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*Full Length Research Paper*

# Antisense-induced suppression of taxoid 14β-hydroxylase gene expression in transgenic Taxus × media cells

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**The enzyme taxoid 14β-hydroxylase (14OH) directs a side-route of taxol pathway to 14β-hydroxy taxoids. Suppression of this side-route could increase the production of taxol. To suppress taxoid 14β-hydroxylase gene (*14OH*) expression in the *Taxus* × *media* TM3 cell line, antisense RNA inhibition approach was used in this study. Following the construction of an antisense RNA expression vector of *14OH* from *Taxus chinensis*, the antisense *14OH* cDNA (as*14OH*) was introduced into TM3 cells by *Agrobacterium tumefaciens*-mediated transformation. Southern blot analysis of hygromycin phosphotransferase gene (*HYG*) revealed that this selection gene was integrated successfully into the genome of *Taxus* × *media* cells. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the *14OH* mRNA level in transgenic cells dropped dramatically, suggesting that the expression of endogenous*14OH* gene was significantly suppressed by the exogenous as*14OH* gene. Correspondingly, the total yield of three major C-14 oxygenated taxoids (yunnanxane, taxuyunnanine C, sinenxan C) was markedly reduced in the silenced cell lines when compared with those of the non-transgenic controls. These results indicated that the antisense RNA strategy is a useful tool in suppressing the expression of genes in *Taxus* and this method could be used to silence other important genes that divert Taxol pathway to side-route metabolites.**

**Key words:** *Taxus* × *media*, taxoid 14β-hydroxylase, antisense, gene suppression.

## INTRODUCTION

Taxol (commonly known as paclitaxel), is a complex diterpenoid originally derived from the pacific yew (*Taxu brevifolia*) and has been approved as an important antitumor and antileukemic drug. Taxol is effective against ovarian, breast and several other carcinomas (Suffness, 1993). It interferes with cell division by blocking the dissolution of the mitotic spindle during mitosis. More recently, Taxol has shown promise in drug-eluting coronary artery stents used in the treatment of vascular disease (Gruchalla and Nawarskas, 2006) and weekly administration of paclitaxel in patients with gemcitabine-refractory pancreatic cancer seems to be very effective. Although, the uses for Taxol are increasing, only limited amounts of Taxol can be obtained from natural sources (Hanson et al., 1994).

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**Abbreviations: RT-PCR,** Reverse transcription-polymerase chain reaction; **HYG,** hygromycin phosphotransferase gene; **BA,** 6-benzyladenine, **2,4-D,** 2,4-dichlorophenoxyacetic acid; **IAA,** indole-3-acetic acid; **14OH,** 14β-hydroxylase.

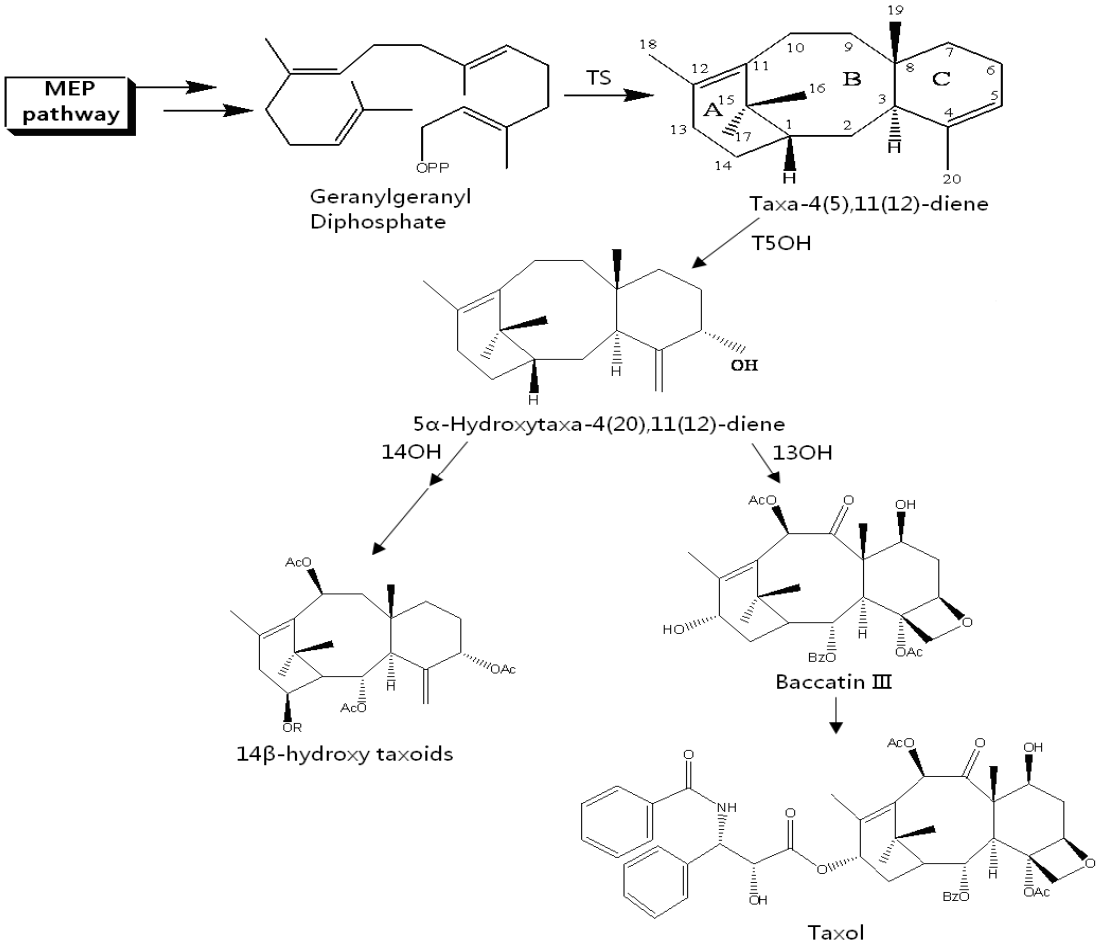


**Figure 1.** Overview of pathway leading to Taxol and 14β-hydroxy taxoids. (MEP: 2-C-methyl-D-erythritol 4-phosphate pathway; TS: taxadiene synthase; T5OH: taxadiene 5α-hydroxylase; 13OH: taxoid 13α-hydroxylase; 14OH: taxoid 14β- hydroxylase).

It is estimated that the biosynthesis of Taxol from the diterpenoid precursor geranylgeranyl diphosphate involves at least 20 distinct enzymatic steps with a similar number of taxoid intermediates (Hezari and Croteau, 1997; Ketchum et al., 2003; Croteau et al., 2006). Many taxoid metabolites are intermediates or products of a network of related pathways. The cytochrome P450 enzyme, taxoid 14β-hydroxylase (14OH) is thought to operate early in the pathway to direct the formation of side-route C-14 oxygenated taxoids (Figure 1) (Jennewein et al., 2003), while the cytochrome P450 enzyme 13α-hydroxylase (13OH) catayzes an early oxygenation step leading to C-13 oxygenated taxoids such as Taxol, baccatin III and 10-deacetylbaccatin III (Jennewein et al., 2001). Both 13α-hydroxylase and 14β-hydroxylase utilize 5α-hydroxytaxa-4(20),11(12)-diene as a substrate (Ketchum et al., 2007). Studies suggested that C-14 oxygenated taxoids can constitute a substantial portion of the total taxoid products mixture. As an example, taxuyunnanine C (2α,5α,10β,14β-tetraacetoxytaxa-4(20),11-diene) accounted for 85% of the mass of the taxoids identified in a *Taxus chinensis* cell culture elicited with 30 mM methyl jasmonate (Menhard et al., 1998).

Three common C-14 oxygenated taxoids are 2α,5α,10-triacetoxy-14β-(3-hydroxy-2-methyl) butyry-loxytaxa-4(20),11-diene (yunnanxane), 2α, 5α, 10β, 14β-tetraacetoxytaxa-4(20),11-diene (taxuyunnanine C), 2α,5α, 10β-triacetoxy-14β-(2-methyl)butyryloxy-taxa-4(20),11-diene (sinenxan C) (Figure 2). If the synthesis of these side-route C-14 oxygenated taxoids is blocked by down-regulating the *14OH* gene, 5α-hydroxytaxa-4(20), the 11(12)-diene might be directed to the production of useful C-13 oxygenated taxoids such as Taxol. Here, we reported a general method for adjusting regulation of the taxoid pathway and provide evidence for the suppression of taxoid 14β-hydroxylase gene expression in transgenic *Taxus* × *media* cell lines.

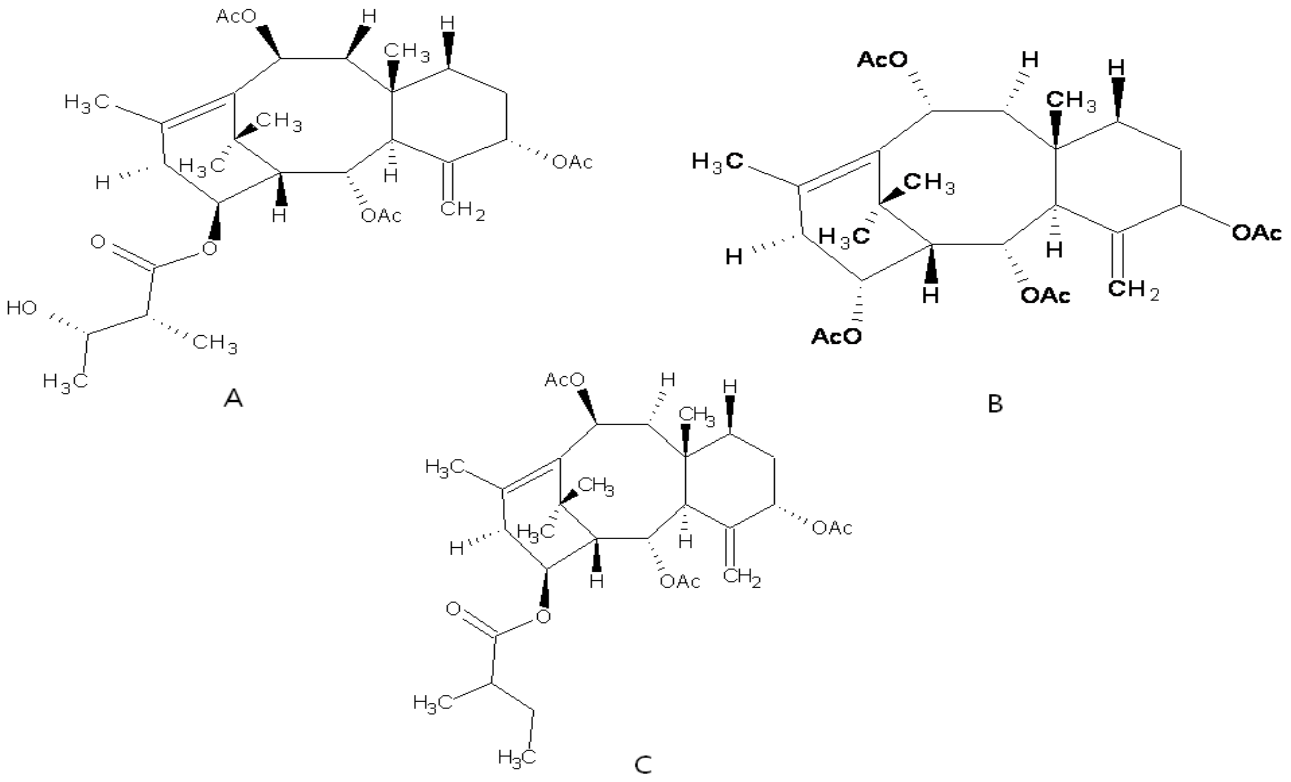


**Figure 2.** The structure of C14 oxygenated taxoids. (A: yunnanxane; B: taxuyunnanine C; C**:** sinenxan C).

**Table 1.** The components of 6,7-V.

| Content | Concentration (mg/l) |
|---|---|
| $KNO_3$ | 800 |
| $MgSO_4 \cdot 7H_2O$ | 250 |
| $CaCl_2$ | 220 |
| $(NH_4)_2SO_4$ | 100 |
| $NaH_2PO_4 \cdot 2H_2O$ | 150 |
| KCl | 200 |
| $Na_2HPO_4 \cdot 12H_2O$ | 20 |
| $H_3BO_3$ | 5 |
| $MnSO_4 \cdot 4H_2O$ | 4.5 |
| $ZnSO_4 \cdot 4H_2O$ | 1.5 |
| $CuSO_4 \cdot 5H_2O$ | 0.25 |
| KI | 0.05 |
| $CoCl_2 \cdot 6H_2O$ | 0.25 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.25 |
| $FeSO_4 \cdot 7H_2O$ | 27.8 |
| $Na_2EDTA$ | 37.3 |
| Thiamine·HCl($VB_1$) | 1.0 |
| Pyridoxine·HCl($VB_6$) | 1.0 |
| Nicotinic acid | 1.25 |
| Myo-Inositol | 50 |
| Agar | 5,000 |
| Sucrose | 30,000 |

## MATERIALS AND METHODS

### Plant cell cultures

Four cell lines (S1, TM3, M3 and TM4) were used for the transformation studies. They were established from *Taxus baccata*, *Taxus* x *media*, *T. chinensis*, *Taxus* x *media,* respectively. All cell lines have been maintained for over 10 years on 6,7-V solid medium (Table1). In this study, *Taxus* cell lines was first cultured on 6,7-V solid medium (0.5% agar) supplemented with 0.2 mg/l BA (6-benzyladenine), 1.5 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and 2 mg/l IAA (indole-3-acetic acid). 4 g of 21 day-old cells were taken from cultures grown on solid medium. Before transformation, these cells were cultured in 15 ml 6,7-V liquid medium at 23°C and 125 rpm for one day. Transgenic cells after stable transformation were grown in solid 6,7-V medium containing 300 mg/l cefotaxime and 2.5 mg/l hygromycin.

### Vectors construction

Two binary vectors were used for the transformation studies. One is pCAMBIA1304, this vector contains a hygromycin phosphor-transferase gene (*hptII*), a kanamycin resistance gene (*Kan*) and a *GUSPlus* reporter gene. The second vector is pZH01 (Figure 3a). It was obtained from professor Li-huang Zhu of Institute of Genetics and Developmental Biology, Chinese Academy of Science. This vector contains the cauliflower mosaic virus 35S promoter (CaMV35S), a kanamycin resistance gene and a hygromycin resistance gene. pZH01 was used in the construction of the antisense RNA vector. To construct the antisense RNA expression

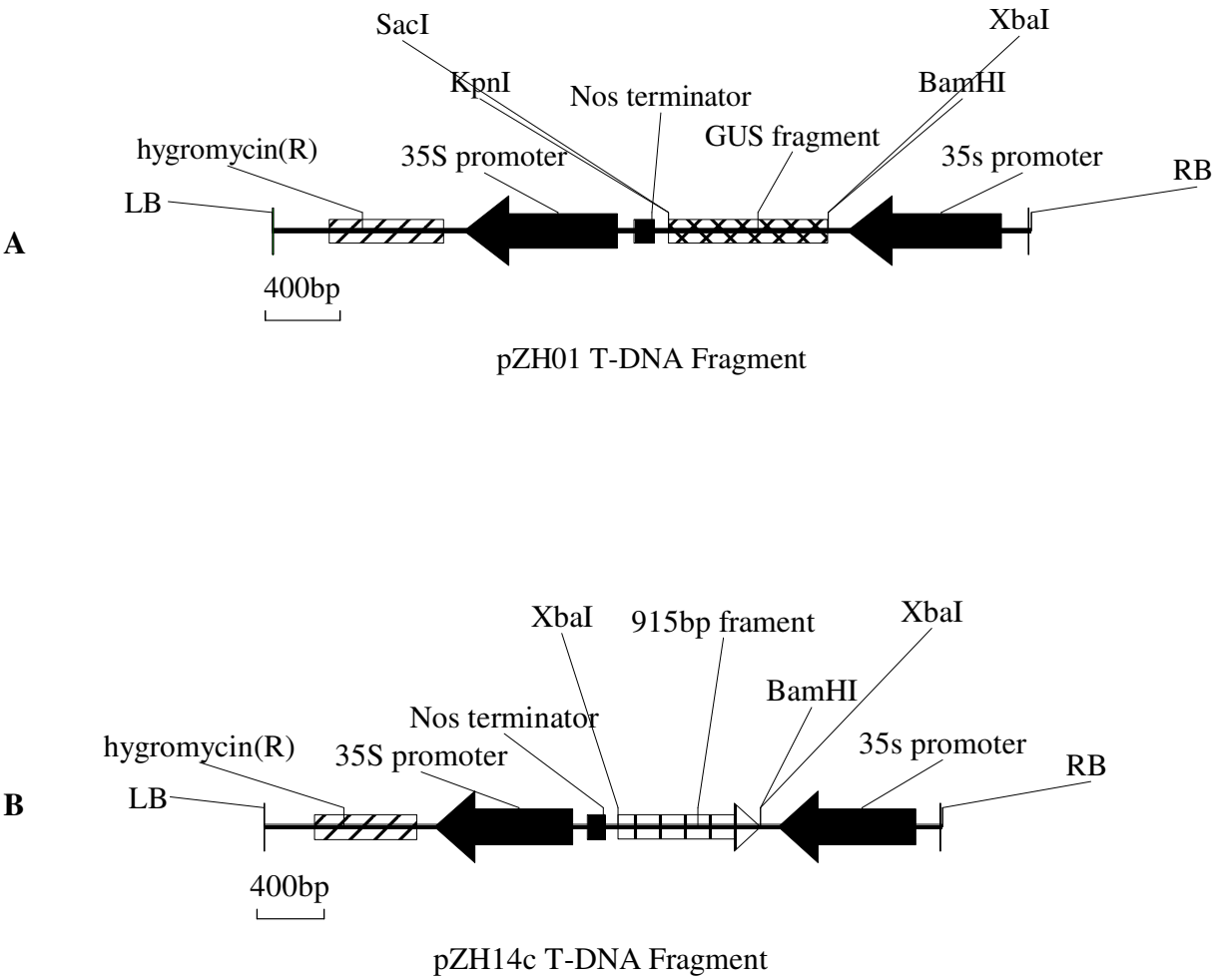


**Figure 3.** T-DNA region of binary vectors. (A: Plasmid pZH01; B**:** Plasmid pZH14c).

vector pZH14c (Figure 3b), A 915 bp fragment of the *14OH* gene from *Taxus chinensis* (Hu et al., 2006) was inserted into pZH01 reversely. The *Agrobacterium tumefaciens* strain GV3101 was then transformed with the plasmid harboring antisense *14OH* cDNA (as*14OH*). The engineered *Agrobacterium* strain was maintained in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 1% agar) containing 50 mg/l kanamycin and 50 mg/l rifampicin. For the control experiments, the *A. tumefaciens* strain GV3101 harboring PZH01 without any exogenous gene was also used to transform the *Taxus* cells.

**Taxus transformation**

A single colony of *A. tumefaciens* was selected from LB medium containing 1% agar and transferred to 2 ml of liquid LB medium supplemented with 50 mg/l kanamycin and 50 mg/l rifampicin. The *A. tumefaciens* was grown for 2 days on a rotary shaker at 28°C and 250 rpm. 100 ul of this 2-day-old culture was inoculated into a tube containing 10 ml of LB medium supplemented with 50 mg/l kanamycin and 50 uM acetosyringone, The *A. tumefaciens* was subcultured overnight at 28°C and 250 rpm. The bacterial medium was removed by centrifugation and half of the resulting bacterial pellet was suspended in 15 ml of 6,7-V liquid medium where 4 g *Taxus* callus were cultured for a day. *Taxus* cells with bacteria were sonicated for 30 s (Kim et al., 2000) and then incubated for 3 days at 125 rpm and 23°C. *Taxus* cells were washed by gently aspirating the medium with a 5 ml pipette. The medium was replaced with fresh 6,7-V medium containing 300 mg/l cefotaxime and shaken for 15 min at 125 rpm and 23°C. This washing procedure was repeated three more times. Following the fourth wash, all of the cells were plated onto a 90 mm Petri dish containing solid 6,7-V medium with 300 mg/l cefotaxime. After 2 weeks of growth, cells were transferred to 6,7-V solid medium with 300 mg/l cefotaxime and 2.5 mg/l hygromycin. The cells were subcultured each month. Cells expressing the reporter gene were stained for GUS activity after 2 weeks of growth on selection medium. The cells were harvested at days 9 and 15 after stable transformation.

**β-Glucuronidase assay**

β-Glucuronidase (GUS) activity was visualized by hydrolysis of X-gluc using the method of Castle and Morris (Castle and Morris (1994).

**Genomic DNA extraction and gene amplification**

Genomic DNA was isolated from 50 mg of each transgenic cell line using a 2% (W/V) CTAB method as described by Hu et al. (2004).

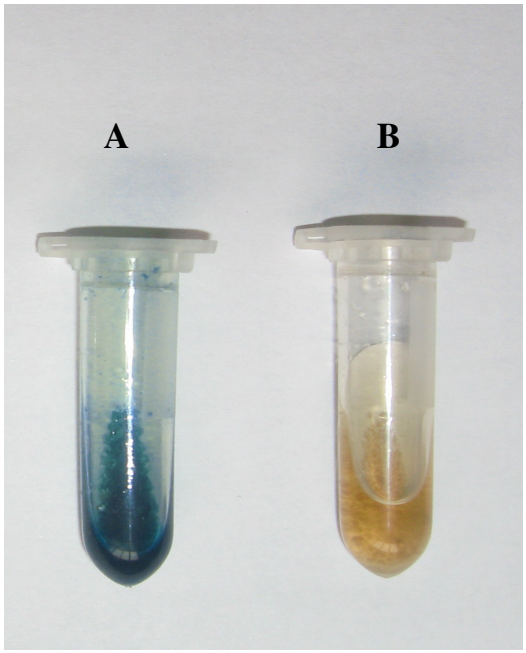


**Figure 4.** β-Glucuronidase assay. (A: TM3 cells transformed with *A. tumefaciens* GV3101 harboring pZH14c; B: TM3 cells transformed with *A. tumefaciens* GV3101 without any exogenous genes).

The P1 and P2 primers were used to amplify the 915 bp DNA fragment of *14OH* from transgenic cell lines and non-transgenic controls which were transformed with GV3101 harboring PZH01.

#### Southern blot analysis

Genomic DNA isolated from both transgenic and control cells were digested with *Xba*I and *BamH*I, separated by electrophoresis on a 0.8% agarose gel and transferred to a Hybond-N+ polyamide membrane. A 500 bp fragment of the *HYG* gene was prepared by PCR amplification of pCAMBIA1301 with the following forward and reverse primers, 5-CATGCCATGGATGCCCTTAAGC-3 and 5-GGACTAGTTTAAGATCTGGAATAGAGT-3, respectively. This DNA fragment was labeled by α-[$^{32}$P]dCTP and then was used as the probe for Southern blot.

#### RT-PCR analysis

Nine and fifteen days after transformation, the total RNA was isolated from transformed cells and treated with RNase-free DNase (Promega, Mannheim, Germany). The first strand of the cDNA was synthesized using 1 ug total RNA, oligo d(T) primer and superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. To confirm the suppression of the *14OH* gene at the molecular level with semi-quantitative PCR, forward primer 5' -GTG CTG TTG CTG GCA TTG TT -3' (P3) and reverse primer 5' -TCC TCA TCG CTG CAT GGA TT -3' (P4) were used, yielding a product of approximately 800 bp. A *Taxus actin* gene as an internal control for loading was prepared using the forward primer 5'-CAA CTG GGA TGA C(t)A TGG AGA- 3' (P5) and reverse primer 5'- CCA(g) ATC CAG ACA CTG TAC TTC C- 3' (P6). The product size was approximately 800 bp.

#### Taxoid extraction and HPLC analysis

About 4 g of fresh cells were freeze dried at -50°C. About 0.2 g of the dried material was ground in a 10 ml centrifuge tube and soaked in 2 ml methanol (purity ≥ 99.5%). The cells were extracted twice with sonication for half an hour. Methanol fractions were centrifuged briefly to remove solids and the supernatant was evaporated to dryness at 28°C. The residue was reconstituted in 2 ml $CH_2Cl_2$ (purity ≥ 99.5%) and 2 ml $H_2O$, then rotated with vortex and centrifuged at 1700 × *g* for 10 min. The water layer was discarded and the $CH_2Cl_2$ layer was evaporated to dryness. The final residue was reconstituted in 0.5 ml methanol for HPLC analysis. The analysis was performed in triplicate.

HPLC analysis of C-14 and C-13 oxygenated taxoids was performed on a Waters 2487 gradient LC system employing empower software and equipped with dual λ absorbance detector operating at $A_{210}$ . Samples were separated on a $C_{18}$ SunFire$^{TM}$ (Waters, Ireland) column (150×4.6 mm, 5 um particle size) using a $H_2O$:$CH_3CN$ gradient from 5 to 100% $CH_3CN$ (purity, 99.9%) at 1 ml/min over 50 min, followed by 5 min hold at 100% (Ketchum and Croteau, 2006; Ketchum et al., 2007). Standards of C14-oxygenated taxoid, yunnanxane, taxuyunnanine C and sinenxan C were obtained from Dr. Jun-Gui Dai of the Institute of Materia Medica, Chinese Academy of Medical Sciences. The amount of each taxoid was calculated from a calibration curve of authentic standards.

## RESULTS

### Selection of Taxus cell lines for transformation

To identify a cell line suitable for transformation, four potential cell lines, S1, TM3, M3 and TM4, were transformed by co-cultivation with *A. tumefaciens* strain GV3101 harboring pCAMBIA1304. Based on the results from the β-glucuronidase assay, the *Taxus* × *media* TM3 cell line was the only one that can be transformed successfully (Figure 4).

### Antisense inhibition of *14OH* gene expression in transgenic cells

The TM3 cell line was co-cultivated separately with *A. tumefaciens* containing the two different plasmids. Cells transformed with pZH01 (Figure 3a) without a *Taxus* gene insert were used as controls and designated CK. The second group of transformed cells contained the plasmid pZH14c (Figure 3b) with the antisense construct of a 915 bp DNA fragment of *14OH* gene sequence. Cells transformed with pZH14c were designated as asRNA (antisense RNA). Using the *HYG* gene as probe, Southern blot analysis showed that the expected hybridization bands corresponding to the probe were observed in the asRNA transgenic cells, but controls lacked the bright hybridization band (Figure 5). This result indicates that this selection maker gene was integrated successfully into the genome of *Taxus* × *media* cells.

Semi-quantitative RT-PCR analysis showed a reduction of *14OH* gene transcript abundance in asRNA cell lines

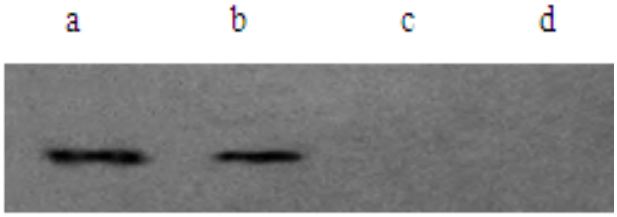


**Figure 5.** Southern blot analysis of *HYG* gene. (a and b are transformed cell lines; c and d are the untransformed controls).

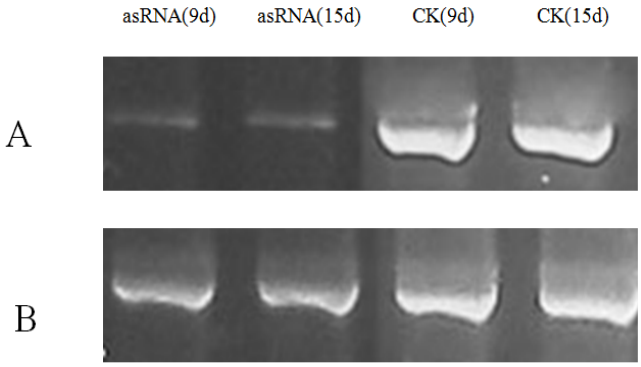


**Figure 6.** RT-PCR results. (A: The expression of *14OH* gene; B: the expression of *actin* gene; asRNA(9 days), asRNA(15 days) represent TM3 cell lines transformed with pZH14c, harvested on day 9 and 15, respectively; CK(9 days), CK(15 days) represent TM3 cell lines transformed with PZH01, harvested on day 9 and 15, respectively).

compared with that in the controls (Figure 6a), whereas the level of transcription for the house-keeping gene *actin* was almost the same for both the transgenic and the control cells (Figure 6b). This result suggests that the expression of endogenous*14OH* gene was significantly suppressed by the exogenous as*14OH* gene.

### Quantification of taxoid products

HPLC analysis of taxoid extracts from 9 and 15 day-old control and antisense transgenic cell lines showed that the total overall yield of three major C-14 oxygenated taxoids (yunnanxane, taxuyunnanine C, sinenxan C) were substantially decreased (Table 2, Figure 7). In asRNA suppression line on day 9, the most abundant C-14 oxygenated taxoid detected by HPLC, taxuyunnanine C, was significantly reduced by about 60.6% when compared with the control ($P < 0.05$). Similarly, the abundance of yunnanxane in asRNA suppression line decreased markedly by about 64.8% and sinenxan C was reduced by approximately 21.0% when compared with the control. For asRNA cells sampled on day 15, taxuyunnanine C was found to decrease significantly by about 43.4% ($P < 0.05$), while the abundance of sinenxan C and yunnanxane increased slightly (Table 2).

## DISCUSSION

### Selection of Taxus cell lines and transformation

The pathway from taxa-4(5),11(12)-diene to make taxoids depends on enzymes in two separate pathways that uses this intermediate to make either the medically useful 13OH taxoid products like baccatin III and Taxol or less useful 14OH products like yunnanxane, taxuyunnaine C or sinenxan C (Figure 1). The main goal of this study was to show that silencing the gene responsible for directing taxa-4(5),11(12)-diene toward the 14OH products will decrease their production. 9 and 15 day-old *Taxus* cells were chosen to transform. Because 9 day-old cells have not yet begun log phase growth and 15 day-old cells are at the beginning of the phase, their production of taxoids may differ. Evidence in Figure 4 shows that *Taxus* × *media* TM3 cell line could be transformed. Although, this cell line could not produce Taxol, we could use it to show a proof of concept that is possible to silence a gene that diverts the Taxol pathway to side-route metabolites in *Taxus* × *media.* Another two important genes that divert pathway flux away from Taxol production have been reported by Hampel et al. (2009) recently. It would be interesting to see what is going to happen in *Taxus* cells when these two genes are inhibited by the antisense RNA approach we described here.

### The expression and products of *14OH* gene

Since there is an endogenous gene for the pathway already in the cloned cells, the silencing strategy was to use a model in which RNA could be inhibited from being expressed as proteins and thus, would not produce the product. This approach has been used for *Taxus* by Ho et al. (2005). However, they did not show enough evidence that they succeeded in suppressing any *Taxus* genes with the antisense RNA method. In our study, the southern hybridization gene confirmed the successful integration of exogenous *HYG* reporter gene in transgenic cell lines (Figure 5). RT-PCR analysis showed that the expression of endogenous*14OH* gene is significantly suppressed by the exogenous as*14OH* gene. Moreover, HPLC analysis revealed that total C-14 oxygenated taxoids did reduce on day 9 in the all transgenic cell lines when compared with those of the controls. These results suggest that the antisense approach in our experiments was a successful one. We observed that yunnanxane and sinenxan C increased slightly in transgenic cell lines on day 15. This phenomenon might be due to the fact that not all cells of *Taxus* suspension were transformed and the variations

**Table 2.** Comparison of the yield of C-14 oxygenated taxoids in transgenic cell lines and the controls.

| Construct | Day after stable transformation | C-14 oxygenated taxoid | | |
|---|---|---|---|---|
| | | Yunnanxane (mg/g DW) | Taxuyunnanine C (mg/g DW) | Sinenxan C (mg/g DW) |
| Antisense | 9 | 0.19±0.06$^{c}$ | 2.96±0.14$^{c}$ | 1.54±0.06$^{c}$ |
| CK | 9 | 0.54±0.03$^{a}$ | 7.51±0.26$^{a}$ | 1.95±0.04$^{a}$ |
| Antisense | 15 | 0.60±0.37$^{b}$ | 3.50±0.28$^{c}$ | 1.69±0.03$^{c}$ |
| CK | 15 | 0.14±0.01$^{a}$ | 6.18±0.22$^{a}$ | 1.14±0.04$^{a}$ |

Within a column, means followed by different letters are significantly different according to the least significant difference (LSD) test ($P < 0.05$). DW: Dry weight; Antisense: TM3 cell lines transformed with GV3101 containing pZH14c; CK: the control TM3 cell lines transformed with GV3101 harboring PZH01.

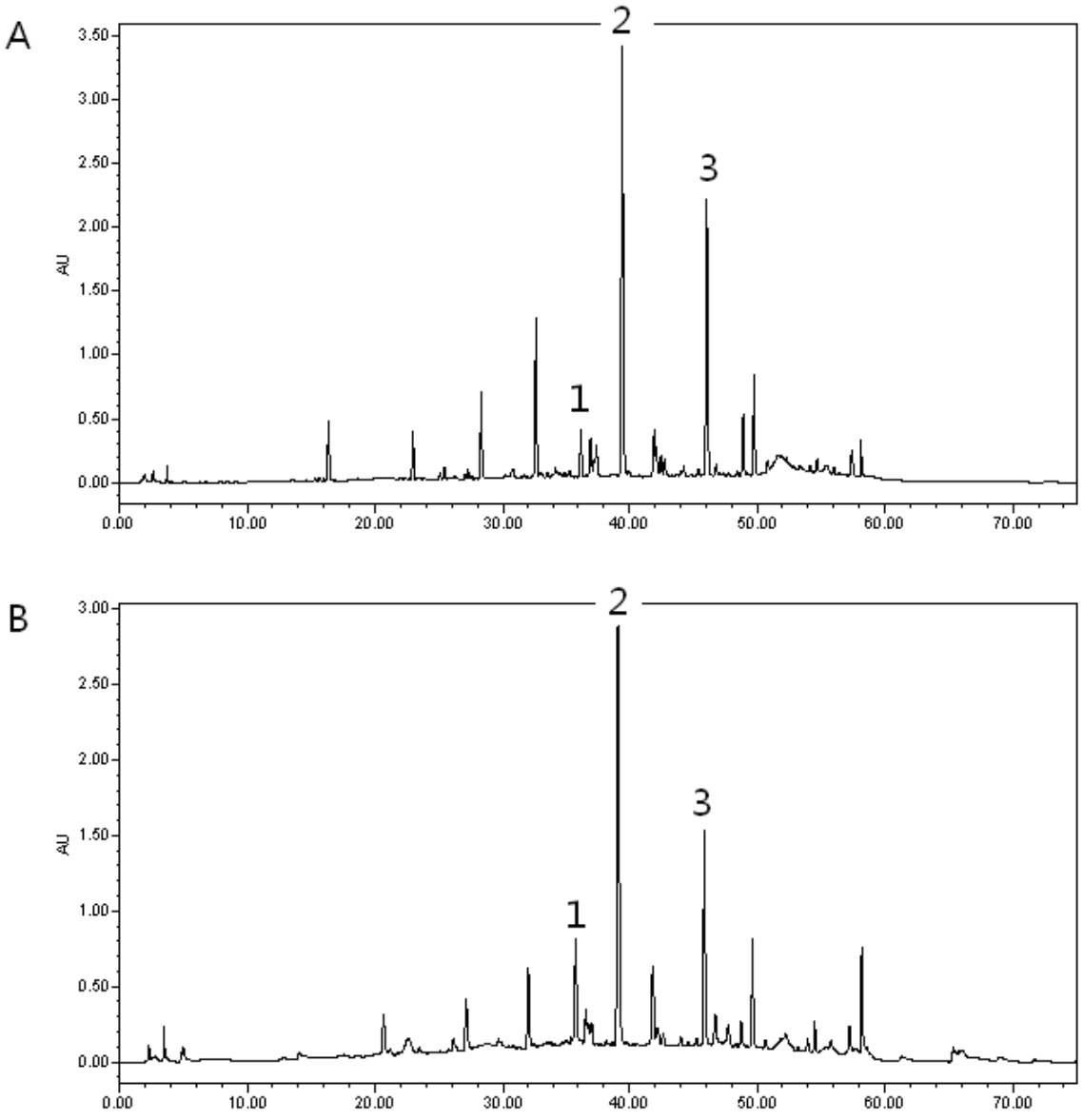


**Figure 7.** HPLC analysis of C-14 oxygenated taxoids. (Peak 1: yunnanxane; Peak 2: taxuyunnanine C; Peak 3: sinenxan C; A: asRNA cell line harvested at day 9; B: asRNA cell line harvested at day 15; C: CK cell line harvested at day 9; D: CK cell line harvested at day 15).

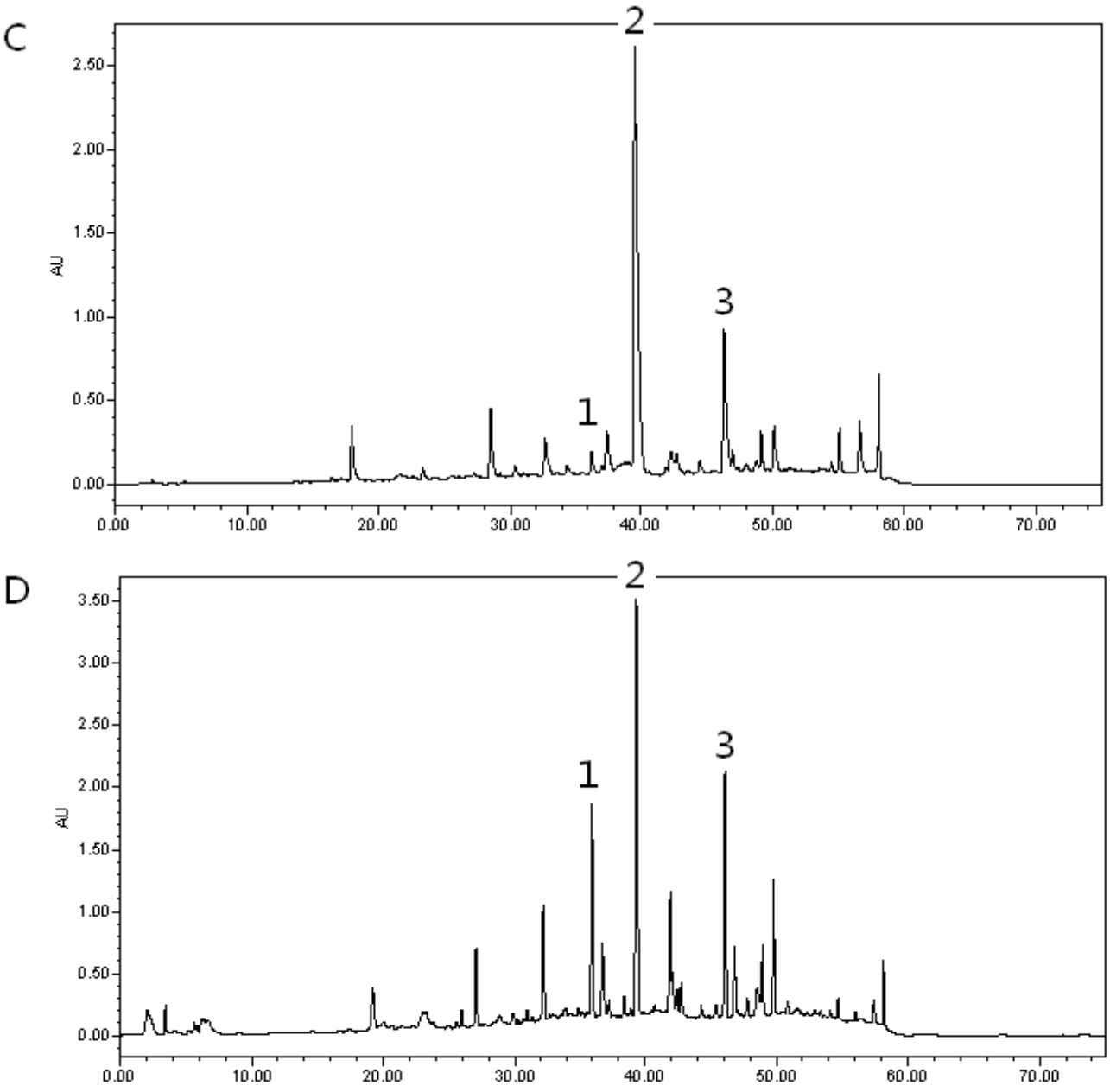


**Figure 7.** Contd.

may come from those untransformed cells. Although, the C-13 oxygenated taxoids baccatin III and Taxol were not detected in any of the cell lines (Figure 7), reduction in the activity of the 14OH gene by the antisense procedure might divert the flow of taxadiene away from “off pathway” taxoids towards Taxol if a Taxol-producing cell line is used in the future.

In summary, our results indicate that antisense suppression approach can repress successfully the expression of the *14OH* gene in yew cell cultures. The method would also be useful to inhibit the expression of other important genes in side-route of Taxol pathway and this might diverts the flow of taxadiene mainly towards Taxol.

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