Development of an Efficient *Agrobacterium*-Mediated Transformation System and Production of Herbicide-Resistant Transgenic Plants in Garlic (*Allium sativum* L.)

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The genetic improvement of garlic plants (*Allium sativum* L.) with agronomical beneficial traits is rarely achieved due to the lack of an applicable transformation system. Here, we developed an efficient *Agrobacterium*-mediated transformation procedure with *Danyang*, an elite Korean garlic cultivar. Examination of *sGFP* (synthetic green fluorescence protein) expression revealed that treatment with 2-(N-morpholino) ethanesulfonic acid (MES), L-cysteine and/or dithiothreitol (DTT) gives the highest efficiency in transient gene transfer during *Agrobacterium* co-cultivation with calli derived from the roots of *in vitro* plantlets. To increase stable transformation efficiency, a two-step selection was employed on the basis of hygromycin resistance and *sGFP* expression. Of the hygromycin-resistant calli initially produced, only *sGFP*-expressing calli were subcultured for selection of transgenic calli. Transgenic plantlets produced from these calli were grown to maturity. The transformation efficiency increased up to 10.6% via our optimized procedure. DNA and RNA gel-blot analysis indicated that transgenic garlic plants stably integrated and expressed the *phosphinothricin acetyltransferase* (*PAT*) gene. A herbicide spraying assay demonstrated that transgenic plants of garlic conferred herbicide resistance, whilst non-transgenic plants and weeds died. These results indicate that our transformation system can be efficiently utilized to produce transgenic garlic plants with agronomic benefits.

INTRODUCTION

Garlic (*Allium sativum*) is a very important and widely cultivated crop that is used for seasoning food and for medicinal purposes. Like all field crops, garlic plants are commonly challenged by competitive weeds. Thus, the production of herbicide resistant garlic plants should help to increase its yield potential. However, despite recent advances (Gosal and Kang, 2012) garlic species have proven to be recalcitrant to genetic transformation tech-

niques such that transgenic garlic plants with agronomic benefits rarely develop to maturity.

In the first report of the development of transgenic garlic, the optimization of the temperatures and duration of *Agrobacterium* co-cultivation with garlic calli derived from the apical meristem of cloves was carried out on the basis of reporter gene β -glucuronidase expression and the *hygromycin phosphotransferase* (*HPT*) selective gene (Kondo et al., 2000). As a subsequent alternative strategy, a particle bombardment-mediated system of garlic transformation was developed using apical meristem-derived calli (Park et al., 2002). In that study, transgenic garlic plantlets resistant to chlorsulfuron, a sulfonylurea herbicide supplement in the growth medium, were obtained with only limited success. More recently, calli derived from different explant sources including roots as the most easily available explant and an *Agrobacterium*-mediated procedure were used to produce agronomically useful transgenic garlic plants resistant to beet armyworm (*Spodoptera exigua*), a polyphagous insect (Zheng et al., 2004). In addition, the *Agrobacterium*-mediated transformation of garlic was also reported using embryos (Eady et al., 2005) and immature leaf materials (Kenel et al., 2010). These recent garlic transformation methods are still inefficient, although more transgenic events were obtained.

In our present study, we established an efficient *Agrobacterium*-mediated genetic transformation procedure in garlic. More specifically, we improved the *Agrobacterium* co-cultivation conditions by adding the antioxidants 2-(N-morpholino) ethanesulfonic acid (MES), L-cysteine and dithiothreitol (DTT). In addition, to increase the transformation efficiency, we employed a two-step selection strategy on the basis of hygromycin resistance and *sGFP* expression. Using our optimized procedure, we successfully produced transgenic garlic plants harboring *phosphinothricin acetyltransferase* (*PAT*), a herbicide resistance gene. Our herbicide spraying assay demonstrated that the transgenic garlic plants are completely resistant to the herbicide Basta. This has not previously been successfully shown.

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MATERIALS AND METHODS

Plant materials

Danyang, a field-grown elite Korean cultivar (cv.) of garlic, was used in this study. Cloves were separated from garlic bulbs, disinfected in 25% (v/v) commercial bleach Clorox (4% sodium hypochlorite; Yuhan, Korea), and rinsed three times with sterile distilled water. Bulbs dissected from garlic cloves were used to produce *in vitro* plantlets as previously described (Ayabe and Sumi, 1998).

Vector construction

We constructed the pPAT-GFP-HPT vector (Fig. 1) for garlic transformation by inserting the *synthetic green fluorescent protein (sGFP)* and *hygromycin phosphotransferase (HPT)* genes into the pB2GW7 vector (Karimi et al., 2002) under the control of the *cauliflower mosaic virus 35S (CaMV35S)* promoter. This vector also contains the bacterial *PAT* gene under control of the *nopaline synthase (NOS)* promoter and terminator (Karimi et al., 2002). More details of this vector construction are available upon request. The vector was then introduced into the supervirulent *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986) using the freeze-thaw method (An et al., 1988).

Callus induction

Calli were induced from the root segments of *in vitro* plantlets of garlic cv. *Danyang* on MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg/L 2,4-D and 0.2 mg/L IAA for 2 months.

Agrobacterium co-cultivation

Agrobacterium cells were grown in LB liquid medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with 10 mg/L hygromycin for 2 days at 28°C with vigorous rotary shaking (120 rpm). The bacteria were collected by centrifugation. Cell pellets were suspended in the co-cultivation medium ($OD_{600} = 1.0$) containing 100 μ M acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone). A 10-ml aliquot of bacterial suspension was mixed with chopped calli and incubated for 10 min. The calli were then transferred onto co-cultivation media with removal of the *Agrobacterium* cells. To investigate the effects of co-cultivation media on gene transfer efficiency, we tested various media with different types of antioxidants [MES (1 mM), L-cysteine (200 mg/L), and DTT (100 mg/L)] in the presence of a half-strength N6 (Chu et al., 1975) (Table 1). The calli were co-cultured in darkness for 4 days at 25°C.

Selection of transgenic calli

Following co-cultivation, *Agrobacterium* cells were thoroughly removed by several washes in sterilized water containing 100 mg/L vancomycin and 200 mg/L cefotaxime. The cells were then placed on MS medium (Murashige and Skoog, 1962) containing 1 mg/L 2,4-D and 0.1 mg/L IAA supplemented with antibiotics (25 mg/L hygromycin, 100 mg/L vancomycin and 200 mg/L cefotaxime). After two subcultures with a 4-week interval in darkness at 25°C, the *sGFP*-expressing pieces of actively growing hygromycin-resistant calli were excised, transferred onto the same fresh medium and cultured for 4 weeks.

Plant regeneration

Actively growing calli were transferred to MS medium containing 5 mg/L kinetin and 1 mg/L NAA supplemented with 25 mg/L hygromycin, and incubated at 25°C under continuous light (40 μ mol/m²/s). Plant regeneration was accomplished in 2 to 6 weeks. Regenerated plantlets were transferred onto hormone-

free MS medium with 25 mg/L hygromycin. After 4 weeks, transgenic plants were acclimated and transferred to soil for further growth to maturity.

GFP observations

sGFP expression was monitored using luminescent-image analyzer (LAS 3000; Fuji, Japan), with 460 nm blue light and a GFP 510E filter set and stereomicroscope (SZX9-3122; Olympus, Japan).

Genomic DNA PCR and DNA gel-blot analysis

Genomic DNA was extracted from the young leaves of transgenic garlic plants using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Dickson, 1987). To identify the presence of *sGFP* and *HPT* genes, 20 ng of genomic DNA from transgenic plants was subjected to genomic DNA PCR using the *sGFP*-F primer 5'-CCACTACCTGAGCACCCAGT-3' and the *HPT*-R primer 5'-CAACGTGACACCCTGTGAAC-3' (for primer positions and orientations, see Fig. 1). For PCR, the cycling conditions were 2 min at 95°C, followed by 30 cycles of 95°C for 30 s; 55°C for 30 s; and 72°C for 1 min, and a final extension at 72°C for 7 min. The amplification products were separated on 1% agarose gel and visualized by EtBr staining.

Approximately 30 μ g of genomic DNA was digested with *Hind*III, which does not cut within the *PAT* gene, and then subjected to electrophoresis on a 0.8% agarose gel. The digested DNA was transferred onto a Hybond N+ nylon membrane (Sambrook et al., 1989), and hybridization was performed using a [α -³²P] dCTP-labeled *PAT* gene-specific probe prepared using the Rediprime DNA Labeling kit (Amersham Biosciences) in accordance with standard procedures for high-stringency hybridization conditions (Yi et al., 2004). The blot was hybridized in solution containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% (w/v) BSA, and 7% (w/v) SDS for 20 h at 60°C. Hybridization signals were recorded with a phosphorimager (Typhoon, Amersham Biosciences).

RNA gel-blot analysis

Total RNA from the leaves of transgenic garlic plants was isolated by the RNA isolation kit (Tri Reagent, Molecular Research Center, USA). A 20 μ g portion of total RNA was fractionated on a 1.3% agarose gel (Sambrook et al., 1989). The blot was hybridized using the *PAT* gene-specific probe as described above.

Herbicide bioassay

Transgenic garlic plants grown on soil for 10 weeks were sprayed with 0.3% (v/v) commercial herbicide Basta (18% glufosinate ammonium; Kyung Nong, Korea) solution. After approximately 10 days, surviving plants were distinguishable from wild type controls. To confirm stable expression of the transgene, harvested bulbs from the transgenic plants were planted with weeds in a greenhouse for 5 months and sprayed with the 0.3% Basta (v/v) solution.

RESULTS AND DISCUSSION

Effect of antioxidants on the transformation of garlic calli

To optimize the efficiency of gene transfer into garlic, we examined the influence of basal salts such as N6 (Chu et al., 1975), MS (Murashige and Skoog, 1962), and B5 (Gamborg et al., 1968) on this process. Our preliminary experiment using calli derived from the roots of garlic cv. *Danyang* indicated that a half-strength N6 is the most efficient basal medium (data not shown). This is consistent with previous reports that the use of

low-salt media during *Agrobacterium* infection improves T-DNA transformation (Cheng et al., 1997; Paz et al., 2004), although the mechanism of transformation enhancement is still unknown.

The use of antioxidants has contributed to the increase in transformation efficiency during *Agrobacterium* co-cultivation. For instance, the use of L-cysteine enhances *Agrobacterium*-mediated transformation of maize (Frame et al., 2006). Moreover, DTT in combination with L-cysteine in soybean has been shown to enhance T-DNA transfer (Paz et al., 2004). We thus tested the effects of three antioxidants, 1 mM MES, 200 mg/L L-cysteine, and 100 mg/L DTT, in a half-strength N6 solution (Chu et al., 1975) (Table 1). To determine the most efficient conditions, we examined the number of *sGFP*-expressing calli produced after 4 days of co-cultivation with *Agrobacterium* in darkness at 25°C (Fig. 2A). The results indicated that treatments with MES and L-cysteine and/or DTT gave the highest efficiency in transient gene transfer during *Agrobacterium* co-cultivation, whereas the other conditions were not reasonably effective (Table 1). Thus, we used the half-strength N6 medium supplemented with the antioxidants MES and L-cysteine for subsequent transformation experiments.

Two-step selection of stable transgenic calli

Selection is an important step in the transformation procedure. It is known that garlic calli are highly tolerant to the antibiotic agent kanamycin or to the herbicide phosphinothricin (Park et al., 2002). In contrast, garlic calli are sensitive to hygromycin and thus the *HPT* gene was chosen as selection marker in our experiments. To precisely select transgenic calli, we employed a two-step selection strategy using the antibiotic agent hygro-

Table 1. Effect of co-cultivation conditions on the *Agrobacterium*-mediated transformation of garlic cv. *Danyang*

Components*	No. of calli tested	No. of <i>sGFP</i> expression
-	236	0
MES	240	0
L-cysteine	187	23
DTT	249	0
MES, L-cysteine	263	818
MES, DTT	259	0
DTT, L-cysteine	192	4
MES, DTT, L-cysteine	203	192

*1 mM MES, 200 mg/L; L-cysteine, 100 mg/L DTT

mycin and *sGFP* expression. For this purpose, we constructed the pPAT-GFP-HPT vector harboring *sGFP* and *HPT* genes under the control of the *CaMV35S* promoter as well as the *PAT* gene. First, after *Agrobacterium* co-cultivation calli were incubated for 2 months on selection medium containing hygromycin. Second, transgenic calli were selected by excising the *sGFP*-expressing portions of hygromycin-resistant calli (Fig. 2B, arrow), and then further incubated on fresh medium for one month. These calli all showed a uniform green fluorescence throughout the entire tissue (Figs. 2C and 2D), indicating that our procedure prevented any escape or formation of chimeras.

Fig. 1. T-DNA structure of the binary vector pPAT-GFP-HPT constructed for garlic transformation. Arrows indicate the primer positions and orientation for PCR amplification. LB and RB, left and right borders; P35S and T35S, *CaMV35S* promoter and terminator; PNOS and TNOS, *nopaline synthase* promoter and terminator; PAT, *phosphinothricin acetyltransferase*; *sGFP*, *synthetic green fluorescent protein*; HPT, *hygromycin phosphotransferase*.

Fig. 2. *sGFP* expressions in transgenic calli, plantlets, and bulbs produced via the *Agrobacterium*-mediated transformation of garlic. (A) Transient expression of *sGFP* in garlic calli co-cultivated with *Agrobacterium* for 4 days. (B) *sGFP* expression (arrow) in transformed hygromycin-resistant calli one month after selection. (C, D) Light (C) and *sGFP* expression (D) in transgenic calli 2 months after selection. (E) Plantlet regenerated from selected calli. (F) Green fluorescence image of a regenerated whole plant. (G and H) Light (G) and fluorescence images (H) of bulbs harvested from transgenic garlic plants.

Table 2. Efficiency of *Agrobacterium*-mediated transformation of garlic cv. *Danyang*

No. of cultured calli (A)	No. of transgenic plants (B)	Transformation efficiency (B/A %)
278	3	1.1
223	4	1.8
278	3	1.1
141	15	10.6

Regeneration of transgenic garlic plants

Actively growing *sGFP*-expressing calli were transferred to regeneration medium and incubated at 25°C under continuous light (40 $\mu\text{mol}/\text{m}^2/\text{s}$). Regenerated shoots were obtained in 2 to 6 weeks (Fig. 2E) and transferred onto hygromycin-containing media to allow further root development. The transgenic whole plantlets that were produced exhibited a uniform green fluores-

cence in all tissues (Fig. 2F) and were transferred to soil and grown to maturity in a greenhouse. Green fluorescence was also observed in the harvested bulbs (Figs. 2G and 2H). We obtained a total of 349 transgenic garlic *Danyang* plants.

Transformation efficiency

We carried out four independent transformation experiments with our established procedure. The transformation frequencies for garlic *Danyang* ranged from 1.1% to 10.6%, which was calculated by dividing the number of hygromycin-resistant and *sGFP* expressing plantlets by the total number of co-cultivated calli (Table 2). This transformation efficiency via *Agrobacterium* mediation is higher than previously reported (Kenel et al., 2010).

Molecular characterization of transgenic plants

To determine if all primary transgenic plants contained the *sGFP* and *HPT* genes, we carried out genomic PCR analysis using *sGFP/HPT*-specific primers. All of the plants were PCR-positive (Supplementary Fig. S1), indicating the accuracy and

Fig. 3. DNA and RNA gel-blot analysis of transgenic garlic plants. (A) DNA gel-blot analysis. (B) RNA gel-blot analysis. The EtBr-stained gel shows equal loading of total RNAs. N, non-transgenic plant; 1-5, independent transgenic plants. The *PAT* gene was used as a probe in the blot analysis.

Fig. 4. Herbicide resistance testing of transgenic garlic plants by spraying with 0.3% (v/v) Basta solution. (A) Non-transgenic plants grown in a pot after spraying with Basta. (B) Transgenic garlic plants grown in a pot after spraying with Basta. (C) Transgenic garlic plants and weeds grown in a greenhouse before spraying with Basta. (D) Transgenic garlic plants and weeds grown in a greenhouse after spraying with Basta. Only transgenic garlic plants survived in the middle of dead weeds.

effectiveness of our selection strategy using hygromycin resistance and *sGFP* expression to generate stable transgenic plants. To determine the transgene copy number, we further selected 5 transgenic plants, and carried out DNA gel-blot analysis using the *PAT* gene as a probe (Fig. 3A). For this purpose, genomic DNA extracted from transgenic plants were digested with *Hind*III which does not cut within the *PAT* gene. Two transgenic lines were found to contain a single copy of the transgene (lines 2 and 3) whereas the other three lines carried 2 (line 5) or 3 (lines 1 and 4) copies. Thus, the average copy number was estimated to be roughly 2 per transgenic line.

To examine the expression of *PAT*, we carried out RNA gel-blot analysis using total RNAs isolated from the leaves of the same five independent transgenic lines (Fig. 3B) with high expression detected in all cases. We did not observe any correlation between the copy numbers of the transgene and the level of transgene expression. These lines were then analyzed using a herbicide resistance assay.

Herbicide resistance test

We tested herbicide resistance by spraying the validated transgenic garlic growing on soil with herbicide Basta. Ten days after spraying, all transgenic plants remained healthy with active growth, whilst the wild type controls all died (Figs. 4A and 4B). We also grew harvested bulbs from transgenic plants together with weeds in a greenhouse and sprayed them with Basta. After the herbicide treatment, only transgenic plants survived and actively grew (Figs. 4C and 4D).

We have established an efficient procedure for the *Agrobacterium*-mediated transformation of garlic. Notably, we have developed optimized co-cultivation conditions through supplementation with the antioxidants MES, L-cysteine and DTT, and also an efficient two-step procedure that is essential for the accurate selection of stable transgenic calli. Using this established method, we have successfully produced herbicide-resistant transgenic garlic plants of the Korean elite cv. *Danyang*. Our new procedure should provide an opportunity for further production of transgenic garlic plants that possess other agronomical important traits.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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