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Identification and characterization of a delta-12 fatty acid desaturase gene from marine microalgae *Isochrysis galbana*

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Abstract

The cDNA of the delta-12 fatty acid desaturase gene, *IgFAD2*, was cloned from the marine microalgae *Isochrysis galbana*, a species capable of producing docosahexaenoic acid. Sequence analysis indicated that the open reading frame measured a length of 1 158 bp and encoded 386 amino acids with a predicted molecular weight of 42.8 kDa and an isoelectric point of 9.2. Computational analysis of the protein sequence of *IgFAD2* showed typical features of membrane-bound desaturase such as three conserved histidine boxes along with four membrane-spanning regions that were universally present among plant desaturases. Quantitative real-time PCR results showed that the abundance of *IgFAD2* transcript was significantly upregulated under different environmental stresses including low temperature (15°C), high salinity (salinity of 62 and 93), and nitrogen starvation (220 μmol/L). Heterologous expression indicated that yeast cells transformed with a plasmid construct containing *IgFAD2* could convert endogenous oleic acid ($18{:}1^{\Delta 9}$, OA) into linoleic acid ($18{:}2^{\Delta 9, 12}$, LA). These findings confirm that *I. galbana IgFAD2* plays important roles in the biosynthetic pathways of unsaturated fatty acids.

Key words: delta-12 fatty acid desaturase, expression analysis, *Isochrysis galbana*

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1 Introduction

Fatty acids in plants, as in all other organisms, are the major structural components of membrane phospholipids and triacylglycerol storage oils (Sørensen et al., 2005; Li et al., 2007). Fatty acid desaturases are enzymes responsible for the production of unsaturated and polyunsaturated fatty acids (Alonso et al., 2003). Among these enzymes, delta-12 fatty acid desaturase is the first and the rate-limiting step enzyme that introduces a double bond between the twelfth and thirteenth carbon atom of fatty acid chain and thereby converts oleic acid ($18{:}1^{\Delta 9}$, OA) into linoleic acid ($18{:}2^{\Delta 9, 12}$, LA) in fatty acid biosynthesis pathway (Wei et al., 2004; Zhang et al., 2009; Khadake et al., 2009).

Changing levels of unsaturated fatty acids (UFA) have a crucial role in maintaining the fluidity of membrane lipids when plants are subjected to abiotic stress (Allakhverdiev et al., 2001; Alonso et al., 2003). The ability of cells to regulate the desaturation of membrane lipids is mainly determined by the action of fatty acid desaturases (Mendes et al., 2012). The transcriptional level of fatty acid desaturases has association with diverse environmental factors, such as temperature, salinity, and nitrogen availability (Allakhverdiev et al., 2001; Kargiotidou et al., 2008; Lu et al., 2009; Zhang et al., 2011). Several fatty acid desaturase genes have been cloned from various organisms such as fungi (Sakai and Kajiwara, 2005; Gostinčar et al., 2009), algae (Domergue et al., 2003; Lu et al., 2009; Iskandarov et al., 2010; Wang et al., 2016), moss (Kaewsuwan et al., 2006; Chodok et al., 2013) and higher plants (Mietkiewska et al., 2006; Cao et al., 2013; Lozinsky et al., 2014). However, as an important player in fatty acid biosynthesis pathway, the effects of abiotic stress on the gene expression of delta-12 fatty acid desaturases and their heterologous expression have yet to be investigated in microalgae without cell wall.

As one of the most promising biofuel producers, *Isochrysis galbana* is receiving increasing attention owing to its high lipid

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content, photosynthesis efficiency, land avoidance and cultivation success (Demirbas and Demirbas, 2011; Sánchez et al., 2013). In addition, *I. galbana*, a unicellular photoautotrophic microalga without cell wall, is rich in ω-3 polyunsaturated fatty acids (PUFAs). In the present work, we reported the isolation and characterization of a delta-12 fatty acid desaturase gene (*IgFAD2*) from *I. galbana* via rapid amplification of cDNA ends (RACE) and analyzed the gene expression levels under stress conditions through quantitative real-time PCR (qRT-PCR). Moreover, the heterologous expression of this gene in yeast *Saccharomyces cerevisiae* was investigated.

2 Materials and methods

2.1 *Microalgae culture and stress treatments*

The microalga *I. galbana* was obtained from the Institute of Oceanology, Chinese Academy of Sciences. The microalgae (initially 2.6×10^5 cell/mL) were grown in liquid f/2 medium (880 μmol/L nitrogen) (Guillard and Ryther, 1962) at 20°C and at a salinity of 31 under a constant irradiation of 100 μmol/(m^2·s).

In order to determine the abundance of *IgFAD2* transcript at different conditions, the microalgae were cultured at 15°C and 25°C under the stable illumination duration for 6, 12, 24, and 48 h; at the salinity of 62 and 93 for 6, 12, 24, 48, and 72 h; or in medium containing 220 μmol/L and 1 760 μmol/L nitrogen for 6, 12, 24, and 48 h.

2.2 *Total RNA extraction*

The microalgae were harvested for extracting cellular total RNA through centrifugation at 12 000× *g* and 4°C for 10 min. Algal cells were ground into powder in liquid nitrogen. Total RNA was extracted by the method of Wang et al. (2016). The concentration of total RNA was determined by measuring the UV absorbance at 260 nm using a Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and the RNA purity was checked by determining the A_{260}/A_{280} ratio and 1.2% denaturing agarose gel electrophoresis.

2.3 *Rapid amplification of IgFAD2 cDNA ends*

The mRNA was purified from total RNA extracted with CTAB method by using an Oligotex mRNA Mini Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The reverse transcription reaction was performed with 3′-RACE CDS Primer and 5′-RACE CDS Primer and PrimeScript™ Reverse Transcriptase (TaKaRa, Tokyo, Japan) following the manufacturer's protocol. The RACE was performed using *IgFAD2* RC1 and RC2 (Table 1) and SMART RACE cDNA Amplification Kit (Clontech, CA, USA) following the manufacturer's instructions. The PCR products were purified with a gel purification kit (Tiangen, Beijing, China), subcloned into pMD18-T vector (TaKaRa, Tokyo, Japan) and sequenced (Sunny Biotechnology Company, Shanghai). The full-length cDNAs were assembled on the basis of the sequences of 3′-RACE and 5′-RACE PCR fragments.

2.4 *Bioinformatics analysis*

The full-length cDNA sequence of *IgFAD2* was assembled with SeqMan software of DNASTAR 7.1. The theoretical MW and pI of *IgFAD2* were calculated with ExPASy Compute pI/Mw tool (http://au.expasy.org/tools/pi_tool.html). Multiple alignments were created using ClustalX and analyzed using BoxShade program (www.ch.embnet.org). Transmembrane regions were predicted through DAS Transmembrane Prediction server (http://www.sbc.su.se/~miklos/DAS/maindas.html). Phylogenetic tree was constructed with Mega 5.0 by using a neighbor-joining algorithm with 1 000 permutations.

2.5 *Quantitative real-time PCR*

The *IgFAD2* expression was analyzed through qRT-PCR with Stratagene Mx3000P® qPCR system and SYBR® PrimeScript™ RT-PCR kit (TaKaRa, Tokyo, Japan). According to the manufacturer's instructions, a pair of primers (RT1, RT2) (Table 1) was designed and used to quantify the abundance of *IgFAD2* transcript. The abundance of *rbc*L (RT3, RT4) was used as an endogenous control. The qRT-PCR was performed for 40 cycles of denaturation at 95°C for 10 s and annealing at 51°C for 10 s and at 72°C for 10 s. All of the reactions were conducted in at least three duplicates. The qRT-PCR data were examined with comparative Ct ($2^{-\Delta\Delta Ct}$).

2.6 *Expression in yeast*

The open reading frames were amplified with specific primers, *IgFAD2* F1 and *IgFAD2* R1 (Table 1), and subcloned into the yeast expression vector pYES2.0 (Invitrogen) under the control of GAL1 promoter. The 5′ end of the F1 and R1 contained an *Eco*RI or a *Bam*HI restriction site (italicized) to facilitate subsequent manipulation. The sequence orientation and identity were confirmed by sequencing and the resulted plasmid was designated pYFAD2. Plasmids pYES2.0 and pYFAD2 were introduced into the *S. cerevisiae* INVSc1 using electroporation. Yeast cultures were grown to logarithmic phase at 28°C in synthetic minimal medium (SC-Ura). The cells were incubated at 15°C for 48 h. The cells were harvested, and fatty acids were assayed.

2.7 *Fatty acid analysis*

Fatty acids were extracted and determined according to the method of Yang et al. (2013). In brief, fatty acid methyl esters (FAME) were identified and quantified after splitless injection and run in temperature programming by using an Agilent 7890A GC instrument equipped with a HP-5MS capillary column (30 m×

Table 1. Primers used in this study

Primer	Sequence (5′-3′)	Application
IgFAD2	F: TATCAGGGCATCAACCTTACCGC	Amplifying cDNA
IgFAD2	R: CGAGTAGAAGTCCTTCCAGTAGCCA	Amplifying cDNA
IgFAD2 RC1	F: CGAAGCACCACCACTACACCAACCA	5′ RACE
IgFAD2 RC2	R: GGCGGTAAGGTTGATGCCCTGATAGA	3′ RACE
IgFAD2 RT1	F: ATCAGGGCATCAACCTTACCG	Quantitative PCR
IgFAD2 RT2	R: CCAGGAGAAGTAGGGCGTCA	Quantitative PCR
*rbc*L RT3	F: TTCACGCAGGTACAGTAGTTGG	Quantitative PCR
*rbc*L RT4	R: GAGCGAAGAATAAACCTTGAGGAA	Quantitative PCR
IgFAD2 F1	F: GAATTC***ATG***GGCAAGGGAGGCTCGGCAGGC	pYFAD2 construction
IgFAD2 R1	R: TCTAGA***TCA***GCGGAACCACATGAAGTTGTCC	pYFAD2 construction

0.25 mm×0.25 μm) coupled to an Agilent 5975C mass spectrometer.

2.8 *Statistical analysis*

Analysis of variance (ANOVA) and *t*–test were conducted with SPSS version 16.0. Observations were expressed as mean±standard error. The probability of <0.05 was used in judging significant difference.

3 Results

3.1 *Cloning and characterization of the IgFAD2 gene from I. galbana*

The cDNA of *IgFAD2* contained a 1 158 bp ORF which encoded a protein of 386 amino acid residues with a deduced molecular mass of 42.8 kDa and the theoretical pI of 9.2. The sequence was deposited in GenBank with an accession number of AFB82638. The assembled full-length cDNA of *IgFAD2* had 81% and 57% similarity to delta-12 fatty acid desaturases of *Emiliania huxleyi* CCMP1516 (XP005759480) and *Chrysochromulina* sp. CCMP291 (KOO28400), respectively. The deduced *IgFAD2* was clustered within microsomal delta-12 fatty acid desaturase (*FAD2*) group. All of the plastidial delta-12 fatty acid desaturases (*FAD6*) are grouped. Based on the sequence similarity and phylogenetic analysis, it implies that *IgFAD2* encodes a microsomal delta-12 fatty acid desaturase (Fig. 1). The deduced *IgFAD2* was highly conserved; it contained three conserved motifs (HECGH, HAKHH and HVVHH), which is a characteristic feature of membrane-bound desaturases (Fig. 2). It also presented four strong hydrophobic transmembrane domains (66–88, 116–135, 174–195 and 230–257) (Fig. 3), which is common to most membrane-bound desaturases and presented well-conserved domains between algae and high plants (Fig. 2).

3.2 *Effects of temperature, salinity and nitrogen concentration stress on the IgFAD2 expression of I. galbana*

In temperature treatments, the abundance of *IgFAD2* transcript increased by 8.6-fold when the alga was cultured at 15°C for 12 h compared with the control (Fig. 4). In salt stress treatments, the *IgFAD2* transcript reached the maximum abundance (5.9-fold of the control) when the alga was cultured at a salinity of 62 for 24 h compared with the control (Fig. 5). In nitrogen concentration treatment, *I. galbana* was cultured at 880 μmol/L treatment (nitrogen concentration in f/2 medium) as control. The qRT-PCR analysis results showed that the maximum *IgFAD2* mRNA expression level was reached (2.6-fold of the control) at 220 μmol/L treatment for 12 h (Fig. 6).

3.3 *Functional analysis in S. cerevisiae*

Heterologous expression in yeast was used to confirm delta-12 fatty acid desaturase regioselectivity and function. To validate the protein activity, both pYFAD2 and pYES2.0 (control) were transformed into *S. cerevisiae* INVSc1. The total lipids of the transformants were subjected to GC-MS analysis. The results showed that a novel peak, corresponding to LA ($18{:}2^{\Delta 9,\,12}$) methyl ester standards, were detected in the transgenic *S. cerevisiae* expressing the *IgFAD2* gene (Table 2). In contrast, the peak normally was not present in the wild-type yeast cells. Four fatty acids were mainly found in *S. cerevisiae*, namely, C16:0, C16:1, C18:0, and C18:1. The finding indicates that pYFAD2 encodes a delta-12 fatty acid desaturase, which can convert C18:1 into C18:2 in yeast.

4 Discussion

delta-12 fatty acid desaturase emerges to be the key enzyme in the synthesis of LA ($18{:}2^{\Delta 9,\,12}$), a crucial precursor for producing subsequent PUFA production. *Isochrysis galbana* is known to contain an essential amount of LA. Although several desaturases and elongases have been elucidated from *I. galbana* (Qi et al., 2002; Wang et al., 2016), the delta-12 fatty acid desaturase associated with LA biosynthesis has not been functionally identified. Currently, delta-12 fatty acid desaturase genes have been isolated from microalgae, including *Phaeodactylum tricornutum* (Domergue et al., 2003), *Chlorella vulgaris* (Lu et al., 2009), *Parietochloris incisa* (Iskandarov et al., 2010), and *Chlamydomonas* sp. (Zhang et al., 2011). In this research, we identified a delta-12 (Δ12) fatty acid desaturase, *IgFAD2*, from *I. galbana* using RACE method. *IgFAD2* contains three histidine boxes (HXXXH, HXXHH and HXXHH) that have similar characteristics to all

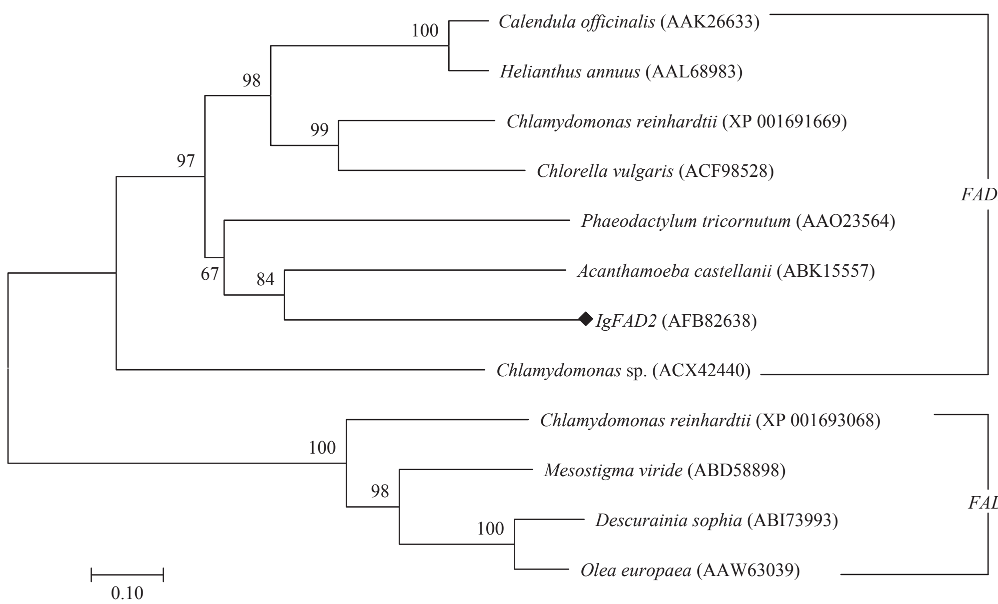

Fig. 1. Phylogenetic tree constructed with the neighbor-joining method. Accession numbers are shown in parentheses.

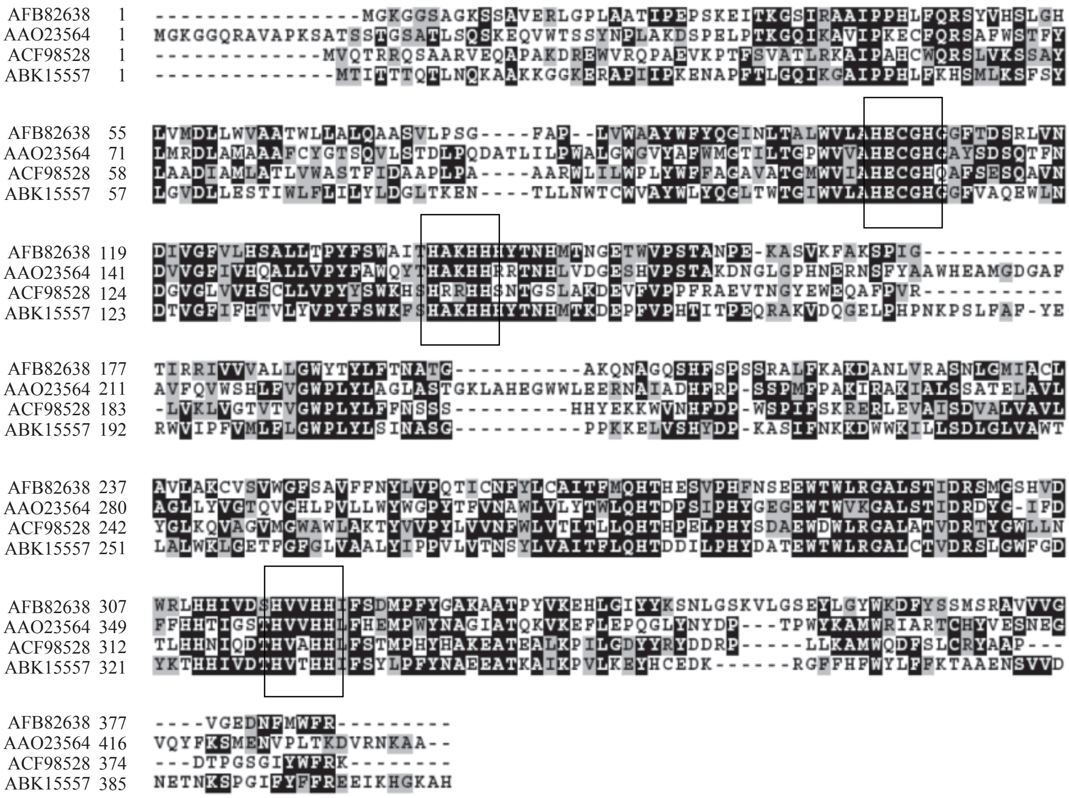

Fig. 2. Alignment deduced *IgFAD2* with those of diverse species. AFB82638 (*I. galbana FAD2*, AFB82638), AAO23564 (*Phaeodactylum tricornutum FAD2*, AAO23564), ACF98528 (*Chlorella vulgaris FAD2*, ACF98528), and ABK15557 (*Acanthamoeba castellanii FAD2*, ABK15557). Black bars show the identical amino acid residues. Deletions are indicated by dashes. Within boxes are three typical histidine motifs.

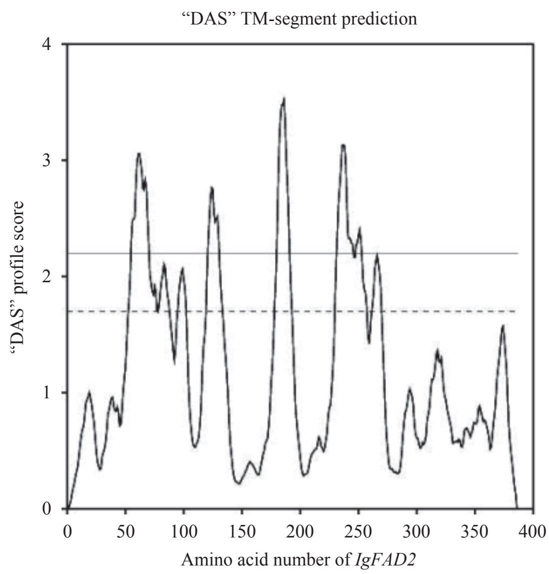

Fig. 3. Transmembrane domains of deduced *IgFAD2*. Four clusters of strong hydrophobic regions representing the putative membrane spanning helices were classed by strict cutoff.

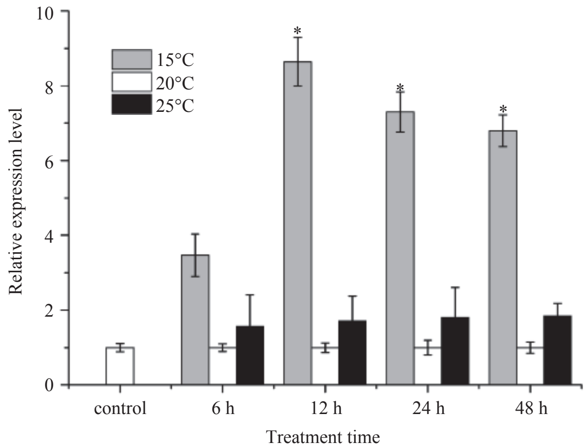

Fig. 4. *IgFAD2* mRNA expression levels relative to *rbc*L mRNA levels under different temperatures and times analyzed by qRT-PCR. Isochrysis galbana were treated with 20°C as control to investigate the mRNA expression levels of *IgFAD2*. Data are presented as the mean±SD (n=3). Asterisks indicated a significant difference from the control value (p<0.05).

membrane-bound desaturases, and *FAD2*s that are also similar to other plant species. The histidine-rich motifs in the sequence are thought to be involved in the oxygen activation and substrate activation process through formation of a di-iron center part (Khadake et al., 2009). Nevertheless, *IgFAD2* did not contain a cytochrome*b5*-like domain including the HPGG motif in the heme-binding region, which is normally present in front-end desaturases.

Changes in temperature can affect the biomass of microalgae,

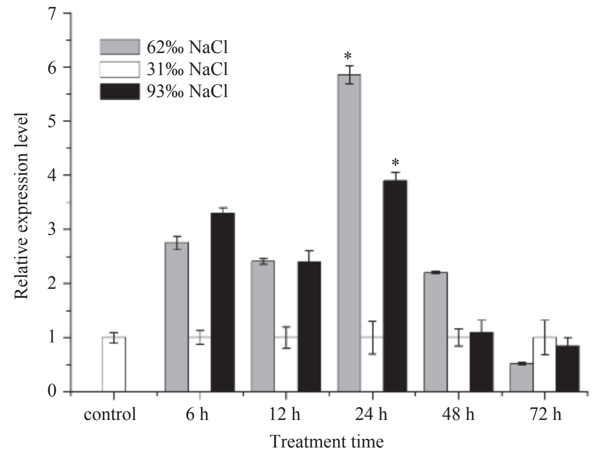

Fig. 5. *IgFAD2* mRNA expression levels relative to *rbc*L mRNA levels under a salinity of 62 or 93 at different times analyzed by qRT-PCR. *Isochrysis galbana* were treated with a salinity of 31 as control to investigate the mRNA expression levels of *IgFAD2*. Data are presented as the mean±SD (n=3). Asterisks indicated a significant difference from the control value (p<0.05).

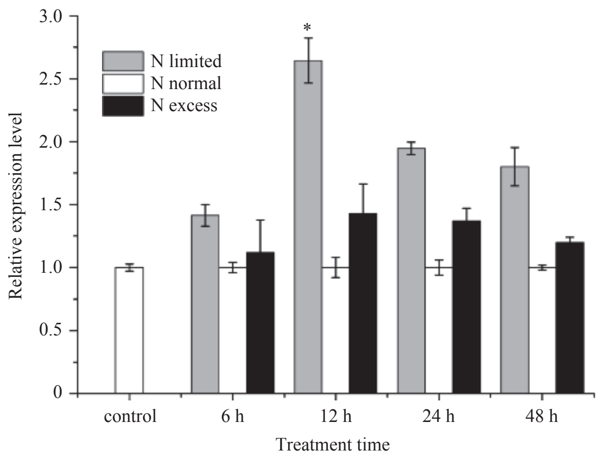

Fig. 6. *IgFAD2* mRNA expression levels relative to *rbc*L mRNA levels at different nitrogen concentrations and times analyzed by qRT-PCR. *Isochrysis galbana* were treated with 880 μmol/L nitrogen concentrations as control to investigate the mRNA expression levels of *IgFAD2*. Data are presented as the mean±SD (n=3). Asterisks indicated a significant difference from the control value (p<0.05).

which associates with gene transcription and related enzyme activity. It has been confirmed that the cell activity of microalgae will decline when the temperature decreases due to the reduction of enzyme activity (Chong et al., 2011). However, the expression of fatty acid desaturases involved in lipid biosynthesis will be upregulated to increase membrane fluidity at low temperature (Zhang et al., 2011; Han et al., 2013). The findings on the mechanisms of temperature-dependent fatty acid composition alterations in plant membrane lipids have provided evidence of control in both the transcriptional and translational levels for delta-12 fatty acid desaturase genes (Chinnusamy et al., 2007). Based on the qRT-PCR analysis results, *IgFAD2* mRNA transcript level was higher at 15°C compared with the algae at 20°C (Fig. 4). The *IgFAD2* mRNA expression levels increase to 8.6-fold at 15°C for 12 h. The *IgFAD2* mRNA transcript expression is consistent with the delta-12 fatty acid desaturase, which was isolated from the Antarctic microalgae *C. vulgaris* NJ-7. The accumulation of delta-12 fatty acid desaturase gene transcripts increased by 2.2-fold at 15°C compared with the algae at 25°C (Lu et al., 2009). Miyasaka et al. (2000) reported that the level of transcript of delta-12 fatty acid desaturase gene of *Chlamydomonas* sp. increased by 2.3-fold at 4°C for 6 h, whereas the transcript level under heat stress (38°C) for 6 h was only 87% of the control.

Salt stress is among the main environmental factors that limit the growth and productivity of plants and microorganisms. Several reports have suggested that lipids might be involved in the protection against salt stress (Turk et al., 2004; Gostinčar et al., 2009). Salt stress induced increases in the unsaturated fatty acids of membrane lipids for the sake of adaptation to a wide range of NaCl concentrations (Lu et al., 2009). This phenomenon has been observed in yeasts (Gostinčar et al., 2009), fungi (Turk et al., 2007; Lin et al., 2017) and cyanobacteria (Allakhverdiev et al., 2001; Kumar et al., 2015). Furthermore, the delta-12 fatty acid desaturase gene is isolated from the Antarctic ice algae *C. vulgaris* NJ-7, and the mRNA accumulation of gene transcripts increased up to 8.5-fold at a salinity of 62 compared with the algae at a salinity of 31 (Lu et al., 2009). Similar results were obtained for the delta-12 fatty acid desaturase gene is isolated from Antarctic microalgae *Chlamydomonas* sp. ICE-L, in which the expression level increased by 3.8-fold at a salinity level of 62 for 2 h (Zhang et al., 2011). In this study, *IgFAD2* was involved in the adaptation to high salinity stress and reached the maximum expression level of 5.9-fold after 62‰ NaCl treatment for 24 h compared with the control (Fig. 5). The unsaturated fatty acid (UFA) content in *I. galbana* increased at a salinity of 62 (data not shown).

Limited nitrogen concentration of the medium prompts fatty acid accumulation in a wide range of microalgal species (Jiang et al., 2012; Griffiths et al., 2012). Lipid content has been documented to increase when *I. galbana* is cultured under nitrogen starvation (Mairet et al., 2011), as it does in *C. Reinhardtii* (Miller et al., 2010). Wang et al. (2016) reported that the level of transcript of delta –6 fatty acid desaturase gene of *Isochrysis* sp. increased by 4.5-fold in nitrogen-deplete medium for 6 h. The results are consistent with our experiments. In nitrogen deficiency, *IgFAD2* mRNA expression level increases by 2.6-fold at 220 μmol/L treatment for 12 h (Fig. 6).

Heterologous expression in yeast was used to confirm delta-12 regioselectivity and function of *IgFAD2*. Both pYFAD2 and empty vector, pYES2.0 (control), were transformed into the *S. cerevisiae* INVSc1. The total lipids of the transformants were determined through GC-MS analysis. The findings demonstrate that a novel peak was present in pYFAD2, which was absent from

Table 2. Composition of the major fatty acids (%, w/w; average±SD, n=3) of pYES2.0 and pYFAD2 yeast transformants by GC-MS analysis

Transformant	C16:0	C16:1	C18:0	C18:1	C18:2
pYES2.0	34.38±0.44	16.67±0.37	3.13±0.77	45.82±0.78	ND
pYFAD2	28.74±2.33	13.79±1.85	5.75±1.64	48.27±1.70	3.45±1.18

Note: ND means not detected; all others significant to <0.05.

the control (Table 2). The novel peak was LA ($18:2^{\Delta 9, 12}$) by comparison of the retention time to FAME standard mixtures (Sigma). The result indicated that *IgFAD2* encodes a delta-12 fatty acid desaturase, which can convert C18:1 into C18:2 in yeast. This finding is consistent with the recent reports on delta-12 fatty acid desaturases from the microalgae *C. vulgaris* (Lu et al., 2009), the fungus *Lentinula edodes* (Sakai and Kajiwara, 2005), *Rhizopus arrhizus* (Wei et al., 2004) and the higher plants *Olea europaea* (Hernández et al., 2005), *Gossypium hirsutum* (Zhang et al., 2009), *Linum usitatissimum* L. (Khadake et al., 2009). In contrast, delta-12 fatty acid desaturases from the higher plants *Gossypium hirsutum* (Pirtle et al., 2001), *Tropaeolum majus* (Mietkiewska et al., 2006), *Physcomitrella patens* (Chodok et al., 2013), *Physaria fendleri* (Lozinsky et al., 2014), and the microalgae *P. tricornutum* (Domergue et al., 2003) have been shown to have specific activity for C16:1 and C18:1 fatty acid substrates. Although the reason remain far to be elucidated, the different hydrophobic profiles of *FAD2*s that indicate diverse transmembrane topologies among various organisms may be useful to clarify this phenomenon (Wei et al., 2004; Chodok et al., 2013).

5 Conclusions

In conclusion, a cDNA of the delta-12 fatty acid desaturase gene (*IgFAD2*) was isolated from *I. galbana*. The full-length cDNA of *IgFAD2* contained a 1 158 bp ORF, which encodes a fatty acid desaturase protein comprising 386 amino acids with a deduced molecular mass of 42.8 kDa and the theoretical pI of 9.2. The computational analysis of the protein sequence of *IgFAD2* revealed three conserved histidine motifs. Under different stress conditions, the results of qRT-PCR showed that the *IgFAD2* expression was upregulated by low temperature (15°C), high salinity (62 and 93), and nitrogen starvation (220 μmol/L). Heterologous expression indicated that the cDNA encoded a delta-12 fatty acid desaturase from *I. galbana* was able to convert C18:1 into C18:2. These findings may clarify the importance of delta-12 desaturase activity in polyunsaturated fatty acids biosynthesis.

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