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Improved *in vitro* Regeneration of Potato (*Solanum tuberosum* cv. Superior) Transformed by *Agrobacterium* Expressing β-Glucuronidase

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In order to enhance the system of potato transformation and further regeneration, potato was transformed using the *Agrobacterium tumefaciens* harboring β-glucuronidase (GUS) gene. We found that a series of modified medium attained 100% shoot regeneration within 5 weeks after the preincubated explants on stage I medium were infected with *Agrobacterium*. Callus appeared at the cut edges of stem segments on stage II medium, mainly at the basal parts. Some explants started to form shoots after two to three weeks on stage III medium containing kanamycin (50 mg/L). When transferred to MS medium containing 200 mg/L kanamycin, 81% of the transformed shoots formed roots at the cut edge of the plantlets. In contrast, untransformed shoots never rooted and became yellowish after few weeks under the same conditions. Southern and northern analysis indicated that transformation of potatoes was successfully proceeded. Consequently, we have improved *in vitro* shoot regeneration on the callus derived from the potato explants, which were incubated with *Agrobacteria*. The regeneration cycle was shortened after the transformation and finally the transformation efficiency was highly enhanced.

Keywords: *Solanum tuberosum*, GUS, regeneration, transformation

Plant genetic techniques such as transformation and protoplast fusion have been widely recognized as tools to improve crops and to elucidate the physiological and biochemical control systems of gene expression in plants.

The recent introduction into plants of agronomically important traits, such as resistance to viruses, insects (Hilder *et al.*, 1987; Fischhoff *et al.*, 1989) and herbicides (Stalker *et al.*, 1988), has shown the large impact of this new technique on the breeding of agronomically important species. But the emphasis has been on annuals and crops that are amenable to manipulation in tissue culture (Chiyomi *et al.*, 1991; Caius *et al.*, 1992; Tsuyoshi *et al.*, 1993).

In spite of the fact that production of transgenic potatoes by *Agrobacterium*-mediated transformation was achieved in several species of poatoes (Gracia, Desiree, Boro), the regeneration and transformation efficiency of potatoes were relatively low. Moreover somaclonal variation and chromosomal aberration has been reported among the regenerated transgenic potatoes (Sree-Ramulu *et al.*, 1985; Sree-Ramulu, 1986). It seems to be due to the time lapse during the shoot regeneration after the *Agrobacterium* infection. Therefore, the culture period should be made as short as possible to avoid the endangerment of genetic variation; the regeneration of putative transformed shoots need also to be improved to enhance the final transformation efficiency.

The main object of this study is to improve the *in vitro* regeneration system after the infection of *Agrobacterium* for the efficient transformation of potato. Briefly we shortened the regeneration cycle using a series of modified medium so that these results would be a basis for potato transformation to introduce agronomically good traits.

MATERIALS AND METHODS

Bacterial strain and plant material

A. tumefaciens, LBA4404, was used for transformation experiments, which contains a pBI121 plasm-

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id. *A. tumefaciens* was grown on YEP medium with kanamycin (50 mg/L) at 28°C. Potato (*Solanum tuberosum* L. cv. Superior) tubers were obtained from the Rural Development Administration, Suwon.

Transformation and regeneration of potato

Stem segments were precultured on stage I medium supplemented with 0.4 mg/L IAA, 2.24 mg/L BAP. After two days, the precultured explants were soaked in overnight cultures of *A. tumefaciens* for 10 minutes, which was diluted to 20 folds with the infection medium (stage I medium without growth regulators). The co-cultured explants were transferred to fresh stage I medium and cultured in the dark. After three days, they were transferred into stage II medium containing 50 mg/L kanamycin, 100 mg/L timentin and cultured with 16 hours of light a day for 10 days. They were then transferred into stage III medium containing 50 mg/L kanamycin, 100 mg/L timentin for shoot regeneration.

Genomic DNA isolation and southern hybridization

Genomic DNA was isolated from *in vitro* shoot cultures according to the method of Cone (1989). 10 μg of genomic DNA was digested with *Eco*RI and was separated on a 0.8% agarose gel followed by transfer into a nylon membranc in 0.4 M NaOH. The membrane was prehybridized at 65°C in Church buffer (7% SDS, 1% BSA, 1 mM EDTA and 250 mM $NaPO_4$ (pH 7.2) for 20 minutes, and hybridized in the same solution containing radioactive labelled GUS probe (Church and Gibert. 1984). The membrane was then washed twice with 2×SSC and 0.5% SDS for 30 minutes at 65°C, and two times with 0. 2×SSC and 0.5% SDS for 30 minutes at room temperature. Membrane filters were exposed to X-ray films with an intensifying screen at −70°C. For the probe, the 1.9 kb fragments that contained the β-glucuronidase gene were separated from pBI121 plasmid and radioactively labelled by a random priming method (Feinberg and Vogelstein, 1983).

Northern hybridization

Northern blot analysis was performed by the method of Sambrook *et al.* (1989). RNA samples (20 μg) were electrophoresed on a 1.0% agarose gel containing 0.67 M formaldehyde and then transferred to nylon membrane. The hybridization conditions were the same as form the southern hybridization, except that in this case, the hybridization temperature was 42°C.

Microtuberization of potato

Propagated shoots, GUS-transgenic shoots and nontransgenic shoots, were transferred into MS medium containing kanamycin (50 mg/L) and 5% sucrose and cultured in chamber with 8 hours photoperiod, 500 lux, 17°C.

Fluorometric and histochemical assay for GUS

Fluorometric GUS assay was carried on the leaf tissues according to the Gallagher (1989). Histochemical determination of GUS activity in the plant tissues was conducted according to the method described by Jefferson *et al.* (1987).

RESULTS

Transformation and regeneration of potato

The transformation of potato was carried out by the cocultivation of stem segments with of *Agrobacteria* containing binary vector pBI121. Calli were observed at the edge of stem segments after one week of culture on stage II medium containing kanamycin (Fig. 1 A2) and the neoplastic growths were observed mainly at the basal part of stem segments.

During the culture period, the petri dishes were ventilated to avoid ethylene accumulation in the culture bottle. The explants that contained calli were cultured for another three days in stage II medium and then transferred into stage III medium. Some calli started to form primordia after one week. The primordia developed into shoots (Fig. 1 A3) and were rapidly grown in hormone-free MS liquid medium (Fig. 1 B1). Four weeks from the start of the experiment, healthy shoots were successfully grown at the stem segment.

Characterization of transgenic shoots

The selection of transgenic shoots was carried out in two steps. As a first step the regenerated shoots in the presence of kanamycin (200 mg/L) were selected during a second step, the plantlets were tested for GUS activity by the means of fluorometric assay or not (Gallagher, 1989).

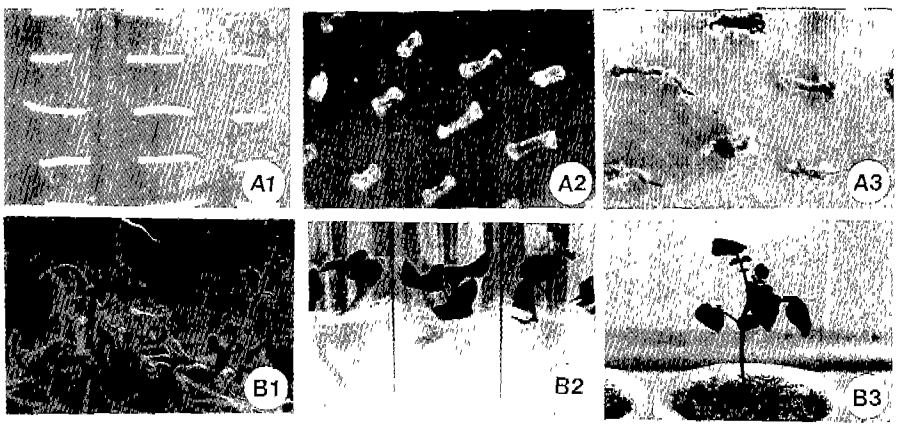

Fig. 1. Regeneration of transformed potatoes. A1, 2 and 3, step of stem segments. B1, propagated shoots. B2 and 3, growth comparation of transformant. B2, after 1 week culture. B3, after 4 weeks culture.

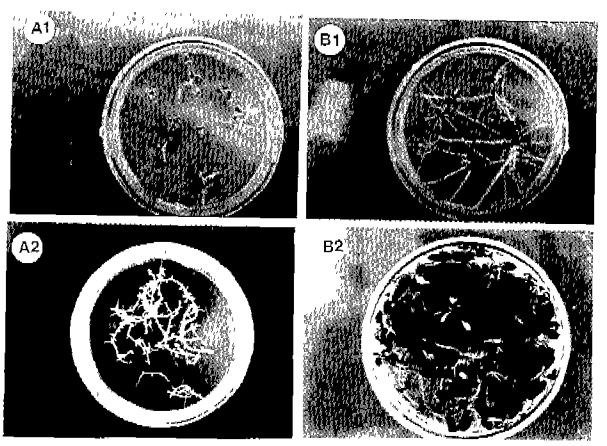

Fig. 2. Test of rooting ability from regenerated shoots on MS medium supplemented with 200 mg/L kanamycin. Al, untransformed shoot. B1, transgenic shoots. A2, untransformed shoots after 4 weeks culture. B2, transgenic shoots after 4 weeks culture.

Putative transgenic shoots were rooted at the cut end of plantlets (Fig. 1 B1) and vigorously grown on MS medium containing 200 mg/L kanamycin (Fig. 1 B2), whereas untransformed shoots regenerated from potato explants that were not infected with *Agrobacterium* did not form roots and became yellowish after a month (Fig. 2 A2). All of the potato explants (432) infected with *Agrobacterium* had regenerated shoots, and 81% of them were rooted on medium containing kanamycin (200 mg/L). When transgenic and nontransgenic plants were transferred into MS medium containing kanamycin (50 mg/L) and 5% sucrose, microtubers were developed (Fig. 3). In the fluorometric assay for GUS activity, most of the plantlet showed a positive result for GUS activity (Data not shown).

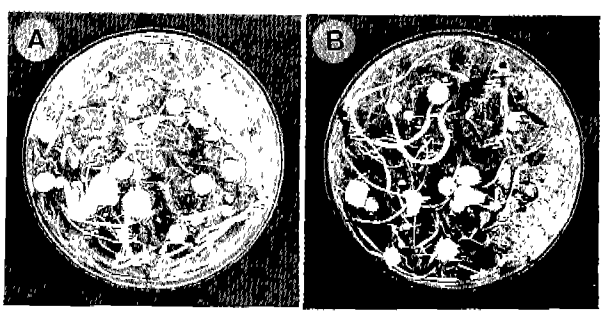

Fig. 3. Microtuberization of transformed potatoes. A, untransformed potato tubers. B, transformed potato tubers.

Southern and northern analysis

To find out whether GUS gene was incorporated into the potato genome, genomic DNA was purified from potato leaves and hybridized with a ^{32}P-labelled probe. The 1.9 kb fragment of GUS coding gene was positively detected in the potato lines which were transformed by *Agrobacterium* harboring pBI121 vector. This result demonstrate that the transformation was successful (Fig. 4). Fig. 5 shows an autoradiogram of a northern blot for the RNA extracted from the leaves of the control and the transformed plants. A single major band appeared at about 2.4 kb from the transgenic potato while there was no northern band observed from the nontransgenic plant.

Histochemical GUS assay in transgenic plants

Potato tissues were stained with X-Gluc. As shown in Fig. 6, microtubers and leaves were in-

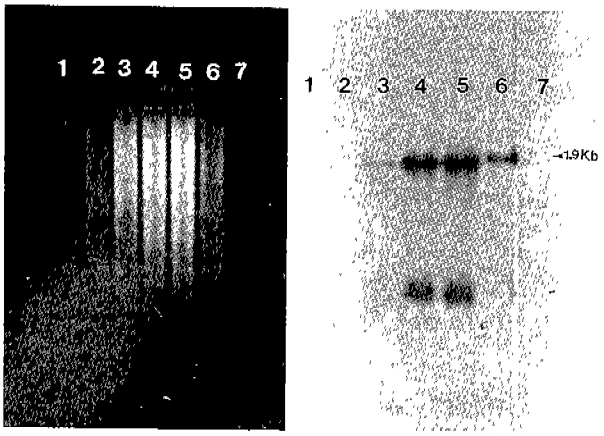

Fig. 4. Southern blot analysis of genomic DNA isolated from β-glucuronidase (GUS) transformants. *Eco*RI digested genomic DNAs of transformed potato were separated and hybridized with ^{32}P-labelled *Hin*dIII and *Eco*RI fragment of pBI121. Lane 1 and 7, 1 kb DNA ladder. Lane 2, untransformed potatoes. Lane 3-6, transformed potatoes (line 1, 2, 3, 4).

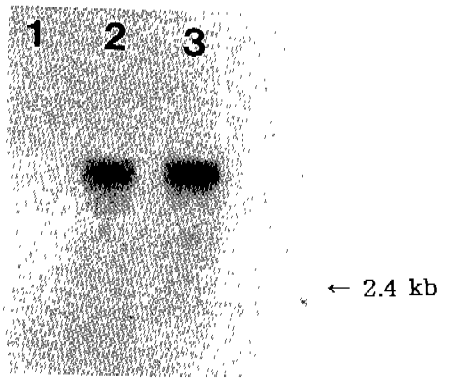

Fig. 5. Northern blot of β-glucuronidase (GUS) transformants. Total RNA from transformants hybridized with ^{32}P-labelled *Hin*dIII and *Eco*RI fragment of bPI121. Lane 1, untransformed potato. Lane 2 and 3, transformed potato.

tensively stained, these results suggest that GUS was expressed with tissue specificity.

DISCUSSION

It is important to note that an efficient regeneration system is needed for the successful transformation of plant matter. Also the culture time must be controlled to avoid the somaclonal variation caused by long-term culture in the callus stage (Stiekema *et al.*, 1988; Ooms *et al.*, 1987). The feeder layer system has been used in rapid regeneration systems but it seems to be difficult to

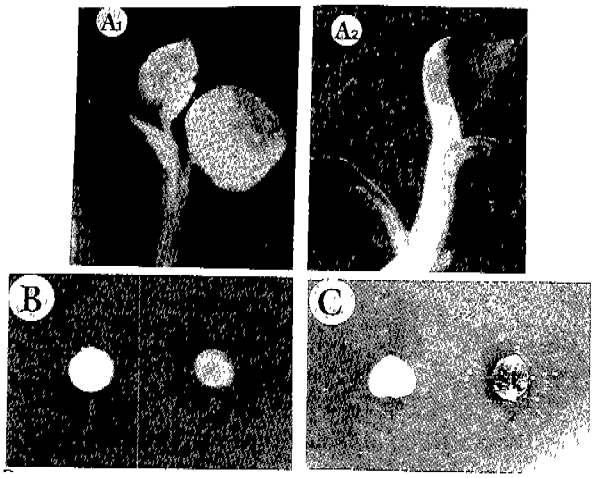

Fig. 6. Histochemical localization of β-glucuronidase (GUS) in potato plants. Explants of potato were incubated in X-Gluc (A1, A2 and B) and Magenta-β-D-Gluc. C, staining solution overnight and photograph under the microscope after chlorophyll removed. Al, untransformed leaves. A2, transformed leaves. B, stained tubers (1, untransformed tuber 2, transformed tuber). C, Magenta-β-D-Gluc stained leaves (1, untransformed leaves 2, transformed leaves).

manipulate the system Sheerman (1988) and Sung (1994).

There are reports that preculturing plant tissues prior to bacterial inoculation increases the frequency of transformants that can be produced (McHughen *et al.*, 1989) and there are suggestions that cells actively undergoing division are more easily infected and transformed than older tissues (Chyi and Phillips, 1987). Preincubation of potato explants before the inoculation and high concentration of cytokinin did help high frequency of shoot formation under the stabilized culture condition by adding MES to the medium.

The selection of transfomant could be carried out in the medium containing appropriate antibiotics (Feher *et al.*, 1991). Herman *et al.*, (1988) reported that 65% of the shoots regenerated on medium containing 50 mg/L of kanamycin sulfate were found to express GUS activity of at least 100 pmol 4-MU (mg/f.w.). Our results showed that 81% of transformed shoots rooted over 200 mg/L of kanamycin and the most plants showed GUS activity even though the degree was variable. This result shows that the regeneration of the transformant was highly enhanced in our experiments.

In addition, De Block (1988) noted that brown-yellow coloration, which made regeration difficult, was

caused by the large amount of ethylene produced during the culture of potato *in vitro*. Browning occured more severely in potato explant when *Agrobacterium* also inoculated. It might helpful that ventilation of the culture bottle and pH control of medium could mitigate the problems.

Southern and northern hybridization confirmed the presence of the GUS gene in the potato genome. To further characterize the transformation system, we investigated histochemical expression of the GUS gene in potatoes. Leaves and sections of transformed microtuber stained intensely. Different cell types within plants are expected to have different metabolic activity with corresponding differences in rates of transcription and translation (Jefferson *et al.*, 1987).

As a result of microtuberization, we obtain many transformed microtubers from a verified transgenic potato in a very short time. It will facilitate the propagation of the transgenic plants and make it amenable to store the plant line.

In conclusion, we shortened the regeneration cycle of potato after *Agrobacterium* mediated transformation. The method for transformation of potato was very rapid and convenient. In addition, our results show that a foreign GUS gene was incorporated into the potato genome and stably expressed in the transgenic potatoes.

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