Plant Biotechnology 26, 243–248 (2009)

Original Paper

Transgenic spinach plants produced by *Agrobacterium*-mediated method based on the low temperature-dependent high plant regeneration ability of leaf explants

Dong Poh Chin, Jin Hua Bao, Masahiro Mii*

Laboratory of Plant Cell Technology, Graduate School of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan

* E-mail: miim@faculty.chiba-u.jp Tel & Fax: +81-47-308-8852

Received December 12, 2008; accepted January 14, 2009 (Edited by S. Ogita)

Abstract *Agrobacterium* strain EHA101 harboring plasmid pIG121-Hm containing the genes for β-glucuronidase (*gus*), hygromycin phosphotransferase (*hpt*) and neomycin phosphotransferase (*nptII*) was co-cultivated with leaf explants from *in vitro* grown spinach plants. Hygromycin-resistant calli were obtained 1 month after selection of transformed cells on 2.5 g l^{-1} gellan gum-solidified MS medium containing 30 g l^{-1} sucrose, 0.5 mg l^{-1} 2,4-D, 2 mg l^{-1} kinetin, 20 mg l^{-1} hygromycin and 20 mg l^{-1} meropenem trihydrate. Regeneration of adventitious shoots was affected by culture temperature, and the highest frequency of shoot formation was 40% when the calli were cultured at 14°C during regeneration process on 2.5 g l^{-1} gellan gum-solidified MS medium containing 30 g l^{-1} sucrose, 0.01 mg l^{-1} 2,4-D, 1 mg l^{-1} kinetin, 1 mg l^{-1} gibberellic acid (GA3), 20 mg l^{-1} hygromycin and 10 mg l^{-1} meropenem trihydrate. Stable expression of *gus* gene was indicated by histochemical GUS assay in the leaves and roots of putative transgenic plants. Southern blot analysis of genomic DNA isolated from T0 plants confirmed the successful integration of T-DNA into the plant genome. Segregation of the *gus* gene in transgenic T1 progeny was confirmed by PCR analysis. These results show that *Agrobacterium*-mediated transformation combined with plant regeneration at a low temperature, 14°C, can be efficiently used for producing transgenic spinach plants with useful genes.

Key words: *Agrobacterium tumefaciens*, genetic transformation, plant regeneration, spinach, *Spinacia oleracea*.

Spinach (*Spinacia oleracea* L.) is one of the major vegetable crops grown for commercial production worldwide and also a rich source of iron, vitamins and minerals (Swiader et al. 1992). Currently, the major purpose of breeding spinach is to develop cultivars with resistance to various diseases and pests, high temperature, late flowering, and low concentrations of nitrate and oxalate in the plant (Pandey and Kalloo 1993). Although a large number of spinach cultivars with some of these traits have been developed through conventional breeding programs, it is still difficult to produce cultivars with all of these desirable traits, because of the lack of available germplasm. Is is now expected to apply genetic manipulation of spinach genome for improvement of this important vegetable crop.

So far, there have been few reports on genetic transformation of spinach. Knoll et al. (1997) reported regeneration and transformation of spinach, by using root explant as a target material for *Agrobacterium tumefaciens* inoculation. Yang et al. (1997) transformed leaf segments of spinach with *A. tumefaciens* using genes for coat proteins of cucumber mosaic virus (CMV) and confirmed the expression of CMV coat proteins in transgenic plants. Zhang and Zeevaart (1999) obtained transgenic spinach plants that over-expressed the green fluorescence protein (GFP) gene in cotyledons after inoculation with *A. tumefaciens*. Genetic transformation of spinach was also achieved by using *A. rhizogenes* by Ishizaki et al. (2002). In these studies, the transformation and regeneration efficiencies were highly affected by cultivar and explants types that used for infection with *Agrobacterium*, which gave a range of 0–87% for transformation and 0–96% for regeneration efficiencies. To further improve the transformation and regeneration efficiency for a wide range of spinach cultivars we developed an *A. tumefaciens*-mediated transformation system with highly reproducible results by using an efficient plant regeneration system with an appropriate temperature treatment and use of hygromycin for

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; *Bt*, *Bacillus thuringiensis*; CTAB, cetyltrimethylammonium bromide; DTT, dithiothreitol; GA_3, gibberellic acid; GFP, Green fluorescence protein; Hm, hygromycin; *hpt*, hygromycin phosphotransferase; IBA, indole-3-butyric acid; *nptII*, neomycin phosphotransferase.

This article can be found at http://www.jspcmb.jp/

selection.

Materials and methods

Plant materials

In vitro plants of the spinach cultivar 'Glory', 'Orai', (Takii Seed and Seedling Co., Kyoto, Japan) 'Aspire', 'Dimple', 'Orion', 'Pandora', and 'Parade' (Sakata Seed Co., Kanagawa, Japan) were used. Seeds were surface sterilized successively with 70% ethanol for 3 min and 1% sodium hypochlorite solution containing 80 μl of Tween-20 for 20 min, followed by rinsing 3 times with sterile water. Seeds were then germinated on hormone-free MS medium (Murashige and Shoog 1962) containing 30 g l^{-1} sucrose and solidified with 2.5 g l^{-1} gellan gum (Gelrite; Wako Pure Chemical Industries, Osaka, Japan) in culture bottles. Cultures were kept in an incubator under 8/16 h (light/dark) photoperiod at 20°C.

Plasmid vector and bacterial strain

A. tumefaciens strain EHA101 (Hood et al. 1986), which harbors a binary vector pIG121-Hm (Ohta et al. 1990) that contains a hygromycin phosphotransferase gene (*hpt*) and an intron-*gus* gene both under the control of a 35S cauliflower mosaic virus promoter, and a neomycin phosphotransferase II gene (*nptII*) under the control of a nopaline synthase promoter in the T-DNA region, was used (Figure 4A).

Influence of culture temperature on plant regeneration

Twenty leaf segments (*ca*. 5 mm in width) of 'Glory', 'Orai' and 'Orion' each from 2–3 week-old *in vitro* plants were cultured on 2.5 g l^{-1} gellan gum-solidified MS medium containing 30 g l^{-1} sucrose, 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D; Wako Pure Chemical Industries, Osaka, Japan) and 2 mg l^{-1} kinetin (Sigma-Aldrich, St. Louis, MO, USA) for callus induction. Cultures were incubated under the 8 h light photoperiod at 20°C and subcultured onto the same medium at 2-week intervals. After 2 months, calli were removed from leaf segments, transferred onto regeneration medium, which was MS medium containing 30 g l^{-1} sucrose, 0.01 mg l^{-1} 2,4-D, 1 mg l^{-1} kinetin, 1 mg l^{-1} gibberellic acid (GA3; Wako Pure Chemical Industries, Osaka, Japan), and incubated at 3 different temperatures, 14°C, 20°C, and 25°C. For each treatment, 30 calli from each cultivar were tested and the mean of the regeneration rate for three experiments was recorded.

Inoculation and co-cultivation with Agrobacterium

Agrobacterium was grown overnight at 28°C in LB liquid medium containing 50 mg l^{-1} hygromycin (Hygromycin B; Wako Pure Chemical Industries, Osaka, Japan), 50 mg l^{-1} kanamycin (Kanamycin sulfate; Wako Pure Chemical Industries, Osaka, Japan) and 25 mg l^{-1} chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA). For preparing a bacterial solution for inoculation, an *Agrobacterium* suspension culture was added to 40 ml MS liquid medium containing 30 g l^{-1} sucrose, 0.5 mg l^{-1} 2,4-D, 2 mg l^{-1} kinetin and 100 μM acetosyringone (3′, 5′-dimethoxy-4′-hydroxy-acetophenone; Sigma-Aldrich, St. Louis, MO, USA) to make a density of OD_{600}=0.4 or 1.0. For inoculation, leaves from 2 to 3 week-old *in vitro* plants of 'Glory' were cut transversely into segments of 5 mm in width, and immersed in the bacterial solution for 10 min. After removing the excess bacterial solution on sterilized filter paper, explants were transferred onto co-cultivation medium consisting of 2.5 g l^{-1} gellan gum-solidified MS medium of the same composition as described above in Petri dishes and maintained at 20°C in the dark.

Selection of transgenic callus and plant regeneration

After 3 days of co-cultivation, the leaf explants were transferred onto the co-cultivation medium but lacking acetosyringone and containing 20 mg l^{-1} meropenem trihydrate (Meropen; Dainippon Sumitomo Pharma, Osaka, Japan; Ogawa and Mii 2007) for bacterial elimination and 20 mg l^{-1} hygromycin for selection of the putative transformed callus. Subcultures were performed every two weeks. After 2 months, hygromycin-resistant calli were removed from leaf explants and transferred to regeneration medium containing 10 mg l^{-1} meropenem trihydrate and 20 mg l^{-1} hygromycin. To test the influence of temperature on the regeneration, the calli were incubated at 14 or 20°C under an 8 h photoperiod.

For rooting, basal part of regenerated shoots were dipped into 20 mg l^{-1} indole-3-butyric acid (IBA) for 2 h and subsequently transferred onto the mixture of vermiculite and paper pulp blocks (Florialite; Sanei Co., Gunma, Japan) containing 7 ml MS liquid medium (MS basal salts and vitamins, 30 g l^{-1} sucrose, hormone-free) in test tubes (25×100 mm). After 6 weeks, rooted plants were transferred into pots with soil and kept in a transparent plastic box for acclimatization. The plants that were successfully acclimatized were then grown in a growth chamber. Throughout root induction and acclimatization, they were kept at 20°C under the 8 h light photoperiod for inducing vegetative growth since flower bud initiation occurred easily under a long day condition. For flowering, spinach plants were transferred under 16 h light photoperiod at 20°C. Since spinach is a dioecious plant, T1 progeny was obtained by crossing the transgenic plants with non-transformed plants.

GUS assay

Histochemical assay to detect GUS activity was conducted following the procedure reported by Jefferson et al. (1987). Plant tissues (calli, leaves and roots) were immersed with 1 mM X-Gluc in 50 mM phosphate buffer (pH7.0) containing 5 mM dithiothreitol (DTT) and 20% methanol, placed under a mild vacuum for 10 min and then incubated overnight at 37°C. The tissues were soaked in 70% ethanol for several hours to remove chlorophylls.

DNA isolation and molecular analysis

Total genomic DNA was extracted from leaf tissues of both T0 and T1 transgenic plants and non-transformed plants using the CTAB method (Murray and Thompson 1980). For Southern blot analysis, 10 μg of genomic DNA was digested with *Xba*I, which cuts a single site within the T-DNA, and fractionated on a 0.9% agarose gel. Separated DNA fragments were then transferred to nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co., Billerica, MA, USA). The *gus* (1.2-

kb), *hpt* (0.6-kb) and *nptII* (0.7-kb) probes were generated by PCR from the plasmid pIG121-Hm by using the following set of primers, 5′-ACAGCGTCTCCGACCTGATGCA-3′ and 5′-AGTCAATGACCGCTGTTATGCG-3′ for *hpt* (Xiao and Ha 1997), 5′-GGTGGGAAAGCGCGTTACAAG-3′ and 5′-GTTTACGCGTTGCTTCCGCCA-3′ for *gus*, 5′-GAGGCT-ATTCGGCTATGACTG-3′ and 5′-ATCGGGAGCGGCGATAC-CGTA-3′ for *nptII* (Hamill et al. 1991), were used. The PCR was performed according to the following thermal cycles: 30 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1.5 min. Hybridization and detection were performed according to the instruction manual of the DIG labeling and Detection System (Roche Diagnostics, Mannheim, Germany). The *gus* probe was striped from the membrane after detection and the blot was re-hybridized with *hpt* as well as *nptII* probes according to the manufacturer's manual.

Results

Effect of culture temperature on shoot regeneration from calli

We examined the effect of culture temperature on shoot regeneration from calli induced from leaves of 3 cultivars by incubating at 14, 20 and 25°C. Calli cultured at the lower temperature, 14°C, showed a higher regeneration rate for all 3 cultivars. The cultivar 'Glory' showed higher regeneration efficiency with well-developed shoots than those of other cultivars (Figure 1). Therefore, 'Glory' was mainly used for the subsequent transformation experiments.

Selection of hygromycin-resistant calli and plant regeneration

The leaf explants from *in vitro* plants of spinach cultivar 'Glory' were co-cultivated for 3 days at 20°C with *A. tumefaciens* EHA101 harboring pIG121-Hm plasmid. After transferring to selection medium containing 20 mg l^{-1} hygromycin (Hm), calli were produced from the sites used for cutting the leaf explants (Figure 2A), while no calli appeared from non-transformed leaf explants that died within 1 month. After 2 months of culture on selective medium, more than 50 Hm-resistant calli (43%) were obtained from 120 inoculated leaf explants for both bacterial densities, these was no significant difference in the transformation efficiency when two densities of bacteria, OD_{600}=0.4 and 1.0, were used for inoculation. When 40 randomly chosen Hm-resistant calli of 'Glory' were subjected to a histochemical GUS staining, more than 90% of them were GUS positive. These results indicate that 'Glory' had high transformation efficiency, since more than 40% of the inoculated explants produced Hm-resistant calli, and over 90% expressed GUS in the leaves. Applicability of this transformation method was confirmed in 5 other cultivars (i.e., 'Aspire', 'Dimple', 'Orai', 'Pandora', 'Parade'), which successfully produced Hm-resistant calli at 20.8–88.3% of inoculated explants (Figure 3). This result indicates that various cultivars of spinach could be used for genetic transformation by *A. tumefaciens* although some modifications of the method might be needed for the cultivars with relatively low transformation efficiency.

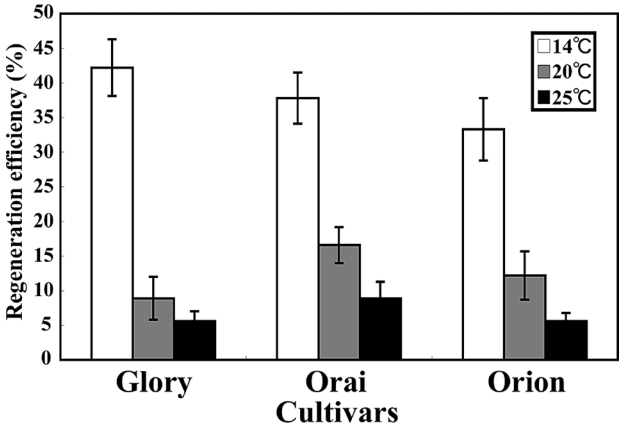

Figure 1. Influence of the culture temperature on plant regeneration. Calli induced from leaf explants of 'Orai', 'Orion' and 'Glory' were incubated at 14, 20 and 25°C. For each cultivar, thirty calli were used, and the percentage of those calli with adventitious shoots was recorded after three months. Each value represents a mean±SE of three independent experiments.

When the Hm-resistant calli were transferred to regeneration medium, approximately 40% of the calli cultured at 14°C initially turned red, and then adventitious shoots emerged (Figure 2B).

Regeneration of roots from hygromycin-resistant shoots

The adventitious shoots regenerated from hygromycin-resistant calli were propagated by stem cuttings using the same culture condition, but it is rather difficult to produce an adequate root system for successful acclimatization to soil. Our preliminary experiments showed that immediate transfer of the shoots onto MS medium containing 1 mg l^{-1} IBA caused re-proliferation of calli near the base of the shoots instead of inducing roots. Therefore, the base of regenerated shoots were treated with IBA (20 mg l^{-1}) for 2 h, and then transferred into test tubes with Florialite and liquid hormone-free MS medium. By using this method, most of the shoots were rooted within 6 weeks (Figure 2C). The regenerated plants were then successfully acclimatized after transfer into pots with soil by first keeping them in a transparent plastic box. After acclimatization, the transformed plants flowered in a growth chamber when the photoperiod was changed from short (8 h) to long (16 h) day condition (Figure 2D). Transgenic plants produced T1 seeds by cross-pollination with non-transgenic female or male plants.

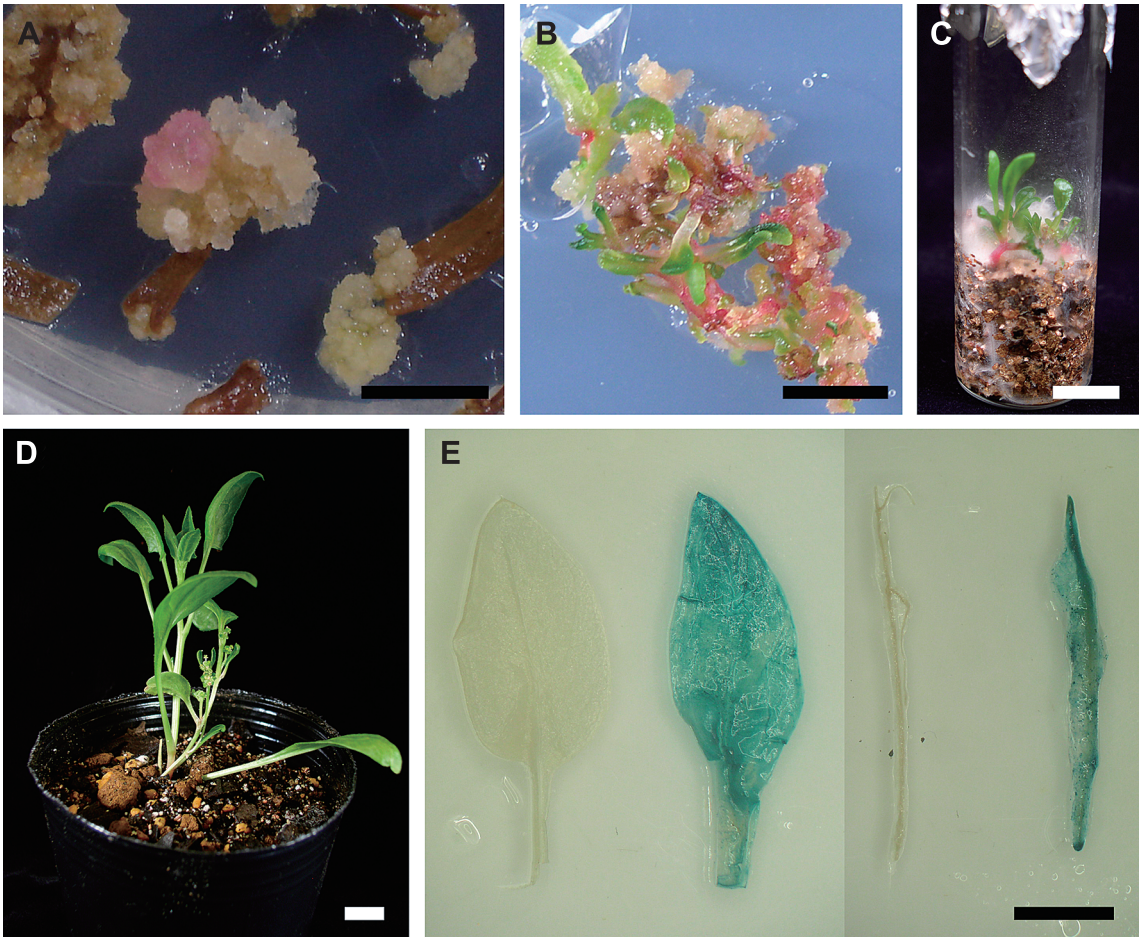

Figure 2. Production of transgenic spinach plants after infection with *A. tumefaciens*. (A) The emergence of Hm-resistant calli from leaf explant 2 months after culture on the selective medium. (B) Regenerating shoots from Hm-resistant callus on regeneration medium at 14°C. (C) Transgenic plantlet rooting and growing on Florialite in test tube. (D) Transgenic plant after acclimatization and growing in pot. (E) Stable GUS expression in leaf and root of transgenic plant (right) and untransformed plant (left) of 'Glory', respectively. Bars 10 mm.

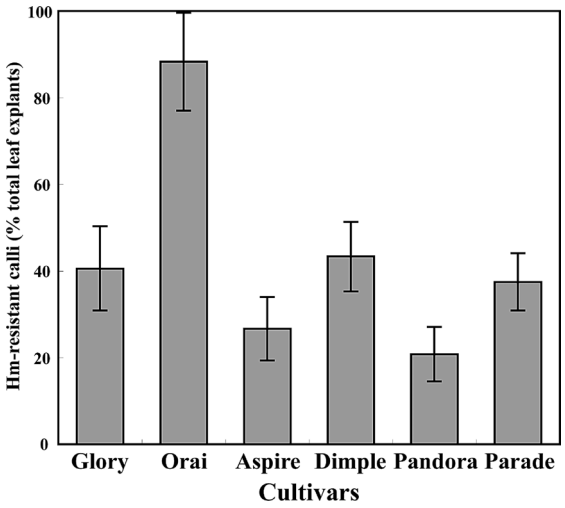

Figure 3. Comparison of the transformation efficiency in 5 cultivars. Leaf explants of 'Aspire', 'Dimple', 'Orai', 'Pandora', 'Parade' were inoculated with *A. tumefaciens* and subsequently transferred onto selection medium containing 20 mg l^{-1} hygromycin. For each cultivar, fifteen leaf explants were used and percentage of those leaf explants emerged Hm-resistant calli was recorded after two months. Each value represents a mean±SE of three independent experiments.

Confirmation of transformation

Histochemical GUS assay was performed to detect the expression of the *gus* gene in leaves and roots of putatively transformed plants. Blue staining was not detected in nontransformed plants (Figure 2E). Southern blot analysis was performed to confirm the integration of *gus*, *hpt* and *nptII* genes into the plant genome using genomic DNA extracted from the leaf tissues of acclimated T0 plants. The hybridization signals were detected from the genomic DNA of 8 randomly chosen transformants. Since *Xba*I cuts once 5′ of the *gus* gene (Figure 4A), the result shown in Figure 4B, C revealed that 7 plants had a single copy and 1 plant had multiple copies of the *gus* and *hpt* genes integrated in the plant genome. Hybridization signals were detected after hybridization with the *nptII* probe in 7 transgenic plants. The *nptII* gene was not detected for the plant shown in lane 2, possibly due to incomplete integration of *nptII* gene (Figure 4D). PCR analysis for the *gus* gene was performed using DNA extracted from some of the T1 plants. As shown in Figure 5, 1.2-kb *gus* signals were

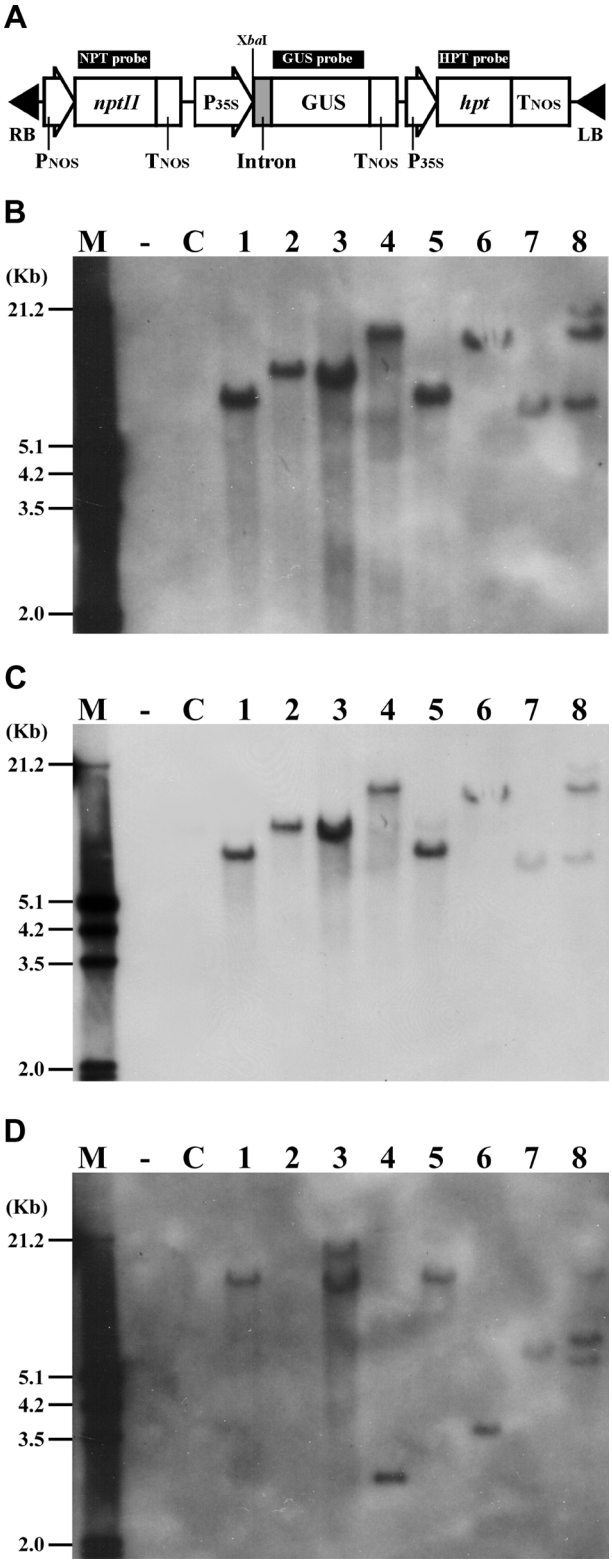

Figure 4. Southern blot analysis of T0 transgenic plants. (A) T-DNA regions of pIG121-Hm and probes shown as bars. DNA samples were digested with *Xba*I, fractionated by electrophoresis, transferred to a nylon membrane and hybridized to *gus* (B), *hpt* (C) or *nptII* (D) probes. Lane M, molecular size marker (λDNA/*Eco*RI, *Hin*dIII); Lane C, untransformed control plant; Lanes 1–8, transgenic plants. Molecular markers are indicated on the left.

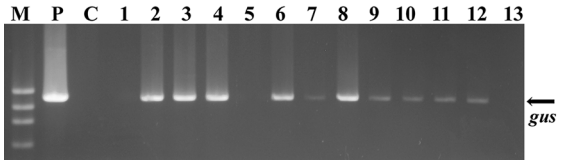

Figure 5. PCR analysis of T1 spinach plants for the *gus* gene. Lane M, molecular size marker (ϕX174/*Hae*III); Lane P, plasmid (pIG121-Hm); Lane C, untransformed control plant; Lanes 1–13, transgenic T1 plants.

only detected in 16 of the 30 T1 plants analyzed, revealing that T1 progeny consisted of untransformed and transformed plants due to the segregation of transgene. The 1 : 1 segregation ratio of *gus* gene (16 GUS^+ and 14 GUS^-) verified by PCR analysis confirmed a single copy insertion for two T0 transgenic plants that shown in result of Southern blot analysis (lanes 1 and 2).

Discussion

Although regeneration of plants from various explants in spinach, such as hypocotyls, cotyledons, roots and leaves have been reported (Al-Khayri et al. 1991; 1992, Mii et al. 1992; Xiao and Branchard 1995; Komai et al. 1996; Knoll et al. 1997; Zdravković-Korać and Nešković 1999; Ishizaki et al. 2001; Leguillon et al. 2003), leaf explants were selected as the material for *Agrobacterium* inoculation in the present study because of the constant availability of enough amount of explants from *in vitro* grown plants. A high number of Hm-resistant calli were obtained from leaf explants of the cultivar 'Glory' and 5 other cultivars tested making it likely that leaf explants from *in vitro*-grown elite cultivars could be used for the introduction of foreign genes with this system. Since the cultivar 'Orai' was revealed to have comparably high shoot regeneration ability as 'Glory' and the highest transformation efficiency compared with other cultivars used in the present study, this cultivar might be useful as a model plant for genetic transformation of spinach.

Previous studies have shown that low temperature had positive effect on regeneration in other plants, such as in *Heloniopsis orientalis*, a Liliaceae ornamental plant (Kato and Ozawa 1979) and wheat (Hou et al. 1997), which enhance regeneration rates in these plants by treatment with at 16°C and 5°C, respectively. The positive effect of low temperature treatment on shoot regeneration observed in this study strongly indicates the importance of culture temperature on regeneration in spinach.

In the previous studies on the transformation of spinach, only kanamycin was used as a selective agent corresponding to the *nptII* gene (Knoll et al. 1997; Yang et al. 1997; Zhang and Zeevaart 1999). Although the plasmid used in the present study containing both *nptII*

and *hpt* genes, we selected *hpt* gene in the present study to confirm its usefulness as a selective marker. As a result, hygromycin was proved to be an alternative antibiotic to kanamycin for selection in spinach transformation since it completely killed leaf explants of non-transformed spinach within 1 month after transfer to the medium containing 20 mg l^{-1}.

The introduction of other useful genes such as nitrate reductase, chitinase, defensin and *Bacillus thuringiensis* (BT) toxin genes are now in progess.

References

AI-Khayri JM, Huang FH, Morelork TE (1991) Regeneration of spinach from leaf callus. *Hort Sci* 26: 913–914

Al-Khayri JM, Huang FG, Morelock TE, Busharar TA (1992) *In vitro* plant regeneration of spinach from mature seed-derived callus. *In Vitro Cell Dev Biol Plant* 28: 64–66

Hamill JD, Rounsley S, Spencer A, Todd G, Rhodes MJC (1991) The use of the polymerase chain reaction in plant transformation studies. *Plant Cell Rep* 10: 221–224

Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T DNA. *J Bacteriol* 168: 1291–1304

Hou BK, Yu HM, Teng SY (1997) Effects of low temperature on induction and differentiation of wheat calluses. *Plant Cell Tiss Organ Cult* 49: 35–38

Ishizaki T, Komai F, Masuda K (2001) Screening for strongly regenerative genotypes of spinach in tissue culture using subcultured root explants. *Plant Cell Tiss Organ Cult* 67: 251–255

Ishizaki T, Hoshino Y, Masuda K, Oosawa K (2002) Explants of Ri-transformed hairy roots of spinach can develop embryogenic calli in the absence of gibberellic acid, an essential growth regulator for induction of embryogenesis from nontransformed roots. *Plant Sci* 163: 223–231

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6: 3901–3907

Kato Y, Ozawa N (1979) Adventitious bud formation on leaf and stem segments of *Heloniopsis orientalis* grown at various temperatures. *Plant Cell Physiol* 20: 491–497

Knoll KA, Short KC, Curtis IS, Power JB, Davey MR (1997) Shoot regeneration from cultured root explants of spinach (*Spinacia oleracea* L.): a system for *Agrobacterium* transformation. *Plant Cell Rep* 17: 96–101

Komai F, Okuse I, Harada T (1996) Somatic embryogenesis and plant regeneration in culture of root segments of spinach (*Spinacia oleracea* L.). *Plant Sci* 113: 203–208

Leguillon S, Charles G, Branchard M (2003) Plant regeneration from thin cell layers in *Spinacia oleracea*. *Plant Cell Tiss Organ Cult* 74: 257–265

Mii M, Nakano M, Okuda K, Iizuka M (1992) Shoot regeneration from spinach hypocotyl segments by short term treatment with 5,6-dichloro-indole-3-acetic acid. *Plant Cell Rep* 11: 58–61

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight DNA. *Nucl Acids Res* 8: 4321–4325

Ogawa Y, Mii M (2007) Meropenem and moxalactam: Novel β-lactam antibiotics for efficient *Agrobacterium*-mediated transformation. *Plant Sci* 172: 564–572

Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol* 31: 805–813

Pandey SC, Kalloo G (1993) Spinach. In: Kalloo G, Bergh BO (eds) *Genetic Improvement of Vegetable Crops*. Pergamon Press, Oxford, pp 325–336

Swiader JM, Ware GW, McCollum JP (1992) *Producing Vegetable Crops*. Interstate Publishers, Danville, Illinois

Xiao L, Ha SB (1997) Efficient selection and regeneration of creeping bentgrass transformants following particle bombardment. *Plant Cell Rep* 16: 874–878

Xiao XG, Branchard M (1995) *In vitro* high frequency plant regeneration from hypocotyl and root segments of spinach by organogenesis. *Plant Cell Tiss Organ Cult* 42: 239–244

Yang YM, Al-Khayri JM, Anderson EJ (1997) Transgenic spinach plants expressing the coat protein of cucumber mosaic virus. *In Vitro Cell Dev Biol Plant* 33: 200–204

Zdravković-Korać S, Nešković M (1999) Induction and development of somatic embryos from spinach (*Spinacia oleracea*) leaf segments. *Plant Cell Tiss Organ Cult* 55: 109–114

Zhang HX, Zeevaart JAD (1999) An efficient *Agrobacterium tumefaciens*-mediated transformation and regeneration system for cotyledons of spinach (*Spinacia oleracea* L.). *Plant Cell Rep* 18: 640–645