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Direct Regeneration of Transgenic Buckwheat from Hypocotyl Segment by *Agrobacterium*-mediated Transformation

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ABSTRACT : Transgenic plants from hypocotyl segments of buckwheat were produced with the *Agrobacterium* strain LBA4404 harboring the binary vector pBI121 containing chimeric genes of *neomycin phosphotransferase II (npt II)* and *β-glucuronidase (gus)*. Two weeks after co-cultivation with *Agrobacterium*, most of the hypocotyl segments gradually became brown and died on the selection medium containing 100 mg/l of kanamycin. Plants regenerated from the hypocotyl explants grown on selection medium were GUS-positive in the leaf, stem and vascular tissues by histochemical assay, and varied in *gus* activity (440~2568 pmol, 4-MU/mg protein) by fluorimetry. The plants showing GUS activity were confirmed of containing GUS and NPT-II genes by polymerase chain reaction (PCR). Within 3 months, transgenic buckwheat plants were able to obtained from the hypocotyl segments.

Keywords **:** buckwheat, hypocotyl, *Agrobacterium tumefaciens*, *β-glucuronidase (gus)*, *neomycin phsphotransferase II (npt II)*, transformation

Genetic improvement of buckwheat (*Fagopyrum esculentum* Moench) through conventional plant breeding has received little attention because of its breeding restrictions due to strong self-incompatibility. Transformation of dicotyledonous plants by *Agrobacterium*-mediated gene transfer is well-established and has produced stable transgenic plants expressing a number of foreign genes (Maria *et al.*, 1996; Geetha *et al.*, 1999; Tjokrokusumo *et al.*, 2000). Transgenic buckwheat plants have been regenerated from cotyledons (Srejovic and Nesskovic, 1985; Miljus-Djukic *et al.*, 1992; Romchatngoen *et al.*, 1998) and apical meristems (Kojima *et al.*, 2000). Buckwheat protein has a desirable amino acid (Choi *et al.*, 1992) and is a potential supplement for foods low in lysine (Maria *et al.*, 1996; Geetha *et al.*, 1999; Tjokrokusumo *et al.*, 2000).

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Due to the restrictions in using conventional breeding, molecular technique combined with *in vitro* methods provides a unique and novel approach in buckwheat genetic improvement.

This study demonstrates a rapid method to incorporate foreign genes in buckwheat through *Agrobacterium tumefaciens*-mediated transformation.

MATERIALS AND METHODS

Plant materials

Decoated buckwheat (*Fagopyrum esculentum* Moench vars. Shinnong No.1 and Yangjul) seeds were surface-sterilized by immersion in 75% ethanol for 30 second followed by continuous agitation in 50% (v/v) sodium hypochlorite (NaOCl) solution for 10 min. and rinsed three times with sterile distilled water. The seeds were germinated *in vitro* on Murashige and Skoog (MS; Murashige and Skoog, 1962) basic solid medium. After a week, the hypocotyls were cut into sections about 0.7 mm in length, and cultured in MS basic solid medium for 4 days prior to infection.

Agrobacterium transformation

The *Agrobacterial* strain LB4404 with the binary vector pBI121 containing the *gus* gene under the regulatory control of CaMV 35S promoter and *nopaline synthase (nos)* terminator, and the *nptII* gene linked to a NOS promoter and terminator (Jefferson *et al.*, 1987) was used for transformation. A single colony of LBA4404/pBI121 was inoculated and grown at 28°C in dark with continuous agitation at 200 rpm overnight in 2 ml of YEB medium containing 100 mg/l of kanamycin. The bacterial cells were then collected by centrifugation at 5000 rpm for 5 min. The pellet was resuspended in MS basic medium with 20 mM acetosyringone. The density of bac-

teria was adjusted to 5×10^8 cells/ml. After 4 days of preculture of the hypocotyl segments, these were infected with *Agrobacterium* for 2 hours, blotted on sterile filter paper and placed onto a co-culture medium of MS containing 0.01 mg/l 2,4-D, 500 mg/l carbenicillin and 100 mg/l kanamycin (selection medium) for 4 times of subcultures before regeneration to plant. Every three weeks the explants were transferred to fresh selective medium as a sulculture. Shoots formed from hypocotyl segments were transferred to MS basic medium with 1 mg/l of kinetin for plant regeneration. The plants were grown under 16 hours of daylength using cool-white fluorescent light at an intensity of 3000 lux.

GUS assay

GUS activity of the regenerated plants was determined by both histochemical and fluorimetric assay as reported by Jefferson *et al.* (1987). Histochemical assay was performed using GUS assay buffer containing 20% methyl alcohol to eliminate the endogeneous GUS activity (Kosugi *et al.*, 1990). Fluorimetric GUS assay was performed using 4-methyl-umbelliferyl-β-D-glucuronidase (MUG) as a substrate using 4 young leaves randomly selected from each transgenic line showing GUS activity in the histochemical assay. Transgenic plant with vector only was used for control. The level of fluorescence was measured with a TK Dedicated Mini-Fluorometer (Hoefer) and compared with 4-methylumbelliferone standards to determine the GUS activity. The fluorescence per hour was converted to specific activity by measuring the protein concentration of extracts using the Bradford (1976) method.

DNA isolation and PCR analysis

Total genomic DNA was isolated from fully expanded leaves of *in vitro*-grown GUS positive plants based on histochemical test and nontransformed control(transformed with vector only) as described by Ueno *et al.* (1996). PCR analysis was carried out to confirm the presence of the *gus* and *nptII* genes. The primers were designed for the two genes followed to those of Ueno *et al.* (1996). The reaction mixture contained 10 ng DNA, 200 mM dNTPs, 0.5 mM of each primer, 1.0 unit of Taq polymerase (Promega) 2 mM of Mg^{2+}, and Taq polymerase buffer (Promega). Samples were heated to 94°C for 5 min. and then subjected to 40 cycles of 1 min. at 94°C, 3 min. at 58°C, and 3 min. at 72°C. Samples of PCR products were separated on a 1.0% (w/v) agarose gel electrophoresis and visualized with ethidium bromide.

RESULTS AND DISCUSSION

Selection and plant regeneration

In preliminary experiments, buckwheat (*Fagopyrum esculentum* Moench var. Shinnong No.1 and Yangjul) cotyledons and hypocotyl explants were used in transformation. The response in culture between cotyledon and hypocotyl segments were different. Unlike previously reports (Srejovic and Nesskovic, 1985; Akashi *et al.*, 1998; Romchatngoen *et al.*, 1998), cotyledon explants responded only for callus induction and not for plant regeneration. On the other hand, hypocotyls explants proved to be efficient in the production and recovery of regenerated plants. Likewise, 2,4-D levels ranging from 0.01 to 2.0 mg/l induced direct shoot regeneration from hypocotyl segments of buckwheat. Initially, embryogenic callus was expected to be induced at the higher levels of concentration of 2,4-D (1-2.0 mg/l), but it was found that only poor and non-embryogenic callus was induced while the frequency of shoot regeneration was obtained at low levels or without (0-0.05 mg/l) 2,4-D through organogenesis regardless of variety used.

On the basis of these preliminary results, a medium supplemented with 0.01 mg/l 2,4-D was chosen for direct regeneration of transgenic plants. Two weeks after inoculation with *Agrobacterium*, hypocotyl explants gradually turned brown and most died on the selection medium containing 500 mg/l carbenicillin and 100 mg/l kanamycin (Km); on the other hand, putative transformed plants grew along the cut surface of infected hypocotyls (Fig 1A); the leaves of the negative control turned brown and died. Transgenic buckwheat plantlets were produced after 3 months in *in vitro* culture. Sensitivity of hypocotyl tissue to *Agrobacterium* has been reported earlier (Maria *et al.*, 1996; Soryu *et al.*, 1996; Ueno *et al.*, 1996). The growth rate of kanamycin-resistant tissues were slower in medium with Km than without Km. In former case, that may be due to damage caused by the bacteria during co-cultivation (Gonzalez *et al.*, 1998). After the 4th subculture on the selection medium, the degree of the survival varied between genotypes; 40.3% and 26.8% in Shinnong No.1 and Yangjul, respectively. All shoots developed into green plants which were morphologically normal in appearance (Fig. 1B); similar to the observation of Miljus-Djukic *et al.* (1992). However, some of them showed the abnormalities such as rolling and translucent leaf and short and thick stem (Fig. 1C). Five shoots were regenerated from Shinnong No.1 and three from YangJul (Table 1).

Analysis of GUS gene assay

Three months after *Agrobacterial* infection, 8 putative

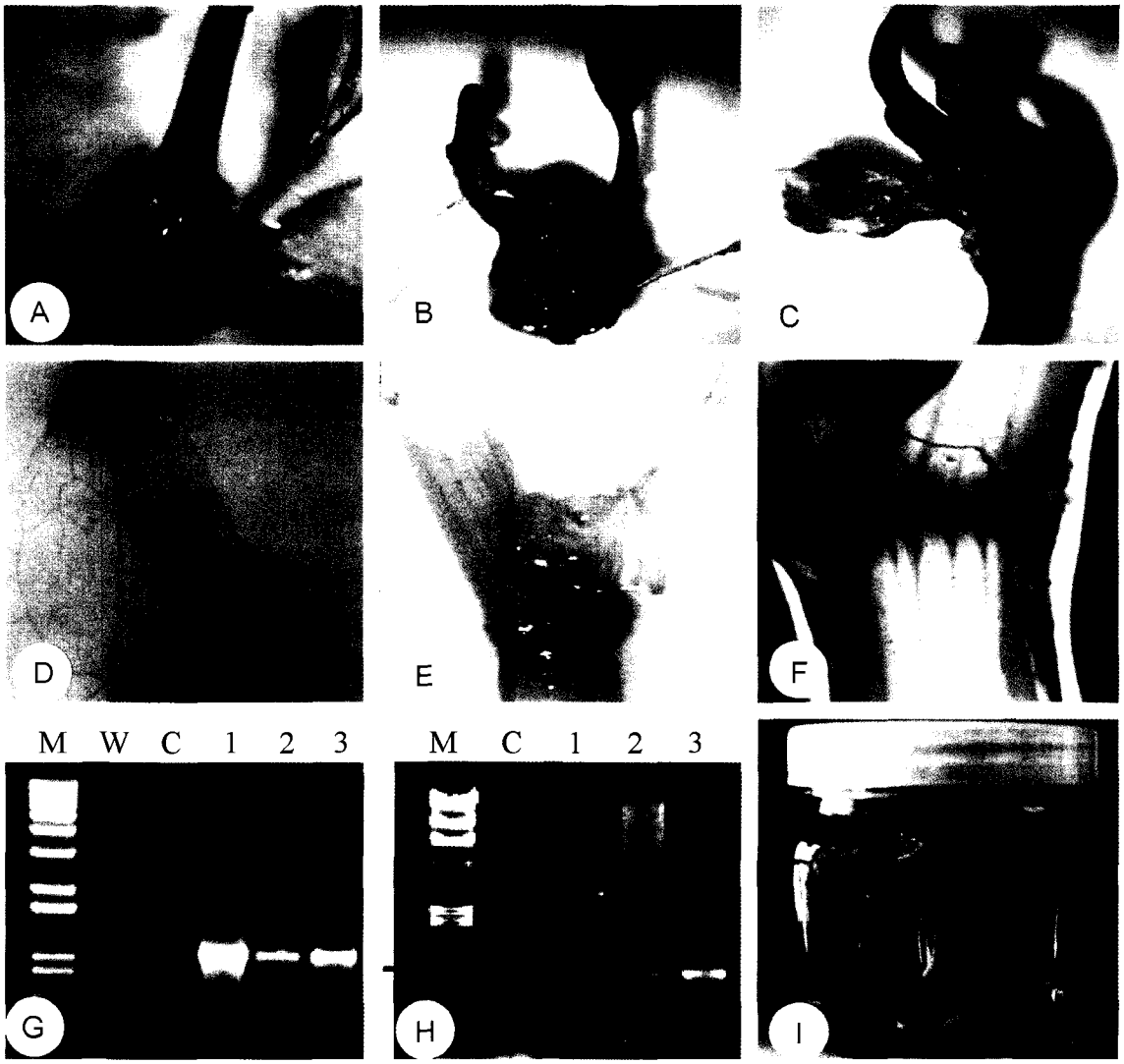

Fig. 1. Plant regeneration and results of histochemical GUS assay and DNA analysis by PCR from *Agrobacterium*-mediated transformation with pBI121 in buckwheat (cv. Shinnong No.1 and Yangjul). A : Regeneration of putative transformed shoot on selection medium (cv. Yangjul). B, C : direct plant regeneration from hypocotyl explant (cv. Shinnong No.1). D-F : Gus activity in leaf, stem and vascular tissues (D: cv. Shinnong No.1; E, F: cv. Yangjul). G, H: Gel electophoresis of PCR products amplified from putative transgenic buckwheat (M: Marker, W: water, C: untransformed control) G, lanes 1-3 : 1.9 kb fragment of *gus*, H: lanes 1-3: 0.8 kb of *nptII* were amplified (arrows indicate the 1.9 and 0.8 kb) I: Regenerated whole plant via *Agrobacterium*mediated transformation of Shinnong No.1.

Table 1. Response of buckwheat hypocotyls after infection with *Agrobacterium Tumefaciens*, pBI121 and GUS activity of the regenerated plants.

Variety	No. of hypocotyls Inoculated	Survival No (%)†	Callus formation No (%)	Regeneration No (%)	GUS+ No (%)‡
Shinnong No. 1	57	23 (40.3)	23 (40.3)	5 (8.7)	2 (3.5)
Yangjul	67	15 (22.3)	15 (22.3)	3 (4.4)	1 (1.4)

†Percentage was based on the no. of hypocotyls inoculated.
‡GUS+: *gus* positive by histochemical assay.

transformants which were cultured in kanamycin-containing selection medium were regenerated from hypocotyl segments. Leaves of these plants were subjected to the GUS histochemical test and 3 plants were shown to express histochemical GUS activity. Plants analyzed histochemically had characteristic blue staining in leaves, stems and vascular

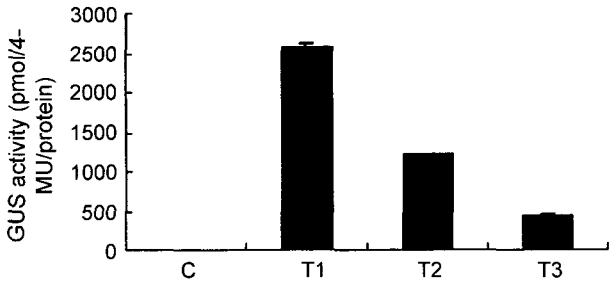

Fig. 2. GUS activity in leaf extracts from control (C) and transformed plants (T1, T2: Shinnong No. 1, and T3 : Yangjul) determined by fluorimetric assay.

tissues (Fig. 1D-F). The intensity of staining varied among transformants, similar to the observation in pigeonpea (Geetha *et al.*, 1999), garlic (Kondo *et al.*, 2000) and petunia (Tjokrokusumo *et al.*, 2000). This observation seems to be a typical expression pattern of CaMV 35S promoter-regulated GUS gene (Jefferson *et al.*, 1987) which has been widely used as a constitutive promoter in the production of transgenic plants (Akashi *et al.*, 1998; Gonzalez *et al.*, 1998). Variation in levels of GUS gene in plants could be due to differences in copy number of inserted genes and position effect arising from the integration of gene at different chromosome locations (Park and Facchini, 2000). *Agrobacterium*-mediated transformation of hypocotyl has become popular due to the simplicity and efficacy of the procedure as compared with using cotyledon segments (Miljus-Djukic *et al.*, 1992; Akashi *et al.*, 1998; Park and Facchini, 2000). The specific activity of GUS in leaves of 3 plants was measured fluorimetrically. Total activity as well as the variation on the quantitative measurement of the GUS gene expression were similar to the results of histochemical assay. This observation is the same as that of other crops like California poppy (Park and Facchini, 2000).

Fig. 2 shows the GUS activity of leaves determined quantitatively in the transformants using 4-MUG. One of three plants showed the high activity (2568 pmol 4-MU/mg protein) in Shinnong No. 1, while the control did not show any activity. Differences in activities shown by different independent transformants were reported previously (Maria *et al.*, 1996; Ueno *et al.*, 1996).

PCR analysis

The plants showing GUS activities in histochemical and fluorimetric assays were confirmed for the presence of GUS and *npt-II* genes by PCR using the NPTII- and GUS-specific primers. Amplification of these two genes were observed in the transgenic plants while no amplification occurred when these primers were used to amplify DNA from non-transformed plants. Analysis revealed that GUS-positive plants displayed a distinct band corresponding to the relevant sequence of the *gus*, 1.9 kb (Fig. 1G; lane : 1, 2 and 3) and *nptII* genes, 0.8 kb (Fig. 1H; Lane : 1, 2 and 3). Foreign DNA delivery to dicot plants via *Agrobacterium tumerfaciens* allows the genetic improvement of diverse varieties, as well as the elucidation of many aspects of molecular biology. It is well known that direct regeneration of shoots from explants give rise to regenerants with unmodified genetic constitution, apart from the inserted gene. In our case, the use of hypocotyl explants in buckwheat allowed the avoidance of time consuming process in regeneration and the development of transgenic shoots from callus usually formed poorly.

The limited genetic base in domestic buckwheat cultivars has restricted the power of traditional breeding methods to develop varieties with improved or value-added traits. The use of this buckwheat transformation protocol is gives the possibility of introducing new agronomic traits into buckwheat.

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