Single Somatic Embryogenesis from Transformant with Proteinase II Gene in *Panax ginseng* C.A. Meyer

Deok-Chun Yang, Se-Young Kim, Yeong-Deok Rho, and Moo-Sung Kim
Center for Oriental Medicinal Materials & Processing, College of Life Science,
KyungHee University, suwon 449-701, Korea

ABSTRACT

Ginseng(*Panax ginseng* C.A. Meyer) is a perennial herbaceous plant which grows very slowly. It takes about 3 to 4 years from seeding to collecting the ripe seeds and the ginseng propagation is very difficult. and so, it is very difficult to breed ginseng plant. Ginseng tissue culture was started from at 1960, and ginseng commercial product by *in vitro* callus culture was saled, however upto now, regenerants were not planted to soil normally. Recently, plant genetic engineering to produce transgenic plants by introducing useful genes has been advanced greatly. In a present paper, transformation of ginseng plants was achieved by co-cultivation with *Agrobacterium* harboring the binary vector coding Proteinase-II gene, which confer resistant or tolerant to insect pests, The binary vector for transformation was constructed with disarmed Ti-plasmid and with double 35S promoter. The NPT II gene and introduced genes of the transgenic ginseng plants were successfully identified by the PCR. Especially the transgenic ginseng plants were regenerated using new techniques such as repetitive single somatic embryogenesis.

Key words **:** Embryogenesis, *Panax ginseng*, PI-II gene, Transformation

INTRODUCTION

Panax ginsengis a traditional medicinal plant known to the world since ancient times. Ginsenoside present in the root is an important saponin having many medicinal properties. These perennial herbaceous plants grow in natural condition very slowly and can be harvested after being cultivated for 4-6 years. The yield of the ginseng roots is also very less due to the various diseases and its peculiarlife style. The ginseng is very expensive because of this long-term conventional and troublesome production cycle. It has shown that plant regeneration from tissue culture of ginseng is also difficult except for direct single somatic embryogenesis from cotyledon explants on hormone free MS medium. The main problem is a lack of root formation or inadequate root formation from regenerants(Butenko, 1993; Yang and Choi, 2000). Most of the genetic transformation in ginseng was for inducing hairy roots with *Agrobacterium rhizogenes* (Yoshikawa and Furuya, 1987; Inomoto, 1993; Bulgakova et al., 1999). Yang and Choi(2000) reported transgenic plants from hairy root derived callus cultures. Recently Choi et al.(2001) reported *Agrobacterium* mediated transgenic ginseng

Corresponding author : **Deok-Chun Yang**, E-mail : dcyang@khu.ac.kr

plants with GUS gene using plasmolysing pretreatment to cotyledons for efficient and high degree of transformation. The present study deals with *Agrobacterium* mediated method of transformation with proteinase inhibitor-II(PI II) gene for increased insect resistance(Kim et al., 2001). Insect predation is a major factor effecting the reduction of the worldwide crop yield. The advantage of using crop varieties, which are inherently resistant or tolerant to insect pests, is of importance. By the adventof genetic engineering crop plants can be make a major contribution for the increased crop production by transient expression. Plants typically respond to environmentally stress such as insect herbivore, mechanical damage and UV irradiation by inducing defense-related proteins(Liu et al., 2001). Among these PI I and PI II are two well-known characterized chymotrypsin inhibitors. Both proteins accumulate in the leaves and are involved in signal transduction pathways in the plant protective response against environmental herbivores and pathogens(Liu et al., 2001). Proteins that form complexes with proteinases and inhibit their proteolytic activity are widespread in nature. These so-called proteinase inhibitors(PI's) can be divided in to four mechanistic classes, viz: serine, cysteine, aspartic and metallo proteinase inhibitors (Brzin and Kidric, 1995). These proteins bind to proteinases in the mid gut of the insects thus interfering with the insect's metabolism. Many plant species accumulate the PI s in seeds and vegetative organs constitutively. Or on wounding fungal elicitor bacterial infection, and plants hormones. It is believed that these inhibitors are involved in the protection of the plants against herbivore insects. There are two families serine PI s in plants. Type 1 inhibitors are small proteins that inhibit chymotrypsin at a single reactive site where as type 2 families contains the reactive sites one of which inhibits chymotrypsin and other trypsin (Kim et al., 2001). An attempt has been made in the present study to transform ginseng with PI II gene for increased insect resistance.

MATERIAL AND METHOD

Cotyledon culture:

Korean ginseng(*Panax ginseng* C.A. Meyer) seeds Cultivar Chunpung were stratified in humidified sand at a temperature of 15℃ since the zygotic embryos just after harvest were at an immature globular stage(about 2mm in length). After stratification about 3 months them 4 month cold treatment, the seeds were immersed in 70% ethanol for 1 min, in 2% sodium hypochlorite for 30 minutes and then washing 4 times with sterile distilled water. Then the zygotic embryos were excised from seeds and kept in MS(Murashige and Skook, 1962) basal media for two days to elongate. The cotyledons were removed and co-cultivated with *Agrobacterium* harboring PI II gene.

Agrobacteriumculture and co-cultivation:

The vector contains the plasmid which carries the PI II gene as the reporter gene driven by the alfalfa mosaic virus(AMV) 35S double promoter and nopaline synthase(Tnos) as the terminator sequence(Yang et al., 1995). *Agrobacterium tumefacians* MP 90 harboring the vector for PI II gene were cultured on LB media containing kanamycin and gentamycin 25㎍/mL each for two days at 27℃ with vigorous shaking. The cotyledons were dippedin bacterial culture for 10 min later drying in a sterile filter paper. And transferred to MS medium containing 100㎛ acetosyringone for co-cultivation. After 3 days of co-cultivation in MS basal medium the cotyledons were transferred to MS+300 ~ 500㎍/mL cefotaxime to remove the extra bacterial growth for 2-3 weeks. Later the cotyledons were transferred to selection media MS+100㎍ /mL kanamycin and 500㎍/mL cefotaxime.

Somatic embryogenesis from callus

Transformants formed on MS medium containing 1.0 mg/L 2,4-D, 100㎍/mL kanamycin and 300㎍/mL cefotaxime were selected and transferred to the same medium for embryogenic callus induction. The embryogenic transgenic callus with PI-II gene was maintained by 5 week subculture intervals. Culture vessels and conditions were the same as those of callus induction. To induce somatic embryos from embryogenic transformants, 10 callus clumps(each about 50 mg) were transferred to a 10×1 cm petri dish containing 30 ml MS medium. After 2 months, the number of somatic transgenic embryos per embryogenic clump with PI-II gene was counted.

Polymerase Chain Reaction for NPT II and Chitinase genes

Genomic DNA from 200 mg control plants and from all putative kanamycin resistant plants were extracted from leaves as described by Edwards et al.(1991). We have utilized the sequence information derived from chitinase, and designed degenerated oligonucleotides of corresponding genes from ginseng genomic DNA for PCR amplification. The oligonucleotide 5' -GGA-AGC-TAC- GGA-GAG-CAG-TG-3' was used as the sense primer and the oligonucleotide 5' -CCG-TTG-ATG-ATG-TTC-GTC-AC-3' as the antisense primer for checking of chitinase gene. The primer for the NPT II gene fragment, 5' -GAG-GCT-ATT-CGG-CTA-TGA-CTG-3' as the sense primer and 5' -ATC-GGG-AGC-GGC-GAT-ACC-GTA-3' as the antisense primer were used for PCR screening of kanamycin resistant plants. Gene Amp PCR system 9600(Perkin Elimer Cetus, Norwalk, USA) was used in a reaction mixture of 20 ㎕ containing 0.1-0.5 ㎍ of genomic potato DNA using a Pre-mix Top(Bioneer). Mineral oil were overlaid with the mixture, and heated to 94℃ for 5 min. The PCR was performed with 35 cycles, each consisting of 1 min at 94℃, 1 min at 60℃ and 1 min 30 s at 72℃. PCR products were electrophoresised in 1% agarose gel and detected by ultraviolet light.

RESULTS AND DISCUSSION

Transformation of ginsegn using chitinase gene

Sterilization of seeds were done with rinsing first with 70% Ethanol for one minute and then washing with 2% Sodium hypochorite (NaOCl) for 30 min and later thoroughly washing with sterile distilled water for 3 times. The cotyledons were carefully excised from the seeds with a sterile blade and kept in MS basal medium as a preculture method. This had two advantages, we can select the sterile cotyledons if at all any contamination is there while excising and also the cotyledons will elongate after two days of culture which will be easier for removing the cotyledon and later for co-cultivation. The precultured cotyledons dipped in *Agrabacterium* cultures harboring PI-II gene for 10 min and later transferring to MS+100㎛ acetosyringone as the co-cultivation medium for 3 days. After three days they were transferred to MS+300㎍/mL cefataxime to eliminate the bacteria for three weeks. After three weeks of culture globular embryos were emerged from cut end portion of the cotyledons(Fig. 1-A,B,C). These cotyledon along with the somatic embryos were transferred to MS+100㎍/mL kanamycin and 500㎍/mL cefotaxime for strong selection. The same procedures were adapted for PI II gene transformation. The percentage of embryo formation was as follows. In the case of PI II out of 24 cotyledons only 7 could induce embryos (29%). While in the control it was 87%(Table 1). Another important findings are in control all embryos germinated to shootlets while in transformed embryos the germination rate was very less (Fig. 1-D). Some failed to germinate or germinated ones were albinos and some were green in color. Somatic embryogenesis in ginseng via callus culture were reported by Kevers et al.(2000), They have used liquid culture in the presence of BSAA for embryogenesis and

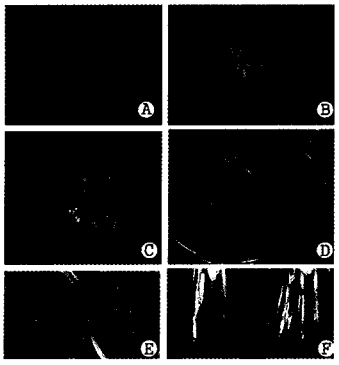

Fig. 1. Single somatic embryogenesis(A,B,C,E) and shoot regeneration(D,F) of *Panax ginseng* C.A. Meyer by transformation using chitinase gene. (A,B,C) Kanamycin resistant single embryos were cultured from ginseng callus transformed by chitinase gene. ginseng zygotic embryo. (D, F) Mature transformants were cultured on the medium containing GA_3. (E) Single embryos from zygotic embryo were formed on the growth regulator free media with kanamycine 100 μg /mL and cefotaxim 250 μg/mL.

further regeneration with the presence of a cytokinin ZR. While in American ginseng Tivarjoh et al(1998) reported SE from root leaf and epicotyl derived callus in the presence of auxins such as dicamba, NAA and 2,4-D and germination of embryos in the presence of BA and GA_3. While in the present study for embryo formation no growth hormones(Fig. 1-E) wee needed and for germiantion of embryos only GA3 was needed(Fig. 1-F). Choi et al(1998) reported that somatic single embryos formed from germinating zygotic embryos were regenerated into normal plants with both roots and shoots but multiple embryos from immature zygotic embryos were developed into only multiple shoots. Therefore, hormone free treatment can be useful tool for the both single cell-derived somatic embryo formation and their transgenic plant regeneration by chitinase gene in *P. ginseng*. Maturation of transgenic somatic embryos was normally proceed until cotyledonary stage on hormone-free MS medium until 2 months of culture. However, further development of somatic embryos such as germination and plant regeneration did not occur, probably indicating dormancy. Although somatic embryos were transferred to fresh MS medium, the embryos did not germinate but stayed in white pigment. There have been no report on the dormancy of somatic embryos of *P. ginseng*. However, in many papers, GA_3 treatment in combination of BAP or kinetin was commonly used for the maturation and shoot regeneration from ginseng somatic embryos (Chang and Hsing 1980, Shoyama et al. 1987, Arya et al. 1993). GA_3 is the well known substances for dormancy breaking in many plant species (Bewley 1997).

Polymerase chain reaction analysis for selection of transgenic plants

DNA amplification of transgenic plants regenerated in the presence of kanamycin was analysed by PCR for the co-integration of the NPT II(Fig. 2-A) or chitinase(Fig. 2-B). NPT II primers amplified a 700 bp fragment. To show the presence of the chitinase gene from the transgenic ginseng genome, PCR analyses were performed for the genomic DNA isolated from *in vitro* grown shoots. By using internal chitinase primers for DNA amplification, the presence of the 746 bp

Table 1. Frequency of transgenic somatic-embryo(SE) formation and No of SE per explant from zygotic cotyledons on the media with/without any antibiotics using PI-II gene

Treatment	Formation of SE(%)	No of SE per explant	Medium
Hormone-free	21/24(87)	18	w/o-antibiotics
Hormone-free (KM + CT)	7/24(5)	2.3	with antibioticse

KM : kanamycine 100 μg/mL, CT: cefotaxim 250 μg/mL

chitinase fragment was demonstrated in DNA from transgenic ginseng(Fig. 2-B), indicating that the chitinsase gene was incorporated into the genomic DNA of the transformants of *Panax ginseng*. No such fragment was detected in DNA from control ginseng.

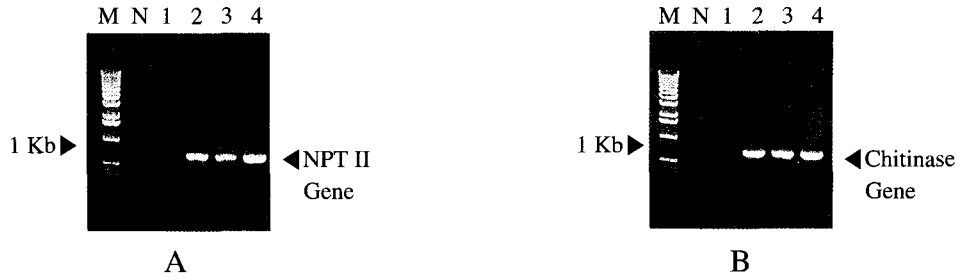

Fig. 2. PCR detection of NPT II(A) and chitinase(B) genes from genomic DNA in transgenic and control ginseng plants. M: 1Kb size marker, N: normal ginseng plant, 1-4; ginseng plants transformed on the growth regulator free media with kanamycine 100 μg/mL and cefotaxim 250μg/ mL. 1; non-transformed ginseng plant, 2-4; real transgenic ginseng plants.

ACKNOWLEDGEMENTS

This research was supported by a grant (code# PF003101-01) form Plant Diversity Research Center of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean government.

REFERENCES

Arya, S., I. D. Arya and T. Eriksson. 1993. Rapid multiplication of adventitious somatic embryos of *Panax ginseng*. Plant Cell Tissue Org. Cult. 34: 157-162.

Brzin, J and Kidric, M. 1995. Proteinases and their inhibitors in plants: role in normal growth and in response to various stress conditions. In: MP Tombs(Ed.) Biotechnology and genetic engineering Review, Intercept, Andover, pp 421-467.

Bulgakov, V. P., M. V. Khadakovskaya, N. V. Lebetskaya, G. K. Chernoded and Y. N. Zhuralev. 1999. The impact of plant rolC oncogenes on ginsenoside production by ginseng hairy root cultures, Phytochemistry 49: 1929-1934.

Butenko, R. G., I. V. Brushwitzky and L. I. Slepyan. 1968. Organogenesis and somatic embryogenesis in the tissue culture of *Panax ginseng* C.A. Meyer. Bot. Zh. 7: 906-913.

Choi YE, Yang DC, Kusano T and Sano H (2001) Rapid and efficient Agrobacterium mediated transformation of *Panax ginseng* by plasmolyzing pre-treatment of cotyledons. Plant Cell Rep 20: 616-621.

Choi YE, Yang DC, Park JC, Soh WY, Choi KT (1998) Regenerative ability of somatic single and multiple embryos from cotyledons of Korean ginseng on hormone-free medium. Plant Cell Rep. 17: 544-551.

Inomoto S, YokoyamaM, Gozu Y, Shimizu T and Yanagi M (1993) Gowth pattern and ginsenoside production of *Agrobacterium* transformed *Panax ginseng* roots. Plant Cell rep 12:681-686.

Kevers C, Gal NL, Monterio M, Donnes J and Gasper T (2000) Somatic embryogenesis of *Panax ginseng* in liquid cultures: A role for polyamines and their metabolic pathways. Plant Cell Tissue and Organ Culture 31: 209-214.

Kim S, Hong YN, An CS and Lee KW (2001) Expression characteristics of serine proteinase

inhibitor II under variable environmental stresses in hot pepper (*Capsicum annum* L.) Plant Science 161:27-33.

Liu G, Chen N, Kaji A, Bode AM, Ryan CA and Dong Z (2001) Proteinase inhibitors I and II from potatoes block UVB-induced AP-1 activity by regulating the AP-1 protein compositional patterns in JB6 cells. PNAS 98: 10, 5786-5791.

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

Shoyama Y, Kamura K, Nishioka I (1987) Somatic embryogenesis and clonal multiplication of *Panax ginseng*. Planta Med. 54: 155-156.

Yang DC and Choi YE (2000) Production of transgenic plants of *Panax ginseng* from *Agrobacterium* transformed hairy roots. Plant Cell Rep. 19: 491-496.

Yang DC, Han SS and Yoon ES (1995) Adenosine deaminase gene: possible slectable marker for tobacco transformation. Korean Journal of plant Tissue Culture 22:235-240.

Yoshikawa T and Furaya T (1987) saponin production by cultures of *Panax ginseng* transformed with *Agrobacterium rhizogenes*. Plant Cell Rep. 6: 449-453.

(Received Oct. 5, 2003)
(Accepted Oct. 30, 2003)