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Agrobacterium tumefaciens-mediated genetic transformation of the Taxol-producing endophytic fungus *Ozonium* sp EFY21

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ABSTRACT. An efficient *Agrobacterium tumefaciens*-mediated genetic transformation method was successfully established for a newly isolated Taxol-producing fungus, *Ozonium* sp EFY21. A specific hygromycin B resistance expression vector, pCAMBIA1304'AN7-1, was constructed for fungal transformation. Key factors affecting transformation efficiency were thoroughly investigated and optimized. PCR amplification and Southern hybridization were used to verify the transformation events. This study should pave the way for future genetic modification studies of *Ozonium* sp EFY21.

Key words: Taxol-producing fungus; Protoplast; Genetic transformation; *Agrobacterium tumefaciens*

INTRODUCTION

Taxol® is a well-known antitumor drug. Since Taxol was first marketed for the treatment of ovarian cancer in 1992, it has been used alone or in combination with other drugs for the treatment of a wide range of cancers (Suffness and Wall, 1995; Amikura et al., 2006; Kumar Naraharisetti et al., 2007; Fu et al., 2009; Oh et al., 2011). Taxol was discovered in the bark of *Taxus brevifolia* Nutt. (Wani et al., 1971) and for decades has been produced mainly by extraction from the bark and needles of yew trees. However, many factors, especially the dwindling numbers of *Taxus* trees and the extremely low Taxol concentration, severely limit its market supply. Due to continuously growing demand, many efforts have been made to find an economical and environmentally friendly way to produce Taxol. The first Taxol-producing fungus that was discovered, *Taxomyces andreanae*, was isolated from the bark of a yew tree (Stierle et al., 1993). Since then, many other Taxol-producing fungi have been reported (Raja et al., 2008; Kumaran et al., 2008, 2009; Geng et al., 2010; Kathiravan and Sri Raman, 2010), thereby opening avenues for the possibility of Taxol production by microbial fermentation.

Ozonium sp EFY21 is a Taxol-producing endophytic fungus isolated in our laboratory from wild *Taxus chinensis* var. *mairei* (Zhou et al., 2007). However, low yield precludes its practical use in industrial-scale production. To solve this problem, strain improvements as well as optimization of fermentation conditions are being exhaustively studied in our laboratory. Molecular biology techniques are powerful tools for strain improvement in the case of microorganisms. The establishment of an effective transformation system is a crucial prerequisite for applying molecular approaches for the improvement of *Ozonium* sp EFY21. A PEG (polyethylene glycol)-$CaCl_2$-mediated transformation method for *Ozonium* sp EFY21 had been established in our laboratory, but the transformation efficiency is low and the transformants are unstable. An effective *Ozonium* sp EFY21 transformation system with high efficiency has yet to be established.

Agrobacterium tumefaciens-mediated genetic transformation (ATMT) was formerly used for plant genetic transformations. Since the time ATMT was used to transform *Saccharomyces cerevisiae* (Schiestl and Petes, 1991), many different types of fungi have been genetically transformed by this technique (Bundock et al., 1999; Aimi et al., 2005; Michielse et al., 2005; Betts et al., 2007). In the present study, ATMT was successfully applied to *Ozonium* sp EFY21 genetic transformation and key transformation parameters were evaluated. The optimized ATMT procedure dramatically enhances the transformation efficiency and should greatly facilitate the introduction of genetic modifications into Taxol-producing fungi for yield improvement studies.

MATERIAL AND METHODS

Fungal culture

Ozonium sp EFY21 was used as a recipient for ATMT and was cultivated in the dark at 28°C on YPS solid medium (20 g/L sucrose, 10 g/L tryptone, 5 g/L yeast extract, 1 g/L $MgSO_4$, 1 g/L K_2HPO_4, 15 g/L agar, pH 6.8).

Bacterial strains

A. tumefaciens EHA105 was used as the host for maintenance of the transformation

vector and for ATMT. It was cultured at 28°C on Luria-Bertani medium supplemented with 50 µg/mL rifampicin and 25 µg/mL streptomycin. *Escherichia coli* DH5α was used for all molecular manipulations and was cultured on Luria-Bertani medium at 37°C.

Plasmid construction

pCAMBIA1304 and pAN7-1 were the two starting plasmids used in this study. pCAMBIA1304 provided the skeleton for transfer DNA (T-DNA) and kanamycin selection in *Agrobacterium*, while pAN7-1 provided an expression cassette for hygromycin B selection in *Ozonium* sp EFY21. First, pCAMBIA1304 was cleaved with *Xho*I to remove the internal hygromycin B-resistance gene, and a 11.2-kb fragment was size selected, self-ligated, and introduced into *E. coli* DH5α, yielding pCAMBIA1304'. pCAMBIA1304' was digested with *Bgl*II and *Hin*dIII and the large fragment was ligated with a 3.9-kb *Bgl*II-*Hin*dIII fragment from plasmid pAN7-1 containing a hygromycin B resistance expression cassette, yielding the target plasmid pCAMBIA1304'AN7-1 (Figure 1).

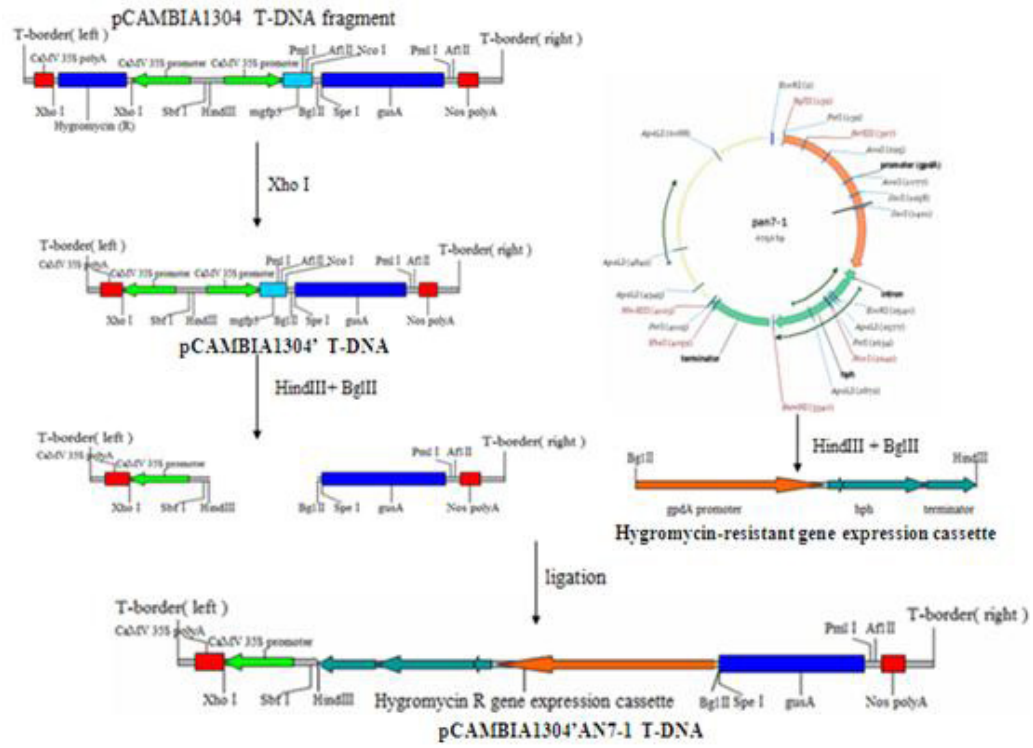

Figure 1. Construction of pCAMBIA1304'AN7-1 for fungal transformation.

Protoplast preparation

Protoplasts of *Ozonium* sp EFY21 were prepared as previously described (Wei et al.,

2010), with some modifications. Cultured EFY21 mycelia were collected by centrifugation and washed once with sterile distilled water, then once with 0.1 M Tris-EDTA solution, and finally once with 0.7 M NaCl solution. Five percent (w/v) Lywallzyme™ solution was mixed with wet mycelia at a ratio of 1 mL/250 mg wet weight. The mixture was incubated in a rotary shaker at 30°C and 150 rpm for 4-4.5 h. After enzyme digestion, the crude mixture was filtered; it was first filtered through 4 layers, then 5, and finally through 6 layers of sterilized abrasive mirror paper. Subsequently, it was centrifuged at 4000 rpm for 5 min. The deposit was washed twice with STC buffer (1.2 M sorbitol, 25 mM $CaCl_2$, and 0.01 M Tris-HCl, pH 7.5) and resuspended in STC for later use.

Genetic transformation of EFY21 protoplasts by ATMT

The ATMT transformation protocol was carried out as previously described (de Groot et al., 1998), with some modifications. *A. tumefaciens* EHA105 harboring pCAMBIA1304'AN7-1 was cultured in 5 mL minimal medium (MM) (Hooykaas et al., 1979), supplemented with 50 µg/mL rifampicin, 20 µg/mL streptomycin, and 75 µg/mL kanamycin at 250 rpm and 28°C for 48 h until its OD_{600} value reached 1.5. The strain was collected by centrifugation and washed once with induction medium (IM) composed of MM with 40 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.3, 10 mM glucose, and 0.5% glycerol (w/v) (Bundock et al., 1995), and then resuspended in IM; its OD_{600} value was adjusted to 0.15-0.20. The bacterial suspension was further cultured until its OD_{600} value reached approximately 0.6. An aliquot of EFY21 protoplasts was mixed with an equal volume of activated *A. tumefaciens* EHA105 and the mixture was spread on a cellophane sheet that was placed on the cocultivation medium (CM) (IM medium with added agar) and cultured at 22-23°C for 48 h. After cocultivation, the cellophane sheet was transferred to select medium (MM supplemented with 150 µg/L hygromycin B, 250 µg/mL carbenicillin, and 250 µg/mL cefotaxime and cultured at 28°C for several days until hygromycin B-resistant transformants appeared.

Stability of hygromycin B-resistant transformants

To evaluate the stability of hygromycin B-resistant transformants, randomly selected putative transformants were cultured on YPS solid medium for five generations in the absence of hygromycin B and then transferred to YPS solid medium containing 150 µg/L hygromycin B to determine mitotic stability.

Fungal genomic DNA extraction, PCR verification, and Southern analysis

Extraction of fungal genomic DNA was carried out as previously described (Sweigard et al., 1990). Confirmation of the presence of a T-DNA fragment was carried out by amplifying the hygromycin B resistance gene (*hph*) of pAN7-1. The PCR conditions were as follows: initial denaturation at 94°C for 5 min followed by 32 cycles of 94°C for 40 s, 56°C for 45 s and 72°C for 1 min, using the primers hphF: 5'-CACTGGCAAACTGTGATGGAC-3' and hphR: 5'-GCTG TATCTGGAAGAGGTAAACCC-3'. PCR products were detected on 1% agarose gels.

Southern blot hybridization was performed to determine the integration pattern of T-DNA. Procedures for genomic DNA digestion, agarose gel electrophoresis, and mem-

brane transfer were those described by Sambrook and Russell (2001). Probe labeling, hybridization and signal detection were carried out based on the recommendations in Gene Image AlkPhos Direct Labeling and Detection System (Amersham, UK). Primers (HygS: 5'-AGGGCGAAGAATCTCGTGCTTTC-3', HygAS: 5'-ATGTTGGCGACCTCGTATTGGGA-3') designed to acquire the correct DNA fragment were used as probes and were based on the sequence of the *hph* gene of pAN7-1.

RESULTS

Sensitivity of wild EFY21 to hygromycin B

The antibiotic sensitivity of wild-type fungi was determined by culturing EFY21 on solid YPS medium supplemented with various concentrations of hygromycin B (0, 25, 50, 100, 125, and 150 μg/L). The growth of wild-type EFY21 was progressively inhibited as the concentration of hygromycin B increased and was completely inhibited at 150 μg/L (Figure 2). Therefore, to ensure that all the transformants selected were authentic, 150 μg/L hygromycin B was used for selection.

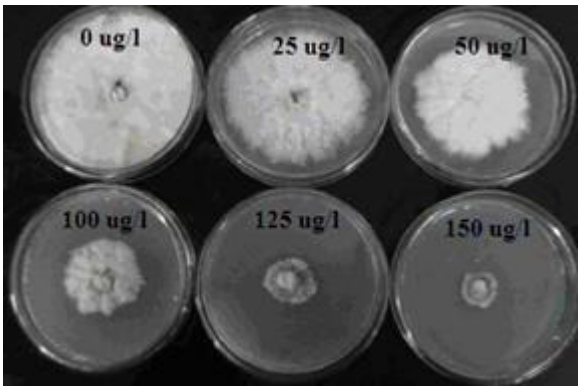

Figure 2. Growth inhibition assay for wild EFY21 cultured on YPS medium supplemented with different concentrations of hygromycin B (0, 25, 50, 100, 125, 150 μg/L).

Optimization of ATMT conditions for EFY21

Cocultivation of EFY21 protoplasts with EHA105 harboring pCAMBIA1304'AN7-1 on CM for 48 h led to the formation of observable transformants about 3-4 days after the cellophane sheet was transferred to select medium (Figure 3).

Many factors were examined, including the use of different *Agrobacterium* strains, acetosyringone (AS) concentration and addition pattern, concentration of fungal protoplasts, and cocultivation conditions. Three different types of *Agrobacterium* strains (AGL-1, LBA4404, and EHA105) were tested, and the results showed that *A. tumefaciens* EHA105 was the most suitable for the ATMT of EFY21; few or no transformants were obtained when AGL-1 and LBA4404 were used (Figure 4). As other studies have shown, successful fungal trans-

formation with *Agrobacterium* requires the right bacterial strain. The first step for a particular fungal transformation by ATMT is therefore to identify an appropriate *Agrobacterium* strain.

Figure 3. Result of *Agrobacterium tumefaciens*-mediated transformation for EFY21 protoplast.

Figure 4. Effects of three different types of *Agrobacterium* strains on *Agrobacterium tumefaciens*-mediated transformation.

AS is another key factor in ATMT transformation. We used 200 μM, the same concentration as that used in many other studies. In this study, the addition pattern of AS was fully investigated. AS could be added at three different times: during *Agrobacterium* pre-culture, during *Agrobacterium* activation, and in the cocultivation period. The results showed that when AS was completely omitted, no transformants were observed. Addition of AS during the cocultivation period was absolutely necessary and was sufficient for obtaining many positive transformants. The addition of AS during the *Agrobacterium* culture cultivation period could increase transformation efficiency to some extent, but was not necessary (Figure 5).

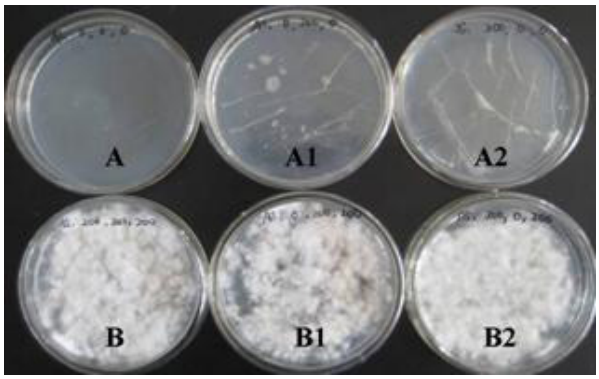

Figure 5. Transformation results by different addition patterns of acetosyringone in μM (**A.** 0, 0, 0; **A1.** 0, 200, 0; **A2.** 200, 0, 0; **B.** 200, 200, 200; **B1.** 0, 200, 200; **B2.** 200, 0, 200).

The effect of protoplast concentration on ATMT was also examined. As anticipated, the number of transformants increased as the protoplast concentration increased (Figure 6). In this experiment, the *Agrobacterium* concentration was set at OD_{600} 0.6, while the protoplasts were diluted to different concentrations. However, to obtain cleaner protoplasts, the filtration process was repeatedly operated so that the resultant protoplast concentration was always 10^7/mL. The effect of *Agrobacterium* concentration was also studied. For OD values between 0.6 and 1.0, no significant increase in transformation efficiency was observed; therefore, the bacterial concentration was set at OD_{600} 0.6.

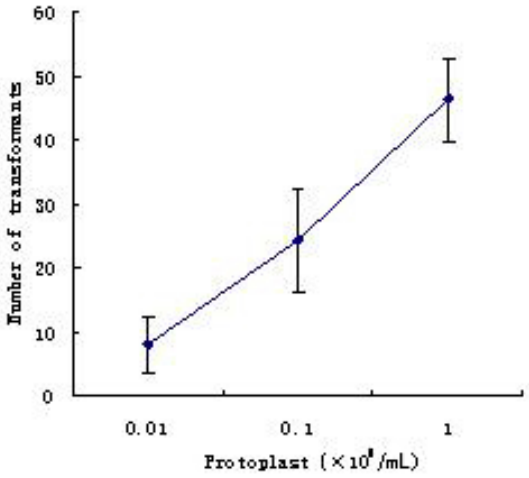

Figure 6. Effects of protoplast concentration on transformation efficiency.

According to our research, cocultivation conditions markedly affected transformation efficiency. In this experiment, different temperature and time combinations were studied. Very few transformants were formed when the cocultivation time was less than 24 h, and the transformation efficiency significantly increased when the cocultivation time was longer than 36 h. The highest efficiency was obtained following cocultivation for 48 h (Figure 7). Because the EFY21 mycelium grows luxuriously when cultivated on CM medium for significantly longer than 48 h and this would not be convenient for selection of transformants, a cocultivation time of 48 h was appropriate for the ATMT of EFY21. The effect of different temperatures was also studied and the results showed that temperatures between 20° and 25°C were most suitable for EFY21 transformation. In this experiment, *A. tumefaciens* EHA105 concentration was set at OD_{600} 0.6, and the protoplast concentration was 1 x 10^7/mL.

Stability of hygromycin B-resistant transformants

Randomly chosen transformants were repeatedly cultured on solid YPS medium in the absence of hygromycin B followed by transferring fungal hyphal tips to YPS medium supplemented with 150 µg/L hygromycin B. The results showed that all the transformants selected maintained hygromycin B resistance (Figure 8).

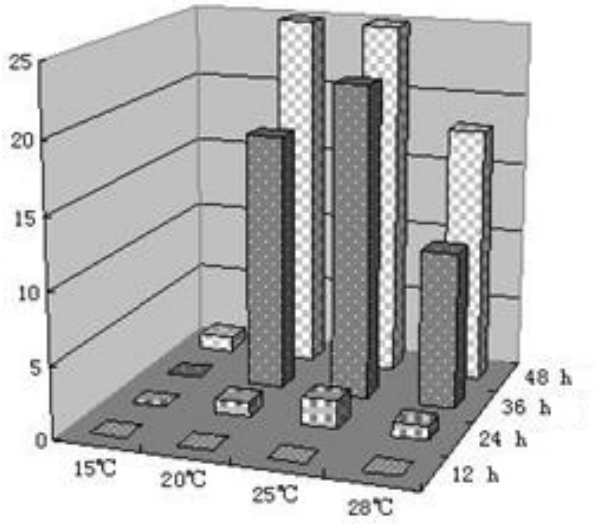

Figure 7. Effects of cocultivation conditions on transformation efficiency.

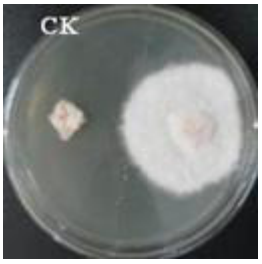

Figure 8. Stably inherited hygromycin B resistance of transformants. CK = wild-type EFY21.

PCR verification and Southern analysis of transformants

To confirm transformation events, PCR amplification was used to identify a specific *hph* gene fragment in the genomic DNA of transformants. The results (Figure 9) showed the presence of a 783-bp fragment of the *hph* gene in all the randomly selected transformants. Wild-type EFY21 was used as a negative control, and plasmid pAN7-1 was a positive control.

Southern hybridization was performed on transformants to analyze the integration mode of the T-DNA. Four transgenic fungal strains were randomly selected and analyzed along with wild-type EFY21 as a control. Genomic DNA was extracted and digested with *Bgl*II. The result showed that there was no hybridization signal with wild-type EFY21, while strong signals were observed with the hygromycin B-resistant fungal strains, demonstrating that T-DNA had inserted into the fungal genome with different numbers of copies (Figure 10).

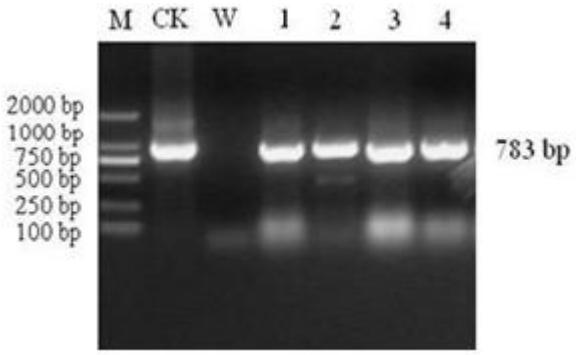

Figure 9. PCR verification of randomly selected hygromycin B-resistant transformants. *Lane M* = DNA marker DL2000; *lane CK* = pAN7-1 as positive control; *lane W* = wild-type EFY21 as negative control; *lanes 1-4* = randomly selected transformants.

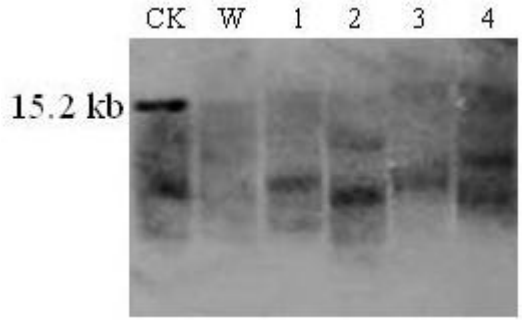

Figure 10. Southern blot analysis of transgenic fungal strains. *Lane CK* = p CAMBIA1304'AN7-1 as positive control; *lane W* = wild-type EFY21 as negative control; *lanes 1-4* = randomly selected transformants.

DISCUSSION

Compared with traditional methods like PEG-mediated protoplast transformation, ATMT methods have many advantages, especially the high transformation efficiency and flexibility in fungal starting materials. In our study, both mycelium and protoplast were successfully used with the ATMT method. However, to gain enough transformants when space on Petri dishes is limited, EFY21 protoplasts were chosen for ATMT transformation.

In this study, an ATMT system with great stability and high efficiency was successfully established for the Taxol-producing endophytic fungus *Ozonium* sp EFY21. Several factors affecting transformation efficiency were tested and optimized. This study should facilitate strain improvement of EFY21 by molecular approaches and is a good reference for genetic study of other fungi.

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