Chiang Mai J. Sci. 2018; 45(7) : 2554-2565
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Unfolded Protein Response (UPR) Induced Hybrid Promoters for Heterologous Gene Expression in *Pichia pastoris*

Niwed Kullawong [a], Sutipa Tanapongpipat [b], Lily Eurwilaichitr [b] and Witoon Tirasophon* [a]

[a] Institute of Molecular Biosciences, Mahidol University, Salaya, Nakhon Pathom, 73170, Thailand.

[b] BIOTEC Central Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani, 12120, Thailand.

* Author for correspondence; e-mail: witoon.mu@gmail.com

Received: 24 March 2017
Accepted: 7 June 2017

ABSTRACT

Inducible promoters constitute a powerful tool in controlling gene expression of heterologous proteins in *Pichia pastoris*. To expand a native promoter activity, we created an inducible hybrid promoter consisting of a constitutive transcription elongation factor 1 (*TEF1*) promoter (P_{TEF1}) and a 22-bp multimer of unfolded protein response element (UPRE) from *Saccharomyces cerevisiae KAR2* gene. The four copies of the UPRE sequence were located immediately upstream to the P_{TEF1}. The activity of the hybrid promoter for driving expression of green fluorescent protein (GFP) was three to four times higher than its native counterpart without any chemical supplement. Upon adding dithiothreitol (DTT) supplement, the promoter activity increased an additional three-fold within nine hours. The activity was also effective with recombinant human erythropoietin (EPO) expression. Hence this platform may have applications in metabolic engineering, where it can be used as an alternative system for fine-tuning gene expression of heterologous protein in *P. pastoris*.

Keywords: *Pichia pastoris*, inducible gene expression, hybrid promoter, unfolded protein response element (UPRE), endoplasmic reticulum stress

1. INTRODUCTION

Heterologous gene expression in methylotrophic yeast, *Pichia pastoris*, is an outstanding system for producing recombinant proteins in large quantity. This is possible because of the following properties: efficient protein synthesis, good secretion capacity and ability to grow at very high cell density in affordable and simple media[1]. Hence this expression system is very attractive for biotechnology applications including metabolic engineering for high value biochemical products [2]. The success rate in heterologous gene expression in *P. pastoris* greatly depends on the efficiency of the expression system [3]. However the platform for gene expression in *P. pastoris* is constrained to only certain designs, which limit promoter and overall

expression system usage. Two major types of promoter have been utilized in *P. pastoris* expression vectors both of which have been isolated from its native genes: (i) constitutive promoters offering dynamic expression efficiency and (ii) inducible promoters that offer well defined expression output through specific inducers.

Constitutive promoters are usually obtained from isolation of native promoters and can drive transcription constitutively with relatively constant level of gene expression and no requirement for specific inducer or media formulation. However, this type of promoter is not suitable for expressing toxic proteins or enzymes. Many robust constitutive promoters (P_{GAP}, P_{ENO1}, P_{GPM1}, P_{HSP82}, P_{ILV5}, P_{KAR2}, P_{KEX2}, P_{PET9}, P_{PGK1}, P_{SSA4} and P_{TEF1}) express high level of heterologous proteins in *P. pastoris* [3]. Commonly used constitutive promoters include the glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}) [4], translation and elongation factor 1 promoter (P_{TEF1}) [5], and phosphoglycerate kinase promoter (P_{PGK1})[6]. Constitutive promoters exhibit varying efficiency due to different regulation mechanisms and culturing conditions.

Inducible promoters (P_{AOX1}, P_{AOX2}, P_{DAS}, P_{FLD1}, and P_{PHO89}), on the other hand, offer a well-defined method to control gene expression through addition of a small molecule inducers [3]. The best known inducible promoter in *P. pastoris* is the alcohol oxidase I (P_{AOX1}) [7]. P_{AOX1} activity is completely repressed in media containing carbon sources like glucose and glycerol. Upon switching to media with methanol as a sole carbon source, P_{AOX1} activity switches on allowing for continuous transcriptional induction of the heterologous gene at high level. Nonetheless, under certain circumstances, a wider range of promoter types may be required to allow for flexibility of gene expression. Genes in the unfolded protein response (UPR) pathway provide good examples. Specific signaling cascades modulate these genes, allowing them to fine-tune their expression and maintain homeostasis in the endoplasmic reticulum (ER) [8].

The UPR has been shown to play important roles in overexpression and secretion of some recombinant proteins [9]. Manipulating the level of transcription factors and ER chaperones in the UPR pathway improved secretion of recombinant proteins [10, 11]. An important characteristic of the UPR is that it is directly linked to transcription activation processes governed by ER stress conditions. In *Sachcharomyces cerevisiae* this process is mediated by a unique mechanism involving cooperative action of Hac1 transcription factor (TF) and a short conserved DNA sequence referred to as unfolded protein response element (UPRE) in the promoter of UPR target genes [12]. The UPRE regulatory *cis* acting element lies within 22-bp upstream of the promoter of UPR responsive genes, which is crucial for transcriptional induction under ER stress [13]. The best characterized UPRE core sequence was UPRE-1 (**CA**N**C**N**TG**) from *S. cerevisiae* (for examples from *KAR2*, **CA**G**C**G**TG** and *PDI1*, **CA**C**C**G**TG**) [13, 14]. The similar UPRE-1 is also found in the promoter region of the *P. pastoris KAR2* (**CA**G**C**G**TG**), *INO1* (**CA**A**C**T**TG**) and *HAC1* (**CA**A**C**T**TG**) genes [15]. The presence of an *HAC1* UPRE implies that Hac1p can up-regulate its own transcription. Unconventional splicing of *HAC1* mRNA after ER stress signaling generates the active form of basic leucine zipper (bZIP) transcription factor Hac1p, which binds to the UPRE [16]. Since the UPR pathway is conserved in all eukaryotic species, *P. pastoris*, possesses a similar

transcriptional activation cascade. Therefore, developing an alternative gene expression platform that combines high basal activity from a constitutive promoter with inducible properties would be highly desirable.

In this study, we mimicked ER stress conditions to develop an alternative platform for gene expression in *P. pastoris*. By combining the constitutive promoter P_{TEF1} of *P. pastoris* with 22-bp UPRE repeating units from *KAR2* gene of *S. cerevisiae*, we demonstrated that the hybrid promoter exhibited high basal promoter function for heterologous expression and was further induced upon challenging with an ER stressor in modulating heterologous gene expression in *P. pastoris*.

2. MATERIALS AND METHODS

2.1 *Escherichia coli* and *P. pastoris* Strains

E. coli strain DH5α [*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsd*R17] was used as the host strain in all cloning and plasmid propagation. Bacteria harboring the desired plasmid were grown in low salt Luria-Bertani (LB) broth [1% (w/v) tryptone, 1% (w/v) yeast extract and 0.5% (w/v) NaCl] at 37 °C. *E. coli* transformation was carried out by a standard method [17]. *E. coli* strains transformed with plasmid pGEM-T backbone (Promega) were selected under 100 µg/ml amplicilin while the strain under pPICZ backbone were selected under 25 µg/ml Zeocin (Invitrogen). *P. pastoris* KM71 (*his4, arg4, aox1Δ::ScARG4*) (Invitrogen) was used for all recombinant protein expression experiments. The yeast strain was cultured in yeast peptone dextrose (YPD) medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose] containing with 100 µg/ml Zeocin (Invitrogen) at 30 °C until the desired cell density or time point.

2.2 Recombinant Plasmids Construction

2.2.1 Green fluorescent protein reporter plasmid

A reporter plasmid with yeast-enhanced UV-excitable green fluorescent protein (GFP) under constitutive *TEF1* promoter (P_{TEF1}) was constructed as follows. The GFP cDNA was amplified from pKT150 plasmid (EUROSCARF) using yESapF and yESapR primers and cloned into pGEM-T vector (Promega). The reactions were set up in 1X Phusion™ HF buffer, 200 µM each of dNTPs, 0.2 µM each of primers, 0.02 U/µl of Phusion DNA polymerase (Finnzymes) in total volume of 50 µl. The reaction was amplified with initial denaturing at 94 °C for 2 min, followed by 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 47 °C for 30 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 2 min. The fragment was excised from the plasmid with *Eco*RI digestion then subcloned into pPICZαA (Invitrogen). P_{TEF1}, position -602 to -1 relative to its translational start site, was amplified from *P. pastoris* genomic DNA using pTef1F and pTef1R primers. The PCR fragment was digested with *Hin*dIII and *Nhe*I and cloned into *Hind*III/*Spe*I sites upstream of the GFP gene in the pPICZαA plasmid backbone (Invitrogen). Then a 0.6 kb fragment of CDS1067 locus of *P. pastoris* GS115 genome (currently designated as PAS_Chr1-4_0291 on NCBI Ac. FN392319.1) was PCR amplified with 1067F and 1067R primers as previously described and with cycling condition at annealing temperature of 50 °C. The CDS1067 fragment was cloned into the reporter plasmid at *Nsi*I/*Hin*dIII sites upstream to P_{TEF1} to serve as the integration site to the yeast genome. Finally an extra *Xho*I site in the plasmid was eliminated by *Sfi*I and *Sac*II digestion then joined by blunt end ligation resulting in P_{TEF1}-GFP. To construct

a GFP reporter plasmid under P_{TEF1} and UPRE, P_{TEF1}-GFP was digested with *Xho*I then randomly ligated with oligonucleotide duplexes containing UPRE sequence ($UPRE_{KAR2}$ or $UPRE_{PDI1}$) from *S. cerevisiae* (Figure 1). All restriction enzymes were from New England Biolab. Synthetic oligonucleotides including primers and oligonucleotides were custom synthesized by Tech Dragon Limited, Hong Kong. The copy number of the UPRE sequence and their arrangement in the plasmids were determined by restriction endonuclease digestion and automated DNA sequencing (Tech Dragon, Hong Kong).

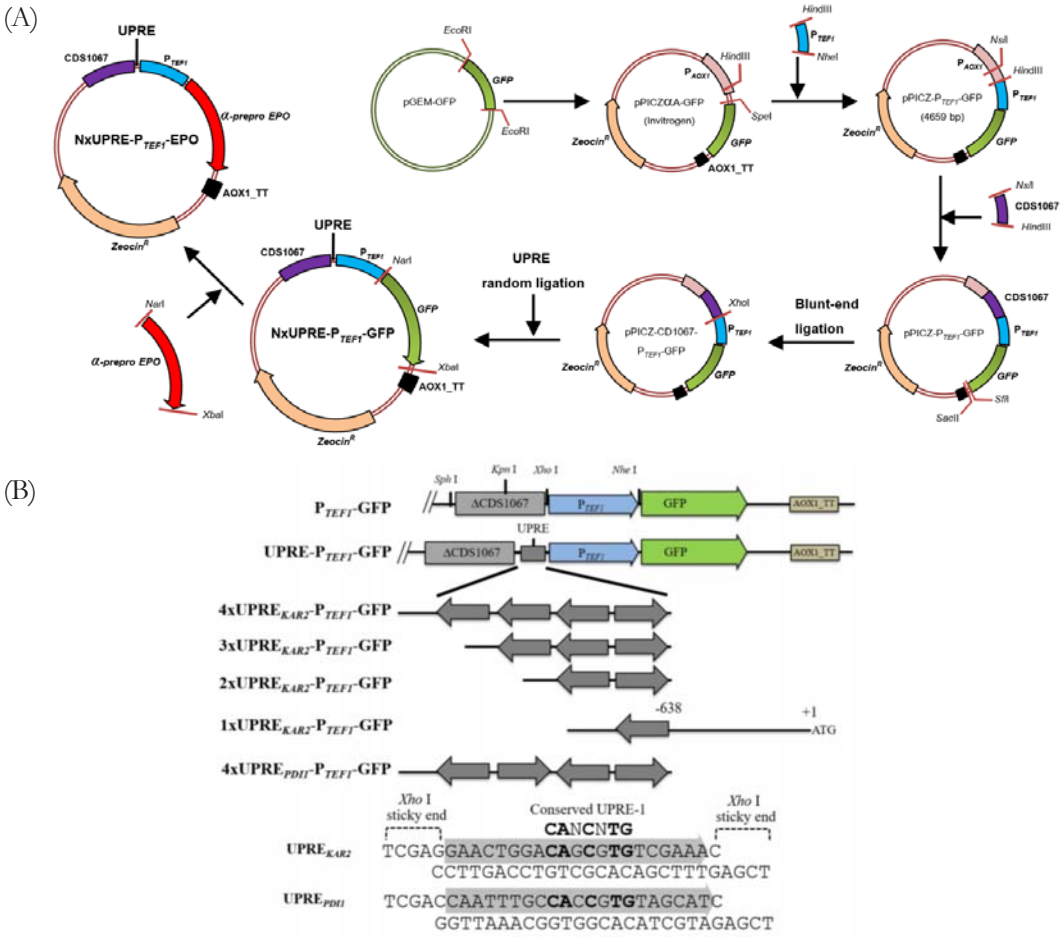

Figure 1. Schematic of UPR inducible expression cassettes with GFP reporter for *P. pastoris* with and without UPRE multimers. (A) Cloning strategy is shown: GFP, green fluorescent protein (yESapphire); P_{AOX1}, *AOX1* promoter; P_{TEF1}, *TEF1* promoter; $Zeocin^R$, drug selectable marker (*Sb Ble*) for Zeocin (Invitrogen); AOX1_TT, *AOX1* transcriptional terminator; CDS1067, PAS_Chr1-4_0291 on NCBI Ac. FN392319.1; α-prepro EPO, human EPO fused with leader sequence for secretion in *P. pastoris*. UPRE is unfolded protein response responsive element from *S. cerevisiae*. (B) The sequences of the UPREs from *KAR2* and *PDI1* genes are shown. Number and direction of the UPRE is indicated. The synthetic oligonucleotides of UPREs from *KAR2* and *PDI1* are indicated [14]. Letters in bold indicate conserved sites between the UPREs. DCDS1067 is the integration site.

2.2.2 Human erythropoietin (EPO) expression plasmid

Human EPO is secreted glycoprotein (~35 to 45 kDa) containing 3 N-linked glycosylation sites which are hyper-glycosylated (>50 kDa) when expressed from yeast cell. The cDNA encoding mature human EPO protein was first cloned in frame with α factor secretion signal peptide sequence of pPICZαA. The entire DNA fragment covering signal peptide sequence and EPO cDNA was then digested with *Nar*I and *Xba*I. The fragment was subcloned into the recombinant plasmid ρP_{TEF1}-GFP and $4xUPRE_{KAR2}$-P_{TEF1}-GFP at appropriate sites to replace the GFP cDNA in the plasmids.

2.3 *P. pastoris* Transformation and Screening

The recombinant plasmid was linearized by *Sph*I digestion and transformed to *P. pastoris* KM71 by electroporation using MicroPulser (BioRad). The transformants were selected on YPD agar containing with 100 µg/ml Zeocin. To screen for the expected transformants, a small part of colony was resuspended in 20 µl of SCED buffer (1 M sorbitol, 10 mM sodium citrate, pH 7.5, 10 mM ethylene diamine tetraacetic acid (EDTA) and 10 mM DTT) supplemented with 10 units of lyticase (Sigma). The suspension was incubated at 30 °C for 30 min, boiled for 10 min and centrifuged at 12,000 rpm for 10 min. One microliter of the supernatant was added to a total of 25 µl PCR reaction containing 1X ViBuffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.1 µM InPGK1F and pTef1R primers and 1 unit of *Taq* DNA polymerase (Vivantis). The reaction was amplified with 94 °C initial denaturing for 2 min and 30 cycles of 94 °C denaturing for 30 s, 50 °C annealing for 30 s and 72 °C extension for 60 s, and 72 °C final extension for 2 min. The PCR product was analyzed by agarose gel electrophoresis. Selection of the transformants carrying comparable copy of plasmid intregrant was performed by multiplex PCR. Genomic DNA from the transformants was extracted with standard phenol/chloroform method [17]. 100 ng genomic DNA was used in 25 µl PCR reaction in which components were similar to those described earlier except that four oligonucleotide primers were used: yESapF, AOX1TTR, ACT1F and ACT1R with annealing temperature of 50 °C. The PCR product of actin gene (*ACT1*) was used as internal control. Transformants that showed PCR product intensity of the plasmid integrant comparable to actin were selected for further characterization.

2.4 Green Fluorescent Protein Expression and Measurement

Overnight culture of the transformed *P. pastoris* in YPD medium was diluted in 50 ml fresh medium to 1 OD_{600}/ml and further grown for 2 h at 30 °C. Ten ml of the culture was then added to a 50-ml flask for cultivation. For applying ER stress conditions, DTT was added to the culture for indicated time. The cells were harvested by centrifugation at 4,000 rpm for 10 min at 4 °C. The packed cells were washed twice with ice-cold phosphate buffered saline (PBS) and then resuspended in the PBS at density of approximately 3.33 OD_{600}/ml. 150 µl of cell suspension was loaded into OptiPlateTM-96 F (PerkinElmer) for actual OD_{600} measurement in 5 wells. Maximum and minimum OD_{600} value of the results were excluded for calculation. The relative fluorescence intensity unit (RFU) was measured by Fluorescent ELISA reader model DTX880 Multimode Detector (Beckman Coulter). All RFU measurements were performed in triplicate.

2.5 Reverse Transcription PCR (RT-PCR)

To isolate total RNA, the packed cells were resuspended in TRI reagent® (Molecular Reseaech Center) and disrupted by vortexing in the presence of acid-washed glass beads according to manufacturer's recommendation. RNA was extracted by chloroform, precipitated with isopropanol and washed with 75% Ethanol. The RNA pellet was dissolved in DEPC-treated water. RQ1 RNase-Free DNase (Promega) was used to remove DNA contaminants according to manufacturer's recommendation. Complementary DNA was synthesized from 1 µg total RNA using oligo(dT) primer and ImPrompt-^{II}TM Reverse transcriptase (Promega). Two µl of the first-strand cDNA reaction was used for PCR amplification. All reagents were similar to those described earlier except that HAC1F and HAC1R primers and annealing temperature at 60 °C were used.

2.6 Quantitative PCR (qPCR)

Real-time quantitative PCR was performed in triplicate using the KAPA™ SYBR FAST qPCR Kit (Kapa Biosystems) and Realplex4 machine (Eppendorf) in 20 µl reaction containing 1X Mastermix, 200 nM of each primer and 10 ng cDNA. Reaction conditions were as follows: initial denaturation at 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 20 s. GFP-F and GFP-R primers were used for GFP target whereas EPO-F and EPO-R were used for EPO target. Actin was used as reference. The data were calculated using the $2^{-\Delta\Delta Ct}$ method [18].

2.7 Western Blot Analysis

Total protein from recombinant *P. pastoris* was prepared as follows. Yeast cells were washed once with ice-cold PBS then resuspended in 100 µl of cell lysis buffer (50 mM sodium phosphate, pH 7.4, 1 mM, 2 mM EDTA and 5% glycerol) containing 1X of Protease Inhibitor Cocktail IV (A.G. Scientific). Ice-cold glass beads (425-600 microns) (Sigma Chemical) were added and the cells were disrupted by vortexing for 30 s and cooling for 8 cycles. The samples were centrifuged at 12,000 rpm at 4 °C for 10 min to collect supernatant. Total protein concentration was quantified by Bio-Rad *DC*™ Protein Assay (Biorad). Equal amounts of protein (50 µg) in 2X sample buffer were resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) prior to transferring to polyvinylidene difluoride (PVDF) membrane (BioRad). The membrane was blocked with 5% (w/v) skim milk in PBS containing 0.2% (v/v) Tween-20 for 1 h at room temperature. The membrane was probed with mouse monoclonal antibody to EPO (1:4000) (Santa Cruze) for 1 h followed with horseradish peroxidase conjugated goat anti-mouse polyclonal antibodies (1:4000) (Sigma Chemical). EPO corresponding signal was detected using the ECL Plus Western Blotting Detection Reagent (GE Healthcare).

3. RESULTS AND DISCUSSION

3.1 Hybrid UPRE-*TEF1* Promoter Confers ER Stress Response in *P. pastoris*

Previous studies in *S. cerevisiae* demonstrated that a single copy of 22-bp UPRE inserted upstream of *CYC1* minimal promoter mediated an 8-fold increase of a reporter gene (β-galactosidase) activity under ER stress induction [12, 13]. When 2 to 4 repeating units of UPRE were used, approximately a 40-fold increase was observed under ER stress [12]. Although postulated as a highly conserved mechanism in all eukaryotes, little information is known about the UPR pathway and UPRE in *P. pastoris*. An earlier study demonstrated that

the transcriptional response of *P. pastoris* UPR is not identical but does overlap to that of *S. cerevisiae* [15].

To assess function of the UPRE to the P_{TEF1} and GFP reporter gene, relative fluorescence signal from yeasts transformed with the reporter plasmids with and without 4 copies of $UPRE_{KAR2}$ were compared. The recombinant *P. pastoris* expressing GFP reporter gene under P_{TEF1} alone (P_{TEF1}-GFP) showed relatively stable fluorescence signal over the period of 14 h (Figure 2, A). Addition of 5 mM DTT to the culture did not clearly alter the magnitude of the fluorescence signal in this recombinant yeast. In the absence of DTT, *P. pastoris* transformed with GFP reporter gene under 4 copies of $UPRE_{KAR2}$ and P_{TEF1} ($4xUPRE_{KAR2}$-P_{TEF1}-GFP) showed relatively higher and stable value of the fluorescence signal. Initially, the fluorescence signal increased at 2 h post DTT supplementation and continued to increase with a 3-fold peak increase at 9 h post DTT supplementation. Taken together, these results clearly indicated that the presence of the heterologous sequence of $UPRE_{KAR2}$ upstream of the constitutive P_{TEF1} enables upregulation of GFP reporter gene expression in the *P. pastoris* during ER stress condition by DTT.

This is the first study demonstrated modulation of transcriptional induction in *P. pastoris* by $UPRE_{KAR2}$ sequence from *S. cerevisiae* and indicated that Hac1p transcription factors are interchangeable between the two yeast species and the UPR pathways of the two yeasts share conserved components.

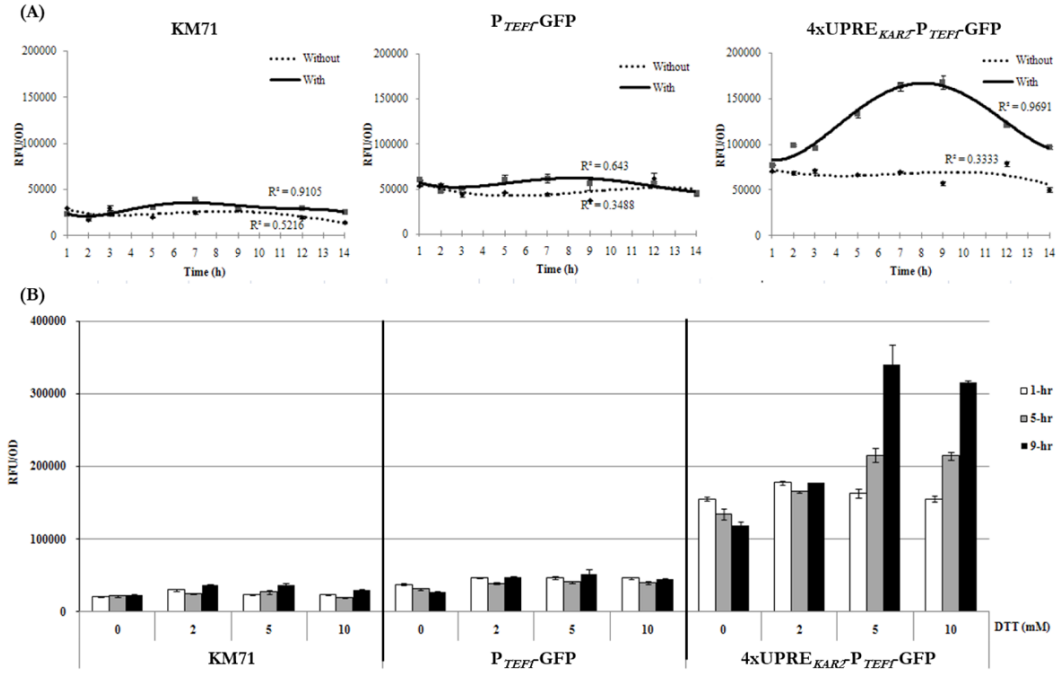

Figure 2. Average relative fluorescent intensity unit per cell density (RFU/OD) from three independent cultures over 14 h from (A) parental *P. pastoris* KM71, *P. pastoris* transformed with P_{TEF1}-GFP or *P. pastoris* transformed $4xUPRE_{KAR2}$-P_{TEF1}-GFP. "With" and "Without" are the conditions treated with or without 5 mM DTT, respectively. (B) The effect of DTT concentration (0, 2, 5 and 10 mM) on GFP reporter gene expression of different constructs and at different time points (1, 5 and 9 h).

3.2 Optimal Induction of UPRE-P_{TEF1} Hybrid Promoter

Next, we investigated the influence of the number of UPRE repeating units on the overall activity of the hybrid promoter. The presence of $UPRE_{KAR2}$ repeating units in the hybrid promoter enhanced the reporter gene signal upon DTT treatment (Figure 3, A). The overall activities of hybrid promoters with 1, 2 and 3 $UPRE_{KAR2}$ repeating units were only minimally enhanced in both baseline and ER stress conditions. However, the presence of 4 $UPRE_{KAR2}$ repeating units in the hybrid promoter drastically increased the GFP signal to approximately 6-fold and 13-fold without and with DTT treatment, respectively. Surprisingly, there was no elevated promoter activity when the 4 repeats of $UPRE_{KAR2}$ were replaced with 4 repeats of $UPRE_{PDI1}$ (Figure 3, B). Indeed the overall activity of the hybrid promoter carrying 4 $UPRE_{PDI1}$ repeats was extremely low in both baseline and under DTT stress conditions. The dramatic difference in DTT induced transcriptional activation of the GFP reporter through UPRE sequence from *KAR2* and *PDI1* of *S. cerevisiae* in our study emphasized that Hac1p transcription factor of *P. pastoris* exhibits different sequence preference between these two targets [13].

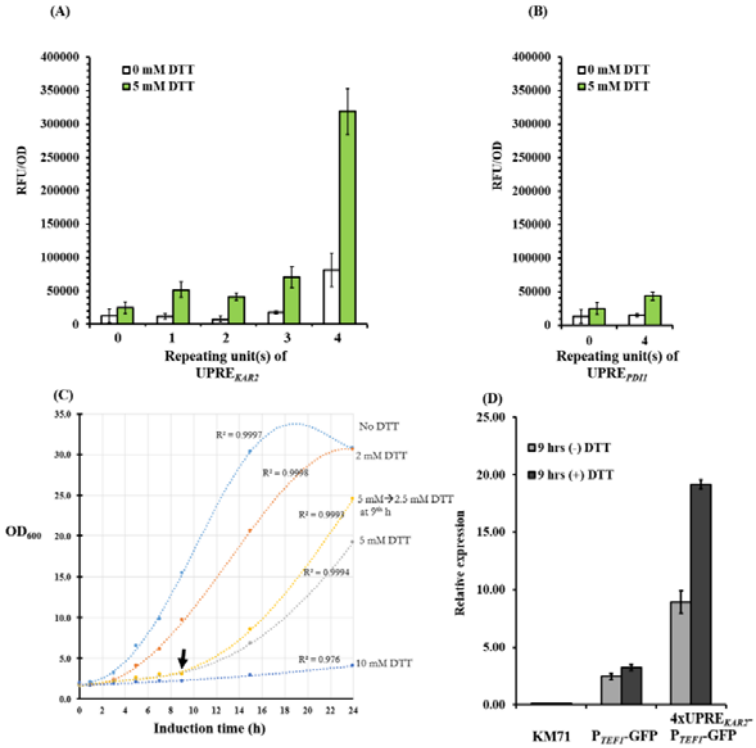

Figure 3. The response of UPR inducible reporter gene under DTT stressor. (A and B) The relative fluorescent signal unit per OD cells (RFU/OD) of *P. pastoris* carrying GFP reporter gene with various copies of $UPRE_{KAR2}$ or $UPRE_{PDI1}$ in response to DTT stressor. The RFU/OD were reported as means with standard error from 3 independent cultures of each construct. (C) Growth curves of *P. pastoris*, the medium containing various concentration of DTT (0, to 10 mM). Arrow indicates the time of reduction of DTT concentration from 5 to 2.5 mM. (D) Relative expression of GFP mRNA level expressed from indicated constructs was determined by qPCR. The value was means with standard error from three independent qPCR experiments.

The ability of 4xUPRE$_{KAR2}$-P$_{TEF1}$ hybrid promoter to enhance GFP reporter gene expression upon DTT treatment prompted us to investigate an optimal condition for its induction. Firstly, the effect of various concentrations of DTT to the *P. pastoris* culture was tested. Supplementation with DTT up to 2 mM had no effect on GFP expressed from the 4xUPRE$_{KAR2}$-P$_{TEF1}$ hybrid promoter (Figure 2, B). However, increasing DTT concentration to 5 or 10 mM significantly enhanced GFP signal at 5 h and peaked at 9 h strongly suggesting that the hybrid promoter activity is DTT dose-dependent. DTT is a small-molecule redox reagent and a strong ER stressor that can severely disturb ER homeostasis and perturb overall cellular activities.

We therefore monitored the effect of DTT concentration on *P. pastoris* growth rate (Figure 3, C). Supplementation with 2 mM DTT slowed down *P. pastoris* growth rate by approximately 30% during the first 16 h compared to the control culture (No DTT). However the growth rate returned to normal by 24 h. An approximately 80% growth inhibition was observed during the first 16 h of cultivation with 5 mM DTT. Nevertheless, cells cultured in this condition still showed a clear growth recovery at later time points and better recovery when DTT concentration was reduced to 2.5 mM. On the other hand, supplementation of the *P. pastoris* culture with 10 mM DTT severely impaired cell growth for the entire period with no sign of growth recovery, thus the effect was irreversible. Collectively, our results suggested that 5 mM DTT is the most suitable condition to induce the function of the UPRE$_{KAR2}$-P$_{TEF1}$ hybrid promoter in *P. pastoris*.

By comparison with the P$_{TEF1}$, conventional P$_{AOX1}$ promoter causes continuous transcription activation of the target gene by using an explosive methanol inducer for a period of days. The transcription induction by our hybrid promoter lasted for a shorter period (<10 h) by non-explosive DTT supplement. However the transcription activation of the 4xUPRE$_{KAR2}$-P$_{TEF1}$ hybrid promoter could be prolonged by additional DTT supplementation (data not shown). Such property may be desirable for fine-tuning the expression of recombinant protein targets in specific time points rather than obtaining large amounts of protein product. An example of this is cellular toxic proteases or enzymes for toxic by-products. It is worth noting that all the experiments conducted in this study were performed at low cell density. It would therefore be interesting to investigate the efficiency of our hybrid promoter platform at high cell density culture to determine whether the expression level will be improved.

In addition, qPCR demonstrated the level of GFP transcript in each condition ensuring that the difference in GFP signal expressed from the 4xUPRE$_{KAR2}$-P$_{TEF1}$ hybrid promoter was a direct consequence of the promoter function. The GFP transcripts expressed from the P$_{TEF1}$ alone, relative to *ACT1* (actin) transcript, was lower and did not significantly change by DTT supplementation (Figure 3, D). In contrast, the transcript from the GFP reporter gene under the 4xUPRE$_{KAR2}$-P$_{TEF1}$ hybrid promoter was increased to 3.6 (without DTT) and 5.9 (with DTT) times compared to the P$_{TEF1}$ alone in the same condition confirming that the hybrid promoter is inducible by the ER stressor. The finding that basal activity of the 4xUPRE$_{KAR2}$-P$_{TEF1}$ hybrid promoter surpassed the P$_{TEF1}$ was unexpected as this was not observed in baker's yeast. It is unlikely that this observation resulted from variation in gene dosage among transformants. Indeed, all recombinant *P. pastoris* used in this study were selected from transformants

carrying comparable copies of plasmid integrant (relative to actin). Moreover, similar scenarios were observed in several independent transformants carrying 4xUPRE$_{KAR2}$-P$_{TEF1}$-GFP. We presume that the enhancement of basal level of expression from the constructs carrying 4xUPRE$_{KAR2}$-P$_{TEF1}$ was associated with the status of UPR in the *P. pastoris* strain.

3.3 *HAC1* mRNA was Constitutively Spliced in the *P. pastoris*

The elevated basal expression of GFP reporter gene from the 4xUPRE$_{KAR2}$-P$_{TEF1}$ hybrid promoter connected to the status of UPR in *P. pastoris*. *HAC1* mRNA splicing (removing 322-bp intron) is a key regulatory step for UPR activation as it generates spliced *HAC1* mRNA (347-bp *HAC1*^{S}), whose protein product directly modulates transcription of several downstream UPR responsive genes (including *KAR2* and *PDI1*) by binding to their UPRE. We monitored the level of *HAC1* mRNA splicing in the recombinant *P. pastoris*. Surprisingly, *HAC1*^{S} was the major form of the *HAC1* transcripts in all conditions, while the unspliced *HAC1* mRNA (699-bp *HAC1*^{U}) was barely observed, even in the absence of DTT (Figure 4, A) implying that the basal activity of the hybrid promoter was already increased in the absence of DTT. The level of *HAC1*^{S} transcript was further increased 3-4-fold after supplementation with 5 mM DTT (Figure 4, B) indicating the very high UPR activation capacity in *P. pastoris*. Our results are in agreement with previous studies, where *HAC1* mRNA was constitutively spliced even without DTT supplementation suggesting that the UPR pathway in this organism is partially turned on [19]. This condition might be sufficient to trigger transcriptional activation of the reporter gene under the control of the hybrid promoter. In addition, it is plausible that the presence of 4 repeating units of UPRE$_{KAR2}$ facilitates recruitment of the Hac1p transcription factor to the hybrid promoter more efficiently, thereby exhibiting stronger basal promoter activity.

3.4 Expression of Human Erythropoietin Under the 4xUPRE$_{KAR2}$-P$_{TEF1}$

To explore the potential application of 4xUPRE$_{KAR2}$-P$_{TEF1}$ hybrid promoter on expression of other heterologous proteins, recombinant *P. pastoris* strains transformed by plasmid expressing human EPO, a hormone stimulating red blood cell production, under the 4xUPRE$_{KAR2}$-P$_{TEF1}$ hybrid promoter or the P$_{TEF1}$ alone were analyzed at both protein and mRNA levels. Due to a low level of EPO secreted to the medium by the yeast, we therefore monitored levels of EPO protein inside the cell by western blot. As a control, the EPO was barely detected from cells harboring the P$_{TEF1}$-EPO construct regardless of DTT supplementation (Figure 4, C). Notably, the basal level of EPO expressed from 4xUPRE$_{KAR2}$-P$_{TEF1}$-EPO construct was clearly higher than that derived from P$_{TEF1}$-EPO, which resembled the earlier finding for the GFP reporter gene. The level of EPO from the hybrid promoter was dramatically increased upon treatment with DTT. The level of gene expression matched that of the protein level in that the presence of 4xUPRE$_{KAR2}$ in the hybrid promoter improved EPO expression approximately 2-fold by DTT supplementation. More importantly, the basal level of EPO transcript derived from the 4xUPRE$_{KAR2}$-P$_{TEF1}$ increased approximately 3-fold compared to the P$_{TEF1}$ alone (Figure. 4, D). This data confirmed that the presence of 4xUPRE$_{KAR2}$ adjacent to the P$_{TEF1}$ not only enhanced the basal activity of the hybrid promoter even in non-stress conditions

(as the UPR pathway in this organism is partially turned on), but also responded to the ER stress by DTT through the UPR activation.

In this study a constitutive promoter P_{TEF1} was chosen for hybrid promoter construction because it could drive expression of the GFP at detectable level by the fluorescent measurement and could be differentiate for auto-fluorescence of the native KM71 strain. The P_{TEF1} was previously reported to possess a wider range of optimal conditions and exhibit two-fold stronger promoter activity in carbon limited fed batch culture compared to P_{GAP} system [5]. In fact, cumulative reports suggested that in certain cases of gene expression using strong constitutive promoters such as P_{GAP} or P_{TEF1} can result in comparable or higher yield than that obtained from inducible P_{AOX1}.

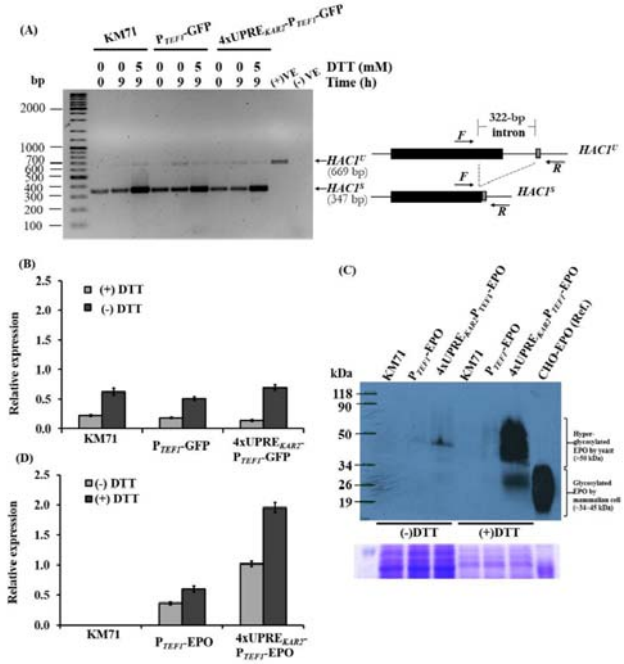

Figure 4. Monitoring of *HAC1* mRNA splicing and recombinant human EPO expression in *P. pastoris*. (A) Detection of unspliced *HAC1* (*HAC1*^{U}) and spliced *HAC1* (*HAC1*^{S}) mRNA transcripts in parental *P. pastoris* KM71 or *P. pastoris* KM71 strain with and without UPR inducible reporter gene by RT-PCR. Arrows indicate binding site of primers across the intron; F and R are HAC1F and HAC1R, respectively. Total RNA was harvested from cells cultured in the presence or absence of DTT for indicated time. (+)VE and (-)VE are RT-PCR reactions with and without genomic DNA of *P. pastoris* KM71. (B) Relative *HAC1* mRNA level compared to ACT1 (actin) was determined by qPCR. (-)DTT and (+)DTT indicate the condition without and with 5 mM DTT induction, respectively. Bars indicate standard error from three independent qPCR experiments. (C) Western blot detection of recombinant human EPO in total cell lysates of recombinant *P. pastoris* (P_{TEF1}-EPO, 4xUPRE$_{KAR2}$-P_{TEF1}-EPO) with or without DTT. CHO-EPO (Ref.) is recombinant human EPO expressed from mammalian Chinese Hamster Ovary cell, which served as positive control. The level of protein loaded into each lane was shown by Coomassie blue stained SDS-PAGE. (D) Relative EPO mRNA level compared to *ACT1* (actin) as determined by qPCR. Means with standard error from three independent qPCR experiments are shown.

4. CONCLUSION

Our hybrid promoter expression platform offers an alternative way of simple static-dynamic expression that is applicable for fine-tuning heterologous gene expression in *P. pastoris* system. Future work should focus on the potential use of our new hybrid promoter in large-scale heterologous gene expression. It would also be of interest to find alternative combinations of the UPRE to other constitutive promoters.

ACKNOWLEDGEMENTS

This work was partially supported by a research grant from Thailand Research Fund (BRG5080023) and Mahidol University to W.T. N.K. was supported by Ph.D. Scholarship from Commission of Higher Education, Thailand.

REFERENCES

[1] Cregg J.M., Tolstorukov I., Kusari A., Sunga J., Madden K. and Chappell T., *Method. Enzymol.*, 2009; **463**: 169-189. DOI s0076-6879(09)63013-5.

[2] Vogl T., Hartner F.S. and Glieder A., *Curr. Opin. Biotechnol.*, 2013; **24**: 1094-1101. DOI 10.1016/j.copbio.2013.02.024.

[3] Vogl T. and Glieder A., *Nat. Biotechnol.*, 2013; **30**: 385-404. DOI 10.1016/j.nbt.2012.11.010.

[4] Waterham H.R., Digan M.E., Koutz P.J., Lair S.V. and Cregg J.M., *Gene*, 1997; **186**: 37-44.

[5] Ahn J., Hong J., Lee H., Park M., Lee E., Kim C., Choi E. and Jung J., *Appl. Microbiol. Biotechnol.*, 2007; **74**: 601-608. DOI 10.1007/s00253-006-0698-6.

[6] de Almeida J.R., de Moraes L.M. and Torres F.A., *Yeast*, 2005; **22**: 725-737. DOI 10.1002/yea.1243.

[7] Tschopp J.F., Brust P.F., Cregg J.M., Stillman C.A. and Gingeras T.R., *Nucleic Acids Res.*, 1987; **15**: 3859-3876.

[8] Kaufman R.J., *J. Clin. Invest.*, 2002; **110**: 1389-1398. DOI 10.1172/JCI16886.

[9] Hohenblum H., Gasser B., Maurer M., Borth N. and Mattanovich D., *Biotechnol. Bioeng.*, 2004; **85**: 367-375. DOI 10.1002/Bit.10904.

[10] Damasceno L.M., Anderson K.A., Ritter G., Cregg J.M., Old L.J. and Batt C.A., *Appl. Microbiol. Biotechnol.*, 2007; **74**: 381-389. DOI s00253-006-0652-7.

[11] Inan M., Aryasomayajula D., Sinha J. and Meagher M.M., *Biotechnol. Bioeng.*, 2006; **93**: 771-778. DOI 10.1002/bit.20762.

[12] Cox J.S. and Walter P., *Cell*, 1996; **87**: 391-404. DOI S0092-8674(00)81360-4.

[13] Mori K., Sant A., Kohno K., Normington K., Gething M.J. and Sambrook J.F., *EMBO J.*, 1992; **11**: 2583-2593.

[14] Mori K., Ogawa N., Kawahara T., Yanagi H. and Yura T., *J. Biol. Chem.*, 1998; **273**: 9912-9920.

[15] Graf A., Gasser B., Dragosits M., Sauer M., Leparc G.G., Tuchler T., Kreil D.P. and Mattanovich D., *BMC Genomics*, 2008; **9**: 390. DOI 10.1186/1471-2164-9-390.

[16] Whyteside G., Nor R.M., Alcocer M.J. and Archer D.B., *FEBS Lett.*, 2011; **585**: 1037-1041. DOI 10.1016/j.febslet.2011.02.036.

[17] Sambrook J.a.R., D.W., *Molecular Cloning: A Laboratory Manual*. 3 ed. 2001, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

[18] Livak K.J. and Schmittgen T.D., *Methods*, 2001; **25**: 402-408. DOI 10.1006/meth.2001.1262.

[19] Guerfal M., Ryckaert S., Jacobs P.P., Ameloot P., Van Craenenbroeck K., Derycke R. and Callewaert N., *Microb. Cell Fact.*, 2010; **9**: 49. DOI 10.1186/1475-2859-9-49.