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Self-excising Cre/mutant *lox* marker recycling system for multiple gene integrations and consecutive gene deletions in *Aspergillus oryzae*

Silai Zhang,[1] Akihiko Ban,[1] Naoki Ebara,[1] Osamu Mizutani,[2] Mizuki Tanaka,[1] Takahiro Shintani,[1] and Katsuya Gomi[1,*]

Laboratory of Bioindustrial Genomics, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan[1] *and Applications Research Division, National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-Hiroshima 739-0046, Japan*[2]

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In this study, we developed a self-excising Cre/*loxP*-mediated marker recycling system with mutated *lox* sequences to introduce a number of biosynthetic genes into *Aspergillus oryzae*. To construct the self-excising marker cassette, both the selectable marker, the *Aspergillus nidulans adeA* gene, and the Cre recombinase gene (*cre*), conditionally expressed by the xylanase-encoding gene promoter, were designed to be located between the mutant *lox* sequences, *lox66* and *lox71*. However, construction of the plasmid failed, possibly owing to a slight expression of *cre* downstream of the fungal gene promoter in *Escherichia coli*. Hence, to avoid the excision of the cassette in *E. coli*, a 71-bp intron of the *A. oryzae xynG2* gene was inserted into the *cre* gene. The *A. oryzae adeA* deletion mutant was transformed with the resulting plasmid in the presence of glucose, and the transformants were cultured in medium containing xylose as the sole carbon source. PCR analysis of genomic DNA from resultant colonies revealed the excision of both the marker and Cre expression construct, indicating that the self-excising marker cassette was efficient at removing the selectable marker. Using the marker recycling system, hyperproduction of kojic acid could be achieved in *A. oryzae* by the introduction of two genes that encode oxidoreductase and transporter. Furthermore, we also constructed an alternative marker recycling cassette bearing the *A. nidulans* pyrithiamine resistant gene (*ptrA*) as a dominant selectable marker.

[**Key words:** *Aspergillus oryzae*; Selectable marker recycling; Cre/*loxP*; Mutant *lox*; Pyrithiamine resistance; Kojic acid; Transporter]

Aspergillus oryzae is an important industrial fungus for production of traditional Japanese fermented beverages and seasonings, such as sake, shoyu (soy sauce), and miso (soybean paste), as well as useful enzymes and valuable heterologous proteins (1–3). In addition, *A. oryzae* is also an attractive host for heterologous secondary metabolite production, as it has less productivity of its own secondary metabolites, which leads to the production of the metabolite of interest at a highly pure grade. Recently, there have been an increasing number of reports on biosynthetic genes involved in fungal secondary metabolite biosynthesis, heterologously overexpressed in *A. oryzae*, to identify the function of the target biosynthetic enzyme by characterization of the resultant product (4–10). The heterologous expression system using *A. oryzae* as a host has been useful for functional analysis of biosynthetic genes. However, secondary metabolite biosynthetic pathways consist of many enzyme reaction steps, for example, the lovastatin or compactin biosynthetic pathway is constituted of at least nine steps (11–13) and aflatoxin biosynthesis occurs via over 25 steps (14,15), with the biosynthetic genes involved being clustered. Thus, to elucidate the function of each biosynthetic gene in each chemical step, or in production of the final chemical compound, a number of or all of the genes in the biosynthetic cluster should be expressed in the heterologous host. However, since fungal structural genes are transcribed as monocistronic operons, each coding region of interest situated between a suitable promoter and terminator of a host fungus should be constructed for efficient expression in the host. To simultaneously introduce such a large number of the resultant expression constructs into *A. oryzae*, several selectable markers are required. Although expression of multiple genes in *A. oryzae* has been successful, such as biosynthesis of tenellin from *Beauveria bassiana* (16) and aphidicolin from *Phoma betae* (17), both of which are synthesized by four biosynthetic gene products, it is difficult to introduce a number of the expression constructs that are all required for biosynthesis of a target metabolite, owing to the limited number of selectable markers available in *A. oryzae*. To overcome this problem, a marker recycling system that enables repeated use of a single selectable marker gene is necessary for introduction of multiple expression constructs in *A. oryzae*.

The application of the Cre/*loxP* recombination system to rescue selectable markers from the genome is an ideal procedure for efficient marker recycling. This system involves site-specific recombination of the DNA fragment between two 34-bp *loxP* sequences catalyzed by Cre recombinase (18,19), and has been used for sequential gene deletions in a wide variety of organisms, including filamentous fungi such as *Aspergillus nidulans*, *A. fumigatus*, *Epichloë festucae*, and *Trichoderma reesei* (20–23). We have previously reported a novel Cre/*loxP*-mediated marker

* Corresponding author. Tel.: +81 22 717 8901; fax: +81 22 717 8902.
E-mail address: katsuya.gomi.a6@tohoku.ac.jp (K. Gomi).

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recycling system in which Cre recombinase is directly introduced into *A. oryzae* cells to excise the marker gene flanked by *loxP* sites (24). Following this procedure, Cre/*loxP*-mediated excision and insertion have been successfully achieved, and thus sequential gene deletions could be conveniently done. On the other hand, since a single *loxP* site is left on the genome by Cre-mediated excision in selectable marker recycling, several rounds of gene deletions or integrations using Cre/*loxP* system may result in the accumulation of numerous *loxP* sites on the genome. In particular, when multiple gene integrations occur in a tandem manner, several *loxP* sequences may remain tandemly in the integrated chromosomal region. Hence, it is be possible that undesirable excision of the chromosomal DNA region may occur between the *loxP* sites located adjacent to each other with an identical orientation by Cre-mediated recombination. To avoid this possibility resulting from the remaining *loxP* sites, it can be favorable to use a pair of mutant *lox* sites, namely *lox66* and *lox71*, which contain 5-bp exchanges at their 5′- or 3′-ends of the native *loxP* sequence, respectively. Cre-mediated recombination of a *lox66*/*lox71* pair yields a double-mutant *lox72* site that is not recognized by Cre (25,26). Thus, subsequent rounds of gene manipulation can be carried out by selectable marker recycling using the Cre-recombination system with such mutant *lox* sites in the same host strain (27–29).

In the present study, we developed the Cre/*loxP*-mediated marker recycling system with mutant *lox* sequences to introduce a number of biosynthetic genes into *A. oryzae*. In addition, we attempted to improve the marker recycling system in a more convenient manner by applying a self-excising marker cassette that allows the removal of selectable marker together with the Cre expression construct. Through the use of the Cre/*loxP*-mediated marker recycling system we constructed, hyperproduction of kojic acid by the overexpression of the genes that encode oxidoreductase and transporter (30) was successfully accomplished. In addition, the importance of a putative kojic acid transporter in kojic acid production was examined by deleting the corresponding gene, using the Cre/*loxP*-mediated marker recycling system.

MATERIALS AND METHODS

Strains, media, and molecular biological techniques An auxotrophic *adeA* mutant of *A. oryzae* was constructed by the disruption of the resident *adeA* gene from the *ligD* disruptant (Δ*ligD*::*sC*, *niaD*$^{-}$) (31), and used as the recipient strain for selectable marker recycling and for overexpression of the genes responsible for kojic acid biosynthesis. *A. oryzae* strains were grown in complete YPD medium containing 0.5% yeast extract, 1% polypeptone, and 1% glucose. Czapek–Dox (CD) medium used as a minimal medium for *A. oryzae* contained 1% glucose, 0.3% $NaNO_3$, 0.1% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4$, trace amounts of $FeSO_4$, $ZnSO_4$, $CuSO_4$, $MnSO_4$, $(NH_4)_6Mo_7O_{24}$, with appropriate nutrient requirements, for example, 70 mM $(NH_4)_2SO_4$ instead of $NaNO_3$, 0.003% L-methionine, 0.01% adenine sulfate for *niaD*, *sC*, and *adeA* deficient strains, respectively. The marker-rescued strains were selected on the CD agar medium with 1% xylose instead of glucose as the sole carbon source. *Escherichia coli* DH5α (*supF44, hsdR17, recA1, endA1, gyrA96, thi-1, relA1, lacU169*/*Φ80lacZ*M15) was used for plasmid propagation. Standard *E. coli* manipulations were performed as described previously (32). The sequences of oligonucleotides used in this study are shown in Supplementary Table S1.

Construction of the *adeA*-disrupted recipient strain To construct an auxotrophic *adeA* mutant of *A. oryzae*, the *adeA* gene was replaced by the pyrithiamine resistant gene, *ptrA* (33,34) in the *ligD* disruptant. A fragment approximately 3 kb in length containing the *adeA* gene was amplified by PCR using primers AoadeAFw and AoadeARv with *Pst*I recognition sites at both 5′-ends. The resultant PCR-amplified fragment was subcloned to pCR2.1-TOPO (Invitrogen Co., Tokyo, Japan), and then the fragment containing the *A. oryzae ptrA* gene obtained by digesting the pPTRI plasmid (Takara Bio, Otsu, Japan) with *Sma*I and *Mfe*I was inserted into the *Nru*I-*Mfe*I digested plasmid. Then the resulting plasmid was digested with *Pst*I and was used for *adeA* disruption. PCR amplification experiments in this study were carried out using KOD FX Neo (Toyobo, Osaka, Japan).

Construction of the plasmids for self-excision of the selectable marker gene First, a cassette consisting of the selectable marker, *A. nidulans adeA*, flanked by the mutant *lox* sites, *lox66* and *lox71*, was amplified by PCR using primers AnadeA-lox66 and AnadeA-lox71 with *Hin*dIII recognition sites at both 5′-ends and the *A. nidulans* genome DNA as template. Then, the PCR-amplified fragment was digested with *Hin*dIII and ligated to pAPLTN (35), which was digested in advance with *Hin*dIII to delete the selectable marker gene, *niaD*, yielding pAANA1 and pAANA2, in which the *lox71-AnadeA-lox66* fragment was inserted in opposite directions. The plasmid pAPLTN contains the *A. oryzae* α-amylase gene (*amyB*) promoter and *A. nidulans amyA* terminator, and was provided by the Noda Institute for Scientific Research (Noda, Japan). On the other hand, to construct the plasmids for conditional expression of the Cre recombinase gene (*cre*) of bacteriophage P1 in *A. oryzae*, the *A. oryzae xynG2* promoter was first used as a controllable expression promoter, which is significantly repressed by glucose and induced by xylan or xylose (36,37). The promoter fragment was amplified by PCR using the *A. oryzae* genome as template and primers PxynG2Fw + PxynG2Rv, which contain the restriction enzyme recognition sites at their 5′-ends. The resulting PCR product was digested with *Pst*I and *Sal*I and cloned into the corresponding sites of pNGA142 (38), in which the *glaA142* promoter was deleted by digestion with the same restriction enzymes, yielding pNXG. The *cre* gene was also amplified by PCR using the plasmid pSH47 (39) as template with primers creFw and creRv (Supplemental Table S1). The resulting PCR product was digested with *Spe*I and *Hin*dIII and cloned into pNXG via the same restriction sites, yielding pNXG-Cre.

We attempted to construct an expression plasmid consisting of a self-excising selectable marker cassette by inserting the *PxynG2::cre::TagdA* fragment obtained by digesting pNXG-Cre with *Kpn*I into the *Kpn*I-digested pAANA2. However, such a plasmid was unobtainable, possibly because the promoter of fungal origin may function in *E. coli*, and the *cre* gene was slightly expressed (see Results and discussion). Hence, to avoid the unexpected excision of the self-excising marker cassette in *E. coli*, a 71 bp intron of the *xynG2* gene was inserted into the coding region of the *cre* gene using fusion PCR. The fragment consisting of the *xynG2* promoter and 1–628th nucleotide of the *cre* coding region attached with 46 nt of the 5′-half region of the first intron of the *xynG2* gene was amplified by PCR with primers PxynG2-IFw + Cre628intronRv. Another DNA fragment consisting of the 629–1250th nucleotides of the *cre* coding region with 46 nt of the 3′-half region of the first intron of the *xynG2* gene and the *agdA* terminator was amplified by PCR with primers Cre629intronFw + TagdA-IRv. The position of the *xynG2* intron inserted in the *cre* coding region was chosen to make the splice site sequence nearly the same as that of the resident *xynG2* gene (5′-AG|gtata…cag|AG-3′), so that the intron splicing could properly occur in *A. oryzae*. Then, the resulting fragments were subjected to fusion PCR using primers PxynG2-IFw + TagdA-IRv, and digested with *Kpn*I and ligated to *Kpn*I-digested pAANA2, yielding pAAAXG-Cre (Fig. 1).

To replace the *xynG2* promoter and *agdA* terminator with the *Penicillium chrysogenum xylP* promoter and terminator (40), they were amplified by PCR using the *P. chrysogenum* genome as template and primers PxylPFw + PxylPRv and TxylPFw + TxylPRv, respectively. The *cre* coding region was obtained by digesting pAAAXG-Cre with *Hin*dIII and *Spe*I. These three DNA fragments and a yeast vector, pYES2 (Invitrogen Co.), digested with *Eco*RI and *Bam*HI, were assembled in *Saccharomyces cerevisiae*, using the endogenous homologous recombination system. Then, the DNA fragment containing the *xylP* promoter, *cre*, and *xylP* terminator was amplified by PCR with the primers IFPxylPFw + IFTxylPRv, and was inserted into *Nde*I-digested pAANA1 by using the In-Fusion HD Cloning Kit (Takara Bio), yielding pAAAXP-Cre (Fig. 1).

To construct the alternative marker recycling cassette, the heterologous *ptrA* gene from *A. nidulans* was utilized as a dominant selectable marker. The *A. nidulans thiA* gene, the wild-type of mutant *ptrA*, was amplified by PCR using the *A. nidulans* genome as template. To introduce the point mutation in the 5′-untranslated region (5′-UTR) of the *thiA* gene to convert *thiA* to the mutant *ptrA*, complementary primers, AnptrAFw and AnptrARv, with a mutation (A/T → G/C) (33) were designed (the mutated nucleotide is indicated in bold in Supplemental Table S1). The promoter and 5′-UTR of the *thiA* gene was amplified with primers AnthiAlox71Fw (containing *lox71* sequence) + AnptrARv, and the downstream region encompassing the coding region and terminator was amplified with AnptrAFw + AnthiARv. DNA amplicons were subjected to fusion PCR with primers AnthiAlox71Fw + AnthiARv. The resulting *A. nidulans ptrA* amplicon was digested with *Hin*dIII and ligated to *Hin*dIII-digested pAAAXP-Cre, yielding pANPXP-Cre (Fig. 1).

Construction of the plasmids for overexpression and deletion of the genes for kojic acid production The plasmids for overexpression of the genes in the kojic acid biosynthetic cluster were constructed using the plasmid pAAAXG-Cre. The *kojA* gene encoding an oxidoreductase and the *kojT* gene encoding a putative transporter (30) were amplified from *A. oryzae* genomic DNA by PCR using primers kojAFw + kojARv and kojTFw + kojTRv, respectively. Both resulting PCR products were digested with *Nhe*I and *Sma*I, and respectively inserted into the corresponding sites of pAAAXG-Cre, generating plasmids pAAAXGCKA and pAAAXGCKT. For the deletion of the *kojT* gene in the *kojA* overexpression strain, a 1-kb fragment upstream of the start codon of the *kojT* gene and a 1-kb fragment downstream of the stop codon were amplified by PCR using primers kojT-upFw + kojT-uploxRv and kojT-downloxFw + kojT-downRv, respectively. The DNA fragment containing the self-excising marker cassette was also amplified using primers adeA-Cre-lox-Fw + adeA-Cre-lox-Rv. Then, the resulting three DNA fragments and a yeast vector, pYES2, digested with *Eco*RI and *Bam*HI, were assembled in *S. cerevisiae*, and used for deletion of *kojT*.

Transformation of *A. oryzae* Transformation of *A. oryzae* was performed according to the conventional procedure as reported previously (41) with slight

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