

# Direct Evaluation of the Effect of Gene Dosage on Secretion of Protein from Yeast *Pichia pastoris* by Expressing EGFP

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Increasing the gene copy number has been commonly used to enhance the protein expression level in the yeast *Pichia pastoris*. However, this method has been shown to be effective up to a certain gene copy number, and a further increase of gene dosage can result in a decrease of expression level. Evidences indicate the gene dosage effect is product-dependent, which needs to be determined when expressing a new protein. Here, we describe a direct detection of the gene dosage effect on protein secretion through expressing the enhanced green fluorescent protein (EGFP) gene under the direction of the  $\alpha$ -factor preprosequence in a panel of yeast clones carrying increasing copies of the EGFP gene (from one to six copies). Directly examined under fluorescence microscopy, we found relatively lower levels of EGFP were secreted into the culture medium at one copy and two copies, substantial improvement of secretion appeared at three copies, plateau happened at four and five copies, and an apparent decrease of secretion happened at six copies. The secretion of EGFP being limiting at four and five copies was due to abundant intracellular accumulation of proteins, observed from the fluorescence image of yeast and confirmed by western blotting, which significantly activated the unfolded protein response indicated by the up-regulation of the BiP (the KAR2 gene product) and the protein disulfide isomerase. This study implies that tagging a reporter like GFP to a specific protein would facilitate a direct and rapid determination of the optimal gene copy number for high-yield expression.

**Keywords:** Copy number, EGFP, qPCR, *Pichia pastoris*

## Introduction

The yeast *Pichia pastoris* has been widely used as a host organism for expressing heterologous protein for decades. Compared with prokaryotic expression systems, *P. pastoris* has many advantages such as protein processing, folding, and post-translational modification, which can help the protein function correctly. Additionally, compared with mammalian expression systems, *P. pastoris* can grow and be easy to scale-up in inexpensive growth media [7]. More importantly, under the control of the strong, tightly regulated methanol-inducible alcohol oxidase (AOX1) promoter, extremely high-level production of recombinant proteins can be achieved. All of these characteristics make *P. pastoris* a useful protein expression system. Although a variety of heterologous proteins have been successfully

expressed using *P. pastoris*, many other proteins were expressed at significantly lower levels [3]. To improve the heterologous protein production level, several genetic factors could be considered. These are codon usage of the expressed gene, the characteristic of signal peptide, copy number of the integrated transgenes, AT composition of cDNA, and mRNA transcriptional and protein translational blocks [4]. Increasing studies have shown that the dosage of expressed gene has an important role on the recombinant protein expression level. Generally, increasing the foreign gene copy number to a certain extent could improve the production of the recombinant protein in *P. pastoris* [13]. The expression level of pandemic H1N1HA was elevated in proportion to increase in copy number, up to six [1]. Multiple gene copy number could improve the secretion of insulin precursor, and the production level increased from

19 to 250 mg/l for the strain including 11 copies of transgene [8]. However, over-increasing the copy number of target gene could lead to a decline of target protein expression level. When the copy number was higher than 12, the expression level of porcine insulin precursor dramatically decreased. The 52-copy strain's production level was not higher than the 3-copy strain [16]. It is noticeable that the effect of gene dosage on heterologous protein expression and secretion was not directly observed from yeast in most studies. Therefore, here, we show a direct visualization of gene dosage effect on protein secretion through a panel of *P. pastoris* clones carrying increasing copies of the enhanced green fluorescent protein (EGFP) gene.

Green fluorescent protein (GFP), cloned from the jellyfish *Aequorea victoria*, exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range [10]. Owing to its stability, high sensitivity, non-biological toxicity, fluorescence reaction without any exogenous reaction substrates, and non-species or tissue specificity, GFP has become a unique tool that enables direct visualization of structures and processes in living cells and organisms [2, 9]. The fluorescence of GFP can be easily measured by fluorescence microscopy or fluorescence-activated fluid spectrophotometry.

In this study, we successfully identified yeast transformants carrying up to a maximum of six copies of the EGFP transgene by screening on an increasing concentration of Zeocin and SYBR green-based quantitative real-time PCR approach. The gene dosage effect on heterologous protein expression and secretion was directly visualized under fluorescence microscopy, and quantified by measuring fluorescence intensity using spectrophotometry. Furthermore, qRT-PCR and Western blotting were performed to confirm the fluorescence analysis results.

## Materials and Methods

### Construction of the Expression Vector

The coding sequence of the EGFP gene with *EcoRI* and *XbaI* sites on each terminus was amplified from pEGFP-N1 (Clontech, USA) using a pair of primers designated as  $\alpha$ -EGFP F and R (Table 1), and then cloned upstream of the His tag in the yeast expression vector pPICZ $\alpha$ A (Invitrogen, CA, USA) to generate the recombinant plasmid pPICZ $\alpha$ -EGFP.

### Transformation of *P. pastoris* and Screening for Multicopy Transformants

Prior to electroporation, approximately 10  $\mu$ g of plasmid pPICZ $\alpha$ -EGFP was linearized with the *SacI* nuclease, and then pulsed into *P. pastoris* X33 competent cells using a Gene Pulser

**Table 1.** List of primers used in this study.

Primer pair	Sequence 5'–3'	Length of amplicon (bp)
$\alpha$ -EGFP F	GAATTC GTGAGCAAGGGCGAGGA	156
$\alpha$ -EGFP R	TCTAGA GCCTTGTACAGCTCGTCCA	
5' AOX1q F	TCCACAGGTCCATTCTCACACA	154
5' AOX1q R	GCTCCAATCAAGCCCAATAACT	
MET2 F	CGTTCTCGCAACTCTTTTCGAA	100
MET2 R	CAATGGCATCAGTTATGACGGAAG	
KAR2	CACTTGGGTGGTGAGGACTT GGCCTTTTCGACCTCTCTCT	132
PDI	GCCGTTAAATTCGGTAAGCA TCAGCTCGGTCACATCTTTG	145

Xcell Electroporation System (Bio-Rad, USA) at 2,000 V and 4.9 ms. Positive transformants were screened on YPDS (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 1 M sorbitol, and 2% agar) containing 100  $\mu$ g/ml Zeocin. Zeocin-resistant transformants were selected for analysis to determine the presence of insert using PCR. Transformants confirmed by PCR were grown overnight in YPD for preparation of competent cells, and used for a second round of electroporation. The transformation mix was then spreaded on YPD plates containing 1,000 or 2,000  $\mu$ g/ml Zeocin for direct selection of potential multicopy recombinants. A pair of primers (5'AOX1 and 3'AOX1) designed to amplify the inserted fragment was used (Table 1). The amplification program was as follows: denaturing for 5 min at 95°C, followed by 32 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2 min, and a final round of elongation at 72°C for 10 min.

### Copy Number Quantification by qPCR

To screen the recombinant clones harboring different copy numbers of the transgenes in positive transformants, a quantitative real-time PCR (qPCR) method was performed [12]. Briefly, the genomic DNA of *P. pastoris* X33 was diluted 1:10 five times. The Ct values of the *AOX1* gene and homoserine-*O*-transacetylase gene (*MET2*) were detected in triplicate in each dilution. Then, the logarithm of the relative template concentration was plotted against the mean of the Ct values. Finally, a standard curve was generated by linear regression of the plotted points. Through single crossover, the pPICZ $\alpha$ A expression cassette can be inserted into the genome of *P. pastoris*, which will add an additional AOX1 promoter into the genome. In this way, the inserted-gene copy

number could be detected by comparing the total number of copies of the *AOX1* promoter with those of the *MET2* gene, which was a single copy within the genome of *P. pastoris*. The primers used for the qPCR are listed in Table 1. The qPCR was performed in a LightCycler 480 (Roche, Basel, Switzerland) under the following program: one cycle of denaturing at 95°C for 10 min, 40 cycles of denaturing at 94°C for 20 sec, and annealing at 60°C for 20 sec.

### Shake-Flask Culture

Colonies of transformants with different copy numbers grown on YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) plates were inoculated into 100 ml shake flasks containing 25 ml of BMGY (1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) glycerol, 1.34% yeast nitrogen base,  $4 \times 10^{-5}$ % biotin, 100 mM potassium phosphate (pH 6.0)) culture medium with shaking at 28°C and 275 rpm until the optical density reached 20 ( $OD_{600}$ ). Then the cells were centrifuged at 4,000  $\times g$  for 5 min at room temperature and resuspended to an  $OD_{600}$  of 1 in 25 ml of BMMY (1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) methanol, 1.34% YNB,  $4 \times 10^{-5}$ % biotin, 100 mM potassium phosphate (pH 6.0)) medium and then were shaken at 275 rpm. During continuous culture of the cells, methanol was supplemented to a concentration of 0.5% (v/v) in BMMY medium every 24 h to maintain the induction during continuous seven days of expression. Then 300  $\mu$ l aliquots of supernatant were collected every 24 h to measure the culture's optical density at 600 nm and analyze the EGFP expression levels.

### Real-Time RT-PCR

The total RNA was extracted from the induced transformants using the hot phenol method [6]. Briefly, RNA was extracted with hot phenol equilibrated in water and precipitated with 3 M sodium acetate and ethanol. The isolated RNA was treated with DNase I (TaKaRa, Japan) and purified by a phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation. RNA integrity was checked on 1.2% agarose gels and quantified using NanoDrop (Thermo Scientific, USA). After heating at 85°C for 10 min to denature RNAs and inactivate RNase, 400 ng of total RNA was subjected to reverse transcription using the PrimerScript RT-PCR Kit (TaKaRa, Japan). The reaction was terminated by heating at 70°C for 10 min.

The 10  $\mu$ l PCR system contained 100 ng of cDNA, 0.2  $\mu$ l of primers designed to amplify *EGFP*, *KAR2*, *PDII*, and *MET2* (Table 1), and 3.6  $\mu$ l of ddH<sub>2</sub>O. The reaction was performed under the following program: denaturing at 94°C for 5 min, 40 cycles of denaturing at 94°C for 20 sec, annealing at 60°C for 20 sec, and elongation at 72°C for 20 sec. All reactions were run in triplicate. The expression of integrated gene relative to the internal reference gene *MET2* was determined by the 2<sup>- $\Delta$ CT</sup> method.

### Western Blot Assay

Recombinant proteins in 20  $\mu$ l of supernatant were separated on

10% (w/v) SDS-polyacrylamide gels and visualized through Coomassie Brilliant Blue R-250 staining. To analyze the intracellular EGFP, the total intracellular proteins were harvested from different copy number transformants and quantified using a BCA protein assay kit. Then 40  $\mu$ g of protein was loaded onto the wells of SDS-polyacrylamide gels for electrophoresis.

The separated proteins on SDS-polyacrylamide gels were transferred to PVDF membranes (Bio-Rad, USA) at 18 V for 50 min and the membrane was blocked using 5% (w/v) skimmed milk powder in TBS for 1.5 h at room temperature, and then incubated overnight at 4°C with a mouse anti-6 $\times$  His-tag polyclonal primary antibody (1:1,000 dilution) (Abcam, UK) and a goat anti-mouse IgG (1:4,000 dilution) secondary antibody conjugated with horseradish peroxidase (HRP). The protein blotting signal was visualized through the catalysis of enhanced chemiluminescence (ECL) reagent (Thermo, USA) with HRP.

### Fluorescence Measurements

Aliquots of 100  $\mu$ l supernatant from cultures of different copy number transformants were transferred into 96-well plates to measure GFP fluorescence using a BioTek Synergy2 with excitation at 485 nm and maximum emission at 528 nm. Samples of transformed yeast cells were centrifuge at 4,000  $\times g$  for 5 min at 4°C, and then the pallets were washed three times with PBS and finally resuspended with PBS [14]. Intracellular EGFP fluorescence was determined by the BioTek Synergy2. In addition, the yeast cells expressing EGFP were examined under a fluorescence microscope.

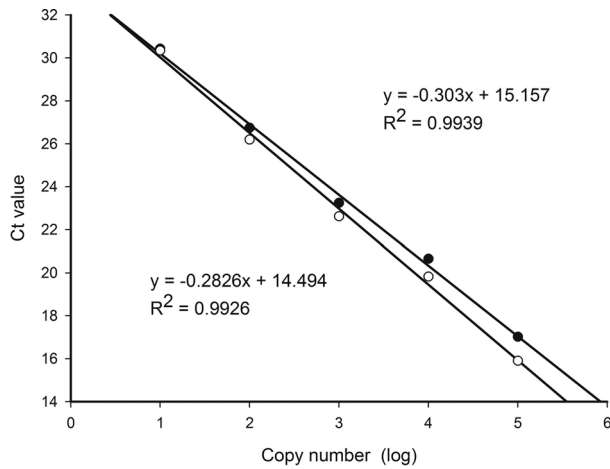
### Statistical Analysis

Quantitative data are presented as the means  $\pm$  SE. Statistically significant differences were evaluated by one-way ANOVA and the Holm-Sidak *post hoc* test for multiple comparisons using SPSS (ver. 16.0).

## Results

### Copy Number Determination

To detect the relationship between the copy number of integrated EGFP gene and the expression level of EGFP protein, positive transformants were first identified by PCR analysis. Next, a real-time quantitative PCR method was employed to quantify the copy number of transgenes within positive transformants. Fig. 1 indicates that reliable standard curves for the *MET2* gene and the *AOX1* promoter were produced by quadruple independent qPCRs ( $R^2 > 0.99$ ). Because the *MET2* gene and the *AOX1* promoter are both single-copy in *P. pastoris*, the insertion of the pPICZ $\alpha$ A expression construct will generate one additional copy of the *AOX1* promoter in the genome. Thus, the transgene copy number can be easily deduced from the copy number ratio of the *AOX1* promoter to the *MET2* gene in each



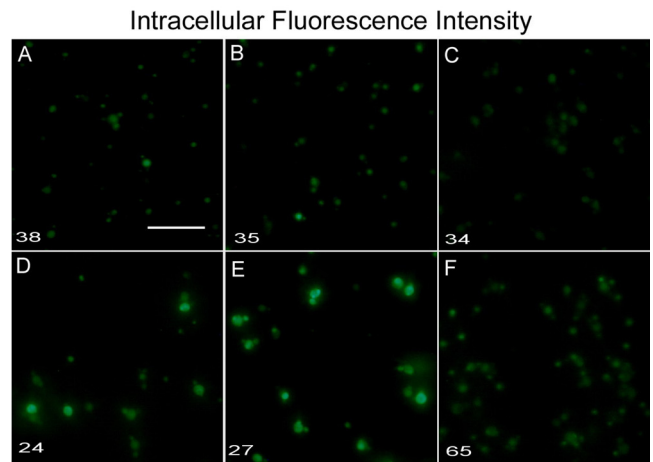
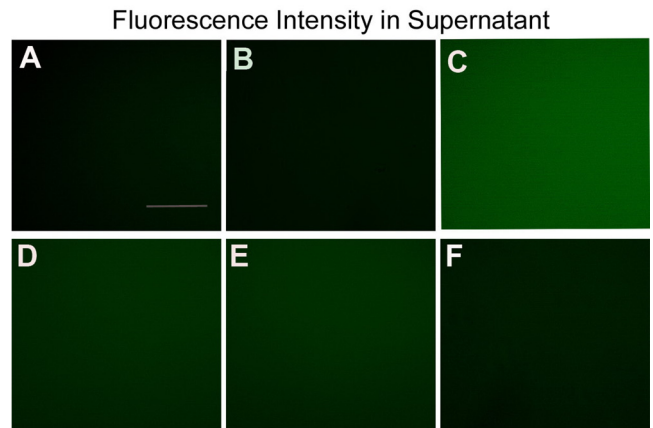
**Fig. 1.** Standard curves for determining the copy numbers of the *AOX1* (●) promoter (○) and the *MET2* gene.

A 1:10 consecutive dilution of genomic DNA from *Pichia pastoris* X33 was arbitrarily assigned to harbor  $10^1$  to  $10^5$  copies of the *MET2* gene and the *AOX1* promoter. Each dilution was amplified by qPCR using *AOX1* and *MET2* primer pairs. For each gene, the mean of the Ct value from quadruple qPCR analysis was plotted against the corresponding logarithm of copy number. A standard curve was then drawn by linear regression through these points.

**Table 2.** Quantification of EGFP gene copy number within positive transformants.

EGFP		EGFP	
Transformant	Copy number	Transformant	Copy number
1	5.90	13	3.20
2	4.04	14	1.13
3	1.25	15	2.29
4	4.69	16	4.09
5	1.60	17	5.74
6	2.66	18	3.63
7	3.14	19	2.97
8	0.99	20	4.94
9	4.28	21	3.84
10	1.93	22	4.33
11	4.53	23	4.99
12	2.01	24	0.96

transformant, as calculated from the standard curves. Finally, 24 positive transformants grown on YPD plates containing 1,000 or 2,000  $\mu\text{g}/\text{ml}$  zeocin were determined to harbor integrated gene ranging from one copy to six copies using real-time quantitative PCR (Table 2).

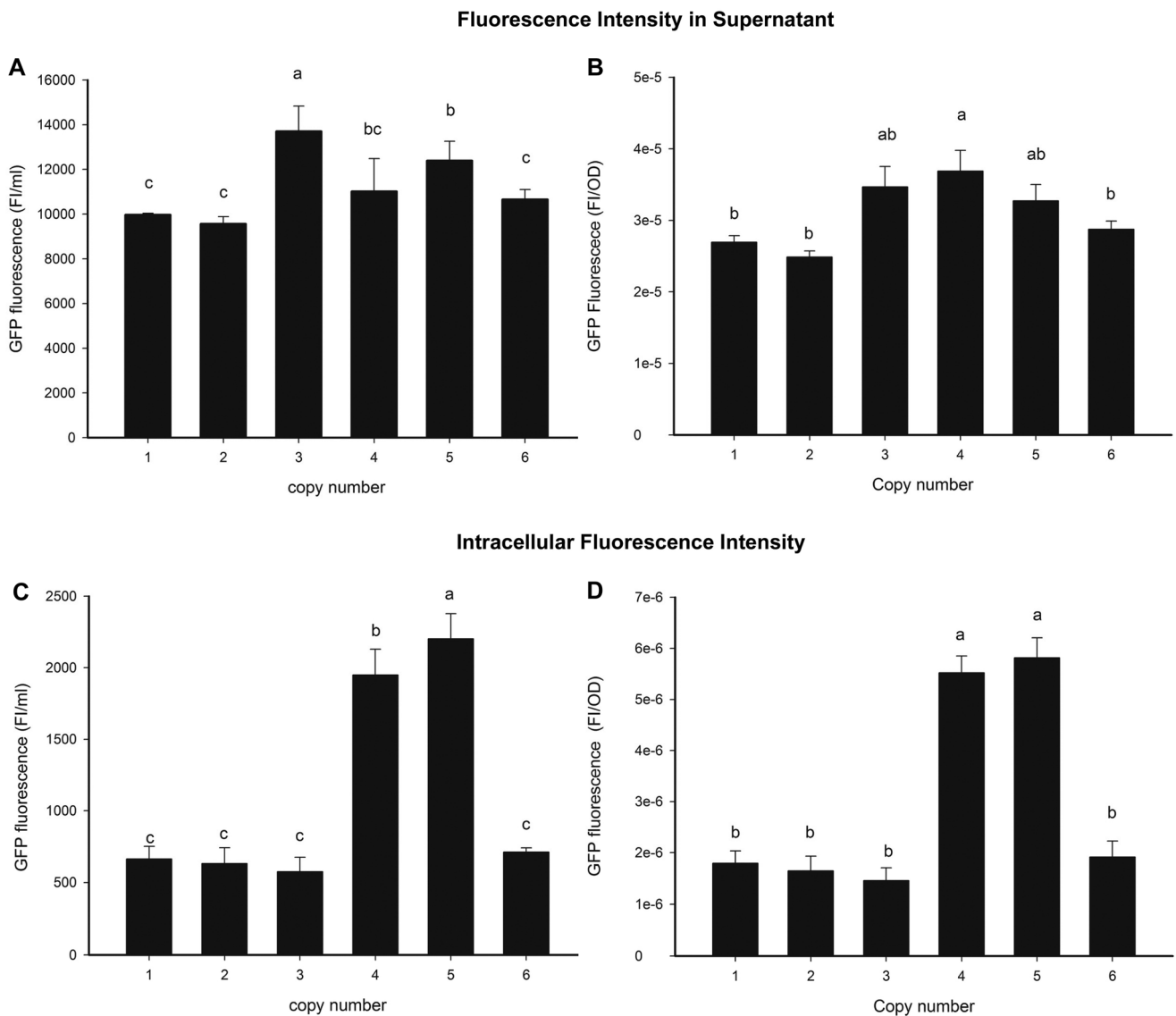


**Fig. 2.** Direct observation of gene dosage effect on EGFP secretion and intracellular accumulation, on the fifth day after methanol induction, through fluorescence microscopy.

A-F stand for transformants that contain transgene from one copy to six copies. The number of yeast cells observed in each case is presented at the lower left corner. Scale bar = 100  $\mu\text{M}$ .

#### Effect of Gene Dosage on Secretion of EGFP from Yeast

Under the control of the  $\alpha$ -factor preprosequence, EGFP was successfully secreted into culture medium. We can directly observe the effect of gene dosage on secretion efficiency by detecting fluorescence intensity in the medium under microscopy. Obviously, EGFP fluorescence varied significantly in the medium of strains with different gene copy numbers (Fig. 2). The fluorescence was very faint at one copy and two copies, and most bright at three copies. However, the fluorescent signal attenuated at four and five copies, and reduced sharply at six copies. Quantification of the fluorescence intensity showed similar results except for a relatively lower value measured from the medium of strains with two copies of transgene (Fig. 3). Examination of the intracellular fluorescence signal showed that the



**Fig. 3.** Quantification of EGFP fluorescence intensity in the supernatant (A and B) and intracellular compartment (C and D) of strains with different copy numbers of transgene on the fourth day after methanol induction.

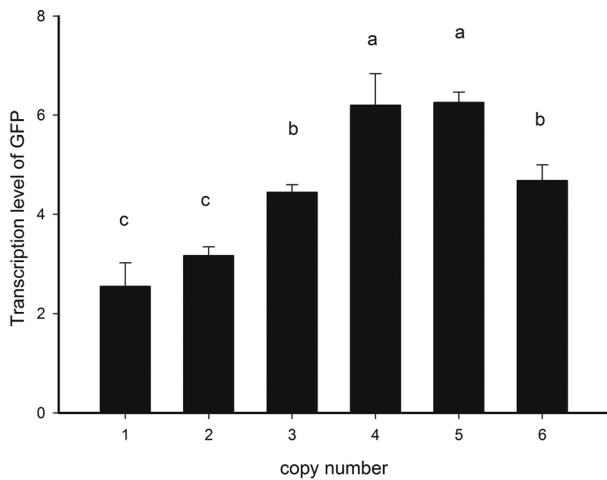
Mean expression levels at different copy numbers were normalized by culture density ( $OD_{600}$ ) (B and D). The value is presented as the mean  $\pm$  SE ( $n = 3$ ). The same letter above the bars indicates the means of expression levels are grouped together.  $P = 0.05$  was used as the level of significance.

most bright GFP was observed within strains with four or five copies of transgene (Fig. 2). Quantification analysis showed a result consistent with microscopic images (Fig. 3). This indicated that abundant GFP was retained in strains with four or five copies of transgene.

To investigate the effect of gene dosage on the GFP secretion level caused by the difference in the transcriptional level in strains with different gene copy numbers, we used the real-time RT-PCR to detect the mRNA synthesis efficiency of EGFP within these strains. The results indicated that the

transcriptional level of EGFP increased gradually from one copy to three copies, peaked at four and five copies, and declined at six copies (Fig. 4). The effect of gene dosage on transcription level was generally correlated to secretory efficiency (Fig. 3B).

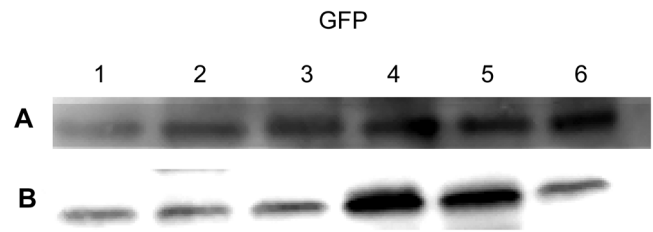
We further carried out Western blot analysis to determine the content of GFP secreted into the medium from strains with different gene copy numbers. To our surprise, the amount of secreted GFP increased gradually from one copy to six copies, where especially extensive blotting appeared



**Fig. 4.** The transcription levels of EGFP gene in strains with different gene copy numbers on the fifth day after methanol induction.

Each qPCR analysis was repeated in triplicate. The value is presented as the mean  $\pm$  SE ( $n = 3$ ). The same letter above the bars indicates the means of expression levels are grouped together.  $P = 0.05$  was used as the level of significance.

at six copies (Fig. 5A). This was inconsistent with the results of the fluorescent images and qRT-PCR analysis. However, the amount of intracellular GFP retained was well correlated with the results of the fluorescent images. Much more abundant GFP was observed in strains with four and

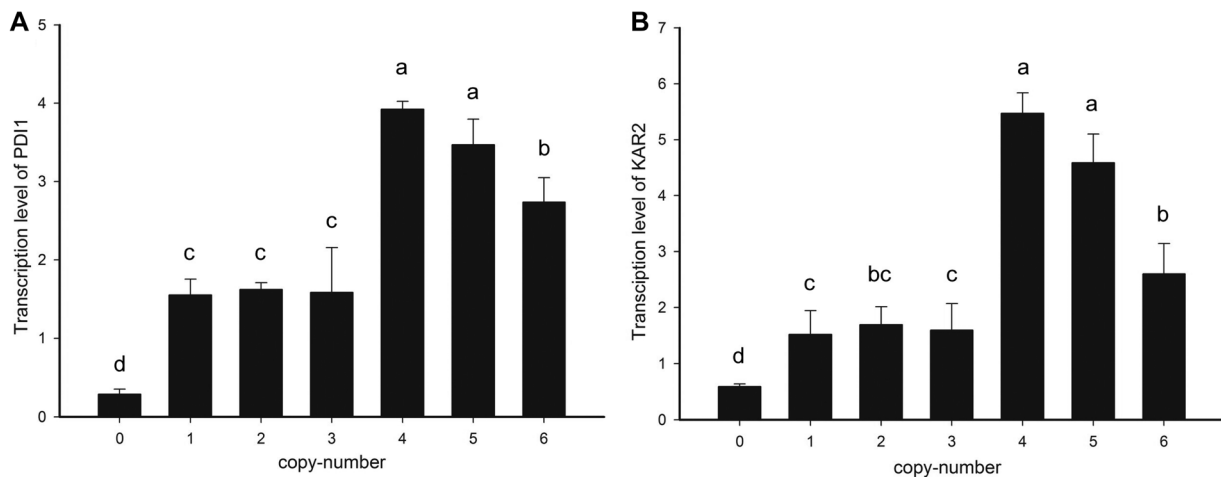


**Fig. 5.** Western blot analysis of the secretion of recombinant EGFP into the supernatant (A) and intracellular recombinant EGFP (B) on the fourth day after methanol induction.

five copies of transgene, and a similar amount of GFP was observed among strains with one copy, two copies, three copies, and six copies of transgene (Fig. 5B).

#### Effect of Gene Dosage on Transcriptional Levels of Unfolded Protein Response Related Genes

The unfolded protein response (UPR) can be triggered by accumulation of overexpressed heterologous proteins in the endoplasmic reticulum (ER) in eukaryotic cells. It was obvious to test whether the retention of GFP was due to folding problems in the ER by measuring the BiP (the *KAR2* gene product) and the protein disulfide isomerase (PDI) concentration in the cells as typical UPR signals [17]. Compared with wild-type yeast, the induction of foreign protein expression in transformants with any copy number



**Fig. 6.** The transcription levels of UPR-related gene *PDI* (A) and *KAR2* (B) in strains with different copy numbers on the fourth day after methanol induction.

The expression levels of tested genes were normalized relative to the internal reference gene *MET2*. Each qPCR analysis was repeated in triplicate. The value is presented as the mean  $\pm$  SE ( $n = 3$ ). The same letter above the bars indicates the means of expression levels are grouped together.  $P = 0.05$  was used as the level of significance.

of transgene significantly up-regulated both PDI and KAR2 transcription levels (Fig. 6). PDI and KAR2 expression levels increased to a similar extent among strains with one, two, and three copies of transgene, peaked in strains with four copies of transgene, and gradually reduced in strains with five and six copies of transgene. This result corresponded to the results observed by Western blot and fluorescent images, which suggests a more abundant GFP retention triggered more severe UPR.

## Discussion

Gene copy number has been proved to be a critical parameter of improving heterologous proteins expression levels in yeast *P. pastoris*. Substantial examples have indicated that increase of gene copy number to a certain extent can significantly increase productivity [15]. However, the correlation between copy number and secretion level usually was not directly observed in most studies. In our study, by expressing GFP, we can directly observe that a positive correlation between copy number and secretion level of biologically active protein existed from one to three copies per cell, bottleneck happened from four to five copies accompanied by significant intracellular accumulation of GFP, and turning to a negative correlation at six copies (Fig. 2). Our observation is different from several studies, which found a linear correlation between copy number and expression up to a relatively higher upper limit (between 12 and 19 copies per cell) [13, 16], but similar to that observed in one study that found an increase of the copy number above two resulted in a decrease of secretion of human trypsinogen [5]. This indicates that the effect of gene dosage on expression level varies significantly from one protein to another protein. Therefore, to improve the expression level of a specific protein in yeast, the optimal gene copy number cannot be directly adopted from other proteins. Thus, we suggest that tagging a reporter like GFP to a specific protein would facilitate a fast and direct determination of the optimal gene copy number for high-yield expression.

An obvious effect of increasing the gene dosage was an increase in the transcriptional level of GFP from one copy to four copies, and a further increase of gene dosage brought about a transition into decrease of transcription (Fig. 4). The limitation of further improvement of the transcriptional level above five copies can be attributed to the activation of UPR. The expression levels of *KAR2* and *PDI* were substantially up-regulated at four and five copies strains (Fig. 6), accompanying the most abundant intracellular

accumulation of GFP (Fig. 2), which indicated a strong activation of UPR in the yeast, which in turn decreased the biosynthetic burden of the secretory pathway by down-regulating the expression of GFP. Therefore, a decreased transcriptional level of GFP was observed at six copies (Fig. 4) accompanying an alleviated UPR (Fig. 6).

The discrepancy between the relatively high-level secretion of GFP from six copies strains detected by Western blot and the relatively low level of fluorescence intensity observed under fluorescent microscopy (Fig. 2) and measured by spectrophotometry (Fig. 3) is unexpected. We speculated a proportion of the GFP was non-functional. One possible explanation is that deprivation of chaperones may happen in six copies strains owing to extensive synthesizing of GFP, which would result in the release of a non-functional protein into the extracellular medium, as ER-mediated membrane translocation occurs in an unfolded state [11]. It will be worthwhile to investigate in the future if the increase of gene dosage can be directly linked to decreased secretion of functional products.

Taken together, it is obvious that ER stress can prevent the improvement of heterologous protein expression levels by increasing the copy number. Our work showed a simple and direct way to examine this bottleneck for optimization of recombinant protein secretion.

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