Insertion Mutation in HMG-CoA Lyase Increases the Production Yield of MPA through Agrobacterium tumefaciens-Mediated Transformation

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Introduction

Mycophenolic acid (MPA) is an antibiotic produced by Penicillium brevicompactum. MPA has antifungal, antineoplastic, and immunosuppressive functions, among others. β-Hydroxy-β-methylglutaryl-CoA (HMG-CoA) lyase is a key enzyme in the bypass metabolic pathway. The inhibitory activity of HMG-CoA lyase increases the MPA biosynthetic flux by reducing the generation of by-products. In this study, we cloned the P. brevicompactum HMG-CoA lyase gene using the thermal asymmetric interlaced polymerase chain reaction and gene walking technology. Agrobacterium tumefaciens-mediated transformation (ATMT) was used to insert a mutated HMG-CoA lyase gene into P. brevicompactum. Successful insertion of the HMG-CoA lyase gene was confirmed by hygromycin screening, PCR, Southern blot analysis, and enzyme content assay. The maximum MPA production by transformants was 2.94 g/l. This was 71% higher than wild-type ATCC 16024. Our results demonstrate that ATMT may be an alternative practical genetic tool for directional transformation of P. brevicompactum.

Keywords: Agrobacterium tumefaciens, Penicillium brevicompactum, directional transformation, mycophenolic acid, HMG-CoA lyase
transform using traditional methods. Furthermore, transformants can be genetically analyzed, and disrupted sequences can be identified more easily because the transformation is highly efficient and the percentage of single T-DNA insertion events is high.

The MPA biosynthetic pathway has been elucidated. Several phenolic intermediates derived from the tetraketide chain have been implicated in MPA synthesis through the farnesyl pyrophosphate pathway. β-Hydroxy-β-methylglutaryl-CoA (HMG-CoA) lyase is a key enzyme in this bypass metabolic pathway [4, 25]. HMG-CoA is an important MPA precursor. The inhibitory activity of HMG-CoA lyase increases the biosynthetic flux of MPA by reducing the generation of by-products.

In this study, we cloned the HMG-CoA lyase gene of *Penicillium brevicompactum* using thermal asymmetric interlaced PCR (TAIL-PCR) and gene walking technology. An efficient ATMT system for *P. brevicompactum* was used to insert the mutant HMG-CoA lyase gene. MPA production from fermentation was effectively improved. ATMT may be alternatively implemented as a practical way for the genetic transformation of *P. brevicompactum* in fermentation studies.

**Materials and Methods**

**Strains and Plasmids**

*Escherichia coli* strain DH5α (Tiangen Biotech, Shanghai, China) was used to propagate plasmid DNA. *A. tumefaciens* LBA4404 cells, *P. brevicompactum* ATCC 16024 cells, and pPK3 plasmids were stored in our laboratory. The pPK3 plasmid was derived from pPK2. The *kan* restriction enzyme site of pPK2 was substituted for SacI on pPK3. The pPK3 plasmid carries hygromycin and kanamycin resistance genes as selective markers. The *hph* gene was amplified from pPK3 by PCR using the following primers: *hph*-U (5'-TTGATAGAGGCGGTGAT-3') and *hph*-D (5'-CGGTCTGCTGCTCCATACAAG-3').

*E. coli* strain DH5α was cultured and manipulated as previously described [32]. The *A. tumefaciens* LBA4404 strain was cultured in yeast extract peptone growth medium containing 100 μg/ml streptomycin in minimal medium (MM) and 30 μg/ml rifampicin in induction medium (IM) (Sigma-Aldrich, USA) at 28°C [2, 6, 14].

**Agrobacterium-Mediated *P. brevicompactum* Transformation**

Modified transformation was performed as described previously [9]. The *A. tumefaciens* strain LBA4404 carrying binary vectors was cultured overnight at 28°C in MM containing 100 g/ml streptomycin and kanamycin. The optical density at OD₆₀₀ of *A. tumefaciens* cells was adjusted to 0.15–0.20 in IM containing 200 μM acetoxyringone (AS). Then, the cells were cultured for 6–8 h at 28°C, 220 rpm.

*A. tumefaciens* and *P. brevicompactum* co-cultures were established. Spores and pre-cultured LBA4404 were mixed and plated onto nitrocellulose filters in a culture plate containing co-cultivation medium (IM plus 5 mM glucose and 2% (w/v) agar, with AS for co-cultivation). After incubating for 24–72 h at 22–28°C, filters containing fungal and bacterial colonies were transferred onto potato dextrose agar (PDA) containing 100 μg/ml cephalexin to kill *A. tumefaciens*. Transformants were selected by 50 μg/ml hygromycin. After an additional incubation for 3–5 days, individual colonies were transferred to hygromycin-containing PDA. The mean data were from independent experiments repeated three times.

**Fermentation**

*P. brevicompactum* was cultured in fresh PDA. The seed and fermentation medium contained the components as described previously [11]. A 7 L fermenter with two 6-bladed disc turbine impellers (Shanghai Baoxing Bio-Engineering Equipment Co., Ltd., China) was used for MPA batch fermentation. We used a working volume of 5 L. The agitation speed was 300 rpm and the aeration rate was 11/min. The temperature was maintained at 28 ± 0.2°C during fermentation. The pH was adjusted with NaOH and HCl and monitored by a pH electrode. Other fermentation parameters were measured at different time points. Measurements were performed in triplicate and the presented data are expressed as mean ± standard deviation [11].

**Gene Cloning**

The *P. brevicompactum* HMG-CoA lyase gene was cloned by TAIL-PCR [18]. Pairs of degenerate primers LY1, LY2, LY3, and LY4 were designed based on the conserved coding regions of published *Penicillium* sequences. Two sequences (GenBank Access Nos. XM_002152582 and XM_002567941) from *Penicillium marneffei* ATCC 18224 and *Penicillium chrysogenum* Wisconsin were aligned using BLAST to identify conserved fragments and design the degenerate primers (Table 1). The TAIL-PCR conditions were as follows: an initial denaturation of 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 59°C for 30 sec, and 72°C for 40 sec, with a final extension at 72°C for 10 min.

The unknown flanking regions of the HMG-CoA lyase gene were identified by genome walking using the primers in Table 1. Three rounds of TAIL-PCR were performed for sequencing. The *hlb* internal fragment and the *hrb* and HMG-CoA lyase genes were amplified by PCR using the primers in Table 1. The genome walking kit, PCR purification kit, plasmid mini kit, and gel extraction kit were purchased from Promega (USA).

**Plasmid construction**

The pPK3 vector harboring the *hlp* fusion gene was constructed as follows (Fig. 1). The *hrb* upstream sequence from the HMG-CoA lyase gene was amplified with *hrb*-1 and *hrb*-2 primers (Table 1), containing XbaI and HindIII restriction enzyme sites. The *hlb* downstream sequence was amplified with the *hlb*-1 and *hlb*-2 primers (Table 1), containing EcoRV and SacI restriction enzyme sites (Fig. 2B). The resulting *hlb* and *hrb* fragments were
Table 1. Primers*

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY1</td>
<td>GTMCGHATCGTGGAAGTCGG</td>
</tr>
<tr>
<td>LY2</td>
<td>ARACAAAGMYCGARYTGATT</td>
</tr>
<tr>
<td>LY3</td>
<td>CCATAABTGATCRTGRAAATG</td>
</tr>
<tr>
<td>LY4</td>
<td>ASWTCTYCTGTSGAVACATT</td>
</tr>
<tr>
<td>L-F1</td>
<td>ACACATCAAAATGCCGCACCAAC</td>
</tr>
<tr>
<td>L-F2</td>
<td>GTCCTTACGAGGGTCCCGGATG</td>
</tr>
<tr>
<td>L-F3</td>
<td>TGTCTGACATCTGCTCAGCCT</td>
</tr>
<tr>
<td>L-R1</td>
<td>TGTCCGCCACCGAGACTTCATC</td>
</tr>
<tr>
<td>L-R2</td>
<td>ATCGGGACCTCTGTAAGACAT</td>
</tr>
<tr>
<td>L-R3</td>
<td>TGTGGGCGCATTTGATGTGTCG</td>
</tr>
<tr>
<td>hlb-1</td>
<td>CGAGCCTCTATGCAATGGTAGACTGGGCTGGT</td>
</tr>
<tr>
<td>hlb-2</td>
<td>GATATCAACCTGTAATGTCTATCT</td>
</tr>
<tr>
<td>hrb-1</td>
<td>TGCTCTAGAAACTAATCTGCTCAGCAGG</td>
</tr>
<tr>
<td>hrb-2</td>
<td>AAGCTCGACTCTCTCCTCCCAAGA</td>
</tr>
<tr>
<td>LQ1</td>
<td>ATGTTTTTAATTGGATTTGAGCCGA</td>
</tr>
<tr>
<td>LQ2</td>
<td>GGCACTCTGACCCAACGGCAACTCG</td>
</tr>
</tbody>
</table>

*L1, L2, L3, and L4 were used for amplifying the internal fragment of the HMG-CoA lyase gene; L-F1, L-F2, and L-F3 for the upstream unknown sequence of the HMG-CoA lyase gene; L-R1, L-R2, and L-R3 for the downstream unknown sequence of the HMG-CoA lyase gene; L-F1, L-F2, and L-F3 for the upstream unknown sequence of the HMG-CoA lyase gene; L-R1, L-R2, and L-R3 for the downstream unknown sequence of the HMG-CoA lyase gene.

Southern Blot Analysis

Southern hybridization was performed using a digoxigenin hybridization system according to the manufacturer’s guidelines (Sigma-Aldrich). An 1.3 kb region of the HMG-CoA lyase gene was amplified using primers hlb-2 and hlb-2. The PCR conditions were as follows: 94°C for 5 min, 30 cycles (94°C, 30 sec, 60°C, 1 min; 72°C, 1 min), with a final extension of 72°C for 10 min. Then, 10 μg genomic DNA from transformants and the pPK3-hlb-hrb vector were digested by restriction enzymes (HindIII/EcoRV) and separated by 0.8% agarose gel electrophoresis. Separated DNA was transferred onto Hybond N+ nylon membranes. The 1.3 kb amplified fragment of the HMG-CoA lyase gene was labeled with a probe to detect the integrated T-DNA in the genome. According to the manufacturer’s instructions, aqueous hybridizations were performed at 42°C overnight and bands were detected by chemiluminescence.

Enzyme Content Assay

The HMG-CoA lyase content assay was detected by the kit (R&D Systems, USA) according to the manufacturer’s instructions. A double-antibody sandwich enzyme-linked immunosorbent assay was performed to measure the level of P. brevicompactum HMG-CoA lyase in the sample. Extracted cellular proteins were added to a plate containing an anti-HMG-CoA lyase polyclonal antibody. After antibody incubation, horseradish peroxidase was added to catalyze the colorimetric reaction. The color intensity correlated positively with the HMG-CoA lyase concentration in the samples. HMG-CoA lyase concentrations were determined by the OD450 value. Assays were repeated three times to ensure reproducibility and the presented data are expressed as the mean ± standard deviation.

Results and Discussion

HMG-CoA Lyase Gene Sequence and Flanking Regions

A HMG-CoA lyase gene sequence of 1.763 kp was obtained by PCR amplification from P. brevicompactum with the LQ1 and LQ2 primers (Fig. 2A). The pPK3 vector harboring the hph fusion gene was constructed (Fig. 1). Gene sequence homology was 87% compared with that of P. chrysogenum Wisconsin 54-1255 according to a NCBI-BLAST analysis.

Analysis of P. brevicompactum Transformants

A tumefaciens has previously been used to successfully transform a variety of filamentous fungi [10]. ATMT can be used to randomly insert T-DNA by insertional mutagenesis into the recipient genome. In this approach, T-DNA carries a selectable marker, which permits random and site-directed mutagenesis and is increasingly being used for the transformation of many fungi [24]. The hygromycin-resistant transformant that produced high levels of MPA was named AHL-4. The HMG-CoA lyase gene sequence was amplified using the LQ1 and LQ2 primers. As shown in Fig. 3A, the amplified PCR product was about 4.5 kb compared with 1.763 kp of the wild type. The 0.6 kb hph gene was PCR-amplified from transformants using the hph-U and hph-D primers. The amplification pattern contained the obvious stripe from the AHL-4 transformant in contrast to the non-transformed wild-type ATCC 16024. Taken together, the PCR results confirmed successful integration of T-DNA into the P. brevicompactum genome.

Genomic DNA and the pPK3-hlb-hrb vector were digested with HindIII and EcoRV restriction enzymes. The amplified 1.3 kb fragment of the HMG-CoA lyase gene was labeled with a probe. The hybridization pattern of the non-transformed wild-type ATCC 16024 contained an obvious band in contrast to the AHL-4 transformant and pPK3-hlb-hrb vector (Fig. 3B). This suggested that the exogenous gene

was integrated into the *P. brevicaulis* genome during T-DNA transfer. The ATMT system was demonstrated to be a high-efficiency mediator for targeted genetic transformation of *P. brevicaulis*.

**HMG-CoA Lyase Content Assay**

Fermentation during the 0 to 96 h time frame of the culture is the exponential growth phase. The samples were harvested by centrifugation when the dry cell weight reached the maximum. The washed cells (100 mg) were ground to a powder in liquid nitrogen, and the HMG-CoA lyase content assay was performed according to the manufacturer’s instructions. As shown in Fig. 4, the concentration of HMG-CoA lyase was 520 ng per 100 mg ATCC 16024 cell weight. However, the bioactivity of HMG-CoA lyase was almost undetectable in AHL-4 transformants. These results show that the mutated HMG-CoA lyase gene was not expressed in the AHL-4 transformants.

**MPA Production Fermentation Assay**

The time course of MPA fermentation is illustrated in Fig. 5. The dry cell weight increased to 40.25 g/l compared with 35.97 g/l in ATCC 16024 cells. A reduction in dry cell weight caused by autolysis was observed between 96 and
During the growth phase, MPA production was low because cell growth was relatively rapid, but production increased sharply during the production phase, particularly toward the end of fermentation. The dry cell weight decreased continuously from 120 to 168 h, but the biosynthesis of MPA increased rapidly. The rate of cell autolysis and pH increase reduced gradually at 180 h, and maximum MPA production (2.94 g/l) was achieved, showing a 71% increase compared with the wild type (1.72 g/l).

Biological fermentation has mainly been performed using liquid-state fermentation. Xu and Yang [39] reported 16.3 mg/l/h MPA production in a rotating fibrous-bed bioreactor, and 5.51 g/l MPA was produced after 338 h. MPA continued to be produced by immobilized mycelia when fresh medium was introduced into the bioreactor [39]. However, industrial fermentation is usually performed in stirred-tank fermenters. We have previously described a combined feeding strategy for enhancing MPA production.
which is suitable for conventional industrial equipment of scale-up production [11].

On the bases of previous research studies, we attempted to explore a directional idea for modifying metabolic pathways to enhance MPA production. The biosynthesis pathway of MPA is shown in Fig. 6 [25]. The farnesyl pyrophosphate biosynthetic pathway has several branches. HMG-CoA is catalyzed by HMG-CoA reductase, and HMG-CoA lyase generates mevalonic acid and acetoacetic acid through different pathways. The MPA biosynthetic flux increases by reducing generation of by-product. Inactivating a gene is a feasible way to create fungal mutants [13]. In this study, we cloned the HMG-CoA lyase gene from P. brevicompactum and showed that it can be used for directional transformation, which will be a good help for further study in the field of MPA fermentation.

In conclusion, our study describes a directional transformation method to express the HMG-CoA lyase gene in fusion with the P. brevicompactum hph gene. After subculture, the transformants were screened with
hygromycin, PCR, Southern blot analysis, and enzyme content assay to confirm that the HMG-CoA lyase gene was successfully inactivated in the AHL-4 transformant. MPA production in the transformant reached 2.94 g/l, which was a 71% increase compared with the wild type. ATMT may be applied alternatively as a practical genetic

**Fig. 6.** Biosynthesis pathway of MPA. The inhibitory activity of HMG-CoA lyase is required to increase the MPA biosynthetic flux by reducing the generation of by-product.
tool for directional transformation of \emph{P. brevicompactum} in the future.

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\section*{References}


