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# Novel elongase of *Pythium* sp. with high specificity on $\Delta^6$-18C desaturated fatty acids

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ABSTRACT

We identified a novel elongase gene from a selected strain of the Oomycete, *Pythium* sp. BCC53698. Using a PCR approach, the cloned gene (*PyElo*) possessed an open reading frame (ORF) of 834 bp encoding 277 amino acid residues. A similarity search showed that it had homology with the PUFA elongases of several organisms. In addition, the signature characteristics, including four conserved motifs, a histidine-rich catalytic motif and membrane-associated feature were present in the *Pythium* gene. Heterologous expression in *Saccharomyces cerevisiae* showed that it was specific for fatty acid substrates, having a double bond at $\Delta^6$-position, which included γ-linolenic acid (GLA) and stearidonic acid (STA), and preferentially elongated the *n3*-18C PUFA. This is an elongase in Oomycete fungi, which displays very high specificity on $\Delta^6$-18C desaturated fatty acids. This will be a powerful tool to engineer PUFA biosynthesis in organisms of interest through the *n*-6 series pathway for producing value-added fatty acids.



## 1. Introduction

Long chain-polyunsaturated fatty acids (LC-PUFAs) have been recognized as nutritionally important lipids with vast applications in the food and feed industries. In particular, the importance of 20-carbon PUFAs as principle precursors of biologically active molecules, called eicosanoids, has been of considerable interest. The series-1 prostaglandins ($PGE_1$) are derived from dihomo-gamma linolenic acid (DGLA, C20:3$\Delta^{8,11,14}$), whereas arachidonic acid (ARA, C20:4$\Delta^{5,8,11,14}$) and eicosapentaenoic acid (EPA, C20:5$\Delta^{5,8,11,14,17}$) are precursors for synthesis of $PGE_2$ and $PGE_3$, respectively [1]. Indeed, the 20-carbon PUFAs are categorized into lipid-based biofunctional product, which display a wide range of beneficial effects in eukaryotic systems, such as the cardiovascular, immune and reproduction systems. In general, LC-PUFAs are synthesized via a series of oxygen-dependent desaturation and elongation reactions. Similar to the fatty acid desaturation systems, there are multiple membrane-bound elongation systems, which are responsible for chain extension of fatty acids. Differentiation of elongase activities relies on their specificity towards fatty acyl substrates [2]. Most PUFA-elongases have broad specificities on various fatty acid substrates, containing different double bond positions and acyl chain lengths. With regard to the biosynthetic pathway of 20-carbon PUFAs, the elongase enzymes with specificity for 18-carbon PUFAs are of particular interest. It has been shown that the $\Delta^6$-18C-elongase is the rate-limiting step in 20-carbon PUFAs biosynthesis [3]. The selection of the elongase enzyme, that is highly specific for a target substrate, is a promising strategy for reducing the bypass of metabolic flux in the biosynthetic pathway.

The Oomycete fungi, such as *Pythium* sp. and *Phytophthora* sp., are able to synthesize 20-carbon PUFAs. However, there is a variation in their fatty acid profiles and contents that is strain-dependent. Previously, we analyzed fatty acid profiles of a number of selected strains of Oomycete fungi (unpublished data). Of them, *Pythium* sp. BCC 53698 contained DGLA, ARA and EPA at high amounts, whereas other $\Delta^6$-elongated PUFAs including eicosadienoic acid (EDA, C20:2$\Delta^{11,14}$) were undetectable, presuming that it might carry an elongase gene with high specificity for GLA (C18:3$\Delta^{6,9,12}$). As a consequence, a PUFA-elongase gene was identified from *Pythium* sp. BCC 53698 in this work. Its functional characteristics in terms of substrate specificity and preference were investigated by heterologous expression in *Saccharomyces cerevisiae*. This is a report describing the identification of a novel

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elongase gene, from an Oomycete fungus with a high specificity for $\Delta^6$-18C PUFAs, thus providing a potential for the redirection of the PUFA pathway in an oleaginous host system for the production of valuable LC-PUFAs and the opportunity for commercial exploitation.

## 2. Material and methods

### 2.1. Organisms and cultivation

*Pythium* sp. BCC53698, used in this study, was grown in a semi-synthetic medium [4] at 30 °C to logarithmic phase. *S. cerevisiae* DBY746 (*MAT*α, *his*3-Δ1, *leu*2-3, *leu*2-112, *ura*3-52, *trp*1-289), was used as a host for heterologous gene expression. The yeast was grown either in a complete medium, YPD (1% bacto-yeast extract, 2% bacto-peptone and 2% glucose), or a selective medium, SD (0.67% bacto-yeast nitrogen base without amino acids and 2% glucose). Appropriate amino acids, including L-tryptophane, L-histidine-HCl and L-leucine were supplemented to SD medium at the concentrations of 20, 20 and 30 mg/l, respectively. *Escherichia coli* DH5α was used for plasmid propagation and grown at 37 °C in Luria–Bertani medium (LB) containing 100 mg $l^{-1}$ of ampicillin [5].

### 2.2. Cloning of the $\Delta^6$-elongase gene from Pythium sp.

Total RNA of *Pythium* sp. was extracted using TRI reagent (Molecular Research Center, Inc., Ohio) according to the manufacturer's instructions. A portion of the *Pythium* $\Delta^6$-elongase (*PyElo*) cDNA was amplified by RT-PCR. Approximately 3 μg of total RNA was reverse-transcribed using the P29-oligo-dT-AP primer (Table 1) and SuperScript II first-strand synthesis system (Invitrogen, CA). Then, approximately 50 ng of the first-strand cDNA were used as templates for PCR using degenerate primers, P25-PyElo-F and P27-PyElo-R (Table 1), which were designed based on conserved amino acid sequences of fatty acid elongases of other fungi, YLSK(M/I)(L/W)(D/E) and MY(T/M)YY(F/G)VS, respectively. The reaction was carried out for 3 min at 94 °C, followed by 35 cycles of 94 °C for 35 s, primer-specific annealing temperature for 40 s and 72 °C for 1 min, and a final extension of 72 °C for 5 min.

To obtain the full length *PyElo* gene, the 5′- and 3′-ends of the cDNA were amplified using the RACE technique. The 5′-RACE cDNA amplification kit was obtained from Invitrogen (CA, U.S.A.). As shown in Table 1, the gene-specific primers were designed from the known sequences of the DNA fragment derived from RT-PCR using the Primer3 software program (http://frodo.wi.mit.edu/primer3/). The 5′-RACE was then carried out using the AAP primer and the antisense primer, P35-PyElo-R1. Nested PCR was also done to obtain the specific product of 5′-end cDNA using P49-PyElo-R2-N and AUAP primers. To amplify the 3′-end cDNA of the *PyElo*, P37-PyElo-F1 and P30-3RACE-AP (Table 1) were used as sense and antisense primers, respectively. A pair of primers (P38-PyElo-F2-N and P30-3RACE-AP) was also used for nested PCR. All PCR fragments were purified using the QIAquick Gel Extraction kit (Qiagen) following the manufacturer's protocol and then were subcloned into TOPO 2.1 vector (TOPO® TA cloning® kit, Invitrogen) for further sequencing using a service of Macrogen (Korea). The obtained sequences were analyzed against nucleotide or amino acid sequences available in GenBank database using the BLASTN and BLASTX programs. The cDNA sequence of *PyElo* has been deposited in GenBank and assigned the Accession Number KJ546459.

### 2.3. Structural characterization and phylogenetic analysis of Pythium $\Delta^6$-elongase

The deduced amino acid sequence of *PyElo* was aligned with the $\Delta^6$-fatty acid elongases of other organisms by using the ClustalW [6] and GeneDoc programs [7]. The conserved characteristics of the $\Delta^6$-elongases, including histidine-rich motifs and transmembrane domains, were determined. Transmembrane prediction was done using HMMTOP program [8,9] and PRODIV-TMHMM method [10] in the Octopus program (http://octopus.cbr.su.se) [11]. Based on amino acid sequence alignment, a phylogenetic tree of the *Pythium* $\Delta^6$-elongase and other elongases was generated using the neighbor-joining method in MEGA5 software [12]. The rate of replication was calculated from bootstrap tests (1000 replicates).

### 2.4. Functional characterization of the Pythium $\Delta^6$-elongase gene by heterologous expression in S. cerevisiae

To verify the function of the cloned gene, the full-length elongase cDNA of *Pythium* sp. was amplified by RT-PCR using high fidelity Taq polymerase (Invitrogen, CA) and a pair of specific primers, P53-PyElo-*Bam*HI-F and P54-PyElo-*Eco*RI-R (Table 1). The 5′-end of each primer contained *Bam*HI or *Eco*RI restriction sites (underlined letters) to facilitate subsequent cloning. The amplified product was subcloned into pYES2 expression vector (Invitrogen, CA) downstream of the *GAL1* promoter to generate PY-PyElo plasmid. The recombinant plasmid and the empty plasmid (pYES2) were then individually transformed to *S. cerevisiae* by the PEG/lithium acetate method following the manufacturer's protocol (Invitrogen). Transformed cells were selected on a uracil-deficient agar medium. Selected yeast transformants were grown in SD medium containing 2% (w/v) raffinose and 50 μM of GLA (Sigma, St. Louis, MO) at 30 °C for 48 h. Subsequently, gene expression was induced by adding galactose to a final concentration of 2% (w/v), and the

**Table 1**
Oligonucleotide primers used for amplifying the $\Delta^6$-elongase gene of *Pythium* sp.

| Primer | Oligonucleotide sequence (5′-3′) | Strand* |
|---|---|---|
| P29-Oligo-dT-AP | GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTT | – |
| P25-PyElo-F | TTYTAYCTSTCCAAGRTSCTSGA | + |
| P27-PyElo-R | GCTSACRAAGTAGTAMRTGTACAT | – |
| AAP | GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG | + |
| AUAP | GGCCACGCGICGACTAGTAC | + |
| P35-PyElo-R1 | CGTAGATGTCGGCGTCGTAG | – |
| P49-PyElo-R2-N | GAGAGCTGGTTCCACTTC | – |
| P30-3RACE-AP | GGCCACGCGTCGACTAGTAC | – |
| P37-PyElo-F1 | TGGACTTCTGCGACACCTTC | + |
| P38-PyElo-F2-N | ACTACATGAACTTCCGCGTG | + |
| P53-PyElo-BamHI-F | CGCGGATCCATGGCGACCGAACTCTGG | + |
| P54-PyElo-EcoRI-R | CCGGAATTCTTAGTTGGTCTCTTCTTGTC | – |

* Sense and anti-sense strands are denoted by plus (+) and minus (–) symbols, respectively.

cultures were further grown at 25 °C for 48 h. Cells were harvested by centrifugation, washed twice with 0.1% TritonX 100, dried and then used for fatty acid analysis. Three independent experiments were carried out for each culture.

### 2.5. Study of substrate specificity and preference of Pythium $\Delta^6$-elongase

Substrate specificity of *PyElo* was studied in *S. cerevisiae* by supplementation with different fatty acid substrates for the elongase enzyme activity. The yeast transformants bearing PY-PyElo and pYES2 (control) were grown in SD medium supplemented with 50 μM of individual fatty acid substrates, which were different in length and double bond position in their acyl chains, including saturated fatty acids (arachidic acid; C20:0, behenic acid; C22:0 and lignoceric acid; C24:0), 18C-PUFAs (linoleic acid; LA; C18:2$\Delta^{9,12}$, GLA; C18:3$\Delta^{6,9,12}$, α-linolenic acid; ALA; C18:3 $\Delta^{9,12,15}$ and STA; C18:4 $\Delta^{6,9,12,15}$) and 20C-PUFAs (ARA and EPA). Rate of substrate conversion was calculated as follows; percentage of conversion rate = [product formed/(substrate + product formed)] × 100.

### 2.6. Fatty acid analysis

Fatty acid methyl esters (FAMEs) were prepared to determine the fatty acid composition of the yeast transformants using the method modified from Lepage and Roy [13]. Wet cells were transmethylated with 2 ml of 5% HCl in methanol at 80 °C for 90 min. After the samples were cooled to room temperature, 1 ml of distilled water and 1 ml of butylated hydroxytoluene (0.01% in *n*-hexane, v/v) were added and the suspensions were shaken vigorously. The *n*-hexane phase containing FAMEs was collected and directly analyzed by gas chromatography using a GC-17A gas chromatograph (Shimadzu, Tokyo) equipped with a capillary column Type OMEGAWAX™250 (Supelco, USA) (30 m × 0.25 μm) and a flame ionization detection. Helium was used as a carrier gas at a constant flow rate of 1.0 ml $min^{-1}$. The temperatures of the column and detector were set at 150–230 °C and 260 °C, respectively. The areas of chromatographic peaks were used to calculate the relative amount of fatty acids. The retention times of individual chromatographic peaks were compared with those of FAME standards (Sigma) for fatty acid identification.

## 3. Results

### 3.1. Cloning and characterization of Pythium $\Delta^6$-elongase gene

The $\Delta^6$-elongase gene of *Pythium* sp. was cloned by a combination of RT-PCR and RACE techniques. Using degenerate primers, approximately 200 bp of DNA fragment was obtained by RT-PCR, and its sequence analysis showed a high similarity to very long-chain polyunsaturated fatty acid elongases (VLC-PUFA elongases) of other related species. Subsequently, the 5′- and 3′-end cDNAs of the *Pythium* gene were amplified by RACE. Sequence analysis by BlastN revealed that the 5′- and 3′-end fragments contained 494 and 462 bp lengths, respectively, which correspond to fungal $\Delta^6$-elongases. Thus, the full-length cDNA of *PyElo* contained an ORF of 834 bp encoding 277 amino acid residues with a calculated molecular mass of 32.1 kDa. The deduced amino acid sequence of the *PyElo* showed the highest similarity (85% sequence identity) to the putative VLC-PUFA elongases of *Pythium aphanidermatum*. It also showed 68 and 65% identity with the $\Delta^6$-elongases of *Phytophthora infestans* and *Pythium irregulare*, respectively [14].

Amino acid sequence alignment showed that *PyElo* rendered common characteristics to the known PUFA elongases, particularly four conserved motifs (KxxExxDT, QxxFLHxYHH, NxxxHxxMYxYY and TxxQxxQ), and a histidine-rich catalytic motif (HVYHH), which are the Elo family signature [15] (Supplementary file 1). Involvement of the four conserved motifs in catalytic ring organization and influencing the activity of very long-fatty acid elongating enzymes, has been proposed [14–16]. It has been postulated that the histidine-rich motif is critically essential for metal ion binding similar to that in other oxidative enzymes, such as fatty acid desaturases [17]. It was also found that there was a variation of amino acid residues located upstream of the histidine-rich motif among various PUFA elongases, which has been proposed to be involved in the optimum enzyme activity for a wide range of organisms [18,19]. Two lysine (K) residues were also found at the C-terminus of the *Pythium* elongase. It has been previously reported that the lysine residues might function as an endoplasmic reticulum (ER) retention signal [20]. Hydropathy analysis of *PyElo* sequence showed a membrane-bound characteristic of PUFA elongases, which contained seven hydrophobic domains (Fig. 1) and the four conserved motifs were located at the cytosolic proximal regions of transmembrane helices 3, 4, 5 and 6, respectively (Supplementary file 1 and Fig. 1). Therefore, PyElo is characterized to be a member of the elongase family by the fact that it contains five to seven transmembrane helices as previously reported [21–24]. The phylogenetic analysis indicated that the *PyElo* belongs to the clade of PUFA elongase family of Oomycete fungi (Fig. 2).

Functional characterization of the *PyElo* gene was done by heterologous expression in *S. cerevisiae* under the control of *GAL1* promoter. Fatty acid analysis of the yeast transformants carrying *PyElo* showed a new chromatographic peak of DGLA (C20:3 $\Delta^{8,11,14}$) in the culture supplemented with GLA substrate, whereas this elongated product was absent in the pYES2 transformant (Fig. 3 and Table 2). As a result, the *PyElo* encodes for PUFA elongase, which could elongate the $\Delta^6$-desaturated fatty acid yielding the longer acyl chain. Obviously, there was no extra peak in the *PyElo* transformant cultures without fatty acid addition.

### 3.2. Substrate specificity and preference of PyElo

To determine substrate utilization of the *Pythium* elongase, various fatty acids were fed to recombinant yeast cultures. In addition to GLA, *PyElo* could catalyze the elongation of STA yielding ETA. Considering the conversion rate of both fatty acid substrates, the *Pythium* enzyme preferred STA rather than GLA as shown in Table 2. However, it was unable to elongate endogenous saturated fatty acids (C16:0 and C18:0) and other saturated fatty acids (C20:0, C22:0 and C24:0) supplemented to the cultures. Monoene fatty acids (C16:1$\Delta^7$ and C18:1$\Delta^9$), diene fatty acid (LA), *n*3-triene fatty acid (ALA) and 20C-PUFAs (ARA and EPA) were not substrates for the *Pythium* elongase. These results are similar to previous studies of Δ6-elongases of moss *Physcomitella patens* [25] and microalgae *Parietochloris incise* [26], which have a high specificity for $\Delta^6$-18C PUFAs substrates, but they strongly preferred STA substrate over GLA.

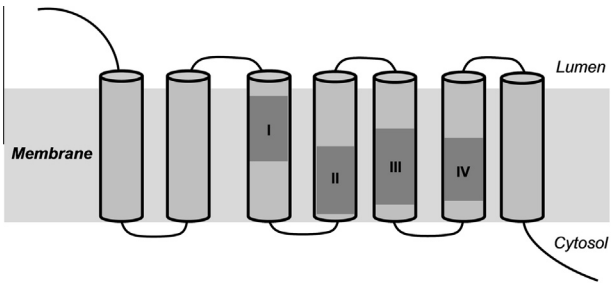


**Fig. 1.** Topology model of PyELO. The enzyme topology was generated by using the HMMTOP and OCTOPUS programs. The transmembrane regions in the enzyme topology are indicated by cylinders, which are four conserved motifs of elongases.

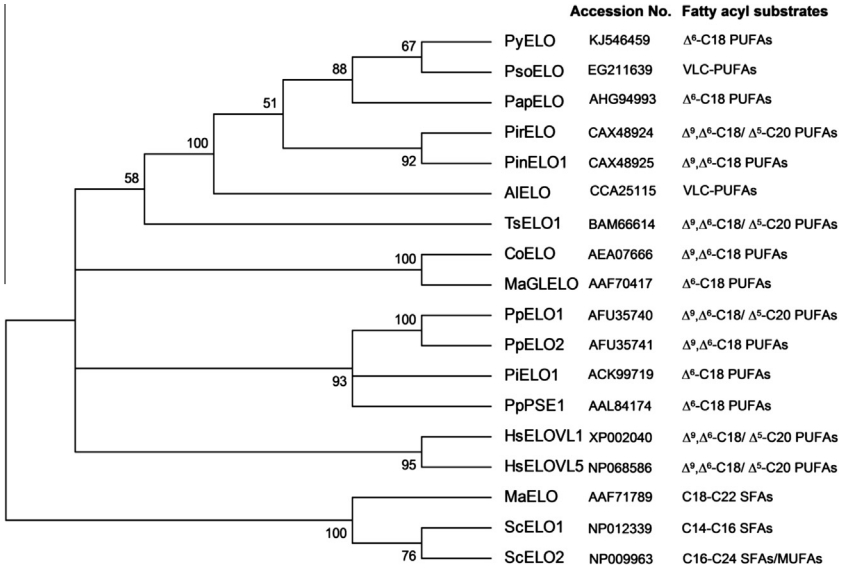


**Fig. 2.** Neighbor-Joining consensus tree showing the relationships of the *Pythium* enzyme (PyELO) with elongases from other organisms. Bootstrap values from 1000 replicates test are shown at individual nodes. Branches corresponding to partitions reproducing less than 50% bootstrap replicates are collapsed. Abbreviations of elongase genes in individual organisms: PyELO, *Pythium* sp. BCC53698; PsoELO, *P. sojae*; PapELO, *P. aphanidermatum*; PirELO, *P. irregular*; PinELO1, *P. infestans*; AlELO, *A. laibachii*; TsELO1, *Thraustochytrium* sp.; CoELO, *Conidiobolus obscurus*; MaGLELO, *M. alpina*; PpELO1 and PpELO2, *P. patens*; PiELO1, *P. incisa*; PpPSE1, *P. patens*; HsELOVL1 and HsELOVL5, *Homo sapiens*; MaELO, *M. alpina*; ScELO1 and ScELO2, *S. cerevisiae*.

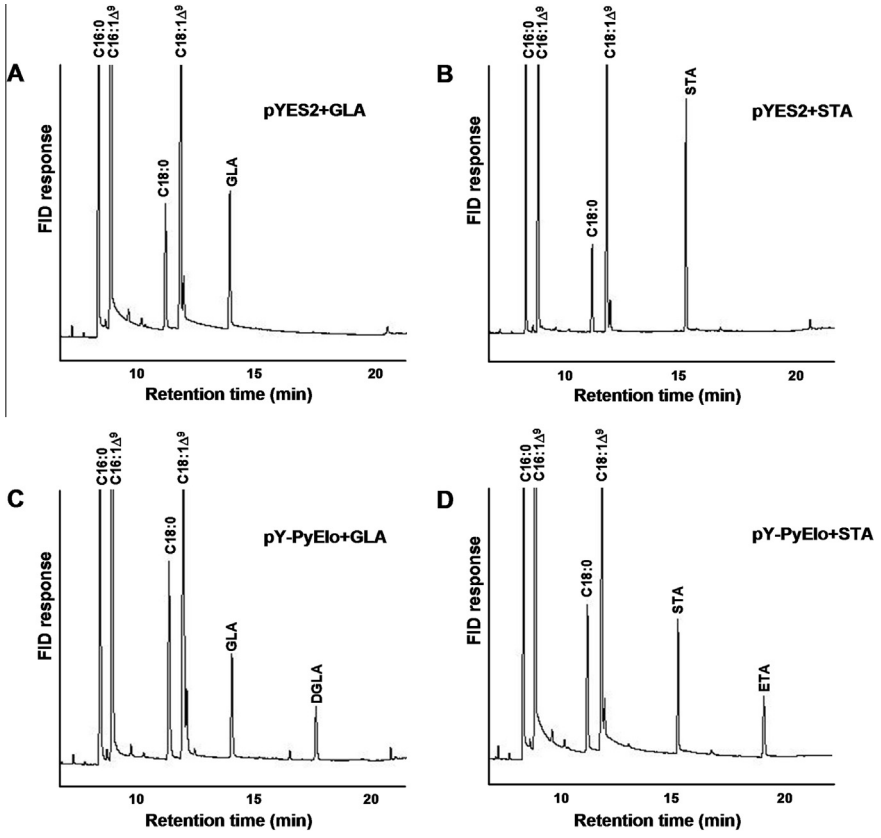


**Fig. 3.** Chromatographic profiles of yeast transformants carrying pYES2 (A and B) and PY-PyElo (C and D). Yeast cultures supplemented with 50 μM of GLA (A and C) and STA (B and D) were grown at 30 °C for 48 h, and then added with 2% (w/v) galactose for inducing the gene expression.

## 4. Discussion

Catalytic diversity of fatty acid elongases exists in living organisms and definitely link to discrimination in metabolic pathway of fatty acid synthesis across biological systems. Several genes coding for the PUFA elongases have been isolated from mammals, fungi, lower plants and algae. However, the common characteristics of the membrane-bound elongases, including histidine-rich motifs, transmembrane regions and homology-based results cannot be used to clearly distinguish their substrate specificities and

**Table 2**
Fatty acid composition of yeast transformants containing the *Pythium* $\Delta^6$-elongase cDNA (PY-PyELO) and pYES2 grown in SD medium supplemented with 50 μM individual fatty acids.

| Yeast transformants | Fatty acid compositions in total fatty acids (%, w/w) | | | | Substrate conversion rate (%) |
|---|---|---|---|---|---|
| | C18:3$\Delta^{6,9,12}$ (GLA) | C18:4$\Delta^{6,9,12,15}$ (STA) | C20:3$\Delta^{8,11,14}$ (DGLA) | C20:4$\Delta^{8,11,14,17}$ (ETA) | |
| pYES2 (control) | | | | | |
| No fatty acid addition | – | – | – | – | |
| +GLA | 8.6 ± 0.5 | – | – | – | |
| +STA | – | 13.9 ± 1.0 | – | – | |
| PY-PyELO | | | | | |
| No fatty acid addition | – | – | – | – | |
| +GLA | 5.9 ± 0.2 | – | 2.4 ± 0.2 | – | 29.3 ± 2.8 |
| +STA | – | 6.1 ± 1.0 | – | 3.5 ± 0.3 | 36.5 ± 3.9 |

preference. Thus functional analysis of the elongases is usually required. Moreover, some elongases are specific for PUFA with variable chain-lengths (multi-step PUFA elongases) [27] or exhibit broad substrate specificities capable of elongating several PUFA substrates, including $\Delta^6$-18C (GLA and STA), $\Delta^9$-18C (LA and ALA) and $\Delta^5$-20C PUFAs (ARA and EPA), such as the two enzymes, MALCE1 of *Mortierella alpina* [28] and PpELO1 of moss *P. patens* [29]. With regard to enzyme engineering, much effort has been done by site-directed mutagenesis to identify amino acid residues which play important roles in catalytic function and substrate recognition; however knowledge on the relationship between enzyme structure and substrate utilization has not been well clarified. As a chemotaxonomic marker, the profiling of fatty acids is a powerful not only for strain identification, but also used for rational selection of a target enzyme/gene involved in PUFA synthesis from an array of organisms. Based on our previous results, we employed *Pythium* sp. BCC 53698 as a genetic resource, which accumulates high levels of 20C-PUFAs (DGLA, ARA and EPA), which are the products derived from the $\Delta^6$-elongation reaction, but does not contain C20:2$\Delta^{11,14}$.

Based on the results of structural and functional characterization, it can be concluded that the cloned *PyElo* gene encoded the $\Delta^6$-elongase, which had very high specificity on 18-carbon PUFAs (GLA and STA) containing $\Delta^6$-double bond in their acyl chains similar to the previous report for MaGLELO from *M. alpina* [30]. Thus, the *Pythium* $\Delta^6$-elongase was categorized into the one-step PUFA-elongase family, which is specific for PUFA of fixed chain-length [27]. One-step 18C-PUFA elongase (PinELO) has been reported in the moss *P. infestans*, enabling it to use $\Delta^6$-18C and $\Delta^9$-18C PUFAs [14]. Although the PyElo shared a high homology with other PUFA elongases of Oomycete fungi, it displayed a different substrate specificity. The PUFA elongase (PirELO) of *P. irregulare* is capable of elongating $\Delta^6$-C18 and $\Delta^5$-C20 fatty acid substrates [14]. The unique characteristic of the identified elongase of *Pythium* sp. BCC 53698, particularly in terms of utilization of specific substrates, has not been previously reported in Oomycete fungi.

Information on substrate specificity and preference of fatty acid elongases is important for engineering the PUFA pathway in a host of choice. There was not much difference of PyElo in the conversion of GLA and STA substrates. This finding indicates that the potent *PyElo* gene can be used for reconstituting a fatty acid profile in transgenic organisms through the *n*-6 series pathway for synthesis of DGLA, which particularly could become interesting by the health care market. This is a report of molecular identification of a novel elongase gene with high specificity only for $\Delta^6$-18C PUFAs from Oomycetes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.004.

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