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Developing polycistronic expression tool in *Yarrowia lipolytica*

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ABSTRACT

Unconventional oleaginous yeast *Yarrowia lipolytica* has gained widespread applications as a microbial cell factory for synthesizing various chemicals and natural products. The construction of efficient cell factories requires intricate metabolic engineering. However, multi-gene expression in *Y. lipolytica* is labor-intensive. To facilitate multi-gene expression, we developed the polycistronic expression tool using 2A peptides. We first compared different 2A peptides in *Y. lipolytica* and identified two 2A peptides with high cleavage efficiency: P2A and ERBV-1. The effect of 2A peptides on the expression level of upstream and downstream genes was then determined. Ultimately, we applied the identified 2A peptides to express four genes in canthaxanthin biosynthetic pathway within one expression cassette for canthaxanthin production. This study enriches the multi-gene expression tools of *Y. lipolytica*, which will facilitate the cell factory construction of *Y. lipolytica*.

1. Introduction

Yarrowia lipolytica, a non-conventional oleaginous yeast, is widely recognized as an attractive microbial cell factory for industrial production. It has been reported to produce many valuable chemicals, such as organic acids [1,2], lipids [3], and terpenoids [4]. Engineering efficient cell factories require systematic and complex genetic modifications. Gene expression in *Y. lipolytica* is commonly achieved through methods such as non-homologous end joining (NHEJ)-mediated random integration [5,6] and CRISPR-mediated targeted integration [7,8]. However, it is difficult to achieve multi-gene expression in either of these ways because the length of gene expression cassettes for multi-gene expression is very long, resulting in the requirement of iterative gene manipulation. Unlike prokaryotic microorganisms, which can structurally express multiple genes in operons, eukaryotic microorganisms lack robust, natural multi-gene expression tools. Therefore, developing robust multi-gene expression tools is necessary to accelerate the efficiency of metabolic engineering.

In *Y. lipolytica*, several DNA assembly approaches, such as the GoldenGate Kit [9], USER Cloning Approach [10], and BioBricks (YaliBricks) [11,12] Systems have been developed. The GoldenGate cloning technique utilizes type IIS restriction endonucleases and T4 DNA ligase to achieve seamless assembly of multiple DNA fragments [9]. USER cloning employs specific enzymes to generate sticky ends at designated sites, facilitating precise end formation and seamless assembly [10]. The modular design of BioBricks system allows for the convenient combination of different functional elements; however, each assembly step requires enzymatic digestion and ligation [11,12]. Anyway, these techniques require promoters and terminators for each gene, increasing operational complexity.

Polycistronic expression systems offer significant advantages as a single promoter can control the expression of multiple genes, simplifying genetic design. Polycistronic expression in eukaryotic organisms generally requires the expression of multiple genes mediated by internal ribosome entry site (IRES) sequences, 2A peptides, or intergenic sequence (IGG) [13]. IRES-mediated translation typically yields lower expression levels for downstream proteins than cap-dependent translation [14,15]. Compared with IRESs, 2A peptide sequences have major advantages in multi-gene expression, such as shorter sequences and higher expression levels of downstream genes [16–19]. The 2A peptide system employs short peptide sequences found in viruses to induce ribosomal skipping during translation, facilitating the co-expression of multiple genes from a single transcript. 2A peptide sequences allow a eukaryotic cell to produce multiple separated peptides from one mRNA through an event called "stop-carry on" (Fig.1a). Research on 2A peptide from the foot-and-mouth disease virus (FMDV) shows that it forms a

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specific secondary structure that interacts with the ribosome, facilitating peptide bond hydrolysis and resulting in cleavage at the 2A site to produce distinct proteins [20] (Fig. 1b). This cleavage does not require additional cofactors, making 2A peptide an effective tool for simultaneously expressing multiple genes. The relatively short length of the 2A sequence allows for consistent expression levels of linked genes, simplifying the construction of expression vectors while avoiding the repetitive usage of promoter and terminator. IGG sequence is from filamentous fungi [13]. Recently, the sequence has been optimized to allow the facile bicistronic expression of genes of interest in a variety of fungi [13]. IGG sequence leads to lower gene expression than 2A peptides and higher expression than internal ribosome entry site (IRES) in bicistrons [13].

Viral 2A peptides have been extensively used as an established tool for polycistronic gene expression in both animals and plants [21–24]. In fungi, 2A peptides have also been employed across various species to facilitate compound production. In *Saccharomyces cerevisiae*, 2A peptides have been utilized to synthesize β-carotene [25], geraniol [26], and friedelin [27]. In *Rhodosporidium toruloides*, 2A peptides have been used for β-carotene production [28], and in *Kluyveromyces marxianus*, they have been applied to enhance the saccharification of corncobs [29]. These studies validate the role of 2A peptides for polycistronic gene expression and also highlight the differences among various 2A peptides in different species. Numerous viruses harbor 2A peptides, each with distinct cleavage efficiencies. Various 2A peptides, such as P2A (2A from Porcine teschovirus-1, also called PTV), E2A (2A from Equine rhinitis A virus, also called ERAV), F2A (2A from Foot-and-mouth disease virus, also called FMDV), T2A (2A from Thosea asigna virus, also called TaV), ERBV-1 (2A from Equine rhinitis B virus), and O2A (2A from Operophtera brumata cypovirus-18, also called OpbuCPV18), have been investigated in fungi. Notably, the cleavage efficiency of a particular 2A peptide can significantly vary across different species. For instance, F2A exhibits high cleavage efficiency in animals but demonstrates low efficiency in fungi [27]. Therefore, systematic comparisons of the cleavage efficiencies of different 2A peptides are required in specific organisms. Yang et al. used 2A peptide to express the genes of lipogenesis pathway in *Y. lipolytica* [30]. However, there is still a gap in the systematic comparison of the efficiency of different 2A peptides in *Y. lipolytica*.

In this study, we systematically compared the cleavage efficiencies of five 2A peptides and identified two 2A peptides with high cleavage efficiency. Then, we successfully applied the screened high-efficiency 2A peptides to the expression of four genes in canthaxanthin biosynthetic pathway within one expression cassette. Here, our work validated the feasibility of the 2A peptide as a tool for multi-gene expression in *Y. lipolytica*, which will facilitate the engineering of *Y. lipolytica* strains to produce various chemicals.

2. Materials and methods

2.1. Strains and media

Y. lipolytica strain PO1f was used as chassis strain. *Y. lipolytica* was cultivated in YPD medium (20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone) or SC-LEU medium (1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, and 0.69 g/L complete synthetic mixture minus leucine). *Escherichia coli* strain DH5α was used for plasmid construction. *E. coli* was cultivated in LB medium (5 g/L yeast extract, 10 g/L peptone, 10 g/L NaCl) supplemented with 0.2 mg/mL ampicillin. Agar concentration was maintained at 20 g/L in solid media formulations.

2.2. Plasmid construction

Inverse PCR was utilized to modify the plasmid YLEP-ubi-hrGFP and a 2A peptide sequence was inserted between the *ubi* sequence and *hrGFP* [31], resulting in the construct of YLEP-ubi-2A-hrGFP (2A represents a series of different 2A peptides). The *hrGFP* gene and *mCherry* gene [31] were amplified from the plasmid UAS1B8-TEF-hrGFP and UAS1B8-TEF-mCherry by polymerase chain reaction (PCR) using Phanta Max Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China). The *hrGFP* and *mCherry* fragments were ligated via the 2A peptide sequence by using fusion PCR and inserted into the YLEP-LEU2 vector to generate the plasmids YLEP-hrGFP-2A-mCherry (2A represents a series of different 2A peptides). The *SDCrtW* gene (GenBank accession number AB181388.1) was amplified from the plasmid YLEP-SDC. Genes *CarRP* (GenBank accession number AJ250827.1), *CarB* (GenBank accession number AJ238028.1), and *GGS1* (NCBI Reference Sequence: NC_089381.1) were amplified from plasmid p-M3. *SDCrtW*, *CarRP*, and *CarB* were linked using P2A and ERBV-1 peptides sequence and cloned into the pUAS1B8-TEF-LEU2 vector, resulting in the plasmid of 2A-C3. *SDCrtW*, *CarRP*, *CarB*, and *GGS1* were similarly ligated and cloned into the same vector to create plasmid 2A-C4. PCR using Phanta Max Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China). Primers were synthesized by TsingKe (Beijing, China). The restriction enzymes were purchased from Thermo Fisher Scientific (Shanghai, China). All strains, plasmids, primers, and 2A peptide sequences used in this study are listed in supplementary (Table. S1, S2, S3, S4).

2.3. Transformation

The lithium acetate method [32] was used to transform *Y. lipolytica*. The episomal plasmids of YLEP-ubi-2A-hrGFP and YLEP-hrGFP-2A-mCherry were transformed into PO1f strain for episomal expression. The plasmids of 2A-C3 and 2A-C4 were linearized through enzymatic digestion and subsequently transformed into the PO1f strain for random integration into the genome. The transformants

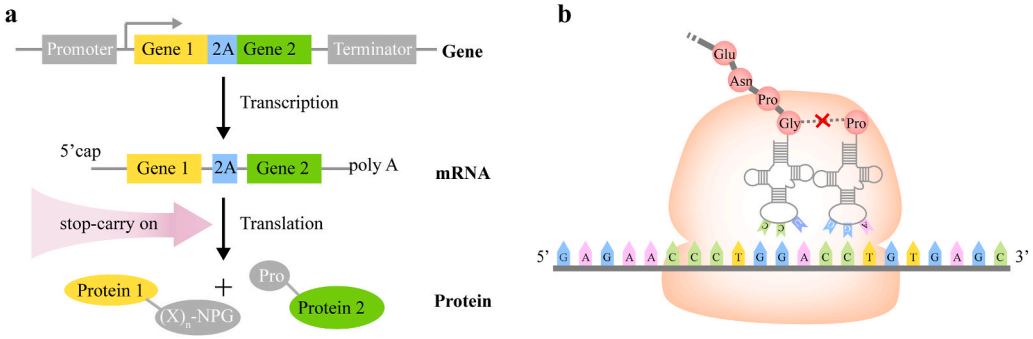

Fig. 1. The mechanism of 2A peptide-mediated self-cleavage. (a) Through an event known as "stop-carry on", the 2A peptide allows a single mRNA in eukaryotes to produce multiple peptides. The protein upstream of the 2A peptide contains most of the 2A peptide sequence, while the protein downstream contains only an additional Pro. (b) When the ribosome translates the NPGP sequence at the C-terminus of the 2A peptide, the secondary structure formed by the translated 2A peptide sequence prevents the formation of the peptide bond between the terminal Gly and Pro.

were selected on the corresponding selection media in plates.

2.4. Fluorescence detection in yeast strains

Strains were precultured at 30 °C and 200 rpm for approximately 24 h. Then, 2 % (v/v) of the seed cultures were transferred to fresh medium and grown at 30 °C and 200 rpm in a deep well plate for 36 h. The cell density at OD_{600} and green fluorescence (excitation, 485 nm; emission, 528 nm) were detected using a 1420 Multilabel Counter (Victor™ 3 V, PerkinElmer, USA). All fluorescence measurements were normalized to OD_{600}.

2.5. Fermentation, extraction, and analysis of canthaxanthin and other carotenoids

The pre-cultured seed of strains was transferred to deep well plates containing 1.5 mL of YPD fermentation medium and cultured at 30 °C and 200 rpm for 3 days. A 50 μL aliquot of the fermentation broth was centrifuged to collect the cells. The cells were resuspended in 0.7 mL of dimethyl sulfoxide, and disrupted by a high-throughput tissue grinding and crushing instrument (Shangkezhi, China). The instrument parameters were set to 60 Hz for 20 cycles of 60 s. Then, the cells were incubated at 55 °C for 10 min and then at 50 °C for 10 min with an equal volume of acetone. Finally, the samples were centrifuged at 12,000×*g* for 5 min.

The samples were analyzed using a Shimadzu LC-20 AT high-performance liquid chromatograph with a 475-nm variable wavelength detector and an XDB-C18 column (Eclipse, USA). One of the mobile phases was methanol, acetonitrile, and dichloromethane (21:21:8), and the other was 80 % acetonitrile. The flow rate was 1.0 mL/min, and the column temperature was 30 °C.

3. Results

3.1. Screening of 2A peptides with high-cleavage efficiency in Y. lipolytica

Previous studies have shown that 2A peptides from different sources exhibit different cleavage efficiency when applied to different microorganisms or mammals. To identify 2A peptides applicable to *Y. lipolytica*, we selected five versions: T2A has been used in *Y. lipolytica* [30], P2A, E2A, F2A, and ERBV-1 were reported to show high cleavage efficiency in other organisms [25–29].

The expression of 2A peptides can lead to three possible outcomes: (1) translation of separate peptide chains, (2) translation of only the upstream protein, or (3) translation of an uncleaved fusion protein. For polycistronic expression, the first scenario is preferred because fusion proteins may not retain the physiological functions of individual proteins. Here, we define the proportion of the first scenario among all translation outcomes as the cleavage efficiency of the 2A peptide.

We utilized a green fluorescent protein degradation assay to compare the cleavage efficiencies of different 2A peptides in *Y. lipolytica*. N-terminus of hrGFP was fused to a ubiquitin degradation tag via different 2A peptides. The strain only expressing hrGFP was used as positive control and the strain expressing hrGFP fused to a ubiquitin degradation tag at the N-terminus was used as negative control (Fig. 2a). We found that the fluorescence level was high in the positive control because hrGFP was expressed normally. While in the negative control, the presence of the degradation tag at the N-terminus resulted in very low fluorescence levels, and it is close to the background fluorescence of the strain, demonstrating that hrGFP protein was completely degraded (Fig. 2b). In the presence of 2A peptides, when the 2A peptide cleaved efficiently, two separate peptide chains were produced, and the N-terminus of hrGFP did not have a degradation tag, preventing its degradation. When uncleaved fusion proteins were produced, the degradation tag at the N-terminus caused hrGFP to be degraded. We found that in the presence of 2A peptides, the fluorescence levels of P2A, T2A, and ERBV-1 were between the negative and positive control. It indicated that only a portion of protein can be cleaved. Among the tested 2A peptides, the strains containing P2A and ERBV-1 had higher fluorescence levels, around 45 % of that in the positive control. The fluorescence level of the strains containing T2A was also much higher than the negative control, while the fluorescence levels of the strains containing F2A and E2A were very low, only slightly higher than the negative control. The higher the cleavage efficiency of the 2A peptide, the greater the proportion of non-degraded GFP, and hence, higher fluorescence levels in the strain (Fig. 2a). Therefore, we determined the relative cleavage efficiencies of the five 2A peptides in *Y. lipolytica* as follows: P2A > ERBV-1 > T2A > F2A > E2A (Fig. 2b).

3.2. Impact of 2A peptides on the expression of upstream and downstream genes

It is reported that 2A peptides affect the translation levels of both upstream and downstream proteins [16]. The translation of downstream proteins decreases with the use of 2A peptides. To explore the effects of 2A peptides on upstream and downstream genes in *Y. lipolytica*, we

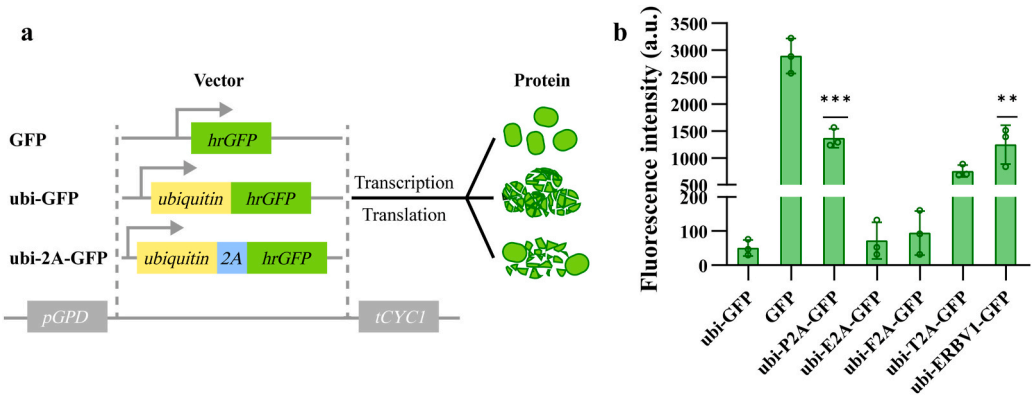

Fig. 2. Comparison of the cleavage efficiency of different 2A peptides using the GFP degradation assay. (a) Principle of the GFP degradation assay. When hrGFP is expressed alone, the hrGFP level is high; when a ubiquitin degradation tag is attached to the N-terminus of hrGFP, hrGFP is degraded; when hrGFP and the ubiquitin degradation tag are fused by a 2A peptide, the hrGFP produced by self-cleavage is not degraded, while the fusion protein is degraded. (b) Fluorescence results of the GFP degradation assay. Fluorescence intensity values for each group are obtained by dividing the fluorescence intensity at 485 nm/528 nm by OD_{600} of the culture and subtracting the background fluorescence of the strain. Three clones were selected for each group. Error bars represent standard deviations. Mean values ± standard deviations are shown (n = 3 independent biological samples). Student's t-test was used to compare the experimental group with the ubi-GFP control group (*p < 0.05, **p < 0.01, ***p < 0.001).

designed a reporter system utilizing an episomal expression vector consisting of the *GPD1* promoter, and the *CYC1* terminator, upstream gene encoding a green fluorescent protein (GFP), the downstream gene encoding a red fluorescent protein (mCherry) and 2A peptides of interest in the middle. The plasmid that expressed *hrGFP* and *mCherry* genes alone was used as a control (Fig. 3a). When using P2A and ERBV-1 to express red and green fluorescent proteins tandemly, the green fluorescence intensity of the strains was similar to that of the strain expressing *hrGFP* alone. However, when using other 2A peptides for tandem expression, the green fluorescence intensity was 25%–30 % lower than that of expressing *hrGFP* alone (Fig. 3b). Comparing with the *mCherry*-only group, the red fluorescence intensity was 16%–32 % lower in tested 2A peptide tandem groups (Fig. 3c). We conclude that 2A peptides P2A and ERBV-1 have a minimal effect on the expression of upstream proteins, and they tend to reduce the expression of downstream proteins.

3.3. Constructing canthaxanthin biosynthesis pathway in Y. lipolytica using high-cleavage efficiency 2A peptides

After identifying two 2A peptides with high cleavage efficiency, we aimed to validate their feasibility as tools for polycistronic expression in *Y. lipolytica*. Canthaxanthin biosynthetic pathway was used as an example (Fig. 4a). To construct the pathway, the *CarB* gene from *Mucor circinelloides*, which encodes phytoene dehydrogenase, the *CarRP* gene from *Mucor circinelloides*, which encodes phytoene synthase/lycopene cyclase [33], and the *SDCrtW* gene from *Brevundimonas* sp. strain SD212, which encodes beta-carotene ketolase [34] were expressed in PO1f to produce canthaxanthin. We constructed a tricistronic expression cassette containing the strong promoter *UAS1B8-TEF* and terminator *CYC1*, with the genes arranged from the N-terminus to the C-terminus as SDCrtW-ERBV-1-CarRP-P2A-CarB (Fig. 4b). Previous studies have shown that the order of genes in polycistron using 2A peptides affected the chemical yield in *S.cerevisiae* [25]. It is suggested that the first and second positions at the N-terminus exhibit better expression efficiency in other species. Therefore, we placed *SDCrtW* in N-terminal position and followed by *CarRP* and *CarB*. The expression cassette containing plasmid was linearized and transformed into PO1f strain for random integration. Different transformants were cultivated in a YPD liquid medium for 96 h. The strains can produce canthaxanthin and different transformants have different production. The canthaxanthin production was 0.96 mg/L-3.975 mg/L (Fig. 4c). We also compared the tricistronic expression with the strains expressing *SDCrtW*, *CarRP*, and *CarB* with individual promoters and terminators (Fig. S1) and found that the canthaxanthin production was slightly lower in tricistronic expression strains (Fig. S1). It may be attributed to the slightly reduced expression of downstream genes in tricistronic expression strains when the genes were linked by the 2A peptide. To further explore the possibility of using 2A peptides for tetracistronic expression, we extended the expression cassette by adding the endogenous *Y. lipolytica GGS1* gene at the C-terminus via an ERBV-1 linkage, creating a tetracistronic cassette (Fig. 4d). GGS1 can increase the supply of the canthaxanthin precursor GGPP. The strain expressing this cassette can produce 6.54 mg/L-19.98 mg/L canthaxanthin (Fig. 4e). The maximum canthaxanthin production of the PO1f strain containing tetracistronic cassette is 4-folds higher than that of the PO1f strain containing tricistronic cassette. We also observed the accumulation of a small amount of lycopene and β-carotene, demonstrating the activity of SDCrtW and CarRP still needs to be improved.

4. Discussion

This study aims to identify 2A peptides with high cleavage efficiency. To screen high-efficiency 2A peptides, we conducted a hrGFP degradation assay. Based on the fluorescence intensities of different groups, we preliminarily determined the relative cleavage efficiencies of the five 2A peptides as follows: P2A > ERBV-1 > T2A > F2A > E2A. Other studies have compared the cleavage efficiencies of different 2A peptides using immunoblotting, calculating the specific cleavage efficiency from the grayscale values of cleaved and uncleaved bands [25–29]. In *R.toruloides*, the cleavage efficiency of P2A is higher than that of F2A [28]. In *S.cerevisiae*, the relative cleavage efficiencies of the five 2A peptides were determined to be ERBV-1 > P2A > T2A > E2A > F2A [27], similar to our findings in *Y. lipolytica*. It is reported in some fungi organisms such as *Ustilago maydis*, *Aspergillus fumigatus,* and *Aspergillus niger*, P2A peptides can result in around 100 % cleavage [35–37], but in yeast *S. cerevisiae* or *Pichia pastoris*, the cleavage efficiency cannot reach to 100 % [27,38]. In this study, we found that similar to other yeasts, in *Y. lipolytica*, the cleavage efficiency also cannot reach 100 %. If the cleavage efficiency is calculated as the ratio of fluorescence intensity of the 2A peptide group to the non-degradation group, the results are lower than those obtained through immunoblotting. We believe the sensitivity of the fluorescence method is different from immunoblotting. Nevertheless, this experiment provides a rapid and stable method for comparing the cleavage efficiencies of different 2A peptides by measuring the fluorescence intensity.

When tandemly linking red and green fluorescent proteins using two highly efficient 2A peptides (P2A and ERBV-1), the green fluorescence intensity of the strains was higher than that of other 2A peptides. The result indicates that the higher cleavage efficiency of the 2A peptide results in higher expression efficiency of the upstream protein. In all 2A peptide tandem groups, the red fluorescence intensity was lower than in

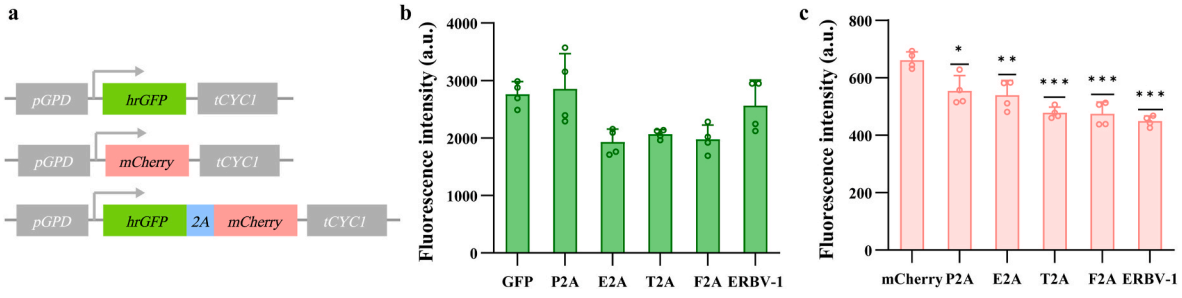

Fig. 3. Exploring the impact of 2A peptides on upstream and downstream proteins. (a) Structure of the expression cassette used in this experiment. The *GPD1* promoter and *CYC1* terminator regulate gene expression. *hrGFP* is positioned upstream of the 2A sequence, and *mCherry* is positioned downstream. (b) Green fluorescence levels in the strain expressing *hrGFP* alone and the strains with tandem genes with 2A sequence. Fluorescence intensity values are obtained by dividing the fluorescence intensity at 485 nm/528 nm by OD_{600} of the culture. Four clones were selected for each group. Error bars represent standard deviations. (c) Red fluorescence levels in the group were expressed by *mCherry* alone and the strains with tandem genes with 2A sequence. Fluorescence intensity values are obtained by dividing the fluorescence intensity at 590 nm/645 nm by OD_{600} of the culture. Four clones were selected for each group. Error bars represent standard deviations. Mean values ± standard deviations are shown (n = 4 independent biological samples). Student's t-test was used to compare the experimental group with the control group (*p < 0.05, **p < 0.01, ***p < 0.001).

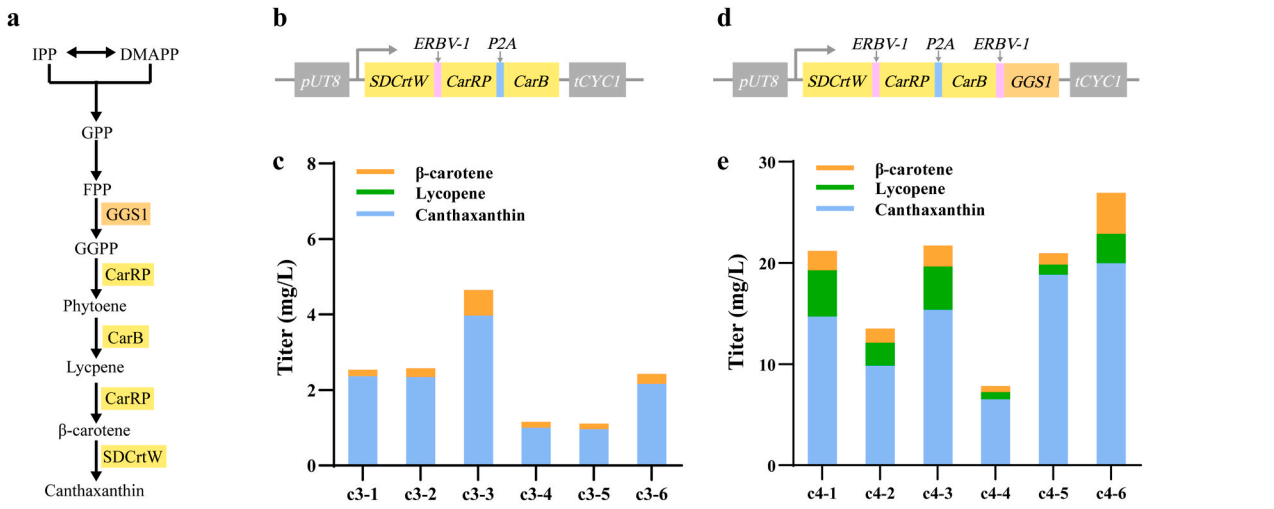

Fig. 4. Polycistronic expression to construct canthaxanthin biosynthetic pathway using 2A peptides. (a) Canthaxanthin biosynthetic pathway. *Y. lipolytica* can synthesize GGPP through an endogenous pathway. Expression of CarRP, CarB, and SDCrtW in *Y. lipolytica* enables the synthesis of canthaxanthin. (b) Structure of the tricistronic expression cassette. The promoter is *UAS1B8-TEF*, and the terminator is *CYC1*. *ERBV-1* was fused between *SDCrtW* and *CarRP*, while P2A was fused with *CarRP* and *CarB*. (c) Carotenoids production in PO1f containing tricistronic expression cassette. c3-1 to c3-6 represent six transformants of PO1f containing a tricistronic expression cassette. (d) Structure of the tetracistronic expression cassette. The promoter is *UAS1B8-TEF*, and the terminator is *CYC1*. ERBV-1 was fused between *SDCrtW* and *CarRP*, P2A was fused between *CarRP* and *CarB*, and ERBV-1 was fused between *CarB* and *GGS1*. (e) Carotenoids production in PO1f containing the tetracistronic expression cassette. c4-1 to c4-6 represent six transformants of PO1f containing the tetracistronic expression cassette.

the group expressing red fluorescent protein alone, suggesting that 2A peptides reduce the expression of downstream proteins.

We validated the feasibility of using 2A peptides as tools for polycistronic expression in *Y. lipolytica* by expressing the genes of canthaxanthin biosynthetic pathway. By constructing a tricistronic expression system with 2A peptides, we successfully synthesized canthaxanthin in *Y. lipolytica*, demonstrating that 2A peptides can be used as tools for polycistronic expression in the production of compounds in *Y. lipolytica*. Studies have shown that the expression efficiency of genes in the third and fourth positions linked by 2A peptides tends to be lower [25]. To verify the potential for tetracistronic expression using 2A peptides, we extended the tricistronic expression system by linking the endogenous *Y. lipolytica* gene *GGS1*, responsible for synthesizing the precursor GGPP, at the end via ERBV-1, creating a tetracistronic vector. The canthaxanthin production of strains containing the tetracistronic plasmid was 4–5 folds higher than that of strains containing the tricistronic plasmid, indicating stable expression of GGS1 in the fourth position.

This study identified two highly efficient 2A peptides for *Y. lipolytica* and demonstrated that a tetracistronic expression system constructed with 2A peptides can be stably expressed in *Y. lipolytica*. Using 2A peptides to construct polycistronic expression cassettes reduces the construction cycle for multi-gene expression. Since multiple genes fused by 2A sequence use the same promoter, it allows for synchronized regulation of multiple genes in *Y. lipolytica*.

CRediT authorship contribution statement

Donghan Li: Writing – original draft, Methodology, Investigation, Data curation. **Jianhui Liu:** Writing – original draft, Methodology, Investigation, Data curation. **Lingxuan Sun:** Methodology, Data curation. **Jin Zhang:** Formal analysis. **Jin Hou:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.09.010.

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