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Improved Transformation of the Filamentous Fungus *Aspergillus niger* Using *Agrobacterium tumefaciens*

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Since it is known that *Agrobacterium tumefaciens*, which has long been used to transform plants, can transfer the T-DNA to yeast *Saccharomyces cerevisiae* during tumourigenesis, a variety of fungi were subjected to transformation to improve their transformation frequency. In this study, I report the *A. tumefaciens*-mediated transformation of filamentous fungus *Aspergillus niger*. Transfer of the binary vector pBIN9-Hg, containing the bacterial hygromycin B phosphotransferase gene under the control of the *Aspergillus nidulans trpC* promoter and terminator as a selectable marker, led to the selection of 50~100 hygromycin B-resistant transformants per 1×10^7 conidia of *A. niger*. This efficiency is improved 10~20 fold more than reported elsewhere. In order to avoid the difficulties in selection transformant from the over-growing non-transformant, I used top agar containing 900 μg/ml of hygromycin. Genomic PCR and Southern analysis showed that all transformants contained single T-DNA insert per fungal genome. This technique offers an easier and more efficient method than that of using protoplast.

KEYWORDS: ***Aspergillus niger*, Fungal transformation**

Filamentous fungi are used in a variety of industrial processes, including the production of fermented foods, primary metabolites such as organic acids and vitamins, and secondary metabolites (Bennett, 1988). Moreover, filamentous fungi have been developed to produce homologous and heterologous proteins due to secretion of a broad spectrum of extracellular enzyme. *Aspergillus niger* is a filamentous fungus that is an attractive host for the large-scale production of homologous and heterologous protein, because it has the capacity to secrete substantial amounts of protein, fermentation is well established, and it is a GRAS (Generally Recognized as Safe) microorganism.

However, transformation frequency of *A. niger* is relatively inefficient due to problems with high concentrations of antibiotic selection. In addition, most protocols involve the transformation of protoplast, which is troublesome to prepare. Recently, *Agrobacterium tumefaciens*-mediated transformation has been used to transform several fungi (Abuodgeh *et al.*, 2000; Chen *et al.*, 2000; Covert *et al.*, 2001; de Groot *et al.*, 1998).

In this study, I improved the *Agrobacterium*-mediated transformation frequency of *A. niger* employing modified selection on hygromycin plate.

Materials and Methods

Strains and culture conditions. For bacterial cloning, the *E. coli* strain DH5α was used. The *A. tumefaciens* strain LBA4404 was used for the transformation of *A. niger* strain ATCC2119. *E. coli* cells were grown on LB medium (Sambrook *et al.*, 1985) supplemented with appropriate antibiotics to maintain plasmids; 25 μg of kanamycin per ml. Culture of *A. tumefaciens* were grown in Luria Broth containing rifampicin 100 ng/ml. *A. niger* was grown on complete medium (CM) or minimal medium (AMM) with a composition according to Pontecorvo (1969), and maintained at 30°C.

Plasmid construction. pBIN19-Hg was constructed by insertion of a 2.5 kb *SmaI*/*Hin*dIII fragment containing the hygromycin B phosphotransferase gene (*hph*) from pSHG25 into the *SmaI*/*Hin*dIII sites of the binary vector pBIN (Bevan, 1984). The pSHG25 was constructed by cloning of 2.5 kb of *Sal*I fragment containing *hgh* gene from pDH25 (Kim *et al.*, 1995) into *Sal*I site of pUC19. The other recombinant techniques were performed as described by Sambrook *et al.* (1985). DNA was introduced into *A. tumefaciens* by tri-parental mating methods (Ditta *et al.*, 1980).

T-DNA transformation. *A. niger* conidia was obtained by growing the strain on a CM plate for several days and washing with a physiological salt solution. *A. tumefaciens* strain containing pBIN19-Hg vector was grown at 28°C for 48 h in minimal medium (MM; Hooykaas *et al.*, 1979) supplemented with kanamycin (50 μg/ml). Bacterial cells in a 2 ml aliquot of this culture were harvested and washed with induction medium (IM; Bundock *et al.*, 1995), and then resuspended with 5 ml of IM in the presence or absence of 200 μM acetosyringone (AS). Suspension of *A. tumefaciens* cells, 100 μl, were mixed with 100 μl of *A. niger* conidia at a concentration of 10^6, 10^7 and 10^8 conidia/ml. Subsequently, each 200 μl of these

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mixtures was plated on IM containing 5 mM glucose instead of 10 mM glucose in the presence or absence of 200 μM AS. The plates were incubated at 30°C for 2 days, and then, 10 m*l* of top agar containing 900 μg/m*l* of hygromycin to select transformant and 200 mM of cefotaxime to inhibit the growth of the *Agrobacterium* was overlaid. The plates overlaid were incubated for 5 or 7 days at 30°C, and the transformants growing on the top agar were transferred to new medium containing hygromycin B.

Mitotic stability. To determine the stability of the hygromycin-resistant phenotype, the transformants growing through the top agar were transferred to PDA containing 100 μg/m*l* of hygromycin B (PDA-Hyg). After third successive transfers on PDA-Hyg, transformants were plated back to PDA medium and transferred three more times on PDA medium. After then, the transformants were transferred again to PDA-Hyg media, and the viability of the transformants were examined and single-spored on fresh PDA-Hyg.

Analysis of transformants. Genomic DNA from *A. niger* was extracted using the method described by Kim *et al.* (1995). In order to determine the T-DNA copy number per genome, genomic PCR and Southern analysis were performed. The genomic DNA (10 μg) was digested with *Eco*RI, separated on 1% agarose gel, and transferred onto the Hybond N^+ membrane (Amersham Pharmacia Biotech, UK). The blot was hybridized with the [α-^{32}P]-dCTP-labeled 2.5 kb *Sal*I fragment of pDH25 (Kim *et al.*, 1995).

Results and Discussion

In order to improve an *Agrobacterium tumefaciens*-mediated transformation of *A. niger*, I constructed the binary vector pBIN19Hg containing the coding region of bacterial hygromycin B phosphotransferase gene (*hgh*) under the control of *A. nidulans trpC* promoter as a selectable marker. Plasmid pBIN19Hg was then transformed to *A. tumefaciens* strain LBA4404 which carries the *vir* genes that code for the T-DNA transfer system. Co-cultivation of *A. niger* asexual conidia with *A. tumefaciens* cells carrying pBIN19Hg led to the formation of hygromycin-resistant colonies in the presence of acetosyringone (AS), a compound inducing the expression of virulence genes in *A. tumefaciens*. In the absence of AS during co-cultivation, no transformants were found, which is consistent with earlier reports showing that the induction of *vir* gene is essential for transfer of the foreign gene in fungal transformation (de Groot *et al.*, 1998).

To avoid the low efficiency caused by hygromycin B selection on agar plate, which is one of the major limits of *A. niger* transformation, I modified selection method using top agar overlaid method which was strong enough to completely inhibit the growth of untransformed *A. niger* as described in Materials and Methods. The results showed that the transformation frequency was highly increased from approximately 50~100 transformants per 10^7 conidia, which is up to 10~20 times higher than that of transformants obtained from previous report (de Groot *et al.*, 1998). To determine the stability of the hygromycin B resistant phenotype, ten transformants were analyzed for hygromycin B resistance through conidiation as described in Materials and Methods. All transformants showed considerable stability of resistance after conidiation.

To determine the integration of T-DNA into the *A. niger* genomic DNA, I isolated genomic DNA from six randomly selected transformants. Genomic PCR using two primers derived from *hph* gene produced a DNA amplicon of 900 bp in size, which indicates the integration of *hph* gene into genomic DNA of *A. niger* (Fig. 1A). Southern blot analysis using the *hph* gene as a probe showed that all transformants have shown two bands indicating a single copy of the T-DNA integrated, because the *hph* gene in T-DNA contains one *Eco*RI restriction site (Fig. 1B).

I also tested the effect of the number of *A. tumefaciens* cells initially used for co-cultivation on the transformation efficiency. Different volumes of *A. tumefaciens* cells from the same culture, ranging from 50 μl to 200 μl, were

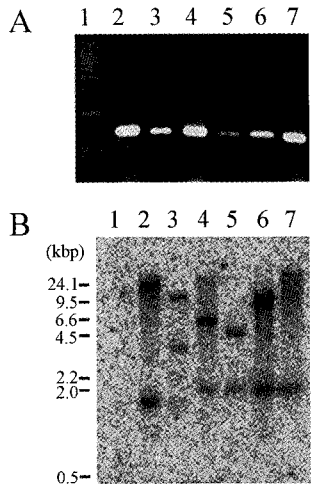

Fig. 1. A, PCR analysis using two primers derived from hygromycin B phosphotransferase gene (*hph*). Lane 1, 1.2 kb ladder (GIBCO BRL, USA); Lanes 2-7, transformants. B, Southern blot analysis of six independent *Aspergillus niger* transformants. The genomic DNA was digested with *Eco*RI that cuts once in the *hph* gene. An α^{32}P-dCTP-labeled *Sal*I fragment containing the *hph* gene from pDH25 (Kim *et al.*, 1995) was used as a probe and detects the DNA fragments of *hph* gene in the genomic DNA. Lane 1, untransformed strain; Lanes 2 to 7, transformants.

adjusted to 100 μl and then co-cultivated with 100 μl of fungal conidia for 48 h. As reported elsewhere (Abuodeh *et al.*, 2000), the transformation efficiency was a little increased as the *A. tumefaciens* cell number increased (data not shown).

The direct transformation of conidia makes *Agrobacterium* mediated T-DNA transfer a powerful and easy tool to use for genetic transformation of the filamentous fungus *A. niger*, which is used in industry for the large-scale production of homologous and heterologous proteins. My results improved the transformation frequency of *A. niger* employing the modified and simplified antibiotic selection, which completely inhibited the growth of untransformed *A. niger*.

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