Full Length Research Paper

Engineering taxol biosynthetic pathway for improving taxol yield in taxol-producing endophytic fungus EFY-21 (Ozonium sp.)

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Key enzyme gene transformation is an ordinary and effective method to increase secondary metabolites of plants and microorganisms. In this work, a fungus expression vector (pV2*-TS-pAN7-1) containing taxadiene synthase gene (ts), a rate-limiting enzyme gene for taxol biosynthesis, was constructed and used in the transformation of taxol-producing endophytic fungus EFY-21. Southern blot analysis demonstrated the integration of the ts gene in the genome of independent transformants and one taxol-increased transgenic strain was screened out by LC-MS analysis. Reverse transcription-polymerase chain reaction (RT-PCR) results proved the over-expression of the ts gene in the transgenic strain. This study provides a possibility to improve the taxol yield of taxol-producing fungi by genetic engineering and a prospect for massive production of taxol by fungal fermentation.

Key words: Taxol, taxol-producing fungi, transformation, taxadiene synthase (TS), LC-MS.

INTRODUCTION

Taxol (paclitaxel) is a plant secondary metabolite originally extracted from the bark of yew tree (Wani et al., 1971). It is a highly effective and broad-spectrum natural anti-cancer drug, which was certificated by FDA as one of the most powerful anti-cancer drugs so far discovered (Goldspiel, 1997; Michaud et al., 2000). Currently, the clinically used taxol is mostly produced by semisynthesis using natural precursors from differentiated yew tissues (Holton et al., 1995). However, low content and slow growth of the precious yew tree have always been the limits for its widespread use (Vidensek et al., 1990; Nadeem et al., 2002).

In the past decades, to expand and find new sources of taxol became a hot research field. In 1993, the first taxol-producing endophytic fungus Taxomyces andreanae was discovered in Taxus brevifolia (Stierle et al., 1993), which led to a whole new prospect of taxol source from fungus fermentation. Subsequently, many other taxol-producing endophytic fungi were reported in succession (Strobel et al., 1996; Li et al., 1998; Shrestha et al., 2001; Gangadevi and Muthumary, 2008). EFY-21 was one of the recently isolated taxol-producing fungus from Taxus chinensis var. mairei in the laboratory (Zhou et al., 2007). However, the taxol yield of these so far discovered fungi is too low to be commercially used, which limits the large-scale production of taxol by industrial fermentation.

Optimization of media and culture conditions is a common way to improve taxol yield in taxol-producing fungi. It was reported that the taxol production of Fusarium mairei strain Y1117 was proved to be 31% higher after optimization of the nutrient levels (Xu et al., 2006) but due to the different nature of different species, each taxol-producing fungus has its own optative media and culture conditions. It has also been shown that the use of elicitors such as methyl jasmonate, which is
usually used for cell culture of *Taxus*, can also improve taxol production in fungi (Wang, 2007). Genetic engineering is another prospective way. In 1998, Long obtained transformants of taxol-producing fungus by PEG-mediated transformation (Long et al., 1998). In 2007, Wang succeeded in the transformation of taxol-producing endophytic fungi by restriction enzyme-mediated integration (REMI) (Wang et al., 2007). However, to the authors’ knowledge, transformation and over-expression of key-enzyme genes involved in the taxol biosynthesis pathway has not been reported yet for taxol-producing fungi. The media and culture conditions for EFY-21 was previously optimized to a taxol yield around 80 μg/l and successfully set up a protoplast transformation system for this strain in the laboratory (Wei et al., 2010). Future researches to further improve the taxol yield of EFY-21 by genetic engineering are anticipated.

Taxadiene synthase is a cyclase for the first slow committed step in taxol biosynthesis, which catalyzes cyclization from GGPP to taxadiene (Ueda et al., 2001) and is also the first major rate-limiting enzyme in the taxol pathway (Hezari et al., 1995; Walker and Croteau, 2001). Ts gene cloned in different species of *Taxus* showed high homology (Wildung and Croteau, 1996; Kai et al., 2005). Therefore, *Ts* was chosen as the target gene for transformation. In this work, transferring and over-expressing the key-enzyme gene *Ts* in taxol-producing endophytic fungus EFY-21 was carried out for the first time to improve taxol yield.

**MATERIALS AND METHODS**

**Fungal strain**

The taxol-producing endophytic fungus EFY-21 was previously isolated from *T. chinensis* var. *mairei* in the laboratory and was identified as *Ozonium* sp. according to its morphological characteristics (Zhou et al., 2007). Strain EFY-21 grows fast on PDA or YPS medium at 27°C in the dark.

**Chemicals**

The reference chemical substance of taxol, 10-Deacetyl Baccatin III (10-DAB) and Baccatin III were purchased from Sigma (United States). Other chemicals used were of reagent grade.

**Construction of fungal expression vector**

*Ts* gene of taxus media was preciously cloned and stored in the laboratory (Kai et al., 2005). Primers of ITS and rTS (Table 1) with restriction sites were used to amplify *ts* gene by polymerase chain reaction (PCR). In order to construct *ts* into a fungal expression vector, the *Bst*I restriction site in *ts* of *Taxus* media which would influence digestion later was first point mutated. A replacement of A by G at the 705th nucleotide of *ts* was conducted by PCR using primers rTSBstI and fTSBstBI (Table 1). The three steps point mutation PCR was performed with KOD plus DNA polymerase (Toyobo) under the following conditions: 94°C for 2 min followed by 32 cycles of amplification (ITS and rTSBstI: 15 s denaturation at 94°C, 30 s annealing at 58°C, 1 min of extension at 68°C; fTSBstI and rTS: 15 s denaturation at 94°C, 30 s annealing at 55°C and 2 min of extension at 68°C; ITS and rTS: 15 s denaturation at 94°C, 30 s annealing at 55°C, and 2 min 40 s of extension at 68°C). The mutated *ts* gene was cloned in pMD18-T simple vector (Takara, Japan) and digested with *Bst*I and *Bam*HI.

**Table 1. Primers used in the present study.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (bold type as restriction site or mutation site)</th>
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<tbody>
<tr>
<td>ITS:</td>
<td>5'-TTCCAAAATGCTCGACCTCTTATTTAATGCAG-3' (bold as BstB I)</td>
</tr>
<tr>
<td>rTS:</td>
<td>5'-GGATCCTCATACTTGAATTTGATCAGTAATAA-3' (bold as BamH I)</td>
</tr>
<tr>
<td>fTSBstI:</td>
<td>5'-GTCGAGAAGATTATCTCGAATCCGGG-3' (bold as mutation site)</td>
</tr>
<tr>
<td>rTSBstBI:</td>
<td>5'-GCGGAAAGATTATCTCGAATCCGGG-3' (bold as mutation site)</td>
</tr>
<tr>
<td>f7-1:</td>
<td>5'-GGGGTACCGAATTCTTTGATCTCTACAGCAGGCT-3' (bold as KpnI)</td>
</tr>
<tr>
<td>r7-1:</td>
<td>5'-GGGAGGCCCCTCGAGGAGTGAGTGAGTGGG-3' (bold as Apa I)</td>
</tr>
<tr>
<td>fhph:</td>
<td>5'-GTCGAGAAGATTATCTCGAATCCGGG-3'</td>
</tr>
<tr>
<td>rhph:</td>
<td>5'-GCTGACACTTGCAGGCGAGTACT-3'</td>
</tr>
<tr>
<td>fTCTS:</td>
<td>5'-GTTTAGTCGTCCAGCAGGGTGAGC-3'</td>
</tr>
<tr>
<td>rTCTS:</td>
<td>5'-AATCTCAAGAATTGATCAGTCCTC-3'</td>
</tr>
<tr>
<td>F-18S:</td>
<td>5'-CCTCTAAATGACCAAGTTCTTG-3'</td>
</tr>
<tr>
<td>R-18S:</td>
<td>5'-GGAAGGGRTGTATTTATAG-3'</td>
</tr>
<tr>
<td>fTS:</td>
<td>5'-CAAACCATCTGCGAATTTGAGAAG-3'</td>
</tr>
<tr>
<td>rTS:</td>
<td>5'-CAAAGTTGCGGACACTTCTCGGATC-3'</td>
</tr>
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pAN7-1 is a common transformation vector for filamentous fungi.
containing the *hph* gene as a dominant selectable marker under transcriptional control of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and *trpC* terminator (Punt et al., 1987). A 4.1 kb fragment harboring the complete *hph* expression cassette was amplified from plasmid pAN7-1 with KOD plus DNA polymerase by PCR using primers f7-1 and r7-1 (Table 1) under the condition of 94°C for 2 min followed by 30 cycles of amplification (15 s denaturation at 94°C, 30 s annealing at 58°C and 4 min 20 s of extension at 68°C) (Figure 1b). The amplified fragment and plasmid pV2'-TS were respectively digested by KpnI and Apal. The flanked *hph* expression cassette was cloned into the vector by ligation.

The resulting pV2'-TS-pAN7-1 (Figure 1c) is a fungal expression vector carrying two separate expression cassettes of taxadiene.

**Figure 1.** The map of plasmid pV2'-TS-pAN7-1 construction. A, replacement of *hph* with *ts* ORF in pV2'; B, clone of *hph* cassette in pAN7-1 by PCR; C, insertion of *hph* cassette in pV2'-TS; D, the map of pV2'-TS-pAN7-1 used in transformation.
synthase gene (ts) driven by trpC promoter and hygromycin B phosphotransferase gene (hph) driven by gbdA. After confirmation by enzymatic digestion analysis and DNA sequencing for the presence of ts and hph gene, it was used for subsequent transformation.

**Transformation of EFY-21**

PEG-mediated transformation was carried out with ts expression vector pV2-TS-pAN7-1 following the previously described method for EFY-21 (Wei et al., 2010). Meanwhile, a transformation with pV2 was taken as a control.

**Screening transformants**

The transformed protoplasts were plated on Czapek medium containing 150 mg/l of hygromycin B. After hygromycin-resistant fungal colonies appeared, each of them was transferred to YPS solid medium with the same hygromycin content and sub-cultured for at least 3 generations for stable transformants.

Genomic DNA of the fungi was isolated by CTAB method (Yang et al., 2006). The DNA of hygromycin resistant transformants was screened by PCR for the presence of hph using primers fhph and rph (Table 1). Then, fTS and rTS were also used to confirm the presence of ts gene. PCR was carried out under the following conditions: 94°C for 3 min followed by 30 cycles of amplification (hph, 45 sec denaturation at 94°C, 45 s annealing at 58°C and 1 min of extension at 72°C; ts, 45 sec denaturation at 94°C, 45 s annealing at 55°C and 2 min 40 s of extension at 72°C). DNA from wild-type EFY-21 served as a negative control and pV2+TS-pAN7-1 was used as a positive control in the PCR analysis.

To analyze the integration of ts in the genome of transgenic fungi, southern blot hybridization was carried out. 20 μg DNA per sample was digested for 36 h at 37°C with BamHI and pV2+TS-pAN7-1 linearized with BamHI was used as a positive control. The samples of the digested DNA were electrophoresed on 1% agarose gel in 1×TAE buffer under 80 V for 3 h. The digested products were transferred by the method of alkaline transfer (Sambrook et al., 1989) to a positively charged nylon membrane (Hybond-N+, Amersham Biosciences). The probe for southern blot assay was generated by primers fTCTS and rTCTS (Table 1), which is a 618 bp fragment of partial trpC gene expression cassette of pAN7.

**LC-MS analysis**

The extraction and purification for transformants and wild-type EFY-21 were based essentially on the method described by Guo et al. (2006). HPLC-MS spectrum was performed in a 2545 binary gradient module with 3100 mass detector (Waters). 10 μl samples were loaded onto an Atlantis™ 4.6x150 mm, 5 μm C18 column (Waters) and eluted with a mobile phase of 50:50 (v/v) H₂O: acetonitrile containing 0.05% trifluoroacetic acid (TFA) at a flow rate of 0.7 ml/min for 10 min. Taxol, 10-DAB and Baccatin III were mixed in methanol as standard at a final concentration of 0.2 mg/ml for each. 2, 5, 10, 15 and 20 μl standards were analyzed by HPLC to set up a standard curve.

For MS analysis, the nebulizer gas flow rate was set at 10 l/min. The voltage of collision-induced dissociation and the capillary voltage were kept at 70 V and 3.0 kV. The molecular weight distribution was from 50 to 2300 Da. Ions of the monitor had H⁺, Na⁺ and K⁺ under 350°C of drying gas temperature, respectively.

**Ts gene expression assay**

The total RNA was extracted by using RNA extraction and purification kit (Huashun, China) from transformants and wild-type EFY-21. After quantitative determination by optical density measurement, reverse-transcription-polymerase chain reaction (RT-PCR) was carried out with ts gene-specific primers fTscore and rTscore (Table 1) by using a one-step RT-PCR kit (Takara). 18 s rDNA served as a marker gene for the expression in fungi by using the primers of F-18S and R-18S (Table 1) (Smit et al., 1999). In parallel, wild-type EFY-21 served as an internal control and pV2 transformant served as a negative control (CK).

1 μg RNA was used as the template in the one step RT-PCR under the following conditions: reverse transcription at 55°C for 30 min, denaturation of 2 min at 94°C, followed by 30 amplification cycles of 94°C for 45 s, 58°C for 50 s, 68°C for 1 min and a final extension of 10 min at 72°C.

**RESULTS AND DISCUSSION**

**Construction of fungal expression vector**

To construct an expression vector containing both ts gene and the selectable marker gene hph, pV2 and pAN7-1 were chosen as the basic vector; they have different fungal promoters but both were proved to be active in taxol-producing fungus *Ozonium* sp. in previous works (Wei et al., 2010; Zhou et al., 2008). It was reported that, about 300 bp homology in the nopaline synthase promoter system could cause gene silencing (Matzke and Matzke, 1993) and the extent even reduced to 90 bp in 35S (Vacheret, 1993). Therefore, to construct different promoters in the expression vector, it was necessary to lower the chances of gene silencing.

In order to replace the hph with ts ORF by Clal-BamHI digestion and ligation, to point mutate the 3 Clal sites in the ts gene from *T. chinensis* var. *mairei* was attempted. However, it was found that there was only one BstBI site in ts, which is the iso-codon of Clal. Therefore, BstBI was point mutated and the mutated ts was cloned into the pV2 vector by BstBI/Clal-BamHI digestion and ligation. The 4.1-kb hygromycin expression cassette of pAN7-1 was amplified by PCR and was inserted into pV2-TS by the site of KpnI and Apal. The resulting vector pV2-TS-pAN7-1 was a 10.7-kb plasmid containing the ts expression cassette and dominant selectable marker hph expression cassette under different fungal promoters.

**Screening of positive and independent transformants**

Comparison of the transformation of EFY-21 with pV2 in the previous work (2.16 ± 0.27 transformants per μg DNA) (Wei et al., 2010), showed that the efficiency was observably decreased with vector pV2-TS-pAN7-1 constructed in this study. Only eight hygromycin positive colonies from 50 μg plasmid DNA transformation
appeared. It may be due to the size of the plasmid (10.7 kb), which was much larger than pV2 (5 kb). Protoplasts are more likely to absorb small exogenous DNA (Krens et al., 1982).

After hygromycin screening for generations, six stable hygromycin-resistant colonies were obtained. Then, PCR was used to detect the presence of hph gene and ts gene in the transformers. As shown in Figure 2, the anticipated 960 bp band of hph gene fragment appeared from all the six samples but only 4 of them showed 2.6 kb band of ts gene as in the positive control of plasmid pV2+TS-pAN7-1, while there was no band amplified from the wild type EFY-21 DNA.

Southern blot analysis was carried out for further detection for the integration of foreign genes in the genome of EFY-21 transformers. 618-bp probe of partial ts and partial trpC promoter sequence was used to detect the presence of ts expression cassette. The data showed that all the selected transformants are independent transformants, and the ts gene was integrated in the fungal genome at low copies (Figure 2c). There was no hybridization signal of wild-type EFY-21. Although, a 632 bp ts core fragment was cloned from EFY-21 in a previous work, the full length sequence has not yet been cloned. The southern blot results of no hybridization signal for the 5’ end sequence of wild-type EFY-21 may be due to the low homology of 5’ end sequence of the ts gene between the plant and fungus. The PCR and southern blot results indicated that, the ts gene was successfully integrated into the fungal genome of the four independent transformants.

Analysis of taxol production

To analyze the influence of taxol production after transformation, all the four independent transformants, one pV2 transformant (CK) as a negative control and wild-type EFY-21 (WT) as an internal control were prepared for LC-MS analysis. Figure 3a shows the HPLC chromatograms of standard taxol, 10-DAB and baccatin III by UV detection at 230 nm. Taxol was eluted at 9.20 min from HPLC column and produced a (M-H)⁺ ion at m/z 854.46 by MS tests (Figure 4a). 10-DAB was eluted at
Figure 3. HPLC analysis of standards (A), wild-type EFY-21 (B) and transformant extracts (C). The peaks of taxol, 10-DAB and baccatin III are indicated by arrows.

Figure 4. Mass spectrum of standard taxol (A, retention time: 9.20 min) and extracts from transgenic EFY-21 (B, retention time: 9.12 min).
now, the integration mechanism of foreign DNA by protoplasts transformation has not been quite clear. The position of integration and the structure of the transgene locus, which vary among independent transformants, can influence the level and stability of the transgene expression (Kohli et al., 2003; Kumar and Fladung, 2001). This may be the reason why only one yield improved transformant was screened out and the two copies integration transformant had even more improved taxol yield than the four copies transformant. Therefore, a more productive strain might be obtained by large transformants screening.

Until recently, the biggest problem in utilizing these taxol-producing fungi had been the low yield and unstable production of taxol. Transferring key enzyme genes involved in taxol biosynthesis is one prospective way to increase taxol yield in fungi. However, there were very few reports about transgenic taxol-producing fungus, which may be due to the difficulties of transformation for the variety of taxol-producing fungi. In this study, a ts gene expression vector was constructed and also succeeded in the transformation of taxol-producing endophytic fungus EFY-21 with it. A taxol increased transformant was obtained which had the content of almost 5 times of the wild-type fungus. However, this level of yield does not meet the needs of the industrial production and the stability had not been tested. There is still a long way to develop a commercial strain for fungal fermentation. Some further work of transformation may help to increase taxol yield in the transgenic fungi. Since the full length of ts from fungus has not been cloned, the ts gene used for over expression was from Taxus. Homogeneous gene expression may be more efficient than heterogeneous expression in this endophytic fungus. Moreover, other important enzyme genes as tat, dbat, bapt, dbntbt, etc. involved in taxol biosynthesis will be hopeful for better improvement of taxol in transgenic fungi. Nevertheless, this work already provides a means of increasing the taxol yield by transformation and may allow for the massive production of taxol by using transgenic endophytic fungi in the future.

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