T-DNA transfer and integration as a tool for insertional mutagenesis in the taxol-producing fungus①

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Abstract

Agrobacterium tumefaciens-mediated DNA transformation method was applied to transform *Nodulisporium sylviforme* fusant HDF-68, a taxol-producing fungus. We constructed a binary vector pBI121-43 carrying a hygromycin-resistant gene cassette between the right and left borders of T-DNA. Optimal co-cultivation of *N. sylviforme* with *A. tumefaciens* containing pBI121-43 led to 110 ~ 130 hygromycin-resistant transformants per million conidia. Putative transformants were found to be mitotically stable. The molecular analysis of transformants demonstrated the random integration of single copy of the T-DNA into the host genome. This transformation system serves as a basic tool for insertional mutagenesis in *N. sylviforme* fusant HDF-68, and the development of such system lays a solid foundation for constructing high-yied gene engineering strain and clarifying taxol biosynthesis pathway in this fungus.

Key words: taxol, T-DNA, insertional mutagenesis

0 Introduction

Taxol is a diterpene alkaloid compound originally isolated from the bark of yew[1]. It is one of the most powerful anticancer drugs so far discovered and is used widely in the treatment of a variety of cancers. However, the low content of taxol in the Taxus species and the limited supply of natural Taxus trees seriously impede its widespread clinical use. Thus, intensive efforts have been made to develop alternate means of taxol production.

The taxol-producing endophytic fungus *N. sylviforme* (strain HQD33) was successfully isolated from *Taxus cuspidata* phloem by our study group in 1993 and was identified as a new genus and species in China[2, 3]. In previous studies, through protoplast fusion with inactivated parents $UV_{40\text{-}19}$ and $UL_{50\text{-}6}$, which were mutants derived from *N. sylviforme* HQD_{33}, we succeeded in obtaining a high yielding fusant, HDF-68, which could produce taxol up to 468.62 $\mu g/L$[4, 5].

In order to further improve the biosynthesis level of taxol to meet industrial demand, new research methods need to be explored by using modern gene engineering techniques to replace traditional mutagenicity. This depends on an understanding of the biosynthetic pathways in fungi. Currently, the taxol biosynthesis pathway in yew has been clarified and the enzymatic genes involved in taxol biosynthesis have been identified and isolated[6]. However, the taxol metabolic pathway in fungi remains to be elucidated.

Insertional mutagenesis is an attractive approach for cloning and studying genes of interest. Restriction enzyme-mediated integration (REMI) is a well-known method for insertional mutagenesis. REMI has contributed to the isolation of a large number of genes in various pathogenic fungi in plants[7]. However, the insertion of multiple copies of a plasmid or untagged mutagenesis including chromosomal rearrangement has often been observed[7].

Agrobacterium-mediated transformation was originally developed for plants, but has been applied successfully to several fungi recently[8-11]. One of the main advantages that *Agrobacterium* offers for fungal transformation lies in that various materials can be transformed, such as mycelium and spores[8, 10, 12], thereby the time-consuming preparation for protoplast can be omitted. This is combined with a 100- to 1000-fold higher efficiency than with conventional methods[9, 13]. T-DNA integrates predominantly as a single copy into the fungal genome at a random position by illegitimate recombination, which allows insertional mutagenesis and the identification of disrupted sequences[14].

In this study, we reported, for the first time, *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *N. sylviforme* fusant HDF-68. This provides an important step towards the detailed analysis of the taxol biosynthesis pathway in microbes and lays a solid foundation for the generation of high yields of taxol strains.

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1 Materials and methods

1.1 Strains and growth conditions

Escherichia coli strain DH5α was used as a host for gene manipulation and *A. tumefaciens* strain LBA4404 was used as a T-DNA donor for fungal transformation. These bacterial strains were maintained on LB medium at 37℃ and on YEB medium at 28℃, respectively. HDF-68, a taxol producing fusant strain was used as a recipient. Fungal strains were cultured on potato dextrose agar (PDA) medium at 28℃.

1.2 Construction of the T-DNA binary vector pBI121-43

The T-DNA binary vector pBI121-43 carrying the hygromycin B phosphotransferase (*hph*) gene cassette (the *hph* gene under control of *Aspergillus nidulans* trpC promoter and the terminator) between the right and left borders was constructed on the backbone of pBI121. The 2.4kb *hph* gene cassette was derived from pCSN43 (kindly provided by the Fungal Genetics Stock Center (FGSC), Kansas City, USA) by *Sal* I digestion and introduced into the *Sal* I site of pUC18 to make pUC18-43. The *Sma* I/*Hin*d III fragment containing the *hph* gene cassette was excised from pUC18-43 after its *Dra* I digestion and ligated with the 11.7kb backbone from pBI121, after *Eco*R I/*Hin*d III double-digestion and blunting the *Eco*R I site, resulting in the binary vector pBI121-43.

1.3 *Agrobacterium*-mediated transformation of HDF-68

The ATMT protocol was a modification of a method described previously[8]. Conidia of HDF-68 were freshly prepared and adjusted to about 10^6/mL. A single colony of *A. tumefaciens* containing the binary vector was grown overnight at 28℃ under agitation (200rpm) in YEB medium supplemented with 250μg/mL spectinomycin and 50μg/mL kanamycin. The culture was diluted to an optical density at 660 nm (OD_{660}) of 0.15 in induction medium (IM) either in the presence (IM + AS) or in the absence (IM-AS) of 200μM acetosyringone (AS)[15]. The cells were grown for additional several hours until the OD_{660} of the culture reached 0.24 ~ 1.53. Subsequently, 100μL of bacterial and 100μL of conidial suspension were mixed and spread onto nylon membranes on IM agar plates with or without AS. Following 24 ~ 96h of co-cultivation under different temperatures, the membranes were transferred onto solid PDA selection medium containing 200μM cefotaxim to kill the bacteria and 30μg/mL hygromycin B (Invitrogen) to select transformants.

Mitotic stability of the integrated T-DNA was tested by subculturing transformants on PDA medium for five times without hygromycin B. Subsequently, resistance of these transformants to hygromycin B was tested by transferring them to PDA medium containing 100μg/mL hygromycin B.

1.4 Molecular analysis of transformants

Fungal genomic DNA was extracted from mycelia by the CTAB method. PCR amplification for detection of the presence of *hph* gene in the putative transformants was carried out using primers: hph-F (5'-GAGCCTGACCTATTGCATCTC-3') and hph-R (5'-CCGTCAACCTTGCTCTGATAG-3'). The PCR program used was one cycle of 1min denaturation at 94℃, followed by 30 cycles of 30s denaturization at 94℃, 30s of annealing at 55℃ and 1min extension at 72℃, followed by a final 7min elongation at 72℃. DNA from untransformed wild-type HDF-68 served as a negative control.

For the hybridization analysis, DNA (about 5μg) from transformed strains and untransformed wild-type HDF-68 was digested for 16h with *Hin*d III, of which the restriction site was not presented inside the T-DNA. The internal 550bp *hph* fragment was used as a probe. The 550bp PCR product that was amplified using hph-F and hph-R as primers and plasmid pCSN43 as template served as a positive control. Hybridization was performed under conditions recommended for the digoxigenin (DIG) hybridization system by Roche (Mannheim, Germany).

2 Results

2.1 Hygromycin B sensitivity of HDF-68

The sensitivity of HDF-68 to hygromycin was tested by bringing mycelium onto PDA agar plates with different concentrations (0, 5, 10, 15, 20 and 30μg/mL) of hygromycin. The results showed that the growth of mycelium was totally inhibited on 15μg/mL (Fig.1). Therefore, 30μg/mL hygromycin B was chosen for the selection of resistant colonies in the following transformation experiments to prevent the growth of false transformants.

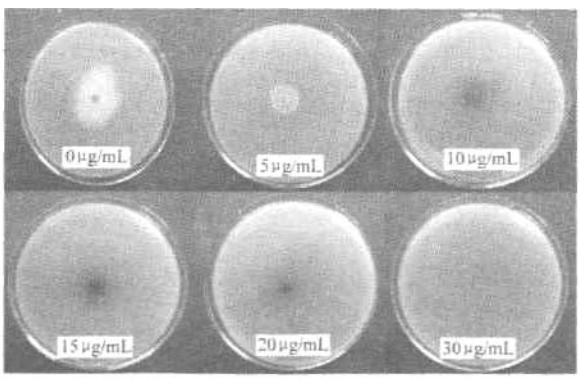
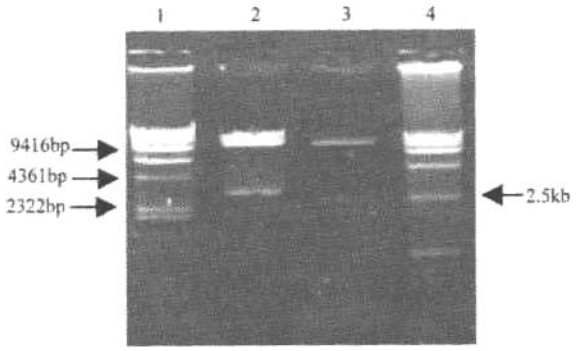


Fig.1 Effect of different concentrations of hygromycin B on HDF-68 mycelium growth

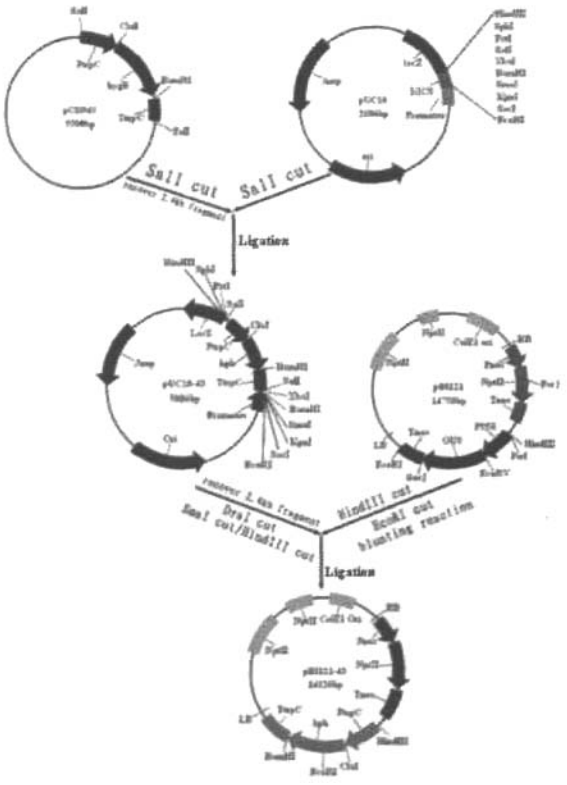
2.2 Identification of the T-DNA binary vector pBI121-43

Enzyme digestion was performed to identify recombinant pBI121-43. Fragments (11.7kb and 3kb) appeared after the original plasmid pBI121was digested with *Eco*R I and *Hin*d III. The 2.4kb fragment was obtained when the recombinant pBI121-43 was digested with *Sal* I, suggesting that T-DNA binary vector, pBI121-43, had been successfully constructed (Fig.2(a), and Fig.2(b)).



Lane 1 : λ *Hin*d III molecular marker; Lane 2 : pBI121 *Eco*R I / *Hin*d III digestion; Lane 3 : pBI121-43 Sal I digestion; Lane 4 : DL15000 molecular marker

(a) Digestion identification of binary vector pBI121-43



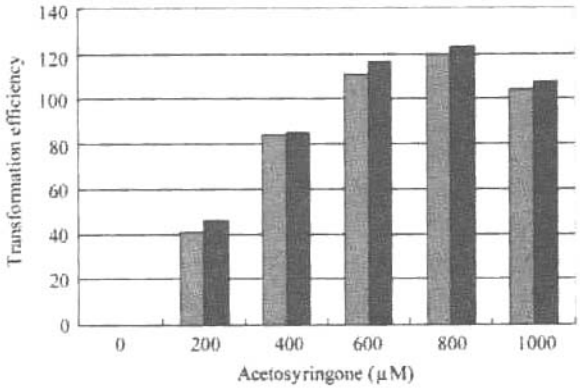
(b) Construction flow of binary vector pBI121-43

Fig.2

2.3 Agrobacterium-mediated transformation of HDF-68

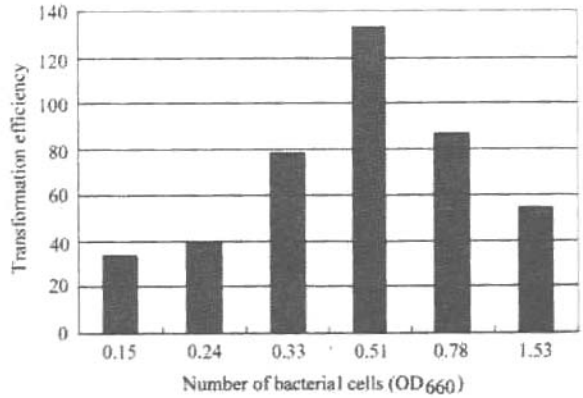
Hygromycin-resistant transformants were obtained after 7 ~ 10 days cultivation on selection medium.

First, we determined whether AS, which is necessary for the induction of *vir* genes[10, 16-18], was essential for the transformation of HDF-68. No transformants were detected when AS was omitted from the co-cultivation treatment. Furthermore, we examined the effect of AS prior to co-cultivation. The number of hygromycin-resistant colonies was not associated with the induction of bacterial cells with AS prior to co-cultivation. The preincubation of *A. tumefaciens* cells with AS displayed little effect on transformation efficiency (Fig.3(a)). Therefore, in the



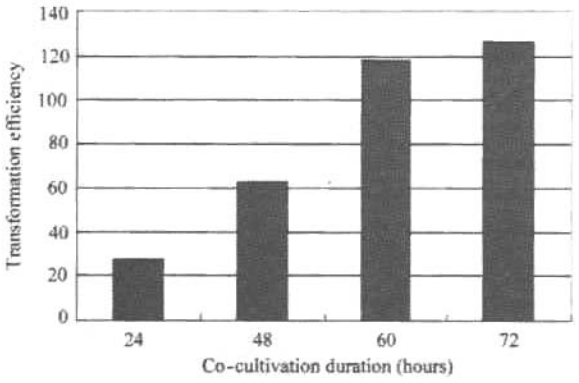
Bacterial cells were incubated in the presence (the left bar/column) of 200 μM AS or absence (the right bar/column) of AS prior to co-cultivation. The OD_{660} of bacterial culture was 0.5. Conidial suspension was adjusted to 10^6/mL conidia. Various concentrations of AS was added to IM plates. The bacterium culture (100μL) was mixed with the same volume of conidial suspension and incubated on each IM plate at 25℃ for 60h.

(a) Effect of AS on transformation efficiency



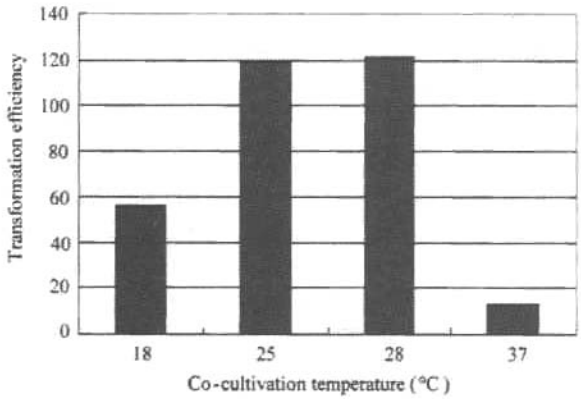
The OD_{660} of bacterium culture (non-preincubated with AS) was 0.15-1.53. The conidial suspension was adjusted to 10^6/mL conidia. The bacterium culture (100μL) was mixed with the same volume of conidial suspension and incubated on each IM plate containing 800 μM of AS for 60h at 25℃

(b) Effect of the number of *A. tumefaciens* cells initially applied for co-cultivation on transformation efficiency



The OD_{660} of bacterium culture (non-preincubated with AS) was 0.5. Conidial suspension was adjusted to 10^6/mL conidia. The bacterium culture (100μL) was mixed with the same volume of conidial suspension and incubated on each IM plate containing 800μM of AS for different duration period at 25℃.

(c) Effect of co-cultivation period on transformation efficiency



The OD_{660} of bacterium culture (non-preincubated with AS) was 0.5. Conidial suspension was adjusted to 10^6/mL conidia. The bacterium culture (100μL) was mixed with the same volume of conidial suspension and incubated on each IM plate containing 800μM of AS for 60 h at different temperature.

(d) Effect of co-cultivation temperature on transformation efficiency

Fig.3 Effect of culture parameters on the efficiency of *Agrobacterium*-mediated *N. sylviforme* fusant HDF-68 transformation. Transformation efficiency, number of transformants per 10^6 conidia

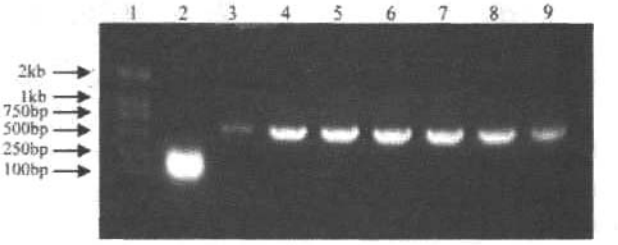
following experiments, we used the *A. tumefaciens* cells that were not induced by AS prior to co-cultivation. The number of transformants dramatically increased with an increasing concentration of AS, reaching a maximum at 800μM. The increase of the concentration above 800μM of AS resulted in a slight decrease in the number of transformants (Fig.3(a)). The effect of the number of *Agrobacterium* cells initially used for co-cultivation on transformation of HDF-68 was assessed. Maximum transformation efficiency was observed at 800μM of AS when the OD_{660} of the *Agrobacterium* culture reached around 0.5. As OD_{660} increased, significant reductions occurred in the transformation efficiency (Fig.3(b)). Subsequently, the effects of co-cultivation time and temperature were assessed. We found that the number of transformants increased with the increasing co-cultivation duration (Fig.3(c)). The optimal temperature of co-cultivation was in the range of 25℃ ~ 28℃ (Fig.3(d)). As a result, the transformation efficiency of HDF-68 was about 110 ~ 130 transformation per 1×10^6 conidia when *A. tumefaciens* cells (0.5 at OD_{660}) non-preincubated with AS were co-cultivated at 25℃ for 60h on the IM containing 800μM of AS.

2.4 Mitotic stability of transformants

HDF-68 transformants did not show phenotypical differences with the wild-type strain under the same physiological conditions. In addition, several transformants were grown on PDA plates in the absence of hygromycin B and replated for five generations, in order to determine whether the T-DNA was maintained or not in the genome of HDF-68. As a result, all of them were able to grow in the presence of hygromycin B (even up to 200μg/mL), thus confirming the genetic stability of the integrated DNA in this fungus.

2.5 Molecular analysis of transformants

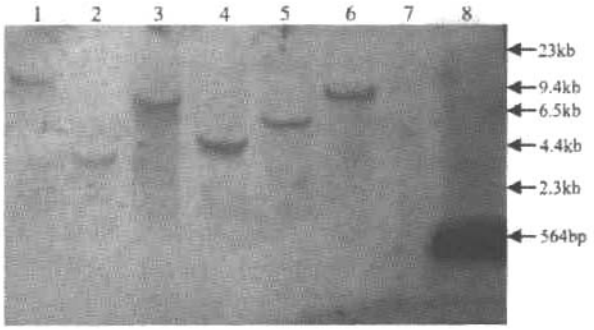
Putative HDF-68 transformants showing resistance to hygromycin were first screened by PCR analysis. Using the *hph*-F and *hph*-R, the expected 550 bp PCR product was amplified from all tested transformants (Fig.4).



Lane 1, DL2000 molecular marker; Lane 2, negative control with wild-type DNA as template; Lanes 3-9, HDF-68 transformants. A band of 550 bp was amplified from all tested HDF-68 transformants.

Fig.4 PCR analysis of HDF-68 transformants

In order to confirm the PCR results and to determine the copy number and fate of the T-DNA, Southern blotting analysis was performed for six randomly selected transformants. All the transformants showed one band suggesting a single copy had integrated into HDF-68 genome. No Hybridizing band was detected in the lane loaded with DNA from the untransformed wild-type fungus (Fig.5). Furthermore, the differences in the DNA fragment size showed that there were at least no strongly preferred integration sites in the fungal genome for the pBI121-43 T-DNA.



Lanes 1-6, transformants T1, T2, T3, T4, T5, and T6; Lane 7, wild-type strain; Lane 8, positive control.

Fig.5 Southern blotting analysis of HDF-68 transformants

3 Discussion

In our study, another binary vector pCAMBIA1301-1003 carrying *hph* gene that was drived by the same promoter as pBI121-43 was also constructed. As a result, transformants were only acquired when *Agrobacterium* carrying pBI121-43 was used. Chen et al. (2000) and Godio et al. (2004) noted differences in the transformation efficiency depending on the particular promoter expressing the *hph* gene[12, 19]. However, the promoters driving the *hph* gene in these two plasmids were identical. Therefore, the different transformation efficiencies of the two plasmids are most likely due to the sole difference between them, namely the structure of the single-strand DNA transferring from *Agrobacterium* to the fungus[20].

Factors like the presence or absence of AS prior to co-cultivation, the concentration of AS, co-cultivation period and temperature and the number of *A. tumefaciens* cells used during co-cultivation, which has already been known been to affect the transformation efficiency in fungi[9, 11, 13, 14, 16] were tested in our study. Despite of optimization of these parameters, the highest transformation efficiency was about 110 ~ 130 transformants per 10^6 conidia, which was slightly lower than those of other fungi such as *Fusarium oxysporum* (300 ~ 500 per 10^6 spores)[9] and *Colletotrichum lagenarium* (150 ~ 300 per 10^6 spores)[17]. In the latter case, the binary vector with the same *hph* gene cassette as pBI121-43 for ATMT was used, suggesting that in addition to the parameters mentioned above, the transformation efficiency also strictly depends on the strain of *A. tumefaciens*[12, 20] and the fungal species itself[8].

As described previously, some factors like the addition of AS to the *Agrobacterium* pre-culture[9, 13, 16] and the length of co-cultivation period[9, 13] have an influence on T-DNA copy number. In our study, we found that the addition of AS to the *Agrobacterium* pre-culture had little effect on the T-DNA copy number, while the co-cultivation period indeed affected the copy number. The multiple-copy T-DNA insert appeared when the duration of the co-cultivation period was extended to 72h, but more transformants were obtained following this period of time. The single-copy T-DNA integration is preferred for the application of ATMT for the generation of mutant banks, as it enables the linkage of an observed phenotype with a single alteration in the genome. Therefore, we chose 60h as the co-cultivation duration in order to obtain the single T-DNA insertion events.

In summary, we successfully transformed the *N. sylviforme* fusant HDF-68 by use of the self-constructed binary vector. The transformation efficiency was 110 ~ 130 transformants per 10^6 conidia. All the randomly selected transformants showed one single copy integration. This transformation system can be considered a powerful tool for insertional mutagenesis studies due to its easy operation, single-copy insertion, high transformation efficiency and the stable transformants acquired. Therefore, genetic transformation in HDF-68 mediated by *A. tumefaciens* may inactivate genes involved in taxol production, thus allowing the selection of blocked mutants and the subsequent isolation of taxol biosynthesis-related gene by the thermal asymmetric interlaced-PCR (TAIL-PCR) method[21]. Following the protocol optimized for HDF-68, a library of *Agrobacterium*-mediated insertional mutagenesis mutants have been generated and the screening of mutants that display the alteration of taxol yield is currently under way.

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