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Agrobacterium Mediated Transformation of *Rehmannia glutinosa* L. with Glutathione S-Transferase Gene (*Gh*-5)

Jung Dae Lim*, Eun Soo Sung**, Deok Chun Yang***, Song Joong Yun****, Ill Min Chung*****, Myong Jo Kim*, and Chang Yeon Yu*†

*College of Agriculture and Life Sci., Kangwon Natl. Univ., Chunchon 200-701, Korea.
**Genome Research Center, KRIBB, P. O. Box 115, Yusung, Taejon 305-600, Korea.
***College of Life Science, Kyung Hee University, Yongin 449-701, Korea.
****Faculty of Biological Research Science, Chonbuk Natl. Univ., Chonju 561-765, Korea.
*****College of Life and Environment Sci., Konkuk University, Seoul 143-701, Korea.

ABSTRACT : Using *Agrobacterium*-mediated transformation method the auxin-regulated cotton GST (*Gh*-5) constructs were used to transform *Rehmannia glutinosa* L. The PCR analysis was conducted to verify transgenicity. Based on the PCR analysis, there was verified that the 988 bp DNA band had showed in transgenic plant genomes in PCR analysis using Gh5-1 and Gh5-2 primers. The effects of cocultivation with *Agrobacterium tumefaciens*, regeneration and selection conditions on the transformation efficiency of Chinese foxglove (*Rehmannia glutinosa* L.) were investigated. Factors such as cocultivation period, use of acetosyringone, postcultivation in darkness, and different kanamycin concentrations for selection were assessed. *In vitro* regeneration, the number of leaves, shoot lengths and numbers on MS medium were superior to on B5 and WPM medium, and the shoot formation rate was highest level of 95% in cultured base part containing leaf stalk. Addition of acetosyringone at concentration of 200 μM to cocultivation medium and 3-day of cocultivation improved transformation frequencies. Exposure of explants to darkness for 4 weeks on selection medium resulted in further increased the regeneration frequency of transgenic shoots. In PCR analysis, the amplified fragments of Gh5 gene were detected (988 bp), and GST-expressing transgenic *R. glutinosa* L. plants had approximately three-fold higher activity in leaf extracts compared with control plant.

Key words : *Agrobacterium, Rehmannia glutinosa* L., glutathione S-transferase, cocultivation, acetosyringone

INTRODUCTION

Glutathione S-transferase (GST: EC 2.5.1.18) are a super family of enzymes that conjugate reduced glutathione to a wide variety of compounds that are lipophilic and have an electrophilic center (Coles & Ketterer, 1990; Mannervik & Danielson, 1988; Pickett & Lu, 1989). Multiple GST isozymes are present in most plants. This reaction yields a GSH conjugate that is often inactive, water-soluble, and is usually less toxic than the parent compound (Droog *et al.*, 1995; Itzhaki & Woodson, 1993).

Almost all of the known cytosolic GSTs are homo- or hetero-dimers of subunits with molecular weights of 25~29 kDa (Droog, 1997). Distribution of GST is ubiquitous and GST presumably evolved with GSH in aerobic organisms to protect the cells from oxidative damage and electrophilic attack. These enzymes are distributed in a wide range of organisms ranged from E. coli to mammals (Mannervik & Danielson, 1988). Also, GSTs serve in the intracellular detoxification of mutagens, carcinogens and other toxic compounds

† Corresponding author : (Phone) +82-33-250-6411 (E-mail) cyyu@kangwon.ac.kr
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(Daniel, 1993; Mannervik & Danielson, 1988; Pickett & Lu, 1989). They catalyze the conjugation of reduced glutathione with reactive electrophilic and hydrophobic molecules, making these compounds inactive and immobilized. In mammalian systems, increased levels of expression of several related GST isozymes are known to protect the cells from structurally diverse electrophiles that are present in our environment or are used in cancer chemotherapy. GSTs are play an important role in the phase II of conjugation with xenobiotics, and it has another role as glutathione peroxidase. Those tagged conjugates are transported to the vacuoles or apoplast in much less toxic than the parent compounds. Oxygen radicals are highly harmful to the cell components. Those toxic reactive oxygen species can change the property of DNA, lipid layer, and protein.

In plants, GSTs play a major role in the detoxification of herbicidal compounds. Recently, a group of GSTs have been reported to be associated with various stress responses in plants including pathogen attack and heavy metal toxicity. They are also involved in the synthesis of secondary products such as anthocyanins and cinnamic acid (Marrs *et al.*, 1995) and are reported to modulate the activity and uptake of auxins from membranes, and trafficking auxins to receptors (Jones, 1994). GSTs have been implicated in protection from oxidative damage. Bartling *et al.* (1993) reported the cloning of a GST from *Arabidopsis thaliana* that also exhibited glutathione peroxidase (GPX: EC 1.11.1.9) activity.

Chinese foxglove, *Rehmannia glutinosa* L. is an important medicinal plant in Korea, Japan, and China and it called di-huang, or "yellow earth". Rehmanniae radix (the dried root of R. glutinosa L.) is a popular herbal medicine and is commonly used in clinics. This herbal medicine used to replenish vital force and helps with diabets, constipation (Oshio *et al.*, 1981; Hasegawa *et al.*, 1982), urinary tract problems, anemia, dizziness, and regulating menstrual flow. Also it is protective to the liver and helps prevent the depletion of stored glycogen, which can make it beneficial for hypoglycemia. It can lower glucose levels and helps to reduce blood pressure while increasing circulation to the brain. Chinese foxglove has an antifungal effect and has been used for Candida, and helps disperse heat from the body and alleviates night sweats and fevers.

In the recent years, advance in plant genetic engineering have opened a new avenue for crop improvement and various transgenic plants with novel agronomic traits have been produced (Dennis & Llewellyn, 1991). At the present time, various methods and different approaches for the genetic transformation of plants have been developed. *Agrobacterium*-mediated gene transfer is one of the major techniques which has allowed the routine production of transgenic plants. Although many examples have been reported *Agrobacterium*-mediated transformation of medicinal plants, there are relatively few reports of *Agrobacterium*-mediated gene delivery to oriental medicinal plants (Park *et al.*, 2002). The development of methods for introducing modified genes into plants could accomplish two main objectives: rational genetic improvement of agronomically important crop species and/or better understanding of plant gene expression and function.

The success of plant genetic engineering since the efficiency of *Agrobacterium*- mediated transformation is considered to be dependent on two primary factors, such as infection frequency of *Agrobacterium* and the regeneration frequency from the infection tissue is determined by an efficiency tissue culture system. However, for certain plant species it is not easy to establish a transformation system which is sufficiently reliable and efficient to be routinely used in the laboratory. This is especially true for medicinal plants.

In this study, experiments were designed to determine the optimal condition for *Agrobacterium*-mediated GST gene transformation and expression and to develop stress resistance plant by overexpression of GST gene on *R. glutinosa* L. This work was to develop a more efficient and reliable transformation system for chinese foxglove that could be routinely used for genetic improvement. For this purpose, factors directly affecting *Agrobacterium tumefaciens* virulence and explant cell competence for transformation, as well as factors affecting the regeneration phase by favoring callus formation and

the development of transformed shoots, were studied in detail.

MATERIALS AND METHODS

Plant material and culture media

Leaves (divided to base part containing leaf stalk, intermediate, tip containing spine) and stem explants obtained from *R. glutinosa* grown *in vitro* were used for this study. *In vitro* regeneration and growth of plant materials were done on MS, WPM and B5 basal medium containing different type and various concentrations of plant growth regulators and maintained with a 16-h photoperiod under illumination at 45 μmol/m^{2}s^1 and 60% relative humidity at 25℃ for 3 additional weeks. The BAP, TDZ, 2iP, kinetin, NAA and 2,4-D were used at the concentration of 0.01, 0.1, 1, 2 mg/ℓ and combination at TDZ 0.1 or 1 mg/ℓ, 2iP 0.5 or 1 mg/ℓ and NAA 0.1 mg/ℓ with 2 concentration levels (0.5, 1 mg/ℓ) of BAP. For selection of putative transgenic plants efficiently during transformation procedure, kanamycin resistance test was performed. The kanamycin were used at the concentration of 0, 20, 30, 50, 75 and 100 mg/ℓ, and it added to MS salt containing 3% Sucrose + 1 mg/ℓ TDZ + 1 mg/ℓ BAP + 0.1 mg/ℓ NAA. Leaf and stem disk were placed to this medium and the survival rate investigated after 30 days.

Gene cloning and construction

A partial GST cDNA fragment Gh5 was identified at the Dr. Allens laboratory in a screen of cotton fiber specific cDNA library. This partial cDNA fragment was used as a probe for Nothern blot analysis to confirm the GST mRNA expression. Both the cDNA library vector primer (T7) and 3 oligonucleotide primer from the partial cDNA fragment were synthesized and used to amplify the putative GST cDNA by PCR-based cDNA library screening. For Gh5 cloning, the 5primer (5'-ATTATGCTGAGTGATATCCCGCT-3') was designed and the 3 primer has the sequence 5'-TGGTCAAGAGCCAAGAAATA-3'. Using a specific cDNA library PCR-based cDNA library screening was performed. PCR analysis was carried out for 35 cycles at 94℃ for 1 min., 55℃ for 1 min., and 72℃ for 1.5 min. The PCR products were analyzed on a 1% agarose gel, and amplified fragments were ligated into the pCR 2.1 cloning vector (Invitrogen, San Diego, CA). Transformation was carried out using DH-5-competent cells (Invitrogen, San Diego, CA) and plated on LB medium with 5-bromo-4-chloro-3-indlyl-B-D-galactosidase (X-gal) and isopropyl B-D-thiogalactopyranoside (IPTG). White clonies were selected for plasmid isolation and digested with *Eco*RI to release the insert. After transformed to DH-5 competent cells, amplified cDNA fragment was used for further analysis, such as to develop gene constructs for the expression in *E coli* and in transgenic plant. The Gh5 gene construct was developed by isolating the cDNA fragments from the pCR 2.1 vector. The cloned cDNA fragment was digested with *Nco*I/*Sac*I and ligated into the *Nco*I/*Sac*I sites of the pRTL 2 expression vector. That vector carries an enhanced CaMV 35S promoter, a tobacco etch' virus robosomal binding sites, and a 35S-terminator polyadenylation signal (Fig. 1.). The NT107 gene cassette was excised as a Sph I fragment and ligated into the binary plant transformation vector pCGN 1578 to create pCGN-G. The pCGN-Gh5 plasmid was transformed into *Agrobacterium tumefaciens* strain EHA 101. A single colony was inoculated into 3

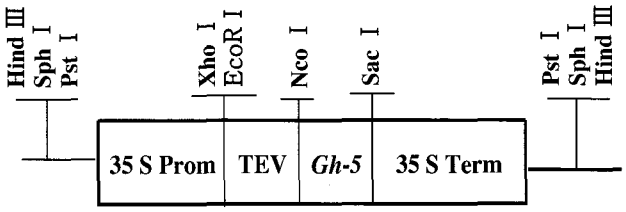

Fig. 1. The development of the sense *Gh*-5 construct. Cloned cDNA fragment was digested with *Nco*I/*Sac*I and ligated into the *Nco*I/*Sac*I sites of the pRTL 2 expression vector. This vector carries an enhanced CaMV 35S promoter, a tobacco etch virus ribosomal binding site, and a 35S-terminator polyadenylation signal. The Gh-5 gene cassette ligated into the binary plant transformation vector pCGN 1578 to create pCGN-Gh-5. The pCGN-Gh-5 plasmid was transformed into Agrobacterium strain EHA 101.

㎖ of LB medium with 100 ㎎/ℓ kanamycin. The culture was grown overnight at 28℃ in a shaking incubator. *Agrobacterium* strain was diluted in 10:1, and used to inoculate *R. glutinosa* L. leaves.

Transformation of *R. glutinosa*

In vitro-cultured *R. glutinosa* were cut in leaf tissues, base part containing leaf stalk segments (7×7 ㎟) transversely from 5 week old. The explants were incubated for 15 min in 10 cm diameter plates containing 15 ㎖ of the bacterial suspension in liquid cocultivation medium, consisting of MS salt, 1 ㎎/ℓ BAP, 1 ㎎/ℓ TDZ, 0.1 ㎎/ℓ NAA, MS vitamin, 3% sucrose, and 0.8% agar at pH 5.8. The infected explants were blotted dry on sterile filter paper and placed horizontally on cocultivation medium. To assess factors affecting the transformation frequency, different treatments were performed. (1) The explants were cocultivated with Agrobacterium on solid cocultivation medium for 1, 2, 3, 4 or 5 days. (2) Cocultivation was done on cocultivation medium or cocultivation medium plus acetosyringone (0~400 ㎛ M). The explants were precultured for 2 days, and then inoculated with the bacteria, blotted dry and transferred to plates for cocultivation. All the experiments were repeated at least twice. After cocultivation, the explants were blotted dry with sterile filter paper and transferred to selection medium, consisting of MS salt, 1 ㎎/ℓ BAP, 1 ㎎/ℓ TDZ, 0.1 ㎎/ℓ NAA, MS vitamin, 3% sucrose, and 0.8% agar at pH 5.8, supplemented with 50 ㎎/ℓ kanamycin for selection and 250 ㎎/ℓ cefotaxime to control bacterial growth. (3) For effect of darkness period during selection, the plate were maintained in the dark for 10 weeks. The plates were transferred to 16 h photoperiod, 45 ㎛ol m^{-2} s^{-1}, 60% relative humidity and 26℃. The explants were subcultured to fresh medium every 4 weeks. Shoots longer than 3 ㎝ were sub-cultured on MS and 1/2 MS media supplemented with kanamycin for root induction.

PCR analysis

In order to determine whether the GST gene was introduced, DNA of kanamycin-resistant plantlets was extracted with CTAB (cetyltrimethyl ammonium bromide) method (Murray and Thompson, 1980). Selection marker *npt*II gene was detected with N-1 (5'-GAAGCTATTCG GCTATGACTG-3') and N-2 (5'-ATCGGGAGCGGCGATACCCTA-3'). PCR reactions were performed with TOUCHDOWNTM (HYBRID), and amplification conditions were: 45 cycles of pre-denature (at 94℃, 5 min.), denaturation (at 94℃, 1 min.), annealing (at 35℃, 1 min.), and extension (at 72℃, 2 min.), 40 cycle of postelongation (at 72℃, 10 min.). To analyse the introduction of Gh5 cDNA fragment in the *R. glutinosa* genome, DNA was extracted and PCR was performed by same method to do *npt*II gene detection. The primers were Gh5-1 primer, 5'-ATTATGCTGAGTGATATCCCGCT-3' and Gh-2 primer, 5'-TGGTCAAG AGCCAAGAAATA -3'. The amplification cycle consisted of denaturation at 94℃ for 1 min., primer annealing at 55℃ for 1 min, and primer extension at 72℃ for 1 min. 30 sec. After 40 repeats of the thermal cycle and final extension at 72℃ for 10 min., the amplified DNAs were run on a 1.5% agarose gel and stained with ethidiumbromide. DNA bands were visualized under long-wavelength ultraviolate light. PCR analysis was carried out to confirm the successful insertion of Gh5 cDNA on the chromosome of transgenic *R. glutinosa* L. plants.

GSt enzyme assay for transgenic plants

Activities of GST enzyme from the transgenic and control *R. glutinosa* L. plants were evaluated by determining kinetic parameters with respect to the model substrate 1-chloro-2,4-dinitrobenzone (CDNB) according to the procedure of Dean *et al.*, (1990). Samples were collected and homogenized by grinding with sodium-phosphate buffer in the microcentrifuge tube. The mixture was then centrifuged at 12,000 rpm for 10 min. at 4℃, and the supernatant was used as crude enzyme extract. The reaction mixture contains final concentrations of 20 mM reduced glutathione (GSH) and 20 mM CDNB, which is dissolved in 2.5% ethanol, and potassium-phosphate buffer (0.1 M, pH 6.5). GST activity was calculated by measuring the absorbance at 340 ㎚ and an extinction coefficient of 9.6 mM/㎝. The protein content of samples was determined by Bradford reagents.

RESULTS AND DISCUSSION

In vitro regeneration

The best combinations of cytokinin and auxin for regeneration were 0.5 ㎎/ℓ of BAP, 0.5 ㎎/ℓ of TDZ, 0.1 ㎎/ℓ of 2iP and 0.1 ㎎/ℓ of NAA for regeneration (data not shown). After 4 weeks culture on MS, B5 and WPM medium combined with growth regulators (1 ㎎/ℓ of BAP and 0.1 ㎎/ℓ of NAA), leaf number, shoot length and number on MS medium were superior to on B5 and WPM medium. The shoot number was highest on MS medium and 4.2 shoots per tube, but the numbers of shoot observed on B5 and WPM, were 3.5, and 3.0 shoots per tube respectively. In an experiment, shoot length and the number of leaf were appeared similar to the result of shoot length (Fig. 2).

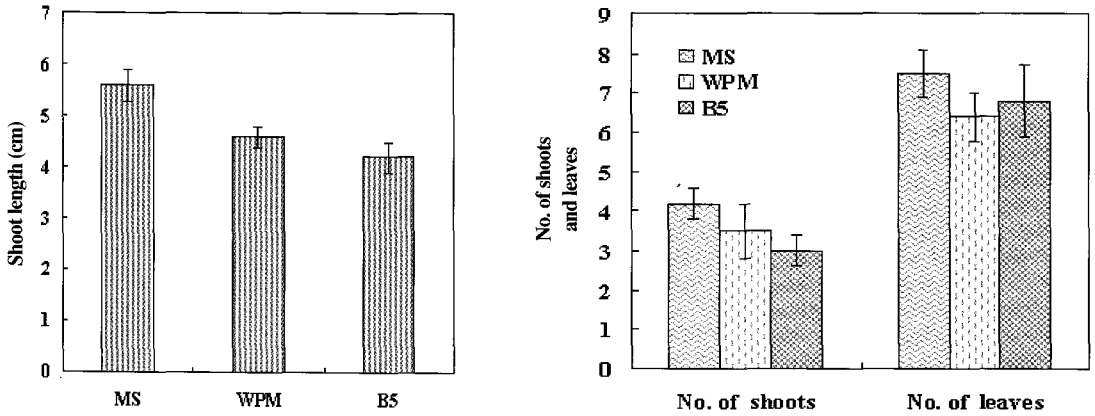

Fig. 2. Effect of media on shoot regeneration from leaf cultured in *Rehmannia glutinosa* for 45 days.

To investigate the shoot formation and regeneration in different part of leaf, it divided to base part containing leaf stalk, intermediate, and tips containing leaf blade and cultured on a half of MS salt containing 0.5 ㎎/ℓ BAP, 0.5 ㎎/ℓ TDZ, and 0.1 ㎎/ℓ 2iP. In base part containing leaf stalk, shoot formation rate was highest level of 95% than other parts. When intermediate part and tips containing leaf blade of leaf were cultured on same media, their shoot formation rate was decreased to 52% and 15% respectively (Fig 3). However, Debergh and Wael (1997) reproted that leaf blade and intermediated containing central vein were optimal parts on shoot formation in Ficus lyratas. In this result, we suggested that each organ part showed different reaction pattern according to organ softness degree in vitro culture system.

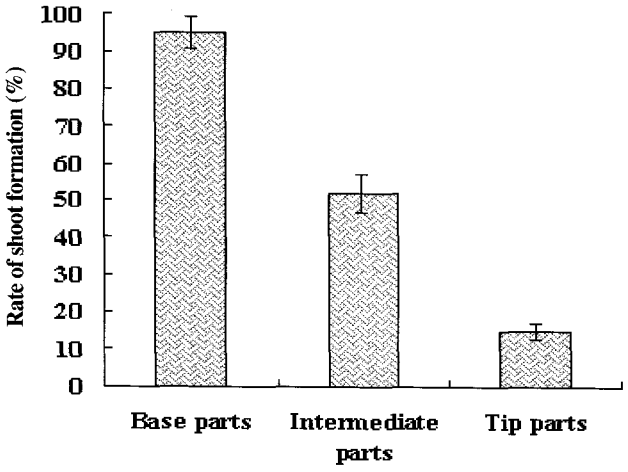

Fig. 3. Comparison of shoot formation rate by different parts of leaf from *Rehmannia glutinosa*.

Kanamycin resistance test

The effectiveness of kanamycin as selective antibiotic in the regeneration of *Agrobacterium*-inoculated and non-inoculated *R. glutinosa* L. explants was shown in Table 1. Whereas 75 or 100 ㎎/ℓ kanamycin completely inhibited shoot formation in noninoculated control explants, but shoots regenerated were very weak and induced necrosis in leaf tissue. Regenerated shoots were obtained at high frequency from explants exposed to 30 ㎎/ℓ kanamycin, but the fragments of Gh5 gene were not detected in the

Table 1. The ratios of kanamycin resistance of leaf and stem explant in *Rehmannia glutinosa*.

Medium	Explant	Kanamycin (㎎/ℓ)	No. of explant	No. of survival explant	Rate of survival (%)
MS + TDZ 0.5 + BAP 0.5 + 2iP 0.1 + NAA 0.1	Leaf	0	75	75	100
		20	75	35	47
		30	75	25	33
		50	75	5	5.3
		75	75	1	1.3
		100	75	0	0
	Stem	0	75	75	100
		20	75	30	40
		30	75	18	24
		50	75	3	4
		75	75	0	0
		100	75	0	0

shoots regenerated from the explants cocultivated with *Agrobacterium* (data not shown). It was suggested that kanamycin at 50 ㎎/ℓ was effective for transformation.

Transformation cocultivation period

Fig. 4 shows the influence of the cocultivation period on transformation of *R. glutinosa* L. explants. When the explants were transferred to selective medium immediately after inoculation with *Agrobacterium* (no cocultivation), no transformation was observed. The transformation frequency was very low after 1 day cocultivation, but increased rapidly when the coculture was prolonged to 2 days, reaching a maximum at day 3. Although prolonged cocultivation periods of more than 3 days have been successfully used for certain plants (Jia *et al.*, 1989; Dong *et al.*, 1991; Mourgues *et al.*, 1996), 2~3 days cocultivation has been routinely used in most reported transformation protocols, since longer cocultivation periods frequently result in *Agrobacterium* overgrowth. In *R. glutinosa* L., 5-day cocultivation also resulted in abundant proliferation of the bacteria and a subsequent decrease in the regeneration frequency of transformed shoots (data not shown). Therefore, a 3 day cocultivation was routinely used.

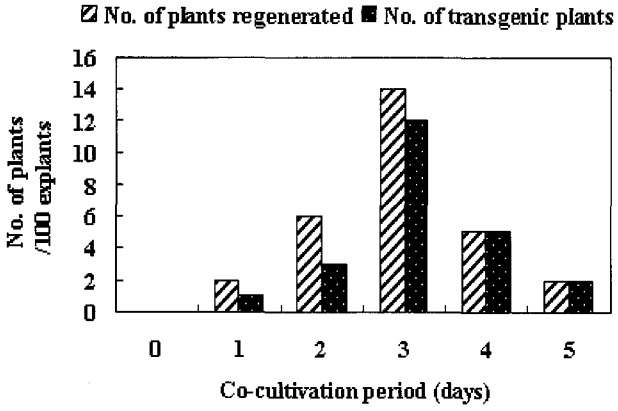

Fig. 4. Influence of co-cultivation period on transformation of *Rehmannia glutinosa* explants.

Influence of acetosyringone as transformation enhancer

The use of acetosyringone during cocultivation has been shown to increase *Agrobacterium*- mediated transformation frequencies (Sheikholeslam & Weeks, 1987). Fig. 5 shows that the addition of acetosyringone at concentration of 200 ㎛M to cocultivation medium increased six fold transformation frequency in *R. glutinosa* L. explants.

Acetosyringone is a phenolic compound produced during wounding of plant cells that induces the transcription of the virulence genes of *A. tumefaciens*. Its beneficial role has been demonstrated in the genetic transformation of some woody fruit species, such as apple (James *et al.*, 1993) and kiwifruit (Janssen & Gardner, 1993). Kaneyoshi *et al.* (1994)

have also reported the use of acetosyringone during cocultivation of *Poncirus trifoliata* explants with *Agrobacterium*. However, its role as a transformation enhancer was not determinated clearly, it was supposed as signals for *VirA* activation. The T-DNA tnransfer is mediated by products encoded by the 30 ~40 kb vir region of the Ti plasmid. This region is composed by at least six essential operons (*virA, virB, virC, virD, virE and virG*) and two non-essential (*virF and virH*). The only constitutive operons are *virA* and *virG*, coding for a two-component (*virA-virG*) system activation the transcription of the other vir genes. *VirA* is a transmembrane dimeric sensor protein that detects signal molecules, mainly small phenolic compounds, released form wounded plants (Pan *et al.*, 1993). The signals for *VirA* activation include acidic, pH, phenolic compounds, such as acetosyringone (Winans *et al.*, 1992), and certain class of monosaccharides which acts synergistically with phenolic compounds (Ankenbauer *et al.*, 1990; Cangelosi *et al.*, 1990; Shimoda *et al.*, 1990). We have shown here that *R. glutinosa* L. explants responded positively to the presence of acetosyringone in activation of various *vir* genes for trasformation frequency.

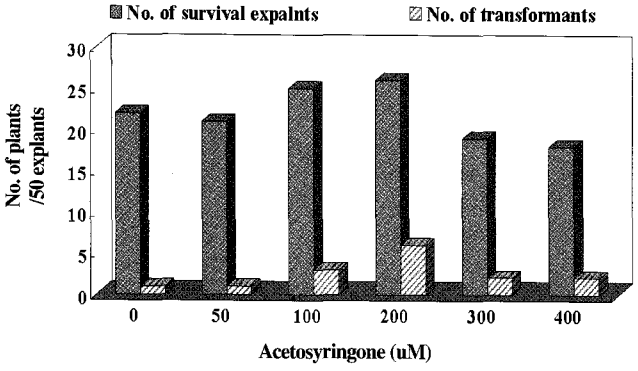

Fig. 5. Effect of acetosyringone concentrations on transformation in *Rehmannia glutinosa*. Explants were cocultured on feeder plates of co-culture for 3 days.

Postcultivation in darkness

As it is shown in Fig. 6, culture in darkness clearly improved shoot induction from non-inoculated *R. glutinosa* L. explants. Callus formation (data not shown) and consequent regeneration were progressively increased by maintaining the explants for 2 and 4 weeks in darkness and then transferring them to the light. However, when the explants were kept in the dark for 8 weeks, excessive callus was formed which was detrimental for shoot regeneration. In transformation experiments, the frequency of transgenic shoots regenerated, as well as shoot regeneration, also increased from 2 to 4 weeks and decreased from 8 to 10 weeks of dark incubation, as did the frequency of total regenerated shoots. However, the highest frequency of shoot regeneration was obtained when the explants were directly transferred to the light after cocultivation with *Agrobacterium* (Fig. 6). In this case, shoots arose from the explants with little or not detected GST gene by PCR.

Exposure of explants to darkness has also been used to stimulate regeneration of transgenic shoots in other plant, sweet orange (Pena *et al.*, 1995), apple (Maheswaran *et al.*, 1992; James *et al.*, 1993) and pear (Mourgues *et al.*, 1996). Here, we have confirmed a stimulatory effect of dark exposure of explants on the regeneration of transgenic shoots. Furthermore, our results suggest that high number of escapes shoots can occured in potential transgenic plants when exposed explants directly to the light after cocultivation.

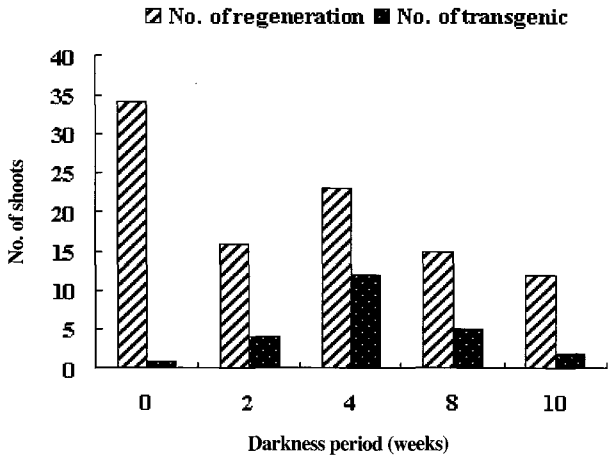

Fig. 6. Effect of darkness on the regeneration and transformation in *Rehmannia glutinosa*. Explants were cocultured on feeder plates for 3 days and then transferred to the selection medium.

PCR analysis

PCR analysis was carried out to confirm the insertion of Gh5 fragment on the chromosome which was isolated from the transgenic and wild type *R. glutinosa* L. plants (Fig. 7). The amplified fragments of Gh5 gene were detected on the agarose gel as the anticipated size from the transgenic plants, but not found from the wild type plant (988 bp).

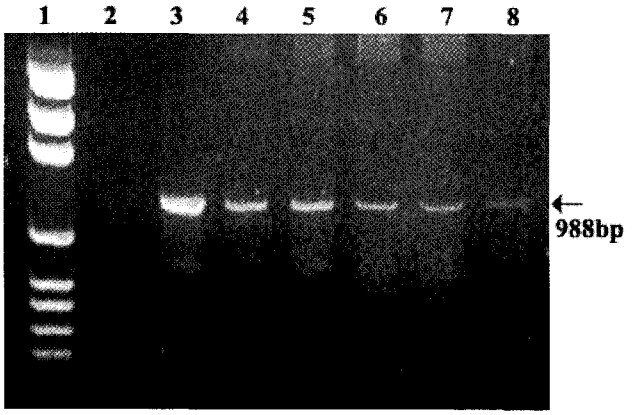

Fig. 7. Detection of C-GST gene in transgenic plants by using *Gh5*-1, and *Gh5*-2 primer.
Lane 1 : pGEM marker DNA.
Lane 2 : PCR product of non-transformant plant.
Lane 3 : PCR product of C-GST gene fragment.
Lane 4~8 : putative transgenic plants selected in medium containing Km 50 mg/ℓ .

GST activity

Levels of total GST specific activity were assayed in transgenic and wild type plants, using CDNB as a substrate. GST-expressing transgenic *R. glutinosa* L. plants had approximately three-fold higher activity in leaf extracts compared with control plant (Table 2). The results from this assay confirmed that Gh5 transgenes encode an active GST in transgenic *R. glutinosa* L. plants.

Table 2. Comparison of GST activities of transformant in *Rehmannia glutinosa* L.

Lines	GST activity (㎛㏖/min. · mg†)
Control	4.73±0.54
Transgenic Plant (GST)	13.58±0.43
LSD.05	0.259

†Enzyme activity: Calculated the enzyme activity using extinction coefficient of 9.6 mM change/min.

In this study, various factors affecting genetic transformation of *Rehmannia glutinosa* L. were investigated in *Agrobacterium* mediated gene transformation system. The results suggested possibilities that overexpression of cotton GST (Gh5) in *Rehmannia glutinosa* L. can improve their resistance against environmental stresses. Further analyses of identification of transformation using other molecular techniques and transgenic plants that overexpress GST will yield fundamental information about the effect of oxidative stress due to pathogen infection, low temperature, herbicides etc. We remain hopeful that investigation of this type will eventually provide significant improvements in the tolerance of cultivated resource plants to environmental stress.

ACKNOWLEDGEMENTS

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