Journal of Biotechnology 146 (2010) 151–159

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Quantitative Y2H screening: Cloning and signal peptide engineering of a fungal secretory LacA gene and its application to yeast two-hybrid system as a quantitative reporter

Takuma Kamiya[a], Teruyo Ojima[b], Kanoko Sugimoto[a], Hideo Nakano[b], Yasuaki Kawarasaki[a,c,*]

[a] Biomolecular Engineering Laboratory, Department Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan
[b] Laboratory of Molecular Biotechnology, Graduate School of Bio and Agricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan
[c] School of Food and Nutritional Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan

ARTICLE INFO

Article history:
Received 9 August 2009
Received in revised form 7 February 2010
Accepted 11 February 2010

Keywords:
Quantitative screening
Y2H
Fungal beta-galactosidase
LacA
Directed evolution

ABSTRACT

A quantitative protein/peptide screening system amenable to high-throughput screening has been developed by furnishing conventional yeast two-hybrid (Y2H) system with an engineered fungal secretory beta-galactosidase gene (designated LacA3). We describe the molecular cloning and signal peptide-optimization of the original fungal LacA gene of which extracellular expression was initially toxic to the host cell. The engineered LacA, LacA3, showed less toxicity, resulting in improved cultural properties of the host. The release of the enzyme to the medium was constant to the cell density under a certain induction condition and independent of the growth phase. The released enzyme kept the wild type properties, was highly glycosylated, stable in a wide pH range and high temperature, and had an acidic pH optimum. In the Y2H system with the novel reporter in combination with the conventional Y2H reporters, the yeast colonies are visibly stained in blue, white or red in the growth context, according to the interaction intensity. The clones with the more stable interactions are easily found as colonies with the larger blue halos, due to the increased extracellular LacA3 expression. A quantitative, high-throughput Y2H screening of cDNA library based on the novel reporter was demonstrated. An application of the novel Y2H system to directed evolution of a peptide fragment was also exemplified.

1. Introduction

The Y2H system is a widely used genetic screening method capable of detecting a protein or peptide that binds to a defined bait protein, based on functional complementation of divided transcription factor and the consequent activation of reporter genes (Chien et al., 1991; Fields and Song, 1989) including His3 and Ade2. Thus, the clones with bait–prey interaction are allowed to grow in the selection medium with the absence of histidine and adenine. However, depending on the bait protein and the promoter intensities for the bait and prey expression, Y2H system generates high rates of false-positives (Vidalain et al., 2004).

The *lacZ* gene from *Escherichia coli* is another reporter gene that has also been used in the conventional Y2H. The use of *lacZ* gene provides a quantitative assess to the interaction between the bait and the prey: the interaction intensity is represented by the amount of the beta-galactosidase accumulated in the host cell. A good correlation between the bait–prey binding affinity and the reporter expression level has been reported (Yang et al., 1995; Möckli and Auerbach, 2004; Estojak et al., 1995; de Felipe et al., 2004). However, unfortunately, canonical *lacZ* assays are not compatible with high-throughput screening because of poor permeability of the X-gal to the yeast cytosol: *lacZ* assay requires multiple steps in sample preparation, including centrifugation of multi-well plates to remove culture media, cell lysis, and re-centrifugation to remove cell debris, which diminish the assay-throughput. Moreover, the multi-step process is not amenable to robotic liquid handling tools and even causes the introduction of unacceptable variations in replicate samples (de Almeida et al., 2008; Serebriiskii and Golemis, 2000). In addition, the suboptimum pH of phosphate-buffered agar plate (pH 7.0), which is the optimum for the *E. coli* enzyme but much higher than yeast's optimum growth (around pH 5–5.5), affects yeast viability so that variability in *lacZ* expression level occurs (Serebriiskii and Golemis, 2000).

Beta-galactosidase [EC 3.2.1.23] is an enzyme capable of hydrolyzing beta-1, 4 galactoside bond and widely distributed from bacteria to higher eukaryotes. Among them, enzymes from

* Corresponding author at: Biomolecular Engineering Laboratory, Department Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan. Tel.: +81 54 264 5540; fax: +81 54 264 5540.
E-mail address: kawarsky@u-shizuoka-ken.ac.jp (Y. Kawarasaki).

0168-1656/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2010.02.007

fungi have been extensively studied because of industrial interests. Particularly, the fungal extracellular enzyme from *Penicillium multicolor* (Takenishi et al., 1983), *Aspergillus oryzae* (Tanaka et al., 1975), or *A. niger* (Widmer and Leuba, 1979; Manzanares et al., 1998) is commercially useful, because of the ease in their isolation from culture, high thermostability and acidic pH optimum (pH 4–5). These fungal enzymes also show high stability in a wide range of pH (from pH 2 to 8). These properties and secretory nature of the fungal enzymes are also promising as the yeast's genetic reporter, if its gene product is compatible with yeast's secretion machineries and released to the culture supernatant.

In this study, we successfully established a novel Y2H system by furnishing a conventional Y2H host strain with a quantitative reporter originated from *A. oryzae* RIB40. We randomized the signal sequence of *A. oryzae* LacA to select a clone with better secretion profile in recombinant yeast. The selected mutant, LacA3, showed desirable properties as a quantitative reporter. The Y2H system with the novel reporter was successfully applied to quantitative high-throughput screening (qHTS). An application of the novel quantitative Y2H system to directed evolution of a peptide fragment was demonstrated in the final section of this report.

2. Materials and methods

2.1. Strains

INVSc1 (Invitrogen, CA, USA) was used both in the signal peptide optimization of fungal LacA and in the extracellular LacA3 production. A cDNA library from HeLa cell and yeast 2-hybrid strains including HF7c, Y187 and AH109 were purchased from Clontech (CA, USA). *A. oryzae* RIB40 and *A. nidulans* G191 were kind gifts from Drs. T. Kobayashi and M. Kato (Nagoya University).

2.2. Culture and medium

For cultivating *E. coli* cells, LB medium (1% Bacto tryptone, 0.5% yeast extract, and 1% NaCl) supplemented with 100 μg ampicillin/mL was used. Yeasts were grown in YPD (2% yeast extract, 1% peptone, and 2% glucose) or minimal synthetic dropout medium containing 2% carbon source (glucose for SD medium, galactose for SG medium, or lactose for SL medium), 0.67% Difco yeast nitrogen base without amino acids. The SD agar plates for Y2H screening were supplemented with 0.002% X-gal.

2.3. Plasmids and primers

Y2H plasmid pGAD-TA3b, GAL4AD-expressing vector, was constructed by introducing *Eam* 1105I sites to the multiple cloning site of pGAD424. The pGBT-Cm, GAL4DBD-expressing vector, was constructed by replacing ampicillin resistant gene in the original pGBT9 with chloramphenicol resistant gene. Yeast expression vector pYES2NT/c, carrying GAL1UAS-GAL1 promoter (P_{GAL1}), was purchased from Invitrogen. The expression vector pBG13 was a derivative of pYES2NT/c (HindIII-XbaI fragment of the original pYES2NT/c was replaced by a double stranded synthetic oligonucleotides yes2-linkerS (AGCTTAAGAAGATCTACCATGGCTCGAGT) and yes2-linkerA (CTAGACTCGAGCCATGGTAGATCTTCTTA)). Plasmid pAUR101 (YIp-type vector) and pAUR112 (YCp-type vector harboring aureobacidin A-resistant gene) were from TaKaRa (Otsu, Japan). Oligonucleotides were synthesized by Fasmac (Atsugi, Japan).

2.4. cDNA cloning

The fungus grown in a rich media with lactose as a sole carbon source was harvested to prepare the total RNAs. The fungal cDNA libraries were synthesized using Superscript III reverse transcriptase (Invitrogen) with oligo-dT16 primer. The LacA cDNAs were amplified with Phusion DNA polymerase (Finnzymes, Espoo, Finland) with their coding-sequence specific primers (Aor_CDS-F1 (ATGAAGCTCCTCTCTGTTGC) and -R1 (TTAGTATGCTCCCTTCCGCT) for *A. oryzae* LacA, or Ani_CDS-F1 (ATGAGACTGTTGCCAGTCTG) and -R1 (CTAATAGACACCCTTCCTAGACTGA) for *A. nidulans* LacA). In the case of the *A. nidulans* putative LacA, the primer sequences were designed to meet with the putative LacA-coding sequence that was deduced from the sequence similarity to the known *A. niger* and *A. oryzae* LacA gene.

2.5. Signal-sequence mutagenesis

Mutant LacA library with a randomized signal sequence was constructed by inverse PCR with the following two phosphorylated primers; vNYT-F (5′-p(NYT)$_8$TCCATCAAGCATCGTCTCAAT-3′, where Y represents pyrimidine (T or C)) and vNYT-R (5′-p(ARN)$_8$CTTCATGGTAGATCTTCTTAAGC-3′, where R represents purine (G or A)), followed by circularization of the amplified fragment with T4 DNA ligase. The underlined nucleotides in the primers indicate the LacA specific sequences. See supplementary Fig. S3 for detail.

2.6. LacA purification

INVSc1 cells harboring pBG-AorLacA or pBG-LacA3 cultured in 100 mL SD-U were harvested in mid-log phase, then suspended to 100 mL SG-U medium to induce gene expression. After dialysis in 20 mM Tris–HCl (pH 8.0) buffer, the culture supernatant was subjected to DEAE-toyopearl (TOSOH, Tokyo, Japan) anion exchange chromatography (ϕ = 15 mm, L = 110 mm). The adsorbed proteins on the column were eluted by linear gradient of NaCl (0–0.5 M).

2.7. Enzyme assays

Amount of extracellularly produced beta-galactosidase was quantified with a colorimetric assay using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. In accordance with Miller Unit in *lacZ* assay (Miller, 1972), one A_{420} unit of the fungal beta-galactosidase was defined as the amount of enzyme that gave absorbance 1.0 at 420 nm in the reaction mixture containing 75 mM acetate buffer (pH 5.0) at 30 °C for 1 min. When used in Y2H as the interaction reporter, the LacA signal intensity was normalized by dividing the A_{420}U by the amount of the cell (the culture turbidity, measured as OD_{600}).

2.8. Yeast transformation and mating

Yeast competent cells were prepared with Frozen EZ Yeast Transformation II Kit (Zymoresearch, CA, USA). Transformation of the competent cells was carried out according to the manufacturer's document. The resulting transformants were selected on SD medium lacking certain amino acids. The cells transformed by pAUR101 or pAUR112-derived plasmid were selected using an SD-agar plate containing 1.0 mg L^{-1} aureobacidin A (TaKaRa, Otsu, Japan). Mating of bait- and prey (library)-strains was carried out as described in "yeast protocols handbook (Clontech, PT3024-1)".

3. Results

3.1. Cloning of fungal LacA cDNAs and their expression in S. cerevisiae

First we sought a robust and versatile enzyme that could potentially be used as an extracellular reporter in yeast genetic assays.