Short Communication

High-level heterologous protein production using an attenuated selection marker in *Aspergillus sojae*

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Yasuko Araki,* Masanobu Yuzuki, Yosuke Masakari, Atsushi Sato, Keiko Gomi, and Seiichi Hara

Research and Development Division, Kikkoman Corporation, 338 Noda, Noda City, Chiba 278-0037, Japan

Filamentous fungi, including *Aspergillus sojae*, are essential for the industrial production of enzymes. Although multi-copy introduction of a gene encoding the protein of interest is useful for increasing protein production, this method has not been established in the case of filamentous fungi. In this study, we aimed to establish an efficient system for multi-copy chromosomal integration and high-level expression of a heterologous gene in *A. sojae* using an attenuated selectable marker. Consequently, by truncating the promoter region of selectable markers, we efficiently introduced multiple copies of a heterologous gene and enhanced the rate of high-level protein-production in the strains. Since the multi-copy strains obtained in this study maintained high productivity even in a non-selective medium, this system could be applicable for industrial protein production.

Key Words: *Aspergillus sojae*; attenuated marker; filamentous fungi; heterologous expression; multi-copy integration; truncated promoter

Abbreviations: DTT, dithiotreitol; EDTA-Na₂, ethylenediamine tetraacetic acid disodium salt; GDH, glucose dehydrogenase; MCS, multiple cloning site

Filamentous fungi have a significant industrial role as cell factories for the production of various enzymes (Sharma et al., 2009). In order to improve protein production, strategies such as promoter engineering (Tsuboi et al., 2005) and the deletion of host proteases, or of a protease-specific transcriptional factor (Punt et al., 2008; Yoon et al., 2010), have been developed. In addition, the ge-

netic fusion of an endogenous highly secreted protein to the N-terminus of a foreign protein (Ward et al., 1990) and the induction of the unfolded protein response (Valkonen et al., 2003) have been attempted in order to maximize protein production in filamentous fungi systems. However, there is still a need for the development of methods to increase protein productivity. In this study, we aimed to develop a high-level recombinant protein production system in *Aspergillus sojae* based on multi-copy chromosomal integration of heterologous genes.

In filamentous fungi, transforming DNA is mainly integrated into the genome at non-homologous sites, while site-specific recombination occurs at low frequencies (Kück and Hoff, 2010). In addition, the multi-copy chromosomal integration of exogenous DNA does not require homologous regions in the transformation vector. In particular, the acetamidase-encoding *amdS* marker gene of *Aspergillus nidulans* is useful for an adequate selection of high-copy transformants, because the copy numbers of the selection marker are reflected by the degree of acetamide assimilation (Hynes, 1996; Kelly and Hynes, 1985; Penttilä et al., 1987; Tilburn et al., 1983). Such multi-copy transformants were reported as being stable throughout hundreds of mitotic divisions and they maintained their copy numbers throughout the fermentation process, even though multi-copy integration occurs in a tandem repeat fashion, which might lead to a risk of significant loss of integrated genes (Davies, 1991).

In yeast, a high-copy number plasmid with a replication origin, such as 2 μ in *Saccharomyces cerevisiae*, can be used for heterologous protein expression. It is well known that control of the plasmid copy number has a great influence on recombinant protein production. A previous report shows that the plasmid copy number depends on its selection marker and can be enhanced by truncating the marker's promoter region in *S. cerevisiae* (Erhart and Hollenberg, 1983). In addition, use of the attenuated

*Corresponding author: Yasuko Araki, Research and Development Division, Kikkoman Corporation, 338 Noda, Noda City, Chiba 278-0037, Japan. E-mail: yaaraki@mail.kikkoman.co.jp

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Fig. 1. Plasmid used for multi-copy integrative transformation of *A. sojae*.

The glucose dehydrogenase gene (*Mpgdh*) is derived from *Mucor prainii*, and the translation elongation factor promoter (*Ptef*, 748 bp) and the alkaline protease terminator sequence (*Talp*, 800 bp) are from *A. sojae* (Satake et al., 2015). The expression cassette, including *Mpgdh* and a selection marker variant of *pyrG* or *adeA*, was inserted into the multiple cloning site (MCS) of the pUC19 vector (Takara Bio Inc., Shiga, Japan).

marker was effective for multi-copy integration of plasmid vector and increase in heterologous protein production in other yeasts (Bergkamp et al., 1992; Du et al., 2012; Le Dall et al., 1994).

We hypothesized that the attenuation of selection marker expression, performed by truncating its promoter region, should be useful for multi-copy chromosomal integration of recombinant genes in filamentous fungi, considering the following assumptions: (1) the strain having only one or few copies of the selection marker would present a deficient expression level and could not grow in selective medium unless another promoter was located in an upstream position; (2) multi-copy (more than dozens of copies) integration of a selection marker with moderately reduced expression could adequately complement the growth defect of the host strain in selective medium; (3) if cloned in the same vector, the target gene expression cassette is integrated with approximately the same copy number as the selection marker.

In order to test the above hypotheses, we employed the *A. sojae pyrG* marker (Satake et al., 2015) encoding orothidine-5'-phosphate decarboxylase, since its orthologs are used for increasing the plasmid copy number in yeast (Loison et al., 1989; Verma and Singh, 2012) and the back-

Table 1. *Aspergillus sojae* strains used in this study.

Strains	Genotype	Reference or source
Parent	Wild-type	NBRC4239
P6-1	<i>niaD</i> Δ <i>pyrG::niaD</i>	This study
KP-del	Δ <i>pyrG</i> Δ <i>ku70</i>	This study
As-p407-1CP	Δ <i>pyrG</i> Δ <i>ku70</i> <i>Asgdh::</i> [<i>Ptef_Mpgdh_Talp_pyrG407</i>]	This study
As-p56-HC	<i>niaD</i> Δ <i>pyrG::niaD</i> pUC19[<i>Ptef_Mpgdh_Talp_pyrG56</i>]	This study
For luciferase reporter assays	Δ <i>pyrG</i> Δ <i>ku70</i> <i>Asgdh::</i> [<i>PadeA_luc_Talp_pyrG407</i>]	This study
Δ <i>adeA</i>	<i>niaD</i> Δ <i>pyrG::niaD</i> Δ <i>adeA::pyrG</i>	This study
As-a80-HC	<i>niaD</i> Δ <i>pyrG::niaD</i> pUC19[<i>Ptef_Mpgdh_Talp_adeA80</i>]	This study

Table 2. Number and rate of transformants with high relative GDH activity obtained using *pyrG* variants with different promoter lengths.

	Number of transformants				Rate of transformants (%)
	Total	10–14-fold*	15–19-fold*	$\geq$ 20-fold*	$\geq$ 10-fold*
<i>pyrG407</i>	48	1	0	0	2
<i>pyrG229</i>	23	4	0	0	17
<i>pyrG129</i>	26	3	2	0	19
<i>pyrG56</i>	18	1	4	1	33
<i>pyrG0</i>	19	2	1	1	21

*The relative GDH activity of transformants was calculated by comparison to the GDH activity of a one-copy GDH strain (As-p407-1CP).

Table 3. Number and rate of transformants with high relative GDH activity obtained by using *adeA* variants with different promoter lengths.

	The number of transformants				The rate of transformants (%)
	Total	5–9 folds*	10–19 folds*	$\geq$ 20 folds*	$\geq$ 5 folds*
<i>adeA126</i>	19	4	0	0	21
<i>adeA120</i>	12	2	1	0	25
<i>adeA80</i>	7	2	0	1	43

*The relative GDH activity of transformants was calculated by comparison to the GDH activity of a one-copy GDH strain (As-p407-1CP).

ground growth of *pyrG*-defective strains in various filamentous fungi is very low on a medium without uridine/uracil. In *A. sojae*, the one-copy integration of *pyrG* with a promoter of 407 bp (*pyrG407*) could effectively complement the growth defect of a *pyrG*-defective strain on Czapek-Dox minimal medium without uridine/uracil; however, that was not the case with a promoter length of 229 bp (*pyrG229*) (data not shown). Therefore, we transformed a *pyrG*-deleted strain (P6-1; for a description of strain construction see File S1) using a plasmid (Fig. 1) containing *pyrG407*, or each truncated *pyrG* marker variant (*pyrG229*, *pyrG129*, *pyrG56*, and *pyrG0*; promoter lengths of 229, 129, 56 and 0 bp, respectively; for sequences, see File S2) and an expression cassette of the reporter gene, glucose dehydrogenase (GDH) from *Mucor prainii* (*Mpgdh*, Satake et al., 2015). The transformation was performed according to Takahashi et al. (2004), using 5×10^7 protoplasts and 10 μ g of each vector. In addition, a control strain harboring one copy of the *Mpgdh* expression cassette (As-p407-1CP, Table 1) was constructed by transforming the strain KP-del (for a description of strain construction, see File S1) with a DNA fragment containing the *Mpgdh* expression cassette and *pyrG407* inserted between the 5' upstream and 3' downstream regions of the GDH-like gene from *A. sojae* (*Asgdh*; homolog of AO090103000214, protein sequence identity 95%) for directing homologous recombination. Transformants of the truncated *pyrG* markers and As-p407-1CP were cultured at 30°C for 4 days on Czapek-Dox minimal medium agar plates containing 50 mM glucose and 0.33 mM 2,6-dichlorophenol indophenol dehydrate. These plates were used for halo formation assays and the strains showing larger halos than those formed by As-p407-1CP were selected. The selected transformants, as well as As-p407-1CP, were cultured at 30°C at 180 rpm for 3 days in GPY medium containing 2% glucose, 1% hipolypepton (Nihon Pharmaceutical, Tokyo, Japan), 0.5% yeast extract (Becton, Dickinson and Co., Franklin Lakes, USA), 0.5% KH_2PO_4 , and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Cultivated mycelia were harvested using filter paper and dried with paper towels. An equal amount (50 mg) of mycelium of each strain was frozen in liquid nitrogen and treated with beads cell disrupter MS-100R (Tomy Seiko Co., Ltd., Japan), and then suspended in 1 mL of a 100 mM potassium phosphate buffer (pH 7.0). GDH activity was assayed in the soluble fraction of homogenates according to a method described previously (Satake et al., 2015). GDH activity was undetectable in the host strain and not influenced by *Asgdh* disruption, and, thus, the relative GDH activity of each transformant was calculated by comparison with the GDH activity of As-p407-1CP. The transformation with shorter *pyrG* promoters tended to provide fewer transformants in total, but a larger proportion of them presented higher GDH activities (Table 2).

Collectively, when the vector harboring *pyrG56* was used for transformation, the ratio of transformants with high GDH activity was the highest, and we obtained a strain (As-p56-HC) with a GDH activity 30-fold higher than that of As-p407-1CP (Table 2). The number of integrated *Mpgdh* copies in As-p56-HC, estimated by qPCR, was 27. In another additional transformation experiment

using the same vector harboring *pyrG56*, we obtained a strain with 60-fold relative GDH activity and a *Mpgdh* copy number of 55. These results indicated that truncated versions of the *pyrG* promoter, especially *pyrG56*, led to multi-copy integration and higher target gene expression.

Next, we investigated whether multi-copy strains could also be obtained by using an attenuated *adeA* marker encoding phosphoribosylaminoimidazole-succinocarboxamide synthase. First, we predicted the *adeA* promoter length that would be appropriate for obtaining multi-copy strains using a luciferase reporter assay. The codon-optimized luciferase gene (*luc*) from *Luciola lateralis*, in which the Ala residue at position 217 was replaced by Leu for enhancing thermal stability (Kajiyama and Nakano, 1994), was cloned next to each *adeA* promoter (*PadeA*) variant with different lengths. The KP-del strain was transformed with a DNA cassette containing a *PadeA* variant, *luc*, *Talp*, and *pyrG407* inserted between sequences of the *Asgdh* ORF and its terminator. This cassette directed integration to a locus directly downstream of the *Asgdh* ORF, in a region predicted not to contain any promoters. Each strain harboring one copy of the luciferase expression cassette was then selected by semi-quantitative PCR. The selected transformants were cultured at 30°C for 3 days in Czapek-Dox minimal medium. An equal amount (50 mg) of mycelium of each strain was disrupted as described above, and then suspended in 200 μ L of 50 mM tricine-NaOH buffer (pH 7.8) containing ethylenediamine tetraacetic acid disodium salt (EDTA-Na_2) and 1 mM dithiothreitol (DTT). Finally, the soluble fraction of each strain homogenate was measured for intracellular luciferase activity in 50 mM tricine-NaOH buffer (pH 7.8) containing 4 mM ATP, 10 mM MgSO_4 , and 2 mM D-luciferin using Lumitester C-100N (Kikkoman Biochemifa Co., Tokyo, Japan). The one-copy integration of the *adeA* marker with a promoter length of 642 bp (*adeA642*) effectively complemented the Δ *adeA* strain's auxotrophy on Czapek-Dox minimal medium without adenine (data not shown). In *S. cerevisiae*, it was reported that the high copy number of plasmids containing a defective *leu2-d* marker gene with a promoter length of 29 bp could be explained by its weak expression of less than 5% of the wild-type *LEU2* (Erhart and Hollenberg, 1983). Therefore, we targeted the *adeA* promoter length that provided less than 5% relative luciferase activity when compared with *adeA642*. As a result of gradually reducing the *adeA* promoter length, the luciferase activity measured for promoters with lengths of more than 144 bp was almost the same as that of *adeA642*. The luciferase activity measured for promoter *adeA126* (126 bp) was 1% of that of *adeA642*, while those of *adeA128* and *adeA127* (128 and 127 bp) were about 40% and 20%, respectively. In addition, the relative activity shown by the *adeA120* promoter (120 bp) when compared with *adeA642* was about 0.02%, while luciferase activity under promoter *adeA80* (80 bp) was almost the same as that obtained without a promoter.

We also transformed the Δ *adeA* strain with a vector containing the *Mpgdh* expression cassette and each truncated *adeA* marker variant (*adeA126*, *adeA120*, and *adeA80*; for sequences see File S2) that presented a relative activity of less than 5% in the luciferase assay. As expected, trans-

formation with shorter versions of the *adeA* promoter increased the rate of transformants with high GDH activity (Table 3). When the vector harboring *adeA80* was used for transformation, the ratio of transformants with high GDH activity was the highest, and a strain (As-a80-HC) having about a 20-fold relative GDH activity was obtained. In the luciferase reporter assay, the relative activity of the *pyrG56* promoter was almost the same as those of the *adeA80* promoter and the control without a promoter (*pyrG0*). This indicates that the use of a selective marker without any promoter sequence can be considered to be an efficient tool for the high-level production of heterologous proteins. In this investigation, the proportion of strains with a high GDH activity obtained by transformation with *pyrG56* was slightly higher than that obtained with *pyrG0* (Table 2). Further studies will be required to determine whether there is a significant difference between *pyrG56* and *pyrG0* in the rate of acquisition of strains with high production levels of recombinant protein.

In conclusion, the attenuation of the expression levels of *pyrG* and *adeA* markers by truncating their promoter was an effective method to obtain multi-copy and high-expressing strains of heterologous genes in *A. sojae*. Considering that these markers have been used in various filamentous fungi, this system for multi-copy integration will also be applicable in other species. In fact, we found that multi-copy integrated strains were efficiently obtained by the transformation of a *pyrG*-deleted *Aspergillus oryzae* with a vector containing *pyrG56* (data not shown).

In yeast, a previous report showed that the strain harboring a plasmid that contained the *URA3* marker gene with a promoter of 17 bp maintained its productivity in chemostat cultivation using a medium containing yeast extract (Kazemi Seresht et al., 2013). Remarkably, the multi-copy strains obtained in this study showed a high production of heterologous protein, even in a non-selective rich medium, such as GPY medium. For culture scale-up of an industrial process, repeated subculture in a rich medium is required. In the future, we will investigate whether the high-copy strains obtained by this method can maintain the productivity of heterologous protein, even after repeated subculture using a jar fermenter.

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Supplementary Materials

Supplementary materials are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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