Enhancement of Pellets Formation of Marine Aspergillus Niger with Deficiency of Plasma Membrane ATPase

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Abstract

Morphology of filamentous fungi in submerged fermentation has a huge impact on product yield. In order to discover the genes related to pellets formation, the method of insertion mutation by T-DNA was employed to construct the mutagenesis library of marine Aspergillus niger. In this study, 125 positive transformants were identified by detecting the expression of enhanced green fluorescence and one mutant easier to pelletize was selected by flask-shaking test. The insertion site of T-DNA, determined by hiTAIL-PCR, was located in the promoter of plasma membrane ATPase gene. The mutant exhibited decreased production of spores, weaker salt- and acid-tolerance and a broader range of optimal growth temperature.

Keywords: T-DNA, mutagenesis library; ATPase; Aspergillus niger; Pellet

Introduction

Filamentous fungi are morphologically complex microorganisms, exhibiting different structural forms throughout their life cycles [1]. Morphology of filamentous fungi in submerged fermentation has a great influence on product yield. Disperse form of Aspergillus Niger is preferred for gluconic acid production [2], whereas pelleted form is preferred for citric acid production [3]. Also, pelleted form of Penicillium chrysogenum was desired for production of penicillin [4]. The particular forms of filamentous fungi are determined not only by the chemical (medium constituents) and physical (temperature, pH, mechanical forces) culture conditions but also the genetic material of different fungal species [5]. Some studies have verified the genes related with cell wall synthesis and vesicular trafficking were involved in pellets formation [6,7]. The Microascus brevicaulis mutant exhibited hairy pellets and the proteomics study revealed hundreds of proteins were downregulated or upregulated [8]. So, there will probably be other genes involved in pellets formation. Thus, an efficient method should be developed for the screening of the genes involved pellets formation [9,10].

Recently, T-DNA insertion technology with Agrobacterium tumefaciens-mediated transformation (AMT) has been widely used in the construction of mutant libraries in Arabidopsis and other model plants. Interestingly, AMT can also be applied to filamentous fungi. Thus, T-DNA insertion technology can be used for the construction of mutation libraries of A. niger [11]. The insertion sites of T-DNA exhibit high insertion frequencies in the regions with rich genes and high transcriptional activity, untranslated regions and promoter regions, low insertion frequencies in repeat regions, and no bias in genotypes [12]. In addition, T-DNA is generally inserted into the genome with a single copy, which also facilitates the study on the function of single gene.

Materials and Methods

The marine A. niger ZJUBE-1, screened and isolated from the sludge of the east China sea, exhibits a salt-tolerant growth characteristic [13,14]. The mini-Ti plasmid pCAMBIA-egfp-hph was constructed using seamless cloning (Figure 1) and then transformed into marine A. niger by AMT [15]. Subsequently, positive transformants were identified by detecting the expression of enhanced green fluorescence (EGFP) and the mutants easier to pelletize were selected by flask-shaking test with LB medium. Then the mutants were cultivated on PDA slant for studying their morphology on solid medium. Subsequently, the insertion site of T-DNA was determined by hiTAIL-PCR [16]. Lastly, the effects of culture conditions including NaCl concentration, pH and temperature on biomass were studied by flask-shaking test. The medium components, culture conditions and their purposes were listed in Table 1.

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Table 1: Medium for studying the effects of culture conditions on biomass.

| Medium components | Culture conditions | Purposes                              |
|-------------------|-------------------|---------------------------------------|
| GNY medium:       | Different NaCl concentration (0 - 180 g/L), 30 °C, 180 rpm for 1.5 d | Studying the effect of NaCl concentration on biomass |
|                   | Different pH (2 - 9, contain 0.1 M sodium citrate and adjust with HCl, tris or NaOH), 30 °C, 180 rpm for 1.5 d | Studying the effect of pH on biomass |
|                   | Different temperature (20 - 45 °C), 180 rpm for 1.5 d | Studying the effect of temperature on biomass |

Results and Discussion

In this study, 125 positive transformants were identified from 140 transformants resistant to hygromycin according to the expression of EGFP (Figure 2) and one mutant exhibiting stronger pellets formation ability was screened by flask-shaking test. After the mutant was cultivated in LB broth at 30 °C 200 rpm for 3 d, hyphae fragments were significantly less than those of wild-type strain (WT) (Figure 3a). Observation by microscope suggested the pellet of mutant was compact while the WT pellet was hairy (Figure 3b). In addition, after the mutant was cultivated on PDA slant at 30 °C for 5 d, its spores were infrequent compared with the WT spores (Figure 3c). The gene sequence flanked by T-DNA was obtained by hiTAIL-PCR. The insertion site was located in the promoter of plasma membrane ATPase gene by alignment the sequence with the genome of A niger.

Figure 2: The expression of EGFP in marine A niger.

Figure 3: Phenotypic changes of mutant (a) property of fermentation broth; (b) morphology of pellet; (c) production of spores.

Figure 4: Effect of culture conditions on biomass, (a) NaCl concentration; (b) pH; (c) temperature.
The analysis of the mutant cDNA further revealed that the ATPase gene was not transcribed. The results of growth characteristics (Figure 4) revealed that salt- and acid-tolerance of mutant decreased compared with WT. Plasma membrane ATPase is important for adjusting the concentration of intracellular Na+, K+ and H+. Deficiency of ATPase may decrease the tolerance to salt and acid. In addition, the range of optimal temperature for mycelium growth of mutant was wider than WT. The deficiency of ATPase may decrease the consumption of ATP and then more energy can be used in mycelium growth, especially in low and high temperature.

Conclusion

In this study, the method of insertion mutation by T-DNA was employed to construct the mutagenesis library for screening of the genes involved pellets formation. In addition, constitutive expression of EGFP was used for identification of positive transformants, which was much more efficient than PCR identification. In order to obtain large enough mutagenesis library, the transformation efficiency should be improved by optimization. 125 positive transformants were deemed insufficient for discovery more genes involved pellets formation. Besides, phenotypic screening to mutagenesis library was laborious, thus the volume of screening system should be reduced or new screening method should be developed. For the limitation of mutagenesis library, only one mutant exhibited a stronger ability of pellets formation. Analysis on the insertion locus suggested the promoter of plasma membrane ATPase might be disrupted by T-DNA, which was then proved not transcribed. The subsequent studies on growth characteristics established the relationship between other phenotypes and the ATPase.

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