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ORIGINAL RESEARCH PAPER

Establishing an efficient gene-targeting system in an itaconic-acid producing *Aspergillus terreus* strain

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Abstract
Objectives To develop an efficient gene-targeting platform in an excellent itaconic acid producing strain *Aspergillus terreus* CICC40205.
Results The frequency of homologous recombination was improved by deleting the *ku80* gene. A nutritional transformation system based on the bidirectionally selectable marker, $pyrG_{An}$, was established in the *ku80-/pyrG*-double mutant which is convenient for following marker rescue. The modified Cre/*loxP* recombination system was applied for the excision of the $pyrG_{An}$ marker by directly introducing Cre recombinase into the protoplasts.
Conclusions This gene-targeting system is an efficient platform for sequential and multiple genetic modifications in *A. terreus* and is conducive to study biosynthesis mechanisms and to improve the production ability of itaconic acid and other products.

Keywords *Aspergillus terreus* · Cre/*loxP* system · Gene-targeting · Itaconic acid · Non-homologous end joining · *pyrG*

Introduction

Aspergillus terreus is a ubiquitously distributed saprobiotic mold fungus but can cause life-threatening invasive aspergillosis in immunocompromised patients (Slesiona et al. 2012). Importantly, *A. terreus* is a significant species with biotechnological and medical values and has been commercially developed as an excellent producer for lovastatin (e.g. Merck's Mevacor) (Barrios-Gonzalez and Miranda 2010) and itaconic acid (Klement and Buchs 2013). Lovastatin is

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an efficient cholesterol-lowering agent, with worldwide sales topping $10 billion annually (Barrios-Gonzalez and Miranda 2010). Itaconic acid is widely used as a monomer or co-monomer in manufacturing plastics, resins etc. (Klement and Buchs 2013).

With the rapid increase of the fungal omics data, genetic and metabolic engineering approaches have become powerful for functional characterization of the genes and strain improvement. Gene targeting is one of the most important methods for genetic engineering. However, as with other *Aspergillus* spp, two limiting factors hamper the studies towards genetic engineering of *A. terreus*: (1) low frequency of homologous recombination (HR); (2) limited suitable resistance markers. Considering that a large number of genes will be functionally analyzed and manipulated, it is important to develop an efficient platform facilitating this process.

In filamentous fungi, integration of a DNA fragment into the genome is mainly mediated by non-homologous end joining (NHEJ) pathways, resulting in low frequency in gene-targeting. Hence, a multitude of candidate transformants have usually to be analyzed in order to identify the correct one, which makes fungal transformation laborious, tedious and time-consuming. The expression cassette of target gene is randomly integrated into the genome which is not ideal for the comparison of phenotypes between different mutants (Huang et al. 2014a). However, exogenous NDA can be mainly integrated into the genome through HR in the NHEJ-pathway defective mutant (Ishibashi et al. 2006). The frequency of gene-targeting can be significantly improved by deleting the genes *ku70*, *ku80* or *lig4*, the key components of the NHEJ-pathway (Ishibashi et al. 2006; Mizutani et al. 2008; Honda et al. 2011). The NHEJ-pathway defective mutants have been used as the parental strains to study the biosynthesis of secondary metabolites in *A. terreus* (Gressler et al. 2011; Guo et al. 2013).

Selectable marker genes are also essential for genetic transformation. The modification of numerous genes by individual gene targeting with different selectable markers, however, is not appropriate for *A. terreus* being grown commercially because of the high costs involved. Moreover, mutants with resistance markers when used on an industrial scale may also raise public concerns on biosafety. Therefore, recyclable marker modules that allow repetitive rounds of transformations by marker rescue are essential for sequential genetic modifications and environmental security. Site-specific recombination is an important molecular tool for functional genetic studies and has been applied for marker rescue in various microorganisms, including *Aspergillus* sp. (Forment et al. 2006; Florea et al. 2009; Kopke et al. 2010). In the traditional strategy, the recombinase expression cassette should be introduced into the cells and induced (Florea et al. 2009; Kopke et al. 2010). Mizutani et al. (2012) investigated a simple method to use modified Cre/*loxP* recombination system in *Aspergillus oryzae* by directly introducing Cre recombinase into protoplast.

Although there have been some examples of improving the itaconic acid accumulation through genetic engineering (Lin et al. 2004; Tevz et al. 2010), just overexpressing the related genes individually in an industrially-used *A. terreus* strain could not improving the itaconic acid productivity significantly (Huang et al. 2014b). So the genetic engineering of multiple genes with multiple modification approaches would be expected. Here, with a view to studying the regulation mechanisms of itaconic acid biosynthesis and improve the ability of itaconic acid production, we developed an efficient marker-free gene-targeting system with high HR frequency and friendly marker rescue in an excellent itaconic acid production strain *A. terreus* CICC40205. The system should be also valuable to explore and engineer other *A. terreus* strains producing other products.

Materials and methods

Materials

Cre recombinase (M0298L) was obtained from NEB (USA). Lysing enzymes (1412) and cellulose (C1184) were from Sigma-Aldrich (USA), and the snailase was from Sangon (Shanghai, China). The DIG High Prime DNA Labeling and Detection Starter Kit I was purchased from Roche.

Strains, medium, and cultivation conditions

A. terreus strains used in this project are listed in Table 1. *A. terreus* CICC 40205 was obtained from China Centre of Industrial Culture Collection. The spores were cultured on AMMB agar [*Aspergillus*

Table 1 Strains used in this study

Strain	Genotype
CICC40205	*A. terreus* CICC40205 (wild type)
At-Δku80	*A. terreus* CICC40205, *Δku80::ptrA*
At-ΔpyrG	*A. terreus* CICC40205, *Δku80::ptrA, ΔpyrG*
At-loxP-pyrG$_{An}$	*A. terreus* CICC40205, *Δku80::loxP-pyrG$_{An}$-loxP-PalcA::sgfp, ΔpyrG*
At-loxP-ΔpyrG$_{An}$	*A. terreus* CICC40205, *Δku80::loxP-PalcA::sgfp, ΔpyrG*

sgfp the gene encoding synthetic green fluorescent protein, *pyrG$_{An}$* functional *pyrG* cassette of *A.niger*, P*alcA alcA* promoter of *A. nidulans*, *loxP* the *loxP* sequence, *CICC* China Centre of Industrial Culture Collection

minimal medium (AMM, http://www.fgsc.net/methods/anidmed.html) with 5 g wheat bran l^{-1}] at 32 °C for 7 days. Further cultivation was carried out in 500 ml non-baffled shake-flasks containing 55 ml itaconic acid production medium (IPM) on a rotary shaker at 200 rpm and 37 °C (Huang et al. 2014c).

Construction of the *ku80* deletion mutant At-Δku80

The primers used in this study are listed in Supplementary Table 1. The *ku80* (ATEG_06919) was deleted using the split-marker approach. The flanking regions of *ku80* with length of about 1.5 kb were amplified from the genome of *A. terreus* CICC40205 using primer pairs Uku80-F/Uku80-R and Dku80-F/Dku80-R respectively, and then fused with selectable marker *ptrA* by fusion PCR. Two hybrid split-marker constructs, ku80-A containing the 5′-flank of *ku80* and 3/4 of the *ptrA* from the 5′-end, and ku80-B containing the 3′-flank of *ku80* and 1/2 of the *ptrA* from the 3′-end, were amplified from the fusion PCR products using primer pairs Cku80-F/ptrA-744R and ptrA-177F/Cku80-R respectively (Fig. 1a).

2 μg ku80-A and 1.5 μg ku80-B constructs were transformed together into *A. terreus* CICC40205 using the protoplast-PEG method as described (Blumhoff et al. 2013). The transformants were screened on AMMS (AMM with 1.2 M sorbitol) agar plates supplemented with 0.1 mg pyrithiamine (PT) l^{-1}, and purified by single spore isolation. The genotypes of *Δku80*::*ptrA* putative mutants were examined by genomic PCR using primer pairs ptrA-F/ptrA-R, Uku80-F/ptrA-R, ptrA-F/Dku80-R, and Sku80-F/Sku80-R. The genomic DNA of the candidate mutants were digested with *Bam*HI respectively, and used for

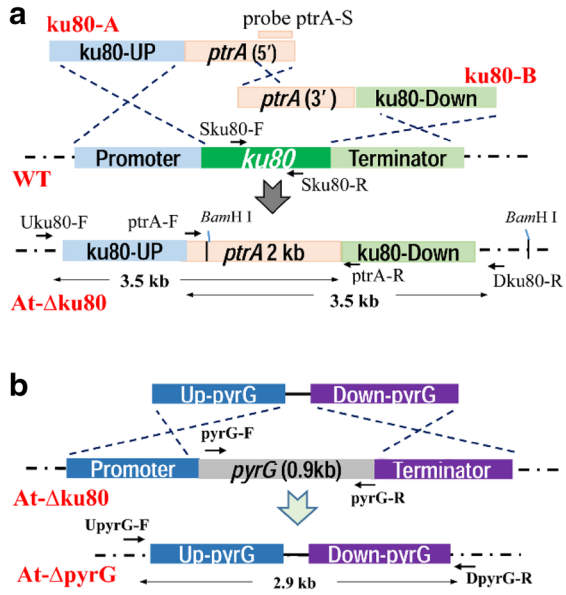

Fig. 1 Schematic model of the construction of mutants At-Δku80 and At-ΔpyrG. **a** The deletion of *ku80* gene using split-marker approach. **b** The deletion of *pyrG* gene in At-Δku80 strain

Southern blot analysis. The probe ptrA-S used for hybridization was obtained by PCR with primer ptrA-F171/ptrA–R744.

Construction of the uracil auxotrophic mutant At-ΔpyrG

The upstream and downstream sequences of *pyrG* (ATEG_09675) were amplified using primer pairs UpyrG-F/UpyrG-R and DpyrG-F/DpyrG-R respectively, and joined together using fusion PCR. The gene-targeting construct was amplified using primers CpyrG-F/CpyrG-R and transformed into At-Δku80

mutant (Fig. 1b). The transformants were selected on AMMS plates with 10 mM uracil and 0.5 g 5-fluoroorotic acid (5-FOA) l^{-1}, and purified by single spore isolation. The *pyrG*-deficient candidates were incubated on the AMM plate without uracil to test the uracil dependence. The genotypes were confirmed by genomic PCR using primer pairs UpyrG-F/D-pyrG-R and pyrG-F/pyrG-R respectively.

Complementation of At-ΔpyrG mutant with $pyrG_{An}$ of *Aspergillus niger*

The DNA fragment comprising *loxP-pyrG*$_{An}$*-loxP*, *PalcA-sgfp* expression cassette, and the flank sequences of *ku80* was amplified from plasmid pXH106 (Supplementary Fig. 1), and transferred into the protoplasts of At-ΔpyrG mutant. The transformants were selected on AMMS plates and purified by single spore isolation. The genotypes were verified by PCR analysis using primer pairs $pyrG_{An}$-F/TtrpC-R, Uku80-F/$pyrG_{An}$-R, $pyrG_{An}$-F/Dku80-R, ptrA-F/ptrA-R respectively.

Direct introduction of Cre recombinase into At-loxP-$pyrG_{An}$ cells for maker rescue

Cre recombinase was introduced into the At-loxP-$pyrG_{An}$ cells as described previously with some modifications (Mizutani et al. 2012). The protoplasts of At-loxP-$pyrG_{An}$ were prepared and adjusted to 5×10^8 cells ml^{-1} in STC buffer. 100 µl protoplast solution was mixed well with 15 µl PSTC buffer, 10 µl Cre recombinase, and 4 µg plasmid pXH-$pyrG_{An}$ (Supplementary Fig. 1). The mixture was incubated on ice for 30 min and 1 ml PSTC was added. The transformants were selected on AMMS plates with 10 mM uracil and 0.5 g 5-FOA l^{-1}, and purified by subculturing for times. The uracil dependence of the transformants were confirmed by incubating on the AMM plate. The genotypes were confirmed by PCR using primer pair Uku80-F/TtrpC-R, and the PCR product were sequenced using primer s-ku80-F.

Characterization of the mutants

To measure the HR frequency in the *ku80* deletion mutant, the ATEG_04633 gene was deleted by gene-targeting in mutant strain At-Δku80. The gene-targeting element of ATEG_04633 was constructed as follows: The selectable marker *hph* was amplified from pG3H (Huang et al. 2014c) using primer hph-F/hph-R. The flanking regions of ATEG_04633 were amplified from the genome of *A. terreus* CICC40205 using primer pairs U4633-F/U4633-R and D4633-F/D4633-R respectively, and then fused with *hph* by fusion PCR. The gene-targeting element was amplified by primers C4633-F/C4633-R and transformed into At-Δku80 mutant. The candidates were selected on potato/dextrose/agar plates with 1.2 M sorbitol and 100 mg hygromycin B l^{-1}, and analyzed by PCR. To examine the uracil auxotrophy, 5-FOA and PT resistant of mutants, the WT, At-Δku80, At-ΔpyrG, At-loxP-$pyrG_{An}$, At-loxP-Δ$pyrG_{An}$ strains were incubated on AMM plates for 4 days, AMM plates supplemented 0.1 mg PT l^{-1} and 10 mM uracil for 7 days, and AMM plates supplemented 10 mM 5-FOA and 10 mM uracil for 7 days. The itaconic acid productivities and growth rates of the WT, At-Δku80, At-loxP-$pyrG_{An}$ mutants were investigated by cultivation in shake flasks as described previously (Huang et al. 2014c).

Results and discussion

Deletion of *ku80* by the split-marker approach

To avoid the ectopic integrations of the DNA fragment containing the intact *ptrA* marker, the split-marker approach was adopted to disrupt the *ku80* gene (ATEG_06919) of *A. terreus* CICC40205 as illustrated in Fig. 1a (Fu et al. 2006; Aragona and Valente 2015). The split-marker constructs ku80-A and ku80-B which contain a 597-bp length overlap region located in the coding sequence of the *ptrA* marker were transformed into *A. terreus* CICC40205. Three transformants with PT resistance were confirmed by genomic PCR, and showing that the *ku80* were deleted completely in transformants #1 and #2 (Supplementary Fig. 2a).

The DNA fragments can yield unexpected ectopic integrations because of the domination of NHEJ, even in the transformants with deletion of *ku80*. These unexpected integrations may cause unpredictable influence on the strain. To identify the completely correct deletion of *Δku80* mutant, the probe ptrA-S was designed within the overlap region of two split-

marker constructs and applied in the southern blot analysis. The result showed that only the band of correct integration (4.4 kb) appeared in the lane of transformant 2# (Supplementary Fig. 2b), which demonstrated that the transformant 2# with *ku80* deletion is the anticipated and exact At-Δku80 mutant without any ectopic integration.

Characterization of the *ku80* deletion strain At-Δku80

To evaluate the influences of *ku80* deletion, the phenotypic characteristics of At-Δku80 were assessed. In comparison to the wild-type, At-Δku80 mutant did not show any significantly difference in the growth rates, conidiation or pigmentation on AMMB agar plates (Fig. 2a). These results were similar to those of *A. fumigatus* and *A. niger* (Krappmann et al. 2006; Honda et al. 2011). Furthermore, no deviations in itaconic acid productivity and vegetative growth were observed in the itaconic acid fermentation, indicating that the deletion of the *ku80* gene in *A. terreus* CICC40205 did not have adverse effect on itaconate production (Fig. 2b). Similar results were also observed in the citric acid producing *A. niger* strain (Honda et al. 2011).

To test the HR frequency of the mutant At-Δku80, the *pdc* gene (ATEG_04633) encoding pyruvate decarboxylase was targeted for the deletion in *ku80*-deficient mutant, At-Δku80. Pyruvate decarboxylase is proposed as the mainly competitive enzyme for the metabolism of pyruvate, the precursor of itaconic acid biosynthesis. Although the fermentation result showed that the deletion of the *pdc* gene did not benefit the production of itaconate (data not shown), the HR frequency of the mutant At-Δku80 during the experiment of *pdc* deletion was significantly improved. In 19 out of 20 transformants, the gene targeting constructs were correctly integrated at the *pdc* loci through HR (Fig. 3). In wild type the HR frequency is very low, about 10 % (date not shown). These results clearly showed that the frequency of homologous integration had been improved to 95 % in the *ku80*-

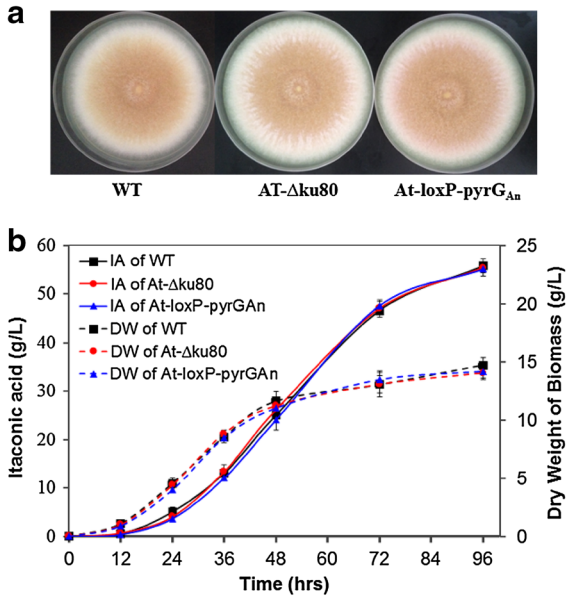

Fig. 2 Comparison of the mutants used in this study. **a** Comparison of cell growth on AMMB plates. WT, AT-Δku80 and At-loxP-pyrG$_{An}$ strains were compared in the growth rate and spore morphology. **b** Comparison of itaconic acid production of the WT, AT-Δku80 and At-loxP-pyrG$_{An}$ strains. The conidia were inoculated in IPM medium and cultivated on a rotary shaker at 37 °C for 4 days. Three independent experiments were performed for each sample. The dry weight of biomass and itaconic acid titers were measured

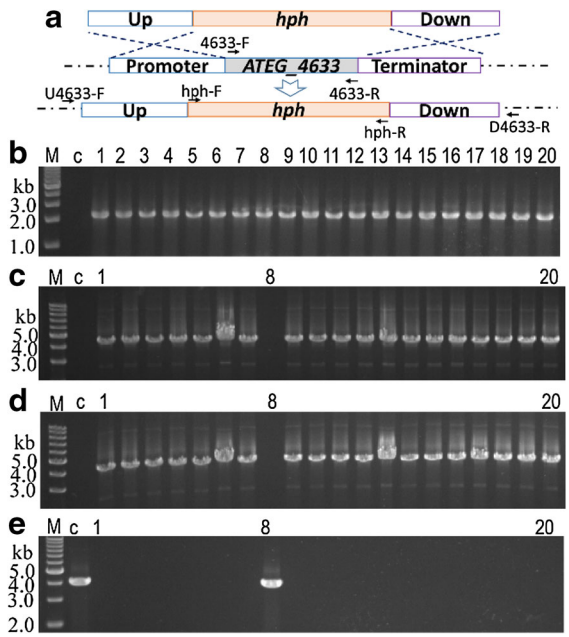

Fig. 3 Deletion of *ATEG_04633* gene in *A. terreus* At-Δku80 strain. **a** Strategy of deleting *ATEG_04633* gene in At-Δku80 strain. The genotypes of 20 transformants were verified by genomic PCR using primer pairs hph-F/hph-R (**b**), U4633-F/hph-R (**c**), hph-F/D4633-R (**d**), and 4633-F/4633-R (**e**) respectively. *Lane M* 1 kb DNA marker; lane c, the negative control, At-Δku80 strain; 1–20, the transformants

deficient mutant, and that we generated an efficient recipient for gene-targeting.

Construction of a bidirectional selection system based on *pyrG*

The bidirectional selection system based on nutritional marker *pyrG* was successfully applied in several kinds of filamentous fungi (Mattern et al. 1987; Kanamasa et al. 2003; Wang et al. 2010; Guo et al. 2013). In this study, the uracil auxotrophy strain At-ΔpyrG was generated by deleting the whole *pyrG* gene in mutant At-Δku80 (Fig. 1b). Two candidates which could not grow on the AMM plate without uracil were characterized by genomic PCR, which confirmed that the *pyrG* were deleted by HR in both two candidates (Supplementary Fig. 3). Besides, we also found that the mutant At-ΔpyrG grew slowly than At-Δku80 on the AMM plate containing PT and uracil, indicating that the deletion of *pyrG* gene had adverse effect on the growth of *A. terreus* even on the medium containing uracil (Fig. 4d).

To confirm that the At-ΔpyrG strain can be used as a recipient in the transformation using the *pyrG* gene as a selectable marker. The functional *pyrG* expression cassette of *A. niger* Co827, $pyrG_{An}$, was cloned and used for the complementation test of mutant At-ΔpyrG (Fig. 4a). The result showed that it transformed At-ΔpyrG to uracil prototrophy (Fig. 4d). Importantly, the At-loxP-pyrG$_{An}$ grew normally as the At-Δku80 and WT strains on the plates without uracil, and showed similar itaconic acid production level in the fermentation test (Fig. 2). Thus, these results demonstrated that the adverse effects caused by *pyrG* deficiency could be completely repaired by complementing the $pyrG_{An}$ marker.

In addition the PCR analysis result confirmed that the *loxP-*$pyrG_{An}$*-loxP* selectable marker was integrated at *ku80* loci of At-ΔpyrG mutant and replaced the *ptrA* gene through HR in 20 out of 22 transformants (Supplementary Fig. 4). This information further demonstrated that the gene-targeting frequency had been improved from10 to 91 % by deleting the *ku80*.

Elimination of $pyrG_{An}$ marker by directly introducing Cre recombinase into the cells

The mutant At-loxP-pyrG$_{An}$ integrating the *loxP-*$pyrG_{An}$*-loxP* cassette was used as the parental strains

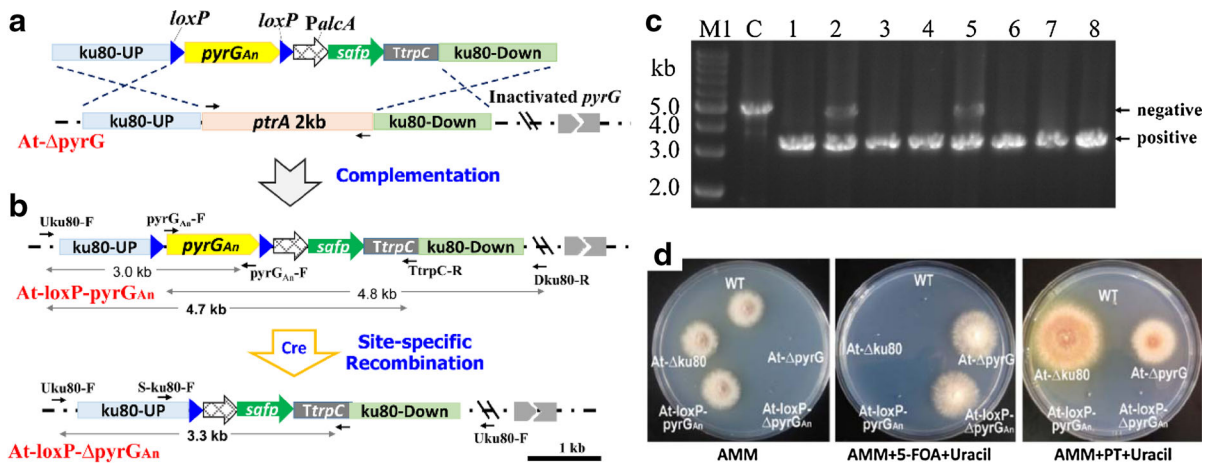

Fig. 4 Strategy for the transformation and marker rescue based on $pyrG_{An}$ marker. **a** Complementation of At-ΔpyrG strain with $pyrG_{An}$. The uracil auxotroph At-ΔpyrG strain was transformed to uracil prototrophy by complementing the *loxP-*$pyrG_{An}$*-loxP* marker at the *ku80* loci, and the *ptrA* marker was replaced. **b** Schematic representation of $pyrG_{An}$ marker rescue from the At-loxP-pyrG$_{An}$ strain. The $pyrG_{An}$ marker was rescued using the Cre/*loxP* system by directly introducing Cre into the protoplast cells. The $pyrG_{An}$ marker located between the two *loxP* sites was excised and only one *loxP* site was left. **c** Genomic PCR analysis of $pyrG_{An}$ marker rescue from the At-loxP-pyrG$_{An}$ mutant using primers Uku80-F/TtrpC-R. C, At-loxP-pyrG$_{An}$ strain; *1–8*, the At-loxP-ΔpyrG$_{An}$ mutants with $pyrG_{An}$ marker rescue. **d** The phenotype of the mutants on different agar plates. The WT, At-Δku80, At-ΔpyrG, At-loxP-pyrG$_{An}$, At-loxP-ΔpyrG$_{An}$ strains were incubated on AMM plates for 4 days, AMM plates supplemented 0.1 mg l^{-1} pyrithiamine and 10 mM uracil for 7 days, and AMM plates supplemented 10 mM 5-fluoroorotic acid (5-FOA) and 10 mM uracil for 7 days

to test the *pyrG*$_{An}$ marker elimination. The protoplasts of At-loxP-pyrG$_{An}$ were incubated with Cre recombinase and pXH-pyrG$_{An}$ plasmid harboring the functional *pyrG* of *A. niger* on ice, and selected on AMMS plates with 10 mM uracil and 0.5 g 5-FOA l^{-1}. More than 100 transformants were obtained, and eight of them were selected randomly and analyzed by genomic PCR with primer pairs as indicated (Fig. 4b). A 3.3-kb length fragment which could be amplified only after excision of *pyrG*$_{An}$ was identified in all of the eight transformants, indicating the *pyrG*$_{An}$ gene was rescued successfully in these transformants, designated as At-loxP-ΔpyrG$_{An}$. While a 4.7-kb length fragment could be obtained when if the *pyrG*$_{An}$ cassette was still present in the genome, such as the mutant At-loxP-pyrG$_{An}$ (Fig. 4c). According to the DNA sequencing result of the 3.3 kb fragment, the *loxP-pyrG*$_{An}$*-loxP* marker located between the ku80-UP and P*alcA* disappeared and only one *loxP* site was left (Supplementary Fig. 5): that is, two *loxP* sites were exactly cut and ligated together by the Cre recombinase, and the *pyrG*$_{An}$ marker was simultaneously cleanly excised. The phenotype test showed that the At-loxP-ΔpyrG$_{An}$ mutant can grow on the AMM plates containing 5-FOA and uracil but not on AMM plates without uracil (Fig. 4d). Therefore the resulting At-loxP-ΔpyrG$_{An}$ mutant strain was transformable using *pyrG*$_{An}$ marker again. The possible positive occurrence of marker rescue in the transformants was 100 % which was consistent with the results observed in *A. oryzae* (Mizutani et al. 2012).

In the conventional marker rescue method based on the site-specific recombination, the recombinase was expressed intracellularly by introducing an inducible recombinase expression cassette into the cell. This is a complex and time-consuming process (Krappmann et al. 2005; Forment et al. 2006; Florea et al. 2009). In this modified Cre/*loxP* system, the Cre recombinase, which is commercially available, was directly introduced into the cells using DNA as a carrier, and the inducible promoter or self-replicating plasmid is no longer needed, and the process of plasmid loss is also eliminated (Mizutani et al. 2012). In this study, a convenient and effective method was introduced into *A. terreus*. Compared with the dominant marker, the bidirectional selectable marker *pyrG*$_{An}$ brings great convenience for marker extinction because the marker-free mutants can be screened out efficiently and easily on the AMM plates supplemented with 5-FOA and uracil. To avoid the unexpected ectopic integrations of the carrier DNA, the pXH-pyrG$_{An}$ plasmid containing the functional *pyrG*$_{An}$ expression cassette was used as the carrier DNA, because the transformants could not grow healthily on the selection plate AMMS while the *pyrG*$_{An}$ DNA was integrated into the genome. In addition to the recyclable marker modules based on the site-specific recombination, the marker gene flanked with two direct repeats could also been self-excised by the internal HR of two repeats. The NHEJ-deficient parental strain and bidirectional selectable marker are the critical parts for this method (Maruyama and Kitamoto 2008; Tani et al. 2013).

In conclusion, an efficient gene-targeting platform was developed in an itaconic acid-producing *A. terreus* strain. This system possesses three prominent features: a chassis cell with high HR frequency, a transformation system based on bidirectionally selectable marker *pyrG*, and an efficient module for selectable marker rescue by directly introducing Cre recombinase into cells. This gene-targeting system is an efficient platform for multiple genetic modifications in *A. terreus*, which is significantly conducive to study biosynthesis mechanisms and improve the production ability of itaconic acid, lovastatin, and other high-valued compounds by genetic engineering *A. terreus*.

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Authors contributions XL, XH and JL designed the experiments. XH, MC performed the experiments. XH, XL, JL and MC drafted the manuscript.

Supporting information Supplementary Table 1—Primers used.

Supplementary Fig. 1—Construction of the plasmid pXH106

Supplementary Fig. 2—Characterization of the genotypes of At-Δku80 mutant strain.

Supplementary Fig. 3—Genomic PCR analysis of the At-ΔpyrG mutant strain.

Supplementary Fig. 4—Genomic PCR analysis of the At-loxP-pyrG$_{An}$ mutants obtained in the complementation test of At-ΔpyrG.

Supplementary Fig. 5—The DNA sequencing result of the PCR-amplified fragments from the *pyrG*$_{An}$ marker region of At-loxP-ΔpyrG$_{An}$ mutant.

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

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