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Full Length Research Paper

Conditions for regeneration and transformation of *Solanum tuberosum* cultivars using the cotton glutathione S-transferase (Gh-5) gene

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Our aim was to produce multiple stress-resistant plants containing the glutathione-S-transferase (Gh-5) auxin-regulated gene, which is related to the presence of the stress resistance protein and is expressed in the transgenic plant genome. We used the Agrobacterium-mediated transformation method. In all tested cultivars (Chubaek, Jasim, Jopoong, Namjak and Sumi), there were higher regeneration frequencies from internode explants than from leaf explants. Incorporation of the GST gene into potato was confirmed by PCR analysis of genomic DNA that demonstrated the 585 bp band. Southern blot analysis confirmed the integration of the Gh-5 gene into the genomic DNA of both forms. No such bands were obtained from non-transformed potato DNA; double bands were detected in digested DNA from transformed potato plants.

Key words: Glutathione-S-transferase gene (Gh-5), potato, transformation, transgenic plant.

INTRODUCTION

Potatoes (Solanum tuberosum L.) are a major food crop in many parts of the world (Gilani and Nasim, 2007). However, yields can be greatly reduced by drought, high salinity, or dramatic temperature changes (Tang et al., 2007a, b). Although, many efforts have aimed at increasing potato plant pathogen resistance, there has been limited application of molecular breeding technology for enhancing environmental stress tolerance (Perl et al. 1993; Jeong et al., 2001) Potatoes in culture generally depend on a source of organic carbon such as sucrose (George, 1986), but there are also reports of autotrophic growth *in vitro* (Kozai and Iwanami, 1988; Pruski et al., 2002). Success in regenerating large numbers of transgenic plants differs among varieties of potato (De Block, 1988; Ottaviani et al., 1993). Transgenic plants have been produced that are resistant to herbicides, insects, and diseases; these properties have been transferred to plants through the application of several genetic transformation protocols using leaf discs and tuber discs (Beaujean et al., 1998). Nevertheless, these protocols have many problems, including a low frequency of transformation and the development of somaclonal variation as a result of tissue culture (Ooms et al., 1987; Ishida et al., 1989). Routine transformation of potato depends on the use of kanamycin resistance as a

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Abbreviations: IBA, Indole-3-butyric acid; **MS,** Murashige & Skoog; **NAA,** α-naphthaleneacetic acid; **GA_3,** gibberellic acid-3; **IAA,** indole acetic acid.

#These authors contributed equally to this work.

selectable marker (Vayda and Belknap, 1992; Conner et al., 1997; Rockhold et al., 2001).

This study aimed at producing potatoes with tolerance to plant pathogens and environmental stressors; our use of the cultivar Jopoong (developed in South Korea) is a novel aspect of the work.

MATERIALS AND METHODS

Shoot cultures of Chubaek, Namjak, Jasim, Jopoong, and Sumi potatoes were maintained by subculturing every 4 weeks on a shoot proliferation medium (pH 5.8) consisting of MS salts and vitamins (Murashige and Skoog, 1962), 6-benzylaminopurine (BA; 1 mg/L), indole-3-butyric acid (0.3 mg/L), sucrose (30 g/L), and plant agar (8 g/L; Duchefa).

Potato tissue culture procedure

Leaf, internode, and tuber explants were cultured on MS media containing different amounts of auxins and cytokinins to optimize shoot regeneration. Effects of plant growth regulators (BA, GA_3, Zeatin, and IAA) on shoot regeneration were also tested on different types of cultured explants. The pH in all media was adjusted to 5.8 prior to the addition of 0.8% agar, followed by autoclaving for 20 min at 120°C. For each treatment, 60 explants were used, and each experiment was repeated at least three times. Inoculated explants were held at 25 to 27°C in a 16-h light / 8-h dark photoperiod provided by cool white fluorescent lights (30 µmol photons m^{-2} s^{-1}). Well-developed shoots were transferred to rooting medium. Healthy rooted plantlets were separated, washed to remove agar, and transferred to plastic pots containing a sterilized soil mixture (1:1 vermiculite : perlite). Each pot was covered with a polyethylene bag to maintain high humidity, placed in a glasshouse in a 16-h light / 8-h dark photoperiod under reduced light intensity (30 µmol photons m^{-2} s^{-1}) at 25 to 27°C for 1 week, and subsequently transferred to normal light conditions (45 µmol photons m^{-2} s^{-1}).

Degree of kanamycin resistance in potatoes

We applied a kanamycin resistance test for efficient selection of putative transgenic plants during the transformation process. Leaves and internodes excised from normal plants were cultured on pH 5.8 MS media containing 0.1 mg/L NAA, 2.2 mg/L BAP, 3% sucrose, and 0.8% agar, with 0, 20, 30, 50, 75 or 100 mg/L kanamycin.

Construction of plant expression vector and bacterial culture

Agrobacterium tumefaciens strain EHA101 carrying the binary vector pCGN1578 was used as the vector system for transformation. The T-DNA region of the vector contains an nptII-based expression cassette for selection; the Gh-5 gene was cloned from cotton. The bacteria were grown in the dark on a gyratory shaker at 220 rpm at 30°C for 24 h in LB liquid medium (Bacto-tryptone 10 g, Bacto-yeast 5 g, NaCl 10 g and pH 7.0) containing kanamycin (50 mg/L). Bacterial cells were harvested by centrifugation and adjusted to an OD of 1.0 by dilution with liquid medium.

Transformation of potato

Leaf, internode, and tuber explants from *in vitro*–grown potatoes 3 to 4 weeks old were slightly scored across the midrib with a sterile scalpel and inoculated with bacterial culture. These plant parts were then transferred into pH 5.8 co-culture media containing 0.01 mg/L IAA + 2.2 mg/L BAP or 1 mg/L Zeatin + 1.5 mg/L IAA, 3% sucrose, 0.8% agar, and acetosyringone for various periods (6, 12, 18, 24 and 30 h). After co-cultivation, the explants were washed in liquid MS medium supplemented with 250 mg/L cefotaxime (filter-sterilized), blotted dry with sterile filter paper, and transferred to pH 5.8 selection media consisting of MS salts, 0.01 mg/L IAA + 2.2 mg/L BAP or 1 mg/L Zeatin + 0.5 mg/L IAA, 3% sucrose, 0.8% agar, 100 mg/L kanamycin for selection, and 250 mg/L cefotaxime to eliminate bacterial growth. The cultures were incubated at 25 ± 2°C for 4 weeks in a 16-h light/8-h dark photoperiod with an illumination of 45 µmol photons $m^{-2}s^{-1}$, transferred to fresh selection medium containing 50 mg/L kanamycin (but no cefotaxime), and allowed to regenerate for 30 days, with subculturing every 2 weeks. The primary regenerated shoots from the leaf explants were then transferred to MS medium supplemented with 0.1 mg/L GA_3 and 50 mg/L kanamycin for root induction. Rooted plantlets were transferred to 85 cm-diameter plastic pots containing a sterilized soil mixture (1:1 vermiculite : perlite), placed inside polyethylene bags to maintain high humidity, incubated at 25 ± 2°C in a growth chamber for 1 week, and transferred to a greenhouse.

DNA isolation and polymerase chain reaction

To verify the presence of Gh-5 genes in the regenerated plants, we used the CTAB (cetyltrimethylammonium bromide) method to extract total DNA from young leaves excised from kanamycin-resistant shoots and control plants (Murray and Thompson 1980). The primers used to amplify a 585-bp fragment of Gh-5 were Gh-5-1 (5´-ATG-GCA-AAG-AGT-GAT-GCC-GAA-GTG-3´) and Gh-5-2 (5´-CCC-ATT-TAA-GCA-GAG-CAG-GAG-TGT-TG-3´) as the antisense primer. Amplification conditions were 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final extension step at 72°C for 10 min. The amplification products were analyzed on 1% (w/v) agarose TBE gels.

Southern blot analysis

Southern blot analysis was performed with genomic DNA extracted from five randomly selected transgenic and non-transformed (control) plants according to the method of Sambrook and Russell (2001). Total genomic DNA from leaf tissue of putatively transformed and non-transformed plants was extracted using the CTAB method and digested with *Hind*III. The digested DNA was separated by electrophoresis on 0.8% (w/v) agarose gels and transferred to positively charged nylon membranes (Hybond-NX, Amersham Biosciences, Buckinghamshire HP7 9NA, UK). The membranes were hybridized overnight at 65°C with a ^{32}P-labeled PCR product of the Gh-5 gene in a buffer containing 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, and 7% SDS. Then membranes were washed at room temperature for 5 min in a buffer containing 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, and 5% SDS. The blot was then washed three times at 65°C with a high-stringency wash buffer (1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 5% SDS), and the dried blots were placed on X-ray film at -80°C for 1 week for radiography.

RESULTS AND DISCUSSION

Conditions of regeneration in *S. tuberosum*

Leaf, internode, and tuber explants of potato were cultured on MS media supplemented with 3.6 mg/L BA

Table 1. Effect of plant growth regulators on shoot regeneration of leaf culture in S. *tuberosum*.

Medium	Cultivar	Number of explant incubated	Number of shoot	Ratio of regeneration (%)
MS	Chubaek	60	18	30
+	Namjak	60	5	8
BA 3.625mg/L	Jasim	60	0	0
+	Jopoong	60	12	20
GA_3 2.525mg/L	Sumi	60	32	53

Table 2. Effect of plant growth regulators on the regeneration of internode culture in S. *tuberosum*.

Medium	Cultivar	Number of explant incubated	Number of shoot	Ratio of regeneration (%)
MS	Chubaek	60	19	32
+	Namjak	60	8	13
BA 3.625mg/L	Jasim	60	0	0
+	Jopoong	60	14	23
GA_3 2.525mg/L	Sumi	60	38	63

Table 3. Effect of plant growth regulators on the regeneration of tuber culture in S. *tuberosum*.

Medium	Cultivar	Number of explant incubated	Number of shoot	Ratio of regeneration (%)
MS	Chubaek	60	17	28
+	Namjak	60	24	40
Zeatin 1.0mg/L	Jasim	60	0	0
+	Jopoong	60	32	53
IAA 0.5mg/L	Sumi	60	0	0

and 2.5 mg/L GA_3 to investigate the effects of plant growth regulators on the induction of adventitious shoots. Frequency of direct shoot regeneration increased significantly in the Sumi cultivar. No shoots of Jasim were induced when the growth regulators were added to the culture medium. The addition of 3.6 mg/L BA and 2.5 mg/L GA_3 resulted in 30% and 20% ratios of shoot regeneration in Chubaek and Jopoong, respectively (Table 1). Internode explant shoot regeneration followed the same trend when regeneration medium was used (Table 2). Internode explants produced better results than leaf explants. Tubers on regeneration medium treated with 1 mg/L Zeatin + 0.5 mg/L IAA had the highest regeneration frequencies (53% in Jopoong, 40% in Namjak, and 28% in Chubaek; Table 3; Figure 1).

Kanamycin resistance test on *S.* tuberosum transformation

To investigate their resistance to the antibiotic, we transferred leaf and internode explants from non-transformed plants onto regeneration media supplemented with kanamycin at various concentrations (0, 20, 30, 50, 75 or 100 mg/L). On medium containing 75 mg/L kanamycin, shoot regeneration was 2 to 4%. In contrast, there was no shoot regeneration on medium containing 100 mg/L kanamycin. Therefore, 100 mg/L kanamycin was used as the minimum concentration required for the selection of transgenic potatoes (Table 4).

The low transformation frequencies in russeted genotypes are related to difficulties in regenerating genotypes from callus. Kanamycin resistance has been verified by rooting putatively transformed shoots on media containing 100 mg/L kanamycin (Park et al., 1995); 100 mg/L kanamycin selection was sufficient to efficiently select transformants. To examine the effects of inoculation time, we incubated tuber explants in the bacterial culture for 6, 12, 18, 24 or 30 h. Transformation efficiencies were highest when the explants were inoculated for 24 h (Figure 2).

Production of transgenic plants

We investigated the effects of acetosyringone

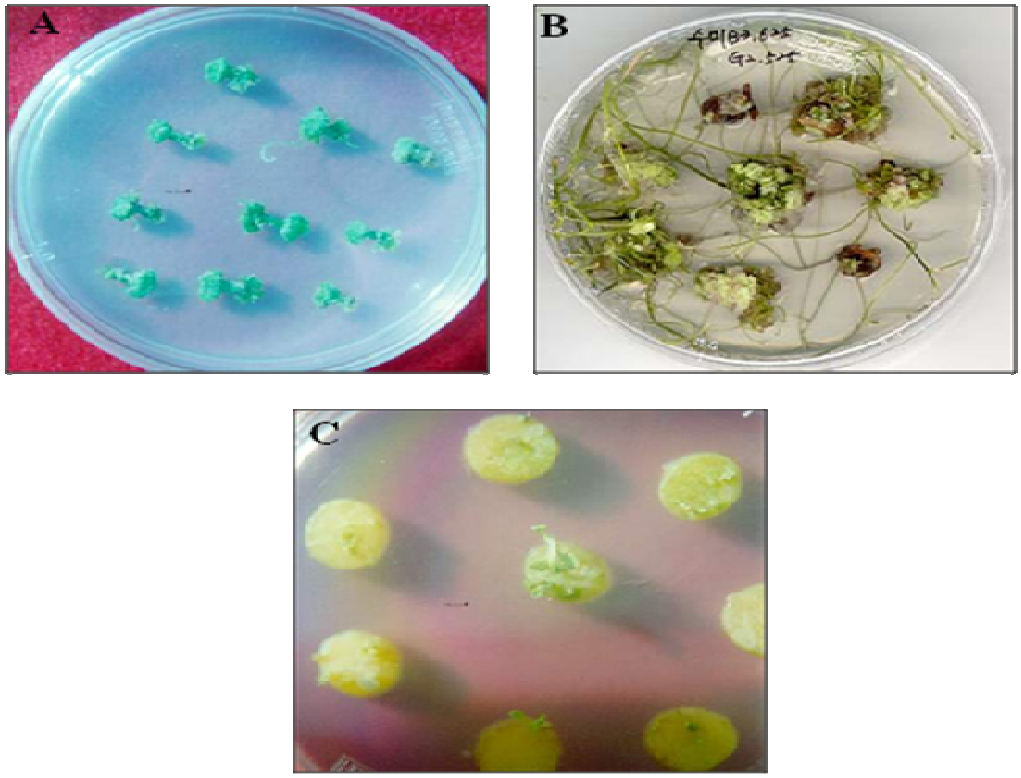

Figure 1. A procedure of plant regeneration in potato explants culture. (A) Callus formation from potato internode explants culture after 3 weeks, (B) Shoot formation from potato internode explants culture after 6 weeks and (C) Shoot formation from potato tuber explants culture after 4 weeks.

Table 4. The ratio of kanamycin resistance of leaf and internode explants in S. *tuberosum*.

Medium	Cultivar	Kanamycin (mg/L)	Number of explant	Number of survival explant	Ratio of survival explant (%)
MS + BA 2.25mg/L + IAA 0.018mg/L	Chubaek	0	60	60	100
		20	60	48	80
		30	60	32	53
		50	60	12	20
		75	60	3	5
		100	60	1	2
	Namjak	0	60	60	100
		20	60	45	75
		30	60	30	50
		50	60	13	22
		75	60	4	7
		100	60	0	0
	Jasim	0	60	60	100
		20	60	43	72
		30	60	34	57
		50	60	15	25
		75	60	2	3
		100	60	0	0
	Jopoong	0	60	60	100

Table 4. Continue.

	20	60	32	100
	30	60	23	38
	50	60	11	18
	75	60	2	3
	100	60	0	0
Sumi	0	60	60	100
	20	60	37	62
	30	60	28	47
	50	60	15	25
	75	60	3	5
	100	60	0	0

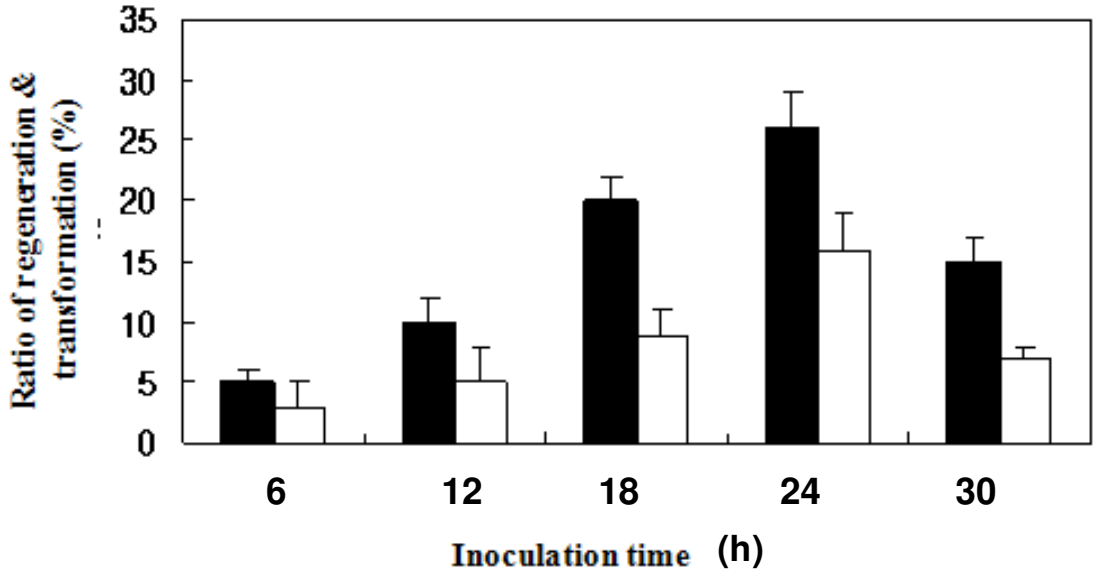

Figure 2. Influence of inoculation time on transformation of potato tuber explants culture. ■: Shoot regeneration, ϒ: transformation.

Table 5. Effect of varying concentrations of acetosyringone (AS) on the transformation efficiency in S. *tuberosum*.

Acetosyringone concentration (µM)	Number of explant incubated	Ratio of regeneration (%)	Ratio of transformation (%)
0	100	7.5	1.2
30	100	11.5	4.3
50	100	18.1	7.0
80	100	27.3	12.2
100	100	32.0	15.0
150	100	24.0	10.7

concentration (0 to 150 µM) on cultured explants. Transformation efficiency was greatly elevated (15%) in co-cultivation conditions (Table 5; Figure 3).

DISCUSSION

Internodal explants are much more resistant to

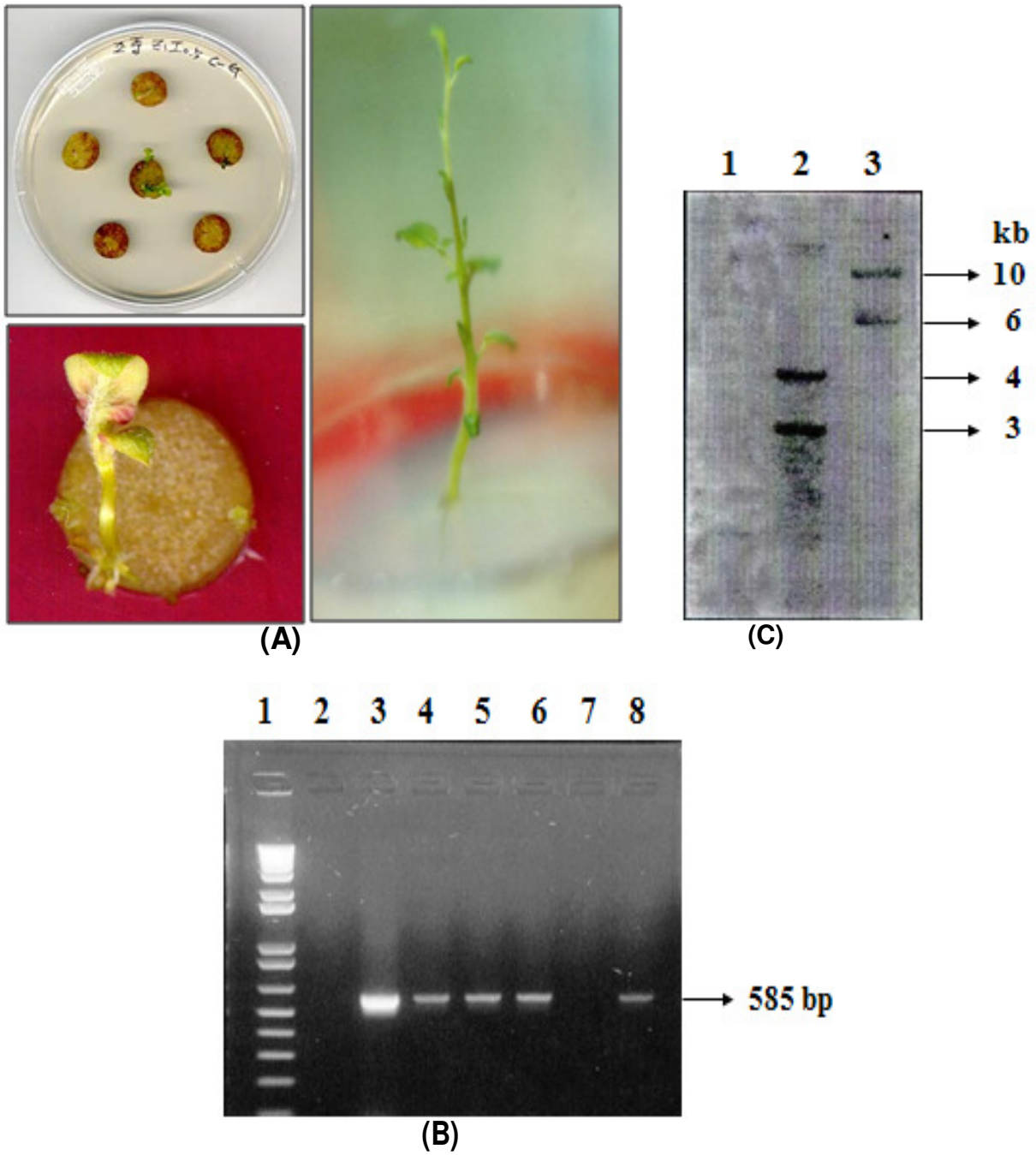

Figure 3. Generation and molecular analyses of potato transgenic plants. (A) Selection and shoot regeneration from transformation with GST on medium containing kanamycin and cefotaxime,(B) PCR analysis were performed on transgenic T_0 potato plants, using Gh-5 gene-specific primers. Lane 1, 1-kb DNA ladder (Invitrogen, San Diego, CA), lane 2, Control plants, lanes 3–6 and 8, nine independent transgenic lines, lane 7, non-transgenic line, (C) Southern blot analysis of T_0 progeny: 20 µg-*Hind*III digest of genomic detected the integrated Gh-5 gene.

manipulation than leaf explants and are more amenable to *in vitro* cultivation (De Block, 1988). Nearly all transformed cells are located near the point of incision in the pericycle tissue (Higgins et al., 1992). Longitudinal incisions on internodes significantly increase the accessibility of the vascular bundle zone to *Agrobacterium*, resulting in a large number of transgenic buds. Explants of cultivated potato *S. tuberusom* cv. Aula and wild *S. stoloniferum* have been established from tubers and subsequently transferred to tissue culture conditions (Hamidoghli and Ward, 2006). Inoculation of explants into an *Agrobacterium* suspension enhances attachment of

the bacterium to plant tissues (Yong et al., 2006). This is also the case for *Cyclamen persicum* (Aida et al., 1999).

Prolonged inoculation (15 min) results in efficient transformation in trifoliate orange (Kaneyoshi et al., 1994; Drake et al., 1997). High transformation efficiencies are also obtained when explants for co-cultivation are inoculated with acetosyringone (Shimoda et al., 1990; Chen and Winans, 1991).

Conclusion

In conclusion, we have developed an efficient system forproducing transgenic potato using *Agrobacterium*-mediated transformation. Acetosyringone concentration and infection time were important factors for increased transformation efficiency. This transformation protocol resulted in the regeneration of morphologically normal plants with over-expression of the Gh-5 transgene. Thus, the protocol presented here may represent a guideline for further stable transformation of various potato cultivars.

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