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Research Article

CONSTRUCTION AND EFFECTS OF TANDEM-REPEAT EXPRESSION GENES FOR *PICHIA PASTORIS*, AND THEIR INCORPORATION EFFICIENCIES AND STABILITIES BY ELECTROPORATION

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ABSTRACT

Expression vectors with polymerized *Green Fluorescent Protein (GFP)* expression genes for *Pichia pastoris* were constructed, and the effects of polymerized genes and their electroporation efficiencies were examined. The *GFP* expression construct was polymerized with up to 5 copies in a vector, and introduced by electroporation. *GFP* expression levels rose with the number of genes in the constructs. Their electroporation efficiencies were almost the same until a repetition number of 4 (≤10.1kbp). However, in the case of a 5-copy construct (=12.2 kbp), the efficacy dropped to 50%. Moreover, in the 4-and 5-copy constructs, colonies with weak or no expression appeared. Those results indicate that polymerization of the target gene is effective for protein production; however, there is a DNA size limitation in *P. pastoris* for the stable incorporation of highly polymerized genes by electroporation.

Keywords: *Pichia Pastoris, Electroporation, Protein Expression, Tandem Genes, GFP, Poxvirus DNA Polymerase*

INTRODUCTION

Many systems for the production of target proteins with recombinant technology have been reported. Typical examples use bacteria (Shibui & Nagahari, 1994), yeasts (Shibui *et al.*, 2013), fungi (Nevalainen *et al.*, 2005), insect cells (Caron *et al.*, 1990), mammalian cells(Kito *et al.*, 2002), plant cells (Hellwig *et al.*, 2004), transgenic insects (Aflakiyan *et al.*, 2014), animals (Yu *et al.*, 2013) and plants (Fussenegger & Hauser 2007). Those systems are basically built up with similar genetic components, as follows: ①Placing a transcriptional promoter with well-controlled and high-transcriptional activity in the 5' upstream direction and a transcriptional terminator sequence to prevent transcriptional read-through in the 3' downstream direction of target genes. ②Inserting sequences for improving the translation of target genes, such as 5'-cap structure, IRES, SD sequences, etc., in the 5' upstream region of target genes. ③Codon optimization of target genes for improved translation in host cells.

Along with those genetic rearrangements, copy numbers of target genes are also known to influence the amount of target proteins produced in host cells (Shibui *et al.*, 1989).

Amongst microbial production systems, yeast production systems are recently being seen as promising substitutes for mammalian cell systems due to their capacity for post-translational modifications and potential to produce large quantities of rather complicated heterologous proteins (Cregg *et al.,* 2000). Especially, *Pichia pastoris* is increasingly being applied to the production of proteins for both diagnostic and therapeutic purposes. Genes of interest are integrated into the *P. pastoris* genome via homologous recombination and stably maintained in the genome. The expressed proteins can either be kept inside the cells or secreted into the culture medium. Since the ability to attain high cell densities by fermentative growth using bioreactors allows for high volumetric productivity (Schilling *et al.,* 2001), *P. pastoris* has become a robust system for the production of recombinant proteins. In this paper, we describe the construction of several multimers of the *GFP* expression gene for *P. pastoris*, their electroporation efficiencies, effects on expression, and stabilities in *P. pastoris*. The results could be applied for efficient productions of recombinant proteins in *P. pastoris*,

MATERIALS AND METHODS

Bacterial and Yeast Strains

E. coli, JM 109, and its competent cells used for plasmid construction were purchased from Toyobo Biochemicals (Japan). *Picha pastoris* strainGS115 for expression of the target genes was from Life Technology (USA).

Plasmids

pEU3-NII (Koga *et al.,* 2009) was kindly provided by Professor Endo at Ehime University, Japan. pPICZ A for the construction of expression vectors was purchased from Life Technology (USA). A map of the pInt 2 TSLR vector is shown in Figure 1 A), and its construction will be described elsewhere.

Reagents

Zeocin was purchased from Life Technology (USA). A DNA polymerase for PCR (Prime Star Max) and the cloning system (Infusion cloning system), which uses unique properties of the 3′→5′ exonuclease activity of *Poxvirus DNA polymerase* (Zhu *et al.,* 2007), were obtained from Clonetech (USA), and used as recommended by the manufacturer unless specified otherwise. DNA purification kits were purchased from Nippon Genetics, Gbm (Japan). Oligo DNAs used in this study are listed in Table 1. They were ordered from and synthesized by Medical & Biological Laboratories Co., LTD. (Japan).KOD FX polymerase used in colony PCR of *P. pastoris* was from Toyobo Biochemicals (Japan). Restriction enzymes were purchased from Takara Bio (Japan).

Culture Media

LB agar medium (Life Technology, USA) supplemented with ampicillin (Meiji Pharmaceuticals) at 40mg/l (LB Amp agar) was used for the construction of plasmids. 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% methanolwas for induction experiments.YPD and YPM plates contained 1.5% agar in YPD and YPM media, respectively.

Transformation of P. Pastoris

The constructed *GFP* expression vectors were linearized by *Nru* I digestion. *P. pastoris* cells were transformed by electroporation according to the manual provided by Life Technology. Transformants that appeared on YPDSZ plates (YPD containing 1 M sorbitol, 1.5% agar, and 100 µg zeocin /ml) were checked for their expression of the*GFP* gene on YPMZ plates (1% yeast extract, 2% peptone, 0.5% methanol, 1.5% agar, and 100 µg zeocin /ml).

RESULTS AND DISCUSSION

Construction of a Plasmid, pINT2GFP1, Containing One Copy of the GFP Expression Gene

The *GFP* gene was amplified from pEU3-E2 by the primers GFP F and GFP R, shown in Table 1, and sub-cloned between *Eco*R I and *Xho* I sites in pPICZA. The resulting *GFP* expression plasmid was digested with *Bgl* II and *Bam*H I to generate a *Bgl* II-*Bam*H I AOX1 Pro-GFP-AOX1 TT fragment. The *P. pastoris* expression vector pInt2 TSLR (Figure 1A) was PCR-amplified by the primers Int2 fusion-AOX1 F and Int2 fusion-AOX1 R (Table1). The 5’ and 3’ ends of the amplified expression vector fragment contained 15 base-pair homologous sequences to the 5’ and 3’ ends of the *Bgl* II-*Bam*H I AOX1 Pro-GFP-AOX1 TT fragment. Those two reaction mixtures were separately purified with a DNA purification kit, mixed, and then treated with the cloning system that utilizes *Poxvirus DNA polymerase* 3’→5’ exonuclease activity (Zhu *et al.*, 2007). JM109 was transformed with the reaction mixture to isolate a plasmid, pInt2 GFP1. This plasmid contains the sequence TSL/R-BH (Figure1 B) for the simplified construction of tandem genes. If a DNA fragment is cloned in a *Bam*H I site in the sequence, it can be easily pluralized by *Eco*R V and *Bam*H I digestion followed by treatment of the cloning system. Since the cloning system requires 15 bp homologous sequences at both ends of the target DNA fragment and the cloning vector, we put TSL and TSL/R-BH sequences at both ends of the *GFP* expression DNA (Figure 1 B) by sub-cloning in the pInt2 TSLR vector. This cloning system also excels in the directional cloning of target genes compared to traditional cloning systems using ligase.

Polymerization of the GFP Expression Gene

With *Eco*R V and *Bam*H I digestions, and the above-mentioned cloning system, pluralized *GFP* expression genes could be sequentially constructed in a vector containing TSL/R-BH. pInt2 GFP1 was completely digested with *Eco*R V and *Bam*H I independently. Each digest was separately purified with the DNA purification kit, mixed, and joined by the cloning system to construct a plasmid, pInt2 GFP2, with the insertion of two copies of the *GFP* expression genes. JM 109 was transformed by the reaction mixture, and selected on an LB Amp plate. The transformants had their plasmids checked by *Eco*R V digestion to confirm two-copy insertion.

In the same manner, pInt2 GFP2 was digested with *Bam*H I and purified. The *Bam*H I-digested pInt2 GFP2 and *Eco*R V-digested pInt2 GFP1was then mixed, and treated with the cloning system to construct a plasmid, pInt2 GFP3, containing three tandem-repeated *GFP* expression genes. Thus, we sequentially constructed the plasmids pInt2 GFP4 from pInt2 GFP3-*Bam*H I and pInt2 GFP1-*Eco*R V digests, and pInt2 GFP5 from pInt2 GFP4-*Bam*H I and pInt2 GFP1-*Eco*R V digests, containing four and five tandem-repeated *GFP* expression genes, respectively (See maps in Figure 1A).

Patterns of *Eco*R V digestion of pInt2 GFP1-5 are shown in Figure 2.The digestion pattern showed that the construction of polymerized genes was successful.

Electroporation of Polymerized GFP Expression Genes

PInt2 GFP1-5 were digested with *Nru* I to linearize the GFP expression genes for electroporation. The linearized *GFP* expression gene fragments contain *AOX*1 promoter 5'- and 3'-region fragments at their 5' and 3' ends of the expression genes, so that a part of the*AOX*1 promoter sequence of the GS115 genome could be replaced with the *GFP* expression fragments by electroporation. Electroporation efficacies of those pluralized *GFP* expression genes are listed in Table 2.

Electroporation efficiencies of pInt2 GFP1-4 were almost the same; however, in pInt2 GFP5, it dropped to approximately 50%. Under our electroporation conditions, the size of 5-times repeated *GFP* expression genes (12.2 kbp) is considered to be close to the size limitation.

Effects of Polymerized Expression Genes on GFP Production

The *GFP* expression levels in colonies that appeared on electroporation of each expression construct are listed in Table 3. The relative expression levels monitored by green fluorescence caused on irradiation with a blue light LED (L=550nm) (Biocraft, Japan) increased in accordance with the copy-number of the *GFP* expression genein the construct (Figure 3).

However, in pInt2 GFP4 and 5, colonies that showed less or no expression appeared (Table 3). The highly polymerized genes became less stable. However, since expressing clones of pInt2 GFP4 and 5 stably expressed *GFP*, when integrated properly, those polymerized genes were considered to be stably maintained in the *P. pastoris* genome.

Relative GFP Expression Levels in Clones of Each Construct

GFP-expressing clones of each construct were cultured on YPD plates for no induction (controls) and YPM plates for induction of the*AOX*1 promoter, and their *GFP* expression levels were measured (Figure 4). The relative expression levels increased almost proportionally with copy-numbers of the *GFP* expression gene in the introduced plasmids. In the clone of pInt2 GFP5, the relative expression levels reached 5.1-fold higher levels than in that of pInt2 GFP1. As *GFP* expression levels in all no-induced clones were almost the same as the background, it was indicated that the*AOX*1 promoter is very strictly controlled with methanol.

Analysis on the GFP Expression Gene in Expressing Clones

As the *GFP* expression gene contains 5' and 3'untranslated region sequences of the *AOX*1 gene at its 5' and 3' ends, both *GFP* and *AOX*1 genes can be amplified by PCR using AOX1 F and R primers (Table 1). Relative copy-numbers of the *GFP* expression gene integrated in the genome of each clone were estimated by comparing the amounts of *GFP* and *AOX*1, which is one copy in the genome and was used as a control, DNA fragments amplified by PCR from each genome (Figure 5). Relative amounts of amplified *GFP* gene increased in accordance with copy numbers of the *GFP* expression gene in incorporated plasmids (Table 4). In the pInt2 GFP5 clone, the ratio of the amplified *GFP* gene was the highest; however, we could not clarify the precise copy-number of the integrated *GFP* gene in each clone with this method. Quantitative PCR might be more suitable for this purpose. In comparing the amount of amplified DNAs, the *AOX*1 gene was found to be more amplifiable than the *GFP* gene, since the intensity of the *AOX*1 DNA band was much higher than that of the *GFP* DNA band. This might be due to the difference in the nucleotide composition of both genes.

Table 1: Synthetic DNAs used in this study

Name of oligo DNAs	Sequence
GFP expression construct:	
GFP F [1)]	5-**TTATTCGAAACGAGG**ATGGTGAGCAAGGGCGAGGAG-3'
GFP R [2)]	5'-**TGGCGGCCGCCGCGGC**TTACTTGTACAGCTCGTCCATGC-3'
Construction of pInt2 GFP1	
Int2 fusion-AOX1 F [3)]	5'-*CGCTAGCGATATCACTAGTCGGATC***GATCTAACATCCAAA-3'**
Int2 fusion-AOX1 R [4)]	5'-*CTAGAGGATCCGACTAGTGATCC***GCACAAACGAAGGTC-3'**
Colony PCR	
AOX 1 F [5)]	5'GACTGGTTCCAATTGACAAGC3'
AOX 1 R [6)]	5'GCAAATGGCATTCTGACATCC3'

[1)] and [2)]: The bold letters are the pPICZ vector sequences required for cloning of the PCR-amplified GFP DNA fragment by the cloning system using Poxvirus DNA polymerase 5'→3' exonuclease activity. The underlined ATG and TTA sequences are the start and stop codons of the GFP gene, respectively.

[3)] and [4)]: The italicized sequences are TSL and TSL/R BH sequences in the pInt2 vector, respectively. Bold letters are the pPICZAAOX1 promoter 5'Bgl II and terminator 3'BamH I flanking sequences required for cloning of the GFP expression gene by the cloning system.

[5)] and [6)]: Sequences located in the AOX 1 promoter 3' and AOX 1 transcriptional terminator 5' regions, respectively.

Conclusion

-A *GFP* expression gene for *Pichia pastoris* was constructed, and it was polymerized up to 5 tandem-repeats in an expression vector to examine the effect and stability of the polymerized expression gene.

-The expression levels of *GFP* in the polymerized-gene-integrated clones increased in accordance with the copy number of the expression gene in the vector.

-The efficiency of electroporation of tandem-gene vectors dropped about 50% in the 5 tandem-repeat constructs (= 12.2 kbp).

-The stability of the polymerized genes upon electroporation decreased in 4 and 5 tandem-repeat constructs, and less or no expressing colonies appeared. However, their *GFP*-expressing patterns indicated that when once integrated properly, those highly polymerized genes were also stably maintained in their genome DNA.

-The results of this study may be effectively used for expressions of foreign genes in *P. pastoris* especially using multiple *in vitro*-constructed genes for more efficient expressions.

Table 2: Electroporation efficacies

Plasmids	**Colonies[1])**
pInt2 GFP 1	345
pInt2 GFP 2	300
pInt2 GFP 3	270[2)]
pInt2 GFP 4	300
pInt2 GFP 5	145[2)]

Pichia pastoris competent cells (8.0 x 10^8 cells were transformed with 10^{12} molecules of each construct linearized with Nru I.)

[1]): Number of colonies appearing on YPDSZ plates.

[2)]: Average of triplicate experiments. Others were conducted once

Table 3: Relative fluorescence intensities of cells transformed with each construct

Plasmids	**Relative fluorescence intensities**						**Total**
	-	+	++	+++	+++ +	+++ ++	
pInt2 GFP 1		16					16
pInt2 GFP 2			16				16
pInt2 GFP 3				16			16
pInt2 GFP 4	7	1			8		16
pInt2 GFP 5	17	2				5	24

Sixteen (pInt2 GFP1-4) and twenty-four (pInt2 GFP5) colonies from each electroporation were picked up, and their GFP expressions were examined on YPMZ plates.

Table 4: Analysis of PCR-amplified DNA fragments in Figure 3

Clone name	Relative intensities of areas [1)]		GPP / AOX1	Relative ratio
	AOX1	GFP		
GS115 (no GFP)	10.0	ND[2)]	-	-
GFP1	6.1	2.9	0.47	1.00
GFP2	8.5	6.6	0.83	1.76
GFP3	10.5	9.0	0.86	1.83
GFP4	10.6	10.1	0.95	2.02
GFP5	8.3	8.6	1.04	2.21

[1)]: The photos of AOX1 and GFP DNA bands amplified from the genome DNA of each clone in Figure 3 were analyzed with Image J (NIH, USA). The intensity of each DNA band is shown in values relative to that of the AOX1 DNA band in GS115 as 10.0.

[2)]: Not detected

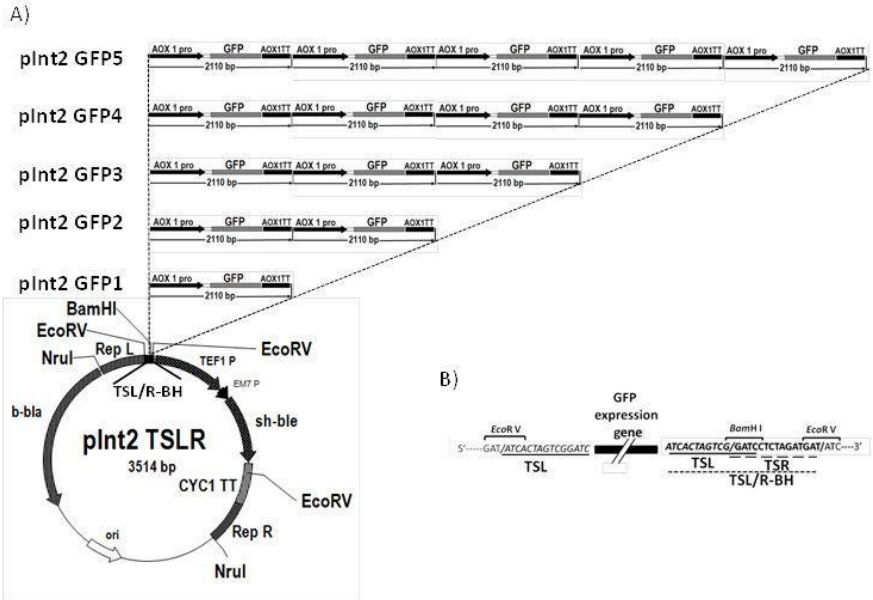

Figure 1: Maps of pInt2 GFP1-5 and sequences at both ends of *GFP* expression gene

A) GFP Expression Vectors for P. Pastoris.

Abbreviations: TSL/R: cloning sequence for tandem-repeat genes, TEF1 P: *TEF*1 promoter, EM7 P: *EM7* promoter for *E. coli* expression, sh-ble: zeocin-resistance gene, CYC TT: transcription termination region,

Rep R: 3' replacement sequence (*AOX*1 promoter 3' region), ori: pUC 18 replication origin, b-bla: ampicillin-resistance gene. AOX 1 pro: *AOX*1 promoter region, GFP: green fluorescent protein gene, AOX 1 TT: *AOX*1 transcription terminator region. Rep L:5' replacement sequence (*AOX*1 promoter 5' region).

B) DNA sequences of TSL and TSL/R-BH at 5' and 3' ends of the *GFP* expression gene.

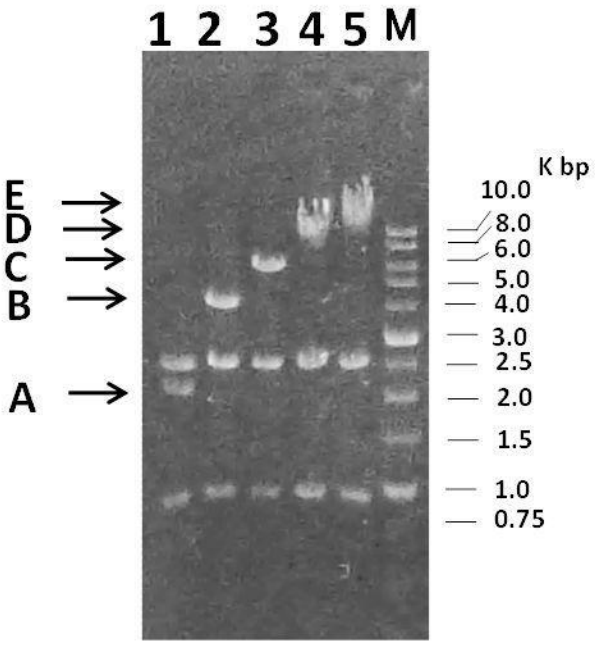

Figure 2: Agarose gel electrophoresis-based analysis of *GFP* expression plasmids

pInt2 GFP1, 2, 3, 4, and 5 were digested with *Eco*R V, and analyzed by 1% agarose gel electrophoresis. Arrows A, B, C, D, and E indicate the *GFP* expression DNA fragments in each plasmid, and contain 1, 2, 3, 4, and 5 copies of the expression construct, respectively.

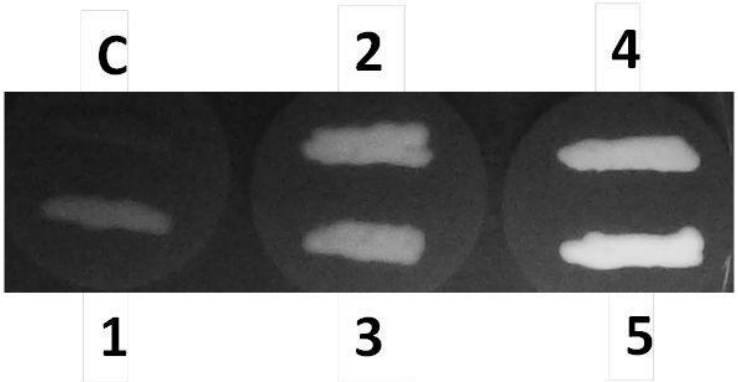

Figure 3: *GFP* expression in *P. pastoris* transformed with pInt2 (GFP) 1, 2, 3, 4, and 5

P. pastoris cells containing each *GFP* expression vector were streaked on disk membrane filters (diameter = 2.3 cm) on a YPD plate, and incubated for 6 hours at 30 ℃. Then, the filters were transferred to a YPM plate, and incubated for 48 hours at 30 ℃. Cells were irradiated with a blue LED (550-nm wavelength), and their fluorescence was recorded with a digital camera through an orange filter.

C: negative control (GS115). 1: pInt2 GFP1. 2: pInt2 GFP2. 3: pInt2 GFP3. 4: pInt2 GFP4. 5: pInt2 GFP5.

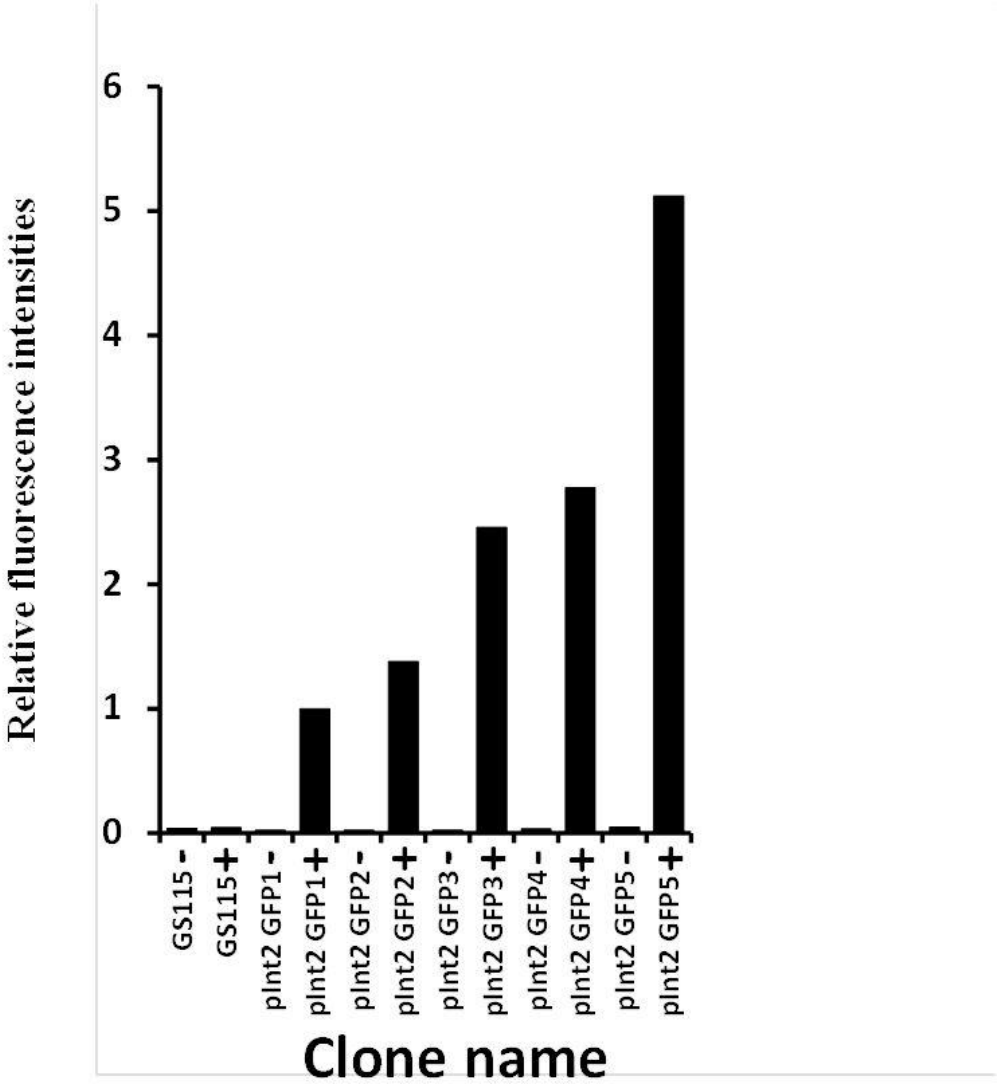

Figure 4: Relative fluorescence intensities of *P. pastoris* transformed with pInt2 GFP 1, 2, 3, 4, and 5

Cells were cultured on YPD (no induction) and YPM (methanol induction) plates at 30℃ for 48 hours, and cell-pellets were collected 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 to 100-fold dilution of the cell suspensions with PBS, and measured in triplicate.
GS115: negative control. -: cells without methanol induction. +: cells with methanol induction. Clones were named after the names of the plasmids used for their electroporation.

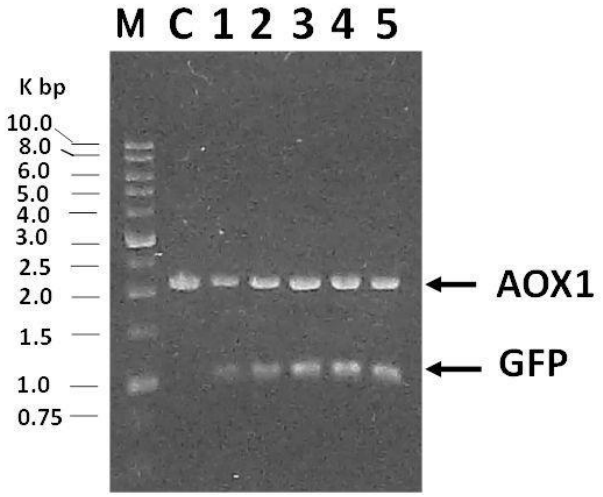

Figure 5: PCR analysis of copy-numbers of the integrated *GFP* gene in cells transformed with pInt2 GFP 1, 2, 3, 4, and 5

*AOX*1 and integrated *GFP* gene fragments were amplified using AOX1 5' F and 3' primers (Table 1) from genomic DNA with colony PCR. The amplified DNA was separated by 1% agarose gel electrophoresis, and visualized with the EZ-vision DNA Stain kit (Amresco, USA). Arrows indicate positions of the *AOX1* and *GFP* DNA fragments amplified by PCR with AOX1 5'F and 3'R primers (Table 1) from each genomic DNA.

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