

## *Agrobacterium*-mediated Transformation of *Rehmannia glutinosa* L. with Resveratrol Gene (RS3) of Peanut

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

\*College of Agriculture and Life Science, Kangwon National University, Chunchon 200-701, Korea.

\*\*College of Life Science, Kyung Hee University, Yongin 449-701, Korea.

\*\*\*Faculty of Biological Research Science, Chonbuk Natl. Uni., Chonju 561-765, Korea.

\*\*\*\*College of Life and Environment Science, Konkuk University, Seoul 143-701, Korea.

\*\*\*\*\*Genome Research Center, KRIBB, P. O. Box 115, Yusong, Taejeon 305-600, Korea.

**ABSTRACT** : The objectives of this study were to establish the genetic transformation system of stilbene synthase in *Rehmannia glutinosa*. Resveratrol, which is both a phytoalexin with antifungal activity and a phytochemical associated with reduced cancer risk and reduced cardiovascular disease, is synthesized in a limited number of plant species including peanut. Resveratrol synthesis is catalyzed by the enzyme stilbene synthase including resveratrol synthase (RS). Stilbene synthase gene (RS3) obtained from peanut, *Arachis hypogaea*, Fabaceae has been transferred into chinese foxglove, *Rehmannia glutinosa* by using *Agrobacterium* mediated transformation. PCR analysis with RS3 primer confirmed that the targeted gene was introduced into the plant genome, 904 bp in size. Further analyses of identification of transformation using developed other molecular techniques and transgenic plants that RS t-DNA introduced to chinese foxglove (*R. glutinosa* L) and its reaction product, stilbene such as resveratrol will be isolate and characterize using NMR, MS, and HPLC.

**Key words** : *Agrobacterium*, *Rehmannia glutinosa* L., resveratrol synthase

### INTRODUCTION

Stilbene synthase (STS) is plant-specific polyketide synthases that play a pivotal role in the biosynthesis of stilbenes (Schröder, 1999). Stilbene synthases convert p-coumaroyl-CoA to the major products resveratrol, the phenolic compound resveratrol (3,5,4'-trihydroxystilbene) is a non-flavonoid phytoalexin produced by plants in response to fungal infection or stress (Langcake, 1981). They occur in widely unrelated plant families and in some cases only a few species of a large family are able to synthesize these substances. STSs are rare in higher plants and occur in distantly related species such as peanut

(*Arachis hypogaea* L.) (Schoppner & Kindl, 1984), grapevine (*Vitis vinifera* L.) (Sparvoli *et al.*, 1994), pines (*Pinus sylvestris* L., *Pinus strobus* L.) (Schanz *et al.*, 1992; Raiber *et al.*, 1995), and rhubarb (*Rheum palmatum* L.) (Kashiwada *et al.*, 1988).

This enzyme (STS) catalyze a sequential condensation of the C6-C3 unit of 4-coumaroyl-CoA as a starting material with three C2 units from malonyl-CoA. The reaction is thought to be initiated by binding of 4-coumaroyl-CoA followed by three rounds of stepwise decarboxylative condensation with malonyl-CoA to form a tetraketide intermediate, followed aldol-type cyclization and decarboxylation lead to formation of trans-3,4',5-trihydroxystilbene (resveratrol).

† Corresponding author : (Phone) +82-33-250-6411 (E-mail) cyyu@kangwon.ac.kr

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Resveratrol synthase (RS) among STS is present only in the plant species where resveratrol is found (Fritzscheier & Kindl, 1981; Rolfs *et al.*, 1987). Four RS genes are known in peanuts (Lanz *et al.*, 1990; Schroder *et al.*, 1988). The regulation of the RS gene expression was investigated using cell cultures from peanuts and grapevines. The transient RS gene expression was also demonstrated with the same factors by which resveratrol synthesis is induced (Lanz *et al.*, 1990). The induction of RS genes by stresses suggests the possible involvement of stress hormones in the RS gene expression (Boller & Kende, 1980; Dong, 1998).

In plants, stilbenes and their derivatives are regarded as phytoalexins that contribute to the defense against fungal infection (Hain *et al.*, 1993). Stilbenes have significant roles in the resistance of wood against microbial degradation. Phytoalexins are benzo- $\gamma$ -pyranderivatives such as stilbene which are ubiquitous in photosynthesizing cells and they have been used for centuries in folk medicine to treat human diseases such as inflammation, allergy, headache, parodontosis, virus and fungal infection, stomach or duodenal ulcers, and even cancer (Adrian *et al.*, 2000; Palomino *et al.*, 2000). A book (Gorham, 1995) covers most of the information on stilbenes and their functions, STS in transgenic plants has also been used to introduce the capacity to produce new phytoalexins in species that otherwise do not synthesize stilbenes (Hain *et al.*, 1990), and sometimes with the side effect of male sterility (Fischer *et al.*, 1997).

Much attention has been focused on resveratrol and RS (resveratrol synthase) genes because of their implication as an important part of the plant defense system. RS genes were transferred into the plants in which RS is absent in order to provide a defense system against fungal infections. Production of resveratrol has been associated with an increased resistance to various fungal pathogens in transgenic tobacco (Hain *et al.*, 1990; 1993), tomato (Thomzik *et al.*, 1997), rice (Stark-Lorenzen *et al.*, 1997), and wheat (Fettig & Hess, 1999). This finding has directed a lot of attention toward the RS gene expression in other medicinal plant tissues.

Chinese foxglove, *Rehmannia glutinosa* L. is an

important medicinal plant in Korea, Japan, and China and it called "di-huang", or "yellow earth". *Rehmannia radix* (the dried root of *Rehmannia glutinosa* L.) is a popular herbal medicine and is commonly used in clinics. Dried or steamed roots have been used to regulate the immune response, and still occupy an important place in traditional oriental medicine. 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. They were also used as a haemostatic, cardiotonic, and diuretic agent. Recently, it has been reported that the herb possesses antibacterial and anti-inflammatory properties (Huang, 1993; Kubo *et al.*, 1994; Tomoda *et al.*, 1994). Many constituents have been isolated from both the *Rehmanniae Radix* and the fresh plant. The major constituents of the herb are h-sitosterol and mannitol. The other constituents include a small amount of stigmasterol and a trace amount of campesterol, catalpol, rehmannin, and vitamin A (Chang & But, 1986; Ni *et al.*, 1992). Recently, Kim *et al.* (1999) demonstrated that *R. glutinosa* Libosch inhibits the secretion of both interleukin-1 (IL-1) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) from mouse astrocytes.

The biosynthesis of stilbene-like phytoalexins is catalyzed by the key enzyme-stilbene synthase. Although the action substrate of STS generally exists in plants, most of the plant are short of stilbene synthase gene, so they can not synthesize phytoalexins (Rupprich & Kindl, 1978). Experiments with transgenic tobacco suggested that STS formation is mainly regulated at the transcriptional level. The stress-induced signal transduction pathway seems to be conserved between different species (Hain *et al.*, 1990). Transgenic tobacco plants are an excellent model system for investigating the underlying mechanisms of STS gene regulation. Two STS genes (*Vst1* and *Vst2*) from grapevine have been isolated and transferred into tobacco (Hain *et al.*, 1993). Transgenic plants containing these two genes were more resistant to infection by *Botrytis cinerea* (Hain *et al.*, 1993). STS genes had been transferred into oilseed rape (Thomzik, 1993), rice (Stark-Lorenzen *et al.*, 1997), barley (Leckband & Lorz, 1998), etc. by

other researchers, and transgenic plants with enhanced disease resistance were obtained. Although the STS gene was induced from *Vitis* spp., which has been successfully transferred into major crops, reports on RS gene isolated from *Arachis hypogaea* L., transferred into medicinal plants have not been found.

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. However, *Agrobacterium*-mediated gene transfer is one of the major techniques which has allowed the routine production of transgenic plants. More than 100 species have been transformed by this method. Although many examples have been reported of *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 success of plant genetic engineering since the efficiency of *Agrobacterium*-mediated transformation is considered to be dependent on two primary factors: one being the infection frequency of *Agrobacterium* and the other, the regeneration frequency from the infection tissue, is determined by several factors, among which an efficiency tissue culture system, in which plants can be regenerated from cultured cells and tissues at high frequencies is crucial one.

The application of molecular technique to the study of stilbene backbone-polyketide biosynthesis will expand the frontiers of our ability to understand and manipulate these pathway in plants. The prospect to engineer stilbene backbone polyketide metabolism of plants for 'systemic' biosynthesis of pharmaceuticals will be required both a thorough knowledge of the regulation of biosynthetic enzymes and gene, and an availability of cloned genes for the genetic transformation of plants.

## MATERIALS AND METHODS

### Plant materials

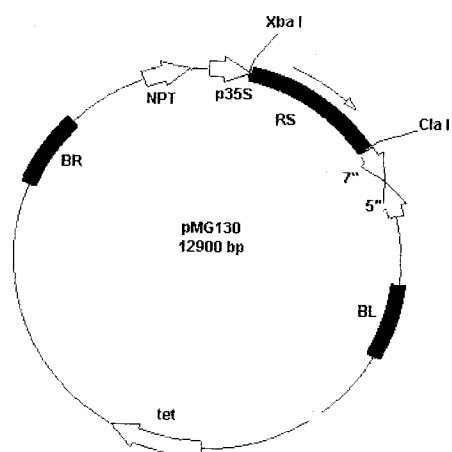
Chinese foxglove plants (*Rehmannia glutinosa* L.)

were grown at the green house. Young leaves of *R. glutinosa* were taken from grown plants. In vitro regeneration and growth of plant material were done on MS (Murashige & Skoog, 1962) basal medium and 2,4-D, NAA, TDZ, and BAP were used at a concentration of 0.01, 0.1, 2, 4 mg/l, respectively. Explants were surface sterilized by a 10 sec immersion in 70% (v/v) ethanol and for 10 min. in aqueous solution of 1% (v/v) NaClO<sub>3</sub> containing a few drops of Tween 20. After rinse five times in sterilized water, leaves were cut aseptically at the ends, in sections 7 × 7 mm<sup>2</sup> in size.

### Expression and transformation vectors

A partial STS cDNA fragment RS3 (AF227963) was identified at Dr. Yun's laboratory in a screen of peanut (*A. hypogaea*) pods and leaves specific cDNA library (Chung *et al.*, 2001) *Escherichia coli* strain XL1 Blue (Stratagene, USA) was used for cloning by the standard techniques (Sambrook & Russell, 2001). Putative RS genomic and cDNA sequences were amplified from peanut genomic DNA and total RNA using the forward (5'-ATAACTAGTATGGTGTCTGTGAGTGGAA TTCG-3') and reverse (5'-GATCTCGAGTTATATGG CCACACTGCGGAG-3') primers that were targeted at the 5'- and 3'-ends of the open-reading frame (ORF) in the peanut RS mRNA sequences. Genomic DNA was prepared from peanut leaves and total RNA from the developing shells that contained seeds as described (Roger & Bendich, 1989; Sambrook & Russell, 2001).

Genomic PCR and RT-PCR were performed using the TaKaRa *Ex Taq* (Bohan Biomedical, Korea) and Titan RT-PCR system (Roche, Germany), respectively, with a primer annealing at 57°C for 30 sec. The specifically amplified DNA and cDNA fragments were cloned into a pGEM-T Easy vector (Promega, USA). The nucleotide sequences of the resulting clones, pPRS3G and pPRS3C, were determined as described (Sanger *et al.*, 1977). The RS3 cDNA fragment was isolated from the pGEM-T Easy vector by digesting with *Xba* I/*Cla* I and introduced into the *Xba* I/*Cla* I sites of the expression vector pGA643 to produced a recombinant plasmid named as pMG130. Subsequently, *E. coli* strain JM109 was transformed with pMG130 (Fig. 1).



**Fig. 1.** Diagram of the T-DNA region of pMG130 BR, right border; BL, left border; *nptII*, neomycin phosphotransferase II; p35s, CaMV 35S promoter; and RS, coding region of RS3.

#### Agrobacterium-mediated plant transformation

For plant transformation, RS3 expression cassette was excised as a *Xba*I/*Cla*I fragments and ligated into the binary plant transformation vector pGA643 to created pMG135. RS3 gene construct in pMG135 was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by direct transformation. Inoculation of chinese foxglove (*Rehmannia glutinosa* L.) leaf disk was performed following the protocol of Park *et al.* (2002). A single colony was grown overnight at  $28 \pm 1^\circ\text{C}$  with shaking (180 rpm) in liquid Luria-Bertani medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, pH 7] containing 50 mg/l of kanamycin, until mid-growth phase. The *A. tumefaciens* cells were collected by centrifugation for 10 min. at 1500 g and resuspended in liquid inoculation medium (Murashige & Skoog salt with 20 g/l of sucrose). The *A. tumefaciens* cell density was adjusted to  $\text{OD}_{600\text{nm}}$  1 for inoculation. In vitro-cultured *R. glutinosa* was cut into leaf tissues, and cultured for two to three days in a pre-treatment MS medium containing 1 mg/l BAP, 2 mg/l TDZ, 0.2 mg/l NAA, MS vitamin, 3% sucrose, and 0.8% agar (pH 5.2). Pre-treated leaves were dipped into the *Agrobacterium* suspension in liquid inoculation medium for 10~15 min., blotted dry on sterile filter paper and incubated on shoot induction medium (MS medium containing 2 mg/l BAP, 1 mg/l TDZ, 0.2 mg/l NAA, MS vitamin, 3%

sucrose, and 0.8% agar at pH 5.2.) in dark treatment. After cocultivation for two days, explants were transferred to shoot induction medium containing 50 mg/l kanamycin and 200 mg/l timentin (selection medium) and were transferred to fresh selection medium every two weeks. Putative transgenic shoot regeneration was observed at 6~8 weeks after the first sub-culture. Shoots longer than 3 cm were sub-cultured in the MS and 1/2 MS media supplemented with kanamycin and timentin for root induction. Putative transgenic shoots were incubated at  $23 \pm 1^\circ\text{C}$  in a growth chamber with a 16 hr photoperiod under standard cool white fluorescent tubes ( $35 \mu\text{mol/s}^{-1} \cdot \text{m}^2$ ) for 30 days. Putative transgenic plantlets were then transferred to the pots containing autoclaved vermiculite, covered with polyethylene bags to maintain high humidity and kept at  $23 \pm 1^\circ\text{C}$  in a growth chamber for one week. After one week, the bags were perforated and these plants were then transferred to the green house.

#### PCR analysis

In order to determine the RS3 gene was introduced, genomic DNA from 200 mg each of control (non-transgenic) plants and all putative of kanamycin-resistant plantlets were extracted from leaves with CTAB (cetyltrimethyl ammonium bromide) method. The sequence information derived from RS3 gene (resveratrol synthase 3) and designed degenerated oligonucleotides of corresponding genes from *R. glutinosa* L. genomic DNA for PCR amplification were utilized. Selection marker *nptII* gene was detected with N-1 (5'-GAAGCTATTCGGCTATGACTG-3') as the sense primer and N-2 (5'-ATCGGGAGCGCG ATACCCTA-3') as the antisense primer. The primers RS3-1 (5'-AGGCACCGTCGTTGGATGCAAGG-3') as the sense primer and RS3-2 (5'-GGCCACACTGCG GAGAACAACGG) as the antisense primer, were used to yield a 904 bp internal resveratrol synthase 3 fragment. PCR reactions were performed with PCR Express (HYBRID) in reaction mixture and heated to  $94^\circ\text{C}$  for 5 min. Amplification conditions were 35 cycle each consisting of 1 min. at  $94^\circ\text{C}$ , 1 min. at  $60^\circ\text{C}$  and 1 min. 30 sec. at  $72^\circ\text{C}$  for *nptII* gene. For resveratrol synthase 3, PCR was performed with denaturation at

94°C for 1 min., annealing at 59°C for 1 min., and extension at 72°C for 1 min. After 35 repeats of the thermal cycle and final extension at 72°C for 5 min., amplification products were analyzed on 1% (w/v) agarose gels. Gels were stained with ethidium bromide and visualized with UV light.

## RESULTS AND DISCUSSIONS

### Transformation with *Agrobacterium*

Four to eight weeks old explant tissues were pre-treated for two days before they were co-cultured with *Agrobacterium* for two days and incubated for 8 weeks followed by selection of regenerated plantlets. Roots were induced after the regenerated plantlets were transferred to MS basal medium supplemented with kanamycin. It has been reported earlier that pre-treatment did not affect transformation of *Daucus*, *Nicotiana*, and *Petunia*, and that it enhances transformation rate for *Arabidopsis* and *Datura* (Pawlick *et al.*, 1992). In *R. glutinosa*, pre-treatment seemed to enhanced the transformation rate. Two days of co-culture for *R. glutinosa* was found to be acceptable. GUS activities reached the highest point at 6th day for *Kalanchoe laciniata* (Jia *et al.*, 1989), whereas, the transformation rate for carrots (*Daucus carota* L.) reached the highest point between the 2nd and 3rd day and showed declined rate after the 7th day, and the removal of *Agrobacterium* was difficult thereafter (Pawlick *et al.*, 1992).

The results indicated that 2 or 3 days of co-culture would be enough for transformation (Jia *et al.*, 1989). In general, kanamycin-resistant line selection took 4 weeks for various crops. However, *R. glutinosa* took 8 weeks for the selection. Plant materials such as lower abaxial parts, new shoots, and roots were known to be used for the transformation. However, leaf explants, the most widely used materials, having large surface area, absorbed nutrients and hormones efficiently, and were less stressed compared with the other materials resulting in higher regeneration rates (Barbier & Dulieu, 1883). Various parts of *R. glutinosa* were used for transformation experiments, and, as a result, auxiliary bud culture resulted in 100% regeneration rate in transformation at each kanamycin

concentration. Whereas, the shoot explants culture resulted in failed regeneration, growth retardation, and no root formation. These results indicated that auxiliary buds were not suitable for the selection of kanamycin resistance. PCR analysis also showed that the plantlets regenerated from the auxiliary buds were not successfully transformed (data not shown). There were no changes in the number of calli at each growth stage (data not shown), and the number of new shoots increased 8 weeks after the inoculation and resulted in increased regeneration rate after 8 weeks. Regeneration rate was greater at lower concentrations of kanamycin. Growth regulators used in this experiment included hormones that directly regenerated the explant tissues. 2,4-D, commonly used for callus induction, could cause morphological and genetic variations in plantlet regenerations, and, thus, was excluded. Two kinds of cytokinins were used for effective regenerations. Insertion of foreign genes in plants by the *Agrobacterium tumefaciens* vector system required use of antibiotics in the regeneration medium, in order to eliminate the *Agrobacteria*. Now, cefotaxime and carbenicillin have been the most commonly used antibiotics but these antibiotics have negative effect on plant regeneration. Even timentin is a mixture of ticarcillin, a penicillin derivative, and clavulanic acid with activity against many gram negative bacteria, though they possess  $\beta$ -lactamase activity (Neu, 1985). Penicillins bind to the penicillin-binding proteins in the bacterial periplasm, thereby interrupting the synthesis of peptidoglycan and provoking the death of the bacteria by cell wall lysis. Some bacteria containing  $\beta$ -lactamase can prevent the activity of the antibiotics by hydrolysing the cyclic amide bond of the  $\beta$ -lactam ring in penicillins. This can be counteracted by the addition of clavulanic acid, a competitive inhibitor of  $\beta$ -lactamase. Influence of the antibiotic timentin on plant regeneration has been reported in *Nicotiana tabacum* (Brigitte *et al.*, 1997) which are compared to carbenicillin and cefotaxime in concentration suitable for elimination of *Agrobacterium tumefaciens*. In this study, timentin was used as antibiotics for eliminating the *Agrobacteria*. Previously studied the effects of timentin on eliminate the *Agrobacteria* and shoot regeneration of Chinese

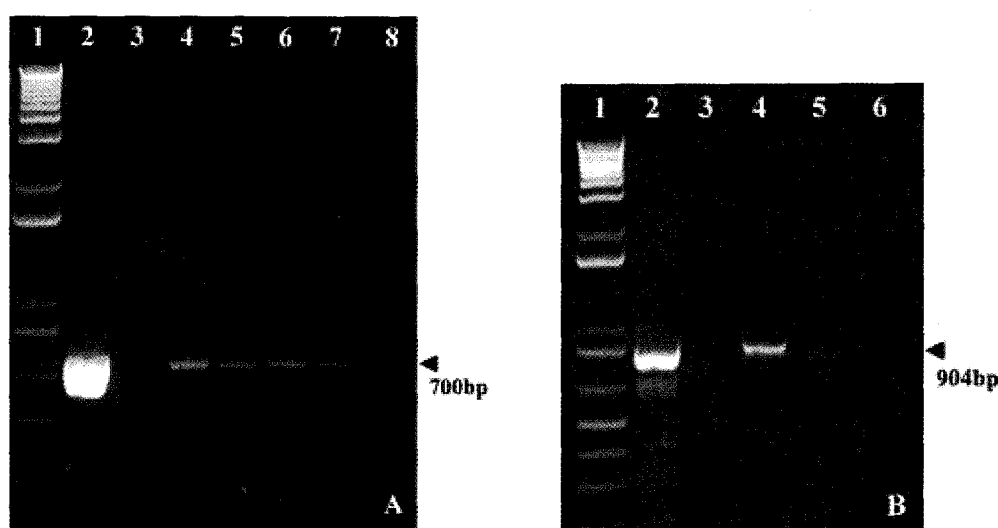
foxglove. Addition of 200 mg/ℓ timentin to regeneration medium appropriated to control the growth of the bacteria (data not shown).

### PCR analysis of transformed plantlets

The introduction of RS3 genes into transformed plant of *R. glutinosa* was demonstrated by PCR analysis. DNA amplification of all thirty-five plants regenerated in the presence of kanamycin was analysed by PCR for co-integration of the *nptII* and stilbene synthase gene. In the PCR analysis of transformation with primers N-1 and N-2, no bands were observed in control plantlets but DNA fragments, 700 bp in size, were observed in all the transformed plantlets (Fig. 2A). Among the plantlets, only in plantlets regenerated from leaf explants and cultured with 50 mg/ℓ of kanamycin concentrations, the *nptII* gene was found inserted into the genome. Five among thirty five kanamycin resistance lines showed introduction of

*nptII*, and also PCR analysis with RS3 primer confirmed that the targeted gene was introduced into the plant genome, 904 bp in size (Fig. 2B). Some of the transformed plantlet lines selected on medium supplemented with kanamycin did not show any positive band in PCR analysis of targeted gene. This was possible because, as Socristan & Melchers reported previously (1987), hormones produced from kanamycin-resistant cells caused the neighboring untransformed cells to regenerate into plantlets. As a result of this study, transformation of RS3 gene by *Agrobacterium* and transplanting in the field was successfully established in *R. glutinosa*.

These transformed *R. glutinosa* plantlets would be used successfully for additional study, such as production of secondary metabolite, monitoring of gene expression profile, and characterization of gene function and gene structure.



**Fig. 2.** PCR detection of *nptII* (A, selection marker gene) and RS3 (B, stilbene synthase) gene from genomic DNA in transgenic and control chinese foxglove. lane 1: 1 Kb Plus marker DNA (Ready-Load™), lane 2: Positive control (using cloned RS3 gene), lane 3: Non-transformed chinese foxglove plant. lanes 4~8: Transgenic chinese foxglove plants. The size of the amplified DNA fragments are 700 bp (*nptII*) and 904 bp (stilbene synthase), respectively.

Plant transformation acts as a core research tool in plant biology and practical tool for plant improvement. Genetic engineering using plant gene transformation system has already been responsible for the production of plants with enhancement in a range of

desirable traits, notably disease resistance, infect resistance, human health benefit and nutritional and commercial value. Progress is also being made towards long-term aims such as improvement of production of new therapeutics compounds in other

traditional medicinal plant.

This study cautions that overexpressing RS t-DNA in Chinese foxglove (*R. glutinosa* L.) by transformation may be more difficult than expected, because both *E. coli* and transgenic chinese foxglove (*R. glutinosa* L.) contained factors which influenced enzyme activity, synthesis process modification more importantly, and the apparent substrate preference of STS. However the overexpression of the RS gene by transformation was investigated in transgenic *R. glutinosa* L.

Further analyses of identification of transformation using developed other molecular techniques and transgenic plants that RS t-DNA introduced to chinese foxglove (*R. glutinosa* L) and its reaction product, stilbene such as resveratrol will be isolate and characterize using NMR, MS, and HPLC. We remain hopeful that investigation of biological activity from transgenic plant will be useful in terms of the dual roles of resveratrol-forming stilbene as a phytoalexin for plant health, as well as a phytochemical for human health.

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