Plant Cell Rep
DOI 10.1007/s00299-014-1568-9

ORIGINAL PAPER

Metabolic engineering of *Nicotiana benthamiana* for the increased production of taxadiene

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Received: 8 October 2013 / Revised: 8 January 2014 / Accepted: 9 January 2014

Abstract
Key message **We report the production of taxadiene by transformation of N. benthamiana with a taxadiene synthase gene. The production was significantly increased by an elicitor treatment or metabolic pathway shunting.**
Abstract Paclitaxel (Taxol®) was first isolated from the bark of the pacific yew tree as an anticancer agent and has been used extensively to treat various types of cancer. Taxadiene, the first committed product of paclitaxel synthesis is cyclized from geranylgeranyl diphosphate (GGPP), and further complex hydroxylation and acylation processes of the unique taxane core skeleton produce paclitaxel. To accomplish de novo production of taxadiene, we transformed *Nicotiana benthamiana* with a taxadiene synthase (*TS*) gene. The introduced *TS* gene under the transcriptional control of the CaMV 35S promoter was constitutively expressed in *N. benthamiana*, and the de novo production of taxadiene was confirmed by mass spectroscopy profiling. Transformed *N. benthamiana* homozygous lines produced 11–27 μg taxadiene/g of dry weight. The highest taxadiene production line TSS-8 was further treated with an elicitor, methyl jasmonate, and metabolic pathway shunting by suppression of the phytoene synthase gene expression which resulted in accumulation of increased taxadiene accumulation by 1.4- or 1.9-fold, respectively. In summary, we report that the production of taxadiene in *N. benthamiana* was possible by the ectopic expression of the *TS* gene, and higher accumulation of taxadiene could be achieved by elicitor treatment or metabolic pathway shunting of the terpenoid pathway.

Keywords Metabolic engineering · Taxol · Taxadiene synthase · Methyl jasmonate · Metabolic pathway shunting

Abbreviations

TS	Taxadiene synthase
GGPP	Geranylgeranyl diphosphate
MJ	Methyl jasmonate
PSY	Phytoene synthase
PDS	Phytoene desaturase
GFP	Green fluorescent protein
NAA	1-Naphthaleneacetic acid
BAP	6-Benzylaminopurine
YEP	Yeast extract peptone
MS	Murashige and Skoog
RT-PCR	Reverse transcription polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
VIGS	Virus-induced gene silencing

Communicated by M. Jordan.

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Published online: 25 January 2014

Introduction

The natural diterpenoid paclitaxel (Taxol®) was first isolated from the bark of the pacific yew tree *Taxus brevifolia* as an anticancer agent (Wani et al. 1971). Paclitaxel was approved by the United States Food and Drug Administration in 1982, and has since shown excellent efficacy for the treatment of different types of cancer including refractory ovarian cancer, metastatic breast cancer (Charles et al. 2001; Hata et al. 2004), lung cancer (Francis et al. 1995), and AIDS-related Kaposi's sarcoma (Gill et al. 1999). Paclitaxel exerts anticancer activity by inducing tubulin polymerization and inhibiting microtubule disassembly (Kumar 1981; Parness and Horwitz 1981); therefore, paclitaxel can block mitosis and inhibit cell proliferation even at very low concentrations (Ahn et al. 2010). Paclitaxel is also effective against many other diseases including kidney failure, rheumatoid arthritis, and restenosis (Arsenaulta et al. 1998; Heldman et al. 2001; Woo et al. 1994). Due to its effective treatment of a wide variety of diseases, demand for paclitaxel is increasing worldwide.

Taxus spp. grows very slowly, and the recovery of paclitaxel from their bark is only 0.01 % (Vidensek et al. 1990) which has hampered industrial production of paclitaxel. Therefore, many attempts to develop economically viable methods for the production of paclitaxel have been made including chemical and biological approaches (Cragg et al. 1993). However, commercially viable production of paclitaxel has not yet been achieved due to the complexity and high costs of production (Danishefsky et al. 1996; Nicolaou et al. 1994). Some naturally occurring endophytic fungi such as *Taxomyces andreanae*, *Taxodium distichum*, and *Corylus* sp. also produce paclitaxel; however, the yield of paclitaxel from these endophytes is too low to be commercially viable (Li et al. 1996; Stierle et al. 1993). Semisynthetic methods for the production of paclitaxel using 10-deacetylbaccatin III as a precursor have been successfully employed (Castor and Theodore 1993). Attempts to cell suspension culture of many species of *Taxus* have also been made but there have been difficulties maintaining successive culture of productive lines (Brunakova et al. 2005; Cusidó et al. 2002; Hara et al. 2008; Srinivasan et al. 1995; Tabata 2006). Recently, the increased accumulation and recovery of paclitaxel in cell suspension culture were achieved by different elicitors or media composition but still need efforts to further increase the production of paclitaxel (Exposito et al. 2009; Kajani et al. 2012; Onrubia et al. 2013). However, *Agrobacterium rhizogenes* induces hairy roots that promote various important secondary metabolites in the roots (David et al. 1984), and paclitaxel was successfully produced by the hairy roots of *T. cuspidata* (Kim et al. 2009).

Several genes encoding enzymes for the biosynthesis of paclitaxel have been cloned to understand the entire paclitaxel synthesis pathway (Walker and Croteau 2001; Wildung and Croteau 1996). The biosynthesis pathway of paclitaxel mainly consists of three steps, synthesis of geranylgeranyl diphosphate (GGPP), cyclization of GGPP to taxa-4(5)-11(12)-diene (taxadiene, the unique taxane core skeleton), and hydroxylation and acylation of the taxane core skeleton to make paclitaxel (Hezari et al. 1995) (Fig. 1). Taxadiene, the first committed product of paclitaxel synthesis, is isolated from the bark of yew trees in very low levels (Koepp et al. 1995); however, taxadiene synthase (*TS*) cyclizes GGPP to taxadiene (Hezari et al. 1995; Wildung and Croteau 1996). The cloning of *TS* genes from *T. brevifolia*, *T. cuspidata* Sieb. Et Zucc, and *T. chinensis* was successful, and the gene products were functional in *Escherichia coli* (Wang et al. 2002). Since metabolic engineering of paclitaxel depends on the first committed step of taxadiene production, many efforts have been made to transfer the *TS* gene into *E. coli* and *Saccharomyces cerevisiae* (Ajikumar et al. 2010; Boghigian et al. 2012; DeJong et al. 2006; Huang et al. 2001). Production of taxadiene has also been attempted by expressing the *TS* gene in different plant systems such as *Arabidopsis*, moss, tomato, and ginseng roots (Anterola et al. 2009; Besumbes et al. 2004; Cha et al. 2012; Kovacs et al. 2007).

Nicotiana benthamiana is a rapidly growing model plant related to tobacco. Here we report the successful transformation of *N. benthamiana* with the *TS* gene derived from *T. cuspidata* Sieb. Et Zucc. Through transformation, the transformed lines were selected and screened for the production of taxadiene. Increased production of taxadiene in the homologous transformed line was further attempted by the treatment of an elicitor or metabolic pathway shunting for the terpenoid pathway.

Materials and methods

Plant materials

Seeds of wild-type *N. benthamiana* were surface-sterilized with 70 % alcohol for 3 min, then submersed in clorox bleach for 10 min and washed five times with sterile distilled water. Seeds were sown in Magenta boxes filled with fresh Murashige and Skoog (MS) medium containing 1 % (w/v) sucrose and 0.8 % agar at pH 5.7 and incubated at 23 °C under dark conditions. After germination of the seeds, the Magenta boxes were kept under a 16 h light and 8 h dark regime for 1 month. All chemicals and media were purchased from Duchefa (Haarlem, The Netherlands) or Sigma-Aldrich (St. Louis, MO., USA).

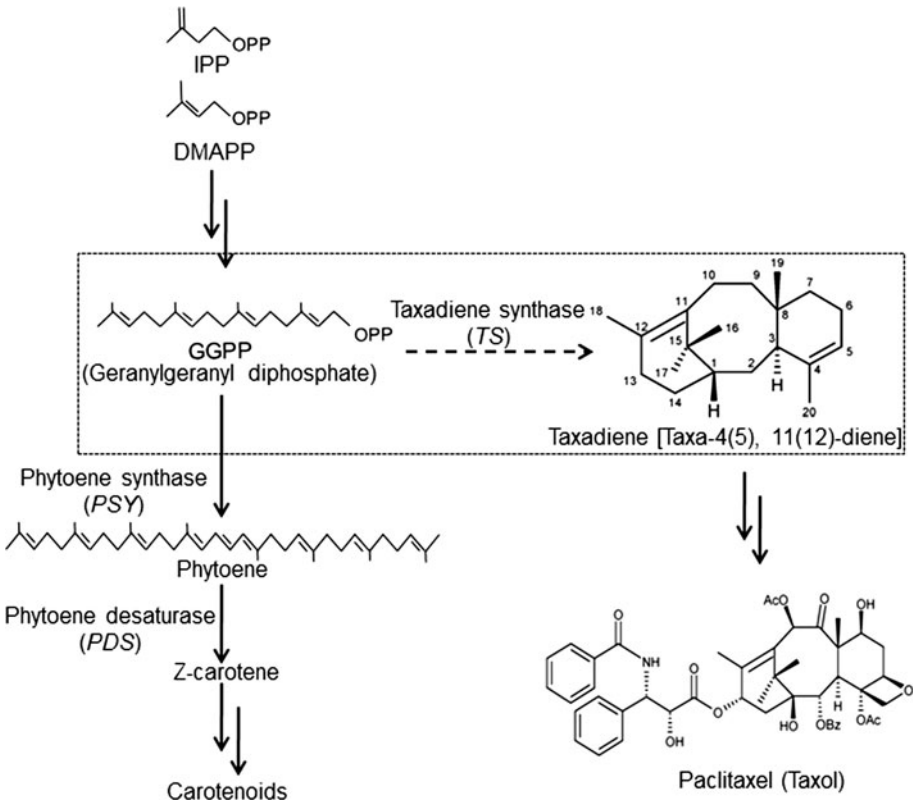

Fig. 1 Taxadiene [Taxa-4(5), 11(12)-diene] or carotenoids biosynthesis from the precursor GGPP

Construction of the transformation vector

TSS vector containing the *TS* gene was constructed using the method described by Cha et al. (2012). Briefly, the vector was constructed by ligating only the coding region of the *TS* gene in the *pBluescript* SK (+) vector into 23001 Ti plasmid vector. The *TS* gene was kindly provided by Dr. Croteau at Washington State University, USA and the 23001 Ti plasmid vector obtained from KRIBB, Korea was a derivative of the pCAMBIA 2300 vector. The coding region of the *TS* gene was obtained by PCR using the gene specific primers shown in Table 1 and the template of the *TS* gene in the *pBluescript* SK (+) vector. The PCR product was purified with a DO-KDO-Prep Gel Extraction Kit (Elpis, Korea), digested with the *Bam*HI and *Kpn*I restriction enzymes, ligated into the 23001 Ti plasmid vector, and transferred into *E. coli* strain DH5α. The TSS vector plasmid was isolated from the transformed *E. coli* using a HiYield plasmid Mini Kit (RBC, Taiwan) and introduced into a KM (s) *A. tumefaciens* strain EHA105 by the freeze–thaw method (Holsters et al. 1978; Hood et al. 1993). The successful transformation of *A. tumefaciens* with the TSS vector was confirmed by PCR using the *TS* gene specific primers shown in Table 1.

Transformation of *N. benthamiana* by *Agrobacterium* infection

Transformation of *N. benthamiana* with the *TS* gene was accomplished by the leaf disc method (Wang 2006) with slight modification. Briefly, leaves from *N. benthamiana* grown for 1 month were aseptically cut to approximately 0.5 cm^2 without the veins using a scalpel. The leaf explants were then precultured in MS media containing 3 % sucrose (w/v), 0.1 mg/L NAA, 1 mg/L BAP, and 0.8 % (w/v) plant agar, after which they were incubated in the dark at 25 °C for 24 h. The *A. tumefaciens* transformed with the TSS vector was grown overnight at 28 °C and 120 rpm in yeast extract peptone (YEP) medium containing 50 mg/L kanamycin, after which it was centrifuged at 3,500 rpm for 10 min. The pellet was then dissolved in 10 mM MES and $MgCl_2$ until an absorbance of 0.5 at OD_{600} was obtained. Acetosyringone was added to the resuspended culture to a final concentration of 100 μM, after which it was incubated for 4 h at 22 °C with gentle shaking. *A. tumefaciens* infection was carried out by dipping the leaf explants in the suspension for 15 min.

Selection of transformed *N. benthamiana* on antibiotic selection media

Following infection with *A. tumefaciens*, the leaf explants were briefly blotted on sterile filter paper and then placed on co-cultivation medium [MS media containing 3 % sucrose (w/v), 0.1 mg/L NAA, 1 mg/L BAP, 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 transferred to regeneration medium [MS media containing 3 % sucrose (w/v), 100 mg/L cefotaxime, 50 mg/L kanamycin,

Table 1 Primers for construction of *N. benthamiana* transformed with the *TS* gene and for virus-induced gene silencing of the *PSY* and *PDS* gene in *TS*-transformed *N. benthamiana*

Gene	Primer	Sequence (5′–3′)	Description
TS	TSClonF	*AGGATCC*ATGGCTCAGCTCTC	Transformation vector construction for the *TS* gene
	TSClonR	*AGGTACC*TCATACTTGAATTGGATC	
	TS5M	GCCAATTATCATGGCGATCT	Confirmation of transformation of *Agrobacterium* with the *TS* gene
	TS3M	CTGCCGCAGAAACATCTGTA	
	TS5D	CGTGACATGCTCGCTCAC	Confirmation of transformation of *N. benthamiana* with the *TS* gene
	TS3D	TCATACTTGAATTGGATC	
	TSqF	AGAGACAGCCTTGTTCCAG	Selection of transformed homozygous line by qPCR
	TSqR	GTCCATAGCTCTTTCGTCAG	
PSY	PhytoSF	*CGGAATTC*TGCTGCTTTGTCCGATACTG′	Primers for VIGS construct
	PhytoSR	*TCCGCTCGAG*GCCTTTGAATCAGGTGCAAT	
	PsyF1	GGAACTATGCTAATGACTCC	Confirmation of silencing of the upstream region of the *PSY* gene
	PsyR1	CCGGTCTTCCCACCTATCTAA	
	PsyF2	GTATGGGCATCTTTGCTGTTG	Confirmation of silencing of the downstream region of the *PSY* gene
	PsyR2	TGTAGGGGGCACAAGAGATT	
PDS	PhytoDSF	*GGTCTAGA*CTGACGAGCTTTCGATGCAGTGCAT	Primers for VIGS construct
	PhytoDSR	*CGGGGATCC*ATATATGGACATTTATCACAGGAAC	
	PdsF1	CGTACTCCAAGTGCCACGA	Confirmation of silencing of the upstream region of the *PDS* gene
	PdsR1	GGCGTGAGGAAGTACGAAAC	
	PdsF2	CATGTTGTCAAAACCCCAAG	Confirmation of silencing of the downstream region of the *PDS* gene
	PdsR2	CCTGATAAGACAGCACCTTCC	

Restriction site sequences are indicated by italic letters and underlined

0.1 mg/L NAA, 1 mg/L BAP, and 0.8 % (w/v) plant agar] and incubated at 25 °C under a 16 h light and 8 h dark regime. After 2 weeks, subcultures of the explants were conducted on the same medium. The calluses raised from the explants were excised and transferred to the rooting medium [MS medium containing 1 % sucrose (w/v), 50 mg/L kanamycin, 0.1 mg/L NAA, and 0.8 % (w/v) plant agar]. After 1 month, the acclimatized transformed plants were transferred and grown on standard greenhouse soil mix.

Molecular analysis of transformed *N. benthamiana* plants

Successful integration of the *TS* gene into the *N. benthamiana* genome was confirmed by one-step RT-PCR. Total RNA was isolated from the transformed *N. benthamiana* leaves using TRI Reagent (Molecular Research Center, Inc. Ohio, USA) and one-step RT-PCR was carried out using the *TS* gene specific primers shown in Table 1. Reverse transcription was carried out at 45 °C for 45 min followed by PCR. The PCR cycle consisted of 35 cycles of denaturing at 94 °C for 30 s, annealing at 50 °C for 40 s, and extension at 72 °C for 1.5 min followed by final elongation at 72 °C for 5 min using an XP Thermal Cycler (BIOER, Japan). The PCR products were then visualized by 1.2 % agarose gel electrophoresis.

Selection of homozygous T1 *N. benthamiana* plants

Seeds from transformed T0 *N. benthamiana* plants were surface-sterilized and sown on MS media containing 50 mg/L kanamycin, after which they were incubated at 25 °C in the dark. After germination, the Magenta boxes were kept under a 16 h light and 8 h dark regime at 25 °C until the plants were large enough to be transferred to a growth chamber. Following acclimatization, the T1 plants were transferred to standard greenhouse soil mix. DNA was then isolated from the leaves of T1 plants following the method described by Dellaporta et al. (1983). Quantitative PCR (qPCR) was conducted to select homozygous transformed lines. *TS* gene specific primers designed to amplify a 216 bp sequence were used for qPCR (Table 1), which was conducted by subjecting the samples to 35 cycles of denaturing at 95 °C for 15 s, annealing at 52 °C for 40 s and extension at 72 °C for 40 s using a real-time PCR machine (Rotor-Gene™ 600, Corbett Life Science, Australia). Seeds were collected from selected homozygous lines and further tested by growth on MS media containing 50 mg/L kanamycin. The homozygosity of all selected lines was confirmed by observing 100 % germination on kanamycin selection media.

Methyl jasmonate (MJ) treatment to elicit the production of taxadiene

To increase the accumulation of taxadiene, 3-week-old transformed T1 plants were sprayed with 100 μM solution of MJ (Duchefa, The Netherland) with 0.05 % Tween-80. Each plant was sprayed with 40 mL of MJ solution to ensure even and complete coating of the plants in a ventilated fume hood and kept in a fume hood for 30 min before shifting to a growth chamber to allow evaporation of the excess solution. *N. benthamiana* plants were sprayed with MJ two times with an interval of 1 week, and the leaves were collected 15 days after the second spray to measure the amount of taxadiene.

Metabolic pathway shunting to increase the precursor GGPP for taxadiene synthase by the application of virus-induced gene silencing (VIGS)

GGPP, the precursor for both diterpenoids and tetraterpenoids, was accumulated by suppressing the pathway to carotenoids. Two genes for the pathway, phytoene synthase (*PSY*) and phytoene desaturase (*PDS*) in the first and second step for the pathway of carotenoid synthesis (Fig. 1), respectively, were silenced. Briefly, the partial products of *PSY* and *PDS* were prepared by PCR using the primers listed in Table 1 using the sequence information of the *N. tabacum PSY* gene (NCBI accession No. JX101475) and *PDS* gene (NCBI accession No. AJ616742), respectively. The amplified PCR products were then digested with respective restriction enzymes and cloned into pTRV2 vector to produce the pTRV2:*PSY* and pTRV2:*PDS* plasmid. Transformation of *A.tumefaciens* strain GV2260 with the constructs of pTRV2:*PSY*, pTRV2:*PDS*, and pTRV2:*GFP* was successfully accomplished by the freeze–thaw method (Chung et al. 2004; Liu et al. 2002). Overnight cell cultures of *A. tumefaciens* transformed with pTRV2:*PSY*, pTRV2:*PDS*, and pTRV2:*GFP* and with pTRV1 were then centrifuged at 5,000 g, after which the pellets were resuspended in 10 mM MES and $MgCl_2$ to give an absorbance of 0.5 at OD_{600} for pTRV2 constructs and 0.1 for pTRV1. Following incubation of the *A. tumefaciens* cultures with acetosyringone at a final concentration of 100 μM for 4 h at 22 °C, the *Agrobacterium* transformed with pTRV1 and pTRV2 constructs was mixed at a ratio of 1:1, after which the mixed cells were infiltrated into the lower leaves of 18-day-old four-leaf-stage *N. benthamiana*. The inoculated plants were then grown under a 16 h light and 8 h dark regime at 23 °C.

To confirm successful silencing of the *PSY* and *PDS* gene, total RNA was isolated from the leaves of VIGS plants over 4 weeks using TRI reagent (Molecular Research Center, Inc., Taiwan). Semiquantitative RT-PCR of the total RNA was then conducted using an Opti-Script™ One-step RT-PCR kit (Intron Biotechnology, Korea). Two sets of primers from upstream and downstream of the VIGS construct region of *PSY* and *PDS* were designed, respectively (Table 1). Reverse transcription was carried out for 45 min at 45 °C followed by PCR amplification of 25 cycles with denaturation at 95 °C for 30 s, annealing 50 °C for 40 s, extension at 72 °C for 40 s followed by a final elongation at 72 °C for 5 min using a PCR machine (XP Thermal Cycler, BIOER, Japan). The PCR products were then visualized by 1.2 % agarose gel electrophoresis.

Analysis of the amounts of taxadiene

Leaves from the transformed T0, homozygous T1, MJ-sprayed, and *PSY* and *PDS* gene-silenced *N. benthamiana* were collected, freeze-dried for 24 h, and then ground to fine powder with a mortar and pestle. The extraction was done following the published protocol of Besumbes et al. (2004) with little modification. The extraction was done by adding 5 mL of hexane containing 20 μg/mL nonadecane into 0.5 g of powdered tissue and sonicated for 20 min at room temperature using a sonic cleaner at 40 kHz (Branson, model-8510, Fredericksburg, VA, USA). The extract was then filtered through a cotton plug, after which the remaining leaf tissues were re-extracted four successive times using the same volume of hexane alone. Each of these subsequent extracts were pooled and dried under N_2 gas. For taxadiene analysis by GC–MS, 1 mL of hexane was added to the dried extracts, and 1 μL of each sample was injected into an Agilent 6890 N GC (Agilent Technologies, Santa Clara, USA) linked to a JMS 700 MS (Jeol, Japan) at the Korea Basic Science Institute at Daegu, Korea. All extracts were 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 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. The concentration of taxadiene was then determined based on comparison to a nonadecane internal standard (100 μg/mL).

Results

Transformation of *N. benthamiana*

TSS vector and empty vector 23001 were used for transformation of the *N. benthamiana* plant with the *TS* gene by co-localization with *A. tumefaciens*. Agro-infected leaf explants were then placed on MS agar media supplemented with BAP, cefotaxime, and kanamycin. Next, 200 calluses

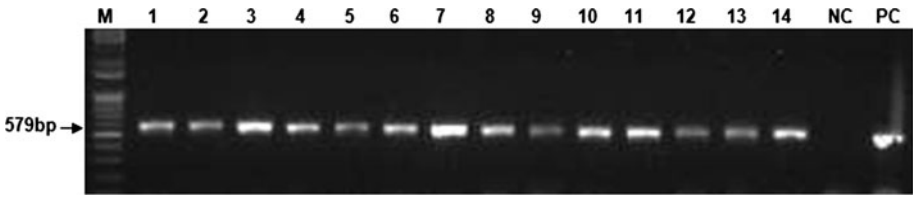

Fig. 2 Selection of *N. benthamiana* plants expressing the taxadiene synthase (*TS*) gene. RT-PCR was performed using total RNA. *Lane 1*: 1 kb + DNA marker, *Lanes 2–14*: *N. benthamiana* plants transformed with the *TS* gene, *Lane 15*: *N. benthamiana* plants transformed with the empty 23001 vector and *Lane 16*: TSS vector plasmid as the positive control

were obtained from the agro-infected leaf explants and transferred to MS agar containing NAA and kanamycin for root formation. A total of 110 transformed lines were subsequently transplanted to soil and grown in a walk-in chamber, after which they were analyzed to confirm successful integration of the *TS* gene. Finally, the survived 14 transformed plants indicated the successful integration of the *TS* gene (Fig. 2).

Measurement of the taxadiene amounts

Hexane extracts from the 14 transformed *N. benthamiana* T0 lines were analyzed for the de novo production of taxadiene. Identification and quantification of the amounts of taxadiene were conducted by GC–MS using the method described by Kovacs et al. (2007) and Cha et al. (2012). When compared with the chromatogram for the extract of the wild-type *N. benthamiana* (Fig. 3a), seven independent T0 transformed lines, TSS-2, 3, 5, 6, 7, 8, and 10, showed a GC peak at 13.09 min (Fig. 3b), and the mass spectrum for the peak exactly matched with the mass spectroscopy profile of taxadiene (Fig. 3c). These data confirmed the de novo production of taxadiene in the seven lines out of total 14 lines, producing 4–18 μg taxadiene per gram of dried leaves (Fig. 4). The top four T0 lines (TSS-5, 7, 8, and 10) which accumulated the highest amounts of taxadiene were selected, self-pollinated, and analyzed for the T1 homozygous lines by quantitative PCR. The leaves of selected T1 homozygous lines were extracted to be the measured amounts of taxadiene, and the TSS-5, 7, 8, and 10 homogeneous lines accumulated 22, 13, 27, and 11 μg taxadiene per gram of dried leaves, respectively (Fig. 4).

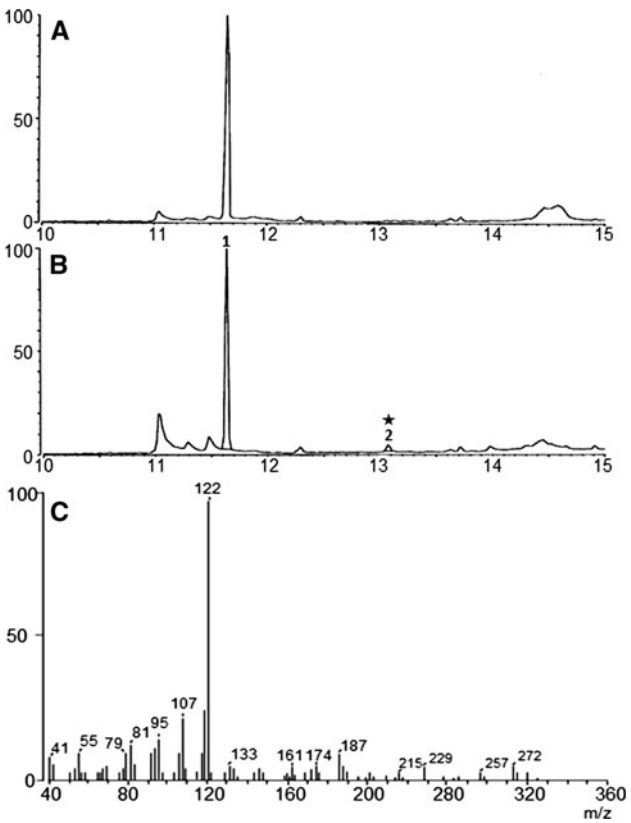

Fig. 3 Measurement of taxadiene by gas chromatography/mass spectroscopy. **a** Crude hexane extracts from leaves of non-transformed wild-type *N. benthamiana* not showing any *peak* corresponding to taxadiene, **b** *peak* at 13.09 min in the gas chromatograph indicating the de novo production of taxadiene in correspondence with taxadiene in the taxadiene synthase transformed *N. benthamiana*, and **c** mass spectra profile matched exactly the taxadiene mass spectra profile

Increasing the production of taxadiene by MJ treatment and metabolic pathway shunting

We selected the TSS-8 line accumulating the highest amounts of taxadiene to determine if taxadiene production could be improved by employing MJ application or diverting the metabolic flow into the carotenoid pathway. GGPP is the substrate for diterpenoids including taxadiene and tetraterpenoid carotenoids; therefore, we attempted to increase the pool of GGPP by reducing the metabolic flux to carotenoids through silencing of the genes in the first and second step of the carotenoid biosynthetic pathway. To accomplish this, a VIGS vector was constructed by insertion of 198 and 369 bp partial products of the *PSY* and *PDS* genes into pTRV2 vector, respectively (Fig. 5a, b). After 2 weeks of VIGS treatment, semiquantitative RT-PCR was conducted to confirm the successful silencing of the *PSY* and *PDS* genes. In addition, the *GFP*-silenced plants were

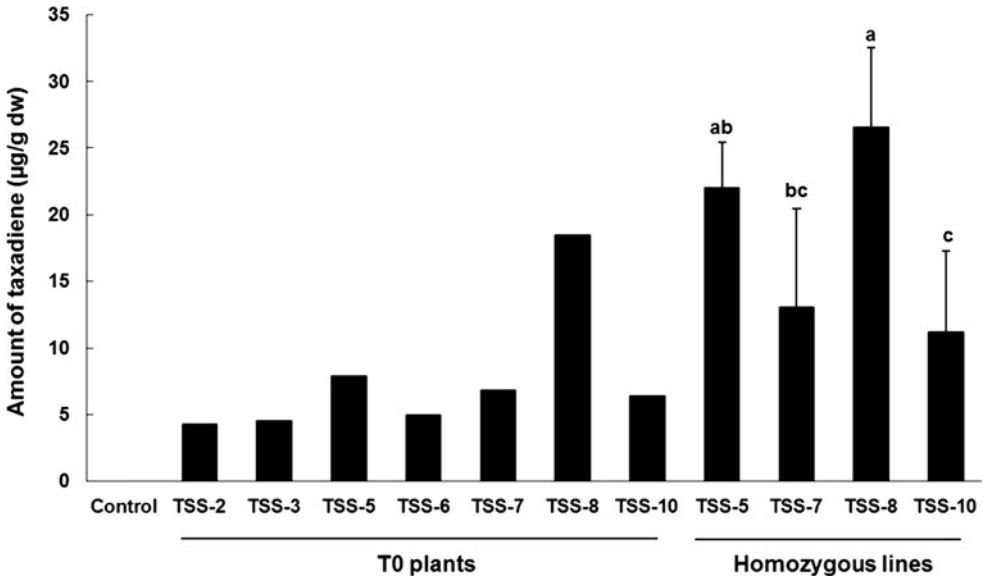

Fig. 4 Accumulation of taxadiene in the leaves of the transformed *N. benthamiana* T0 plants and the homozygous T1 lines. The *vertical bars* represent mean ± standard deviations ($n = 3$)

used to compare the effects of silencing the *PSY* and *PDS* genes. *PSY* and *PDS* gene expression was significantly reduced or almost non-detectable following gene silencing (Fig. 5c, d). *PSY*-silenced plants showed a yellowish- green phenotype, whereas *PDS*-silenced plants showed bleached leaves (Fig. 5e). Both the *PSY*- and *PDS*-silenced plants showed reduced growth relative to the wild-type and TSS-8 line (Fig. 5e). MJ-treated plants showed yellowish leaves with reduced growth (Fig. 5e).

MJ treatment increased the accumulation of taxadiene to 35 µg/g of dried leaves from 25 µg/g in the control TSS-8 line (Fig. 6). Silencing the *N. benthamiana PSY* gene and diverting the pathway for carotenoid biosynthesis resulted in accumulation of 48 µg taxadiene/g of dried leaves, which was 93.58 % higher than the control TSS-8 line (Fig. 6). Conversely, silencing the *PDS* gene by VIGS decreased the accumulation of taxadiene by 47.59 % relative to the control TSS-8 line (Fig. 6).

Discussion

Paclitaxel is a polycyclic diterpene produced by various species of yew trees, *Corylus avellana*, and some fungal species and is used as a potent anticancer drug and marketed as Taxol® (Bestoso et al. 2006; Li et al. 1996; Stierle et al. 1993; Wani et al. 1971). Paclitaxel is available by direct extraction from the bark of yew trees or by semi-synthesis from the extracted precursor of 10-deacetyl-baccatin III. Due to low recovery from natural sources and the high cost of synthetic production, metabolic engineering for paclitaxel is desperately required, and taxadiene, the taxane skeleton core structure is the first committed step for the biosynthesis of paclitaxel.

There have been many attempts to maximize production of taxadiene in different heterologous plants or microbial systems by using metabolic engineering (Anterola et al. 2009; Besumbes et al. 2004; Boghigian et al. 2012; Huang et al. 2001; Kovacs et al. 2007). The successful integration and expression of the *TS* gene into the genome of *N. benthamiana* and the concomitant *de novo* formation of a compound with the same mass spectroscopy profile as taxadiene confirmed the successful production of taxadiene in *N. benthamiana* for the first time in the present study. Our *TS*-transformed homozygous *N. benthamiana* TSS-8 line produced 27 taxadiene µg/g dw, which was higher than previously reported taxadiene production from *TS*-transformed moss (5 µg/g dw; Anterola et al. 2009), *Arabidopsis* (0.6 µg/g dw; Besumbes et al. 2004), ginseng roots (9.6 µg/g dw; Cha et al. 2012) or normal tomato (20 µg/g dw; Kovacs et al. 2007), but lower than that of *TS*- transformed *yellow flesh* tomato (160 µg/g dw; Kovacs et al. 2007). Yellow flesh tomato is a mutant that lacks phytoene synthase activity, the first committed step in the carotenoid synthesis pathway, which might allow the diversion of GGPP to produce taxadiene in place of its use as a substrate for the production of carotenoid (Fray and Grierson 1993; Kovacs et al. 2007).

TS-transformed *N. benthamiana* had late seed germination and reduced growth compared with wild-type *N. benthamiana* (data not shown). Similarly, reduced growth was reported in *TS*-transformed *Arabidopsis* and tomato plants (Besumbes et al. 2004; Kovacs et al. 2007), suggesting that the newly introduced *TS* gene interferes with the biosynthesis of gibberellins possibly by reducing the pool of available GGPP. Indeed, during the synthesis of gibberellins, the pool of GGPP is one of the most important factors; however, ectopic expression of the *TS* gene causes

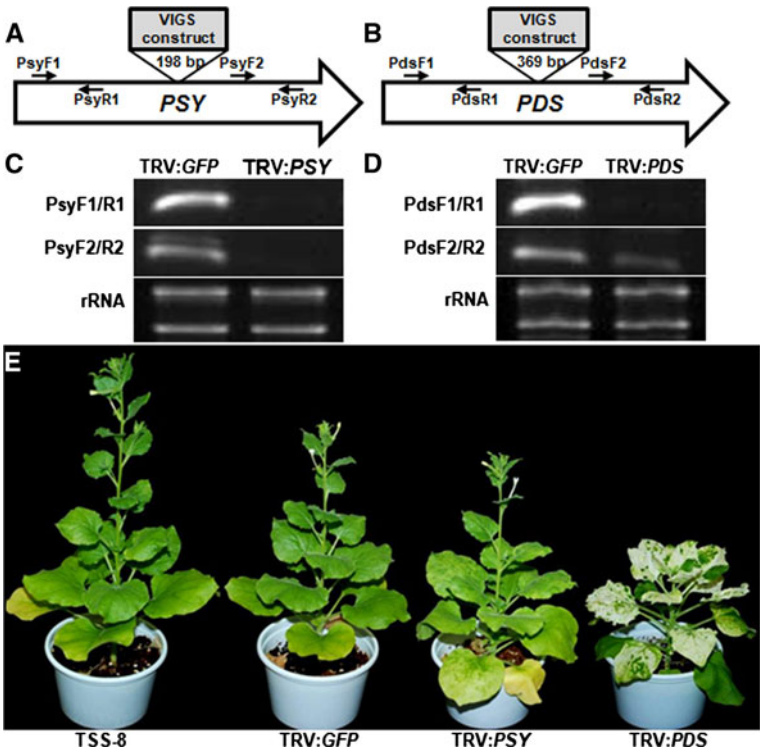

Fig. 5 Virus-induced gene silencing of *PSY* and *PDS* genes in the *TS*- transformed *N. benthamiana* TSS-8 line. **a** Location of the primers used for the VIGS construct for the *PSY* gene (198 bp) and detection of silencing of the *PSY* gene. **b** Location of the primers used for the VIGS construct for the *PDS* gene (369 bp) and for detection of silencing of the *PDS* gene. Semiquantitative RT-PCR confirmed silencing of the **c** *PSY* gene and **d** *PDS* gene using two primer sets for detection of gene expression from both the *upstream* and *downstream region* of the VIGS construct, respectively. **e** Phenotypes of *N. benthamiana* TSS-8 line non-silenced or silenced with *GFP*, *PSY*, and *PDS* gene, respectively

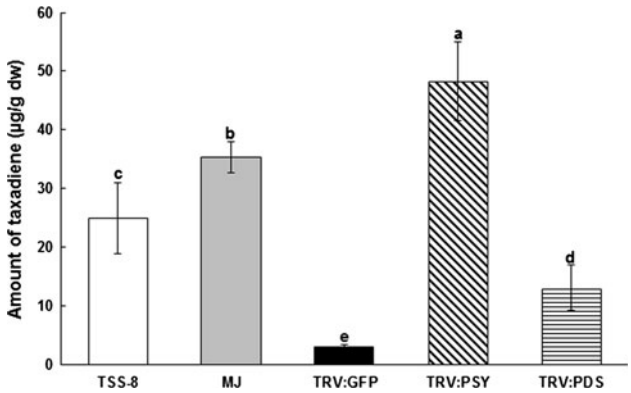

Fig. 6 Effect of methyl jasmonate (MJ) spray or metabolic pathway shunting by virus-induced gene silencing of the *PSY* and *PDS* gene on the accumulation of taxadiene in the leaves of the *TS*-transformed *N. benthamiana* TSS-8 line. The *vertical bars* represent mean ± standard deviations ($n = 3$)

gibberellins synthesis to be competitive since it is also produced from the same precursor GGPP (Kovacs et al. 2007). Similarly, reduced height of tomato and tobacco plants was observed in conjunction with constitutive overexpression of *PSY*, which catalyzes the conversion of GGPP into phytoene, thereby reducing the pool of GGPP available for the synthesis of gibberellins (Fray et al. 1995).

After acquiring the *N. benthamiana* lines producing taxadiene, we further attempted to increase the amounts via two major approaches, treating the plants with an elicitor or diverting the pathway for carotenoid biosynthesis. MJ is known to increase the production of terpenoids in plants (Besumbes et al. 2004; Kim et al. 2009), and a previous study showed that taxadiene production by *TS*-transformed ginseng roots increased by 1.6-fold following treatment with MJ at 50 μM (Cha et al. 2012). As shown in other reports, *TS*-transgenic *N. benthamiana* TSS-8 line showed a 1.4-fold increase in taxadiene production after 15 days of treatment with MJ at 100 μM (Fig. 6).

We also attempted to increase the pool of GGPP by silencing the *PSY* and *PDS* genes, which catalyze the conversion of GGPP into phytoene and phytoene into zeta-carotene, respectively, in the carotenoid synthesis pathway. When the first committed step of carotenoid synthesis was suppressed by silencing of the *PSY* gene through VIGS, the *TS*-transgenic *N. benthamiana* TSS-8 line increased the accumulation of taxadiene by 1.9-fold (Fig. 6). However, silencing of *PDS*, the second committed step for carotenoid synthesis, decreased the accumulation of taxadiene relative to the control TSS-8 line. These data suggest that the *PSY* gene is the most appropriate target for increasing the production of taxadiene in *N. benthamiana*, even though the VIGS vector itself significantly reduced the production of taxadiene due to a metabolic effect like interruption in the newly formed taxadiene synthesis pathway or any other unknown reasons.

Plant metabolic engineering has been extensively investigated for the production of beneficial plant secondary metabolites due to its effective and sustainable features. Through transformation of *N. benthamiana* with a *TS* gene,

we successfully produced taxadiene in *N. benthamiana* for the first time, and the yield was higher than that observed for any transgenic organism to date except for *TS*-transgenic yellow flesh tomatoes. The highest production of taxadiene in *N. benthamiana* and tomato indicates that the Solanaceae family is currently the best option for taxadiene metabolic engineering and future paclitaxel production. Furthermore, *N. benthamiana* or *N. tabacum* is the best candidate for paclitaxel metabolic engineering due to its capacity of the highest production taxadiene, relatively easy gene transformation and silencing, and high biomass production. However, potato plants might not be an appropriate system for the production of taxadiene. We previously transformed potatoes with the same *TS* vector but could not detect the production of taxadiene in the leaves and tubers, which might have been due to unknown problems associated with expression or to technical difficulties analyzing potato plants rich in secondary metabolites (data not shown).

Based on the successful de novo production of taxadiene in *N. benthamiana*, further transformation of genes in the paclitaxel biosynthetic pathway and crossing of lines to accumulate genes are warranted to form a solid basis for the successful production of paclitaxel in heterologous plant systems.

Acknowledgments This work was supported by a grant from the Systems and Synthetic Agro-biotech Center through the Next-Generation BioGreen 21 Program (PJ00950603), Rural Development Administration, Korea. We appreciate Dr. Rodney Croteau for kindly providing the cloned taxadiene synthase gene.

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