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Full Length Research Paper

An efficient transformation system of taxol-producing endophytic fungus EFY-21 (*Ozonium* sp.)

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EFY-21 (*Ozonium* sp.) is a newly isolated taxol-producing endophytic fungus from *Taxus chinensis* var. *mairei*. In this study, an efficient PEG-mediated transformation of EFY-21 was established and conditions for transformation were evaluated. By the optimized enzyme system, mycelium age, digesting temperature and time, over 7 × 10^7 ml protoplasts were obtained and protoplast regeneration frequency was more than 6%. Plasmid pV2 containing the hygromycin-B phosphotransferase gene driven by a fungal promoter (trpC) was used to transform EFY-21 and 50% PEG with 20 mM Ca^{2+} was found to be suitable for the transformation. Southern blot analysis revealed that the transforming DNA was successfully integrated into the EFY-21 genome. By the optimized procedure, over two transformants per µg DNA could be obtained. The establishment of efficient transformation system of taxol-producing endophytic fungus enables us to improve taxol production of the fungus by engineering the taxol biosynthetic pathway genes in the future.

Key words: Endophytic fungus, taxol, PEG-mediated transformation, protoplast, regeneration, *Ozonium* sp.

INTRODUCTION

Taxol is one of the most potent natural anti-tumor drugs originally extracted from the bark of a treasured tree yew (Wani et al., 1971; Goldspiel, 1997; Michaud et al., 2000). Despite of its excellent cytotoxic activity to tumor cells, the commercial taxol production has been always limited by its perceived supply problem. As the first taxol-producing endophytic fungus was isolated from *Taxus brevifolia* in 1993 (Stierle et al., 1993), it provided us a prospect to obtain taxol from fungi. Since then, many other endophytic fungi from various yews and other plant species have been subsequently reported to produce taxol (Li et al., 1996; Strobel et al., 1996; Metz et al., 2000; Wang et al., 2000; Shrestha et al., 2001; Strobel, 2002; Guo et al., 2006). EFY-21 (*Ozonium* sp.) was one of the taxol-producing endophytic fungi recently isolated from *Taxus chinensis* var. *mairei* in our laboratory (Zhou et al., 2007). However, limited taxol production in these fungi has been a main obstacle precluding the use of microbial taxol for a commercial reality. Along with the discovery of so many taxol-producing fungi, how to meliorate and utilize them for improved taxol production became an important issue and hot research field.

The use of molecular approaches for genetic improvement of taxol-producing fungi is a potential way to improve taxol yield. Protoplast production and PEG-mediated transformation are traditional, convenient and relatively inexpensive methods for genetic manipulation of fungi. Many species of fungi have been successfully transformed based on this method (Ballance et al., 1983; Crowhurst et al., 1992; Lorito et al., 1993; Valadares-Inglis and Inglis, 1997; Kim et al., 2000; Fitzgerald et al., 2003). However, since the first successful PEG-mediated transformation of taxol-producing endophytic fungus *Pestalotiopsis microspora* had been reported (Long et al., 1998), the practical gene transformation system for other

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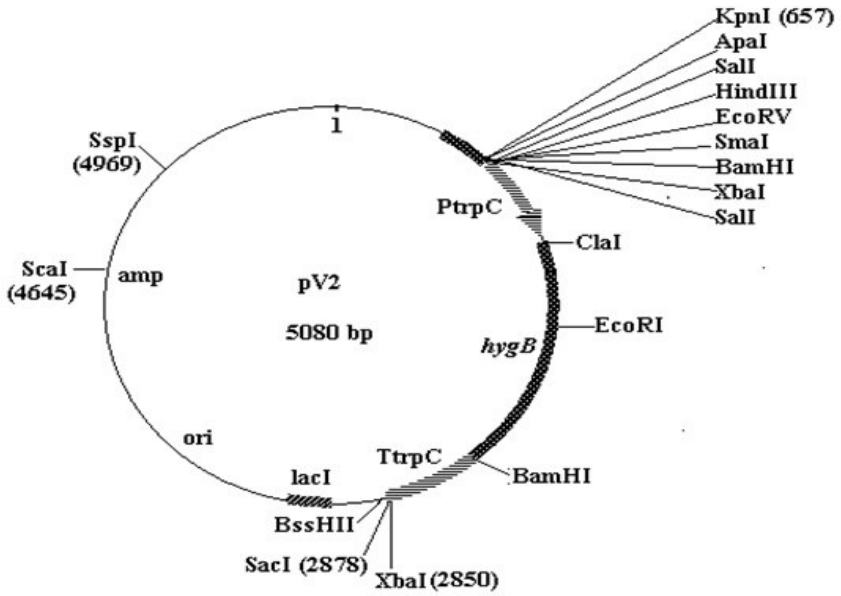

Figure 1. The schematic map of transforming vector pV2.

species of taxol-producing fungi is still yet to be established. This might be hampered by the relatively low efficiency of PEG-mediated transformation.

Successful protoplast production is a prerequisite for transformation with protoplasts. However, almost every fungus requires its own specific enzyme system and digestion condition for the isolation of protoplasts (Peng et al., 1993; Rohe et al., 1996; Chitnis and Deshpande, 2002; Li et al., 2003; Zhou et al., 2008). In the present study, the key factors for production and transformation of EFY-21 protoplasts were evaluated and a feasible way to obtain protoplasts and transformants of the taxol-producing fungus EFY-21 was established, paving the way to engineer this taxol-producing fungus for improving taxol production by transforming genes encoding rate-limiting enzymes in the taxol biosynthetic pathway in the future.

MATERIALS AND METHODS

Chemicals and enzymes

Snailase, cellulose R-10 and lysozyme used for protoplast isolation were purchased from Beijing Dingguo biotechnology Co. Ltd (China) and lywallzyme was obtained from Institute of Microbiology, Guangdong Province, China. The solution of lytic enzyme used in the study was dissolved in 0.7 M NaCl and filtrated with 0.45 μm microporous filtering film. Other chemicals used were reagent grade.

Transformation vector

Vector pV2 was used in fungus transformation. The pV2 (Figure 1) is a transformation vector for filamentous fungi containing the *Escherichia coli* hygromycin B phosphotransferase gene as a dominant selectable marker, under transcriptional control of *Aspergillus nidulans* trpC promoter and trpC terminator signals, which was a gift of Prof Youliang Peng from Agricultural Ministry Key laboratory of Molecular Plant Pathology, China agricultural university.

Fungal strain and growth condition

The microorganism EFY-21 used in the study was endophytic fungus (*Ozonium* sp.) from *T. chinensis* var. *mairei* previously isolated and stored in our laboratory (Zhou et al., 2007). The wild-type EFY-21 was grown in liquid YPS medium (2% sucrose, 1% peptone, 0.5% yeast extract, 0.1% $MgSO_4$, 0.1% K_2HPO_4 and pH 6.8) on a rotary shaker (150 rpm) at 28℃ in the dark.

Protoplast preparation

A modification of the procedure of Churchill et al. (1990) was used. The mycelia were harvested by centrifuged at 5000 g for 5 min and washed with sterilized distilled water. After centrifugation, the mycelia were washed with 0.1 M Tris-EDTA, and subsequently washed with 0.7 M NaCl. An aliquot of 250 mg mycelia (wet weight) was transferred to a 15 ml Falcon tube and 1 ml lytic enzyme solution was added. The mixture was incubated at 30℃ with shaking for 4 h. Mycelial debris was then separated from protoplasts by filtering the suspension through 4 layers of sterilized abrasive mirror paper and the protoplasts were counted by a hemacytometer. Subsequently, the protoplasts were collected by centrifugation at 2000 g for 5 min, and the pelleted protoplasts were washed twice with STC buffer (1.2 M Sorbitol, 25 mM $CaCl_2$, 0.01 M Tris-HCl, pH 7.5), and suspended at the titer of 5×10^7 ml in STC buffer.

Protoplast regeneration

Czapek medium was used as the regeneration medium, which composed of 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$,

0.001% $FeSO_4$, 3% sucrose, and 2% agar. The additional supplement, 0.001% sodium deoxycholate as colony restrictor, was also added in the medium. The essential requirement for obtaining growing colonies from protoplasts is the maintenance of the osmotic stabilizer in the growth medium until the cell wall has been regenerated (Valadares-Inglis and Inglis, 1997). NaCl at the concentration of 0.7 M was chosen as the osmotic stabilizer and added in the medium. The prepared protoplasts were diluted serially to 10^3, 10^4 and 10^5 ml by STC, and 50 µl protoplast suspensions with different concentrations were plated into the regeneration medium in Petriplates. As control, protoplasts were also suspended in sterilized distilled water for 10 min, and then plated on the growth medium without osmotic stabilizer. The cultures were incubated at 28℃ for 3 - 5 d before colonies became visible.

PEG-mediated transformation with pV2

Transformations were carried out according to a modification of the method described by Rohe et al. (1996). Fresh protoplasts were suspended at the density of 5×10^7 ml in STC. An aliquot of 200 µl suspension was transferred to a 15 ml falcon tube containing 10 µg plasmid DNA (pV2), gently mixed and incubated at room temperature for 30 min. Subsequently, aliquots of 100, 250 and 900 µl of PEG buffer (50% PEG-4000, 20 mM Ca^{2+}, 10 mM Tris-HCl, pH7.5) were added stepwise and gently mixed with the protoplast suspension before the next aliquot was added. The mixture was again incubated at room temperature for 10 to 20 min. Then, an aliquot of 6 ml STC was added to facilitate sedimentation of protoplasts by centrifugation (5 min, 2800 g). The deposition of protoplasts was re-suspended in 3 ml liquid growth medium (YPS with 1.2 M sorbitol) and was grown on a rotary shaker (150 rpm) at 28℃ for 3 h. The protoplasts were then plated on the Czapek medium containing 150 µg/ml of hygromycin B. After 4 - 5 d culture, the hygromycin-resistant colonies became visible, and they were transferred to YPS solid medium containing 150 µg/ml of hygromycin B and sub-cultured for at least 3 generations to obtain stable transformants.

Molecular analysis of fungal transformants

Genomic DNA was isolated from wild-type EFY-21 and randomly selected transformants with CTAB method (Yang et al., 2006) and used for PCR analysis for the presence of the hph gene. Primers (fhph: 5'-GTCGAGAAGTTTCTGATCG-3'; rhph: 5'-GTTTCCACTATCGGCGAGTACT-3') were synthesized according to the published hph gene sequence (Gritz and Davies, 1983) and PCR was carried out under following condition 94℃ for 3 min followed by 30 cycles of amplification (45 s denaturation at 94℃, 45 s annealing at 58℃, and 1 min of extension at 72℃). DNA from untransformed EFY-21 (wild-type) served as negative control and pV2 served as positive control was also used in PCR analysis.

Southern blot hybridization was carried on for further analysis. 60 µg DNA per sample was digested overnight at 37℃ with BamH I, which does not cut within the probe region. 5 µg plasmid pV2 as a control was digested with BamH I for 3 h, which cut twice in it. The samples of digested DNA were electrophoresed on 1% agarose gel in 1 × TAE buffer. The gel was transferred by the method of alkaline transfer (Sambrook et al., 1989) to a positively charged nylon membrane (Amersham biosciences hybond-N+, UK). 623 bp probe including part of hph and promoter trpC gene was generated by PCR using plasmid pV2 as template with primers (HygF: 5'-GTTTAGTCGTCCAGGCGGTGAGC-3'; HygR: 5'-AACAGCGGGCAGTTCGGTTTCAG-3'). DNA probe was labeled with alkaline phosphatase for use in conjunction with chemiluminescent detection with CDP-star chemifluorescent detection with ECF (Amersham gene images AlkPhos direct labelling and detection system). After hybridization and post hybridization stringency wash, signal was generated and detected on X-ray film (Kodak).

RESULTS AND DISCUSSION

Optimization of the conditions for protoplast formation

Stable and viable protoplast formation system is the basis for protoplast-based studies such as transformation, fusion and mutation. To prepare transformable protoplasts, several key factors as enzyme system, mycelium age, digesting temperature and time were evaluated. Enzyme used to digest the cell walls is the most important factor for protoplast formation; for that almost each fungus needs its own specialized enzyme system because there is a great variation in structure of cell wall between one fungus and another (Hamlyn et al., 1981). Here we chose 4 lytic enzymes: Lywallzyme (A), snailase (B), cellulose (C), and lysozyme (D), which are commonly used in protoplast isolation of fungi to test *Ozonium* sp. EFY-21, and we applied conventional analytic methods of orthogonal trial (Table 1) to evaluate weight of each factor and get the most efficient combination of enzymes for protoplast yield. The result showed that $R_A > R_B > R_D > R_C$, meaning that factor A (Lywallzyme) had the most effect on protoplast yield while factor C (cellulose) was the least influential (Table 1). Under 3 levels of each factor, the data suggested that $A_3B_1C_3D_3$ was the best combination of lytic enzymes for enhancing protoplast production, which composed of 1.5% lywallzyme, 0.5% snailase, 1.5% cellulase and 1.5% lysozyme. This result of enzyme system was similar to that of taxol-producing fungus BT2 which was also *Ozonium* sp. ,but the protoplast yield was over 20 times higher than BT2 (Zhou et al., 2008).

According to the principles of enzymatic reaction kinetics, the speed of enzymatic reaction is directly influenced by temperature and the suitable temperature of digestion for most fungi is between 24 and 35℃ (Sun et al., 2001). We compared the digestion efficacies under different temperature (27, 30 and 33℃) and the result was shown in Figure 2. Although protoplasts could be yielded under all the temperatures tested, 30℃ was the most suitable temperature for the enzyme system.

The effect of digesting time on protoplast yield was also evaluated. Samples were observed under the discrepancy microscope and protoplasts were counted by a hemacytometer every 1.5 h during digestion. Along with the increased digestion time, protoplast production was gradually increased. The curve of time (Figure 3) illustrated that the maximum protoplast yield ($7.05 \pm 0.60 \times 10^7$ ml) was obtained after 4.5 h incubation.

Mycelium age is another influential factor for protoplast isolation. Acceleration phase cultures were the best for protoplast isolation, and the protoplast yield dropped as exponential growth proceeded (Harling et al., 1988). With the same digestion condition, the mycelia of different ages

Table 1. Design of $L_9(3^4)$ and trial results[a].

Item	Factor (%)				Protoplasts production (×10^7 ml)
	Lywallzyme (A)	Snailase (B)	Cellulase (C)	Lysozyme (D)	
1	0.5	0.5	0.5	0.5	2.07[b]± 0.31[c]
2	0.5	1.0	1.0	1.0	2.20 ± 0.50
3	0.5	1.5	1.5	1.5	1.87 ± 0.50
4	1.0	0.5	1.0	1.5	3.70 ± 0.20
5	1.0	1.0	1.5	0.5	2.67 ± 0.42
6	1.0	1.5	0.5	1.0	2.13 ± 0.15
7	1.5	0.5	1.5	1.0	5.48 ± 0.45
8	1.5	1.0	0.5	1.5	4.39 ± 0.18
9	1.5	1.5	1.0	0.5	1.97 ± 0.25
$\overline{K}_1$	2.04	3.75	2.86	2.23	
$\overline{K}_2$	2.83	3.09	2.62	3.27	
$\overline{K}_3$	3.95	1.99	3.34	3.32	
R	1.91	1.74	0.72	1.09	

[a] All values are adjusted to represent the number of protoplasts from 1 ml enzyme solution and 250 mg mycelia (wet weight).
[b] Each value represents the means based on three experiments.
[c] Standard deviation of the means.

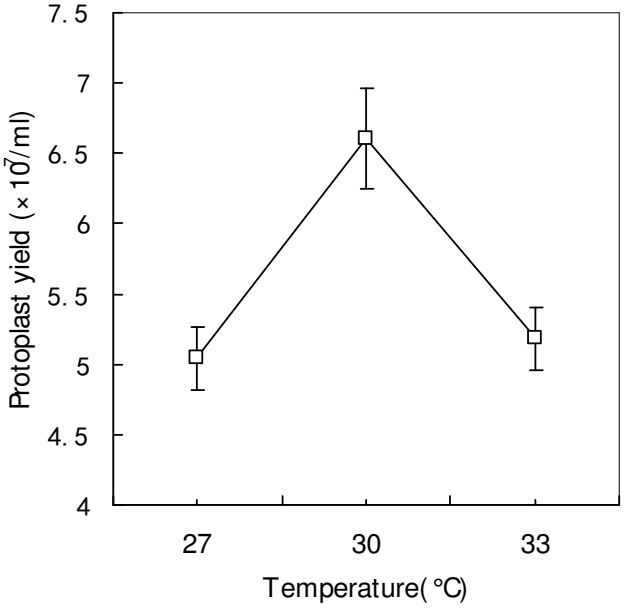

Figure 2. Effect of temperature on enzymatic digestion. All values are adjusted to represent the number of protoplasts from 1 ml enzyme solution and 250 mg mycelia (wet weight). Bars are standard deviations of means based on three replications for each determination.

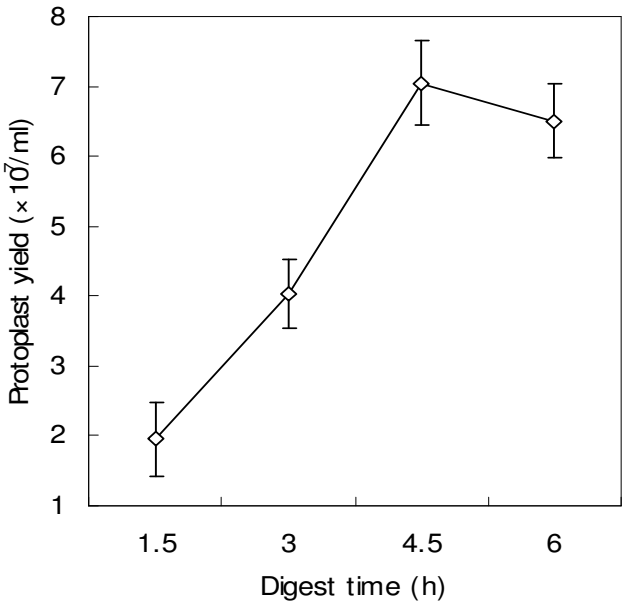

Figure 3. Effect of digestion time on the production of protoplasts. All values are adjusted to represent the number of protoplasts from 1 ml enzyme solution and 250 mg mycelia (wet weight). Bars are standard deviations of means based on three replications for each determination.

ages were used for isolation and regeneration of protoplasts. It was found that the optimal protoplast yield (7.17 ± 0.40 × 10^7 ml) and protoplast regeneration frequency (6.52 ± 0.25%) were obtained using 48 h old cultured mycelium (Figure 4) which was also at acceleration phase of its growth curve (data not shown). Though the older mycelium showed appreciable protoplast formation, they were more resistant to enzymatic hydrolysis and

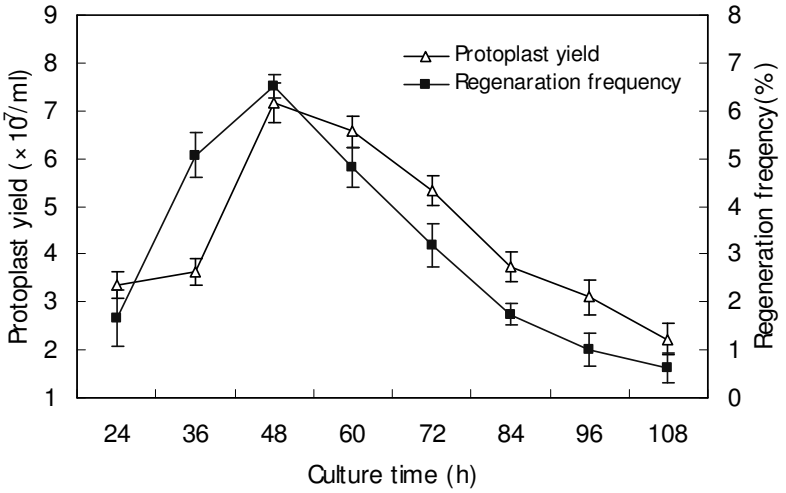

Figure 4. Effect of mycelium age on protoplast formation and regeneration. All values are adjusted to represent the number of protoplasts from 1 ml enzyme solution and 250 mg mycelia (wet weight). Bars are standard deviations of means based on three replications for each determination.

more difficult to regenerate.

The stable and optimized protoplast formation system provided solid basis for further research of protoplast transformation, mutagenesis and fusion.

Optimization of the conditions of PEG- mediated transformation

The effect of PEG is to cause the treated cells to clump, and this may facilitate the trapping of DNA (Fincham, 1989). PEG-4000 was usually used for the PEG-mediated transformation of fungus and PEG-6000 was used for plant transformation. However, some fungi could also be effectively transformed by PEG-6000 (Rohe et al., 1996). In our experiments both PEG-4000 and PEG-6000 were tested and PEG-4000 was found to be more effective than PEG-6000 in the transformation of fungi (data not shown).

PEG concentration is a pivotal factor for PEG-mediated transformation and so is Ca^{2+} which can promote the effect of PEG. In the present study, 30, 40, 50, 60% PEG and 5, 20, 50,100 mM Ca^{2+} were tested, and the result showed that 50% PEG was the most suitable concentration to transform EFY-21 (Figure 5). When the PEG concentration went up to 60%, transformants could be hardly obtained. And for the best Ca^{2+} concentration, our study demonstrated that the transformation frequency was limited in the presence of low or high concentrations of Ca^{2+} (Figure 6). Ca^{2+} at the concentration of 20 to 50 mM induced the best transformation frequency of EFY-21.

Given that long time PEG treatment could make the protoplast lose the ability of regeneration, optimized time of PEG treatment was also needed (Li et al., 2003). According to our pilot experiments, we tried the different duration of PEG treatment including 10 and 20 min, and the result showed that 10 min of PEG treatment (2.16 ± 0.27 transformants per µg DNA) had a dominant superiority to 20 min (1.28 ± 0.13 transformants per µg DNA) for transformation.

To investigate if the hph gene can be used as a dominant selectable marker for fungi transformants, the

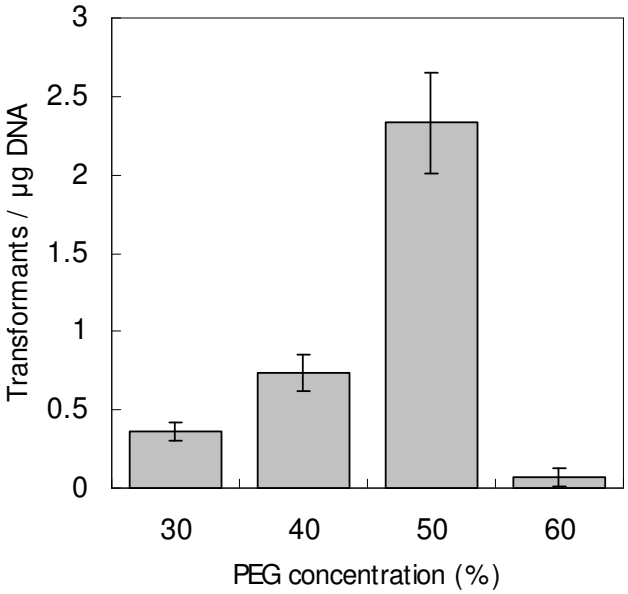

Figure 5. Effect of PEG concentration (%) on the transformation efficiency of EFY-21. Bars are standard deviations of means based on three replications for each determination.

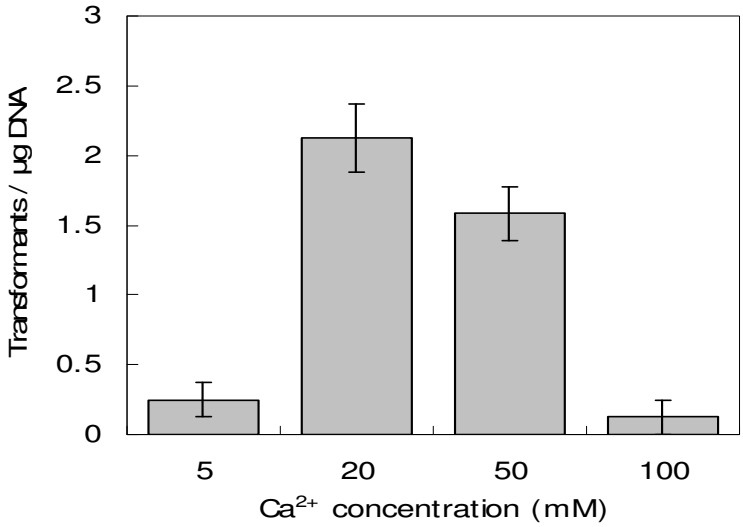

Figure 6. Effect of Ca^{2+} concentration (%) on the transformation efficiency of EFY-21. Bars are standard deviations of means based on three replications for each determination.

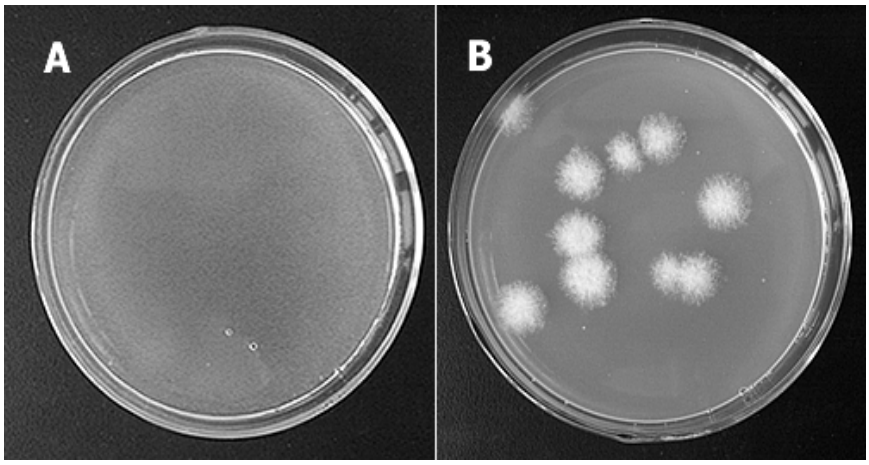

Figure 7. A. Protoplasts without PEG treatment on Czapek medium containing 150 μg/ml of hygromycin after 4 d of transformation (negative control). B. Hygromycin-resistant colonies appeared on Czapek medium containing 150 μg/ml of hygromycin after 4 d of transformation.

inhibitory effect of hygromycin B on the growth of wild-type EFY-21 was tested. The growth of wild-type of EFY-21 was totally inhibited on YPS medium at the concentration of 150 μg/ml hygromycin B (data not shown). On the contrary, stable transformants could grow on such medium (Figure 7).

PCR and Southern blot analyses of transformants

PCR was used to demonstrate the transgenic status of the hygromycin-resistant fungus colonies genomic DNA was isolated from 6 randomly selected hygromycin-resistant colonies as well as from wild-type EFY-21. As expected, 960 bp DNA fragment was amplified from all the 6 colonies and it was confirm to be the expected partial hph sequence by sequencing, while no band was amplified from the wild type EFY-21 DNA (Figure 8).

To determine the plasmid, DNA was integrated in the genome of EFY-21, Southern blot analysis was carried on. The result showed that all the six randomly selected transformants were independent transformants (Figure 9), and the integrated copies were not more than three. The band at 1.4 kb in all transformants and plasmid

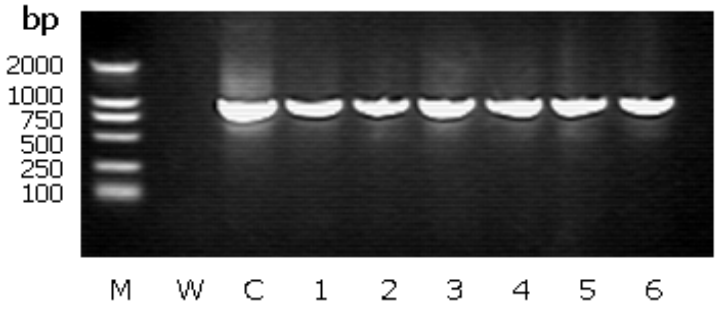

Figure 8. PCR analysis of the randomly selected hygromycin-resistant colonies for the presence of the *hph* gene. M: DL2000 (DNA molecular Marker). W: Wild-type of EFY-21 (negative control). C: pV2 (positive control). 1-6: Independent hygromycin-resistant colonies.

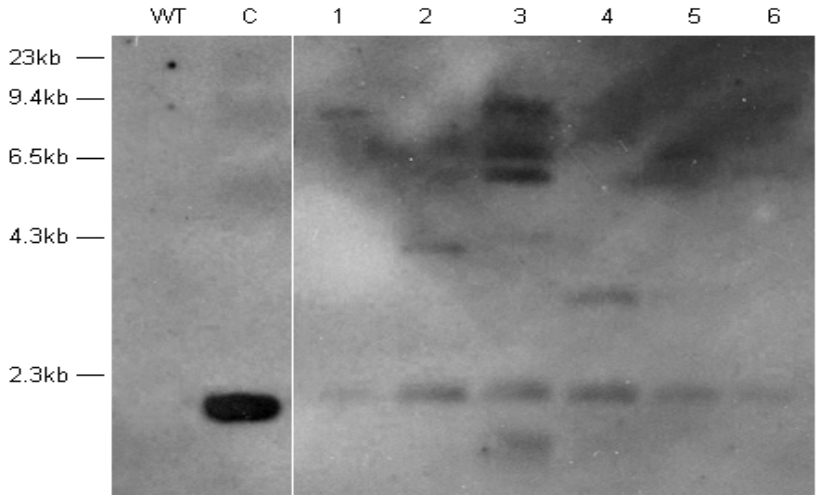

Figure 9. Southern blot analysis of 6 randomly selected transformants of EFY-21. Lanes: 1-6 Transformants, WT: wild-type strain EFY-21, C: pV2. The position of DNA molecular size markers (kb) are indicated on the left.

control corresponded to the internal BamH I fragment of the plasmid pV2. There was no hybridization signal of wild-type EFY-21, which demonstrated that the transgene (hph) has been integrated into the fungus genome.

Conclusion

In conclusion, an efficient and practical procedure for transformation of taxol-producing endophytic fungus *Ozonium* sp. EFY-21 has been firstly established, and many key factors had been evaluated and optimized. This success provides a basis for further genetic manipulation of this taxol-producing fungus for improved taxol production by transforming genes encoding rate-limiting enzymes in the taxol biosynthetic pathway.

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