Construction of an Engineering Strain Producing High Yields of α-Transglucosidase via Agrobacterium tumefaciens-Mediated Transformation of Asperillus niger

Ming Li, Liying ZHOU, Meng LIU, Yunyan HUANG, Xin SUN, and Fuping LU†

Key Laboratory of Industrial Fermentation Microbiology, Education Ministry of China, College of Biotechnology, Tianjin University of Science and Technology, No. 29, 13 Main Street, Economic and Technological Development Zone, Tianjin, 300457, China

Received April 3, 2013; Accepted May 30, 2013; Online Publication, September 7, 2013
[doi:10.1271/bbb.130281]

In this study, Agrobacterium tumefaciens-mediated transformation (ATMT) was used in breeding industrial strains for the purpose of improving α-transglucosidase production. Firstly, an efficient ATMT system for Asperillus niger was established by optimization of several influencing factors, in which transformation efficiency was improved up to 14-fold compared with the initial conditions. Furthermore, binary vector pBI-Glu containing an α-transglucosidase expression cassette was constructed and transferred into Agrobacterium tumefaciens LBA4404 in order to infect A. niger. By the efficient ATMT method, the gene for α-transglucosidase, driven by strong promoter P_gla (the glucoamylase gene promoter), had a high expression level in A. niger A-8 (25.02 U/mL). The optimized ATMT system was found to be effective and suitable for A. niger, and should be a useful tool for studying the function of A. niger genes and for industrial breeding of this strain.

Key words: Agrobacterium tumefaciens-mediated transformation; Aspergillus niger; optimization; α-transglucosidase

The filamentous fungus Aspergillus niger, widespread in foods, plant products, and the soil, is one of the most important microorganisms in biotechnology. Owing to its high secretion capacity and being generally regarded as safe (GRAS) status, A. niger has been in use for many decades to produce a large number of enzymes (e.g., α-transglucosidase, glucoamylase)1) and organic acids (e.g., citric acid).2,3) The long history of using A. niger on an industrial scale makes it well developed as a transformation host to overexpress foreign proteins. In recent years, a number of genes have been cloned and expressed in the A. niger expression system.4–6) α-Transglucosidase (EC 3.2.1.20) is a group of enzymes that catalyze the hydrolysis of α-glucosidic linkages from the non-reducing terminal of substrates releasing α-glucose or transferring a glucosyl residue to the 6-OH of the accepting glucose unit, yielding isomaltooligosaccharide (IMO).7,8) It is widely distributed in microorganisms, animals, and plants. Since an α-transglucosidase-producing strain was selected by Hayashibara Biochemical Laboratories in 1982, it has been widely used in the food industry and has attracted much attention. In the past few years, studies of α-transglucosidase have focused mainly on the screening of production strains and medium optimization,9–11) but the low yield of α-transglucosidase from wild-type strains makes them uneconomical for extensive industrial application, and thus, with the development of genetic engineering techniques, constructing and using a genetic engineered strain as a host for large-scale production is a new way to study the enzyme.12–15) To date, α-transglucosidase gene has been cloned and expressed in A. niger,16) E. coli,17) A. nidulans,13,17) and S. cerevisiae.18) These studies indicate that among all of the hosts, the expression level of α-transglucosidase was the highest in A. niger. Nevertheless, the yield obtained with the A. niger was still low and did not reach the standard for industrial application. Hence it is necessary to use A. niger as a host for the further study to improve the production of α-transglucosidase. In order to characterize the α-transglucosidase gene, and to construct a prolific secretion strain of α-transglucosidase by recombinant DNA techniques, a highly efficient, stable, and convenient transformation method for A. niger must be established to make genetic manipulation possible in this strain.

Agrobacterium tumefaciens, a plant pathogenic bacterium, transfers part of its Ti plasmid (T-DNA) to the host cell and then inserts the T-DNA region into host genome.19,20) Using this property, Agrobacterium tumefaciens-mediated transformation (ATMT) is widely applied as a transformation method for plants. It can be used to achieve targeted gene disruption and random insertional mutation. Since de Groot et al.21) reported that ATMT can be used for the transformation of filamentous fungi, it has been considered to be more convenient, stable, and efficient than traditional transformation methods used in fungi. Reports on ATMT indicate that the transformation efficiency of filamentous fungi via A. tumefaciens is about 300–9,000 transformants per 10⁷ spores, which was improved up to 600-fold as compared to results for PEG transformation of protoplasts.19,22) To date, a wide range of fungi species...
(e.g., Aspergillus fumigatus,\textsuperscript{23} Aspergillus awamori,\textsuperscript{19} Aspergillus japonicus,\textsuperscript{24} and Trichoderma viride\textsuperscript{25}) have been established ATMT system, but the transformation efficiency of \textit{A. niger} by ATMT was very low, only five transformants per 10\(^7\) spores.\textsuperscript{21} Thus, it is essential to establish an efficient ATMT method for \textit{A. niger}.

The transformation efficiency of ATMT differs in different fungal species. Several factors, including spores freshness, the concentration of \textit{A. tumefaciens}, the spore concentration, co-culture temperature, and time affect efficiency to a great extent.\textsuperscript{12,23,26} In this study, therefore, an efficient ATMT method for \textit{A. niger} was established by optimization of these influencing factors. Then an \textit{A. niger} genetic engineered strain expressing \textalpha-transglucosidase at a high level was constructed and selected from a transformant library by means of the improved transformation method.

Materials and Methods

\textbf{Strains, media, and plasmids.} \textit{A. niger} TCCC41056, screened as a strain producing glucoamylase and intracellular \textalpha-transglucosidase by our laboratory, was used as host strain for ATMT. \textit{A. niger} UV-11,\textsuperscript{27} preserved in our laboratory, was used for amplification of the glucoamylase gene promoter (\textit{P}_{\text{plas}}). These strains were maintained on PDA medium at 27 °C. \textit{A. tumefaciens} LBA4404, stored in our laboratory, was used as a T-DNA donor for fungal transformation, and grown on YEB medium (0.5% peptone, 0.5% yeast extract powder, 2% Na\textsubscript{3}PO\textsubscript{4}, 50 mmol 2-(N-morpholino)ethanesulfonic acid (MES), and 1.5% agar) supplemented with 200 μmol/L of acetosyringone (AS), at 28 °C. \textit{E. coli} DH5\textalpha was used for gene cloning. It was grown in LB medium at 37 °C. Fermentation medium (3% soluble starch and 3% bran) was used to produce \textalpha-transglucosidase. The recombiant plasmids used in ATMT were constructed with the vectors pAN7-1,\textsuperscript{28} pBI121,\textsuperscript{29} and pT-Z, a covalently closed circular pUCm-T by ligating open circular pUCm-T. Vector pAN7-1 contains the Hygromycin B resistance gene (\textit{hph}) driven by the Aspergillus nidulans \textit{gpdA} promoter and terminated by the \textit{A. nidulans trpC} terminator. Plasmid pBI121 possessing the T-DNA border repeat sequence and the kan-resistance gene was used for genetic transformation. Vector pT-Z containing the MCS region was used in plasmid construction. All plasmids were preserved in our laboratory.

\textbf{Sensitivity of \textit{A. tumefaciens} LBA4404 to cephapmycin.} Two hundred microlitre of \textit{A. tumefaciens} culture (OD\textsubscript{600} = 1.0) was spread on YEB agar plates supplemented with various concentrations of cephapmycin (0, 20, 40, 60, 80, and 100 μg/mL) and this was incubated at 28 °C for 4 d. The growth of \textit{A. tumefaciens} was observed every day.

\textbf{Sensitivity of \textit{A. niger} TCCC41056 to hygromycin B.} \textit{A. niger} spores were incubated for 4 d on PDA medium were harvested with sterile water. The spore concentration was counted by hemocytometer and diluted from 10\(^7\) spores per milliliter to 10\(^6\) spores per millilitir. Two hundred microlitre of \textit{A. niger} spore suspension (10\(^6\) spores per milliliter) was spread on PDA agar plates supplemented with various concentrations of hygromycin B (0, 40, 60, 80, 100, and 120 μg/mL) and this was incubated at 28 °C in dark for 4 d. The growth of \textit{A. niger} was recorded every day.

\textbf{Construction of binary vector pBI-hph.} The pBI121, EcoRI, and XbaI sites lay between the left and the right border of T-DNA, and hence the binary vector pBI-hph was constructed by insertion of a 2.6-kb EcoRI/XbaI fragment from pAN7-1 containing the \textit{hph} cassette into the EcoRI/XbaI restricted pBI121 plasmid.

\textbf{Constitution of a Strain Producing \textalpha-transglucosidase} 1861

\textit{A. tumefaciens-mediated transformation.} ATMT was developed to introduce DNA into filamentous fungi. In this study, all the transformation experiments were carried out according to the previously described protocol unless otherwise noted.\textsuperscript{21} Initially, binary vector pBI-hph, having the \textit{hph} gene as selection marker, was introduced into \textit{A. tumefaciens} LBA4404 by electroporation.\textsuperscript{20} The transformants were isolated on YEB agar plates supplemented with Rif (50 μg/mL), Str (20 μg/mL), and Kan (50 μg/mL), and confirmed by PCR and enzyme digestion. Positive \textit{A. tumefaciens} transformants were cultured in 5 mL of YEB on a rotary shaker (180 r/min) at 28 °C for 20 h. \textit{A. tumefaciens} cells were collected by centrifugation (5,000 r/min, 10 min) and resuspended in fresh YEB containing AS (200 μmol/L) at a density of OD\textsubscript{600} = 0.15. After preincubation for 6–10 h at 28 °C on a shaker to an OD\textsubscript{600} of 0.4 to 1.0, 100 μL of the cell suspension was mixed with an equal volume of spore suspension at a concentration of 10\(^7\) spores per millilitir to 10\(^6\) spores per millilitir, and then the mixture was spread onto filter paper placed on IM agar plate (with 200 μmol/L AS). The plates were incubated at 20 °C to 28 °C in the dark for 1 to 4 d. After co-cultivation, the filter paper was transferred onto PDA plates containing hygromycin B and cephapmycin to inhibit the growth of \textit{A. tumefaciens} and to select transformants. After an additional incubation at 28 °C for 4 d in the dark, the single colony was transferred to new PDA agar plates and confirmed. All the results presented below were obtained from three independent experiments.

\textbf{Extraction of genomic DNA and total RNA.} \textit{A. niger} spor suspension was inoculated into PDA liquid medium at 28 °C on a rotary shaker (180 r/min). After 4 d, the mycelia were collected with sterile gauze and washed with sterile water. Genomic DNA was extracted by the CTAB method.\textsuperscript{31} Total RNA was extracted according to the specifications of the Trizol kit.

\textbf{Analysis of transformants.} The \textit{hph} gene in the putative transformants was confirmed by PCR using primers hphF and hphR with genomic DNA as template. For Southern blot analysis, genomic DNA from the transformants and the wild-type strain was digested with \textit{XbaI} overnight. The treated fragments were separated by electrophoresis on 0.8% agarose and transferred to a nylon membrane. Pre-hybridization, hybridization, and washing of the membrane were carried out at 68 °C following the instructions in the DIG High Prime DNA Labeling and Detection Start Kit I.

\textbf{Mitotic stability of the transformants.} To determine the stability of the inserted T-DNA in transformants, hygromycin B resistant clones on selection plates were selected randomly and cultured on PDA plates without hygromycin B at 28 °C for 10 successive generations. Spores of the transformants were inoculated on a new PDA plate and cultured 4 d for one generation. Then the transformants were transferred to PDA plates with 100 μg/mL of hygromycin B and this was incubated at 28 °C for 4 d.

\textbf{Construction of binary vector pBI-Glu.} Binary vector pBI-Glu was constructed on the backbone of pBI-hph by inserting an \textalpha-transglucosidase expression cassette, as follows: Strong promoter \textit{P}_{\text{lac}} amplified with sense primer \textit{PgF} containing \textit{KpnI} and \textit{HindIII} and antisense primer \textit{PgR} containing \textit{NcoI} from the genomic DNA of \textit{A. niger} UV-11. An 820-bp PCR fragment was digested with \textit{KpnI} and \textit{NcoI} and ligated to pT-Z digested with the same restriction sites to construct plasmid pT-P. The \textit{A. nidulans trpC} terminator gene was obtained by PCR, using plasmid pBI-hph as template and primers TrF and TrR, which contained the \textit{RsgI} II and the \textit{XbaI} I restriction site respectively. After digestion with \textit{RbiI} II and \textit{XbaI} I, this fragment was inserted into pT-P treated with the same restriction sites, resulting in pT-P-\textalpha-transglucosidase DNA was amplified with sense primer \textit{TGF} containing the glucoamylase signal peptide sequence and antisense primer \textit{TGR} by RT-PCR using the total RNA of \textit{A. niger} TCCC41056 as template. These primers were designed according to the DNA sequence of the \textit{A. niger} mRNA for \textalpha-glucosidase in NCB1 (no. AB285123.1), and contained the \textit{NcoI} I and the \textit{RbiI} II restriction site respectively. The resulting 3.0-kb fragment was cloned into vector pT-P-C between the \textit{glaA} promoter and the \textit{tryC} terminator. This plasmid was named pT-P-\textalpha-C. Then, binary vector pBI-Glu was
constructed by insertion of a 4.4-kb *Hind* III/Xba I fragment from pT-P-α-C containing the α-transglucosidase gene expression cassette into the *Hind* III/Xba I restricted pBI-hph plasmid. The sequences of primers are listed in Table 1.

Construction and selection of the engineered strain. Binary vector pBI-Glu, including the hph gene and the α-transglucosidase expression cassette, was transferred into *A. tumefaciens* LBA4404 via electroporation, and the positive transformants were used to infect *A. niger* TCC41056 by ATMT, which was established and optimized in the above experiments for random insertional mutagenesis.

*A. niger* transformants were identified by PCR with sense primer PgF and antisense primer TrR. The copy number of the α-transglucosidase gene in the *A. niger* transformants was determined by Southern blot analysis according to the instructions in DIG High Prime DNA Labeling and Detection Start Kit I, in addition to digestion of genomic DNA from the putative transformants with *Hind* III.

To determine the activity of α-transglucosidase, the spores of each positive transformant and the wild-type strain were inoculated into 50 mL fermentation medium in 250 mL flasks and shaken (180 rpm/min) at 28 °C for 3 d. α-Transglucosidase from wild-type *A. niger* is intracellular enzyme. Hence, the fermentation broth of wild-type strain must be disrupted by ultrasonication. After centrifugation, the supernatant was used as the enzyme source with increasing concentrations, and that growth of the growth of *A. niger* was completely inhibited when the concentration of cephemycin reached 20 μg/mL. Hence, cephemycin at 20 μg/mL was chosen and used in the following transformation experiments.

Hygromycin B, a very convenient dominant selection marker, is widely used in fungal genetic transformation. In this study, the sensitivity of *A. niger* was tested in PDA medium with various concentrations of hygromycin B. The growth of *A. niger* was affected by the increase in hygromycin B and completely inhibited in medium when the concentration reached 100 μg/mL. These results suggest that hygromycin B is a suitable marker for *A. niger*, and that 100 μg/mL of hygromycin B is an effective concentration for the selection of transformants.

**Construction of binary vector pBI-hph**

In order to establish a transformation system for *A. niger* mediated by *A. tumefaciens*, binary vector pBI-hph was constructed and identified by PCR and enzyme digestion analysis. Then the recombinant plasmid was transferred into *A. tumefaciens* LBA4404 and positive clones were used during ATMT.

**Effect of spore freshness on transformation frequency**

To analyze the effect of spore freshness on transformation efficiency, a spore suspension was prepared from *A. niger* stored at 4 °C refrigerator about two weeks and a fresh strain cultivated at 28 °C for 4 d. Then the spore suspension (10^7 spores per milliliter) was mixed with an equal volume of *A. tumefaciens* culture (OD600= 0.6) and co-cultivated at 20 °C for 1 d. Under the same experimental conditions, it was obvious that transformation efficiency was higher for the fresh strain used as host for ATMT (Table 2). Currently, there is no research in this regard, and the main reason for the difference in transformation efficiency is not very clear. Perhaps the reason is the fresh spores can adapt to the environment quickly and their growth cycle is shorter.

### Table 1. Sequences of Primers Used in This Study

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Primer sequence (5′–3′)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>PgF</td>
<td>GGCGGTACGAGCCTGAGGGGTCIGGCTGAGGGGG</td>
<td>Km I, Hind III</td>
</tr>
<tr>
<td>PgR</td>
<td>CGCGGTACGAGCCTGAGGGGTCIGGCTGAGGGGG</td>
<td>Nco I</td>
</tr>
<tr>
<td>TrF</td>
<td>GAAGATCCGAGGCGCATGATGATGATGATGATGATGATGATG</td>
<td>Bgl II</td>
</tr>
<tr>
<td>TrR</td>
<td>GGGCTCTAGAAAGAAGGATTACCTCTAACAAGATG</td>
<td>Xba I</td>
</tr>
<tr>
<td>TGF</td>
<td>TTAAACGACTGGTGTCGACGTTACTCTACTGCGCTGGCGG</td>
<td>Nco I</td>
</tr>
<tr>
<td>TGR</td>
<td>GGTTGGCATCTCCACACTGCCCCGTTCCGCA</td>
<td>Bgl II</td>
</tr>
<tr>
<td>hphF</td>
<td>CATGCCATGCTGCTAGAATCATCCAGCGCT</td>
<td></td>
</tr>
<tr>
<td>hphR</td>
<td>GGAGATCCGCGGTCGACATCTACCTAT</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. The Effect of Spore Freshness on Transformation Frequency

<table>
<thead>
<tr>
<th>Spore freshness</th>
<th>Number of transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh spores</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Spores saved for 2 weeks</td>
<td>6 ± 2</td>
</tr>
</tbody>
</table>

Each value was the mean ± SD of triplicate experiments.

**Results and Discussion**

**Determination of antibiotic sensitivity**

During the selection phase of ATMT, cephemycin was used to inhibit the growth of *A. tumefaciens*. The sensitivities of *A. tumefaciens* LBA4404 were tested, and the results showed that the inhibition by cephemycin

of the growth of *A. tumefaciens* was gradually strengthened with increasing concentrations, and that growth was completely inhibited when the concentration of cephemycin reached 20 μg/mL. Hence, cephemycin at 20 μg/mL was chosen and used in the following transformation experiments.

**Construction of binary vector pBI-hph**

In order to establish a transformation system for *A. niger* mediated by *A. tumefaciens*, binary vector pBI-hph was constructed and identified by PCR and enzyme digestion analysis. Then the recombinant plasmid was transferred into *A. tumefaciens* LBA4404 and positive clones were used during ATMT.

**Effect of spore freshness on transformation frequency**

To analyze the effect of spore freshness on transformation efficiency, a spore suspension was prepared from *A. niger* stored at 4 °C refrigerator about two weeks and a fresh strain cultivated at 28 °C for 4 d. Then the spore suspension (10^7 spores per milliliter) was mixed with an equal volume of *A. tumefaciens* culture (OD600= 0.6) and co-cultivated at 20 °C for 1 d. Under the same experimental conditions, it was obvious that transformation efficiency was higher for the fresh strain used as host for ATMT (Table 2). Currently, there is no research in this regard, and the main reason for the difference in transformation efficiency is not very clear. Perhaps the reason is the fresh spores can adapt to the environment quickly and their growth cycle is shorter.
Effect of the A. tumefaciens concentration on transformation efficiency

The effect of the A. tumefaciens concentration on transformation efficiency was tested as follows: Four different concentrations (OD$_{600}$ = 0.4, 0.6, 0.8, and 1.0) were chosen and co-cultivated with fresh spore suspension. As shown in Fig. 1A, transformation efficiency improved with increases in the A. tumefaciens cell concentration from 0.4 to 0.8. However, when the concentration was above 0.8, the number of transformants decreased. The increased bacterial concentration, resulting in an overgrowth of A. tumefaciens, might have led to fungal growth limitation. In addition, massive growth of A. tumefaciens also gives rise to difficulty at the selection stage. Similar results have been reported for Coleophoma empetri (OD$_{600}$ = 0.6) and A. awamori (OD$_{600}$ = 0.8). These studies indicated that a higher concentration of cells can improve transformation efficiency.

Effect of the spore concentration on transformation efficiency

The effect of the spore concentration was tested. Transformation efficiency consistently increased with the increases in the spore concentration within a certain range of concentration (Fig. 1B). But there was no statistically significant difference in transformation efficiency when the spore concentration was at $10^7$ spores per milliliter (55 transformants) or $10^8$ spores per milliliter (59 transformants). Thus, $10^7$ spores per milliliter was the optimal spore concentration in this experiment. This result agrees with other ATMT results for fungi. In Trichoderma atroviride, more transformations were obtained when the spore concentration was at $10^8$ spores per milliliter. In the ATMT of A. japonicus, the optimal spore concentration was $10^8$ spores per milliliter. Guo et al. found that transformation efficiency began to decrease at very high spore concentrations, which may lead to nutrient limitation.

Effect of co-culture temperature on transformation efficiency

Co-cultivation is a critical step in transformation. In order to analyze the effect of co-culture temperature on transformation efficiency, three temperatures (20 °C, 24 °C, and 28 °C) were tested. The results indicated that the optimal temperature for this ATMT system was 24 °C (Fig. 1C). The different efficiencies among these temperatures suggested that a relatively low temperature during co-culture is beneficial for A. tumefaciens to transfer its T-DNA to the host, consistently with Hebeloma cylindrosporum, Leptosphaeria maculans, A. awamori. In the experiments of Michielse et al., four temperatures (20 °C, 22.5 °C, 25 °C, and 28 °C) were tested. The results indicated that co-cultivation at 20 °C resulted in variable transformation frequencies, which hampered meaningful statistical analysis, while at 28 °C, excessive growth of A. tumefaciens resulted in a decrease in transformation efficiency. These studies indicate different temperatures significantly influence the number of transformants and background growth. Although 28 °C was the optimal temperature for the growth of A. tumefaciens, it was not appropriate for the transformation mechanism. This...
exorbitant temperature might induce loss of the transformation function of *A. tumefaciens* and thus lead to the decreasing of transformation efficiency.

Effect of co-culture time on transformation efficiency
Transformation efficiency is closely related with co-culture time. In this study, optimal transformation frequency was attained when the mixture was co-cultivated for 2 d, and extending the incubation time led to reduced transformation efficiency (Fig. 1D). Perhaps a decreased co-culture time goes against the transfer of T-DNA, and increased co-culture time might lead to reduced transformation efficiency (Fig. 1D).

Analysis of transformants
To determine whether the *hph* cassette was integrated in the genome, five putative transformants were selected randomly and detected by PCR and Southern blot. The PCR results revealed that 1-kb fragments were present in all five transformants, but not in the wild-type strain (Fig. 2A). Southern blot was performed using the *hph* gene as probe. As shown in Fig. 2B, all the resistant strains harbored single hybridizing fragment signals at different sites, with no signal at any position in the wild-type strain. These results indicate that hygromycin B resistant clones were obtained by ATMT.

Mitotic stability of the transformants
In order to confirm the stability of the transformants, 20 hygromycin B resistant clones obtained by ATMT were selected randomly and grown in PDA (hygromycin B-free) medium for 10 successive generations. Then they were transferred to resistant plate (PDA medium containing 100 µg/mL of hygromycin B) and cultured for 4 d. The genomic DNA of these transformants was extracted and tested by PCR. The results showed that the *hph* resistances of all the transformants were heritable and stable (date not shown).

Establishment of ATMT for *A. niger*
Based on the above described results, a transformation system for *A. niger* mediated by *A. tumefaciens* was established and optimized. One hundred microlitre of fresh spore suspension (10⁷ spores per millilitre) was mixed with an equal volume of *A. tumefaciens* cell suspension (OD₆₀₀ = 0.8) and spread onto filter paper placed on IM plates (200 µmol/L AS added). After co-cultivation at 24 °C for 2 d, the filter paper was transferred onto PDA agar plates containing hygromycin B (100 µg/mL) and cephamycin (20 µg/mL) to select transformants. Transformation efficiency reached 83 transformants per 10⁷ spores by the established ATMT for *A. niger* in this study, which improved by 16 times as compared with that previously reported.

Construction of binary vector pBI-Glu
According to "Materials and Methods," P<sub>glu</sub>A, α-transglucosidase, and the trpC terminator gene were cloned and sequenced respectively. The sequencing results showed that the cloned sequences were consistent with the reported sequences, excepting that the α-transglucosidase gene did not contain signal sequence. Then binary vector pBI-Glu was constructed by cloning P<sub>glu</sub>A, α-transglucosidase, and the trpC terminator gene into pBI-hph.

In improving the production of heterologous gene products in filamentous fungi, the promoter, an essential part of the expression vector in genetic engineering, has a great influence on the expression levels of heterologous proteins. The P<sub>glu</sub>A cloned from glucoamylase-producing *A. niger* UV-11, which can drive the expression of foreign genes effectively, is frequently used to construct expression vectors. It can be used to construct industrial strains, too. Hence, P<sub>glu</sub>A was cloned into pBI-Glu to drive the expression of α-transglucosidase gene to a high level.

α-Transglucosidase derived from *A. niger* is an intracellular enzyme, and it is difficult to extract. In this research, the glucoamylase signal peptide was used to guide the intracellular enzyme to the extracellular matrix. To some extent, this might reduce costs in industrial production. Thus the glucoamylase signal peptide sequence was fused to the N-terminus of the α-transglucosidase gene.

Construction of the engineered strain
*A. tumefaciens* LBA4404, harboring binary vector pBI-Glu, was used to infect *A. niger* TCCC41056 by the optimized transformation method. *A. niger* transformants, obtained by ATMT, were selected and confirmed by PCR. The results revealed that 4.4-kb fragments...
(including the sequences of \(P_{\text{glaA}}\), \(\alpha\)-transglucosidase and terminator of trpC) were present in all transformants, but not in the wild-type strain (data not shown). Then, 400 positive transformants, confirmed by PCR, were randomly selected to analyze mitotic stability, and 230 mitotically stable transformants were obtained (about 57%). This suggested that the \(\alpha\)-transglucosidase gene in most of the transformants was heritable and stable.

**Expression of \(\alpha\)-transglucosidase in the engineered strain**

All stable transformants and the wild-type strain were cultured in fermentation medium for 3 d, and then the enzyme activity was measured (data not shown). Among the transformants, the productivity of \(\alpha\)-transglucosidase of 13 transformants was higher than that of the wild-type strain (Fig. 3). This indicates that genetic engineered strains with high enzyme activity can be obtained by ATMT. As shown in Fig. 3, it was obvious that highest enzyme activity reached 25.02 U/mL, about 12.1-fold of the control strain. One of the reasons for the high expression level of \(\alpha\)-transglucosidase in the engineered strains might be the multiplication of a region in the DNA with a strong promoter. The enzyme assay showed that \(\alpha\)-transglucosidase was secreted into the extracellular matrix by the glucoamylase signal peptide, confirming that the glucoamylase signal peptide contributed to the secretion of \(\alpha\)-transglucosidase in the *A. niger* engineered strain.

In previous studies, the \(\alpha\)-transglucosidase gene was cloned and expressed in different hosts. Nakamura et al. cloned the \(\alpha\)-transglucosidase gene from *A. niger* GN-3 and introduced it into *A. nidulans*, but the expression level in the transformants (highest activity 18 mU/mg) was lower than in *A. niger* GN-3 (21 mU/mg). The activity was still lower when the same gene was inserted into *Emericella nidulans* JVM10259, at only 0.962 mU/mg. When the \(\alpha\)-transglucosidase gene from *A. niger* GN-3 was cloned into the same *A. niger* strain, the activity of the recombinant \(\alpha\)-transglucosidase rose 240 mU/mg. Based on previous research results, there is no doubt that enzyme activity is higher with *A. niger* used as host. Therefore, in order to compare enzyme activities, the activity of \(\alpha\)-transglucosidase from *A. niger* A-8 was determined by the method of Lee et al. The result, that the enzyme activity was 57 U/mg, suggests that *A. niger* engineered strain A-8 is poten-

![Fig. 3. Determination of \(\alpha\)-Transglucosidase Activity. Each value represents the mean ± SD of triplicate experiments. A, wild type; A-1–A-13, transformants.](image)

![Fig. 4. Southern Blot Analysis of *A. tumefaciens* Wild-Type and Transformants. A-1–A-13, transformants; A, wild type.](image)

...tiably valuable for producing high yields of \(\alpha\)-transglucosidase.

The 13 transformants were then tested by Southern blot using the \(\alpha\)-transglucosidase expression cassette (including the sequences of \(P_{\text{glaA}}\), \(\alpha\)-transglucosidase, and the terminator of trpC) as probe, and the wild-type strain was used as control. The genome from each strain was hybridized with the entire \(\alpha\)-transglucosidase expression cassette gene. As shown in Fig. 4, all the transformations had 1–2 \(\alpha\)-transglucosidase fragment signals at various positions, without any signal at any position in the wild-type strain. Twelve transformants were single copies and only one transformant was a double copy. A-1, A-2, A-6, and A-9 produced hybridizing fragments of approximately the same size.

Based on the above results, the highest enzyme activity was obtained from double-copy transformant A-8 at 25.02 U/mL. It can be seen that the insertion of foreign gene can affect the expression mechanism to a certain extent. In fact, many studies have found that the expression is influenced by gene copy number and insert locus. Gene dosage has an important effect on the expression of heterologous proteins. Some experiments found that one copy of the foreign protein expression cassette was sufficient for expression, and others that an increase in copy number had a positive effect on expression. Sreekrishna et al. integrated 20 copies of the *tnf* gene into the chromosome of yeast, and the expression of recombinants was 200 times that of the single-copy strain. When Pichia pastorii was used as host for the expression of Bovine lysozyme, the expression level decreased with increasing of copy numbers, from 0.46 g/L (one copy) to 0.25 g/L (three copies). Therefore, there is no definite conclusion as to whether the influence of gene dosage on the expression of a heterologous protein is a positive effect or a negative one, but in this study, the copy number of \(\alpha\)-transglucosidase gene proved beneficial to the expression.

In addition, single-copy transformants A-1, A-2, A-6, and A-9 showed almost the same activity (12.26 U/mL, 11.82 U/mL, 11.75 U/mL, and 11.09 U/mL respectively), whereas the activity of \(\alpha\)-transglucosidase from the other single-copy transformants was different. These differences suggest that the integration site of the foreign gene also affected the expression level. The insert locus of the foreign gene in the fungal genome is so random that any chromosome can be used as an integration site and the sites are different in the same chromosome. When a foreign gene was transferred to another species and inserted into the chromosome, the change in the structure of host genome affected the expression of the host and foreign genes. Kong and Liu concluded that...
the chromosomal microenvironment near the insert locus influences the activity of the promoter of foreign gene, thereby affecting the expression level. On the basis of the above results, different insertion sites lead to difference in enzyme activity, but the reason for these differences needs future research on the molecular level.

Conclusions

In this study, we established an efficient ATMT system for A. niger. The factors affecting transformation efficiency were optimized, and the highest transformation efficiency was obtained (83 transformants per 10^7 spores) when a fresh spore suspension (10^7 spores per milliliter) was co-cultivated with an equal volume of A. tumefaciens (OD600 = 0.8) in IM medium at 24 °C for 2 d. By this transformation method, α-transglucosidase-producing engineered strains were constructed. The enzyme activity of mutant A-8 was 12.1-fold of the wild-type strain. Thus the ATMT method is not only an effective method for the transformation of filamentous fungi, but also an efficient tool for random integration in A. niger. Besides, ATMT provides a favorable reference for gene engineered strain breeding. More importantly, the A. niger engineered strain is potentially useful for producing high yields of α-transglucosidase.

Acknowledgments

This work was supported by the Tianjin Research Program of the Application Foundation and Advanced Technology Program (no. 11JCYBJC09600), the Key Technology Research and Development Program of Tianjin, China (no. 11ZCKFSY00900), the Program for Changjiang Scholars and the Innovative Research Team in University (IRT1166), the National Natural Science Foundation of China (no. 21176190), and the Chinese Program for High Technology Research and Development (2013AAA102106).

References