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Two Co-Expression Strategies for Eicosapentaenoic Acid Production in *Mortierella alpina*

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Abstract: The omega-3 long-chain Polyunsaturated Fatty Acid (PUFA) Eicosapentaenoic Acid (EPA) has beneficial effects on human health, leading to its use in health products and foods. The oleaginous microorganism *Mortierella alpina* has been used in the industrial production of Arachidonic Acid (AA). The omega-3 desaturase oPpFADS17 is a key enzyme in the bioconversion of AA to EPA from *Phytophthora parasitica* and Glucose-6-Phosphate Dehydrogenase (G6PD2) is a critical enzyme that provide reducing power NADPH for lipid biosynthesis. In this study, we used a double expression cassette and 2A peptide strategies to co-express the these two critical enzymes. Subsequently, we investigated the effects of these strategies on total lipid and EPA accumulation. The recombination strains generated using the above-described strategies exhibited no differences in cell growth, compared with the control strain. Recombination strains generated using the double expression cassette exhibited an increase in total lipids to 43% of the cell dry weight, but did not accumulate EPA. Recombination strains generated using the 2A peptide strategy exhibited increased EPA accumulation, such that this PUFA accounted for 30% of the total lipid content. These co-expression strategies provide the improvements of multi-gene modification in PUFA accumulation in *M. alpina*.

Keywords: Co-expression, Double Expression Cassette, 2A Peptide, *Mortierella alpine*, EPA

Introduction

Eicosapentaenoic Acid (EPA, C20:5), an omega-3 long-chain Polyunsaturated Fatty Acid (LC-PUFA), has attracted considerable attention due to its beneficial effects on human health, including the prevention and treatment of diabetes, immune disorders, cardiovascular disease and cancer (Hirahashi *et al*., 2014; Maehre *et al*., 2015; Xue *et al*., 2013). Within the food chain, EPA is mainly accumulated in marine fish. However, the market demand for this food source exceeds the supply because of human population growth, environmental pollution and limitations associated with the harvest season and location (Ji *et al*., 2015; Tang *et al*., 2018; Xie *et al*., 2017).

Various microorganisms, including *Mortierella alpina* and *Yarrowia lipolytica*, have been investigated as potential hosts for the sustainable commercial production of EPA (Ando *et al*., 2009; Xue *et al*., 2013). *M. alpina*, an oleaginous filamentous fungus currently used to produce commercial Arachidonic Acid (AA, C20:4), can generate EPA from AA via an omega-3 desaturase (EC 1.14.19.-) (Ge *et al*., 2017; Ji *et al*., 2014; Okuda *et al*., 2015). Our latest research identified a new omega-3 desaturase, oPpFADS17, from *Phytophthora parasitica*. This enzyme converts AA to EPA at a conversion rate of 70%. When expressed in a recombinant strain of *M. alpina*, oPpFADS17 increased the proportion of EPA among the Total Fatty Acids (TFA) content to 31.5% while the control

strain without the recombinant enzyme didn't accumulate EPA (Tang *et al*., 2018). Although this recombinant strain represents a breakthrough in EPA production, further enhancement of the TFA content could potentially increase the EPA yield further. Another previous study demonstrated that the Pentose Phosphate Pathway (PPP), especially Glucose-6-Phosphate Dehydrogenase (G6PD2, EC1.1.1.49), plays a major role in the accumulation of total lipids (Hao *et al*., 2016). Accordingly, the co-expression of oPpFADS17 and G6PD2 may represent a good strategy toward improving EPA production in *M. alpina*.

The double expression cassette strategy can be used to co-express multiple copies of a gene or multiple genes with independent promoters (Bouabe *et al*., 2008). Another multi-gene construction strategy, the self-splicing 2A peptide, is one of the most widely used tools for the construction of polygenic vector and has also attracted extensive attention (Park *et al*., 2009; Szymczak *et al*., 2004). The 2A peptide strategy exhibits obvious advantages with respect to protein activity and the expression of multiple downstream genes. Accordingly, it appears to be an ideal gene expression strategy (Hu *et al*., 2009; Trichas *et al*., 2008). Previous studies revealed that 2A peptide could be applied successfully in a broad range of organisms, including fungi (de Felipe *et al*., 2003), plants (Halpin *et al*., 1999; Park *et al*., 2009), mammalian cells (Hu *et al*., 2009; Szymczak *et al*., 2004) and transgenic animals (Trichas *et al*., 2008). In this study, we co-overexpressed oPpFADS17 and G6PD2 using both the double expression cassette and 2A peptide strategies with the aim of improving EPA production in *M. alpina*.

Materials and Methods

Strains and Culture Media

M. alpina strains were maintained on GY medium, which contained 0.3 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L NaH_2PO_4, 2 g/L KNO_3, 5 g/L yeast extract and 30 g/L glucose. Uracil (0.05 mg/mL) and 5-fluoroorotic acid (5-FOA, 0.5 mg/mL) were added to the medium when culturing uracil auxotrophs. *Agrobacterium tumefaciens* AGL-1 was cultured in YEP medium, which contained 5 g/L NaCl, 10 g/L yeast extract and 10 g/L tryptone. Synthetic Complete (SC) medium, Minimal Medium (MM) and Induction Medium (IM) were described previously (Hao *et al*., 2014).

Growth Conditions

Escherichia coli DH5α was cultivated at 37°C on LB agar medium containing 10 g/L NaCl, 10 g/L tryptone and 5 g/L yeast extract. *A. tumefaciens* AGL-1 was grown at 28°C in YEP agar medium. *M. alpina* strains were cultured at 28°C in broth medium at 200 rpm for 168 h (Wang *et al*., 2018).

Construction of the Transfer DNA Binary Vector Using the Double Expression Cassette Strategy

The pBIG2-ura5s-*G6PD2* plasmid was extracted (Hao *et al*., 2016) and the entire *G6PD2* expression unit (including the promoter and terminator) was obtained using Polymerase Chain Reaction (PCR) with *G6PD2*-F1/R1 primer sequences. The underlined regions of the sequences indicate the *XbaI* restriction site (Table 1). Subsequently, the purified G6PD2 expression unit fragment and the extracted plasmid expression vector pBIG2-ura5s-*oPpFADS17* (Tang *et al*., 2018) were digested using the restriction endonuclease *XbaI*. Recombinant ligase (Exnase) was then used to construct the binary expression vector pBIG2-ura5s-*oPpFADS17-G6PD2* using one step cloning kit (Takara).

Construction of the Transfer DNA Binary Vector Using the 2A Peptide Strategy

The 2A sequences are listed in Table 1 (produced by GenScrip.China). The target gene was digested with *HindIII*/*XmaI* and ligated into the binary vector pBIG2-ura5s-Its (Chen *et al*., 2015). According to the 2A peptide co-overexpression strategy, *oPpFADS17* and *G6PD2* were amplified using the *oPpFADS17*-F/R and *G6PD2*-F2/R2 primer pairs, respectively. First, *oPpFADS17* and pBIG2-ura5s-2A were separately digested with *HindIII* and *NheI* and then ligated to construct pBIG2-ura5s-*oPpFADS17-2A*. *G6PD2* and pBIG2-ura5s-*oPpFADS17-2A* were then digested using *KpnI* and *XmaI* and ligated together to generate pBIG2-ura5s-*oPpFADS17-2A-G6PD2*.

A. Tumefaciens-Mediated Transformation (*ATMT*)

ATMT was performed as described previously (Hao *et al*., 2014). Briefly, *M. alpina* uracil-auxotrophic strain spores were harvested and diluted with fresh liquid GY medium to a concentration of 10^8 spores/mL. *A. tumefaciens* AGL-1 was electro-transformed at 2.5 KV and 5.0 ms, then the transformants were cultured and diluted to an OD_{600} of 0.3 with fresh IM. Subsequently, the cells were incubated to an OD_{600} of 0.9, mixed with spore suspension and spread onto on solid IM with uracil of 0.05 g/L and transferred onto uracil-free SC medium with 100 μg/mL cefotaxime and 100 μg/mL spectinomycin. The obtained transformants were sub-cultured on uracil-free SC agar in three times to confirm the stability.

Genomic DNA Preparation

M. alpina strains were cultivated in liquid GY medium and the collected, then washed twice with sterile water. After removing most of the water, the mycelia were snap-frozen in liquid nitrogen. The DNA of *M. alpina* genomic was extracted as described previously (Wang *et al*., 2011).

Table 1: Primers used in this study

Primer	Sequence (5´–3´)a
G6PD2-F1 for double	AGGTACACTTGTTTAGAGGTCTAGAT
expression cassette strategy	TTAGTTGATGTGAGAGTTGTGAGATTCG
G6PD2-R1 for double	AGGGAGTCACGTTATGACCTCTAGAC
expression cassette strategy	CTCTAAACAAGTGTACCTGTGCATTCTG
oPpFADS17-F for 2A peptide strategy	ATCTTGAAGCTTCAATGGCTACCAAGCAGGCC
oPpFADS17-R for 2A peptide strategy	TCTAGAGCTAGCGGTGGACTTGGTCTTGACAG
G6PD2-F2 for 2A peptide strategy	GCACGGGGTACCATGTCTGAGAAGAAGAAGCATCTTT
G6PD2-R2 for 2A peptide strategy	GCTCCCCCCGGGTTAATGGTCAGTCCTTGTGTCCT
2A	CTCTCCTATGAGTCGTTTACCCAGAATGCACAGGTACAAAGCTTTCCCA
	AGCGAATTTGTCATCTCGACTGGTGCAAACTGCGCAAACGGCTGACTCA
	TTGCCCA TGCTTTTCTTCTCCACGCCTATCGTCTGTTACTGCATCTCTGTC
	GTGTTGAATGCGTAACTGATGGACTTCCGTATGGCTAGCGGCTCGGGAG
	CCACCAACTTCTCCCTGCTCAAGCAGGCAGGAGACGTCGAGGAGAAC
	CCCG GACCTGGTACCATGTCTGAGAAGAAGAAGCATCTTTACACTGTG
	GTTGTGCTCGGTGCATCGGGAGACCTAGCCAAGAAGAAAACCTTCCCG
	GCGCTCTTTGGACTCTTCAAAAACCAATATCTGGATCAAAACACGCA
	CATC GTCGGCCCCGGG
Hispro-F	CACACACAAACCTCTCTCCCACT
TrpC-R	CAAATGAACGTATCTTATCGAGATCC
oPpFADS17-RTF	TCTTCCCCACCCTCACCG
oPpFADS17-RTR	CAAGCCACGAGCGTAGTTCA
G6PD2-RTF	GCGTACAAAGATGGATCGG
G6PD2-RTR	TGAAAGCCGTCGTCTGTG
18S-RTF	CGTACTACCGATTGAATGGCTTAG
18S-RTR	CCTACGGAAACCTTGTTACGACT

Reverse Transcriptase Quantitative PCR (RT-qPCR) Analysis

RT-qPCR was carried out as described previously (Chen *et al*., 2015). Total RNA was extracted from the *M. alpina* using TRIzol reagent and reverse transcribed by the PrimeScript RT reagent kit. The primer pairs used for RT-qPCR were shown in Table 1. RT-qPCR was performed using the ABI Prism 7900 sequence detection system and Power SYBR green PCR master mix. The expression levels of all genes were normalised to the expression of the internal control gene (18S rRNA). The date were quantified by the method of $2^{-\Delta\Delta Ct}$.

Determination of Enzymatic Activity

Mycelia were collected by filtration, snap-frozen, ground in liquid nitrogen and suspended in extraction buffer (Chen *et al*., 2015). The suspension was centrifuged and collected the supernatant, then the protein concentration was determined by the Bradford method. The enzymatic activities of G6PD were detected as described previously (Tang *et al*., 2015; 2014) and indicated by the NADPH production of per min and per mg protein.

NADPH Quantification

The *M. alpina* mycelia were rapidly collected, frozen and ground in liquid nitrogen. The NADPH levels were determined using a NADPH quantification colorimetric kit (Biovision, Milpitas, CA, USA) according to the manufacturers' instruction.

Cell Dry Weight (*CDW*) *and Fatty Acid Methyl Esters* (*FAME*) *Analysis*

Fungal mycelia were harvested, washed twice with distilled water and frozen in liquid nitrogen. After lyophilisation, the CDW was determined gravimetrically. Approximately 20 mg (dry weight) of mycelia were subjected to fatty acid extraction and methyl esterification. The profiles of FAMEs were analysed using gas chromatography-mass spectrometry as previously (Chen *et al*., 2015).

Statistical Analysis

The experiments were carried out in three biological replicates and the mean values and standard errors were calculated. A statistical analysis of the data was performed by SPSS 20.0. One-way analysis of variance (ANOVA) was conducted on the data and $P < 0.05$ was considered significantly different.

Results

Co-expression oPpFADS17 and G6PD2 in M. Alpina Using the Double Expression Cassette and 2A Peptide Strategies

To generate a high-EPA-producing strain, *oPpFADS17* and *G6PD2* were amplified and co-

overexpressed in *M. alpina* using the double expression cassette strategy. The recombinant plasmid pBIG2-ura5s-*oPpFADS17-2A-G6PD2* was constructed as shown in Fig. 1. The recombinant vectors yielded target gene fragments of 818, 1256 and 1711 bp, indicating that the double expression cassette strategy was used to successfully construct the co-expression vector pBIG2-ura5s-*oPpFADS17-G6PD2*.

To compare the effectiveness of co-expression between the two strategies, *oPpFADS17* and *G6PD2* were also amplified and co-overexpressed in *M. alpina* using the 2A peptide strategy (Fig. 2). The recombinant vector pBIG2-ura5s-*oPpFADS17-G6PD2* yielded target gene fragments of 818 and 2879 bp, indicating the successful construction of the co-expression vector.

The double expression cassette- and 2A peptide-generated recombinant plasmids were transformed into *M. alpina* using ATMT. Six transformants each were generated using the double expression cassette (MA-*oPpFADS17-G6PD2*-1/2/3/4/5/6) and 2A peptide strategies (MA-*oPpFADS17-2A-G6PD2*-1/2/3/4/5/6) (Fig. 3). The double expression cassette transformants yielded three fragments (818, 1256 and 1711 bp), whereas the wild-type control strain did not yield these fragments. These data indicated successful insertion of the cassette into the genome (Fig. 3A). The 2A peptide transformants were shown to harbour the *ura5* and *oPpFADS17-2A-G6PD2* expression cassettes, as indicated by the presence of two inserted T-DNA fragments which of 818 and 2879 bp (Fig. 3B). In other words, both cassettes were successfully inserted into the genomes of the transformants.

Cell Growth and Lipid Accumulation in oPpFADS17 and G6PD2 Co-Expressing Strains

Next, the CDW, TFA content and EPA content of each *M. alpina* recombination strain was measured to investigate the effects of oPpFADS17 and G6PD2 co-expression via both strategies on cell growth and lipid accumulation (Fig. 4 and 5). As shown in Fig. 4A and 5A, neither recombination strategy appeared to have a significant effect on CDW when the recombinant strains were compared with the control strain *M. alpina* ATCC 32222. In other words, the inserted genes did not affect cell growth. However, the recombination strains generated using the double expression cassette strategy exhibited clear increases in the TFA and MA-*oPpFADS17-G6PD2*-1/2 contents to 42.2% and 43.0% (the value of control strain is 34.9%), respectively, whereas the strains generated using the 2A peptide strategy did not differ from the control strain (Fig. 4B). Moreover, EPA was not detected in any of the recombination strains generated using the double expression cassette strategy (Fig. 4C). In other words, the co-expression of oPpFADS17 and G6PD2 via a double expression cassette increased the TFA but had no effect on EPA accumulation. In contrast, the TFA contents did not differ significantly between the recombination strains generated using the 2A peptide strategy and the control strain (Fig. 5B). Meanwhile, the recombination strains generated using the 2A peptide strategy varied widely with respect to the ratio of EPA to the TFA content. Specifically, the EPA contents in the strains MA-*oPpFADS17-2A-G6PD2*-1/2/3/4 increased to 29.4%, 30.6%, 2.4% and 4.1% (EPA is not detected in control strain), respectively, whereas EPA was not detected in MA-*oPpFADS17-2A-G6PD2*-5/6 (Fig. 5C). The differences in the TFA and EPA contents of these 2A peptide-generated recombination strains can be attributed to random integration based on the ATMT method (Ando *et al*., 2009).

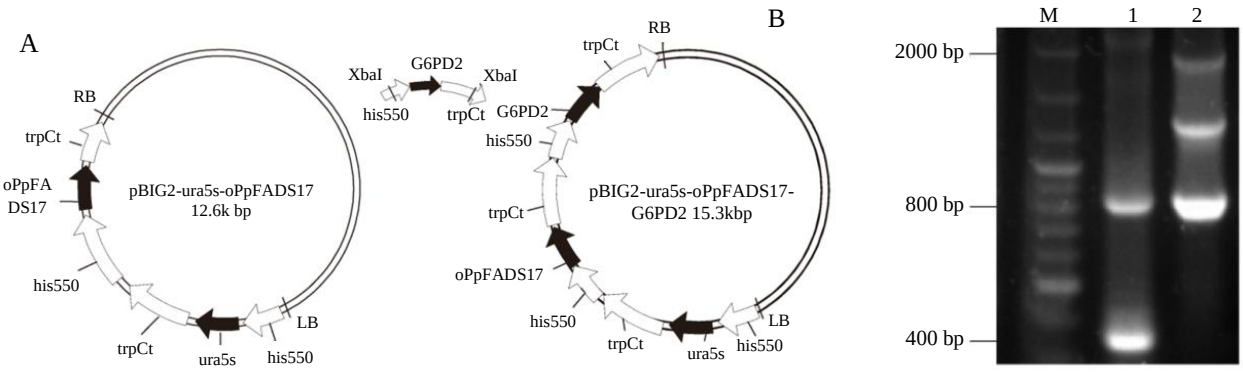

Fig. 1: Construction of the binary vector pBIG2-ura5s-oPpFADS17-G6PD2 using double expression cassette strategy (A) and PCR verification of the recombinant plasmid (B). M, Marker; 1, pBIG2-ura5s-ITs; 2, pBIG2-ura5s-oPpFADS17-G6PD2

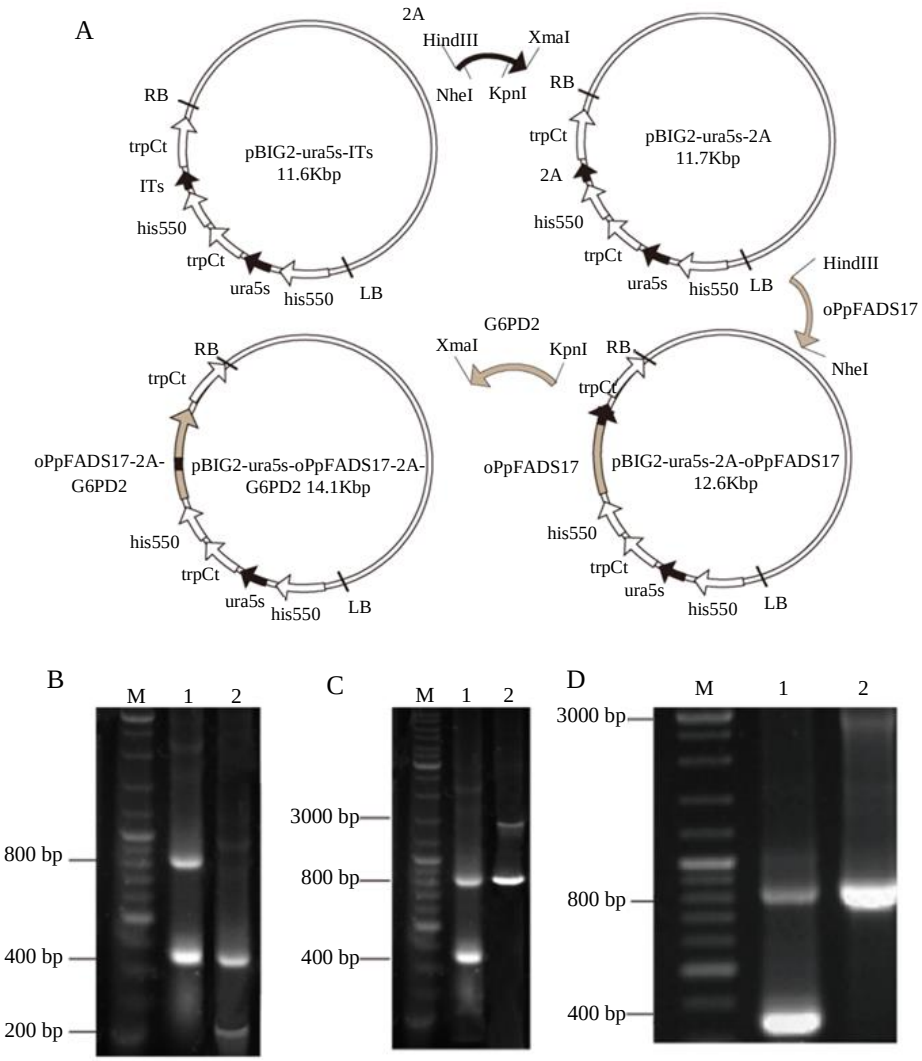

Fig. 2: Construction of the binary vector pBIG2-ura5s-*oPpFADS17-2A-G6PD2* using 2A peptide strategy (A) and PCR verification of the recombinant plasmid [M, Marker; 1, pBIG2-ura5s-Its; 2, pBIG2-ura5s-*2A* (B)/pBIG2-ura5s-*oPpFADS17-2A* (C)/pBIG2-ura5s-*oPpFADS17-2A-G6PD2* (D)]

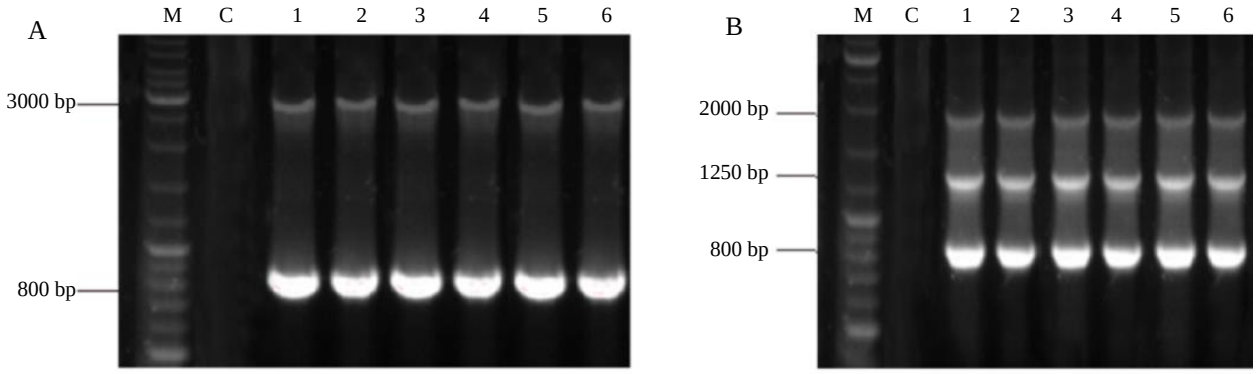

Fig. 3: PCR verification of the transformants. The presence of integrated *oPpFADS17* and *G6PD2* was verified by PCR with the primer pair Hispro-F/TrpC-R. Lane M, marker; lane C, *M. alpina* ATCC 32222 (negative control); lanes 1/2/3/4/5/6, MA-*oPpFADS17-G6PD2*-1/2/3/4/5/6 using double expression cassette strategy (A), MA-*oPpFADS17-2A-G6PD2*-1/2/3/4/5/6 using 2A peptide strategy (B)

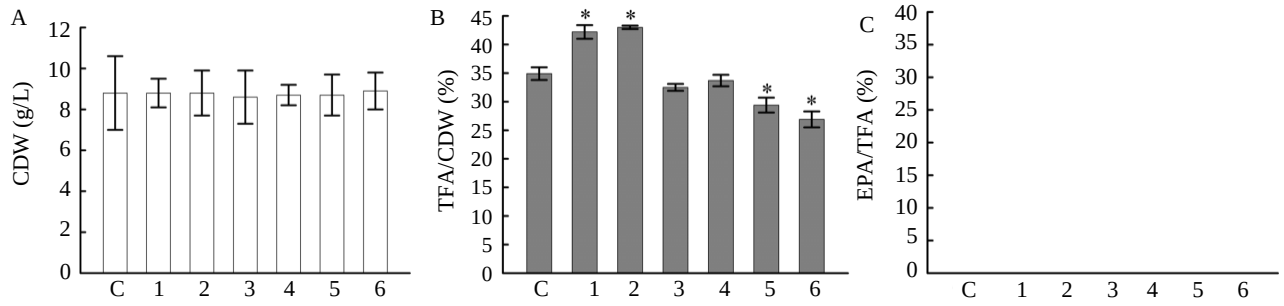

Fig. 4: Cell dry weight (CDW), total fatty acids (TFA) content (w/w, TFA/CDW) and EPA content (w/w, EPA/TFA) in *oPpFADS17* and *G6PD2* co-expressing strains generated using the double expression cassette strategy. C, *M. alpina* ATCC 32222 (negative control), 1/2/3/4/5/6, MA-*oPpFADS17-G6PD2*-1/2/3/4/5/6. Strains were cultured at 28 °C in 250-mL shaking flasks containing 100 mL of broth medium at 200 rpm for 168 h. Three independent experiments were performed and the error bars represent the standard deviations.* $P < 0.05$ indicates a significant difference relative to the C group

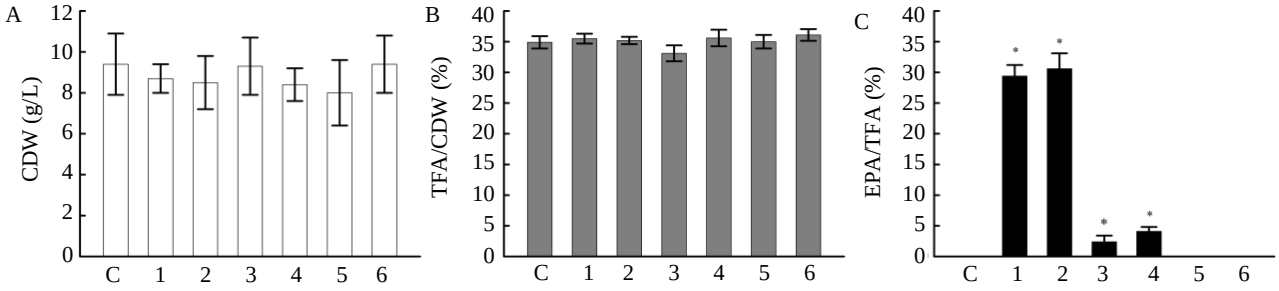

Fig. 5: Cell dry weight (CDW), total fatty acids (TFA) content (w/w, TFA/CDW) and EPA content (w/w, EPA/TFA) in *oPpFADS17* and *G6PD2* co-expressing strains generated using the 2A peptide strategy. C, *M. alpina* ATCC 32222 (negative control), 1/2/3/4/5/6, MA-*oPpFADS17-2A-G6PD2*-1/2/3/4/5/6. Strains were cultured at 28°C in 250-mL shaking flasks containing 100 mL of broth medium at 200 rpm for 168 h. Three independent experiments were performed and the error bars represent standard deviations. * $P < 0.05$ indicates a significant difference relative to the C group

Expression Levels of the oPpFADS17 and G6PD2 in M. Alpina

The transcription levels of *oPpFADS17* and *G6PD2* in the MA-*oPpFADS17-G6PD2*-1/2 (double expression cassette strategy) and MA-*oPpFADS17-2A-G6PD2*-1/2 strains (2A peptide strategy) grown in broth liquid medium for 168 h were determined using RT-qPCR (Fig. 6). As wild-type *M. alpina* does not express *oPpFADS17*, we used a single gene-overexpressing strain (MA-*oPpFADS17*) from the previous study (Tang *et al*., 2018) as the control strain (Fig. 6A). In the transformants generated using the double expression cassette strategy, the respective level of *oPpFADS17* transcription in MA-*oPpFADS17-G6PD2*-1 and -2 either did not differ significantly or was 16% lower when compared with the control strain. In the transformants using the 2A peptide strategy, the respective level of *oPpFADS17* transcription in MA-*oPpFADS17-2A-G6PD2*-1 and -2 either did not differ significantly or increased by 26% when compared with the control strain. The results showed that the transcription level of the *oPpFADS17* occurred in these transformants. The levels of *G6PD2* transcription in the MA-*oPpFADS17-G6PD2*-1/2 transformants were approximately five- and three-fold higher than that in the wild-type strain *M. alpina* ATCC 32222. However, the levels of *G6PD2* transcription in MA-*oPpFADS17-2A-G6PD2*-1/2 did not increase relative to that of the wild-type strain (Fig. 6B). These results suggest that the double expression cassette strategy led to the successful insertion and transcription of *G6PD2*, whereas the 2A peptide strategy had no significant effect on *G6PD2* transcription.

To investigate whether the enzyme encoded by *G6PD2* retained its activity in the transformants generated using the two strategies, the activity of G6PD and the production of NADPH, a product of catalysis supplied directly for lipid synthesis, were determined (Fig. 7). The levels of G6PD activity and NADPH in the transformants generated using the double expression cassette strategy increased significantly when compared with the wild-type strain. In contrast, neither level was affected in the transformants generated using the 2A peptide strategy, compared with the wild-type strain. These results were consistent with the transcription level data.

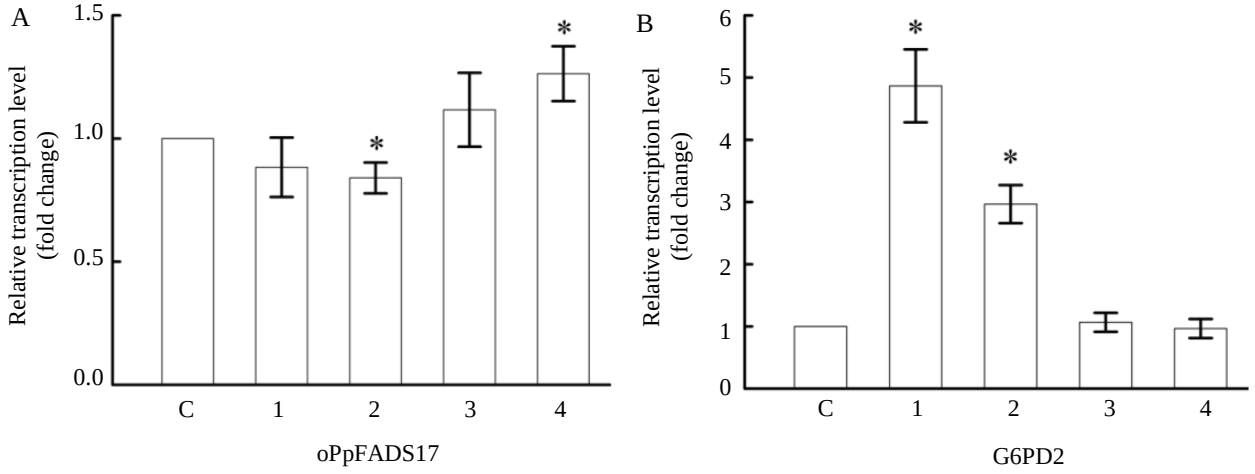

Fig. 6: Relative levels of *oPpFADS17* and *G6PD2* transcription in *M. alpina* transformants. C, MA-*oPpFADS17* (A)*/M. alpina* ATCC 32222 (B); 1, MA-*oPpFADS17-G6PD2*-1 using double expression cassette strategy; 2, MA-*oPpFADS17-G6PD2*-2 using double expression cassette strategy; 3, MA-*oPpFADS17-2A-G6PD2*-1 using 2A peptide strategy; 4, MA-*oPpFADS17-2A-G6PD2*-2 using 2A peptide strategy. Strains were cultured at 28°C in 250-mL shaking flasks containing 100 mL of broth medium at 200 rpm for 168 h. Three independent experiments were performed. Error bars represent standard deviations. * indicates a significant difference from the C group ($P < 0.05$)

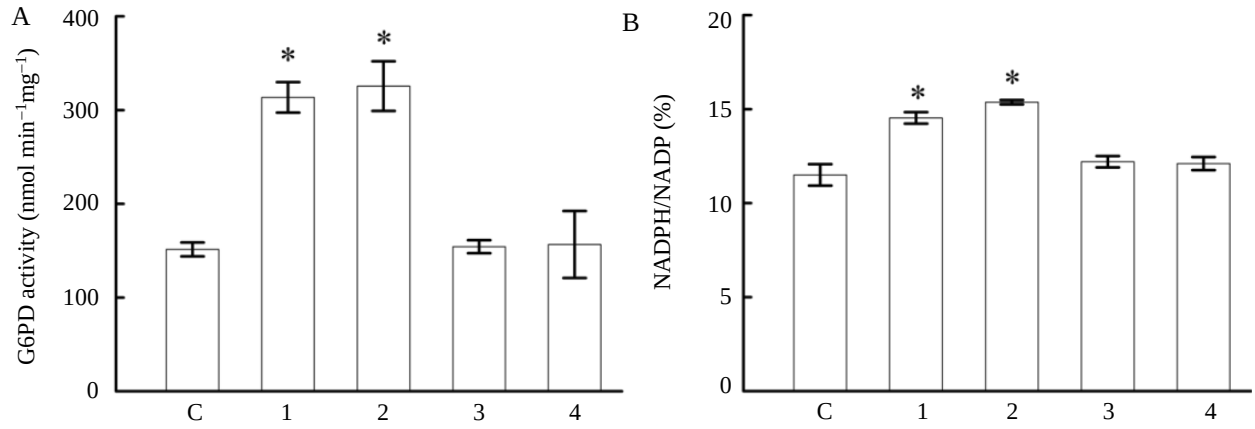

Fig. 7: The G6PD activity (A) and NADPH levels (B) in *M. alpina* transformants. C, *M. alpina* ATCC 32222; 1, MA-*oPpFADS17-G6PD2*-1 using double expression cassette strategy; 2, MA-*oPpFADS17-G6PD2*-2 using double expression cassette strategy; 3, MA-*oPpFADS17-2A-G6PD2*-1 using 2A peptide strategy; 4, MA-*oPpFADS17-2A-G6PD2*-2 using 2A peptide strategy. The strains were cultured at 28°C in 250-mL shaking flasks containing 100 mL of broth medium at 200 rpm for 168 h. Three independent experiments were performed and the error bars represent standard deviations. * indicates a significant difference when compared with the C group ($P < 0.05$)

Discussion

The omega-3 desaturase oPpFADS17 is the key enzyme in the bioconversion of AA to EPA and G6PD2 can provide reducing power NADPH for lipid biosynthesis (Hao *et al*., 2016; Tang *et al*., 2017; 2018). In this study, we co-expressed oPpFADS17 and G6PD2 in *M. alpina* using both the double expression cassette and 2A peptide strategies and studied the effects of co-expression on total lipid and EPA accumulation. In the transformants generated using the double expression cassette strategy, the total lipid content increased but no accumulated EPA was detected. Possibly, the promoters in the double expression cassette strategy interfered with each other and affected the expression of the target gene. Additionally, excessively long target fragments may have increased the burden on the host cells and affected cell metabolism. Several studies showed that the target product expression level correlated with the copy number of the corresponding expression cassette (Fang *et al*., 2014; Mansur *et al*., 2005; Per *et al*., 2011; Wu *et al*., 2014; Zhu *et al*., 2009). According to Yu *et al*. (2003)

different combinations of genes and promoters have important effects on promoter activity and high combinatorial matching could better achieve gene expression. In addition, the enhancer element of one transcription unit may activate the distal promoter of another unit, resulting in cross-interference between two adjacent expression units. Although insulators can be used to prevent this interference, the integration of primitive insulators with internal promoters/enhancers may also reduce the potential activities of adjacent genes. Consistent with this possibility, the first expression unit in our study did not achieve the desired effect and was likely controlled by the enhancer of another expression unit, resulting in abnormal expression.

In many organisms, the 2A peptide facilitates the long-term stable co-expression of multiple genes (de Felipe *et al*., 2006). Indeed, we used the 2A peptide strategy to establish a feasible system for the co-expression of multiple genes in *M. alpina*. Here, the EPA content increased to as much as 30% of the TFA level and the level of *oPpFADS17* transcription was higher than that in the wild-type strain. However, the TFA content did not increase significantly in transformants generated using this strategy, possibly due to the relatively low shear efficiency of the 2A peptide in the recombinant strains. Previous studies indicated that different 2A peptide exhibited various shear efficiencies in different hosts (Donnelly *et al*., 2001; Doronina *et al*., 2008; Luke *et al*., 2008; Szymczak *et al*., 2004). The shear efficiency of the 2A peptide may be associated with two problems. First, the shear efficiencies of polyproteins translated before and after the 2A peptide may differ. Second, the fusion of 2A peptide residues to the C-terminal of the upstream translation protein may interfere with the upstream protein function. The shear activity of the 2A peptide may be related to the total amount of precursor protein. Moreover, G6PD2 plays an important role in the pentose phosphate pathway and its transcription and enzyme activity levels are high. In the presence of a high total amount of precursor protein, the shear activity of the 2A peptide may decrease and the recombinant bacteria would not achieve the expected increase in total lipids. The polycistron employed a single promoter to drive mRNA transcripts containing multi-coding areas and therefore the strength of promoter is very important for the expression of target mRNA. In the 2A peptide strategy of this study, the promoter successfully drived the transcription of *oPpFADS17* but may be unable to drive another G6PD2 co-expression gene. This observation is in accordance with other studies that showed the efficiency of heterologous expression and gene transformation were influenced by different promoter (Cheng *et al*., 2009; Kilaru *et al*., 2006). Also, the mRNA stability is another important factor in gene expression (Hargrove and Schmidt, 1989). The longer mRNA fragments were more likely to experience endonucleolytic attacks by RNA endonucleases and/or mechanical damage (Feng and Niu, 2007; Trcek *et al*., 2011). A nearly 3000-bp length might exceed the capacity to maintain mRNA stability.

Conclusion

In this study, we used double expression cassette and 2A peptide strategies to co-express the omega-3 desaturase oPpFADS17 and G6PD2 in *M. alpina* and investigated the effects on the accumulation of total lipids and EPA. In summary, recombination strains generated using the double expression cassette strategy exhibited an increase in the total lipids but no change in EPA accumulation, whereas those generated using 2A peptide exhibited an increase in EPA accumulation up to 30% of the total lipid content. These multiple gene co-expressing strategies are expected to further enhance the accumulation of PUFAs in *M. alpina*.

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Author Contributions

Xin Tang and Xiaoke Zhang: Carried out the experiments and drafted the manuscript.

Haiqin Chen and Zhennan Gu: Analyzed the data and helped to draft the manuscript.

Haiqin Chen, **Hao Zhang**, **Yong Q. Chen and Wei Chen:** Conceived and designed the study and revised the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors have no conflict of interest.

Ethics Statement

The article has no study within human participants or animals.

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