Amplification of an MFS Transporter Encoding Gene penT Significantly Stimulates Penicillin Production and Enhances the Sensitivity of Penicillium chrysogenum to Phenylacetic Acid

Jing Yang, Xinxin Xu, Gang Liu

ABSTRACT

Penicillin is historically important as the first discovered drug against bacterial infections in human. Although the penicillin biosynthetic pathway and regulatory mechanism have been well studied in Penicillium chrysogenum, the compartmentation and molecular transport of penicillin or its precursors are still poorly understood. In search of the genomic database, more than 830 open reading frames (ORFs) were found to encode transmembrane proteins of P. chrysogenum. In order to investigate their roles on penicillin production, one of them (penT) was selected and cloned. The deduced protein of penT belongs to the major facilitator superfamily (MFS) and contains 12 transmembrane spanning domains (TMS). During fermentation, the transcription of penT was greatly induced by penicillin precursors phenylacetic acid (PAA) and phenoxyacetic acid (POA). Knock-down of penT resulted in significant decrease of penicillin production, while over-expression of penT under the promoter of trpC enhanced the penicillin production. Introduction of an additional penT in the wild-type strain of P. chrysogenum doubled the penicillin production and enhanced the sensitivity of P. chrysogenum to the penicillin precursors PAA or POA. These results indicate that penT stimulates penicillin production probably through enhancing the translocation of penicillin precursors across fungal cellular membrane.

KEYWORDS: Penicillium chrysogenum; penT; Penicillin production; Phenylacetic acid; MFS transporter

1. INTRODUCTION

The β-lactam antibiotic penicillins are the first drugs for the treatment of infectious diseases and still widely used today. Penicillins are produced by some filamentous fungi, such as Aspergillus nidulans and Penicillium chrysogenum. The industrial penicillin-producing strain P. chrysogenum has been improved impressively by classical breeding and genetic engineering during the past 60 years (Schmidt, 2010). Now the penicillin titers and productivities have been boosted more than 1000 times (Elander, 2003). The penicillin biosynthetic pathway and regulatory mechanism have been studied in past decades in both A. nidulans and P. chrysogenum due to successful application of genetic techniques in these fungi (Brakhage, 1998; Brakhage et al., 2004). The great progress on penicillin biosynthesis and regulation is well reviewed elsewhere (Aharonowitz et al., 1992; Martín et al., 1999; Brakhage et al., 2009).

The genes involved in the biosynthesis of penicillin are arranged in a cluster located in the P. chrysogenum chromosome, which contains three structural genes (pcbAB, pcbC and penDE) (Díez et al., 1990). In the first two steps of the penicillin biosynthesis pathway, three amino acid precursors were condensed to tripeptide and oxidized closure to generate β-lactam ring. The reactions are catalyzed by δ-(L-α-amino-adipyl)-L-cysteine-D-valine synthetase and penicillin N synthetase, which are encoded by pcbAB and pcbC separately.
Phenylacetic acid (PAA) is the side precursor of penicillin G. As a weak organic acid, PAA affects the growth of cells as well as influences transcription of genes on genome-wide levels (Harris et al., 2009). Not only penicillin G biosynthesis is dependent on PAA, but also the transcriptions of those transporter encoding genes are up-regulated in both wild-type and high-producing strains grown in the presence of PAA. Thus, these genes might be directly related to penicillin production (van den Berg et al., 2008; Harris et al., 2009). In this study, one of these transcriptional up-regulated genes was selected and designated as penT. The physiological role of penT which encodes an MFS transporter was studied, especially in the penicillin production of \textit{P. chrysogenum}.

## 2. Materials and Methods

### 2.1. Microorganisms and Culture Media

\textit{P. chrysogenum} CGMCC 3.5129 was used as the wild-type strain in this study. \textit{P. chrysogenum} and its derivatives were grown on the 60 mm Petri dishes containing LYP medium (15 g/L lactose, 5 g/L yeast extract, 5 g/L peptone, 10 g/L NaCl, 0.05 g/L MgSO\textsubscript{4}-7H\textsubscript{2}O, 0.06 g/L KH\textsubscript{2}PO\textsubscript{4}, 0.05 g/L FeCl\textsubscript{3}, 0.02 g/L CuSO\textsubscript{4}, 5H\textsubscript{2}O, agar 25 g/L) at 28\textdegree C for 5 days. Then, the fresh spores (about 1 × 10\textsuperscript{8} spores) were collected from one plate and inoculated into 40 mL of seed medium (20 g/L corn steep liquor, 20 g/L sucrose, 5 g/L yeast extract, 5 g/L CaCO\textsubscript{3}, pH 5.8) in 250 mL flask. After incubated at 26\textdegree C for 24 h in an orbital incubator at 200 r/min, aliquot seed culture (4 mL) was used to inoculate 40 mL of fermentation medium (30 g/L corn steep liquor, 35 g/L lactose, 5 g/L (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 1 g/L KH\textsubscript{2}PO\textsubscript{4}, 10 g/L CaCO\textsubscript{3}, 6 mL/L corn soil, pH 6.0) in a 250 mL shake-flask. After incubated for 24 h, the fermentation cultures were supplemented with PAA or POA to the final concentration of 0.2% and continued to incubating at 26\textdegree C for 6 days (Wang et al., 2007). One microliter of sample was taken every 24 h and the fermentation supernatant was used for measuring penicillin production by bioassay using \textit{Micrococcus luteus} CGMCC 1.1848 as the indicator strain. \textit{Escherichia coli} JM109, which was used for cloning experiments, was grown in LB medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) supplemented with the appropriate antibiotics.

### 2.2. Primers and PCR

The sequences and specificities of all oligonucleotides used in this study are listed in Table 1. The PCRs were performed using Taq DNA polymerase (TaKaRa, Japan) or KOD FX (Toyobo, Japan). An initial denaturation at 95\textdegree C for 5 min was followed by 30 cycles of amplification (94\textdegree C for 50 s, 55\textdegree C for 30 s, 72\textdegree C for 1 min) and an additional 10 min at 72\textdegree C (68\textdegree C for KOD FX). The annealing temperature and time, as well as the elongation time were changed to accommodate different templates and primers.

### 2.3. Plasmid Construction for Gene Knock-down and Over-expression

The plasmids pEasyBlunt and pEasyT3 (Transgen, China) were used for sub-cloning experiments. Plasmid pUAC43
which contains the bleomycin resistant gene (ble) under the promoter of pcgB was used for over-expression of penT. The plasmid pJL43-RNAi was used for the gene knock-down experiments.

The promoter of trpC was amplified from the plasmid pBHI2 (Mullins et al., 2001) by using primers TrpC-F and TrpC-R and subsequently cloned into pEasyT3. A 360 bp Sac II-Xba I DNA fragment containing the trpC promoter was subsequently ligated into the corresponding site of pUAC43 to give the plasmid pAC::PtrpC. The pAC::PtrpC was digested with BamHI and subsequently blunted by Klenow in the presence of dNTPs. Finally, a 1.9 kb DNA fragment contained the intact penT was amplified from P. chrysogenum using primers penT-F and penT-R and was cloned into pAC::PtrpC to generate the plasmid pAC::PtrpC-penT (Fig. 1A).

To introduce an additional penT into the genome of P. chrysogenum, plasmid pAC::penT-UF2 was constructed as follow: penT with its flanking sequences containing its own promoter region was amplified using primers penT-UF2 and penT-UR, and the genomic DNA of P. chrysogenum was used as a template. The amplified DNA fragment was sub-cloned into pEasyBlunt and sequenced. Subsequently, a 3254 bp Hind III-Sac II DNA fragment containing the intact penT and its own promoter region was inserted into the corresponding sites of pUAC43 (Fig. 1A).

To knock-down penT, a plasmid was constructed based on pJL43-RNAi (Ullán et al., 2008). The primers penT-F and penT-R were used to amplify the intragenic DNA fragment of penT from P. chrysogenum. The amplified DNA fragment was digested with Nco I to release a 470 bp DNA fragment, which was subsequently inserted into the same site of pJL43-RNAi to generate the penT knock-down plasmid pT-RNAi in which the 470 bp DNA fragment was inserted into the promoters of glyceraldehyde-3-phosphate dehydrogenase gene (gpd) and pcgB in opposite orientation.

### Table 1

<table>
<thead>
<tr>
<th>Primers used in this study</th>
<th>Sequence (5′→3′)</th>
</tr>
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<tbody>
<tr>
<td>penT-01</td>
<td>TCCACGGTCAGACACCAGC</td>
</tr>
<tr>
<td>penT-02</td>
<td>CGCGGACTGGTAGTGGAGG</td>
</tr>
<tr>
<td>penT-F</td>
<td>CTGCAAG(Pst I)TAGGGAGCTTACACG</td>
</tr>
<tr>
<td>penT-R</td>
<td>AGGCTT(Hind III)TATGGCTTGTAGC</td>
</tr>
<tr>
<td>TrpC-F</td>
<td>CCGGGG(Sac II)GATATGGAAAGAGGCAATTTTGG</td>
</tr>
<tr>
<td>TrpC-R</td>
<td>TCTAGA(Taq I)GCTCTGGTAAATAGTGAAGTC</td>
</tr>
<tr>
<td>penT-UF2</td>
<td>AGGCTT(Hind III)CGAAAGATGCTGTGGTGGCAGGAG</td>
</tr>
<tr>
<td>penT-UR</td>
<td>CGCGGG(Sac II)GGCTGGTTAAGGCTCCATACAG</td>
</tr>
<tr>
<td>qpcbABSC</td>
<td>ACGTTCATGGCTCTGATAC</td>
</tr>
<tr>
<td>qpcbABA</td>
<td>CGTGCATTTGCGACCTGTTG</td>
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<tr>
<td>qpcbCS</td>
<td>ACGGCACCAAATTTGAGTTT</td>
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<tr>
<td>qpcbCA</td>
<td>GGTTGAGTTGACCATGTAGC</td>
</tr>
<tr>
<td>actF</td>
<td>CCCAATCTAGGAGGTTTCT</td>
</tr>
<tr>
<td>actR</td>
<td>ATGTCAACGGAGGTTTCA</td>
</tr>
</tbody>
</table>

Restriction sites used for cloning are in italics.

#### 2.4. PEG-mediated protoplast transformation of P. chrysogenum

PEG-mediated protoplast transformation of P. chrysogenum was performed with some modifications as described previously (Cantoral et al., 1987). To obtain the protoplasts, lysing enzyme from Trichoderma harzianum (L1412, Sigma, USA) (10 mg/mL in lysis buffer) was used instead of Novozym 234. The lysis mixture was incubated at 30°C and 80 r/min for 2 h. Transformants were selected in LYP medium supplemented with sucrose (10.3%) and bleomycin (10 μg/mL).

#### 2.5. RNA isolation and real-time quantitative PCR

In order to isolate total RNA, P. chrysogenum strains were grown in fermentation medium. Fungal mycelia were harvested and washed with sterile water and ground to powder in liquid nitrogen. Total RNA was isolated using Trizol reagent (Invitrogen, USA) as described previously (Liu et al., 2005). cDNA was prepared from 5 μg of total RNA with Transcript First-Strand cDNA Synthesis SuperMix according to the instructions (Transgen). The first strand cDNA product was diluted with RNase-free water before used as template for real-time quantitative PCR (qPCR). Quantitative PCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo) using Mastercycler EP realplex (Eppendorf, Germany). The qPCR thermal cycling conditions were: 95°C for 30 s, followed by 40 cycles of 94°C for 15 s, 59°C for 20 s, 72°C for 30 s; then, an additional melting curve cycle (95°C for 15 s, 59°C for 1 min and 95°C for 15 s) was progressed for testing specificity of amplifications. Data analysis was done with the 2−ΔΔCT method (Livak and Schmittgen, 2001), and the actin mRNA was used as an internal control to normalize the data of each sample. Primers penT-01 and penT-02 were used in this experiment to detect the transcriptional level of penT, while actin-F and actin-R were used for amplifying the actin gene. The study was repeated in three independent experiments.

#### 2.6. Absolute quantification of gene copy number

Absolute quantification was performed to quantify the penT gene copies in genome DNA of the wild-type strain and its transformants. Plasmid pAC::PtrpC-penT of known quantity was serially diluted and amplified to generate a standard curve. Genomic DNA of the wild-type strain and its transformants was quantified based on the standard curve. The PCR composition and conditions were the same as used for qPCR.

#### 2.7. Penicillin bioassay

Penicillin bioassay using M. luteus as indicator was carried out as described previously (Ullán et al., 2008). Dry weights of the cultures were determined based on previous protocols (Harris et al., 2009). Pure penicillin G (JingKeHongDa Biotech Inc, Beijing) was used as control to determine the antibiotic concentration. The cultures were incubated in
Fig. 1. Schematic of penT gene and alignment of the deduced PenT with fungal transporters.

Organization of penT in the chromosome and plasmid construction (A) and alignment of the deduced amino acid sequence of penT with CefT from Acremonium chrysogenum, TPO2 from Saccharomyces cerevisiae, TPO3 from Cryptococcus gattii, polyamine transporter from Aspergillus niger (B). A 3254 bp Hind III-Sac II DNA fragment containing the intact penT was inserted into pUAC43 to generate pAC::penT-UF2. A 1.9 kb DNA fragment contained the intact penT under the promoter of trpC was cloned into pAC::PtrpC to generate pAC::PtrpC-penT. The 12 transmembrane spanning domains (TMS) are indicated with solid bars. The identical amino acids are shaded. The proteins were aligned using ClustalX 1.83 program and displayed using GeneDoc.
parallels in triplicate flasks and the same fermentation experiments were repeated three times.

2.8. Sensitivity analysis of \textit{P. chrysogenum} to phenylacetic acid

A spot test was used for measuring the sensitivity of different strains to PAA or POA. The spores of different strains were collected from 5-day-old cultured plates. The concentration of spore suspensions was measured and dropped onto LYP plates containing PAA or POA with indicated serial dilutions. The plates were incubated for 3 days at 28°C. PAA or POA was added into LYP medium at a final concentration of 0.4%, 0.8% and 2%, respectively.

3. RESULTS

3.1. Cloning and characterization of the penT gene

In search of the genomic databases of \textit{P. chrysogenum} Wisconsin 54-1255, more than 830 predicted genes were found to encode transmembrane proteins which mainly belong to the ABC transporter family and MFS transporter family. Two ABC transporters (XM_002565335 and XM_002565893) homologous to the \textit{AtrD} in \textit{A. nidulans} possess a putative nucleotide-binding domain and a pair of transmembrane domains. Our experiments showed that the RNAi transformants of both XM_002565335 and XM_002565893 displayed lower penicillin production, but the reduction was not remarkable (data not shown). Thus, four of MFS transporters (Pc21g01300, Pc15g00120, Pc16g11960, Pc16g12280) were selected. Pc21g01300 exhibits the highest identity and similarity with \textit{P. chrysogenum} Pc21g01300, Pc15g00120, Pc16g11960, Pc16g12280) were selected. Based on its nucleotide sequence, four of MFS transporters (Pc21g01300, Pc15g00120, Pc16g11960, Pc16g12280) were selected. Pc21g01300 exhibits the highest identity and similarity with \textit{CefT} from \textit{A. chrysogenum} (Ullán et al., 2002) and was selected for further study. Based on its nucleotide sequence, primers penT-F and penT-R were designed. A 1813 bp DNA fragment was amplified using the genomic DNA of \textit{P. chrysogenum} CGMCC 3.5129 as template and it was cloned into pEasyBlunt (Transgen). Sequencing analysis of the DNA fragment revealed it contained one ORF which was designated as \textit{penT} (accession number HQ694735). The sequence of \textit{penT} was found to be 99.9% identical with Pc21g01300. Using total RNA as template, the cDNA of \textit{penT} was amplified by RT-PCR with primers penT-F and penT-R. It contains three introns (sites 455–507, 1112–1165, 1498–1556) in the ORF as verified by sequencing the cDNA (Fig. 1A).

As shown in Fig. 1B, the deduced amino acid sequence of \textit{penT} showed strong similarity throughout its entire length with MFS multidrug transporters \textit{CefT} from \textit{A. chrysogenum} (61% identity), TPO2 from \textit{Saccharomyces cerevisiae} (40% identity), TPO3 from \textit{Cryptococcus gattii} (47% identity), polymyamine transporter from \textit{A. niger} (47% identity). By using the TMHMM-2.0 programme (http://www.cbs.dtu.dk/services/TMHMM-2.0/), PenT was predicted to contain 12 transmembrane spanning domains (TMS) with a cytoplasmic loop joining two spans. Based on the structural similarity with the MFS multidrug resistance (MDR) protein in \textit{S. cerevisiae}, PenT belongs to the drug:H\textsuperscript{+} antiporter DHA 1 family (Sá-Correia et al., 2008). In \textit{P. chrysogenum}, peroxisomes are essential for penicillin production as several enzymes involved in the biosynthesis of penicillin are located at these organelles (Martín et al., 2012). The sorting of matrix proteins to peroxisome depends on the consensus peroxisomal targeting sequence (PTS). Kiel et al. (2009) reported their work about characterization of the peroxisomal matrix protein implied that PenT was not included. Consistent with that, PenT does not contain PTS, suggesting that it may not be located at the peroxisomal membrane. Probably there are other membrane proteins for the transport of penicillin precursors from the cytosol to the peroxisome and PenT is responsible for transporting the precursors across the fungal cellular membrane.

3.2. Transcription of \textit{penT} was up-regulated by PAA and POA

As the transcription of \textit{Pc21g01300} was significantly induced by PAA stress (van den Berg et al., 2008), we speculated that \textit{penT} expression could be altered with penicillin precursor PAA or POA treatment. Considering the side-chain precursors PAA and POA are weak acids and they are likely to inhibit the growth of \textit{P. chrysogenum} in fermentation medium, the precursors were added to the fermentation broth only after the strain grew for 24 h when it reached the stationary phase of growth. The transcriptional levels of \textit{penT} under PAA or POA stress were analyzed by using quantitative PCR (Fig. 2). After incubation in 0.2% PAA, the transcription of \textit{penT} was obviously induced and reached a peak level at 48 h fermentation culture in the wild-type strain and its derivatives (Fig. 2A, the left panels). Without precursors, the transcriptional levels of \textit{penT} were almost the same from 48 h to 72 h fermentation in the wild-type strain and its derivatives (Fig. 2, the white columns, and Fig. S2). The relative transcriptional level of \textit{penT} dramatically declined after 72 h fermentation, even with the extension treatment of PAA or POA (Fig. 2A and B, the right panels).

3.3. Knock-down of \textit{penT} decreased penicillin production

To investigate the function of \textit{penT} in vivo, the plasmid pT-RNAi was constructed and introduced into \textit{P. chrysogenum} by PEG-mediated protoplast transformation. The bleomycin resistant transformants were selected and confirmed by PCR. All the transformants had similar phenotype as the wild-type strain in morphological differentiation and growth in LYP medium (data not shown). Nine of them were selected randomly for further fermentation. After incubation for 96 h in the fermentation medium, all the \textit{penT} knock-down mutants showed the reduction of penicillin production in various degrees (Fig. 3). The control strain could produce the same level of penicillin as the wild-type, indicating that the control transformant with an empty RNAi plasmid had little impact on the penicillin production (Fig. 3 and Fig. S3). These results demonstrated that \textit{penT} was related to penicillin production in
3.4. Over-expression of \textit{penT} increased penicillin production

As the transcription of \textit{penT} was induced by PAA and penicillin production was influenced by knock-down of \textit{penT}, we examined the effect of over-expression \textit{penT} on penicillin production. To over-express \textit{penT}, the plasmid pAC::PtrpC::\textit{penT} was constructed and transformed into \textit{P. chrysogenum}. The bleomycin resistant transformants were selected and confirmed by PCR. One \textit{penT} over-expressed transformant (T-29) was selected randomly and the copy number of \textit{penT} in this strain was measured by quantitative PCR. The results showed that T-29 contained an extra \textit{penT} which is under control of the promoter of \textit{trpC} (Fig. 4A). Fermentation results demonstrated that over-expression of \textit{penT} caused more than
The induction of PAA on the wild-type penT was considerable, but transcription of penT under the promoter of trpC could not be induced by PAA or POA. This might be the reason that overexpression of penT under the promoter of trpC did not result in a significant increase of penicillin production.

As the transcription of penT was induced by PAA or POA, the effect of increasing the copy number of penT was measured. The plasmid pAC::penT-UF2 was constructed and transformed into P. chrysogenum. The bleomycin resistant transformants with an additional copy of penT in the chromosome were confirmed by PCR (data not shown). The transformant T2-6 was chosen randomly for fermentation in the subsequent experiments. When strains were grown in fermentation medium, strain T2-6 containing an extra copy of penT showed a slightly reduction of biomass than the wild-type strain. The production of penicillin in liquid cultures supplemented with PAA was examined for a continuous 6-day period from 24 h to 144 h. Introduction of an additional copy of penT in chromosome significantly enhanced the production of penicillin, in contrast with the penT knock-down mutant T-I-2 in which the penicillin production obviously reduced to 50% of that in the wild-type strain (Fig. 4B). The penicillin production of T2-6 was doubled comparing with the wild-type strain (Fig. 4B, the hollow triangle). These results indicated that amplification of penT could dramatically stimulate penicillin production in P. chrysogenum.

3.5. Transcription of penT was positively related with penicillin production

To confirm that expression of penT does affect the penicillin production, we examined the transcription of penT by quantitative RT-PCR in the penT mutant (T-I-2), penT over-expressed strain (T-29), transformant T-2-6 and the wild-type strain. RNA samples were prepared from mycelia incubation for 24 h, 48 h, 72 h, 96 h and 144 h and PAA was supplemented in fermentation culture after 24 h incubation. The transcription of penT was induced dramatically by the precursor PAA in all the strains, especially in T2-6 (4640-fold increase at 48 h with induction of PAA). In an attempt to identify the induction patterns, we detected the mRNA levels of penT in 36 h RNA sample. Examination of the transcription levels of penT by qPCR revealed that the transcription of penT were remarkably induced by PAA addition (Fig. S1). The relative mRNA of penT in all strains declined with the
extension of fermentation, although the decrease was slower in T-29 than other strains (Fig. 5). Notwithstanding the transcriptions of penT in the wild-type strain, T-29 and T-I-2 were similar at 48 h incubation with induction of PAA, the transcriptional level of penT in T-I-2 was declined sharply after 48 h incubation and the penT transcription was always lower than that in the wild-type strain from 72 h to 144 h, suggesting that the knock-down of penT gene was effective. It is probably that the inhibition via knock-down of penT is not strong against the induction of PAA as the DNA fragment used for knock-down of penT is under the promoters of gpd and pcbC which are not induced by PAA. The decline of penT transcription in T2-6 was obviously faster, but its remained level in T2-6 was still much higher than that in the wild-type strain and T-29. At 96 h incubation, the transcription level of penT in T-I-2 was almost 2-fold of that in T-29 and more than 10-fold of that in the wild-type strain. The transcriptional levels of penT were relative low and altered little in all the strains without PAA addition, suggesting that the induction pattern was dependent on PAA (Fig. S2). Based on the fermentation results that over-expression of penT increased penicillin production, the penT transcriptional analysis revealed that the expression of penT played a key role during penicillin production.

3.6. Over-expression of penT enhanced the sensitivity of P. chrysogenum to PAA

Because PAA and POA could inhibit the growth of P. chrysogenum and the transcription of penT was induced by PAA stress, we tested whether penT has any role in PAA transportation and tolerance. Five-day-old fresh spores of P. chrysogenum were transferred onto plates containing varying concentration of PAA and grew for 3 days. The penT knock-down mutant (T-I-2) exhibited slightly better growth than the wild-type strain in the presence of PAA (Fig. 6, the bottom line). In contrast, the penT over-expressed strain T2-6 showed more sensitive to PAA (Fig. 6, the middle line). Without PAA, all the strains grew normally and showed similar phenotype. When POA was added instead of PAA, all strains showed similar patterns as in presence of PAA (data not shown). All these results indicate that penT could stimulate the sensitivity of P. chrysogenum to the penicillin precursors PAA or POA, probably through enhancing their translocation across the fungal cellular membrane.

4. DISCUSSION

In this study, we cloned and characterized an MFS transporter encoding gene penT from P. chrysogenum. Our results indicated that penT was involved in penicillin production, probably through translocation of penicillin precursors across the cellular membrane of this fungus. penT did not affect penicillin biosynthesis as the transcription of pcbAB and pcbC was not obviously difference among the wild-type strain, penT knock-down mutant and penT over-expressed strain (data not shown). This is the first report that an MFS transporter is associated with penicillin production in P. chrysogenum. It should be mentioned that no specific transporter for the translocation of penicillin or its precursors in P. chrysogenum has been reported up to date and penicillin secretion might be resulted from the cooperative activity of multiple transporters.

So many MFS transporters in the filamentous fungi bring out question of their natural physiological roles. The MFS-MDR transporters are traditionally described as drug pumps or tolerance to chemical stress in S. cerevisiae and expression of some MFS-MDR transporter encoding genes is controlled by transcriptional regulatory network. For instance, in yeast the transcription of pleiotropic drug resistance protein TPO1 is activated by several zinc-cluster transcriptional factors, including Pdr1p and Pdr3p (Alenquer et al., 2006). This mode has been studied in A. chrysogenum that CefR acts as a repressor of cefT gene which encodes a transporter of intermediates of the cephalosporin C (Teijeira et al., 2011). It is possible that these transporters have their specific physiological substrates, whereas drugs would be transported opportunistically (Sá-Correia et al., 2008). Unlike cephalosporin biosynthesis, no probable transporter gene is situated within the penicillin biosynthetic gene cluster. Thus, it is difficult to know how the penicillin and its precursors are transported across the fungal cellular membrane.

Sequence analysis revealed 416 putative MFS transporter genes were distributed in P. chrysogenum genome, representing 3.2% of the genome (van den Berg et al., 2008). However, few of them are studied in function. It is well known that the penicillin biosynthetic pathway occurs in different cellular compartments and penicillin can be effectively secreted into the culture broths (García-Estrada et al., 2007). That the transcription of penT was enhanced dramatically after addition of side-chain precursors in the culture broth indicated that PenT might be a sensor for signals triggering penicillin.

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**Fig. 6. Sensitivity of the wild-type strain (WT), penT amplified strain T2-6 and penT mutant T-I-2 to PAA.**

The numbers of spores used in the spot test are indicated on the top.
biosynthesis. It was showed that PAA and POA caused varying degrees of transcriptional induction of penT (Fig. 2), while both compounds lead to similar other effects in all the transformants. This could be resulted from precursor differences in molecule. Both PAA ($pK_a = 4.3$) and POA are weak acids and are likely to uncouple the cell membrane (Harris et al., 2009), while POA was considered to be more toxic for its lower $pK_a (= 3.1$). As the precursor of penicillin G, PAA may have pleiotropic effects on P. chrysogenum, and there may be another gene in P. chrysogenum genome which can be more sensitive to POA stress. PenT shows the characteristic of 12 membrane spanners (TM) as the MFS-MDR transporters which belong to the DHA1 family. Members of the DHA1 drug-efflux family recognize wide range of compounds in yeast. Like CefT which is localized at the plasma membrane of A. chrysogenum (Nijland et al., 2008), PenT is speculated to be localized at the plasma membrane of P. chrysogenum.

In A. chrysogenum, strains without cefT gene could not grow in the medium supplemented with PAA or POA (Ullán et al., 2002). PAA and POA are not the precursors of cephalosporin, so it does not need to add PAA or POA in the fermentation medium for cephalosporin biosynthesis. Unlike cefT, the penT over-expressed strain was more sensitive to PAA than the wild-type strain. In contrast, the penT knock-down mutant (T-I-2) showed higher tolerance to PAA or POA as it grew better than the wild-type strain in presence of PAA or POA. The difference of penT expression with induction of PAA between T-29 and T2-6 implied that the sequence in the upstream of penT might play significant role in response to the precursor (PAA or POA) and the inductive expression of penT in P. chrysogenum. The difference between CefT and PenT implied penT might not take part in detoxification of these chemicals. In fact, detoxification of PAA and POA are mainly performed by phenylacetyl-CoA ligase (PhlA) which is localized in peroxisome of P. chrysogenum (Martín et al., 2012). It is possible that PenT is mainly responsible for the translocation of penicillin precursors across the cellular membrane of P. chrysogenum.

It was said that the side-chain precursors of penicillin crossed fugal plasma membrane by passive diffusion in P. chrysogenum (Hillenga et al., 1995; Eriksen et al., 1998). This mechanism was supported by a recent report that the transport of PAA in a high penicillin-producing strain of P. chrysogenum occurred by passive diffusion of undissociated acid (Douma et al., 2012). However, our results indicated that a protein (PenT)-mediated transport system for dissociated PAA uptake and secretion existed. Considering that many genes encoding transmembrane proteins including penT were up-regulated in response to PAA (Harris et al., 2009), PAA maybe transported via both passive diffusion and protein-mediated transport system in P. chrysogenum.

**ACKNOWLEDGEMENTS**

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**SUPPLEMENTARY DATA**

Fig. S1. Expression of penT was induced by PAA during fermentation.

Fig. S2. Transcriptional level of penT in different strains without PAA addition.

Fig. S3. Penicillin G production in the control transformants (containing an empty plasmid) and wild-type strain. Supplementary data related to this article can be found in the online version at http://dx.doi.org/10.1016/j.jgg.2012.08.004.

**REFERENCES**


Alenquer, M., Tenereiro, S., Sá-Correia, I., 2006. Adaptive response to the antimarial drug artesunate in yeast involves Pdr1p/Pdr3p-mediated transcriptional activation of the resistance determinants TPO1 and PDR5. FEMS Yeast Res. 6, 1130—1139.


Neyfakh, A.A., 2002. Mystery of multidrug transporters: the answer can be
Hillenga, D.J., Versantvoort, H.J.M., Vandermolen, S., Driessen, A.J.M.,
Kiel, J.A., van den Berg, M.A., Fusetti, F., Poolman, B., Bovenberg, R.A.,
Kiel, J.A., van den Berg, M.A., Fusetti, F., Poolman, B., Bovenberg, R.A.,
exploring genome-wide gene expression responses of
Penicillium chrysogenum to phenylacetic acid consumption and penicillin G
production. BMC Genomics 10, 75.

Hillenga, D.J., Versantvoort, H.J.M., Vandermolen, S., Driessen, A.J.M.,
Konings, W.N., 1995. Penicillium chrysogenum takes up the penicillin-G
61, 2589–2595.

Kiel, J.A., van den Berg, M.A., Fusetti, F., Poolman, B., Bovenberg, R.A.,
Veenhuis, M., van der Klei, I.J., 2009. Matching the proteome to the
genome: the microbody of penicillin-producing Penicillium chrysogenum

regulatory gene for nikkomycin biosynthesis in Streptomyces ansochromogenes
that also influences colony development. Mol. Microbiol. 55, 1855–1866.

using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25,
402–408.

Penicillin and cephalosporin biosynthesis: mechanism of carbon catabolite

metabolites: how producer cells send out messages of intercellular

the biosynthesis and secretion of β-lactams and other secondary metabolites.

Agrobacterium-mediated transformation of Fusarium oxysporum: an efficient

Neyfakh, A.A., 2002. Mystery of multidrug transporters: the answer can be

Nijland, J.G., Kovalchuk, A., van den Berg, M.A., Bovenberg, R.A.,
Driessen, A.J., 2008. Expression of the transporter encoded by the cefT
gene of Acremonium chrysogenum increases cephalosporin production in

Roze, L.V., Chanda, A., Linz, J.E., 2011. Compartmentalization and molecular
traffic in secondary metabolism: a new understanding of established

Sá-Correia, I., dos Santos, S.C., Teixeira, M.C., Cabrito, T.R., Mira, N.P.,

prospects in manufacture and therapy. In: Hofrichter, M. (Ed.), The

Teijeira, F., Ullán, R.V., Guerra, S.M., García-Estrada, C., Vaca, I., Matín, J.F.,
2009. The transporter CefM involved in translocation of biosynthetic
intermediates is essential for cephalosporin production. Biochem. J. 418,
113–124.

modulates transporters of β-lactam intermediates preventing the loss of
penicillins to the broth and increases cephalosporin production in Acre-
monium chrysogenum. Metab. Eng. 13, 532–543.

Ullán, R.V., Liu, G., Casqueiro, J., Gutiérrez, S., Bañuelos, O., Matín, J.F.,
2002. The cefT gene of Acremonium chrysogenum C10 encodes a putative
multidrug efflux pump protein that significantly increases cephalosporin C

Ullán, R.V., Godio, R.P., Teijeira, F., Vaca, I., García-Estrada, C., Feltser, R.,
Kosalkova, K., Matín, J.F., 2008. RNA-silencing in Penicillium chryso-
ogenum and Acremonium chrysogenum: validation studies using beta-

ization and transport in β-lactam antibiotic biosynthesis by filamentous
fungi. Antonie Van Leeuwenhoek 75, 41–78.

van den Berg, M.A., Albang, R., Albermann, K., Badger, J.H., Daran, J.M.,
Driessen, A.J., García-Estrada, C., Fedorova, N.D., Harris, D.M.,
Heijne, W.H., Joardar, V., Kiel, J.A., Kovalchuk, A., Martín, J.F.,
Nierman, W.C., Nijland, J.G., Pronk, J.T., Roubos, J.A., van der Klei, I.J.,
van Peij, N.N., Veenhuis, M., von Dohren, H., Wagner, C., Wortm, J.,
Bovenberg, R.A., 2008. Genome sequencing and analysis of the filament-

cloning and functional identification of a novel phenylacetyl-CoA ligase
360, 453–458.