Transgenic Res (2009) 18:655–660
DOI 10.1007/s11248-009-9252-5

BRIEF COMMUNICATION

Production of taxa-4(5),11(12)-diene by transgenic *Physcomitrella patens*

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Received: 4 August 2008 / Accepted: 4 February 2009 / Published online: 25 February 2009

Abstract Taxadiene synthase gene from *Taxus brevifolia* was constitutively expressed in the moss *Physcomitrella patens* using a ubiquitin promoter to produce taxa-4(5),11(12)-diene, the precursor of the anticancer drug paclitaxel. In stable moss transformants, taxa-4(5),11(12)-diene was produced up to 0.05% fresh weight of tissue, without significantly affecting the amounts of the endogenous diterpenoids (*ent*-kaurene and 16-hydroxykaurane). Unlike higher plants that had been genetically modified to produce taxa-4(5),11(12)-diene, transgenic *P. patens* did not exhibit growth inhibition due to alteration of diterpenoid metabolic pools. Thus we propose that *P. patens* is a promising alternative host for the biotechnological production of paclitaxel and its precursors.

Keywords Taxa-4(5),11(12)-diene · Taxadiene synthase · *Physcomitrella patens* · Taxol · Paclitaxel · Cancer

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Introduction

Paclitaxel (more commonly known as Taxol^{TM}) is a widely prescribed anticancer agent that inhibits cell proliferation by stabilizing microtubules (Schiff et al. 1979). It was originally isolated as a bioactive component from the bark of Pacific yew (*Taxus brevifolia*; Suffness and Wall 1995), and later structurally defined as a very complex multi-substituted taxane diterpenoid (Wani et al. 1971). Extremely low levels of paclitaxel in slow-growing Pacific yews (~0.015% dry weight; Vidensek et al. 1990), prompted several laboratories to develop chemical methods to totally synthesize paclitaxel (reviewed in Kingston et al. 2002), but none of these methods are suitable for large-scale commercial production of the drug. Paclitaxel is currently manufactured from an advanced paclitaxel precursor 10-deacetylbaccatin III (Holton et al. 1995), which is extracted from needles of European yew (*Taxus baccata*; Chauviere et al. 1981).

As with any perennial crop, the yields of 10-deacetylbaccatin III from European yews are highly variable (ElSohly et al. 1997; Zhao et al. 2006) and affected by environmental conditions (Wheeler et al. 1992; Hook et al. 1999). Yews also require substantial land area for cultivation (Kikuchi and Yatagai 2003), long time prior to harvest (Thomas and Polwart 2003), intensive labor (Smith and Cameron 2002), and additional costs for extraction and semisynthesis of the drug. Thus, efforts have been made by several groups to find alternative sources of paclitaxel.

Plant tissue culture is a potential source of paclitaxel. Liquid cell suspension cultures of *Taxus* species can be induced to produce paclitaxel using various elicitors and treatments (Hezari et al. 1997). Studies to improve production levels of paclitaxel in plant tissue culture have been conducted (reviewed in Tabata 2006), but the reproducibility of various induction treatments from one cell line to another have yet to be demonstrated. Moreover, *Taxus* cells (like other cultured plant cells) undergo epigenetic and mutational changes while in culture (Kaeppler et al. 2000; Cassells and Curry 2001) which can change their totipotency and ability to produce paclitaxel.

Another potential source of paclitaxel is endophytic fungi living inside *Taxus brevifolia*, which have been found to produce paclitaxel (Stierle et al. 1994). However, these endophytic fungi do not grow very well in culture and produce only minute amounts of paclitaxel (Li et al. 1998). Various approaches including genome shuffling (Zhao et al. 2008), insertional mutagenesis (Chi et al. 2008), and restriction enzyme mediated integration (Wang et al. 2007) have been used to overexpress paclitaxel biosynthetic genes in endophytic fungi to improve paclitaxel production, but their slow growth imposes limitations on their productivity.

Faster-growing heterologous systems such as bacteria and yeasts have been used to overexpress paclitaxel biosynthetic genes. Overexpression of taxadiene synthase (which catalyzes the first step in paclitaxel biosynthesis) resulted in appreciable levels of taxa-4(5),11(12)-diene in both *E. coli* (1.3 mg/l culture, Huang et al. 2001) and *Saccharomyces cerevisiae* (8.7 mg/l culture, Engels et al. 2008). Coexpression of taxadiene synthase with taxadiene-5α-hydroxylase in *S. cerevisiae* (DeJong et al. 2006) resulted in ample amounts of taxa-4(5),11(12)-diene (0.5% dry weight of cells) but very low levels of taxadiene-5α-ol (25 μg/l culture). Simultaneous expression of taxadiene synthase and taxadiene-5 α-hydroxylase with downstream enzymes (i.e. taxadiene-5α-ol acetyltransferase and taxane 10β-hydroxylase) of the paclitaxel pathway did not produce detectable downstream products beyond taxadiene-5 α-ol. Thus, neither yeast nor bacteria has been successful so far in producing advanced paclitaxel precursors.

With higher plants as heterologous hosts, overexpression of taxadiene synthase produced up to 0.00006 and 0.016% dry weight of taxa-4(5),11(12)-diene in *Arabidopsis thaliana* (Besumbes et al. 2004) and tomato fruits (Kovacs et al. 2007), respectively. These transgenic tomato fruits thus contained taxa-4(5), 11(12)-diene at about the same level as paclitaxel found in Pacific yew bark. However, these transgenic tomato and arabidopsis plants reportedly grew more slowly than wild type (Kovacs et al. 2007; Besumbes et al. 2004) presumably due to disruption in gibberellin biosynthesis. Furthermore, coexpression of taxadiene-5α-hydroxylase with taxadiene synthase in tobacco did not produce taxadiene-5α-ol, but rather an unexpected product 5(12)-oxa-3(11)-cyclotaxane (Rontein et al. 2008). Thus, transferring paclitaxel biosynthetic genes into another plant cannot be assumed to lead automatically to paclitaxel formation.

In this paper, we report the heterologous overexpression of taxadiene synthase in the moss *Physcomitrella patens*. Our results show that the transgenic *P. patens* is able to produce taxa-4(5),11(12)-diene without any deleterious phenotypic consequences, and thus provides an appropriate and promising alternative to the production of paclitaxel.

Methods, results and discussion

The coding region of taxadiene synthase from *Taxus brevifolia* was amplified by PCR using oligonucleotides 5′-CACCATGGCTCAGCTCTCATTTAAT-3′ and 5′-TCATACTTGAATTGGATCAATATAAAC TTT-3′ as primers, and taxadiene synthase cDNA (provided by Dr. Rodney Croteau, Washington State University, Pullman, WA) as template. The amplified product (~2.6 kb) was gel purified and cloned into a pENTR vector (Invitrogen) via a Topoisomerase-mediated ligation reaction, and then subcloned into an expression vector (pTHUBIgateway, constructed from pGEMT-easy of Promega) via a Gateway LR reaction using LR clonase II, to generate the plasmid pTHUBI:TS. This plasmid placed taxadiene synthase under the control of a ubiquitin promoter.

After verification of the inserted taxadiene synthase gene in pTHUBI:TS by DNA sequencing, pTHUBI:TS was linearized using the restriction enzyme *Swa*I and then transformed into *Physcomitrella patens* (Gransden strain) protoplasts using standard polyethylene glycol-mediated transformation procedure (Schaefer and Zryd 1997). Hygromycin resistant *P. patens*

transformants were isolated and grown according to Perroud and Quatrano (2006). After screening transformants for targeted gene insertions by PCR, two transgenic lines (TS3 and TS9) showed the presence of the transgene at the targeted locus. Southern blot analysis performed independently with hygromycin and taxadiene synthase probes confirmed the presence of multicopy insertion of the vector in both lines. Presence of taxadiene synthase protein ($\sim$75 kDa) was verified by western blot (data not shown) using rabbit polyclonal antibody raised against taxadiene synthase (provided by Dr. Croteau). TS3 and TS9 lines were then further analyzed, along with a wild type control, for the presence of taxa-4(5),11(12)-diene, as follows.

Hexane (1 ml) was added to $\sim$100 mg (fresh wt) transgenic or wild type *P. patens* tissues, which were then homogenized in the presence of 1 g Zirconia beads (Fisher) by shaking on a FastPrep instrument (setting 6.0, 3 × 20 s). After centrifugation at 16,000*g* for 2 min, the hexane supernatant (0.5 ml) was transferred into a glass vial, from which 5 µl was injected (in splitless mode) by a Varian CP-8410 autoinjector into a FactorFour™ 5 ms capillary column that was installed on a Varian 3900 gas chromatograph connected to a Saturn 2100T ion trap mass spectrometer. Using a temperature program that runs from 50°C (initially held for 1 min) to 250°C at a rate of 20°C per minute, a unique peak at $\sim$11 min (Peak 1, Fig. 1) was observed in extracts from both TS3 (data not shown) and TS9 (Fig. 1). The mass fragmentation pattern of this peak had the same diagnostic ions (*m*/*z* 107, 121, 122, 123, 229, 257 and 272) as that reported for taxa-4(5),11(12)-diene (Wildung and Croteau 1996). The amounts of *ent*-kaurene (Peak 2, Fig. 1) and 16-hydroxykaurane (Peak 3, Fig. 1) in the samples have been quantified by comparison of peak areas with authentic standards provided by Robert Coates (University of Illinois).

In the absence of taxa-4(5),11(12)-diene standard, we were unable to verify an identical retention time for this peak with taxa-4(5),11(12)-diene. Nevertheless, the presence of a taxadiene synthase gene and protein in the transgenic moss (confirmed by Southern and western blots, respectively) and the de novo formation of a compound with the same fragmentation pattern as taxa-4(5),11(12)-diene in transgenic mosses (but not in the wild type) constitute sufficient evidence to suggest that *P. patens* has for the first time been metabolically engineered to produce taxa-4(5),11(12)-diene. Using nonadecane as an internal standard, we estimated that taxa-4(5),11(12)-diene could be produced by *P. patens* up to 0.05% of its fresh weight (maximum value among 12 independent extractions of TS3 and TS9).

The amount of taxa-4(5),11(12)-diene that can be produced by transgenic *P. patens* is still below those that can be achieved using bacteria and yeast. However, yeasts and bacteria do not have the same post-translational modification mechanisms as plants, which may be required by plant enzymes for optimal activity. Plant cells also utilize metabolons (multi-enzyme complexes) for coupling consecutive steps in a pathway (Ralston and Yu 2006; Wu and Chappell 2008), which may not form properly in bacterial and yeast cells. This is probably the reason why individual expression of taxadiene synthase (Huang et al. 2001), taxadiene-5α-hydroxylase (Hefner et al. 1996), taxadiene-5α-ol acetyltransferase (Walker et al. 2000), taxane 10*β*-hydroxylase (Schoendorf et al. 2001), and taxane 13α-hydroxylase (Jennewein et al. 2001) in yeast or bacteria resulted in active enzymes, yet simultaneous expression of these genes in yeast (which has not yet been attempted to our knowledge in bacteria) did not result in the production of the expected downstream products, except for taxadiene 5α-ol (DeJong et al. 2006). It is more likely for *P. patens* than for yeasts and bacteria not to have these problems in post-translational modification of heterologously expressed plant enzymes and formation of multi-enzyme complexes because it is a plant cell.

There were no phenotypic differences observed between transgenic *P. patens* and the wild type when examined under 10× to 40× magnification (data not shown). In contrast, both arabidopsis and tomato had reduced growth when they overexpressed taxadiene synthase (Besumbes et al. 2004; Kovacs et al. 2007). Presumably, the introduced taxadiene pathway in arabidopsis and tomato interfered with endogenous gibberellin biosynthesis (Besumbes et al. 2004), which used the same precursor (geranylgeranyl diphosphate) as taxa-4(5),11(12)-diene. Mosses do not require gibberellins for growth (Yasumura et al. 2007; Hirano et al. 2007; Vandenbussche et al. 2007), which is probably why growth was not inhibited in transgenic *P. patens*.

P. patens produces two diterpenoids (*ent*-kaurene and 16-hydroxykaurane) as secondary metabolites

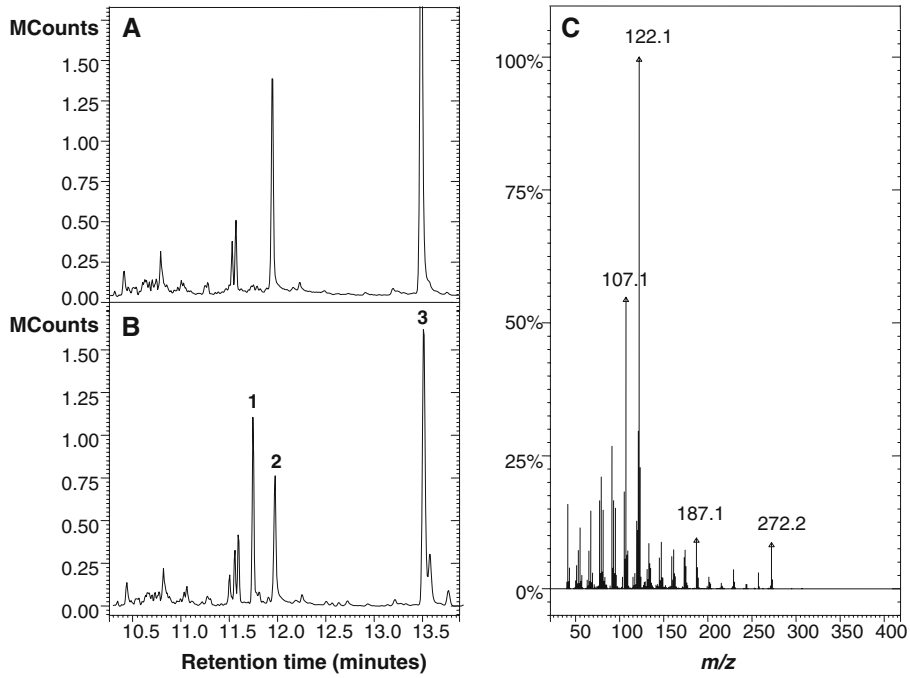

Fig. 1 GC–MS analysis of hexane extracts from wild type (**a**) and transgenic (**b**) *Physcomitrella patens* overexpressing taxadiene synthase. A new peak labeled *1* is found in transgenic moss but not in wild type. The mass spectral fragmentation pattern (panel C) of peak *1* matches that of taxa-4(5),11(12)-diene. Peaks *2* and *3* have been identified as *ent*-kaurene and 16-hydroxykaurane, respectively, by comparison of their retention times and mass spectra with authentic chemical standards provided by Dr. Robert Coates (University of Illinois, USA)

(von Schwartzenberg et al. 2004), which together can comprise up to 0.2% of its fresh weight (based on GC–MS analysis). Since the production of *ent*-kaurene and 16-hydroxykaurane still constitute a major portion of the diterpenoid pool in transgenic *P. patens* (Fig. 1), taxa-4(5),11(12)-diene production can still be increased by eliminating *ent*-kaurene and 16-hydroxykaurane formation, i.e. by knocking out the moss bifunctional diterpene synthase gene (Hayashi et al. 2006). Thus, *P. patens* can potentially be genetically engineered to produce higher levels of paclitaxel (or its precursors) than what is currently obtained. Since *P. patens* is already being used to express human proteins in bioreactors (Decker and Reski 2008), it may even be possible in the future to use metabolically engineered moss for the commercial production of paclitaxel and its precursors.

Acknowledgments We thank Dr. Rodney Croteau (Washington State University) for the taxadiene synthase gene and antibody used in this study, as well as Dr. Robert Coates (University of Illinois) for *ent*-kaurene and 16-hydroxykaurane standards. This work is supported by the Southern Illinois University SEED Grant (to A.A.) and Southern Illinois University REACH Award (to E.S.).

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