Appl Microbiol Biotechnol
DOI 10.1007/s00253-014-5727-2

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Evaluation and screening of efficient promoters to improve astaxanthin production in *Xanthophyllomyces dendrorhous*

Kiyotaka Y. Hara · Toshihiko Morita · Yusuke Endo · Masao Mochizuki · Michihiro Araki · Akihiko Kondo

Received: 16 February 2014 / Revised: 11 March 2014 / Accepted: 25 March 2014

Abstract Astaxanthin is a valuable carotenoid that is widely used in the aquaculture, food, pharmaceutical, and cosmetic industries. *Xanthophyllomyces dendrorhous* is a carotenoid-synthesizing yeast strain that produces astaxanthin as its main pigment. Although metabolic engineering using gene manipulation is a valuable way to improve astaxanthin production, a gene expression system for *X. dendrorhous* has been poorly developed. In this study, three known promoters of *X. dendrorhous*, glycerol-3-phosphate dehydrogenase (*gpd*) promoter (P*gpd*), glucose dehydrogenase (*gdh*) promoter (P*gdh*), and actin (*act*) promoter (P*act*), were evaluated for use in the overexpression of target proteins using green fluorescence protein (GFP) as an expression level indicator protein. The actin promoter, P*act*, showed the highest expression level of GFP when compared with P*gpd* and P*gdh*. Additionally, to obtain new promoters for higher expression of target protein in *X. dendrorhous*, intracellular GFP intensity was evaluated for 13 candidate promoters. An alcohol dehydrogenase promoter, P*adh4*, showed more efficient expression of GFP rather than P*act*. Overexpression of *crtE* gene encoding rate-limiting enzyme of carotenoid synthesis under the *adh4* promoter yielded an increase in intracellular astaxanthin content of about 1.7-fold compared with the control strain. The promoters identified in this study must be useful for improving carotenoids production in *X. dendrorhous*.

Keywords Promoter · GFP · *Xanthophyllomyces dendrorhous* · *Phaffia rhodozyma* · Carotenoid · Astaxanthin

Kiyotaka Y. Hara and Toshihiko Morita contributed equally in this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-5727-2) contains supplementary material, which is available to authorized users.

K. Y. Hara · M. Mochizuki · M. Araki
Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodaicho, Nada, Kobe 657-8501, Japan

T. Morita · Y. Endo · A. Kondo (✉)
Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodaicho, Nada, Kobe 657-8501, Japan
e-mail: akondo@kobe-u.ac.jp

Introduction

Astaxanthin (3, 3′-dihydroxy-β, β-carotene-4, 4′-dione; $C_{40}H_{52}O_4$) is an orange-red carotenoid pigment that is used for the coloration of salmon, lobster, and other seafood species. Astaxanthin also acts as a protective agent against oxidative damage to cells in vivo (Satoh et al. 2009; Tripathi and Jena. 2009). Therefore, astaxanthin is one of the most valuable carotenoids and is widely used not only in the aquaculture industry but also in other fine chemical industries such as food, healthcare, cosmetics, and pharmaceuticals (Miao et al. 2011); demand for astaxanthin is growing in global markets (Schmidt et al. 2011). At present, most of the commercial astaxanthin used in the aquaculture industry is chemically synthesized from petrochemical sources. Few organisms capable of synthesizing astaxanthin *de novo* have the potential to produce astaxanthin more efficiently than the chemical synthesis process. But the heterobasidomycete, *Phaffia rhodozyma* (sexual form, *Xanthophyllomyces dendrorhous*), is a very promising candidate for maximizing astaxanthin production using a microorganism from biomass resources for, especially, use in fine chemical markets (Rodríguez-sáiz et al. 2010).

Improving astaxanthin production in *X. dendrorhous* was carried out by the following three methods: optimization of cultivation conditions, screening of astaxanthin-hyperproducing strains from random mutant strains library, and cloning and overexpression of genes involved in

Published online: 16 April 2014

astaxanthin synthesis. Most of the previous improvements to astaxanthin production in *X. dendrorhous* were carried out using the first and the second methods; however, fewer improvements were carried out using the third method because of the poor development of a target protein expression system in *X. dendrorhous* (Schmidt et al. 2011; Rodríguez-sáiz et al. 2010). For instance, geranylgeranyl-pyrophosphate (GGPP) is known as one of the rate-limiting metabolites for astaxanthin biosynthesis in *X. dendrorhous* (Breitenbach et al. 2011), and it was already reported that self-cloning and overexpression of *X. dendrorhous crtE*, which catalyzes biosynthesis of GGPP from farnesyl-pyrophosphate (FPP), increased the production of carotenoids, including astaxanthin (Breitenbach et al. 2011). In these studies, overexpression of *crtE* was independently carried out using different promoters: glycerol-3-phosphate dehydrogenase (*gpd*) (Verdoes et al. 2003) and glucose dehydrogenase (*gdh*) (Rodríguez-sáiz et al. 2009). Actin (*act*) promoter was also used for integration of target genes into genome of *X. dendrorhous* (Wery et al. 1997). However, there is no information about the relative expression level of *crtE* using these different promoters. To develop a system that most highly expresses target proteins, obtaining the strongest promoters is required. Additionally, to further improve astaxanthin production, identification of new, better promoters are required in promoting efficient expression of target proteins without disturbing the cell physiology and growth of the organism.

In this study, the abilities of the *gpd* promoter (P*gpd*), *gdh* promoter (P*gdh*), and actin promoter (P*act*) to overexpress a target protein were compared using green fluorescence protein (GFP) as an expression level indicator. Additionally, new promoters were identified as candidates for overexpression, and astaxanthin production in *X. dendrorhous* was improved by expression of *crtE* gene under P*adh*4, the best promoter identified in this study.

Materials and methods

Strains and media

NovaBlue (Novagen, Madison, WI, USA) was used as the *Escherichia coli* host strain for recombinant DNA manipulation. *X. dendrorhous* (NBRC 10129) was used as the parental host strain for gene expression. *E. coli* transformants were grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride) supplemented with 100 μg/mL ampicillin. Transformants of *X. dendrorhous* were cultured in YM medium (5 g/L tryptone, 3 g/L yeast extract, 3 g/L malt extract, and 10 g/L glucose). Yeast extract and malt extract were purchased from Becton Dickinson (Sparks, MD, USA). Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) or Wako Chemicals (Osaka, Japan).

Plasmid construction and yeast transformation

Candidate promoters were cloned by PCR. KOD-Plus-Neo (TOYOBO, Osaka, Japan) was used as the DNA polymerase. DNA sequences of cloning primers for promoters (accession AB915561-AB915576) and terminators (accession AB915577, AB915578) are shown in Table S1. Construction of examples of plasmids is shown in Fig. 1. GFP was cloned from the pAcGFP1 vector (Takara, Shiga, Japan). Transformation was carried out based on the method described in previous reports (Wery et al. 1997; Niklitschek et al. 2008) with some modifications for construction of competent cells; *X. dendrorhous* parental strain was grown in 5 mL liquid YM medium at 22 °C with agitation at 250 rpm for 24 h. An adequate volume of each culture was inoculated into 150 mL liquid YM medium to achieve an initial OD_{600} value of 0.03. Cultures were then grown at 22 °C with agitation at 120 rpm for 16.5 h.

Growth of *X. dendrorhous* strains

X. dendrorhous strains expressing GFP were grown in 5 mL liquid YM medium containing 40 μg/mL G418 at 22 °C with agitation at 250 rpm for 72 h. An adequate volume of each culture was inoculated into 80 mL liquid YM medium containing 40 μg/mL G418 to achieve an initial OD_{600} value of 0.15. Cultures were then grown at 22 °C with agitation at 120 rpm for less than 72 h.

Promoter analysis

Cell concentration was measured as the OD_{600} after growth for the appropriate length of time. Cells from the culture were harvested by centrifugation at 8,000×*g* at 4 °C for 3 min. Fluorescence intensity of GFP from harvested cells was measured by an EnVision 2104 micro-plate reader (Perkin Elmer, MA, USA) or a FACS CantoII flow cytometer (Becton

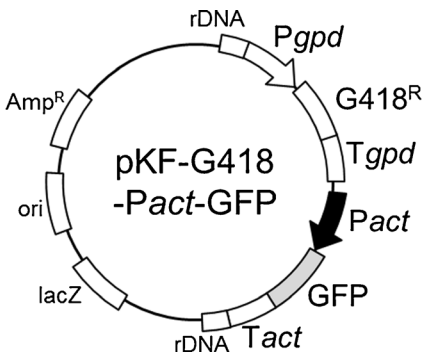

Fig. 1 Schematic illustration of pKF-G418-P*act*-GFP as an example of plasmids constructed in this study for GFP expression under each promoter. *rDNA* ribosomal DNA sequence, MCS multi cloning site, $G418^R$ G418 resistant gene, P*act* actin promoter, T*act* actin terminator

Dickinson) after dilution into an appropriate volume of distilled water. The number of GFP gene copies integrated into the genome of the *X. dendrorhous* transformants was measured by real-time PCR.

To measure the astaxanthin concentration of *X. dendrorhous*, harvested cells were suspended into 1 mL acetone. The cells were broken using a bead shocker with zirconia beads. The cell extract was centrifuged at 15,000×*g* at 4 °C for 10 min, and then the supernatant was diluted into acetone. Astaxanthin concentration was determined using a high-performance liquid chromatography (Shimadzu, Kyoto, Japan) equipped with a Develosil ODS-HG-5 column (Nomura Chemical, Aichi, Japan). The operating conditions were 25 °C, with acetonitrile/methanol/2-propanol (85/10/5 (*v*/*v*)) as the mobile phase at a flow rate of 0.8 mL/min, and the detection was performed at 471 nm with a UV detector SPD-20A (Shimadzu).

Results

Quantitative evaluation of known promoters in *X. dendrorhous*

It has been independently reported that P*gpd*, P*gdh*, and P*act* promoters can be used for overexpression of target proteins in *X. dendrorhous*. To compare the ability of these promoters to overexpress target proteins, GFP was used as an expression level indicator protein. To express GFP in *X. dendrorhous*, plasmids with the GFP gene under the control of P*gpd*, P*gdh*, or P*act* were constructed as the example of Pact-GFP shown in Fig. 1. To select the transformants with the maximum copy number of integrated GFP for each strain, more than eight transformants were selected, and GFP intensity was compared after these strains were grown for 24 h. The transformants that showed the highest intracellular GFP intensity (GFP intensity per dried cell weight (DCW)) were selected as maximally expressing GFP strains. By averaging multiple replicates of the intracellular fluorescence intensity from the maximally expressing GFP strains (Fig. 2a), we determined the relative promoter strengths to be P*act*>P*gdh*>P*gpd*.

Screening novel promoters for efficient expression of target proteins

To screen for novel strong promoters in *X. dendrorhous*, 13 candidate promoters were selected (Fig. 2b). The lengths of these candidate promoters were less than 1,000 bp, and they were located upstream of genes whose promoter is known to be strong promoter in *Saccharomyces cerevisiae* (Partow et al. 2010). These strong candidate promoters of *X. dendrorhous* included one phosphoglycerate kinase (*pgk*), four translational elongation factors (*tef1* - *4*), one pyruvate decarboxylase (*pdc*), one triose-phosphate isomerase (*tpi*), five alcohol dehydrogenases (*adh1* - *5*), and one hexose transporter (*hxt*). The *X. dendrorhous* strains expressing the GFP gene under each promoter candidate were constructed, and the fluorescence intensity of GFP from these strains was measured after growth for 72 h. Cell concentration and GFP intensity of more than eight transformants of each strain were measured, and the transformants showing the highest intracellular GFP intensity were selected to compare the intracellular GFP intensities. As shown in Fig. 2b, P*tpi* showed an equal GFP expression level to P*act*, and P*adh*4 showed a higher intracellular GFP expression level than P*act*. P*hxt* and P*adh5* showed lower GFP expression levels than P*gdh*, although the levels were higher than P*gpd*. The remaining nine candidates that we tested showed lower intracellular GFP expression levels than P*gpd*.

Time dependence of selected GFP promoters

To test the stability of selected promoters for expression of a target protein, cell concentration and intracellular GFP intensity from the strains expressing GFP under P*gpd*, P*act*, P*tpi*, or P*adh4* were compared after a time course of flask-scale cultivation. As shown in Fig. 3a, the cell growth properties were similar among all tested strains. By contrast, P*adh4* showed a continuous increase in the intracellular GFP expression level for more than 72 h, although P*act* and P*tpi* ceased to increase the intracellular GFP expression level after less than 48 h.

Next, to compare the maximum copy numbers of GFP genes integrated into genome of the selected GFP-expressing strains, copy numbers of GFP genes were measured by real-time PCR using the number of *act* genes as a standard. As shown in Table 1, the maximum copy number of P*act*-GFP, P*tpi*-GFP, P*adh4*-GFP, and P*gpd*-GFP-expressing strain were 5, 15, 16, and 16, respectively. The intracellular GFP intensity at 72 h from the P*act*-GFP (58×10^4/g-DCW), P*adh4*-GFP (66×10^4/g-DCW), and P*gpd*-GFP (40×10^4/g-DCW) expressing strains were 12.3-fold, 14.0-fold, and 8.5-fold higher than that of P*gpd*-GFP (4.7×10^4/g-cell) expressing strain, respectively.

Conversely, populations of the P*act*-GFP-, P*adh*4-GFP-, and P*tpi*-GFP-expressing strains were compared using flow cytometer to measure their intracellular GFP intensity as shown in Fig. 4. The population of the P*adh4*-GFP strain showed Gaussian distribution (Fig. 4c), and the P*tpi*-GFP and P*act*-GFP strains showed double-peak distributions (Fig. 4a, b). The lower peak of P*act*-GFP-expressing cells showed almost equal intracellular GFP intensity as the higher peak of P*tpi*-GFP-expressing cells. By contrast, the higher peak of P*act*-GFP-expressing cells showed almost equal intracellular GFP intensity as the single peak of P*adh4*-GFP-expressing cells.

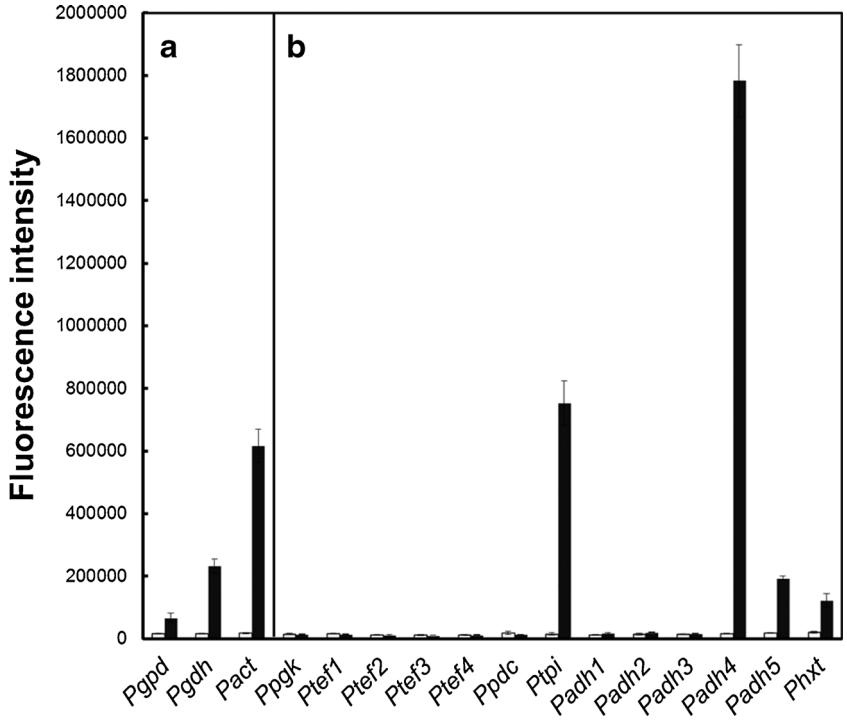

Fig. 2 Evaluation and screening of *X. dendrorhous* promoters for overexpression of target proteins using GFP. Cell concentration (OD_{600}) and GFP intensity was measured after growth for 72 h. Intracellular GFP intensity of each strain expressing a negative, no-gene control (*white bar*) and GFP (*black bar*). GFP gene expression under 3 known promoters or 13 candidate promoters is calculated and shown. **a** Quantitative evaluation of expression ability of known promoters. **b** Screening of new high expression promoters. The values are means, and the *error bars* show the SD (n=3)

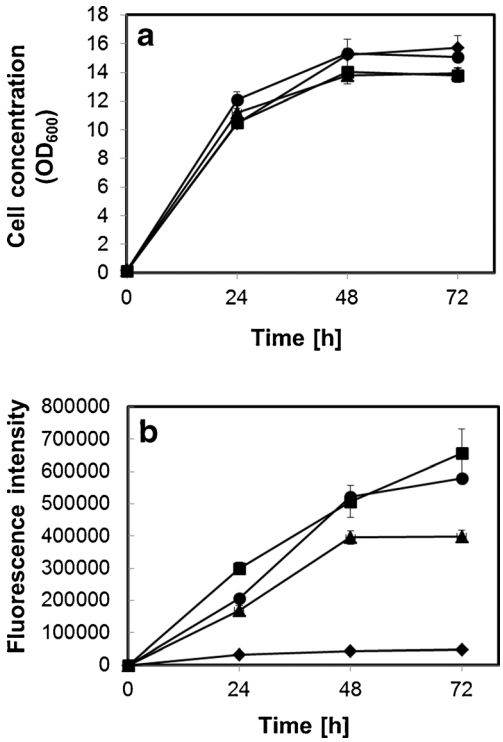

Fig. 3 Time courses of cell concentration and fluorescence intensities of GFP in *X. dendrorhous* strains expressing the GFP gene under the selected promoters or *gpd* promoter as control. *Diamonds*, *triangles*, *circles*, and *squares* represent P*gpd*, P*tpi*, P*act*, and P*adh4*, respectively. **a** Cell concentration (OD_{600}). **b** Intracellular fluorescence intensity of GFP. The values are means, and the *error bars* show the SD (n=3)

Using the *adh*4 promoter to enhance astaxanthin production

It has already been reported that astaxanthin production was enhanced by the overexpression of *crtE*, which is a gene in the mevalonate pathway (Breitenbach et al. 2011). To use P*adh4*, the strongest overexpression promoter identified in this study, to enhance astaxanthin productions, *X. dendrorhous* strains expressing the *crtE* gene under the *adh4* promoter and a negative, no-gene control (P*adh4-crtE* and P*adh4*-(−)), were compared. The cell concentration, volumetric astaxanthin concentration, and intracellular astaxanthin content of the P*adh4-crtE* strain and P*adh4*-(−) strain are shown in Fig. 5. As shown in Fig. 5a, the cell concentration of the P *adh4-crtE* strain at 72 h (14.9) was 0.92-fold lower than that of the P*adh4*-(−) strain (16.2). In contrast, as shown in Fig. 5b, the

Table 1 Growth, copy number, and GFP intensity in engineered *X. dendrorhous* strains

Promoter	Maximum copy no. of GFP	Cell growth [OD_{600}]	GFP intensity [$\times 10^5$]	Intracellular GFP intensity [$\times 10^4$/g-cell]
P*act*	5	15.1±0.14 (9.6)	33±0.58 (1.2)	58±0.96 (12.3)
P*adh4*	15	13.8±0.51 (8.8)	34±0.41 (1.2)	66±0.75 (14.0)
P*tpi*	16	13.9±0.42 (8.9)	21±0.64 (7.5)	40±0.20 (8.5)
P*gpd*	16	15.7±0.85 (1.0)	2.8±0.22 (1.0)	4.7±0.59 (1.0)

The culture conditions are described in "Materials and methods." Parentheses represent the relative values. The values are represented as mean± SD (n≥3)

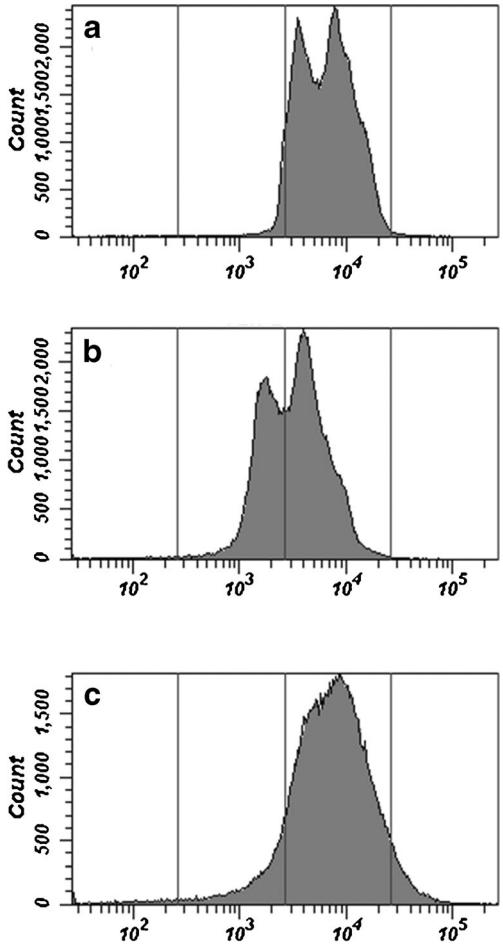

Fig. 4 Intracellular fluorescence intensities of GFP in populations of *X. dendrorhous* expressing GFP gene under selected efficient expression promoters, P*act* (**a**), P*tpi* (**b**), and P*adh4* (**c**). Intracellular GFP fluorescence from individual cell was detected by flow cytometer. Each sample included 1,000,000 cells

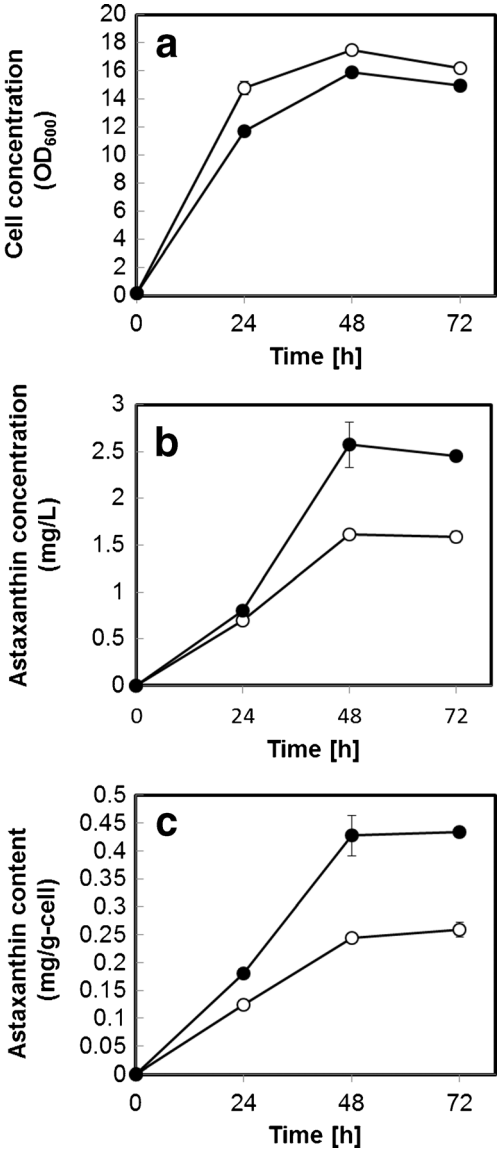

Fig. 5 Astaxanthin production by *X. dendrorhous* expressing *crtE* under the *adh4* promoter. **a** Cell concentration (OD_{600}). **b** Astaxanthin concentration (mg/L). **c** Intracellular astaxanthin content (mg/g-cell). *Closed* and *open symbols* represent the astaxanthin fermentation of *X. dendrorhous* expressing P*adh4-crtE* and its control P*adh4*-(−), respectively. Measurement method for astaxanthin concentration was described in "Materials and methods." The values are means, and the *error bars* show the SD (n=3)

astaxanthin concentration of the P*adh4-crtE* strain at 72 h (2.45 mg/L) was 1.54-fold higher than that of the P*adh4*-(−) strain (1.59 mg/L). The intracellular astaxanthin content of the P*act-crtE* strain at 72 h (0.43 mg/g-DCW) was 1.65-fold higher than that of the control strain (0.26 mg/g-DCW) as shown in Fig. 5c. These results show that expression of *crtE* under the *adh4* promoter clearly enhances astaxanthin production in *X. dendrorhous*.

Discussion

In this report, we carried out quantitative evaluation of three known promoters, P*gpd*, P*gdh*, and P*act* in *X. dendrorhous* using GFP as an indicator for expression level of a target protein. The fluorescence intensities from intracellular GFP expressed under each candidate promoters were compared. Using this method, P*act* was identified as the most highly expressing promoter among the known promoters in *X. dendrorhous* (Fig. 2a). This is the first study to compare the relative expression level of target protein between different known promoters. We also tried to identify new promoters with more efficient protein expression capabilities than the *act* promoter. To achieve this, we screened 13 candidate promoters of less than 1,000 bp in length and located upstream of genes whose promoter was previously shown to be a strong promoter in *S. cerevisiae*. As shown in Fig. 2b, *X. dendrorhous* P*adh4* showed higher GFP expression than

P*act*, and P*tpi* showed almost equal to P*act*. These results indicate that the *adh4* promoter is stronger than the *act* promoter for target protein expression. Additionally, as shown in Fig. 3b, a time course of the intracellular GFP intensity of all strains expressing GFP under the selected promoters showed a continuous increase of the intracellular GFP level after 24 h, although their cell growth stopped around 24 h (Fig. 3a). In the case of the *adh4* promoter, the increase in the intracellular GFP level continued after more than 72 h of growth, but the *act* and *tpi* promoters ceased to increase the intracellular GFP level after 48 h of growth (Fig. 3b). This result indicates that the *adh4* promoter enhanced not only the expression level of the target protein but also the stability of promoter activity compared with the *act* promoter. This insight is strongly supported by the result of population distribution of intracellular GFP fluorescence (Fig. 4).

The comparative expression abilities of *S. cerevisiae* promoters were evaluated to be P*tef*~P*pgk*>P*tpi*>P*adh*>P*hxt* (Partow et al. 2010). Especially, P*tef* has been a well-known constitutive strong promoter in *S. cerevisiae* (Gatignol et al. 1990). However, in the case of *X. dendrorhous*, the expression level of P*tef* and P*pgk* were lower than that of P*adh4* and P*hxt* (Fig. 2b). This P*adh* is a promoter of an alcohol dehydrogenase (Adh). It has been reported that ethanol addition increases Adh activity in *X. dedrorhous* (Gu et al. 1997) like that observed in *S. cerevisiae* (Young et al. 1982). High expression level of P*adh4* shown in this study indicates that one of the reasons of the high performance of ethanol production in *X. dendrorhous* during the astaxanthin production (Nghiem et al. 2013) is due to the high expression level and positive feedback regulation of Adh by ethanol. In contrast, high expression level of P*hxt*, which is a promoter of hexose transporter, indicates the strong hexose utilizing activity of *X. dendrorhous* when glucose is supplied as carbon source.

As the results in Table 1 show, the maximum copy number of P*act*-GFP was only five. Transformants possessing 11 copies of P*act*-GFP could be obtained; however, these transformants had altered growth (data not shown). In contrast, even though introducing five copies of P*act*-GFP showed acceptable cell growth, the double-peak distribution of GFP intensity (Fig. 4a) indicates unstable expression of GFP gene under the *act* promoter. This result indicates that the introduction of a high copy number of *act* promoter-driven genes competes with expression of the endogenous actin protein and causes changes in cytoskeleton formation due to a decrease in actin filament synthesis (Slaninová et al. 1999). These results indicate that the *adh4* promoter is a more efficient promoter for target genes than the *act* promoter and promotes more stable expression of target proteins without altering normal cell growth. In contrast to the *adh4* promoter, the *tpi* promoter showed lower intracellular fluorescence over time (Fig. 3b) and a similar population distribution of GFP fluorescence (Fig. 4b) to the *act* promoter, indicating that the *tpi* promoter has lower expression and equal stability of the target protein compared with the *act* promoter.

We also tested astaxanthin production using the *adh4* promoter, which we identified as the most efficient promoter in this study. Expressing the *crtE* gene under the *adh4* promoter yielded an increase in intracellular astaxanthin content (0.43 mg/g-DCW) of about 1.7-fold, although the cell growth was slightly decreased compared with the control strain (Fig. 5). Conversely, expressing the *crtE* gene under the *gpd* promoter yielded an equal intracellular astaxanthin content, even though it yielded an increase in a total carotenoid content (Breitenbach et al. 2011). By contrast, expressing the *crtE* gene under the *gdh* promoter yielded an increase in intracellular astaxanthin content (0.20 mg/g-DCW) of about 1.4-fold (Rodríguez-Sáiz et al. 2009). These results indicate that the *adh4* promoter is acceptable for overexpression of target proteins for metabolic engineering in *X. dendrorhous*. In this study, through the quantitative evaluation of known promoters and new candidate promoters of *X. dendrorhous*, we identified *act*, *tpi*, and *adh4* as the most efficient promoters for overexpression of target proteins. In the future, these efficient promoters can be used for the expression of multiple genes involved in astaxanthin biosynthesis, improving overall astaxanthin production. Furthermore, by considering the higher potential of terpene production in *X. dedrorhous* than in *E. coli* and *S. cerevisiae* (Melillo et al. 2013), the metabolic engineering system developed in this study is a new, useful tool for improving not only astaxanthin production but also carotnoids production in *X. dendrorhous*.

Acknowledgments This study was supported by the Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe, iBioK), MEXT, Japan. Hara KY was supported by a Grant-in-Aid for Young Scientists (B) (22760608).

References

Breitenbach J, Visser H, Verdoes JC, van Ooyen AJ, Sandmann G (2011) Engineering of geranylgeranyl pyrophosphate synthase levels and physiological conditions for enhanced carotenoid and astaxanthin synthesis in *Xanthophyllomyces dendrorhous*. Biotechnol Lett 33: 755–761

Gatignol A, Dassain M, Tiraby G (1990) Cloning of *Saccharomyces cerevisiae* promoters using a probe vector based on phleomycin resistance. Gene 91:35–41

Gu WL, An GH, Johnson EA (1997) Ethanol increases carotenoid production in *Phaffia rhodozyma*. J Ind Microbiol Biotechnol 19: 114–117

Melillo E, Setroikromo R, Quax WJ, Kayser O (2013) Production of α-cuprenene in *Xanthophyllomyces dendrorhous*: a step closer to a potent terpene biofactory. Microb Cell Fact 12:13

Miao L, Chi S, Tang Y, Su Z, Yin T, Guan G, Li Y (2011) Astaxanthin biosynthesis is enhanced by high carotenogenic gene expression and decrease of fatty acids and ergosterol in a *Phaffia rhodozyma* mutant strain. FEMS Yeast Res 11:192–201

Nghiem NP, Kim TH, Yoo CG, Hicks KB (2013) Enzymatic fractionation of SAA-pretreated barley straw for production of fuel ethanol and astaxanthin as a value-added co-product. Appl Biochem Biotechnol 171:341–351

Niklitschek M, Alcaíno J, Barahona S, Sepúlveda D, Lozano C, Carmona M, Marcoleta A, Martínez C, Lodato P, Baeza M, Cifuentes V (2008) Genomic organization of the structural genes controlling the astaxanthin biosynthesis pathway of *Xanthophyllomyces dendrorhous*. Biol Res 41:93–108

Partow S, Siewers V, Bjørn S, Nielsen J, Maury J (2010) Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*. Yeast 27:955–964

Rodríguez-Sáiz M, Godio RP, Alvarez V, de la Fuente JL, Martín JF, Barredo JL (2009) The NADP-dependent glutamate dehydrogenase gene from the astaxanthin producer *Xanthophyllomyces dendrorhous*: use of its promoter for controlled gene expression. Mol Biotechnol 41:165–172

Rodríguez-Sáiz M, de la Fuente JL, Barredo JL (2010) *Xanthophyllomyces dendrorhous* for the industrial production of astaxanthin. Appl Microbiol Biotechnol 88:645–658

Satoh A, Tsuji S, Okada Y, Murakami N, Urami M, Nakagawa K, Ishikura M, Katagiri M, Koga Y, Shirasawa T (2009) Preliminary clinical evaluation of toxicity and efficacy of a new astaxanthin-rich *Haematococcus pluvialis* extract. J Clin Biochem Nutr 44:280–284

Schmidt I, Schewe H, Gassel S, Jin C, Buckingham J, Hümbelin M, Sandmann G, Schrader J (2011) Biotechnological production of astaxanthin with *Phaffia rhodozyma*/*Xanthophyllomyces dendrorhous*. Appl Microbiol Biotechnol 89:555–571

Slaninová I, Kucsera J, Svoboda A (1999) Topology of microtubules and actin in the life cycle of *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Antonie Van Leeuwenhoek 75:361–368

Tripathi DN, Jena GB (2009) Intervention of astaxanthin against cyclophosphamide-induced oxidative stress and DNA damage: a study in mice. Chem Biol Interact 80:398–406

Verdoes JC, Sandmann G, Visser H, Diaz M, van Mossel M, van Ooyen AJ (2003) Metabolic engineering of the carotenoid biosynthetic pathway in the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Appl Environ Microbiol 69:3728–3738

Wery J, Gutker D, Renniers AC, Verdoes JC, van Ooyen AJ (1997) High copy number integration into the ribosomal DNA of the yeast *Phaffia rhodozyma*. Gene 184:89–97

Young T, Williamson V, Taguchi A, Smith M, Sledziewski A, Russell D, Osterman J, Denis C, Cox D, Beier D (1982) The alcohol dehydrogenase genes of the yeast, *Saccharomyces cerevisiae*: isolation, structure, and regulation. Basic Life Sci 19:335–361