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Introduction and Expression of a Thaumatin-like Protein from Rice in American Ginseng Following *Agrobacterium*-mediated Transformation

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ABSTRACT : *Agrobacterium*-mediated transformation of American ginseng (*Panax quinquefolius* L.) with strain LBA 4404 containing a rice thaumatin-like protein gene is described. The selectable markers used were phosphinothricin acetyltransferase and hygromycin phosphotransferase genes. Epicotyl explants from seedlings were precultured for 5-7 days on Murashige and Skoog medium with α-naphthaleneacetic acid and 2,4 dichlorophenoxyacetic acid at 10 μM and 9 μM, respectively (ND medium), prior to *Agrobacterium* infection. The explants were immersed in a bacterial suspension for 20 min. A post-infection co-culture period of 3-4 days was provided on ND medium. Selection for transformed calli was conducted on ND medium with 20 mg/L phosphinothricin followed by 100 mg/L hygromycin over an 8-month period. A transformation frequency of 24.8% was achieved at the callusing phase. The presence of the transgenes in calli was confirmed by Southern hybridization and polymerase chain reaction analysis. The expression of the thaumatin-like protein gene in ginseng calli was demonstrated by Western blot analysis. Somatic embryos were produced from both transgenic calli and suspension cultures, and plantlets were recovered that expressed the transgenic thaumatin-like protein gene.

Key words : *Panax quinquefolius,* genetic transformation, pathogenesis-related protein.

INTRODUCTION

American ginseng (*Panax quinquefolius* L.) is a highly valuable medicinal plant. The potential yield of ginseng plants can be reduced by root and stem-infecting fungi (Punja 1997, Reeleder and Brammall 1994). Developing ginseng cultivars with enhanced disease resistance by breeding methods is presently not undertaken, since ginseng plants produce seeds after 3-4 years of growth and the seeds require over 12 months of stratification before germination can occur (Proctor and Bailey 1987). To potentially enhance tolerance of American ginseng to fungal diseases, we introduced a rice thaumatin-like protein (TLP) gene (*tlp*) into this medicinal plant. Thaumatin-like proteins are pathogenesis-related (PR) proteins, which previously gave enhanced resistance to sheath blight of rice and scab of wheat in transgenic plants (Chen et al. 1999, Datta et al. 1999). In this paper, we report *Agrobacterium*-mediated transformation of American ginseng with the *tlp* gene regulated by the maize *ubiquitin1*(*ubi1*) promoter. The *bar* (coding phosphinothricin acetyltransferase) and *hpt* (coding hygromycin phosphotransferase) genes, which confer resistance to phosphinothricin (PPT) and hygromycin B (Hygromycin), respectively, were used as the selectable markers. This report shows the potential for introduction of foreign genes into American ginseng through genetic engineering.

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MATERIALS AND METHODS

1. Plant materials

Stratified American ginseng seeds were provided by Shu's Ginseng (Wisconsin, USA). The seeds were planted into Sunshine Mix medium (Sun Gro Horticulture, Inc., Surrey, BC, Canada) and grown in the dark for 2-3 weeks. When the epicotyls were 4-6 cm long, they were excised and dipped in 70% ethanol for several seconds and disinfected with NaOCl (0.5%) for 15 min, and then rinsed three times with sterile water. The 0.5-1.0 cm long basal portion of the epicotyl was discarded, and the remainder was cut into 2-3 mm long segments. The segments were pre-cultured for 5-7 days on MS salts medium (Murashige and Skoog 1962) with full complement of vitamins, 3%

sucrose, and NAA at 10 μM and 2,4-D at 9 μM (ND medium) with 0.3% Phytagel™ (Sigma, Oakville, ON, Canada). The pH was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. The ginseng cotyledons in the seeds were also used as explants for transformation. Ginseng seeds were dehusked and dipped in 70% ethanol for 1 min, followed by a soak in 1.7% NaOCl for 15 min and then rinsed three times with sterile water. The cotyledons were dissected out and cut in half and cultured on ND medium. All tissue culture dishes (100×15 mm) with explants were incubated at ambient temperature (20-23°C) in the dark.

2. Transformation procedure

Construct pCambia-bar-tlp (13.7 kb) harbored in strain LBA4404 of *Agrobacterium tumefaciens* was provided by Dr. S. Muthukrishnan (Biochemistry Department, Kansas State University, USA). Selectable markers were the phosphinothricin acetyl-transferase gene (*bar*) and the hygromycin phosphotransferase gene (*hpt*) (Gritz and Davies 1983, Thompson et al. 1987, White et al. 1990). Both selectable marker genes were driven by the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al. 1985). The *tlp* gene (1.0 kb), which was cloned from rice (Velazhahan et al. 1998), was controlled by the maize *ubi1* promoter (2.0 kb) (Christensen et al. 1992, Christensen and Quail 1996). The fragment *ubi1-tlp* (3.0 kb) can be released by digesting the construct with *Hin*d III. The remaining part of the construct was 10.7 kb in size. Bacteria were cultured for 30 h on a shaker (200 rpm) at 28°C in AB medium (Chilton et al. 1974) with 17 mg/L $MnSO_4$, 50 mg/L kanamycin and 50 mg/L streptomycin. Acetosyringone (200 μM) and ascorbic acid (100 mg/L) were added to the medium 5 h prior to infection. The bacteria were collected by centrifugation for 15 min at 2500 rpm and resuspended in 1/10 strength MS medium containing 10 μM NAA, 9 μM 2, 4-D, 1% glucose, 2% sucrose, 200 μM acetosyringone and 100 mg/L ascorbic acid, pH 5.2. The explants were pre-cultured for five days and infected with an *Agrobacterium* suspension (OD_{600} = 0.3) by immersion for 20 min. All explants were co-cultured on NB medium, pH 5.6, for 3-4 days at 22°C. After co-culture, explants were washed with sterile water and transferred to NB medium with 300 mg/L Timentin (Smithkline Beecham Pharma Inc., Oakville, ON, Canada) (N10D9T) with either 1 mg/L PPT or 10 mg/L hygromycin (Oakville, ON, Sigma) and cultured for one week. Subsequently, the concentration of PPT was increased to 5 mg/L and then to 20 mg/L after two months in successive transfers on fresh media. Hygromycin levels were increased to 20 mg/L and then to 100 mg/L in a similar fashion. Calli on selection medium with somatic embryo-like structures were transferred to RM medium (half-strength MS medium with 3% sucrose, 1% activated charcoal and no growth regulators) for plantlet regeneration (Tirajoh et al. 1998). To propagate calli of selected lines through suspension culture, MS medium containing 2.5 μM of NAA and 2.25 μM of 2, 4 D was used, with 10-20 mg/L PPT. Subcultures were made every week. Somatic embryos in suspension culture were also induced to form plantlets like the embryos on calli.

3. Molecular analysis of calli resistant to PPT and hygromycin

DNA was extracted as described by Riede and Anderson (1996) for Southern blot and PCR analysis from putatively transgenic calli lines growing on selection medium and any polysaccharides were removed with 2 M NaCl and ethanol (Fang et al. 1992). The DNA was digested with *Hin*d III and electrophoresced in a 0.7 % agarose gel. DNA fragments were transferred to a nylon membrane (Hybond-N^+, Boehringer, Laval, QC, Canada), and hybridized with a digoxigenin-labeled *tlp* fragment, which was amplified by PCR, to detect the presence of the *tlp* gene as described by Tingay et al. (1997). To detect the polymorphism between transgenic lines, the whole construct (pCambia-bar-tlp) was labeled with ^{32}P to hybridize with the Southern blot. The genomic DNA was digested with *Hin*d III.

The DNA used for Southern analysis was also used for PCR analysis of the transgenic callus lines. To amplify the *bar* gene from transgenic plantlets, small pieces of leaves (about 4 mm) were used in PCR reactions as described by Klimyuk et al. (1993). PCR was performed in a volume of 50 μl with 1.5 mM of $MgCl_2$ using Taq DNA polymerase and the corresponding buffer from GIBCO-BRL (Burlington, ON, Canada) or Pharmacia (Quebec, Canada). The reaction buffer contained 1.0 M Betaine for *bar* gene detection or 1.6 M Betaine for the *tlp* gene detection, but no Betaine for *hpt* gene detection. Betaine was used to improve PCR amplification efficiency of GC-rich DNA sequences (Henke et al. 1997). The primer sequences for PCR were as follows: *hpt* forward sequence (F) 5'-CGTCTGTCGAGAAGTTTCTGAT-3', reverse sequence (R) 5'-TACTTCTACACAGCCATCGGTC-3' to yield a 947 bp fragment; *bar* (F) 5'-ACTGGGCTCCACGCTC-TAC-3', (R) 5'-GAAGTCCAGCTGCCAGAAAC-3' to yield a 202 bp fragment; *tlp* (F) 5'-AACAGGTGCCAG-

TACACGGTGT-3', (R) 5'-CACGGTTACATCCACACA-TGCA-3' to yield a 691 bp fragment. The DNA was denatured at 94°C for 5 min followed by 35 cycles of amplification [1 min at 94°C; 1 min at 57°C (*hpt*), 58°C (*bar*) or 60°C (*tlp*); 1 min at 72°C]. The final incubation at 72°C was extended to 7 min, and the reaction was cooled and kept at 4°C. The GeneAmp® PCR System 9700 was used (Perkin-Elmer Corporation, Norwalk, CT).

Western analysis was conducted as described in Chen et al. (1999) to demonstrate the expression of the *tlp* gene. Total proteins were extracted from transgenic calli and measured according to the method of Bradford (1976).

RESULTS AND DISCUSSION

1. Production of transgenic calli

After eight months of culture on the selection medium following *Agrobacterium* infection, a callusing frequency

Table 1. Callusing efficiency of American ginseng on selection media after transformation with *Agrobacterium tumefaciens* LBA 4404 (pCambia-bar-tlp). The calli were cultured on callusing medium with 20 mg/L PPT and then 100 mg/L Hygromycin. The results were obtained 8 months after transformation. The non-transformed control explants were cultured without selection agents

Explant source	No. of calli	No. of explants	frequency (%)
Cotyledons	0	483	0.0
Cotyledons (control)	82	130	62.8
Epicotyls	79	319	24.8
Epicotyls (control)	81	105	77.1

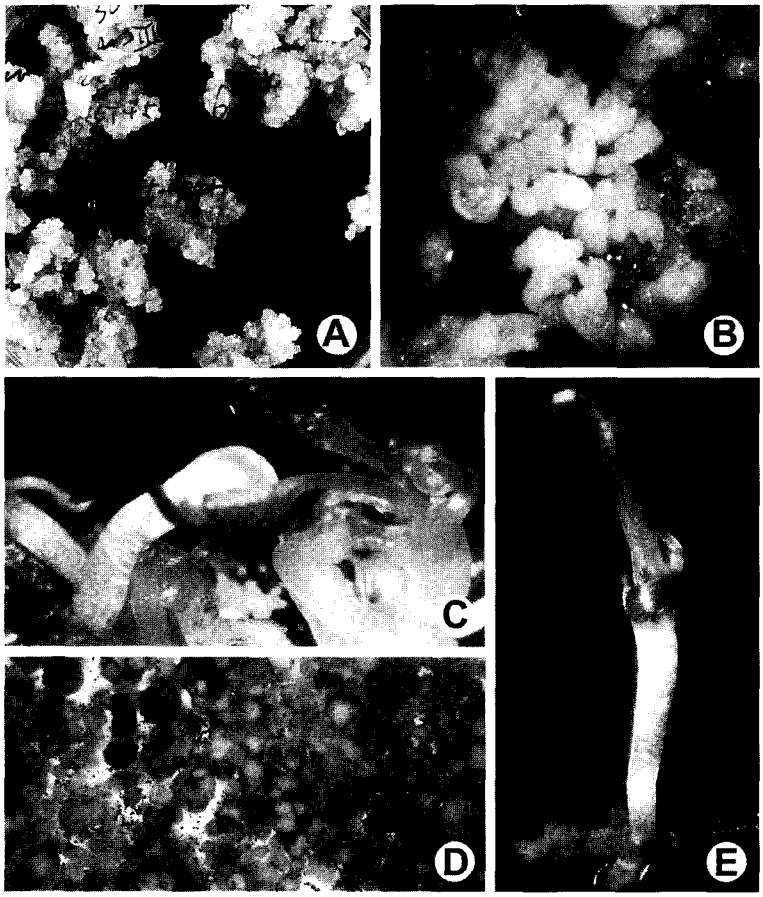

Fig. 1. Callus and plantlet development of American ginseng following *Agrobacterium*-mediated transformation. (A) Resistant calli formed from epicotyl explants, 8 months after infection. (B) Somatic embryo clusters developing from transformed callus. (C) Plantlets recovered from embryos on the transgenic calli. (D) Somatic embryos produced in a suspension culture. (E) A somatic embryo developing into a plantlet.

of 24.8% was obtained using epicotyls as explants (Fig 1A), but no resistant calli were produced from cotyledons (Table 1), indicating that epicotyls are the preferred choice as explants compared to cotyledons for *Agrobacterium*-mediated transformation of American ginseng. In American ginseng tissue culture, epicotyls were observed to be the best source of explant for inducing calli (Tirajoh et al. 1998).

Both *bar* and *hpt* genes were used as selectable markers for American ginseng transformation. In the *bar*-PPT selection system, there can be a cross-protection of non-transgenic cells by transgenic cells (Casas 1995), frequently making non-transgenic calli indistinguishable from transgenic calli. Therefore, selection with PPT alone could produce some escapes after regeneration. The sections of some calli after PPT-selection turned brown after the calli were subsequently selected on hygromycin-containing medium, indicating the calli on PPT-medium could be chimerically transgenic, and using both selectable markers reduced the possibility of escapes. In parallel experiments, the growth of putatively transformed calli appeared to be more vigorous on selection medium with PPT than with hyg. The use of hygromycin alone slowed the growth of callus during selection; however, this antibiotic selection could be used alone and was found to be far superior to kanamycin (data not shown).

The calli with somatic embryos (Fig 1B), which were confirmed to contain the transgenes by PCR and Southern analysis, were transferred to RM medium, and some produced plantlets (18.5% frequency) (Fig 1C). The embryogenic calli were cultured in liquid medium and suspension cultures were formed in three months. To promote somatic embryo formation in suspension cells, one line was cultured for two weeks in suspension culture medium with 1% activated charcoal, then subcultured without the charcoal. Somatic embryos which developed (Fig 1D) were placed on RM medium for plantlet regeneration. After one month, some of the embryos (1.2%) of one line developed into plantlets (Fig 1E). Southern blot and PCR analysis confirmed that this line was transgenic (data not shown).

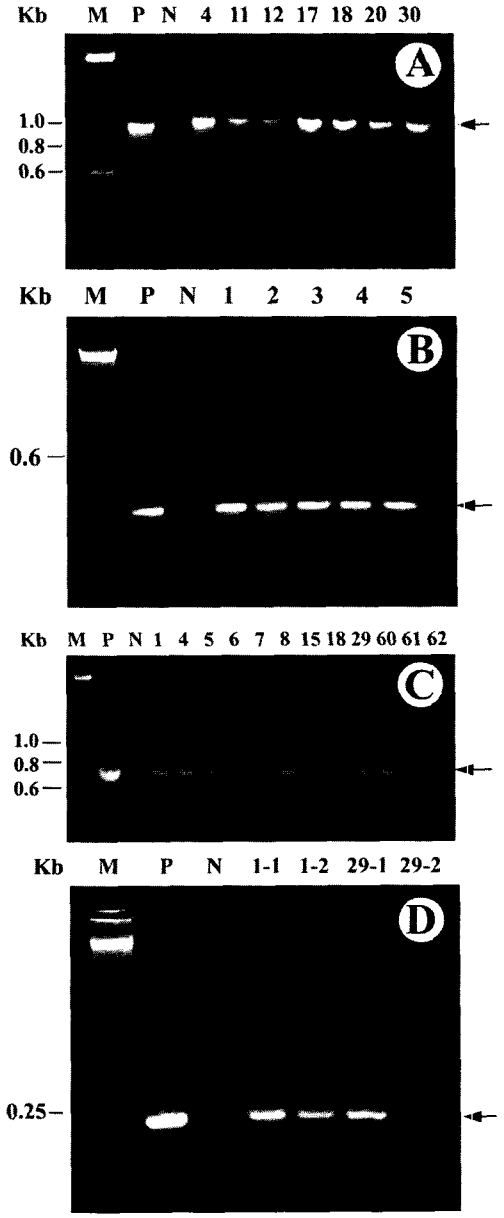

Fig. 2. Detection of the transgenes in transgenic calli and plantlets by PCR analysis. M, 100 bp (A, B, C) or 50 bp (D) DNA ladder; P, positive control using 0.2 ng construct DNA as a template for the *hpt* (A), *bar* (B, D) and *tlp* (C) gene amplification; N, non-transformed ginseng DNA as a template; Other lanes, transgenic lines. Reaction solution contained 100 ng genomic DNA. The sizes of amplified fragments (arrowed) are 947 (A), 202 (B, D) and 691 (C) bp.

2. Integration and expression of the transgenes in calli resistant to PPT and hygromycin

Resistant callus lines were analyzed for transgene integration by PCR and Southern blot hybridization. All lines contained the *tlp*, *bar* and *hpt* transgenes with the expected sizes (Fig 2A, B, C). The transgenic *bar* gene was also amplified from four plantlets of two transgenic lines (Fig 2D). Southern blot showed the expected 3.0 kb fragment of *ubi1* promoter (2.0 kb) and the *tlp* gene (1.0 kb) using *tlp* gene as the probe (Fig. 3A). When the whole

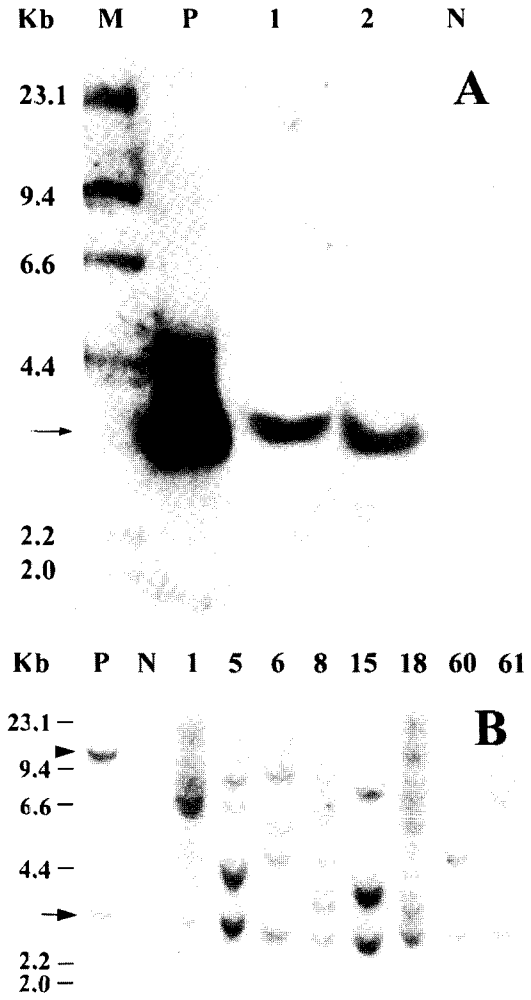

Fig. 3. Southern blot analysis of transgenic callus lines. Plant and plasmid DNA were digested with *Hind* III. N, DNA from non-transformed ginseng calli; P, a mixture of pCambia-bar-tlp (250 pg for A and 100 pg for B) and non-transformed ginseng DNA; M, Lamda-*Hind* III marker; the other lanes show the hybridization patterns of transgenic calli. The probe was PCR-amplified *tlp* coding region (A) or whole construct pCambia-bar-tlp (B). The arrows indicate the sizes of positive control DNA fragments (*ubi-tlp*), and the arrowhead indicates 10.7 kb fragment of the construct after releasing *ubi-tlp*.

construct was used as the probe, not only the band of *ubi-tlp* appeared but other bands showed polymorphism different from the positive control (10.7 kb) (Fig. 3B), indicating the ginseng transgenic lines were from various independent transformation events and the transgenes were integrated into the plant genome.

The expression of the *tlp* gene was studied by Western

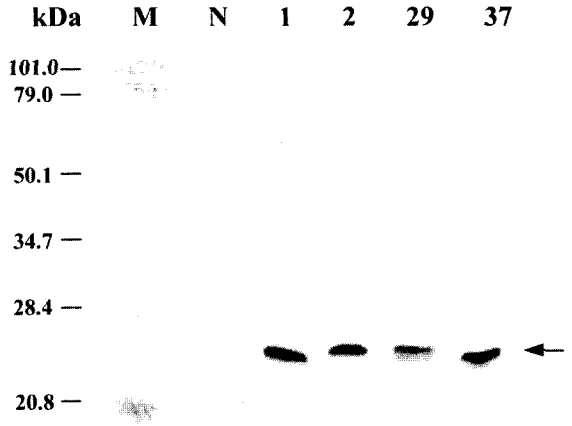

Fig. 4. Detection of TLP expression by Western analysis in transgenic callus lines. Each lane was loaded with 25 μg protein. M, protein size marker; N, non-transformed callus protein. The TLP band (about 23 kDa) is indicated by the arrow.

blot analysis using total protein extracted from transgenic calli. The band with expected size (about 23 kDa) appeared after detection with a TLP antibody. Four of these lines are shown in Fig. 4.

A few plantlets have been recovered to date from the transgenic calli of American ginseng in this study. When a large number become available, the regenerated plants will be further characterized and also challenged with fungal pathogens to determine if enhanced tolerance to diseases has been achieved. Since the ubiquitin promotor has also been shown to be induced by biotic and abiotic stresses (Takimoto et al. 1994), it may prove useful for transgene expression in ginseng tissues upon pathogen infection. This promoter has been used for transgene expression in other dicotyledonous plants, such as canola (Fukuoka et al. 1998) and carrot (Chen and Punja 2002).

The procedure for American ginseng transformation developed in this study is summarized in Fig. 5. An average of 8 months is needed for a transformation experiment to be completed due to the slow growth rate of ginseng tissues.

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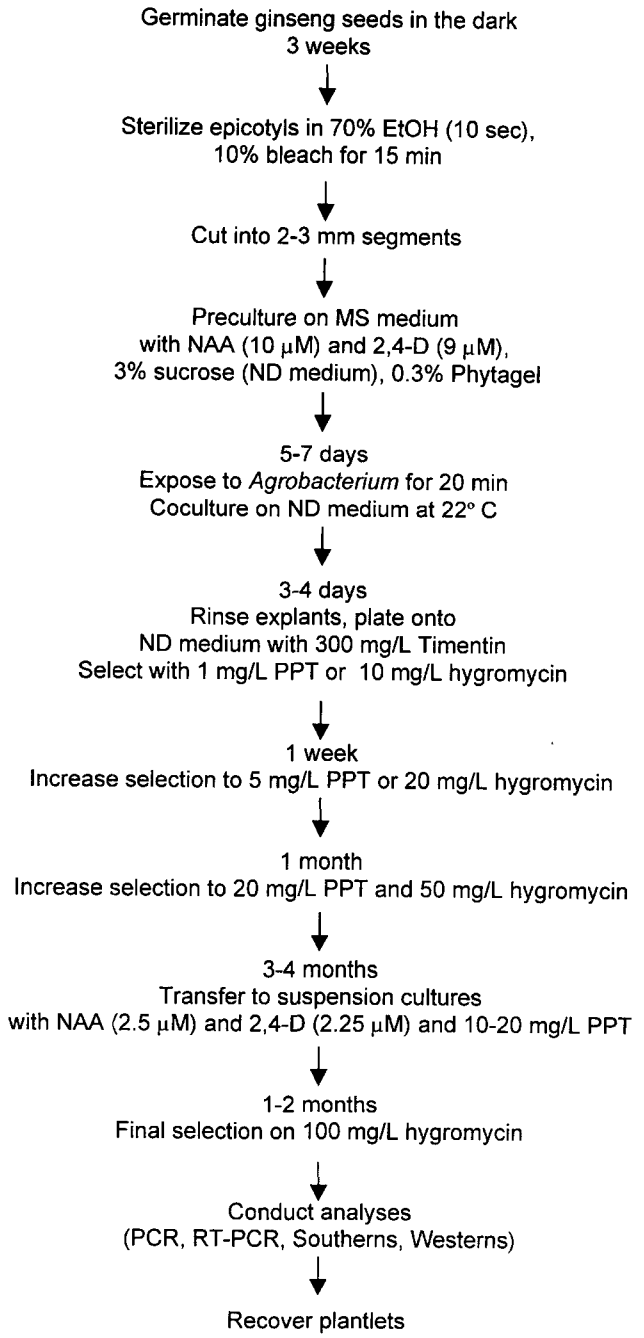

Fig. 5. Summary of American ginseng transformation developed in this study.

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