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Production of taxadiene from cultured ginseng roots transformed with taxadiene synthase gene

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Paclitaxel is produced by various species of yew trees and has been extensively used to treat tumors. In our research, a *taxadiene synthase* (*TS*) gene from *Taxus brevifolia* was used to transform the roots of cultured ginseng (*Panax* ginseng C.A. Meyer) to produce taxadiene, the unique skeletal precursor to taxol. The *TS* gene was successfully introduced into the ginseng genome, and the de *novo* formation of taxadiene was identified by mass spectroscopy profiling. Without any change in phenotypes or growth difference in a *TS*-transgenic ginseng line, the transgenic TSS3-2 line accumulated 9.1 μg taxadiene per gram of dry weight. In response to the treatment of methyl jasmonate for 3 or 6 days, the accumulation was 14.6 and 15.9 μg per g of dry weight, respectively. This is the first report of the production of taxadiene by engineering ginseng roots with a taxadiene synthase gene. [BMB Reports 2012; 45(10): 589-594]

INTRODUCTION

Paclitaxel (Taxol), a natural diterpenoid alkaloid was identified as the active anticancer constituent from the bark extract of the Pacific yew tree *Taxus brevifolia* (1). Anticancer activity is induced by promoting tubulin polymerization and inhibiting microtubules disassembly (2, 3). Therefore, at subnanomolar concentrations, paclitaxel can block mitosis and inhibit cell proliferation (4). Since the Food and Drug Administration's approval of paclitaxel in 1982, it has also been approved to treat refractory ovarian cancer, metastatic breast cancer (5), AIDS-related Kaposi's sarcoma (6), and non-small cell lung cancer in many countries (7). It is commonly used as one the most potent antimiotic agents for a variety of cancers locations including: lymphoma, esophageal, bladder, endometrial, cervical, head, and neck cancers (7, 8). Several studies have reported using paclitaxel to treat other diseases including kidney disease and rheumatoid arthritis (9, 10).

Paclitaxel was initially isolated from the bark of yew trees. Thus its supply is limited due to the very slow growth rates and the ability to contain of only about 0.01% of paclitaxel in the bark on a dry weight basis (11). A method was developed to synthesize paclitaxel, but it was not practical for industrialization due to the complex reaction steps and the high synthetic cost (12-15). Semi-synthetic methods to produce paclitaxel have since been developed, in which a natural precursor, 10-deacetylbaccatin III, was extracted from yew tree needles and used (16). Explorations of microorganisms such as the taxol-producing fungi *Taxomyces andreanae* and *Taxodium distichum* have been explored to determine if they produce paclitaxel, however, yields are too low to be commercially viable (17, 18). Cell cultures of various Taxus species have been explored to efficiently produce paclitaxel and related taxane compounds with the problem of maintaining productive cell lines over successive cultures (19-22). Hairy roots induced by an infection of *Agrobacterium rhizogenes* contain many valuable secondary metabolites (23), therefore, paclitaxel has been successfully refined using hairy roots of Taxus species (24).

Several genes encoding enzymes responsible for paclitaxel biosynthesis have been cloned in a move to further undertand the synthetic pathway (25). The biosynthetic route to paclitaxel can be divided into three steps: the synthesis of geranylgeranyl pyrophosphate (GGPP, the universal precursor of diterpenoids), the cyclization of GGDP to taxa-4(5)-11(12)-diene (taxadiene, the unique taxane skeleton), and the hydroxylation and acylation of the taxane skeleton to produce paclitaxel (3) (Fig. 1). Taxadiene synthase (TS) is the key enzyme that catalyzes the initial cyclization of GGPP to taxadiene to establish the taxane core structure (25, 26). Several *TS genes* have been cloned from *T. brevifolia, T. cuspidata* Sieb. Et Zucc and *T. chinensis*, and have been functionally expressed in *Escherichia coli* (27).

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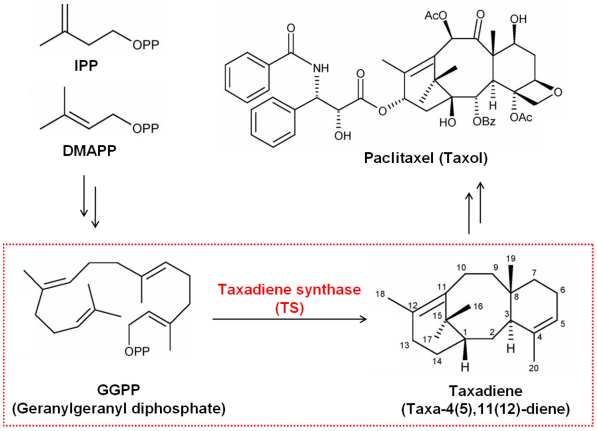

Fig. 1. Taxadiene [taxa-4 (5),11 (12)-diene] synthesis by taxadiene synthase in the taxol biosynthesis pathway.

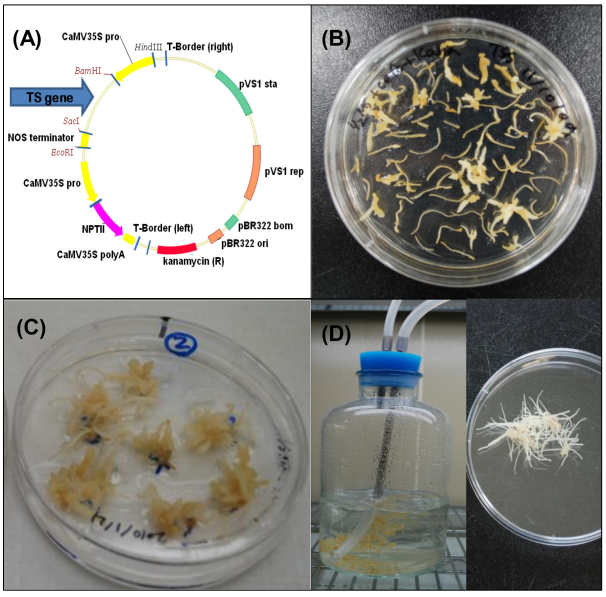

Fig. 2. Vector construction and ginseng transformation. (A) The taxadiene synthase gene sized 2.6 kb was ligated into the Ti 23001 plasmid vector. Ginseng roots transformed (B) and sub-cultured with the taxadiene synthase gene on 1/2MS agar medium (C) and grown in 1/2MS broth medium in an aerated incubator and the grown adventitious roots (D).

Ginseng (*Panax ginseng* C.A. Meyer) has traditionally been used to treat many diseases in Oriental medicine due to the high amounts of triterpenoid saponins and many secondary metabolites (28). Due to the containment of active terpenoid pathways and the establishment of the massive root culture methods, ginseng can be a good candidate for taxadiene metabolic engineering. Here we report the establishment of transformation of the cultured ginseng roots with a *TS* gene derived

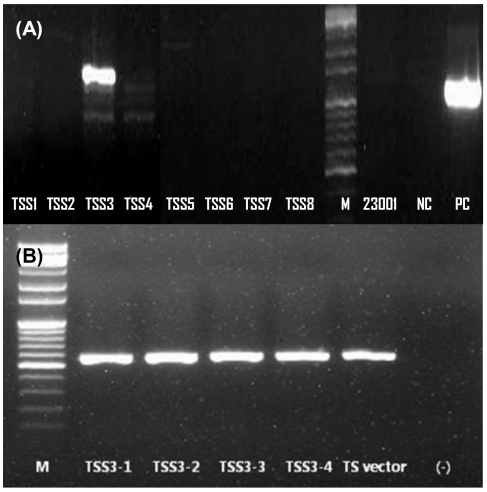

Fig. 3. Selection of ginseng roots expressing the taxadiene synthase (TS) gene. (A) PCRs were performed with the genomic DNA or plasmid. Lanes 1-8: transformed ginseng roots with the TS gene. Lane 9: 1 kb plus DNA ladder. Lane 10: transformed ginseng root lines with the empty 23001 vector. Lane 11: negative control PCR. Lane 12: positive control PCR (B) Expression levels of the TS gene by RT-PCRs. Purified total RNAs from the lines of transgenic TSS3 ginseng were used to make the complementary DNA, and used to perform RT-PCRs. Lane 1: 1kb plus DNA marker. Lane 2-5: TSS3 lines. Lane 6: positive control RT-PCR. Lane7: negative control RT-PCR.

from *T. cuspidata* Sieb. Et Zucc. One stable and fast grown transformant root line was selected and subsequent sub-cultures over 10-months period, and indeed, the line produced taxadiene.

RESULTS

Transformation of cultured ginseng roots

Cultured ginseng roots grew most actively in 1/2 MS media with IBA. The TSS vector and the empty vector 23001 were used to transform cultured ginseng roots by co-localization with *A. tumefaciens*. The transformed ginseng roots were selected on 1/2 MS media with cefataxime and kanamycin (Fig. 2A, B). A total of 98 transformants were selected and analyzed for further *TS* gene confirmation. Among the selected lines, only TSS3, TSS41, and TSS43 showed the existence of the *TS* gene. When the transformants were cultured in 1/2 MS broth media, the TSS3 line displayed the best *TS* gene growth and expression levels. Further successive incubations of the transformed TSS3 cultured ginseng roots produced several lines of TSS3, and we named the lines as TSS3-1, TSS3-2, TSS3-3, and TSS3-4. All of these lines showed high levels of *TS* gene expression, and the TSS3-2 line was finally selected due to the relatively high *TS* gene expression (Fig. 3B).

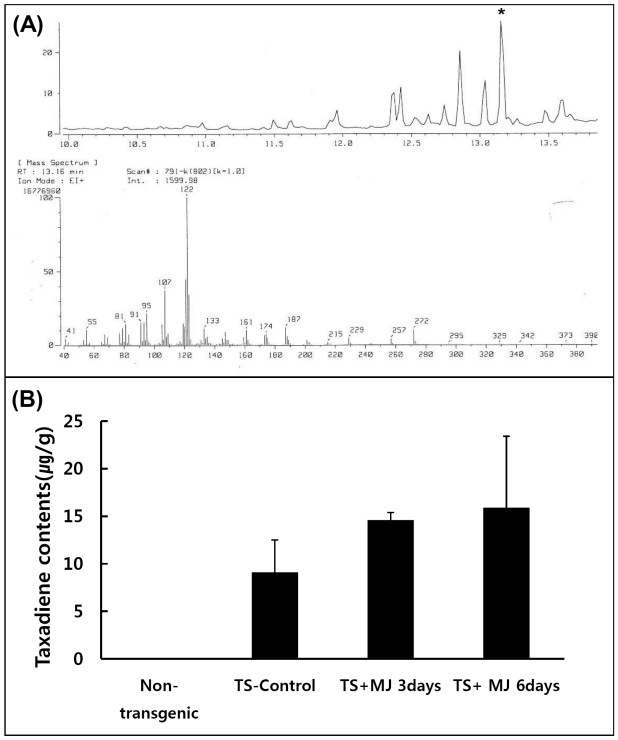

Fig. 4. Analysis of taxadiene by gas chromatography/mass spectroscopy. Crude hexane extracts from cultured wild-type ginseng or taxadiene synthase transformed ginseng roots were analyzed. (A) The peak at 13.16 min in the gas chromatogram had the mass spectra profile matched exactly with the taxadiene mass spectra profile of Kovacs *et al.* (2007). (B) Taxadiene levels in the taxadiene synthase gene transformed ginseng roots. Cultured transgenic ginseng roots were treated with or without methyl jasmonate for 3 days or 6 days. The vertical bars represent means ± standard errors (n = 3).

Measurement of the taxadiene amounts in the transformed cultured ginseng roots

The amount of taxadiene in control and the transgenic TSS3-2 line was analyzed by GC/MS as per the conditions set out by Kovacs *et al*. (29). In the initial attempt, the taxadiene existence was identified by a comparison of the peaks of the MS profile as was reported by Kovacs *et al*. (29). The control wild-type cultured ginseng plants displayed no peak for taxadiene. However, the transformed TSS3-2 line had the peak of taxadiene at 13.16 min of gas chromatogram (Fig. 4A) and the MS profile exactly matched the MS profile of taxadiene as reported in Kovacs *et al*. (29) (Fig. 4A).

The transgenic line TSS3-2 was further analyzed the taxadiene contents after treating methyl jasmonate (MJ) for 3 or 6 days. The transformed cultured ginseng roots contained 9.1 μg per gram of dry weight, and the treatment of MJ treatment increased the production of taxadiene, 14.6 or 15.9 μg taxadiene per gram of dry weight, respectively (Fig. 4B).

DISCUSSION

Several studies have indicated methods to taxadiene in tomato and Arabidopsis using metabolic engineering due to the high price (29-31). The presence and expression of the *TS* gene in the transgenic cultured ginseng root and the de novo formation of a compound with the same fragmentation pattern as taxadiene confirmed the successful transformation of cultured ginseng roots to produce taxadiene. The taxadiene yield from the transformed ginseng roots with the *TS* gene was 9.10 μg/g dry weight, which was higher than the 5 μg/g dry weight produced in moss (30), but lower than the 20 μg/g dry weight produced in transgenic tomato fruits (29).

In contrast to the reduced growth shown in the *TS*-transformed Arabidopsis and tomato (29, 31), no phenotypic or growth differences were observed between the cultured wild-type and the transgenic ginseng roots. Presumably, the newly introduced *TS* gene in Arabidopsis and tomato might convert GGPP to taxadiene, and therefore lower the amount of endogenous gibberellin (31). The moss engineered to produce taxadiene had no growth- retardation (30), because mosses do not require gibberellins for growth (32). The ginseng cultured roots probably do not require gibberellins for the growth, and instead, they should be supplied exogenous auxin for active root growth.

Taxadiene is synthesized directly from GGPP found in most plant tissues, which serves as a common precursor for many metabolites in the terpenoid pathway. MJ increases terpenoid metabolites (24, 31), and indeed, MJ treatment increased the yield of taxadiene in the cultured *TS*-transgenic ginseng roots, increasing by 1.6 times following only a 3-day MJ treatment (Fig. 4B).

Plant metabolic engineering is a potent way to produce beneficial metabolites. Using ginseng transformation with the *TS* gene, we successfully produced taxadiene in cultured ginseng roots for the first time. Production of taxadiene can be the first step of synthesizing paclitaxel in cultured ginseng roots, therefore, future works are required for further fulfillment of the whole metabolic engineering of paclitaxel synthesis in ginseng roots.

MATERIALS AND METHODS

Plant materials

Cultured ginseng adventitious roots were kindly provided by the Ginseng and Medicinal Plants Research Institute, Korea. The ginseng adventitious roots were sub-cultured every 3 weeks on a fresh half-strength Murashige and Skoog (1/2 MS) media with 3% (w/v) sucrose, pH 5.7, 3 mg/L of indole-3-butyric acid (IBA) and 0.34% (w/v) Gelrite at 23°C in dark. All chemicals and media were purchased from Duchefa (Haarlem, The Netherlands) or Sigma-Aldrich (St. Louis, MO, U.S.A).

Construction of the transformation vector

TSS vector, the transformation vector, was constructed. The *TS* gene used in this experiment was kindly supplied by the Dr. Rodney Croteau and acquired from the Washington State University Research Foundation (U.S. Patent Nos. 5,994,114; 6,114,160 and 6,610,527). The TSS vector was constructed by ligating only the coding region of *TS* gene (Fig. 2a) in the pBluescript SK (+) vector into the 23001 Ti plasmid vector. The 23001 Ti plasmid vector was derived from the pCAMBIA 2300, and was obtained from the Korea Research Institute of Bioscience and Biotechnology. The construction of the TSS vector was as follows; First, the coding region was acquired by performing a PCR with the forward primer (5′-aggatc**cATG**GCT CAGCTCTC-3′) and the reverse primer (5′-aggtac**cTCA**TACTT GAATTGGATC-3′), and the TS gene in the pBluescript SK(+) vector as the template. To construct the TSS vector, only the coding region of the *TS* gene in the PCR product was digested with *Bam*HI and *Kpn*I restriction enzymes, extracted with a DOKDO-Prep Gel Extraction kit (Elpis Biotechnology, Taejon, Korea), and ligated into the 23001 vector. The TSS vector was transferred into *E. coli* strain DH5ą using the heat shock method. The TSS vector plasmid was sequenced to verify the successful integration of the *TS* gene into the vector 23001.

The TSS vector plasmid was purified from *E.coli* using the HiYield Plasmid Mini kit (RBC, Taiwan), and transferred into *A. tumefaciens* strain EHA105, a Km (s) derivative of EHA101, using the freeze-thaw method (33).

Transformation of cultured ginseng roots by Agrobacterium

The *A. tumefaciens* with the TSS vector was used for the cultured ginseng roots transformation. The transformed *A. tumefaciens* was grown overnight at 28°C with 120 rpm of agitation in a yeast extract peptone medium containing 50 mg/l kanamycin. After incubation, cells were centrifuged at 3,500 rpm for 10 minutes, and the pellet was resuspended in10 mM MES buffer (pH 6.5) and 10 mM $MgCl_2$ to 0.5 at OD_{600}, and then acetosyringone was added to a final concentration of 100 μM and incubated for 4 hr at 22°C. The *A. tumefaciens* infection was conducted with approximately 1-cm of dissected ginseng adventitious roots using a liquid co-culture method by soaking the roots in the *A. tumefaciens* suspension for 24 h at 25°C with shaking in the dark (34).

Selection of transformed ginseng roots on antibiotic selection media

After the *A. tumefaciens* infection, the ginseng roots were washed with double-distilled water and transferred to on a fresh 1/2 MS media containing 500 mg/L cefotaxime, 50 mg/L kanamycin, 3 mg/L IBA and 0.8% (w/v) plant agar. The roots were sub-cultured on fresh cefotaxime-supplemented media every 3 weeks and maintained at 23°C in the dark for 15 weeks to sterilize the *A. tumefaciens* on the infected roots (Fig. 2b). The survived roots were then transferred to fresh 1/2MS solid media supplemented with only 50 mg/L kanamycin and 3 mg/L IBA.

Molecular analysis of the transformed ginseng roots

The successful transformation of cultured ginseng roots was verified by PCR analysis using genomic DNA. Genomic DNA was isolated from the cultured ginseng roots following the method of Dellaporta et al. (35). PCRs were performed with the primers designed to detect the *TS gene*, the forward primer (TS5C), 5′-TGCAGAACCATATCTTAG-3′ and the reverse primer (NosR), 5′-ATGCTTAACGTAATTCAACAG-3′. PCRs were conducted for 35 cycles with denaturing at 95°C for 20 sec, annealing at 50°C for 40 sec, and extension at 72°C for 1 min 30 sec, followed by a final elongation at 72°C for 5 min on the XP Thermal Cycler (BIOER, Tokyo, Japan). The PCR products were visualized by 1.2% agarose gel electrophoresis.

Culture of transformed ginseng roots

The selected transformed ginseng roots were sub-cultured on 1/2 MS agar media containing 50 mg/L kanamycin and 3 mg/L IBA. The cultures were maintained at 23°C in the dark and sub-cultured every 4 weeks for 4 months to obtain stable transformants. The transformed ginseng roots were then incubated for testing the growth rate in 1/2MS broth media containing 50 mg/L kanamycin and 3 mg/L IBA with 80 rpm shaking speed. Only the TSS3 transformant grew vigorously in the broth media, therefore, the transformant was dissected to several lines for large scale culture in broth media. The lines were tested for the expression levels of the *TS gene* by a semi-quantitative RT-PCR using the TS5M (5′-GCCAATTATCATGGCGAT CT-3′) and the TS3M primer (5′-CTGCCGCAGAAACATCTGT A-3′).

To make the cDNA for the template, total RNA was purified from the cultured ginseng roots using the Trizol solution (Invitrogen, Grand Island, USA) and used to construct cDNA using a cDNA kit (Elpis Biotechnology, Korea). The selected line of TSS3 maintained at 23°C in the dark was sub-cultured every four weeks for 3 months to obtain a stable transformant.

The large scale culture of the transformants was conducted in one liter of 1/2 MS broth media containing 50 mg/L kanamycin and 3 mg/L IBA in air-supplied glass incubators (15-cm diameter and 25-cm height) for 3 weeks (Fig. 2c). The large-scale cultured ginseng roots were divided roughly 10 g to 500 ml flasks and incubated with shaking at 25°C and 80 rpm of agitation in the dark for 7 days (Fig. 2d). To achieve a high production of taxadiene, final concentration of MJ at 50 μM was added to the culture media, and incubated for 3 and 6 days, respectively.

Taxadiene analysis

Cultured transgenic and control adventitious roots were harvested, dried at room temperature for 2 days, and ground to a fine powder with a mortar and a pestle. Five ml of hexane was added to 1 g of powdered tissue and shaken by a sonicator for 20 min at room temperature. The extract was filtered and the root tissues re-extracted 4 times additionally with a similar hexane volume. Each of these subsequent extractions

was pooled and dried. Hexane was added to the dried residue at 1 mg/ml concentration. One μl of each sample was subjected to gas chromatography-mass spectrometry [GC/MS, 6890N gas chromatograph (Agilent Technologies, Santa Clara, USA) linked to a JMS 700 mass spectrometer (Jeol, Japan)] at the Korea Basic Science Institute located at Kyungbuk National University. Samples were injected in a 10 : 1 split mode (injector 280°C) onto a 30 m × 0.25 mm ID DB-5 fused silica capillary column with a 0.25m film thickness. The initial oven temperature was 120°C, which was ramped to 250°C for 5 min at 10°C/min after a 2 min delay (using helium as the carrier gas). Taxadiene content was calculated for the control and MJ-treated transformed ginseng lines by comparison to a nonadecane internal standard.

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