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ACCUMULATION OF TAXADIENE BY ROOT CULTURE OF *NICOTIANA BENTHAMIANA* DOMIN TRANSFORMED WITH TAXADIENE SYNTHASE

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Abstract

Nicotiana benthamiana was transformed with a taxadiene synthase (*TS*) gene for the *de novo* synthesis of taxadiene. The successfully transformed lines of *N. benthamiana* producing taxadiene was further cultivated for root culture to produce taxadiene in *in vitro* system. The roots of lines TSS-5, TSS-7, TSS-8 and TSS-10 accumulated 14.6 - 22.5 µg/g (dw) taxadiene. Methyl jasmonate treatment to the TSS-8 line for 7 or 14 days increased the accumulation of taxadiene by 1.17 and 1.88-folds, respectively. Data suggested that the root culture of *TS* gene transformed *N. benthamiana* is an ideal system for the stable and efficient production of taxadiene.

Introduction

Taxadiene [taxa-4(5), 11(12)-diene] formation is the initial step in the biosynthesis cycle of an anticancer agent paclitaxel (taxol) from the universal precursor geranylgeranyl diphosphate (GGPP) (Hezari *et al.* 1995). Paclitaxel is a strong chemotherapeutic agent showing tremendous activity to treat breast, ovarian and lung cancer (Hata *et al*. 2004, Pezzutto 1996).

The recovery of paclitaxel form *Taxus* plants is very less (0.01% of bark) (Vidensek *et al.* 1990) but its world-wide demand go beyond the supply both from natural sources as well as semi-synthesis. Therefore, to maximize the production of paclitaxel, several efforts together with biological as well as synthetic method have been tried (Cragg *et al*. 1993). However, due to high cost of production and complex reaction steps, paclitaxel production was not up to the satisfactory level to be commercially viable (Danishefsky *et al.* 1996, Nicolaou *et al*. 1994). So, intensive and efficient efforts need to develop an alternative means for the higher production of paclitaxel. However, production of taxadiene is the key to synthesis of paclitaxel via using biological system or semi-synthesis.

Taxadiene was isolated from the Yew tree in less amount (Koepp *et al.* 1995) and its increased production is an urgent need. Taxadiene synthesizes from GGPP by taxadiene synthase (*TS*); however, the cloning of *TS* genes from *T. brevifolia, T. cuspidata* Sieb. Et Zucc and *T. chinensis* were found effective and the products of the gene were functional in *Escherichia coli* (Wang *et al*. 2002). Since metabolically engineered production of paclitaxel depends on the production of taxadiene, the first committed step of paclitaxel synthesis, several attempts have been tried to transfer the *TS* gene into *E. coli* and *Saccharomyces cerevisiae* (Huang *et al*., 2010, Boghigian *et al*. 2012, DeJong *et al*. 2006). The increased production of taxadiene has also been tried by expressing the *TS* gene in various plant systems including *Arabidopsis*, moss, tomato and ginseng roots (Anterola *et al*. 2009, Besumbes *et al*. 2004, Cha *et al*. 2012, Kovacs *et al*. 2007). However, continuous efforts need to the commercial viable production of taxadiene by using easy and short duration plant system.

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Nicotiana benthamiana related to tobacco plant is a rapidly growing model plant. Here we report the successful transformation of *N. benthamiana* with the *TS* gene derived from *T. cuspidata* Sieb. Et Zucc.Transformed *N. benthamiana* by the *TS* gene from *T. cuspidata* Sieb. Et Zucc can produce taxadiene. Therefore, we transformed *N. benthamiana* with a *TS* gene, measured the amount of taxadiene from leaf and then cultured the roots for producing higher amounts of taxadiene in very short time. However, massive root culture systems can be used for producing useful secondary metabolites stably and efficiently regardless of the adverse conditions arisen by climatic challenges.

Materials and Methods

A TSS vector was constructed by ligating 2.6 kb taxadiene synthase (*TS*) gene in the pBluescript SK (+) vector into 23001 Ti plasmid vector following the protocol of Cha *et al*. (2012). In brief, the coding region of the *TS* gene was ligated in the pBluescript SK (+) vector into 23001 Ti plasmid vector. Dr. Croteau from Washington State University, USA kindly provided the *TS* gene and the 23001 Ti plasmid vector collected from KRIBB, Korea was a derivative of the pCAMBIA 2300 vector. By using the gene specific primers (Forward: 5'-*AGGATCC*A TGGCTCAGCTCTC-3'; Reverse: 5'-*AGGTACC*TCATACTTGAATTGGATC-3'), coding region of the *TS* gene was obtained and DOKDO-Prep Gel Extraction Kit (Elpis, Korea) was used to purify the PCR product which were digested with the *Bam*HI and *Kpn*I restriction enzymes, ligated into the 23001 Ti plasmid vector and transferred into *E. coli* strain DH5α. By using a HiYield plasmid Mini Kit (RBC, Taiwan), the plasmid of TSS vector was isolated from the transformed *E. coli* and introduced into a Km (s) *A. tumefaciens* strain EHA105 by the freeze-thaw method (Hood *et al*. 1993). The transformed *A. tumefaciens* with the TSS vector was confirmed by PCR using the *TS* gene specific primers (Forward: 5'-GCCAATTATCATGGCGATCT-3'; Reverse: 5'-CTGCCGC AGAAACATCTGTA-3').

The transformation of *N. benthamiana* was done following the method of Wang (2006) with slight modification where leaves of aseptically grown *N. benthamiana* were collected and cut to approximately 0.5 cm^2 without the veins. The leaf explants were then pre-cultured in MS media containing 3% sucrose (w/v), 0.1 mg/l NAA, 1 mg/l BAP and 0.8% (w/v) plant agar followed by incubation in dark at 25°C for 24 hrs. The transformed *A. tumefaciens* with the TSS vector was grown overnight at 28°C and 120 rpm in yeast extract peptone (YEP) medium enriched 50 mg/l kanamycin which was centrifuged at 3,500 rpm for 10 min to get the pellet. The pellet was then resuspended in 10 mM MES and $MgCl_2$ for the final concentration of 0.5 at OD_{600}. Acetosyringone was added to the resuspended culture to a final concentration of 100 µM followed by incubation for 4 hrs at 22°C with mild shaking. The infection of the leaf explants were done by dipping in *A. tumefaciens* for 15 min followed by blotter dry and placed on co-cultivation medium [MS media containing 0.1 mg/l NAA, 1 mg/l BAP, 3% sucrose (w/v) and 0.8% (w/v) plant agar overlaid with the sterile Whatman filter paper] for 3 days in the dark at 25°C. The explants were then shifted to regeneration medium (MS media containing 100 mg/l cefotaxime, 50 mg/l kanamycin, 0.1 mg/l NAA, 1 mg/l BAP, 3% sucrose (w/v) and 0.8% (w/v) plant agar) and incubated at 25°C under a 16 hrs light and 8 hrs dark regime. The calluses originated from the explants were collected and transferred to root formation medium [MS medium containing 1% sucrose (w/v), 50 mg/l kanamycin, 0.1 mg/l NAA and 0.8% (w/v) plant agar]. The transformed plants were then shifted to the green house in pot soil. Successful *TS* transformed T1 *N. benthamiana* roots were screened for the *de novo* formation of taxadiene.

We measured higher amount of taxadiene from leaf of transformed homozygous (T1) *N. benthamiana* lines TSS-5, TSS-7, TSS-8 and TSS-10 (Hasan *et al*. 2014). For the production of

taxadiene from root, we collected seeds from three plants of each of above transformed lines, surface sterilized and sown in fresh Murashige and Skoog (MS) medium supplemented with 50 mg/l kanamycin in magenta box and incubated at 23°C in dark. Following germination, the magenta boxes were kept in 16 hrs light and 8 hrs dark regime for one month to get sufficient amount of roots (Fig. 1A). The roots were then harvested and cultured on MS agar media supplemented with 50 mg/l kanamycin and 0.1 mg/l NAA (Fig. 1B). All chemicals and media were purchased from Duchefa (Haarlem, The Netherlands) or Sigma-Aldrich (St. Louis, U.S.A).

Successful integration of *TS* gene in the roots of *TS* transformed *N. benthamiana* plant was censured by one step RT-PCR using total RNA as template. By using TRI reagent (Molecular Research Center, Inc. Ohio, USA), total RNA was isolated from the cultured roots. Gene specific primers to amplify 576 bp of *TS* gene were used for the one step RT-PCR (Forward: 5'-CGTGACATGCTCGCTCAC-3', and Reverse: 5'-AGGTACCTCATACTTGAATTGGATC-3'). After making cDNA at 45°C for 45 min, PCR amplification was done 30 cycles for 30 sec at 94°C, 40 sec at 50°C, and 1 min at 72°C by a PCR machine (XP Thermal Cycler, BIOER, Japan). PCR products were then visualized by using 1.2% agarose gel electrophoresis (Fig. 1E).

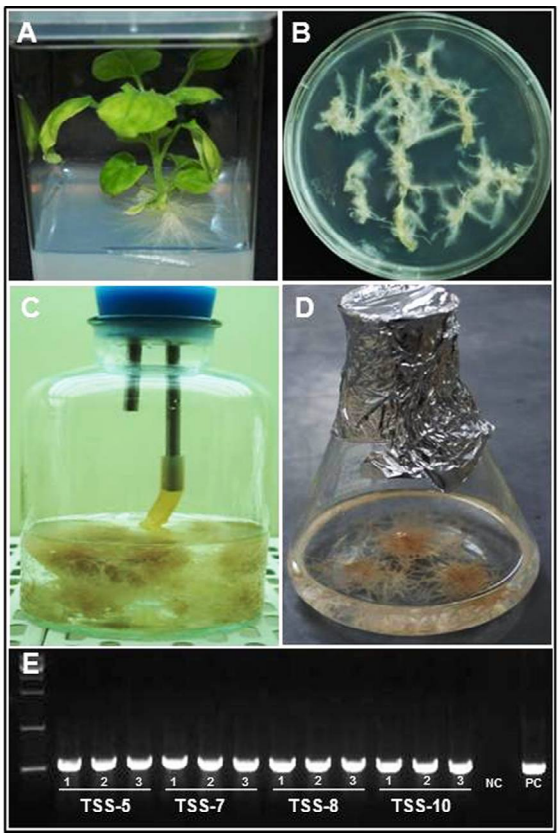

Fig. 1. Root culture for the production of taxadiene. (A) selection of roots on kanamycin-enriched MS agar media, (B) aerial roots on MS agar medium, (C) root culture in MS broth medium in an aerated incubator, (D) root culture in MS broth medium for the methyl jasmonate treatment and (E) RT-PCR profile for identifying the expression of taxadiene synthase in different transformed root lines. Lane 1: 1 kb plus DNA marker; Lane 2-12: *TS* transformed roots line; Lane 13: wild-type control; Lane 14: empty vector; Lane 15: positive control.

Roots from the solid cultures were sub-cultured in MS broth media contained 50 mg/l kanamycin and 0.1 mg/l NAA and maintained at 23°C in darkness at 80 rpm agitation. In every 3 weeks, sub-cultures were done in the same media to get the stable transformants. The roots were then analyzed to identify and quantify the *de novo* production of taxadiene. The root line TSS-8 produced highest amount of taxadiene among all the homozygous lines, therefore, it was further selected to analyze the effect of methyl jasmonate (MJ) on the accumulation of taxadiene. To observe the effect of MJ, the roots were cultured in air-supplied culture system (15 cm diameter and 25 cm height) maintained the same media and conditions for 3 weeks (Fig. 1C). The cultured roots were then divided into 10 g to 250 ml flask and incubated for 7 days with 23°C and 80 rpm of shaking in dark (Fig. 1D). In order to accumulate the high amount of taxadiene, MJ was added to the root culture at a final concentration of 50 µM and incubated for more 7 and 14 days, respectively.

Cultured transgenic and wild-type control roots of *N. benthamiana* were harvested, freeze-dried and ground to fine powder by using a mortar and a pestle. Five (5) ml hexane supplemented with 4 µg/ml nonadecane (Sigma-Aldrich, St. Louis, USA) was added to 0.5 g powdered root tissue. Following vortexing for 30 sex, the root powders were shaken by a sonicator for 20 min at room temperature. The extracts were filtered through cotton plug and re-extracted successively four more times with the same volume of hexane only. All the subsequent extracts were then pooled, dried under N_2 gas and measured the weight. One ml hexane was added to the dried extracts prior to GS-MS analysis. GC-MS analysis was performed following the published protocol of Cha *et al.* (2012) where following the addition of 1 mL of hexane to the dried extracts, injection was done with 1 µL of each sample into an Agilent 6890N GC (Agilent Technologies, Santa Clara, USA) linked to a JMS 700 MS (Jeol, Japan). Every extract was injected in 10:1 split mode (injector 280°C) onto a 30 m × 0.25 mm ID DB-5 fused silica capillary column with a 0.25 m film thickness. The primary oven temperature was maintained at 120°C followed by rising to 250°C for 5 min at 10°C/min after a 2 min delay by helium as the carrier gas. The measurement of taxadiene was then calculated based on comparison to a nonadecane internal standard (100 µg/ml).

Results and Discussion

Roots from three plants of each transformed homozygous line TSS-5, TSS-7, TSS-8 and TSS-10 were analyzed and identified for the successful integration *TS* gene in the genome (Fig. 1E). The artificial culture system for the roots of *TS* transformed *N. benthamiana* was successful for the *de novo* production of taxadiene which were analyzed by a GC-MS following the conditions of Kovacs *et al.* (2007) and Cha *et al.* (2012) where non-transformed wild-type control root of *N. benthamiana* did not show any peak corresponding to taxadiene (Fig. 2A). However, the roots from transformed *N. benthamiana* showed the peak of taxadiene at 13.11 min of the gas chromatogram (Fig. 2B) and the MS profile of taxadiene (Fig. 2C) matched exactly with the MS profile of taxadiene as reported by Kovacs *et al.* (2007). The root lines of *TS* transformed *N. benthamiana* TSS-5, TSS-7, TSS-8 and TSS-10 were produced 14.6 - 22.5 µg taxadiene/g of dw (Fig. 3).

Among the tested root lines, TSS-8 produced highest amount of taxadiene (22.5 µg/g dw) which was lower than the amount produced in leaf using the same line (27.0 µg/g dw; Hasan *et al.* 2014). However, the production of taxadiene in root was higher than the previously reported taxadiene amount in the *TS* transformed ginseng roots (9.1 µg/g dw), *Arabidopsis* (0.6 µg/g dw), moss (5.0 µg/g dw) and normal tomato (20.0 µg/g dw) but less than *TS* transformed *yellow flesh* tomato mutant which is lack of carotenoid synthesis (160 µg/g dw) (Anterola *et al.* 2009, Besumbes *et al.* 2004, Cha *et al.* 2012, Kovacs *et al.* 2007).

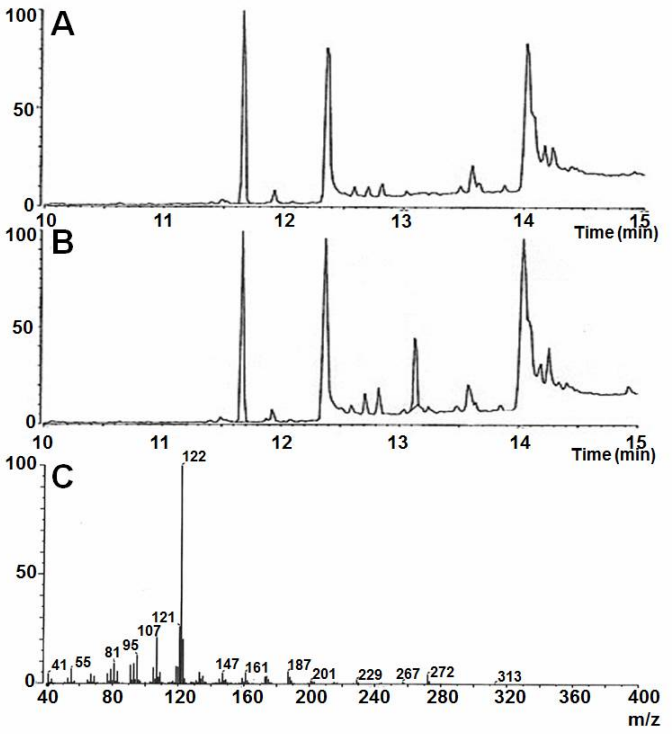

Fig. 2. GC-MS analysis for the identification of taxadiene production. (A) GC chromatogram from non-transformed *N. benthamiana*. (B) GC chromatogram from transgenic of *N. benthamiana* with the taxadiene peak at 13.11 min. (C) Mass spectra profile for the taxadiene pick at 13.11 min, which completely matched with the taxadiene mass spectra profile reported by Kovac *et al*. (2007)

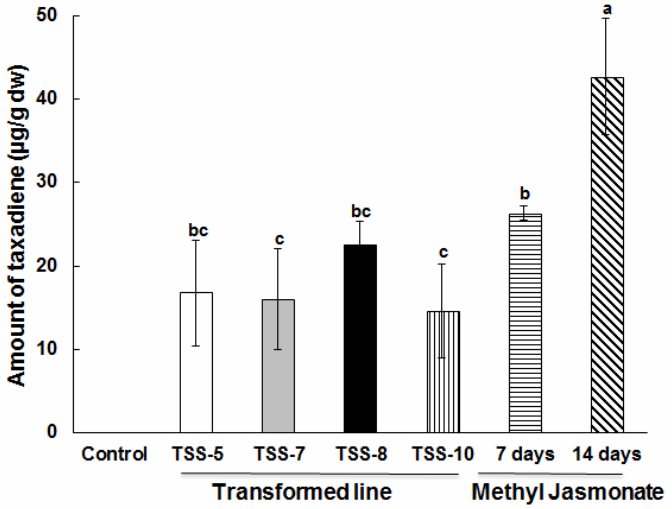

Fig. 3. Accumulation of taxadiene in the cultured transformed roots of lines TSS-5, 7, 8 and 10. The Methyl jasmonate lines indicated the increased amount of taxadiene in transformed root line TSS-8 treated with methyl jasmonate for 7 or 14 days, respectively. The vertical bars represent means ± standard deviations (n = 3).

A common precursor GGPP used to produce many metabolites by the terpenoid pathway of plant system and taxadiene is also directly synthesized from GGPP. MJ is commonly known as the elicitor for the increased production of terpenoids in plants (Besumbes *et al*. 2004). When the elicitor MJ was applied in root culture of TSS-8 line for 14-days, taxadiene accumulation significantly increased by 1.88-folds, indicating tobacco roots were also reacted to MJ to increase the taxadiene amount same as previous study where taxadiene production in *TS*-transformed ginseng roots were increased by 1.6-fold following treatment with MJ at 50 µM (Cha *et al.*2012).

Nicotiana benthamiana was transformed with *TS* gene, induced roots, and produced taxadiene by the root culture aseptically. The cultured tobacco roots accumulated stably and highly taxadiene. In future, in order to complete the synthesis of paclitaxel in *N. benthamiana*, further understanding for the whole genes related to the paclitaxel biosynthesis will be required.

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References

Anterola A, Shanle E, Perroud P and Quatrano R 2009. Production of taxa-4(5),11(12)-diene by transgenic *Physcomitrella patens*. Transgenic Res. **18**: 655-660.

Besumbes O, Sauret-Güeto S, Phillips MA, Imperial S, Rodriguez-conception M and Boronat A 2004. Metabolic engineering of isoprenoid biosynthesis in *Arabidopsis* for the production of taxadiene, the first committed precursor of taxol. Biotechnol. Bioeng. **88**: 168-175.

Boghigian BA, Salas D, Ajikumar PK, Stephanopoulos G and Pfeifer BA 2012. Analysis of heterologous taxadiene production in K- and B-derived *Escherichia coli*. Appl. Microbiol. Biotechnol. **93**:1651-1661.

Cha M, Shim SH, Kim SH, Kim OT, Lee SW, Kwon SY and Baek KH 2012. Production of taxadiene from cultured ginseng roots transformed with taxadiene synthase gene. BMB Rep. **45**: 589-594.

Cragg GM, Schepartz SA, Suffness M and Grever MR 1993. The taxol supply crisis. New NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents. J. Nat. Prod. **56**: 1657-1668.

Danishefsky SJ, Masters JJ, Young WB, Link JT, Snyder LB, Magee TV, Jung DK, Isaacs RCA, Bornmann WG, Alaimo CA, Coburn CA and Di Grandi MJ 1996. Total synthesis of baccatin III and taxol. J. Amer. Chem. Soc. **118**: 2843-2859.

DeJong JM, Liu Y, Bollon AP, Long RM, Jennewein S, Williams D and Croteau RB 2006. Genetic engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae*. Biotech. and Bioeng. **93**:212-224.

Hasan MM, Kim H-S, Jeon J-H Kim SH, Moon B, Song J-Y, Shim SH and Baek K-H 2014. Metabolic engineering of *Nicotiana benthamiana* for the increased production of taxadiene. Plant Cell Rep. **33**: 895-904.

Hata K, Osaki M, Dhar DK, Nakayama K, Fujiwaki R, Ito H, Nagasue N and Miyazaki K 2004. Evaluation of the antiangiogenic effect of Taxol in a human epithelial ovarian carcinoma cell line. Cancer Chemother. Pharmacol. **53**: 68-74.

Hezari M, Lewis NG and Croteau R 1995. Purification and characterization of taxa-4(5),11(12)-diene synthase from pacific yew (*Taxus brevifolia*) that catalyzes the first committed step of Taxol biosynthesis. Arch. Biochem. Biophys. **322**: 437-444.

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res. 2: 208-218.

Huang Q, Roessner CA, Croteau R and Scott AI 2001. Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol. Bioorganic Med. Chem. **9**:2237-2242.

Koepp AE, Hezari M, Zajicek J, Vogel BS, LaFever RE, Lewis NG and Croteau R 1995. Cyclization of Geranylgeranyl Diphosphate to Taxa-4 (5) ,11 (12)-diene Is the Committed Step of Taxol Biosynthesis in Pacific Yew. J. Biol. Chem. **270**: 8686-8690.

Kovacs K, Zhang L, Linforth RST, Whittaker B, Hayes CJ and Fray RG 2007. Redirection of carotenoid metabolism for the efficient production of taxadiene [taxa-4(5),11(12)-diene] in transgenic tomato fruit. Transgenic Res. **16**: 121-126.

Nicolaou KC, Yang Z, Liu JJ, Ueno H, Nantermot PG, Guy RK, Claiborne CF, Ronaud J, Couladouros EA, Paulvannan K and Soronsen EJ 1994. Total synthesis of Taxol. Nat. **367**: 630-634.

Pezzutto J 1996. Taxol production in plant cell culture comes of age. Nat Biotechnol. **14**: 1083.

Vidensek N, Lim P, Campbell A and Carlson C 1990. Taxol content in bark, wood, root, leaf, twig, and seedling from several *Taxus* species. J. Nat. Prod. **53**: 1609-1610.

Wang K 2006. *Agrobacterium* protocol, volume (II), 2nd edition. Humana press, Totowa, New Jersy, USA. pp. 143-154.

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