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ORIGINAL PAPER

Assessment of genetic and epigenetic variation during long-term *Taxus* cell culture

Chunhua Fu · Liqin Li · Wenjuan Wu · Maoteng Li · Xiaoqing Yu · Longjiang Yu

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Abstract Gradual loss of secondary metabolite production is a common obstacle in the development of a large-scale plant cell production system. In this study, cell morphology, paclitaxel (Taxol®) biosynthetic ability, and genetic and epigenetic variations in the long-term culture of *Taxus media* cv Hicksii cells were assessed over a 5-year period to evaluate the mechanisms of the loss of secondary metabolites biosynthesis capacity in *Taxus* cell. The results revealed that morphological variations, gradual loss of paclitaxel yield and decreased transcriptional level of paclitaxel biosynthesis key genes occurred during long-term subculture. Genetic and epigenetic variations in these cultures were also studied at different times during culture using amplified fragment-length polymorphism (AFLP), methylation-sensitive amplified polymorphism (MSAP), and high-performance liquid chromatography (HPLC) analyses. A total of 32 primer combinations were used in AFLP amplification, and none of the AFLP loci were found to be polymorphic, thus no major genetic rearrangements were detected in any of the tested samples. However, results from both MSAP and HPLC indicated that there was a higher level of DNA methylation in the low-paclitaxel yielding cell line after long-term culture. Based on these results, we proposed that accumulation of paclitaxel in *Taxus* cell cultures might be regulated by DNA methylation. To our knowledge, this is the first report of increased methylation with the prolongation of culture time in *Taxus* cell culture. It provides substantial clues for exploring the gradual loss of the taxol biosynthesis capacity of *Taxus* cell lines during long-term subculture.
Key message DNA methylation maybe involved in the regulation of paclitaxel biosynthesis in *Taxus* cell culture.

Keywords *Taxus media* · Paclitaxel biosynthesis · Cell culture · DNA methylation · AFLP · Somaclonal variation

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C. Fu · L. Li · W. Wu · M. Li · X. Yu · L. Yu (✉)
Department of Biotechnology, College of Life Science and Technology, Institute of Resource Biology and Biotechnology, Huazhong University of Science and Technology, 430074 Wuhan, China
e-mail: yulongjiang@mail.hust.edu.cn; biofch@hotmail.com

C. Fu · L. Li · W. Wu · M. Li · X. Yu · L. Yu
Key Laboratory of Molecular Biophysics of Ministry of Education, 430074 Wuhan, China

Present Address:
L. Li
Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, 313000 Huzhou, Zhejiang Province, China

Introduction

The taxane, paclitaxel (Taxol®) is an effective antineoplastic agent originally isolated from the bark of *Taxus brevifolia*, a slow growing yew that is native to the Northwest region of North America (Wani et al. 1971). Paclitaxel is a successful anticancer drug that has been approved for the treatment of numerous tumor types, such as breast, ovarian, and lung cancer. It is also approved for the treatment of AIDS-related Kaposi's sarcoma, and other applications, such as multidrug resistance inhibition and apoptosis inhibitor binding, are currently being investigated (Suyama et al. 2009). Expanding the use of paclitaxel in early intervention therapies and in combination with

other chemotherapeutic agents has resulted in a growing demand on the supply of paclitaxel. In nature, the productivity of paclitaxel is very low because of its low content in the bark of *T. brevifolia* (approximately 0.01 % on a dry weight basis) as well as the extreme slow growth of the yew tree. To facilitate greater production of paclitaxel, four alternative methods have been developed: total chemical synthesis, semi-synthesis from its natural precursor (10-deacetylbaccatin III), production using fungi or bacteria, and plant cell cultures. Among these methods, plant cell culture using well-established procedures is regarded as an environmentally friendly option obtaining a highly pure product. It has met with some commercial success, for example in the case of Shikonin, Ginsenoside, Ajmalicine (Zhang et al. 2002). Cell culture of *Taxus* is a promising, reliable, and long-term alternative for the production of paclitaxel and analogue compounds (Georgiev et al. 2009). Through skillful manipulation of culture conditions, such as optimization of culture conditions, selection of high-paclitaxel producing cell lines, the addition of elicitors or precursors, and metabolic engineering, it is possible to produce large amounts of taxane compounds (including paclitaxel), which are not naturally abundant in the bark and needles of *Taxus* plants.

Thus far, two commercial taxol production system using plant cell cultures have been reported (Malik et al. 2011). However, large-scale production of secondary metabolites (e.g., paclitaxel) through plant cell suspension cultures is still challenging because the levels and patterns of secondary metabolite production in cell cultures are often unstable and unpredictable. Hirasuna et al. (1996) and Kim et al. (2004) reported on the instability of paclitaxel production during *Taxus* cell subculture. In our previous research, we also found that after 5 years of subculturing, paclitaxel could no longer be detected in *Taxus chinesesis* cells (Li et al. 2009).

Several studies have been implemented to investigate the mechanism of paclitaxel synthesis and try to realize the stability of paclitaxel production. Studies on paclitaxel biosynthesis identified two different pathways, mevalonate (MVA) and non-MVA pathways, involving over 20 enzymes (Croteau et al. 2006). Among those, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) was identified as the first key enzyme in the classical MVA pathway, and 1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) as one of the key enzymes in the non-MVA pathway. The abundance of DXR and HMGR may reflect isopentenyl diphosphate accumulation in *Taxus* cell lines. Geranylgeranyl pyrophosphate synthase (GGPPS) was demonstrated to participate in the early biosynthesis of paclitaxel (Hefner et al. 1998), and 10-deacetylbaccatin III-10-O-acetyltransferase (DBAT) has been implicated in later biosynthetic steps (Walker and Croteau 2001). Thus, transcriptional levels of genes encoding those enzymes are closely related to paclitaxel biosynthesis in cell culture. On the other hand, many studies have focused on developing strategies for improving the yield of taxanes, for example by modulating elicitors, gas composition, osmotic pressure, and medium condition (Zhong 2002). Other studies have employed bioprocessing strategies, such as the use of a combination of induction techniques, two-phase and two-stage cultivation, feeding precursors or sugars, and semi-continuous or perfusion culture, or by optimizing scale-up techniques using bioreactors (Chen et al. 2003; Zhang and Fevereiro 2006; Hu et al. 2006; Wang et al. 2007; Yu et al. 2002). These strategies were based on the short-term variation and greatly improved paclitaxel production in cell culture. Unfortunately, full understanding of the characteristics and mechanisms of long-term variation during subculture of *Taxus* cells has not yet been realized.

In this study, we examined the morphological characteristics, genetic and epigenetic variations related to paclitaxel biosynthesis during the long-term cell culture of *Taxus media* cv. Hicksii using paraffin sections, amplified fragment length polymorphism (AFLP), methylation-sensitive amplified polymorphism (MSAP) and high-performance liquid chromatography (HPLC) techniques. The investigation was carried out using *Taxus* cultures maintained over a period of 5 years. Samples from different time points were used for morphological and molecular analyses. Continuously maintained in vitro cell culture provides an excellent research material for exploring the fundamental mechanism of somaclonal variation at both the genetic and epigenetic level. The results of this study may provide substantial clues for improving paclitaxel production in *Taxus* cell cultures, as well as for the stable maintenance of the cells during long-term subculture.

Materials and methods

Plant material

The *Taxus media* cv. Hicksii cell line was proliferated from a callus derived from a nascent *Taxus* stem, which was cultured on modified Murashige and Skoog (MS) medium containing 1.0 mg l^{-1} 2, 4-D, 1.0 mg l^{-1} NAA, and 1.0 mg l^{-1} 6-BA in March 2004. The callus was cultured on the modified MS medium at 25 °C in the dark. Subculturing was carried out at 28-day intervals (Li et al. 2009). Samples were collected monthly during the subculture, and named as S1–S66. Thirty-three, even-numbered samples (for example, S2, S4, S6, S8, S10…S66) were subjected to paclitaxel content analysis. Nine samples with distinguishable differences in cell morphology and paclitaxel content were used for the paraffin section, AFLP,

MSAP, and HPLC analyses. Biomass and the content of paclitaxel and 10-DAB were assayed with initial 10 g 100 ml^{-1} (fresh weight, FW) inoculum dosages. All experiments were performed in triplicate.

Histological examination

Histological studies of calluses derived from different culture times were carried out using paraffin sections, according to the methods of Hartweck et al. (1988) with some modifications. The samples were fixed for 24–48 h at room temperature using a formalin–acetic acid–alcohol (FAA) fixative solution (formalin:glacial acetic acid:70 % ethanol, 5:5:90 by volume), followed by dehydration in a graded ethanol series (30, 50, 70, 85, 95, and 100 %) and then embedded in paraffin. Samples were cut into 12 μm thick sections with a KD-1508A rotary microtome, and fixed to glass slides. The sections were dried at 40 °C for 3 days and removed paraffin with 100 % xylene, followed by staining with hematoxylin and examination under a microscope (Nikon 80i). About 20 sections were examined per treatment.

RNA and DNA extraction

RNA was extracted according to the methods of Li et al. (2009), and DNA was extracted using a plant genomic DNA kit (Qiagen, Germany).

Quantitative real-time RT-PCR

One microgram of total RNA was used for the first-strand cDNA synthesis using SuperScriptTM II Reverse Transcriptase kit (Invitrogen, USA) according to the manufacturer's instructions. Real-time PCR was then performed using a DyNAmo SYBR Green qPCR kit (Finnzymes, Finland). The reaction was carried out in a Rotor-Gene 3000 (Corbett, Australia). The parameters of the real-time PCR system were as follows: denaturation at 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, 48 °C (for 18S rDNA and *HMGR*) or 50 °C (for *DXR*, *DBAT* and *GGPPS* for 25 s, and 72 °C for 25 s. The transcript level of each gene was first normalized to a standard (18S rDNA) using the formula ΔCt (specific gene) = Ct (specific gene) − Ct (18S rDNA). Then, the lowest ΔCt value was selected as the standard for calculating the relative mRNA level with the equation $100 \times 2^{-\Delta\Delta Ct}$. In this equation, $\Delta\Delta Ct = \Delta Ct$ (specific gene) − $\Delta Ct\,(\text{specific gene})_{\text{the lowest level}}$ (Amoroso et al. 2004). The reported values are an average of six independent trials (two biological replicates and three technical replicates). Primer sequences were previously reported by Li et al. (2009).

HPLC quantification of paclitaxel and 10-DAB

The analysis of paclitaxel and 10-DAB using HPLC was performed according to the methods of Zhang and Fevereiro (2006) with minor modifications. Briefly, dry samples (0.1 g) were extracted with methanol/methylenechloride (1:1, v/v) by sonication. The methylene chloride phase was separated using a separatory funnel, and then evaporated in a rotary evaporator equipped with a condenser for solvent recovery. The residue was re-suspended in 1 ml of methanol, and HPLC (Agilent 1200, USA) was carried out at 25 °C with a reverse-phase Eclipse XDB-C18 column (4.6 × 150 mm, Agilent, USA) at 227 nm, using methanol/water (65:35, v/v) as the mobile phase. The elution rate was 1.0 ml min^{-1}.

HPLC quantification of global DNA methylation level

HPLC quantification of global DNA methylation level was performed according to the methods of Demulemeester et al. (1999) with minor modifications. Briefly, 20 μg of DNA was hydrolyzed in 50 μl of 70 % perchloric acid (90 °C for 50 min). The pH was adjusted to 5.0 with 1 mol l^{-1} KOH, and the supernatant was filtered (0.2 μm) prior to HPLC analysis. HPLC (Agilent 1200, USA) was carried out at 30 °C using a reverse-phase Kromasil 100 Å C18 column (Dikma Technologies, 250 × 4.6 mm) at 285 nm. The mobile phase was 50 mmol l^{-1} of KH_2PO_4, plus 10 % methanol (v/v), at a pH of 4.5. The elution rate was 0.5 ml min^{-1}. The percentage of 5mdC was calculated using the following formula: 5mdC (%) = 100 × [5mdC]/([5mdC] + [dC]). Where [5mdC] and [dC] were the concentrations of 5-methyldeoxycytidine and deoxycytidine, respectively, as deduced from the calibration curves for external standards of known concentrations, monitored simultaneously with the samples.

Amplification fragment length polymorphism (AFLP)

AFLP was performed according to the methods of Vos et al. (1995) with minor modifications. A total of 200 ng of genomic DNA were digested with *EcoR* I and *Mse* I (New England Biolabs Inc., USA) at 37 °C for 1 h, and then the relevant adapters were ligated to the digested fragments at 37 °C for 3 h. In total, one pair of pre-selective primers and 32 pairs of selective primers (including 8 *EcoR* I selected primer and 4 *Mse* I selected primer) were used for amplification, the primer sequences are listed in supplementary Table 1. After selective PCR, 10 μl of each sample was denatured (95 °C for 10 min followed by quenching at 4 °C) and mixed with 3 μl of 80 % formamide loading buffer in the presence of bromophenol blue and xylene cyanol. Then, PCR fragments were separated using 6 %

polyacrylamide gel electrophoresis and visualized by silver staining. Two independent amplifications were performed for each sample, and only those with consistently reproducible, well-resolved fragments between 200 and 1,000 bp were used for analysis.

Methylation sensitive amplification polymorphism (MSAP)

MSAP was performed based on the methods of Salmon et al. (2008) with minor modifications. Two digestion reactions, i.e., *Hpa*II + *Eco*RI and *Msp*I + *Eco*RI, were set up for each DNA sample at the same time. Incubation time, ligation reaction, pre-selective and selective amplification were as same as those used in the AFLP procedure. Altogether, 32 primer combinations (including 8 *EcoR* I selected primer and 4 *Hpa* II/*Msp* I selected primer) were employed to detect the cytosine methylation status (in the sequence 5′-CCGG-3′) of DNA samples from short- and long-term cell cultures. The sequences of the related primer are listed in supplementary Table 1. Two independent amplifications were performed for each sample, and only those with consistently reproducible, well-resolved fragments between 200 and 1,000 bp were used for analysis.

Hpa II and *Msp* I (New England Biolabs Inc., USA) are isoschizomers that recognize the same tetranucleotide sequence 5′-CCGG-3′ but have different sensitivities to methylation at cytosines (Fig. 1): *Hpa* II will not cut if either of the cytosines is fully (double strand) methylated, but will cut if the external cytosine is hemi-methylated (single strand); in contrast, *Msp* I will not cut if the external cytosine is fully or hemi-methylated (Salmon et al. 2008; Yang et al. 2011). For each sample, MSAP patterns identified four kinds of polymorphism as described in Salmon et al. (2008): non methylation = *Msp* I and *Hpa* II cut = a band in both lanes; CpG methylation = *Msp* I cuts but *Hpa* II does not = a band in the *Msp* I lane but not in the *Hpa* II lane; CpCpG methylation = *Hpa* II cuts but *Msp* I does not = a band in the *Hpa* II lane but not in the *Msp* I lane; hyper-methylation = both *Msp* I and *Hpa* II do not cut = no band in both lanes. For each sample, the total

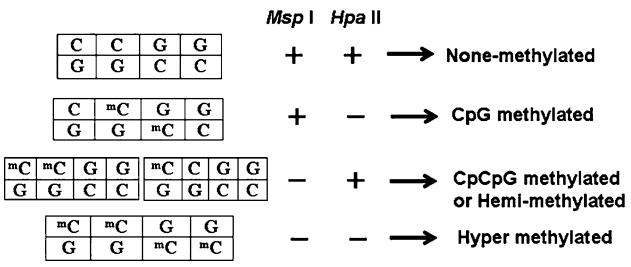

Fig. 1 *Msp* I and *Hpa* II sensitivities to 5-CCGG methylation status (''+'': enzyme cuts; ''−'': enzyme does not cut) (modified on the basis of Salmon et al. 2008)

methylation rate was calculated using the following formula: methylated sites/all sites × 100 %.

Data scoring and analysis

A one-way analysis of variance ([ANOVA] SPSS for Windows version 13.0, SPSS Inc., USA) was performed to determine if significant differences existed among the biomass, taxane concentration, and methylation levels of the nine samples.

Results

Variations in cell morphology and secondary metabolite accumulation

The *Taxus* suspension cultures exhibited various degrees of cell aggregation, friability, sliminess, pigmentation, growth rate, and secondary metabolite accumulation during long-term culture. In the initial culture stage (cells were subcultured for four and five times), the *Taxus* cell cultures were slimy, and had a deep-brown color (Fig. 2a). The cells were of various shapes (Fig. 3a). In this stage, the cells grew slowly, and only increased in biomass 1.25 times (Fig. 4). In the third-year subcultures (cells were subcultured for 39 and 40 times), the cell formed a larger aggregate than initial cultures, and had a light-brown color (Fig. 2b). The cells became loose with a round or oval shape (Fig. 3b). The growth rate of these cells was faster than those in earlier stages, and the biomass had increased 2.51 times (Fig. 4). However, after 1.5 years of subculture (cells were subcultured for 61 and 62 times), the cells became deep-brown in color and grew slowly. At this point, most of the cells became elongated in shape, (Fig. 3c), and these cells died shortly afterward. Fortunately, there were many oyster-white calluses growing on the surface of the deep-brown cells (Fig. 2c). Most of these new cells were spherical and rod shaped (Fig. 3d). These cells were subcultured for long-term stability, and the resulting culture had a pale-yellow color (Fig. 2d). These cells grew quickly, and had a 3.45-fold increase in biomass (Fig. 4).

Conversely, a decrease in the capacity for secondary metabolite biosynthesis was observed with the prolongation of culture time. Specifically, paclitaxel accumulation reduced sharply from an initial level of 452 to 42 μg g^{-1} at the fifth year. Similarly, production of 10-deacetylbaccatin III (10-DAB) was reduced from 741 to 237 μg g^{-1} (Fig. 5). Later, nine samples with distinctly different cell morphologies and paclitaxel content were collected for further genetic and epigenetic variation research. Three samples with a high paclitaxel content (400–450 μg g^{-1}

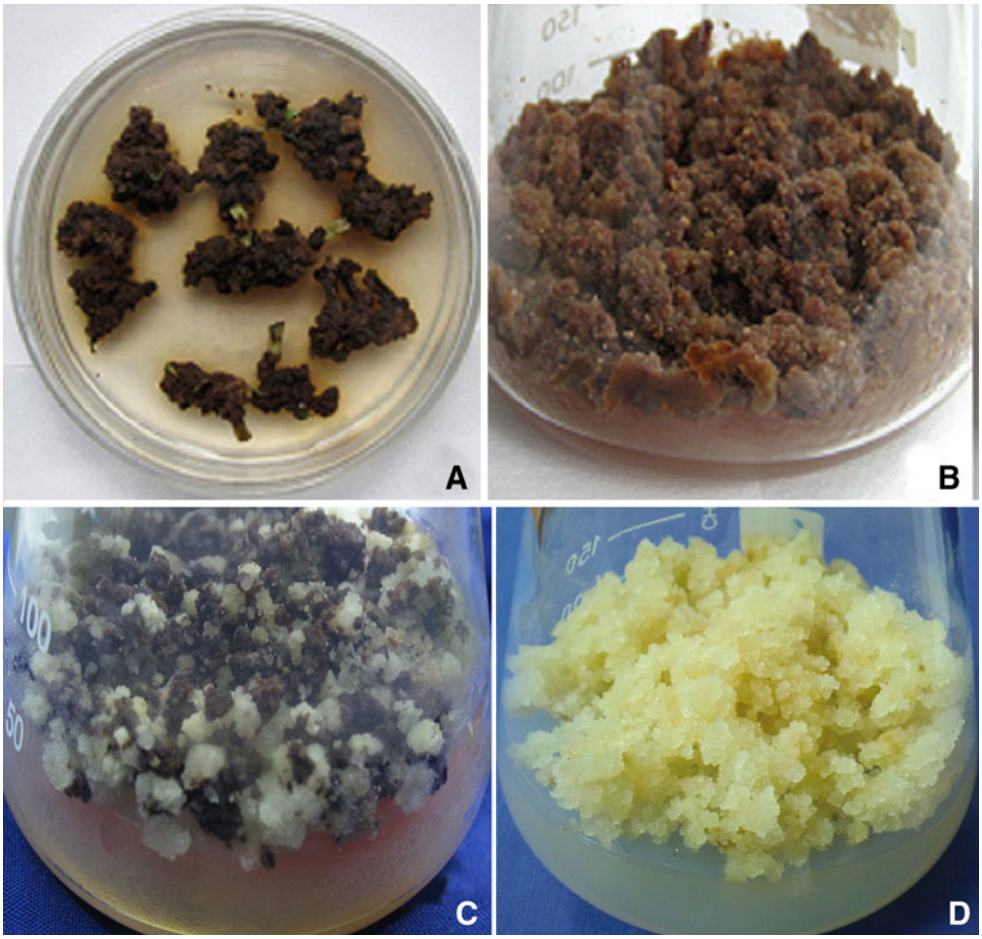

Fig. 2 Phenotypes of the *Taxus media* cv. Hicksii cell line during long-term subculture. **a** initially induced cells; **b** 3-year subcultured cells; **c** 4-year subcultured cells; **d** 5-year subcultured cells 99 × 95 mm (300 × 300 DPI)

[dry weight, DW]) that were derived from the third, fourth and fifth subcultures (named as S3, S4, and S5, respectively) were used as controls. Another three samples with a moderate paclitaxel content (100–150 μg g^{-1} [DW]) that were derived from the 39th, 40th and 41st subcultures (named as S39, S40, and S41, respectively) were used as 3-year subculture samples, and three more samples with a low paclitaxel content (50–70 μg g^{-1} [DW]) that were derived from the 63rd, 64th and 65th (named as S63, S64, and S65, respectively) subcultures were used as 5-year subculture samples.

To further assess the secondary metabolite biosynthesis abilities of the cells during long-term culture, transcription of four genes (*DXR*, *HMGR*, *GGPPS* and *DBAT*) involved in paclitaxel biosynthesis was analyzed using quantitative real-time RT-PCR. The transcriptional levels of these genes varied significantly from the initially induced cell lines to those cultured for 3 or 5 years. The expression level of *GGPP*/DXR decreased nearly 10^3 times, *HMGR* decreased nearly 10^4 times and *DBAT* decreased nearly 10^5 times in the 5-year subcultured cells compared with initially induced cells (Table 1).

Genetic variation assessed by AFLP

The above nine DNA samples taken at different times (initial, and after 3 and 5 years of subculture) were used for AFLP analysis. A total of 1,834 DNA fragments were obtained with 32 primer pair combinations (including 8 *EcoR* I selected primer and 4 *Mse* I selected primer). No changes in DNA fragment patterns or number were observed between the samples in the *Taxus* cell lines over the 5-year culture (Fig. 6).

Epigenetic variation assessed by HPLC and MSAP

The nine samples listed above were used to measure DNA methylation levels via MSAP analysis. In total, 1,552 fragments were amplified with 32 primer pair combinations from the nine samples. The total relative methylation level

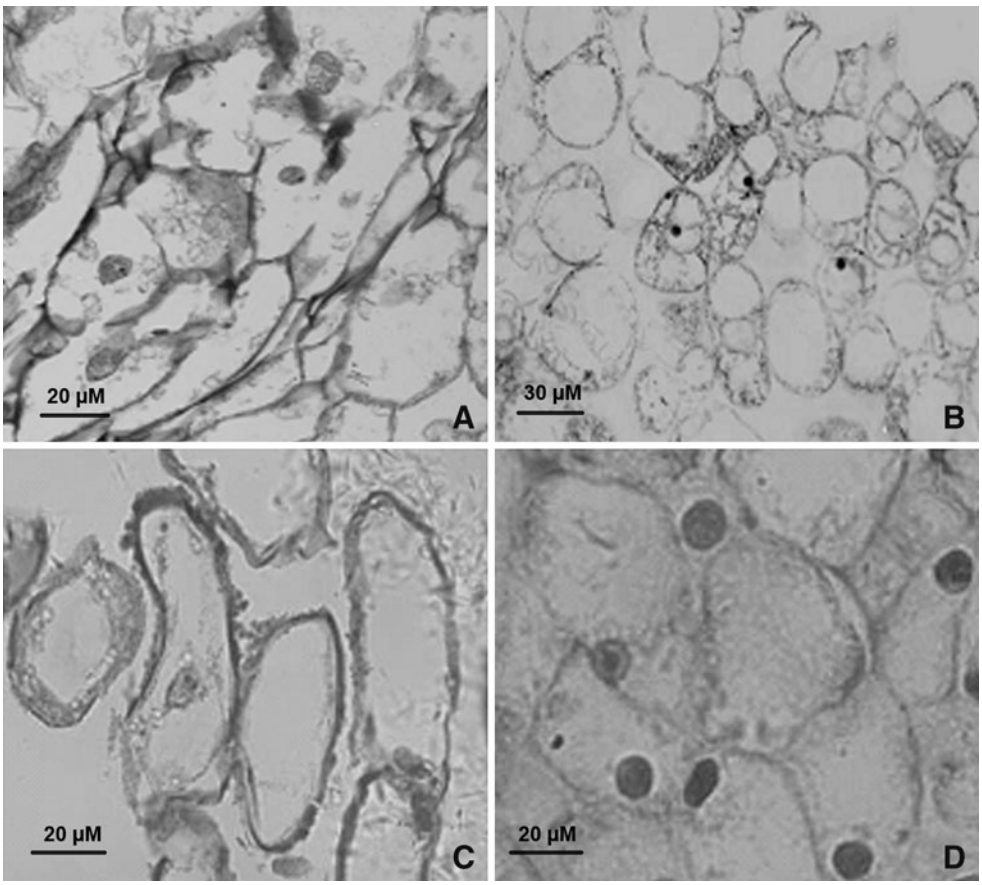

Fig. 3 Characteristics of the cell morphology of *Taxus media* cv. Hicksii by histological observation. **a** initially cultured stage cell; **b** 3-year subcultured cells; **c** dead cells after 4 years of subculture; **d** 5-year subcultured cells 99 × 89 mm (300 × 300 DPI)

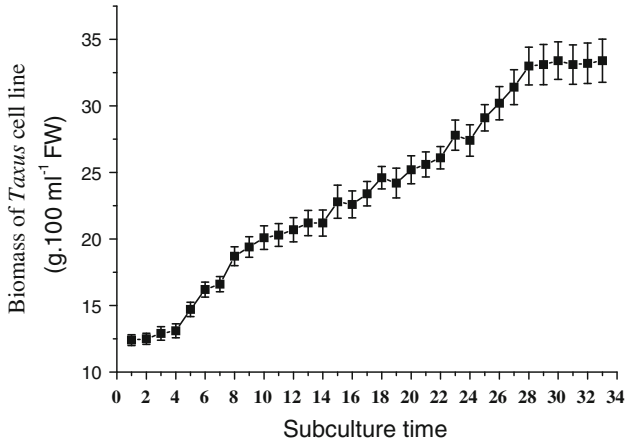

Fig. 4 Biomass accumulation rate of *Taxus media* cv. Hicksii cell line in long-term subculture. 1–33 refer to the samples collected at different subculture time: S2, S4, S6,…,S66

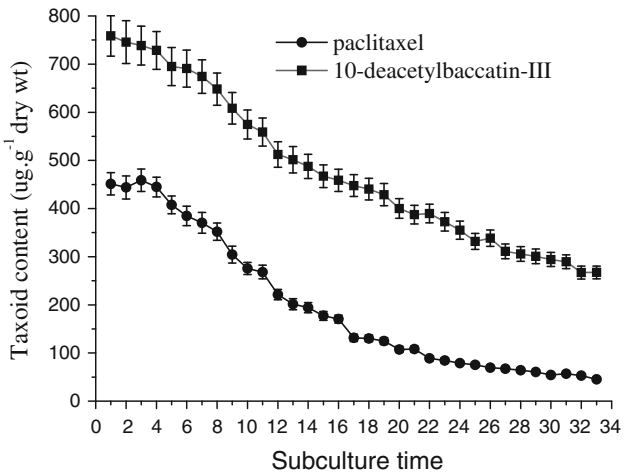

Fig. 5 Taxane content of *Taxus media* cv. Hicksii cell line in long-term culture. 1–33 refer to the samples collected at different subculture time: S2, S4, S6,…,S66

was in the range of 12.2–18.8 %, which comprises 1.4–2.4 % hypermethylation, 4.7–6.9 % CpCpG methylation, and 6.2–9.6 % CpG methylation. Interestingly, the 5-year culture groups yielded the highest total methylation level (18.8 %), while methylation in the initially induced cells was the lowest (12.2 %) (Fig. 7). It should also be noted that both methylation and demethylation occurred during the subculture, but methylation was dominant (as shown in Fig. 8a; one of MSAP patterns using primer pairs

Table 1 Transcriptional levels of four genes in the *Taxus media* cv. Hicksii cell line during long-term subculture

Sample	Relative transcript level (%)			
	GGPPS	*DXR*	*HMGR*	*DBAT*
S3	$(831 \pm 327) \times 10^3$	$(588 \pm 156) \times 10^3$	$(108 \pm 54) \times 10^4$	$(106 \pm 68) \times 10^5$
S4	$(548 \pm 275) \times 10^3$	$(512 \pm 171) \times 10^3$	$(66.3 \pm 21) \times 10^4$	$(114 \pm 59) \times 10^5$
S5	$(891 \pm 413) \times 10^3$	$(512 \pm 138) \times 10^3$	$(83.1 \pm 32) \times 10^4$	$(99 \pm 34) \times 10^5$
S39	696 ± 114	$1{,}834 \pm 765$	$(137 \pm 57) \times 10^2$	918 ± 333
S40	428 ± 108	$2{,}985 \pm 954$	$(222 \pm 72) \times 10^2$	$1{,}392 \pm 476$
S41	857 ± 153	$2{,}785 \pm 654$	$(147 \pm 65) \times 10^2$	$1{,}131 \pm 401$
S63	214 ± 32	141 ± 22	246 ± 84	141 ± 34
S64	162 ± 12	100	100	174 ± 52
S65	100	123 ± 24	131 ± 55	100

"S3, S4, S5" refer to the samples that were initially induced; "S39, S40, S41" represent the 3-year subcultured samples; S63, S64, S65" refer to the 5-year subcultured samples

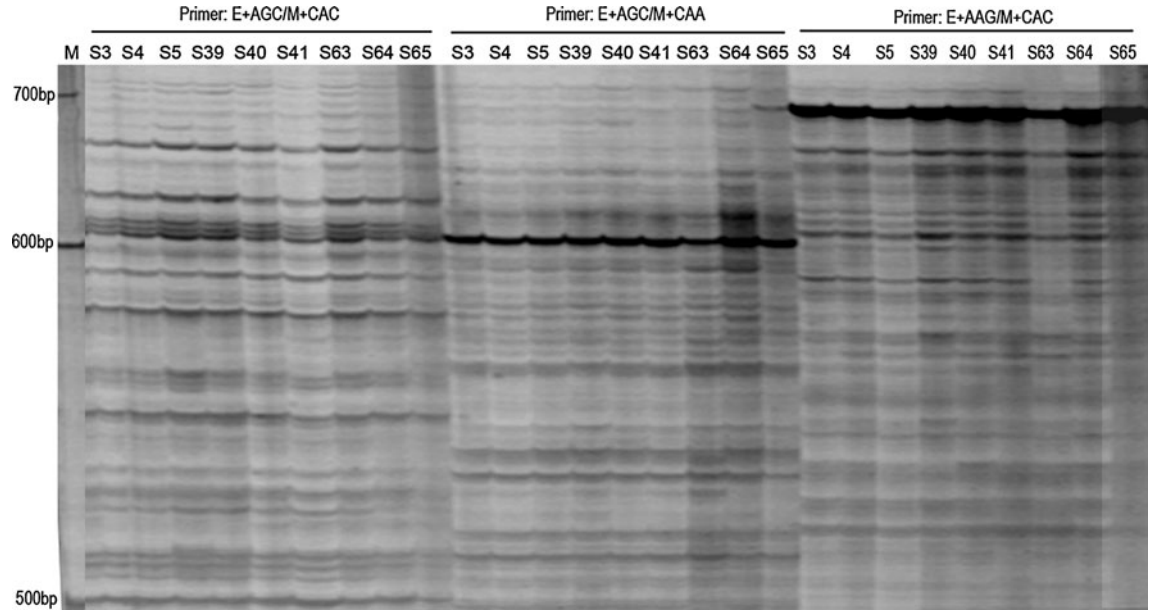

Fig. 6 AFLP patterns of the *Taxus media* cv. Hicksii cell line during long-term subculture by primer pairs of E + AGC/M + CAC. "S3, S4, S5" refer to the samples initially induced; "S39, S40, S41" represent 3-year subcultured samples; S63, S64, S65" refer to 5-year subcultured samples

HM + TAA/E + ACA). Furthermore, minor MSAP pattern variations were detected in the initially induced group. Selective PCR products obtained from the 12 primer pairs yielded 39 polymorphic bands in the initially induced group, however, fewer polymorphic bands were observed in the 3- and 5-year culture samples (Fig. 8b).

At the same time, a direct determination of the 5-methyl-deoxycytidine (5mdC) quantity in genomic DNA was carried out with the same samples using HPLC separation and quantification of nucleosides. The results revealed that a statistically significant increase of ~8.7 % was observed between the initially induced groups and the 5-year subcultured samples, while a minor variation of ~1.5 % was detected in the short-term samples (initially induced samples) (Fig. 9).

In general, a higher level of DNA methylation was observed in the long-term subcultured samples compared with the short-term samples, as determined by either HPLC or MSAP analysis. In addition, methylation levels appeared to fluctuate in the early subculture cycles.

Discussion

Plant cell cultures have become increasingly attractive and cost-effective alternatives to classical approaches for the mass production of plant-derived metabolites. Furthermore, plant cell culture is currently the only economically feasible way of producing some high-value metabolites (e.g., paclitaxel) from rare and/or threatened plants (Georgiev

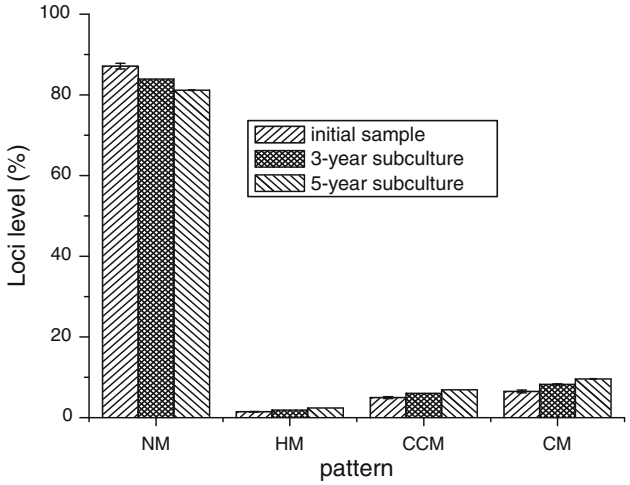

Fig. 7 DNA methylation at the CCGG sites in *Taxus media* cv. Hicksii cell line during long-term subculture. *NM* non methylation, *HM* hyper methylation, *CCM* CpCpG methylation, *CM* CpG methylation

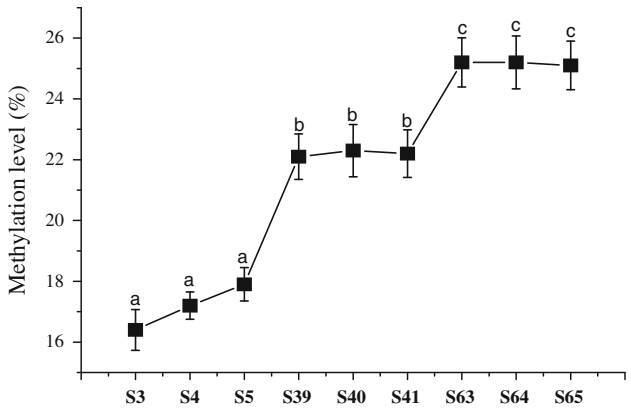

Fig. 9 Methylation level of *Taxus Media* cv. Hicksii in long-term culture assayed by HPLC. "S3, S4, S5" refer to the samples initial induced; "S39, S40, S41" represented 3 years' subculture samples; S63, S64, S65" refer to 5 years' subcultured samples; *Bars* with the *same letter* above them are not significantly different ($n = 3$, $P > 0.05$)

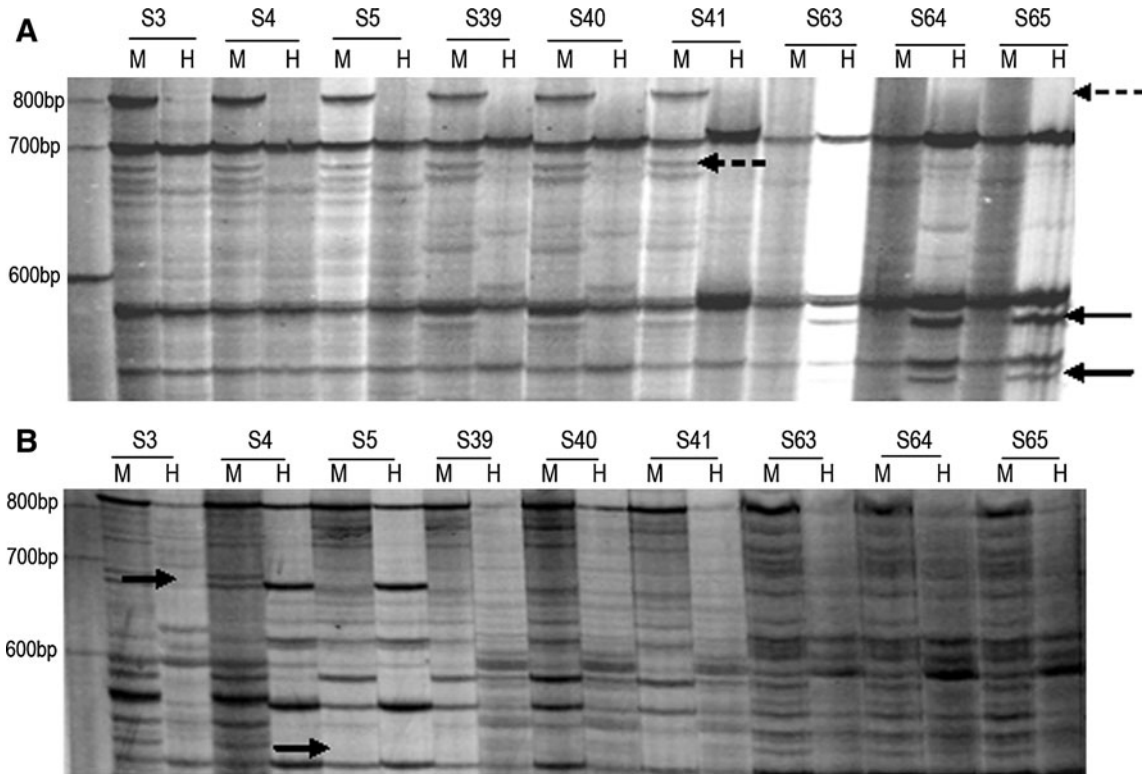

Fig. 8 MSAP patterns of the *Taxus media* cv. Hicksii cell line during long-term subculture. *H* and *M* indicate the enzyme combinations of EcoRI/HpaII and EcoRI/MspI; "S3, S4, S5" refer to the samples that were initially induced; "S39, S40, S41" represent 3-year subcultured samples; S63, S64, S65" refer to 5-year subcultured samples. **a** selective PCR products with primer pairs of HM + TAA/E + ACA, the *dotted arrows* indicate the demethylation and the *solid arrows* indicate methylation. **b** selective PCR products with primer pairs of HM + TCC/E + AAG, the *arrows* indicate the polymorphic bands among the initially induced samples 152 × 107 mm (300 × 300 DPI)

et al. 2009; Malik et al. 2011). However, gradual loss of secondary metabolite production in *Taxus* cell cultures is a common obstacle in the development of a large-scale production system. This has also been observed in other cell culture systems. Neumann and Zenk (1984) reported that loss of alkaloid production in cultured *Catharanthus*

roseus cells occurred, and spontaneous recovery of the initial production rates was never observed over a period of 8 years. Van der Heijden (1988) reported that the alkaloid yields in cell and tissue cultures of *Tabernaemontana* species decreased from the mg l^{-1} range to μg l^{-1} in a period of 18–24 months. Therefore, it is of interest to understand the mechanism of regulation of paclitaxel synthesis in these cell cultures during long-term subculture.

Throughout the history of plant tissue culture, it has been observed that clonally propagated plants often exhibit some level of variation, termed somaclonal variation (Larkin and Scowcroft 1981; Jin et al. 2008; Dann and Wilson 2011; Smulders and Klerk 2011). Thus, much effort has been put into revealing the mechanism behind somaclonal variation during long-term, in vitro plant tissue culture. Accordingly, results have shown that somaclonal variation could be generated via a combination of genetic and epigenetic changes, which were affected by explants' genotypes and environmental conditions such as tissue origin, medium composition, culture temperature and the length of culture time (Chen et al. 2006; Ivan and Peter 2011). Most of the research carried out on the variation of plants was derived in vitro (Kaeppler et al. 2000; Bednarek et al. 2007; Smykal et al.2007; Goodger and Woodrow 2010; Rodríguez López et al. 2010). As for the mechanism behind the gradual loss of secondary metabolite production during plant cell subculture, which only a few reports have mentioned, it is thought to be due to chromosome number instability, selection of a formerly insignificant non-producing but fast-growing cell type, and/or heterogeneity caused by culture conditions such as the presence of regulators in the culture medium (Neumann and Zenk 1984; Morris 1986; Shiio and Ohta 1973; Kawamura et al. 1998). To date, research on the mechanism of morphological, genetic, and epigenetic variations that occur during long-term cell culture of *Taxus* has not been performed.

In this study, the *Taxus media* cv. Hicksii cell line was established 5 years ago, and has been maintained to the present. A continuously maintained in vitro culture such as this provides an excellent research material for exploring the fundamental mechanisms behind somaclonal variation from both the genetic and epigenetic level. The results of the present study revealed that the *Taxus* suspension cultures exhibited various degrees of cell aggregation, friability, sliminess, pigmentation, growth rate, and secondary metabolite accumulation during long-term culture. With the prolongation of subculture time, the cells changed from brown to white or colorless (Fig. 2), and the rate of biomass accumulation increased gradually (Fig. 4). Accompanying the morphological alterations, secondary metabolite (paclitaxel and taxane) bioaccumulation declined gradually (Fig. 5); transcriptional levels of four genes (*DXR*, *HMGR*, *GGPPS* and *DBAT*) involved in paclitaxel biosynthesis also decreased (Table 1). It is well known that secondary metabolites play a major role in the adaptation of plants to the changing environment and in overcoming stress constraints, and higher concentrations of secondary metabolites might result in a more resistant plant, but the production of secondary metabolites is thought to be costly and reduces plant growth and reproduction (Siemens et al. 2002). Paclitaxel is a cytotoxic substance that inhibits cell proliferation by binding to the microtubule surface, specifically to the subunits of the tubulin heterodimers, thus promoting tubulin depolymerization (Kovács et al. 2007). Kim et al. (2004) found that cell cultures of high paclitaxel producing *Taxus* cells demonstrated growth inhibition upon subculture, whereas non-paclitaxel producing ones demonstrated little growth inhibition; therefore, the group speculated that growth inhibition was primarily due to paclitaxel or taxane accumulation. Therefore, we hypothesized that to grow healthily, the cells must initiate a series of mechanisms to restrain the paclitaxel biosynthesis. The detectable genetic and epigenetic variations were studied to explore how the *Taxus* genome adapts paclitaxel biosynthesis during long-term cell culture. Among the molecular stability analysis techniques used for plant cell, tissue and organ cultures, AFLP is a PCR-based assay that can reveal DNA sequence polymorphisms, and has also been widely used for plant DNA fingerprinting (Vos et al. 1995). Molecular marker profiles based on AFLPs may be employed to detect variations at the sequence level (Goodger and Woodrow 2010). The results revealed that no major genetic variation occurred during the subculture (Fig. 6); therefore, the reduction of paclitaxel yield was not ascribed to genomic variation.

Among the factors regulating the gene expression profile, DNA methylation plays an important role in fundamental cellular processes, especially in the repressive control of genome expression, over development, and in maintenance of the overall genomic integrity (Chan et al. 2005). For DNA methylation status exploration, MSAP method could only investigate a small proportion of the cytosine in the genome because *Hpa* II and *Msp* I cannot differentiate distinct methylation states, including unmethylated CCGG, double-strand methylated mCmCGG and double-strand methylated mCCGG (Reyna-López et al. 1997). So, both the MSAP and HPLC techniques were employed to explore the genomic DNA methylation status during long-term subculture in the present study. It was observed that DNA methylation levels increased significantly (Figs. 7, 9), accompanied by losses in paclitaxel yield, over the course of 5 years, and there was a significant negative correlation between them. The data presented here demonstrate that DNA methylation maybe involved in the regulation of paclitaxel biosynthesis in *Taxus* cell

culture. In addition, these results were corroborate the ones from Pennell et al. (2006), who proposed a new method for improving secondary metabolite production by manipulating genomic methylation. To our knowledge, this is the first report of increased methylation with the prolongation of culture time in *Taxus* cell culture. It provides substantial clues for exploring the gradual loss of the taxol biosynthesis capacity of *Taxus* cell lines during long-term subculture.

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