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Scarless gene deletion in methylotrophic *Hansenula polymorpha* by using *mazF* as counter-selectable marker

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ABSTRACT

With increasing application of *Hansenula polymorpha* in fundamental research and biotechnology, many more genetic manipulations are required. However, these have been restricted for the finiteness of selectable markers. Here, MazF, a toxin protein from *Escherichia coli*, was investigated as a counter-selectable marker in *H. polymorpha*. The lethal effect of MazF on yeast cells suggested that it is a candidate for counter-selection in *H. polymorpha*. Markerless or scarless gene deletion in *H. polymorpha* was conducted based on selectable markers cassette *mazF-zeoR*, in which the zeocin resistance cassette and *mazF* expression cassette were used as positive and counter-selectable markers, respectively. For markerless deletion, the target region can be replaced by *CYC1*TT via two-step homologous recombination. For scarless deletion, the innate upstream region (5′UP) of target genes rather than *CYC1*TT mediates homologous recombination to excise both selectable markers and 5′ sequence of target genes. Moreover, scarless deletion can be accomplished by using short homologous arms for the effectiveness of *mazF* as a counter-selectable marker. The applicability of the strategies in markerless or scarless deletion of *PEP4*, *LEU2*, and *TRP1* indicates that this study provides easy, time-efficient, and host-independent protocols for single or multiple genetic manipulations in *H. polymorpha*.

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As a thermotolerant methylotrophic yeast, *Hansenula polymorpha* not only is a useful model system to reveal the molecular mechanisms involved in methanol metabolism, peroxisome biogenesis and degradation, and nitrate transport and assimilation but also is an important biotechnologically applied yeast [1,2]. The availability of strong inducible or constitutive promoters, the formation of recombinants with multiple copies of the target gene, the adequate posttranslational modification of recombinant proteins, and the ease of growth to high cell densities ($\geqslant$100 g dry weight/L) in cheap defined media make *H. polymorpha* a highly competitive system to produce foreign proteins at industrial scales [3,4]. During recent years, more attention has been paid to this yeast as one of the promising producers of other biotechnological products for its specific physiological properties such as functional peroxisome and tolerance to various environmental stresses. For production of β-lactam antibiotics using *H. polymorpha*, various *Penicillium chrysogenum* genes involved in penicillin biosynthesis and the *Bacillus subtilis sfp* gene encoding a phosphopantetheinyl transferase were coexpressed in *H. polymorpha*, and the amount of secreted penicillin was similar to that produced by the original *P. chrysogenum* strain [5–8]. *H. polymorpha* was also reported as a competitive glutathione producer compared with other known yeast and bacteria strains [9]. Moreover, the thermotolerant nature and capability of fermenting D-xylose (as well as glucose and cellobiose) to ethanol make *H. polymorpha* a good candidate to produce fuel ethanol from renewable raw materials by a simultaneous saccharification and fermentation process [10,11].

Regardless of the efficient production of foreign proteins or other biotechnological products using *H. polymorpha*, genetic engineering of the native biological processes or construction of novel metabolic pathways is of the essence and might require manipulation of multiple genes. Moreover, although the genome sequences of *H. polymorpha* have been revealed [12,13], functions for most of the genes have not been identified. Therefore, a large number of genetic manipulations are also needed to identify and characterize the functionally unknown genes. Several genetic tools, such as vectors for expression or deletion of target genes, host strains with auxotrophic markers or deletion of *YKU80*, auxotrophic or dominant markers, and efficient transformation procedures, have been developed during past years [2]. However, the availability of selection markers for genetic manipulation of *H. polymorpha* is still restricted, especially for manipulations involved in multiple genes.

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The biosynthetic marker genes can be used only in the corresponding auxotrophic host strains, whereas the dominant markers can be used in all host strains but cannot be used repeatedly. On the other hand, for safety consideration, the application of strains with dominant markers is limited, especially in food and medicine fields. The application of counter-selectable markers to get marker rescue is a promising and biosafe strategy for multiple genetic manipulations in *H. polymorpha*. The *TRP1* encoding phosphoribosyl anthranilate isomerase was used as the counter-selectable marker in *Saccharomyces cerevisiae* and *Yarrowia lipolytica* for the 5-fluorobenzoic acid resistance of yeast cells with *TRP1* deletion [14,15], but it did not function as a counter-selectable marker in *H. polymorpha* [16]. Orotidine-5′-phosphate decarboxylase encoded by *URA3* can convert 5-fluoroorotic acid (5-FOA)[1] to a cytotoxic compound to inhibit growth of wild-type yeast cells, so *ura3* mutant can be screened for resistance to 5-FOA [16,17]. However, the *URA3* and 5-FOA counter-selectable system can work only in the *ura3* auxotrophic strain.

The *Escherichia coli* protein MazF encoded by *mazF* can cleave messenger RNAs (mRNAs) specifically at ACA to inhibit cell growth by blocking protein synthesis [18]. *mazF* was used as a counter-selectable marker for unmarked genetic manipulation in *B. subtilis* and *Pichia pastoris* [19,20]; however, heterologous fragments were left in the target loci, which might interfere with the subsequent manipulation of other genes in the same host cells. In this study, the availability of *mazF* as a counter-selectable marker in *H. polymorpha* was investigated, and an efficient and simple strategy for scarless gene deletion of *H. polymorpha* was developed by using protease A gene *PEP4*, 3-isopropylmalate dehydrogenase gene *LEU2*, and phosphoribosyl anthranilate isomerase gene *TRP1* as target genes.

Materials and methods

Strains, plasmids, primers, and culture conditions

Strains and plasmids used in this study are listed in Table S1 of the online supplementary material. All primers used in this study are listed in Table S2. *H. polymorpha* Hu11 deposited at the China General Microbiological Culture Collection Center (collection no. CGMCC 1218) is a uracil auxotroph generated by disruption of *URA3* in *H. polymorpha* CGMCC 2.2498. *E. coli* Top10 was used as a general host for plasmid propagation. *E. coli* JM109 was used as the donor of *mazF* gene. Yeast cells were grown generally at 37 °C in YPD medium [21]. To analyze the cytotoxicity of MazF in *H. polymorpha*, YPG medium (10 g/L yeast extract, 20 g/L peptone, and 10 g/L glycerol) and YPM medium (10 g/L yeast extract, 20 g/L peptone, and 5 ml/L methanol) were used. To detect the auxotroph of yeast strains, minimal synthetic defined (SD) medium (6.7 g/L yeast nitrogen base without amino acids and 10 g/L glucose) supplemented with or without 30 mg/L each of uracil and leucine or 90 mg/L tryptophan was used. *E. coli* cells were grown at 37 °C in low-salt Luria–Bertani medium (LLB: 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl, pH 7.5). When necessary, antibiotics were used at concentrations of 100 μg/ml ampicillin or 25 μg/ml zeocin for *E. coli* and 100 μg/ml zeocin for *H. polymorpha*.

General DNA manipulations

General DNA manipulations in *E. coli* or *H. polymorpha* were performed according to standard methods [21,22]. For electroporation of *H. polymorpha*, electrocompetent cells were prepared as described by Faber and coworkers [23]. Yeast transformation and selection of transformants were performed as described previously [24]. Polymerase chain reaction (PCR) was conducted using high-fidelity DNA polymerase KOD Plus according to the manufacturer's instructions (Toyobo, Japan). Purification of DNA fragments was performed using a PCR Clean-up Kit (BGI Life Tech, China). DNA sequencing was completed by Shanghai Invitrogen Biological Technology (China).

Construction of H. polymorpha strain with mazF

The coding region (336 bp) of *mazF* (http://www.ncbi.nlm.nih.gov/nuccore/U00096.2) was amplified from genomic DNA of *E. coli* JM109 by PCR using the primer pair P1/P2. The amplified DNA fragment was digested with *Hin*dIII and *Xba*I and then inserted into the corresponding sites of plasmid pMOXZα-A [24] to generate recombinant plasmid pMOXZ-mazF. The expression of *mazF* is regulated by methanol oxidase gene *MOX* promoter (*MOX*p), which is a regulatable promoter strongly induced by methanol and repressed by glucose. *TEF1*p, the promoter of transcription elongation factor 1 gene of *S. cerevisiae*, drives expression of zeocin resistance gene *zeoR* in *H. polymorpha* to confer zeocin resistance. Plasmids pMOXZα-A and pMOXZ-mazF were linearized with *Sph*I and introduced into *H. polymorpha* Hu11 by electroporation using a Bio-Rad Gene-Pulser apparatus (1.5 kV, 50 μF, 200 Ω, 4–5 ms). Transformants were selected on YPD medium containing 100 μg/ml zeocin and verified by zeocin resistance and PCR analysis. The confirmed transformants with empty vector or recombinant plasmid were named as Hu11-V and Hu11-mazF, respectively.

Construction of plasmids for markerless gene deletion

A general plasmid pEBCMZC was constructed for markerless deletion of target genes in *H. polymorpha*. The transcriptional termination region of *CYC1* of *S. cerevisiae*, *CYC1*TT, and the DNA fragment containing the *mazF* expression cassette and zeocin resistance cassette, *mazF-zeoR*, were amplified from plasmid pMOXZ-mazF with primer pairs P3/P4 and P5/P6, respectively. The amplified *CYC1*TT (370 bp) was cloned into vector pEASY-Blunt (TransGen Biotech, China) to generate pEB-CYC1TT. The amplified *mazF-zeoR* (3558 bp) was digested with *Not*I and *Apa*I and then inserted into the corresponding sites of pEB-CYC1TT to generate plasmid pEBCMZC.

For markerless deletion of protease A gene *PEP4*, regions of homologous 5′ sequence and 3′ sequence of *PEP4* (http://www.ncbi.nlm.nih.gov/nuccore/U67173) were amplified from *H. polymorpha* Hu11 with primer pairs P7/P8 and P9/P10, respectively. There was a 40-bp overlap between the amplified DNA fragments, and a *Sac*I restriction site was introduced. A fusion DNA fragment designated as *PEP4m* with a *Sac*I restriction site replacement of a 6-bp sequence in the coding region of *PEP4* was obtained from the above PCR products via fusion PCR with primer pair P7/P10 and cloned into vector pEASY-Blunt Simple (TransGen Biotech) to generate recombinant plasmid pEBS-PEP4m. A DNA fragment containing *CYC1*TT and *mazF-zeoR* was amplified from pEBCMZC with primer set P11/P12 and designated as *C-mazF-zeoR*. After being digested by *Sac*I and *Bst*EII, the *C-mazF-zeoR* was inserted into the corresponding sites of pEBS-PEP4m to generate recombinant plasmid pEBS-pep4::CMZC, in which 93 bp of the coding sequence of *PEP4* was replaced by the selectable markers cassette *C-mazF-zeoR*. The *PEP4* disruption cassette *pep4::C-mazF-zeoR* was amplified from pEBS-pep4::CMZC with primer pair P7/P10.

For markerless deletion of 3-isopropylmalate dehydrogenase gene *LEU2* (http://www.ncbi.nlm.nih.gov/nuccore/U00889.1), a mutated *LEU2* gene named as *LEU2m* was obtained from *H. polymorpha* Hu11 by PCR and fusion PCR with primer pairs P13/P14,

[1] *Abbreviations used:* 5-FOA, 5-fluoroorotic acid; YPD medium, yeast estract, peptone, and dextrose; YPG medium, yeast extract, peptone, and glycerol; YPM medium, yeast extract, peptone, and methanol; SD medium, minimal synthetic defined medium; PCR, polymerase chain reaction; *MOX*p, *MOX* promoter.

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