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Research Article

ENGINEERING A *PICHIA PASTORIS* STRAIN CAPABLE OF INCORPORATING REPEATEDDNA CONSTRUCTS WITH THE SAME SELECTION MARKER

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ABSTRACT

Using a *Cre*-mutant-*lox*P system, we engineered a *Pichia pastoris* strain that can be used for the repeated incorporation of vectors with the same selection marker. A DNA fragment that contained an inducible *Cre recombinase* expression gene and a selection marker gene between mutant-*lox*P sequences was constructed to transform *P. pastoris*. After the isolation of clones transformed with the construct, the selection-marker gene in their genome was excised by recombination between the mutant-*lox*P sites upon inductions of the *Cre recombinase* gene, preserving the inducible *Cre* gene in the genome. Target genes in vectors with a selection-marker gene sandwiched in between the mutant-*lox*P sequences can be repeatedly introduced into those strains, since the transformants can be selected by the marker, and the marker gene is removed upon induction of the *Cre* gene. In order to verify the efficacy of this system, we put a *Green Fluorescent Protein* (*GFP)*-expressing construct in a vector that contained a zeocin resistance gene in between the mutant-*lox*P sequences and introduced it into the engineered strain three times. The system worked well and the production levels of *GFP* measured based on the fluorescent intensity increased along with the number of introductions. The cells with three introductions showed over a 5-fold higher intensity than those with only one. These results suggest that this strain and vector system will be of marked advantage for the production of larger amounts of target proteins by the repeated incorporations of target genes.

Keywords: *Pichia Pastoris; Cre-loxp; GFP; Protein Production; Recombinant DNA*

INTRODUCTION

There have been many methods reported for the production of target proteins by genetic engineering. Typical systems usually involve bacteria (Shibui and Nagahari, 1992), yeasts(Goodrick *et al.*, 2001), fungi (Nevalainen *et al.*, 2005), insect cells (Caron *et al.*, 1990), mammalian cells (Kito *et al.*, 2002), transgenic insects (Aflakiyan *et al.*, 2014), animals (Yu *et al.*, 2013), and plants (Fussenegger and Hauser, 2007; Strasser *et al.*, 2014). Among them, *Pichia pastoris* provides one of the most useful systems for recombinant protein production (Shibui *et al.*, 2013). As a eukaryotic organism, *P. pastoris* has the potential to produce soluble, correctly folded recombinant proteins with post-translational modifications

often required for functionality. Recently, its glycosylation system has been engineered to modify target protein glycosylation like human proteins (Hamilton *et al.,* 2006). Since it is an organism that is easier to manipulate than mammalian cells, it has a superior capacity to produce proteins for therapeutic and industrial use in concentrations ranging from milligrams to grams per liter (Macauley-Patrick *et al.,* 2005). Many human proteins, such as antibodies or serum albumin, have been reported to be over-produced by it (Shibui *et al.,* 2013). The expressed proteins in *P. pastoris* can either be kept inside the cells or secreted into the culture medium. Since the ability to achieve high cell densities by fermentative growth using bioreactors facilitates high volumetric productivity (Schilling *et al.,* 2001), *P. pastoris* has become a robust system and a consistent choice for heterologous protein production (Cereghino *et al.,* 2002; Cereghino and Cregg, 2000).

Since there are no native plasmids in *P. pastoris,* the expression of target genes is carried out by their chromosomal integration, using integrative plasmids or DNA fragments with sequences required for their integration by homologous recombination between genomic and artificially introduced DNAs. The integration of target gene fragments can occur as gene insertion or replacement. Linearized DNA constructs that contain sequences shared by the host genome at both ends stimulate homologous recombination events that efficiently target the integration of the constructs into specific genomic loci.

Generally, in recombinant protein production, the copy number of target genes affects protein productivity in host cells. Multiply rearranged expression genes of target proteins show a trend of promoting their host cells to produce higher levels of proteins, and such effects are also observed in *P. Pastoris*. However, the introduction of multiply arranged genes into *P. pastoris* with electroporation revealed that there was a size limitation of the constructs (Shibui *et al.,* 2014a). In this paper, we describe the engineering of a *P. pastoris* strain that can repeatedly incorporate target genes with the same selection marker, and its capability to repeatedly incorporate target genes and effects on protein production.

MATERIALS AND METHODS

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).

Kits for DNA Manipulation

The KOD HD 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*(Zhu et al, 2007), 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).

Synthetic DNAs and Plasmids

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

L- and R-*lox*P sequences were previously described as *lox*71 and 66, respectively, by Araki et al., (Araki *et al.*, 1997). They were synthesized by Medical Research Laboratories (Japan), and introduced into 5' and 3' regions of the zeocin resistance gene in pInt2 TSLR (Shibui *et al.*, 2014a), respectively, to construct pInt2 Basic (See the map in Figure 1a). The details on construction will be described elsewhere.

The *CRE* recombines plasmid, pBS185, and *P. pastoris* expression vector, pPICZ A, were purchased from Life Technology (USA).

pPICZ Cre was constructed by inserting the *Cre recombinae* fragment, which was PCR-amplified with primers, Cre F and R in Table 1, in pPICZ A.

The *Green Fluorescent Protein (GFP)*-expressing construct, the G3 expression cassette, was described previously (Shibui *et al.*, 2014a).

Culture Media

LB agar medium (Life Technology, USA) supplemented with ampicillin (Meiji Pharmaceuticals) at 40mg/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.

Transformation of P. Pastoris

P. pastoris and engineered *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).

Assays for GFP Expression Levels in each Clone

Cells were pre-cultured at 30℃ for 24 hours on YPD plates. A portion of 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.

RESULTS AND DISCUSSION

Construction of Direct Int. Cre loxP Zeo by PCR

A *Cre recombinase* expression gene (fragment (A) in Figure 2) was amplified by PCR with primers, AOX1 Pro F and AOX1 TT R in Table 1, from pPICZ Cre. L-loxP Zeo R-loxP DNA (fragment (B) in Figure 2) was amplified by PCR using primers, AOX1 TT F and AmpR in Table 1, from pInt2 Basic. Then, the two fragments, (A) and (B), were mixed, joined, and re-amplified by RCR using primers, AOX1 (528) Fand AOX1 (527) R in Table 1, to construct *DirectInt. Cre loxP Zeo* for *P. pastoris* transformation.

We first tried to construct a plasmid that contained a *Cre* expression gene and a mutant *lox*P-sandwiched zeocin resistance gene. However, we could not obtain the desired plasmid. All the plasmids isolated and detected by agarose gel electrophoreses showed the deletion of the zeocin resistance gene, even though colonies were selected on LB plates containing zeocin. We consider that deletion was caused by a very slight expression of the *Cre* gene with transcriptional read-through from a 5' upstream region of the *Cre* gene, and low-level translation, since the gene does not contain apparent SD-like sequences upstream of the start-methionine codon. Therefore, we stopped using a plasmid vector to engineer *P. pastoris.* Since linearized DNA fragments are generally used in *P. pastoris* transformation, we then decided to apply PCR-mediated ligation of the *Cre* expression DNA and mutant *lox*P-sandwiched zeocin resistance gene to construct a *Direct Int. Cre loxP Zeo* DNA fragment for the transformation of *P. pastoris*.

Construction of an Engineered P. pastoris Strain with Direct Int. Cre loxP Zeo

The *Direct Int. Cre loxP Zeo* DNA fragment constructed and amplified by PCR in the previous section was purified with a DNA purification kit, and directly introduced into *P. pastoris* by electroporation. Since the 5' and 3' ends of the fragment have sequences homologous with the *AOX*1 promoter region, it can integrate into this region. Approximately 500 colonies per microgram of the fragment appeared on YPDZS plates. Two colonies were randomly selected, and incubated in YPM medium at 30℃ for 3 hours to induce *Cre* gene expression. Then, the cells were spread on YPD plates, and colonies that appeared were checked regarding their zeocin resistance on YPDZ plates. About 10% of the colonies were zeocin-sensitive. Integrated DNAs in zeocin-resistant and -sensitive clones were further checked by PCR using primers, AOX1 (528) F and AOX1 (527) R in Table 1. In the zeocin-resistant clone, a DNA fragment of 3.5 kbp (Figure 3 b arrow A) was observed at the same position as the *Direct int. Cre loxP Zeo* fragment. In the zeocin-sensitive clone, a DNA fragment of 2.2 kbp instead of 3.5 kbp was seen (Figure 3 b arrow B) as a result of removing the zeocin resistance gene (1.3 kbp) by expressed *Cre* recombinase. In the genome of the zeocin-sensitive clone, no *Cre* recombinase recognition sequences remain after recombination between L-*lox*P and R-*lox*P by *Cre*. Thus, an engineered *P. pastoris* strain which can accept the re-introduction of the same kinds of vector with selection marker genes flanked by L- and R-*lox*P was constructed, and was named *GS115-CRE*.

The *Cre* gene in the engineered *P. pastoris* genome was well controlled by the *AOX*1 promoter, so we could isolate stable zeocin-resistant colonies. When the clone was induced with methanol, the *Cre* gene was effectively expressed, and the zeocin resistance gene was removed, although the appearance rate of zeocin-sensitive clones was lower than we had expected. We consider that this lower rate may have resulted from the localization of *Cre* proteins in the cell, *i.e.*, most of the expressed *Cre* recombinase probably remained in the cytosol, and a very small amount migrated into the nucleus for recombination.

Multiple Introduction of the same GFP Expression Vector into the Engineered Strain, GS115-CRE

A *GFP* expression cassette, the G3 expression cassette, was sub-cloned in pInt2 Basic with the cloning system using *Poxvirus DNA polymerase 3'→5'exonuclease* activity, as described previously (Shibui *et al.*, 2014b). The constructed plasmid was linearized by *Bam*H I digestion, and introduced into the engineered strain, *GS115-CRE*, by electroporation to create *GFP*-expressing clones. The linearized vector can be integrated by homologous recombination into the *AOX1 TT-Int. seq.* region in the inserted DNA fragment in the *GS115-CRE* genome (See maps a and b in Figure 1). One of the colonies which appeared on YPDSZ plates was selected, and was named *G3x1 Zeo*^{r}. Then, *G3x1 Zeo*^{r}was incubated in YPM medium at 30°Cfor 3 hours for *Cre* gene expression, and the cells were spread on YPD plates. Forty-five colonies were picked up and their sensitivity to zeocin was examined. Four zeocin-sensitive clones were obtained. One of them was selected, and named *G3x1 Zeo*^{s}. The same linearized GFP expression plasmid was re-introduced into *G3x1 Zeo*^{s}, and zeocin-resistant colonies were selected on YPDSZ. One of them was selected and named *G3x2 Zeo*^{r}. *G3x2 Zeo*^{r} was again incubated in YPM at 30°Cfor 3 hours for *Cre* gene expression, the cells were spread on YPD plates, and zeocin-sensitive clones were isolated in a similar manner. One of them was selected and named *G3x2 Zeo*^{s}. Similarly, *G3x3Zeo*^{r}was constructed from *G3x2 Zeo*^{s} by the re-introduction of the same linearized plasmid, and *G3x3 Zeo*^{s}was from *G3x3 Zeo*^{r}.

The *GFP* expression cassette used in our experiment contained three *GFP* expression genes to isolate clearly expressing clones, since we previously reported that only one *GFP* expression gene could not yield sufficient levels of *GFP* expression for detection (Shibui *et al.*, 2014a). In this report, a total of nine copies of the *GFP* expression gene were introduced into *GS115-CRE*. In our previous experiment using tandem *GFP*-expression genes constructed *in vitro* (Shibui *et al.*, 2014a), 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. Using the engineered strain in this report, we could re-introduce the same expression vector as many times as desired without limitation. Thus, we were able to introduce into *P. pastoris* more copies of the expression gene than possible simply by constructing multimers in plasmid vectors.

GFP Expression in the Isolated Clones

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 (Figure 4). *G3x3 Zeo*^{r} and *G3x3 Zeo*^{s} with three introductions showed an approximately 5.3-fold higher intensity compared to the clones with only one. The *GFP* expression levels were not influenced by the removal of the zeocin resistance gene with *Cre* recombinase. Under the no-methanol-induction condition, fluorescent intensities were almost the same as the background in all constructed clones, and the repression of the *AOX*1 promoter was very tightly controlled even in clones with multiple integration of the same expression construct.

Our results show that the repeated introduction of the *GFP* expression cassette led to a greater production of *GFP* in *P. pastoris,* and expression levels rose effectively in association with the number of introductions.

Table 1: Synthetic DNAs used in this study

Name of oligo DNAs	**Sequence**
Cre expression gene	
Cre F [1)]	5-**TCGAAACGAGGAATT**ATTATCTGAGTGTGAAATGTCC-3’
Cre R [2)]	5’-**GCCGCCGCGGCTCGACT**AATCGCCATCTTCCAGCAGG-3’
Construction of Direct Int. Cre loxp Zeo	
1st PCR	
Fragment (A)	5’-GCATACCGTTTGTCTTGTTTGGTATTG-3**’**
AOX1 Pro F [3)]	5’- CTAGTGATCCGCACAAACGAAGG-3’
AOX1 TT R [4)]	
Fragment (B)	5’-CCTTCGTTTGTGCGGATCACTAG-3’
AOX1 TT F [5)]	5’-CGAAAGGGCCTCGTGATACGCC-3’
Amp R[6)]	
2nd PCR and colony PCR	5’-GCATACCGTTTGTCTTGTTTGGTATTG-3’
AOX1 (528) F[7)]	5’-CGACTTTTGGAAGTTTCTTTTTGACC-3’
AOX1 (527) R[8)]	

This engineered strain system can also be applied for the expression of several different kinds of protein in the same cell, if their expression genes are sub-cloned in vectors with a selection marker sandwiched

between the mutant-*lox*P sequences, and the system could be used as one of the mostreliable methods to produce target recombinant proteins in *P. pastoris*. We are planning to apply it to the production of biologics in the future.

[1)] and [2)]: The bold letters are the pPICZA vector sequences required for cloning of the PCR-amplified *Cre* gene fragment from pBS185 using the cloning system described in Materials and Methods. The underlined ATG and CTA sequences are the start and stop codons of the *Cre* gene, respectively.

[3)] and [4)]: Sequences located in the 5' region of the AOX 1promoter and the 3' region sequences in the pPICZA vector, respectively.

[5)] and [6)]: Sequences complementary to AOX 1TT F, and located in the 5' upstream region of the ampicillin resistance gene promoter in pUC18, respectively.

[7)]and [8)]: The sequence at the position of 528-554 and the complementary sequence at the position of 502-527 in the *AOX* 1 promoterregion of the pPICZA vector, respectively.

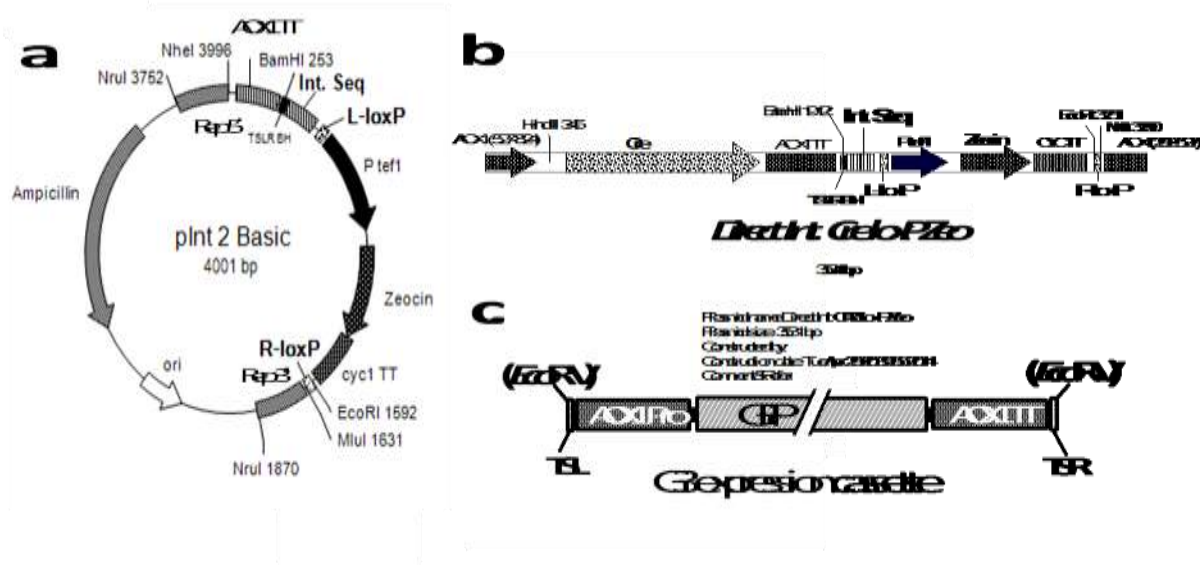

Figure 1: A map of pInt 2 Basic, *Direct Int. Cre loxp Zeo,* and *GFP* expression gene

Abbreviations in maps.

a. Int, seq: A part of the *AOX*1transcriptional termination region. AOX1 TT: The *AOX*1 transcriptional termination region. L-*lox*P and R-*lox*P: Mutated sequences of *lox*P. Ptef1, Zeocin, and cyc1 TT: Zeocin resistance gene from pPICZA. Rep 5' and Rep 3': Homologous sequences of the *AOX*1 promoter for the replacement of target genes in the *AOX*1 promoter region. Ampicillin and ori: Ampicillin resistance gene and plasmid replication origin from pUC18. TSLR BH (Shibui *et al.,* 2014b): A sequence required for the sub-cloning of target genes.

b. AOX1 (527-824) and AOX1 (292-527): *AOX*1 promoter sequences for construct integration into the *P. pastoris* genome. Cre: *cre recombinase* gene. Others are the same as listed above.

c. GFP: *Green Fluorescent Protein* gene. TSL and TSR are required sequences for sub-cloning the expression cassette into pInt2 Basic. Others are the same as listed above. The cassette contains three copies of the *GFP* expression gene for the easier detection of *GFP* expression by green fluorescence.

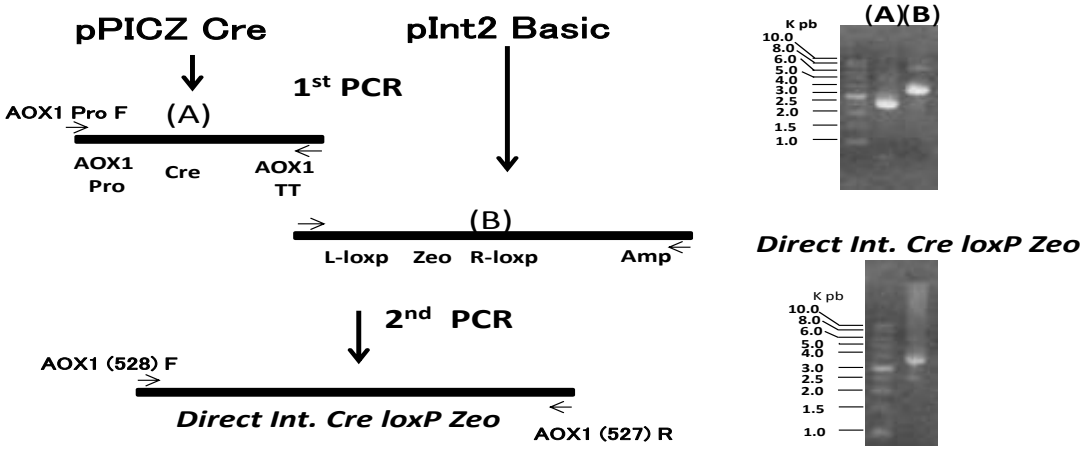

Figure 2: Construction of *Direct Int. Cre loxP Zeo* fragment by PCR

In the 1st PCR step: A *Cre* expression gene fragment (A) from pPICZ Cre and a L-loxP Zeo R-loxP Amp fragment (B) from pInt2 Basic were amplified by PCR using the primers AOX1 Pro F and AOX1 TT R, and AOX1 TT F and Amp R, respectively.

In the 2nd PCR step: *Direct int.Cre loxp Zeo* was constructed by PCR-mediated ligation of (A) and (B) using the primers AOX1(528)F and AOX1(527)R.

The left side: The construction scheme. The right side: Images of agarose gel electrophoresis patterns of the amplified fragments.

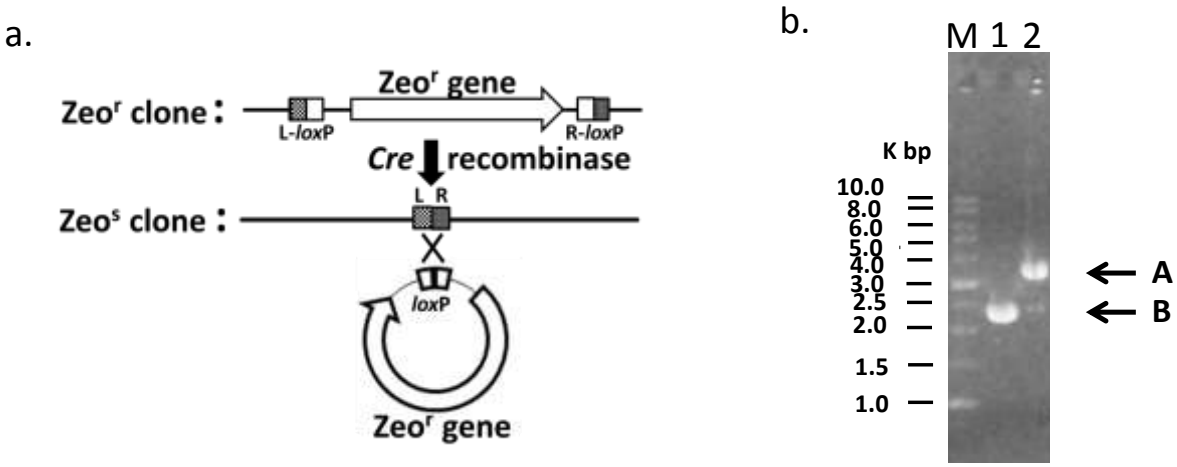

Figure 3: A schematic illustration of the *Cre*-mutant *lox*P system in the engineered *P. pastoris* and PCR analysis of integrated DNA constructs

a. Cre recombinase-catalyzed excision of zeocin resistance (Zeo^r) gene.

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

b. Genomic DNA of both zeocin-resistant and -sensitive clones were directly analyzed with colony-PCR using AOX1 (528)F and AOX1 (527)R primers.

M: Molecular weight markers. 1: Zeo^sclone. 2: Zeo^r clone.

Arrows A and B indicate the positions of the *Direct Int. Cre loxP Zeo* DNA fragment and the *Direct Int. Cre loxP Zeo* minus *loxP Zeo^r gene* DNA fragment, respectively.

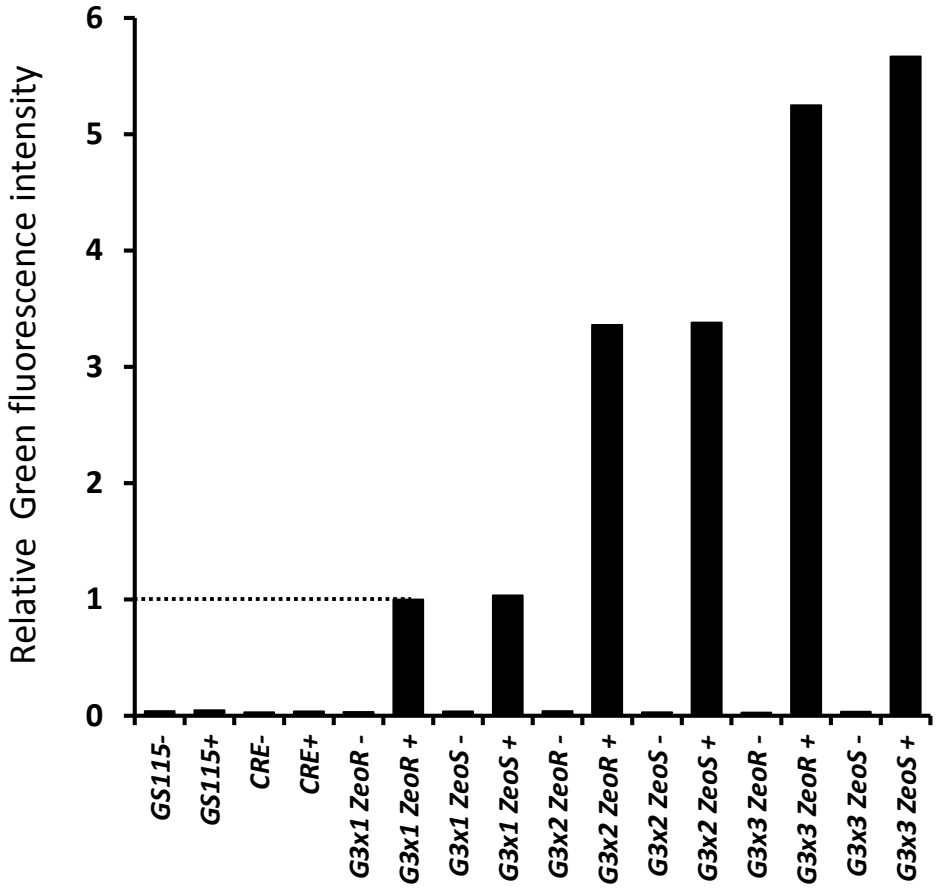

Figure 4: *GFP* expressions of isolated clones constructed from engineered *P. pastoris*

Clones were named after the plasmids used for their electroporation, times of electroporation, and zeocin-resistant phenotypes.

GS115: Host strain (negative control)

CRE: Engineered *P. pastoris* strain in this study (negative control)

G3x1 ZeoR : A clone with a *GFP* expression construct (*Bam* HI-linearized pInt2 Basic G3) introduced into *CRE*.

G3x1 ZeoS: A clone with the zeocin resistance gene removed from *G3x1 ZeoR* by *Cre* recombinase.

G3x2 ZeoR：A clone with the *GFP* expression construct re-introduced into *G3x1 ZeoS*.

G3x2 ZeoS: A clone with the zeocin resistance gene removed from *G3x2 ZeoR* by *Cre* recombinase.

G3x3 ZeoR：A clone with the *GFP* expression construct re-introduced into *G3x2 ZeoS*.

G3x3 ZeoS: A clone with the zeocin resistance gene removed from *G3x3 ZeoR* by *Cre* recombinase.

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

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

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