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A New Efficient Vector for Repeated Use in *Pichia pastoris*

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Abstract: *Using a Cre-mutant-loxP system, we have developed a vector for Pichia pastoris that can be used for the repeated incorporation of target genes. A selection marker gene and an inducible Cre expression gene were placed between mutant-loxP sequences, and target genes can be sub-cloned in the vector to transform P. pastoris by genomic integration. After the isolation of clones transformed with the vector, the selection marker and Cre expression genes in their genome can be excised by recombination between the mutant-loxP sites upon induction of the Cre gene, preserving the target genes in their genome, and eventually they become sensitive to the selection marker. In this manner, target genes in the same vector can be repeatedly introduced into those strains, since the transformants can be re-selectable using the same marker, and the marker and Cre genes between the mutant-loxP sequences can be removed again by inducing expression of the . In order to verify the efficacy of this vector, we put a Green Fluorescent Protein (GFP)-expression gene cassette in the vector, and introduced it into P. pastoris several times. The Cre-mutant-loxP system worked well, as expected, and the production levels of GFP measured based on the fluorescent intensity increased along with the number of vector introductions. The cells with four introductions showed an approximately 4-fold higher intensity than those with only one. The results suggest that this vector could be markedly useful for the production of larger amounts of target proteins in P. pastoris by the repeated incorporations of the same expression vector.*

Keywords: *Pichia pastoris; Cre-loxP; GFP; protein production, recombinant DNA.*

1. INTRODUCTION

Pichia pastoris was initially evaluated as a potential source of single-cell protein (SCP) due to its ability to assimilate abundant methanol made from petrol and achieve continuous culture at high cell densities about fifty years ago [1]. Unfortunately, the oil crisis at that time caused an increase in the cost of sources of methanol. Therefore, SCP production by *P. pastoris* from methanol became unfavorable and declined.

However, since *P. pastoris* strains are easy to manipulate and cultivate as a eukaryotic fungal organism, they are used today as two important biological tools: a model eukaryote used in cell biology research, and a recombinant protein production system [2]. Due to its potential to produce large quantities of heterologous proteins and capability for post-translational modifications [3], *P. pastoris* strains have become increasingly used for both research and the production of recombinant proteins with diagnostic and therapeutic applications [4, 5].

An efficient inducible expression system with methanol was developed using the *AOX*1-promoter that controls the expression of the enzyme alcohol oxidase [6]. The promoter system has been used for the large-scale production of many recombinant proteins. Recently, a constitutive promoter was identified and also used for recombinant protein production [7]. In *P. pastoris* expression systems, the target proteins can either be produced inside the cell or secreted into the media. Especially for secreted production, the purification process is usually simplified by their high quantities in the supernatants: their range of is 30–80% of the total secreted proteins [2].In addition, its ability to achieve very high cell-density cultivation (150–400 g wet cell weight/L) with bioreactors in defined media [8] leads to high volumetric productivities of target proteins, which singles out *P. pastoris* as one of the most promising microbes for the production of recombinant proteins.

Generally in recombinant protein production, the copy number of expressing genes of target proteins affects their protein productivity in host cells. Increasingly arranged expression genes of target proteins show a trend of promoting their host cells to produce higher levels of proteins, and such trends have also been reported in *P. pastoris* [9]. However, the introduction of multiply arranged

genes into *P. pastoris* with electroporation revealed that there was a size limitation of the constructs for incorporation under commonly used electroporation conditions [10]. In order to overcome the size limitation on incorporating multiply repeated target sequences, we describe herein the construction of a new reusable vector for the repeated introduction of target genes using a *Cre*-mutant-*lox*P system for *P. pastoris*, and demonstrate the validity of the repeated incorporation of a target sequence using this vector for efficient protein production.

2. MATERIALS AND METHODS

2.1. Bacterial and Yeast Strains

Escherichia coli, JM 109, competent cells used for plasmid construction were purchased from Toyobo Biochemicals (Japan). *Picha pastoris, GS115,* for expression of the target genes was from Life Technology (USA).

2.2. Kits for DNA Manipulation

The KOD FX polymerase kit used for colony-PCR of *Pichia pastoris* was purchased from Toyobo Biochemicals (Japan). The cloning kit, which utilizes the unique properties of the 3′→5′ exonuclease activity of *Poxvirus DNA polymerase* [11], was from Clonetech (Infusion cloning kit, USA).

PCR for the construction of plasmids and expression constructs was performed with a hi-fidelity DNA polymerase (Prime Max PCR Kit, Clonetech, USA), according to the supplier's manual.

DNA purification kits were purchased from Nippon Genetics, Gbm (Japan).

2.3. Synthetic DNAs and Plasmids

Synthetic DNAs used for PCR amplification were obtained from Sigma Genosis (Japan), and are listed in Table 1.

Plasmids, pInt2 Basic and pPICZ Cre, were previously described [12].

The *Green Fluorescent Protein (GFP)*-expressing construct, the G3 expression cassette, was described previously [10].

2.4. PCR Cloning of E. Coli Lac Operator and OMPA Transcription Terminator

Using primers containing the *lactose operator* sequence (Table 1), a *Cre* expression gene fragment that contained the *lac operator* between the *AOX*1 promoter and *Cre* gene was constructed with PCR-amplified fragments, and re-cloned into the pPICZ vector (Fig. 1).

A DNA fragment containing the *ompA transcription terminator* region (130 bp) was PCR-amplified from the *E. coli JM109* genome. Two types of primer pair (Table 1) were used for cloning. The first one was for the initial cloning of the terminator fragment between the *lac operator* and *Cre* gene, and the second one was for repeated cloning of the terminator fragment between the terminator and *Cre* gene, *i.e.*, tandem-cloning of the terminator.

2.5. Culture Media

LB agar medium (Life Technology, USA) supplemented with ampicillin (Meiji Pharmaceuticals) at 40 mg/L (LB Amp agar) was used for plasmid construction. YPD medium containing 1% yeast extract, 2% peptone, and 0.2% glucose was used for *P. pastoris* cultivation. YPM medium containing 1% yeast extract, 2% peptone, and 0.5% methanol was used for induction experiments. YPD and YPM plates contained 1.5% agar in YPD and YPM media, respectively.

2.6. Transformation of *P. pastoris*

P. pastoris cells were transformed by electroporation according to the manual provided by Life Technology. Transformants were selected on YPDSZ plates (PYD containing 1 M sorbitol, 1.5% agar, and 100 mg zeocin /mL).

2.7. Assays for *GFP* Expression Levels in Each Clone

Cells were pre-cultured at 30℃ for 24 hours on YPD plates. A portion of the cells was spread on YPD (no induction) and YPM (methanol induction) plates, and cultured at 30℃ for 48 hours. Then, cell pellets were scraped by platinum loops and suspended in PBS (10 mM sodium phosphate, pH 7.5, and 150 mM NaCl) at a concentration of 0.1 g wet-cell/mL. The relative fluorescent activity of *GFP*

in each cell suspension was measured with emission light at 535 nm caused by excitation light at 485 nm using a fluorescence spectrophotometer (ARVO MX, Perkin Elmer). All fluorescence spectra were analyzed after a 10- to100-fold dilution of the cell suspensions with PBS, and measured in triplicate.

Table1. *Synthetic DNA primers for PCR used in this study*

Name of oligo DNAs	Sequence
For *Cre* expression construct:	
AOX15' F [1)]	5-**TCATGAGATCAGATC**AACATCCAAAGACGAAAGG-3'
AOX1TT R [2)]	5'-**GGTGTGTGGGGGATC**CGCACAAACG -3'
AOX15' F TSL	5'-**ATCACTAGTCGGATC**AACATCCAAAGACGAAAGG-3'
AOX1TT R TSL	5'-**ATCATCTAGAGGATC**CGCACAAACG-3'
Rv AOX15' F[3)]	5'-**ACGAAGTTATGAATT**CTAACATCCAAAGACGAAAGG-3'
RvAOX1TT R[4)]	5'-**GTACAGACGCGAATT**CACTTAATCTTCTGTACTCTG-3'
Op F[5)]	5'-*GAATTGTGAGCGGATAACAATTCCCC*AGATCTATTATCTGAGTGTGAAATGTCC-**3'**
Op R[6)]	5'-*GAATTGTTATCCGCTCACAATTCCCC*AATTAGTTGTTTTTTGATCTTCTC-**3'**
OmpATT5' F[7)]	5'-**ACAATTCCCCAGATC**CGCAGGCTTAAGTTCTCGTCTGG-3'
OmpATT3' R[8)]	5'-**CTCAGATAATAGATC**TGCTGGGTAAGGAATAACTGACG-3'
OmpATT5' F Tm[9)]	5'-**CTTACCCAGCAGATC**CGCAGGCTTAAGTTCTCGTCTGG-3'
For analysis of genomic DNA	
Zeo F [10)]	5'-CGCCGTACCACTTCAAAACACC-3'
Zeo R [11)]	5'-CTCCTTCCTTTTCGGTTAGAGC-3'
Cre F [12)]	5'-TATTATCTGAGTGTGAAATGTCC-3'
Cre R [13)]	5'-ACTAATCGCCATCTTCCAGCAGG-3'
OCH1 F [14)]	5'-ATCAGACTTTGATTTGATGAGG-3'
OCH1 R [15)]	5'-TGCTTCTTGGTGTTTGTGTTCCG-3'

[1)] and [2)]: The bold letters are the pPICZ vector sequences required for cloning of the PCR-amplified Cre expression DNA fragment in pPICZ by the cloning system using Poxvirus DNA polymerase 5'→3'exonuclease activity. [3)] and [4)]: The bold letters are the pInt2 Basic vector sequences required for cloning of the PCR-amplified Cre expression DNA fragment in pInt2 Basic by the cloning system. [5)] and [6)]: The underlined ATG is the start codon of the Cre gene. The italic sequences are the E.coli lactose operon operator sequences. [7), 8),] and [9)]: Bold letters are sequences between the lac operator and Cre gene required for cloning of the E.coli ompA terminator by the cloning system. [10)] and [11)]: Sequences located in the zeocin-resistance gene 5'and 3'regions in pInt2 Cre Basic, respectively. [12)] and [13)]: Sequences located in the Cre gene 5'and 3'regions in pInt2 Cre Basic, respectively. [14)] and [15)]: Sequences located in the OCH1 region in the P. pastoris, GS115, genome.

3. RESULTS AND DISCUSSION

3.1. PCR-Construction of *Cre* Expression Genes with *E. Coli Lac Operator* and *Ompa Transcription Terminator* and Construction of Reusable Vector, Pint2 Cre Basic

As described in a previous report [12], the *AOX*1 *Cre* expression gene could not be sub-cloned in the pInt2 Basic vector. The *Cre-lox*P system was somewhat induced, and the resulting plasmids showed deleted genes between R- and L-*lox*P sequences.

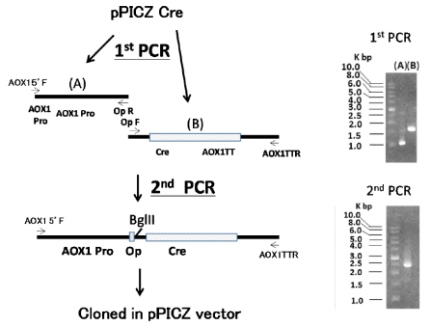

Fig1. *Introduction of a lac Operator sequence before the Cre gene by PCR*

In the 1st PCR step: (A): the AOX1 promoter and untranslated region of the Cre gene were amplified by primers, AOX15' F and Op R, from pPICZ Cre [11], and (B): the Cre gene and AOX1 terminator were amplified by primers, Op F and AOX1TTR from pPICZ Cre [11].

In the 2nd PCR step: AOX1 Pro Op Cre DNA fragment was constructed and amplified from (A) and (B) by PCR using primers, AOX15'F and AOX1TTR.

The left side: the construction scheme. The right side: photos of agarose gel electrophoresis patterns of the amplified fragments in the 1st and 2nd PCR.

This phenomenon was considered to occur with transcriptional read-through from somewhere upstream of the *Cre* gene in *E.coli*. Therefore, in order to terminate this transcriptional read-through upstream of the *Cre* gene, we firstly examined placing a *lactose operator* sequence upstream of the *Cre* gene. Using primers containing the *lactose operator* sequence (Table 1), a *Cre* expression gene fragment (Op-Cre) that contained the *lac operator* between the *AOX*1 promoter and *Cre* gene was constructed by PCR, and cloned into the pPICZ vector (Fig.1). Then, the Op-Cre fragment was PCR-amplified from the vector using primers, Rv AOX15' F and RvAOX1TT R, and sub-cloned into an *Eco*RI site in pInt2 Basic (Fig. 2 a)). However, the resulting plasmids were recombined between L- and R-*lox*P sequences, as was the case without the *lac* operator (Fig. 2 b)). It was considered that a low-level expression of the *Cre* gene might still have taken place based on feint transcriptional read-through via the *lac* operator in *E.coli, JM109,* although the strain contains the *lac* I^q gene for overproduction of the *lac* repressor.

In the next step, we examined the effect of the *omp*A transcription terminator sequence on this transcriptional read-though. A DNA fragment that contained the *omp*A transcription terminator region (130 bp) was PCR-amplified from the *E. coli, JM09,* genome. Two types of primer set (Table 1) were used for the cloning. The first one, OmpATT 5' F and OmpATT 3' R, was for cloning of the terminator at a *Bgl* II site between the *lac operator* and *Cre* gene, and the second one, OmpATT5' F and OmpATT3' R Tm, was for repeated cloning of the terminator at a *Bgl* II site between the terminator and *Cre* gene, i.e., tandem-cloning of the terminator. One to three repeated terminator sequences were introduced between the *lac operator* and *Cre* gene in the pPICZ vector. Then, these *Cre* expression gene fragments containing the terminators were PCR-amplified from the vector using primers, Rv AOX15' F and RvAOX1TT R, and sub-cloned in an *Eco*RI site in pInt2 Basic. The sub-cloning of the *Cre* expression genes was checked by *Eco*RI digestion (Fig. 2 b)). In the case of *Cre* expression genes with 2- and 3-time terminator repeats, precise sizes of *Eco*RI DNA fragments (2.63 kbp for 2- and 2.75 kbp for 3-repeats) were observed in agarose gel electrophoresis, and it was confirmed that those *Cre* expression genes were successfully sub-cloned in pInt2 Basic.

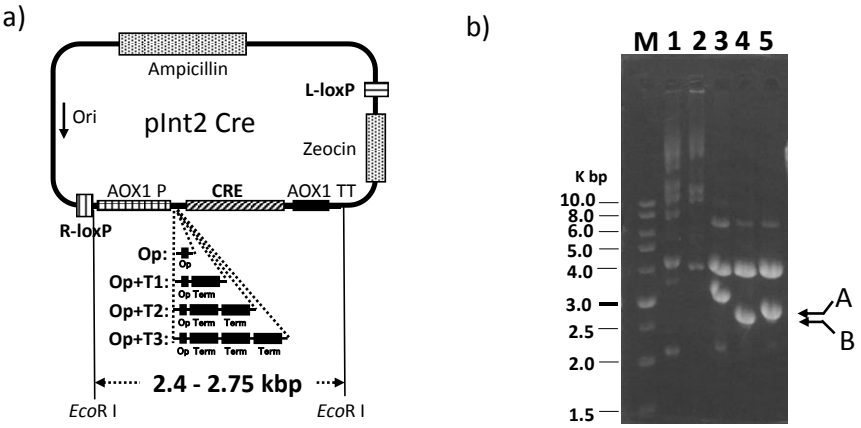

Fig2. *Maps of Cre/loxP plasmids and their stabilities in E. coli*

a) Maps of Cre/loxP plasmids constructed in this study

Abbreviations in maps.

L-loxP and R-loxP: Mutated sequences of loxP. Zeocin: Zeocin resistance gene from pPICZA. Ampicillin and ori: Ampicillin resistance gene and plasmid replication origin from pUC18. AOX1 P and AOX1TT: AOX1 promoter and transcriptional terminator squences. CRE: cre recombinase gene. Op: lactose operator sequence (30 bp). Term: ompA transcriptional terminator sequence (103 bp).

b) Stabilities of plasmids containing a Cre expression construct with various transcriptional control sequences in E.coli.

Lane M: molecular weight markers. Lane 1: a plasmid containing the Cre gene only. Lane 2: a plasmid containing the Cre gene with a lactose operator sequence (Op). Lane 3: a plasmid containing the Cre gene with a lactose operator and ompA transcriptional terminator sequences (Op+T1). Lane 4: a plasmid containing the Cre gene with a lactose operator and two ompA transcriptional terminator sequences (Op+T2). Lane 3: a plasmid containing the Cre gene with a lactose operator and three ompA transcriptional terminator sequences (Op+T3). Arrow A indicates the position of the EcoRI Op+T3 Cre expression fragments (2.75 kbp). Arrow B indicates the position of the EcoRI Op+T2 Cre expression fragments (2.62 kbp).

3.2. Construction of *GFP* Expression Vector that could be used for Multiple Incorporation of *GFP* Expression Gene

A *GFP* expression cassette, the G3 expression cassette, was sub-cloned in pInt2 Cre Basic with the cloning system using *Poxvirus DNA polymerase 3'→5'exonuclease* activity, as described previously [13], to construct pInt2 G3 CRE. The *GFP* expression vector was firstly linearized with *Nru*I digestion. The linearized *GFP* expression fragment, which contains partial sequences of the *AOX*1 promoter at both ends, was replaced inside the *AOX*1 promoter region by double homologous recombination. A zeocin-resistant clone expressing *GFP* was isolated, and named *G3x1 ZeoR*. *G3x1 ZeoR* was then incubated in YPM for 6 hours at 30℃ to express *Cre* recombinase, and spread on YPM plates for single colony isolation. The zeocin sensitivity of those colonies was checked on YPDZ plates. Ninety-six out of one hundred clones picked up were zeocin-sensitive. One of the sensitive clones was chosen, and named *G3x1 ZeoS*. This suggests that the *E.coli omp A* transcriptional terminator did not have any clear effect on *Cre* gene transcription from the *AOX*1 promoter in *P. pastoris*.

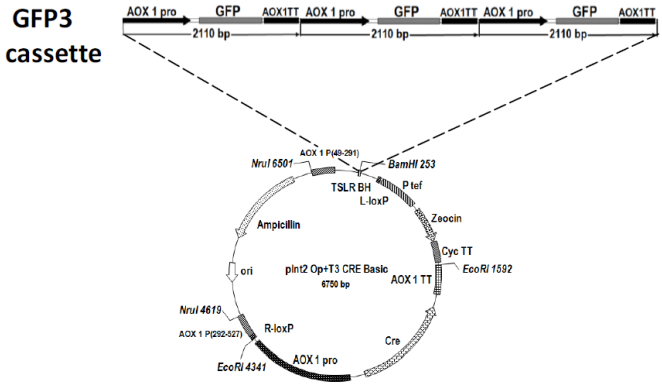

Fig3. *Maps of pInt2 Op+T3 Cre Basic and the GFP expression cassette*

Abbreviations: AOX1 P(49-291) and (292-527): the AOX1 promoter 49-291 and 291-527 region sequences in the pPICZ vector. TSLR BL: a sequence required for the cloning of target genes. Ptef: TEF1 promoter. Zeocin: zeocin resistance gene. Cyc TT: CYC1 transcription terminator. AOX1 TT: AOX1 transcription terminator.

Cre: Op+T3 Cre gene. AOX1 pro: AOX1 promoter. Ampicillin and ori: ampicillin resistance gene and replication origin from pUC18. GFP: green fluorescent protein gene.

3.3. Genomic Analysis of Zeocin-Resistant and-Sensitive Clones

Removal of the sequence between L- and R-*lox*P sequences upon *Cre* gene induction was checked by genomic PCR using primers: Zeo F and Zeo R for the zeocin resistance gene, Cre F and Cre R for the *Cre* gene, and OCH1 F and OCH1 R for the *OCH1* gene as a positive control. As shown in Fig. 3, the zeocin resistance gene and *Cre* gene were not detected in *G3x1 ZeoS*. This indicates that the sequence between L-and R- *lox*P sites, i.e., the zeocin resistance and *Cre* expression genes, should be effectively excised by *Cre* recombinase, and the isolated clone could be used for repeated incorporations of the same kind of vectors.

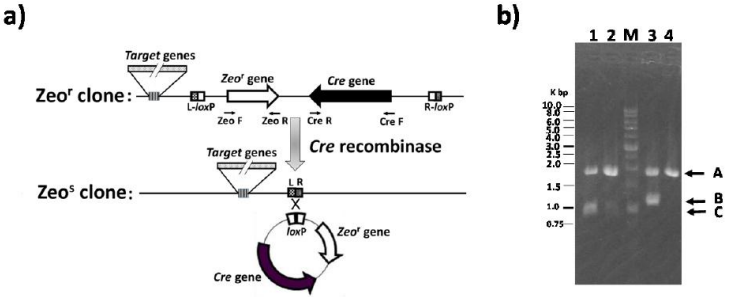

Fig4. *A schematic illustration of zeocin resistance rescue by the Cre-mutant-loxP system in the genome of the vector-integrated P. pastoris and PCR analysis of integrated DNA constructs in the genome*

a) A scheme of Cre recombinase-catalyzed excision of zeocin resistance (Zeo^r) and Cre genes.

Zeo^r: zeocin-resistant. Zeo^s: zeocin-sensitive. Cre: Cre recombinase.

Zeo F and Zeo R: PCR primers for zeocin resistance gene. Cre R and Cre F: PCR primers for Cre recombinase gene. The sequences of primers are listed in Table 1.

b) An agarose gel analysis of genomic DNAs with colony-PCR.

Lane M: Molecular weight markers. Lanes 1 and 3: Zeo^r clone. Lanes 2 and 3: Zeo^s clone.

PCR reactions were done with primers, OCH1 F and R (as an inner control), Zeo F and Zeo R for lanes 1 and 2, and Cre F and Cre R for lanes 3 and 4.

Arrows A, B, and C indicate positions of the OCH1, Cre, and Zeo gene DNA fragments, respectively.

3.4. Multiple introduction of pInt2 G3 Cre into *P. pastoris, GS115*

In the next step, the G3 expression plasmid was linearized by *Bam*HI digestion, and introduced into *G3x1 ZeoS* by electroporation to create higher-level *GFP*-expressing clones. The linearized vector can be integrated by homologous recombination into the sequence at the *Bam*HI *site* in the *Nru*I fragment of pInt2 G3 Cre that was already integrated in the *G3x1 ZeoS* genome (See the map in Fig.3). One of the colonies that appeared on YPDSZ plates was selected, and was named *G3x2 ZeoR*. Then, *G3x2 ZeoR* was incubated in YPM medium at 30℃ for 6 hours for *Cre* gene expression, and the cells were spread on YPM plates. Their sensitivity to zeocin was checked in a similar manner as in the previous step. One of the sensitive clones was selected, and named *G3x2 ZeoR*. The same *Bam*HI-linearized *GFP* expression plasmid was re-introduced into *G3x2 ZeoS*, and zeocin-resistant colonies were selected on YPDSZ. One of them was selected and named *G3x3 ZeoR*. *G3x3 ZeoR* was again incubated in YPM at 30℃ for 6 hours for *Cre* gene expression, the cells were spread on YPD plates, and zeocin-sensitive clones were isolated. One of them was selected, and named *G3x3 ZeoS*. Similarly, *G3x4 ZeoR* was constructed from *G3x3 ZeoS* by the re-introduction of the same *Bam*HI-linearized plasmid, and *G3x4 ZeoS* was from *G3x4 ZeoR*.

The *GFP* expression cassette used in our experiment contained three *GFP* expression genes to obtain clearly expressing clones, since we previously reported that only one *GFP* expression gene could not yield sufficient levels of *GFP* expression for detection [10]. In this report, a total of twelve copies of the *GFP* expression gene were successfully introduced into *P. pastoris*. Making tandem genes *in vitro* using restriction enzymes and ligase and their effects on target gene expression were also reported [14]. However, in our previous experiment using tandem *GFP*-expression genes constructed *in vitro* [10], five tandem-repeats of the expression construct (= 12.2 kbp) were considered to be the maximum DNA size for electroporation in *P. pastroris* under the conditions commonly used for electroporation. Therefore, especially in cases of large expression constructs, multimers of them for a higher expression would not always be successful, although it is possible to construct highly polymerized genes *in vitro*. The *in vivo* construction of multiply integrated strains of target genes using G418 or other resistance marker genes was also reported [14, 15]. However, these methods are not always successful either, and we need to screen many marker-resistant clones to identify a multiply integrated clone, or higher-level producer. Using the vector system in this report, we could strategically increase copy numbers of the target genes in cells by re-introducing the same expression vector as many times as desired without limitation. Thus, this vector system provides a more reliable method to establish *P. pastoris* strains with plural copies of the target genes than other systems do by simply constructing multimers in plasmid vectors or screening more marker-resistant clones to identify multiply integrated strains.

3.5. *GFP* Expression in Clones that Multiply Incorporated Expression Cassette

Clones isolated in the previous section were cultured with or without methanol induction, and their *GFP* expression levels were measured as described in Materials and Methods. When induced by methanol, their fluorescent intensities increased along with the numbers of times the *GFP* expression plasmid was introduced (Fig. 5).

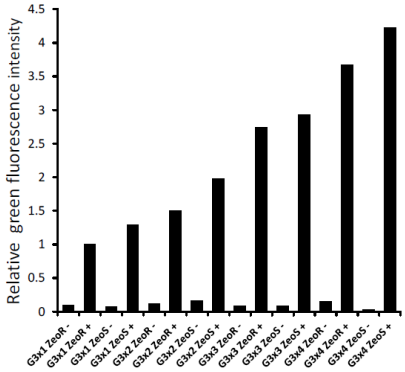

Fig5. *GFP expressions of isolated clones constructed from repeated introduction of pInt2 G3 Cre*

Clones were named after the number of incorporations of pInt2 G3 Cre, and zeocin-resistant phenotypes.

G3x1 ZeoR: A clone transformed with NruI-linearized pInt2 G3 Cre.

G3x1 ZeoS: A clone with the zeocin resistance and Cre expression genes removed from G3x1 ZeoR by Cre recombinase.

G3x2 ZeoR: A clone transformed by incorporation of BamHI-linearized pInt2 G3 Cre into G3x1 ZeoS.

G3x2 ZeoS: A clone with the zeocin resistance and Cre expression genes removed from G3x2 ZeoR by Cre recombinase.

G3x3 ZeoR: A clone constructed with the re-introduction of BamHI-linearized pInt2 G3 Cre into G3x2 ZeoS.

G3x3 ZeoS: A clone with the zeocin resistance and Cre expression genes removed from G3x3 ZeoR by Cre recombinase.

G3x4 ZeoR: A clone constructed with the re-introduction of BamHI-linearized pInt2 G3 Cre into G3x3 ZeoS.

G3x4 ZeoS: A clone with the zeocin resistance and Cre expression genes removed from G3x4 ZeoR by Cre recombinase.

-: Cells without methanol induction. +: Cells with methanol induction.

ZeoR: A zeocin-resistant colne. ZeoS: A zeocin-sensitive clone.

G3x4 ZeoR and *G3x4 ZeoS* with four introductions showed an approximately 4-fold higher intensity compared to the clones with only one. The *GFP* expression levels were slightly increased in the zeocin-sensitive clones. Since the *Cre* recombinase was also expressed during methanol induction of *GFP* expression genes in the zeocin-resistant clones, the removal of the zeocin resistance and *Cre* expression genes during expression also occurred in them, and this event might have affected *GFP* expression levels. These results suggest that the selection marker and *Cre* expression genes are not necessary for stable expression, and their removal is more preferable for target gene expressions. Under the no-methanol-induction condition, fluorescence intensities were almost the same as in the background in all constructed clones, and repression of the *AOX*1 promoter was very tightly controlled even in clones with multiple integration of the same expression construct.

4. CONCLUSIONS

- Using the *Cre*-mutant-*lox*P system, a vector for repeated use in *P. pastoris* was developed by inserting the *E coli omp*A transcriptional terminators between the *AOX*1 promoter and *Cre* gene to terminate the transcriptional read-through upstream of the *Cre* gene.
- Using this vector, the *GFP* expression cassette was repeatedly introduced four times into *P. pastoris*.
- The expression levels increased along with the incorporation times of the vector, and an approximately 4-fold increase was observed in 4-time-incorporated clones as compared to clones with only a single incorporation.
- The zeocin-sensitive clones stably expressed *GFP*, and showed slightly increased *GFP*-expression levels as compared to their parental zeocin-resistant clones.
- As the *GPF* expression cassette contains three copies of the *GFP* expression gene, a total of 12 copies of the *GFP* expression gene were successfully introduced into *P. pastoris* to achieve higher levels of GFP production.
- The reusable vector in this report would be useful for producing a higher level of target proteins in *P. pastoris* by introducing target genes as many times as desired.

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