examined factors were the growth phase and cell concentration of *A. tumefaciens*, cocultivation

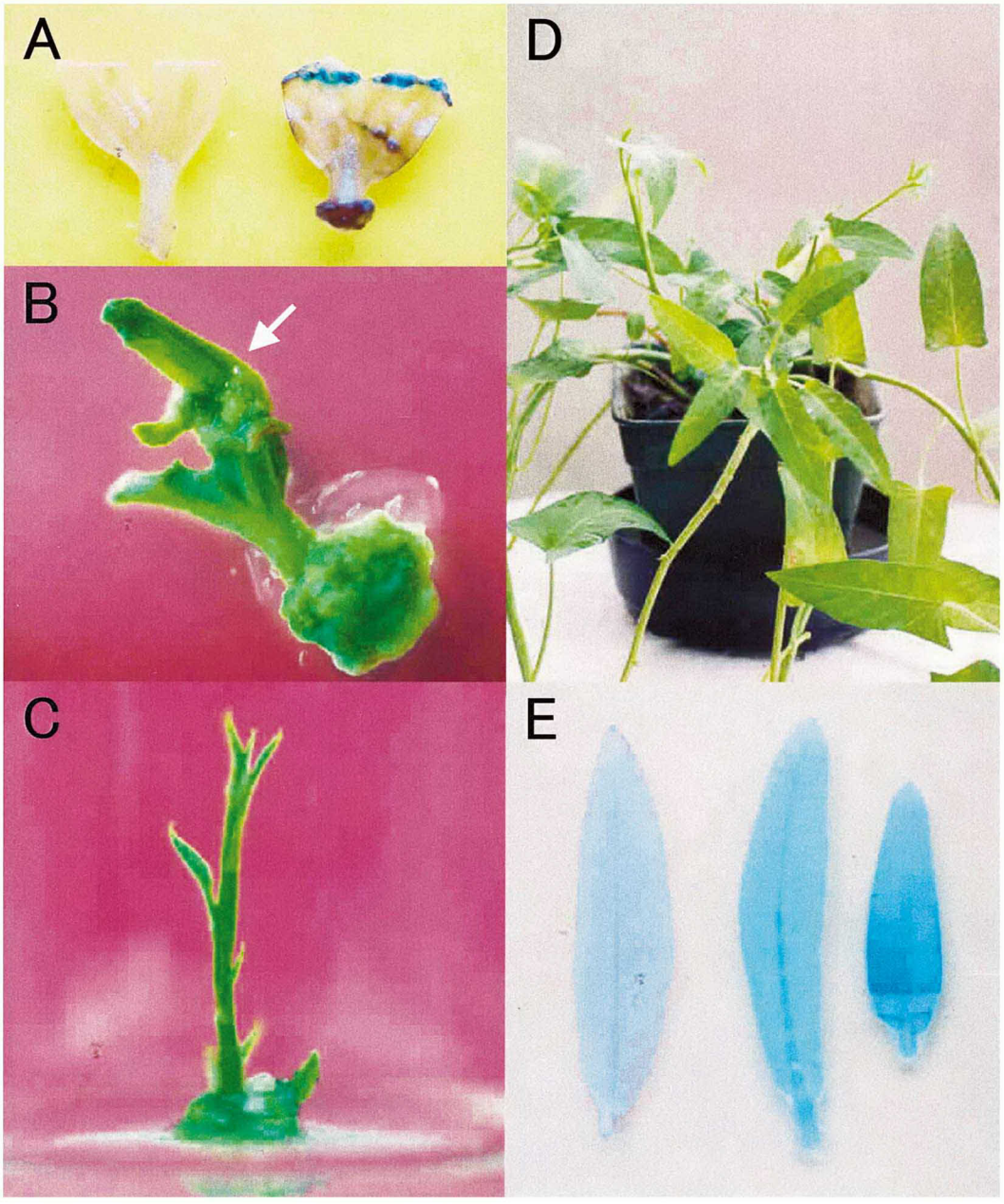

Fig. 1 Transformation of *I. Aquatica*. (**A**) The histochemical GUS assay of cotyledon segments initially infected with *Agrobacterium*. (**B**) An adventitious shoot (arrow) regenerated from cotyledon segment on solid MS medium. (**C**) Hygromycin and kanamycin resistant shoot cultured in a culture bottle. (**D**) Mature plants after transfer onto soil for a month. (**E**) GUS positive leaves identified by blue staining through histochemistry.

Table 1 Cocultivation conditions on transient expression of gus gene

Factor	Condition	% GUS positive
A. tumefaciens growth phase	Mid log phase	17 (51/300)
	Late log phase	41 (123/300)
	Stationary phase	11 (33/300)
A. tumefaciens cell density	4.3x10 cells ml^{-1}	41 (123/300)
	9.0x10^6 cells ml^{-1}	52 (156/300)
	1.8x10^{15} cells ml^{-1}	58 (174/300)
Cocultivation period	30 min	52 (156/300)
	1 h	73 (219/300)
	2 h	75 (225/300)
Acetosyringone conc.	0 μM	31 (93/300)
	50 μM	75 (225/300)
	100 μM	48 (144/300)
	200 μM	31 (93/300)

period, and acetosyringone concentrations.

Regarding the first factor, the highest frequency of GUS positive cotyledon segments (41%) was obtained when late log phase *A. tumefaciens* was used (**Table 1**). Furthermore, the frequency of GUS positive cotyledon segments increased from 41 to 52 and 58% when *A. tumefaciens* cell density during inoculation was increased from 4.5x10^6 to 9.0x10^6 and then 1.8x10^7 cells ml^{-1} (**Table 1**). Based on this finding, *A. tumefaciens* at 9.0x10^6 cells ml^{-1} was used in the following experiments to prevent overdose of infection. With regard to the inoculation period, the frequency of GUS positive cotyledon segments elevated from 52 to 73 and 75% when the time was increased from 0.5 to 1 and 2 h, respectively (**Table 1**). Each cotyledon segment subjected to 2 h inoculation showed more than 2-fold the GUS positive blue spots of those subjected to only 1 h inoculation. Consequently, further experiments were performed with a 2 h inoculation period. The concentration of acetosyringone which gave the maximum (75%) frequency of GUS positive cotyledon segments was then found to be 50 μM (**Table 1**).

Cotyledon segments were subjected to *Agrobacterium* infection under the conditions described above and stably cultivated (**Fig. 1**). To induce adventitious shoots, the infected segments were washed with sterile distilled water containing 300 mg l^{-1} cefotaxime and cultured on modified MS solid medium containing 10 μM TDZ and 300 mg l^{-1} cefotaxime, then incubated at 25 ℃ under 16-h photo period and light intensity of 24 μmol $m^{-2}s^{-1}$ (white fluorescent tubes) for one month. Two hundred adventitious shoots were obtained

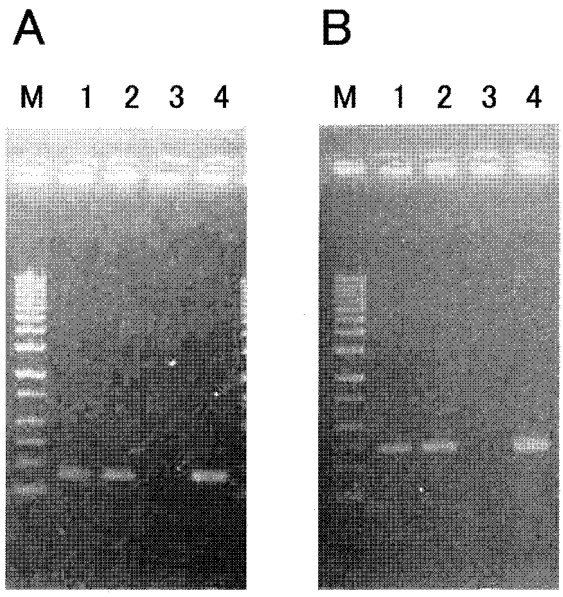

Fig. 2 Identification of introduced genes. PCR analysis of *GUS* (**A**) and *HPT* (**B**) genes in transgenic plants was performed with DNA samples extracted from transgenic plants (lanes 1 and 2) and a non-transgenic plant (lane 3) as the templates. Plasmid pIG121-Hm was used as the template for GUS and HPT genes as the positive control (lane 4).

from 1,250 cotyledon segments infected with *Agrobacterium* (**Fig. 1B**). All adventitious shoots were transferred to culture on a modified MS solid medium containing 10 μM TDZ, 25 mg l^{-1} kanamycin, 25 mg l^{-1} hygromycin and 300 mg l^{-1} cefotaxime, and then incubated under the stated conditions for one month. In this medium, non-transformed adventitious shoots rapidly browned whereas putative transformants grew normally. Two adventitious shoots survived, indicating to be transformants (**Fig. 1C**). Roots developed rapidly at the base of putative transformed shoots after transferred to hormone-free MS solid medium containing 300 mg l^{-1} cefotaxime in a 50 ml plastic bottle. Two plantlets with well developed shoots and roots were transferred to 2-fold diluted modified MS solid medium without supplementation with organic compounds. In order to confirm transformation, DNA was extracted from leaves of transformants and assayed for *GUS* and *HPT* genes by PCR (**Fig. 2**). The results clearly showed an efficient amplification of 390 bp and 713 bp fragments corresponding to *GUS* and *HPT*, respectively. In non-transformed plantlets, neither *GUS* nor *HPT* genes were detectable (**Fig. 2**). The leaves of putative transgenic plantlets were sampled and subjected to GUS enzymatic assay with the X-Gluc reaction. The resulting blue color confirmed the production

of active GUS enzyme (**Fig. 1E**). Explants from non-transformed plantlets did not show GUS activity, indicating that two regenerated plants were indeed stably transformed with the *GUS* gene. Plants were then transferred to the soil, and cultured further to maturity for a month (**Fig. 1D**). Their phenotypes were normal and they grew rapidly at the same rate as wild-type plants. Transformants showed GUS activity for more than a year after transferred to the soil (data not shown).

The current work established a method for stable transformation of *I. aquatica*, which has so far not been the object of molecular breeding. Genetic engineering of plant species for which genomic information is limited usually encounters great difficulty, since no common methods of transformation have been established. In this context, our successful *I. aquatica* transformation is unique and helpful for future breeding. Since *I. aquatica* is a common vegetable, widely used in Southeast Asian countries, its quality improvement is potentially of a great value for phytoremediation as well as substance production. Expected examples include cleaning of waste water and/or production of nutrient-rich ingredients. Up to date, many genes encoding useful proteins have been isolated from a variety of plant species, including *Arabidopsis*, rice, maize and tobacco. Our results make introduction of these genes into *I. aquatica* to confer desirable traits readily achievable. Currently we are attempting to introduce genes involved in sulfur metabolic pathways into this plant in order to cope with sulfur-polluted water environments.

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