Heterologous reconstitution the polyunsaturated fatty acid biosynthetic pathway of *Phaeodactylum tricornutum* in *Arabidopsis thaliana*

Yun-tao Li [1, 2]*, Yuan-min Zhu[1], Chun-hua Fu[1], Mao-teng Li[1**], Long-jiang Yu[1]

[1] College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, 430074, China
[2] College of Life Science, Hubei Normal University, Huangshi, 435002, China
[**] Corresponding author. Address: Institute of Resource Biology and Biotechnology, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, P.R. China. E-mail: limaoteng426@mail.hust.edu.cn. Tel.: +86 (27) 87792264; Fax: +86(27)87792265.

Abstract: The genes encoding Δ6 desaturase, Δ6 fatty acid elongase, and Δ5 desaturase from the alga, *Phaeodactylum tricornutum*, were co-expressed in *Arabidopsis thaliana* under the control of the *Brassica napus* napin promoter to produce arachidonic acid (ARA; 20:4 $\Delta^{5, 8, 11, 14}$) and eicosapentaenoic acid (EPA; 20:5 $\Delta^{5, 8, 11, 14, 17}$). ARA and EPA accumulated up to 0.5% and 0.05% of total fatty acids, respectively, in the transgenic *A. thaliana*. The conversion efficiencies of desaturation steps in n6 (18.6% and 23.8%) and n3 (6% and 9%) were different. The efficiency in n6 pathways was higher than that of in n3 pathways.

[Yun-tao Li, Yuan-min Zhu, Chun-hua Fu, Mao-teng Li, Long-jiang Yu. **Heterologous reconstitution the polyunsaturated fatty acid biosynthetic pathway of *Phaeodactylum tricornutum* in *Arabidopsis thaliana.*** *Life Sci J* 2012;9(4):2561-2565] (ISSN:1097-8135). http://www.lifesciencesite.com. 380

Keywords: Desaturase; Elongase; Arachidonic acid and eicosapentaenoic acid; *Arabidopsis thaliana*; Transformation

1. Introduction

Polyunsaturated fatty acids (PUFAs), such as arachidonic acid (ARA; 20:4 $\Delta^{5, 8, 11, 14}$) and eicosapentaenoic acid (EPA; 20:5 $\Delta^{5, 8, 11, 14, 17}$), are essential for human health and well-being. They can maintain the structure and function of bio-membrane (1). They also have important physiological and medical functions, such as curing of cardiovascular disease anti-inflammatory (2) and promoting brain development (3). Recently people have paid more attention to ARA, EPA and docosahexaenoic acid (DHA; 22:6 $\Delta^{4, 7, 10, 13, 16, 19}$) than other PUFAs because of their special role and influence on human health. At present, the main source of EPA and DHA for human consumption is oily ocean fish (4). The γ-linolenic acid (GLA; 18:3 $\Delta^{6, 9, 12}$) can be found in plant oils derived from evening primrose, borage and black currant (5). ARA is commercially produced by fermentation of oleagenous fungi such as *Mortierella alpina* (6). The increasing demand has raised the interest in obtaining these PUFAs from alternative sources, which are more economical and sustainable than traditional source. One attractive option is to genetically engineer the oil-seed crops like soybean, rape, and others to produce long-chain PUFAs such as GLA, ARA, EPA, and DHA (7).

The production of PUFA such as ARA, EPA and DHA in oilseed crops has been the subject of much recent interest. Abbadi et al. reported 1.5% AA and 1.0% EPA in transgenic flax seeds containing Δ6 and Δ5 desaturases and a Δ6 elongase (8). However, in *Brassica juncea*, the introduction of 5 and 9 structural genes resulted in the production of up to 25% AA and 15% EPA in transgenic seeds (9). Robert et al. used the *Arabidopsis thaliana* as the host plant for biosynthesis of PUFAs using a construct including a dual-purpose Δ5/Δ6 desaturase that acts on acyl-CoA substrates, the production of EPA and AA was 3.2% and 1.6% respectively in transgenic *Arabidopsis* (10). It is clear that some endogenous metabolic bottlenecks need to be overcome to enable transgenic plants to synthesize and accumulate PUFAs to levels equivalent to those found in the marine environment (11, 12, 13). Several different metabolic engineering rationales to increase PUFA production in transgenic seed oils have been suggested. One approach is to find superior desaturases can efficiently use acyl-CoA substrates (11). For example, Δ6-desaturases from liverwort, *Marchantcia polymorpha* and *Mantoniella squamata* can efficiently use acyl-CoA substrates, potentially bypassing the acyl-exchange bottleneck (14, 15, 16). Taken together, these results indicate that both the specific gene combinations used and the choice of host plant are important for VLCPUFA production (17).

In previous, our team has researched the influence of gene copy number on the yield of PUFAs using the Δ6, Δ5 desaturase and Δ6 elongases from *Phaeodactylum tricornutum* in *Pichia pastoris*. Recently our team identified the Δ6 desaturase gene from *M. alpine* W15 (18). In the present study, we report the seed-specific expression of Δ6 desaturase (D6), Δ5 desaturase (D5) (19), and Δ6 elongases (E6) (GenBank accession no. AY746355) from *P. tricornutum* in *A. thaliana* to reconstituted the biopathways for EPA and ARA.

2. Materials and methods

Plant materials

P. tricornutum was grown in f/2 culture medium at 22 °C with photoperiods of 16 h of light, *A. thaliana* ecotype Columbia was grown at 22 °C and used for transformation. *Brassica napus* of B351 which provided by Shanxi Hybrid Rapeseed Research Centre of China was used for the cloning of napin promoter.

Nucleic acids extractions

Total genomic DNA was extracted from leaves of *B. napus* and seedlings of *A. thaliana* using a CTAB method (20). *P. tricornutum* in the logarithmic phase was collected by centrifugation and used for the extraction of total RNA (21). The total RNA of *P. tricornutum* was extracted using RNeasy Maxi Kit (Qiagen, Valencia, CA).

Vector construction

The sequence of napin promoter and the open reading frames of the different desaturases and elongase were modified by PCR to create appropriate restriction sites adjacent to the start codons and stop codons, cloned into the pGEMT-T vector (Promega, Madison, WI), and sequenced to confirm their identity.

For the generation of transformation constructs, a triple cassette containing the napin promoter, the NOS terminator, and three different polylinkers between each promoter and terminator were first inserted into the pUC18 vector, yielding the pUC18*np* plasmid.

The open reading frames of desaturases and elongases were then released using the restriction sites created by PCR and successively inserted into the same restriction sites of the polylinkers of the pUC18*np* plasmid, yielding the pUC18123*np* plasmid. The resulting cassette, containing the three genes each under the control of the napin promoter, was released by digesting the pUC18123*np* plasmid with *Kpn*I and *Bgl*II and cloned into the corresponding sites of the binary vectors pCAMBIA1303 (CAMBIA, Canberra, Australia) , yielding the pC1303*D6E6D5* plasmid. The vectors pC1303*D6E6D5* were transferred into the *Agrobacterium* strain LBA4404.

Plant transformation

A. thaliana transformation was carried out by the floral dipping method of Clough and Bent (22). Seeds from dipped plants (T_1 seed) were collected and plated out on selective media containing hygromycin (40 mg/L). The selected transformed seedlings were transferred to soil to establish T_1 plants.

Molecular analysis of transgenic plants

Transgenic plants were checked by PCR amplification on genomic DNA. In order to detect the expression of target genes, RT-PCR were performed. Total RNAs were isolated from transformant and first-strand cDNAs were synthesized using the First Strand cDNA Synthesis Kit (Fermentas). These templates were used for the amplification of target gene using the same primer sets as those used in genomic PCR.

Fatty acid extraction and analysis

Total seed oil was extracted by a method as previously described (23). The fatty acid methyl esters (FAMEs) were prepared according to Lightner et al. (24) and analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A-5975C GC-MS Network system.

3. Results

Construction of plant expression vectors and molecular analysis of transgenic plants

Restriction and PCR analysis of the resultant clones identified trivalent expression vectors containing *D6*, *E6*, *D5* expression cassette, and the result showed that trivalent expression vectors were successfully constructed (Figure 1).

Hygromycin-resistant seedling lines were obtained after selection in a medium supplemented with increasing concentrations of hygromycin B from 15–45 mg/mL. Genomic PCR analysis revealed that some of the seedlings contained the genes of D6, D5 desaturase, and E6 elongase, while the others contained only one or two genes of them.

RT-PCR analysis showed that plant integrated by the genes of D6, D5 desaturase, and E6 elongase contained the transcripts of the three genes. And the transcripts of the three genes were detected only in the T_1 plant's seeds suggested that the three genes were only expressed in seeds (Figure 2).

Fatty acid analysis of transgenic plants

Following transformation with the trivalent expression vectors and selection on hygromycin, T1 plants were recovered and their seeds were analyzed for fatty acid composition using GC-MS (Figure 3). The result showed that new n3 PUFA, including stearidonic acid (SDA; 18:4 $\Delta^{6, 9, 12, 15}$), eicosatetraenoic acid (ETA; 20:4 $\Delta^{8, 11, 14, 17}$) and EPA, and new n6 PUFA, including GLA, dihomo-γ-linolenic acid (DGLA; 20:3 $\Delta^{8, 11, 14}$) and ARA were synthesized in T_1 plants, conversely these productions were not detected in wild type *A. thaliana*. Thus it is concluded that ARA and EPA biosynthetic pathways were successfully reconstituted in *A. thaliana*.

The fatty acids of the transgenic *A. thaliana* were shown in Table 1. The contents of GLA, DGLA and ARA were 6.2 %, 1.6 % and 0.5 % respectively. The yields of SDA, ETA and EPA accumulated to 0.9 %, 0.5 % and 0.05 %, respectively. The contents of LA and ALA, especially eicosenoic acid (ENA; 20:1 Δ^{11}), were decreased in transgenic *A. thaliana* comparing with the wild type. The decrease of the LA, ALA and ENA contents in transgenic plants indicated that these heterologous genes which were transformed into *A. thaliana* had significant influence on the fatty acid composition.of *A. thaliana*. The conversion efficiency

of each step in the n6 and n3 pathways was calculated as products/ (substrate + products). These results showed that the conversion efficiency of desaturation step in n6 (18.6% and 23.8%) was higher than in n3 pathways (6% and 9%) (Table 2). The low conversion efficiency in n3 pathways is partly because of the low content of EPA.

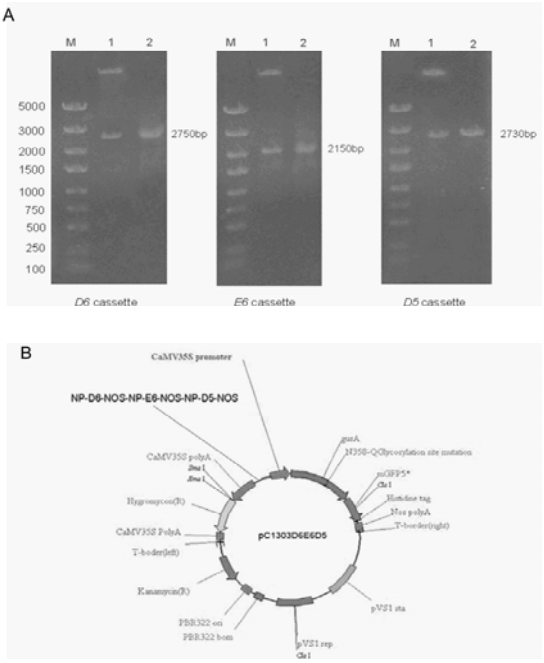

Figure 1. A: Restriction analyses of pC1303*D6E6D5*. M: marker; lane 1: digested recombinant plasmid; lane 2: PCR product of target gene expression cassette. B: The trivalent expression vector.

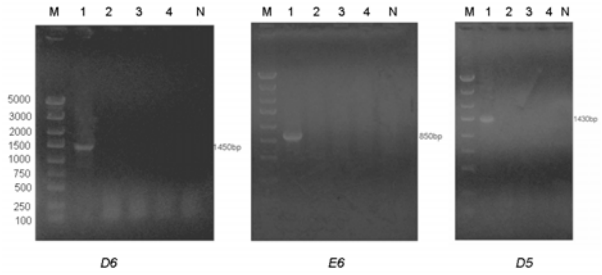

Figure 2. RT-PCR results of transgenic *A. thaliana*. M: marker; lane 1: seed; lane 2: root; lane 3: stem; lane 4: leaf; N: negative control.

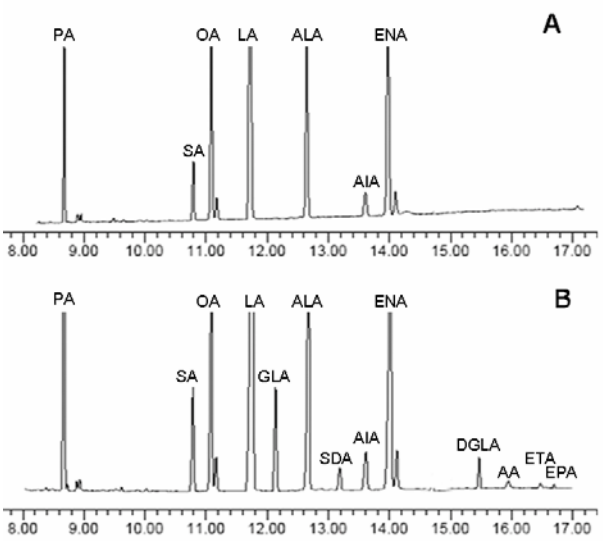

Figure 3. Results of GC-MS analysis of fatty acids in transgenic *A.thaliana*. A, B represent the fatty acids in the wild type and transgenic *A.thali*ana respectively.

Table 1. Fatty acid composition (% w/w) of total fatty acids from transgenic *A.thaliana*.

Fatty acid	*A.thaliana*	transgenic *A.thaliana*
PA [a] 16:0	9.7±0.3[b]	10.1±0.3
SA 18:0	4.3±0.2	6.7±0.2
OA 18:1Δ^{9}	16.6±0.5	15.8±0.5
LA 18:2$\Delta^{9,12}$	28.2±0.6	27.1±0.5
ALA 18:3$\Delta^{9,12,15}$	17.2±0.5	14.0±0.4
AIA 20:0	2.2±0.1	1.7±0.1
ENA 20:1 Δ^{11}	19.7±0.4	10.8±0.3
GLA 18:3$\Delta^{6,9,12}$	ND[c]	6.2±0.4
DGLA 20:3$\Delta^{8,11,14}$	ND	1.6±0.2
ARA 20:4$\Delta^{5, 8, 11, 14}$	ND	0.5±0.1
SDA 18:4$\Delta^{6,9,12,15}$	ND	0.9±0.2
ETA 20:4$\Delta^{8,11,14,17}$	ND	0.5±0.1
EPA 20:5$\Delta^{5, 8, 11, 14, 17}$	ND	0.05±0.0

[a]PA: palmitic acid; SA: stearic acid; OA: Oleic acid; LA: linoleic acid; ALA: α-linolenic acid; AIA: arachidic acid; ENA: eicosenoic acid; GLA: γ-linolenic acid; DGLA: dihomo-γ-linolenic acid; ARA: arachidonic acid; SDA: stearidonic acid; ETA: eicosatetraenoic acid; EPA: eicosapentaenoic acid.
[b]Each value is the mean ± SD from three independent experiments
[c]Not detected

Table 2. Conversion efficiency (%) of *P. tricornutum* PUFAs biosynthetic enzymes in transgenic *A.thaliana.*

Δ6-des		Δ6-elo		Δ5-des	
LA→GLA	ALA→SDA	GLA→DGLA	SDA→ETA	DGLA→ARA	ETA→EPA
18.6 [a]	6.0	20.5	35.7	23.8	9

[a] The conversion efficiency of each step was calculated as 100 × product/(substrate +product) (%)

4. Discussion

In this study, the content of EPA (n3) was lower than that of ARA (n6) in transgenic *A. thaliana*, thus the conversion efficiency of n6 pathways was higher than that of n3 pathways overall. It is partly because of the low content of EPA. The genes of Δ6 desaturase, Δ5 desaturase, and Δ6 elongases were from *P. tricornutum*. The content of EPA was much higher than ARA in *P. tricornutum*. Maybe there was some kind of mechanism by which ARA can be converted to EPA. Recently, some researchers reported that the seed-specific expression of a Δ9 elongase pathway consisted of the *Isochrysis galbana* Δ9 elongase, the *Pavlova salina* Δ8 and Δ5 desaturases in *A. thaliana* and *B. napus,* furthermore the contents of ARA and EPA were 9.7% and 2.4%, respectively in T_2 transgenic *B. napus* seed (25). The efficiency of Δ9 elongase pathway was higher than that of conventional Δ6-pathway, but in the both pathway the content of ARA was much higher than that of EPA. In other study, the content of ARA was higher than that of EPA, too (26).

To obtain a suitable and alternative source of these desired PUFAs, producing PUFAs in the oil-bearing crops would be a preferable alternative. The fatty acids mainly synthesized in the seeds. Napin promoter can control the expression of heterologous genes which were specifically expressed in seeds, prevent foreign gene expression in other parts of the plant, reduce the waste of plant energy and nutrients, and reduce the adverse effects of transformation heterologous genes on plants. So napin promoter was often used in transformation of *A. thaliana* and *B. napus*. *P. tricornutum* has high levels of EPA, and its content exceeds 30% (27). *P. tricornutum* was possibly a good genetic source material. In transgenic *A. thaliana*, the conversion efficiencies of each step in the n6 and n3 pathways were low, so the content of ARA and EPA were low, too. The result showed that heterologous expression of the genes from some material which has high levels of PUFAs may not produce high levels of PUFAs in transgenic material. Hence, it is necessary to take other method to obtain high lever ARA and EPA. For example, using the Δ9 elongase pathway or acyl-CoA-dependent pathway, or using the lyso-phosphatidylcholine acyltransferases gene was optional methods.

Acknowledgements

The investigation was supported by the Key technologies research and development program of Wuhan (201020622246), National Natural Science Foundation of China (Grant No. 20976065) , the Natural Science Funds for Distinguished Young Scholars of Hubei Province (2010CDA097), the New Century Talents Support Program by Ministry of Education of China and National High Technology Research and Development Program of China (2007AA10Z120). The authors are grateful to Ms Cheng of Huazhong University of Science & Technology analytical and testing center for technical assistance.

Corresponding author:

Dr. Mao-teng Li

Institute of Resource Biology and Biotechnology, College of Life Science and Technology, Huazhong University of Science and Technology,

Wuhan, Hubei 430074, P.R. China

E-mail: limaoteng426@mail.hust.edu.cn

Tel.: +86 (27) 87792264; Fax: +86(27)87792265.

References

1. Yehuda S, Rabinovitz S, Carasso LR, Mostofsky DI. The role of polyunsaturated fatty acids in restoring the aging neuronal membrane. Neurobiol Aging 2002; 23: 843-853.
2. Das UN. Estrogen, statins, and polyunsaturated fatty acids: similarities in their actions and denefits: is there a common link? Nutrition 2002; 18:178-188.
3. Benatti P, Peluso G., Nicolai R, Calvani M. Polyunsaturated fatty acids: biochemical, nutritional and epigenetic properties. J Am Coll Nutr 2004; 23: 281-302.
4. Pauly D, Christensen V, Guénette S, Pitcher TJ, Sumaila UR, Walters CJ, Watson R, Zeller D. Towards sustainability in world fisheries. Nature 2002; 418: 689-695.
5. Barre DE, Potential of evening primrose, borage, black currant, and fungal oils in human health. Ann Nutr Metab 2001; 45: 47-57.
6. Streekstra H. On the safety of *Mortierella alpina* for the production of food ingredients, such as arachidonic acid. J Biotechnol 1997; 56: 153-165.
7. Huang YS, Pereira SL, Leonard AE. Enzymes for

transgenic biosynthesis of for transgenic biosynthesis of long-chain polyunsaturated fatty acids. Biochimie 2004; 86: 793-798.
8. Abbadi A, Domergue F, Bauer J, Napier JA,Welti R, Zähringer U, Cirpus P, Heinz E. Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: constraints on their accumulation. Plant Cell 2004; 16:1–15.
9. Wu G, Truksa M, Datla N, Vrinten P, Bauer J, Zank T, Cirpus P, Heinz E, Qiu X. Stepwise engineering to produce high yields of very long-chain polyunsaturated fatty acids in plants. Nat Biotechnol 2005; 23:1013–1017.
10. Robert SS, Singh SP, Zhou XR, Petrie JR, Blackburn SI, Mansour PM, Nichols PD, Liu Q, Green AG. Metabolic engineering of *Arabidopsis* to produce nutritionally important DHA in seed oil. Funct Plant Biol 2005; 32:473–479.
11. Ruiz-López N, Haslam RP, Venegas-Calerón M, Li T-b, Bauer J, Napier JA, Sayanova O. Enhancing the accumulation of omega-3 long chain polyunsaturated fatty acids in transgenic *Arabidopsis thaliana* via iterative metabolic engineering and genetic crossing. Transgenic Res 2012; DOI 10.1007/s11248-012-9596-0.
12. Venegas-Calerón M, Sayanova O, Napier JA. An alternative to fish oils: metabolic engineering of oil-seed crops to produce omega-3 long chain polyunsaturated fatty acids. Prog Lipid Res 2010; 49:108–119.
13. Napier JA, Graham IA. Tailoring plant lipid composition: designer oilseeds come of age. Curr Opin Plant Biol 2010; 13: 330–337.
14. Kajikawa M, Yamato KT, Kohzu Y, Nojiri M, Sakuradani E, Shimizu S, Sakai Y, Fukuzawa H, Ohyama K. Isolation and characterization of Δ6-desaturase, an ELO-like enzyme and Δ5-desaturase from the liverwort *Marchantia polymorpha* and production of arachidonic and eicosapentaenoic acids in the methylotrophic yeast *Pichia pastoris*. Plant Mol Biol 2004; 54:335–352.
15. Hoffmann M,Wagner M, Abbadi A, Fulda M, Feussner I. Metabolic engineering of ω3-very long chain polyunsaturated fatty acid production by an exclusively acyl-CoA-dependent pathway. J Biol Chem 2008; 283:22352–22362.
16. Petrie JR, Shrestha P, Mansour MP, Nichols PD, Liu Q, Singh SP. Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants using an acyl-CoA Δ6-desaturase with omega3-preference from the marine microalga *Micromonas pusilla*. Metab Eng 2010; 12:233–240
17. Cheng BF, Wu GH, Vrinten P, Falk K, Bauer J, Qiu X. Towards the production of high levels of eicosapentaenoic acid in transgenic plants: the effects of different host species, genes and promoters. Transgenic Res 2010; 19:221–229.
18. Liu JM, Li DR, Yin YT, Wang H, Li MT, Yu LJ. Δ6-Desaturase from *Mortierella alpina*: cDNA cloning, expression, and phylogenetic analysis. Biotechnol Lett 2011; 33: 1985-1991.
19. Domergue F, Lerchl J, Zähringer U, Heinz E. Cloning and functional characterization of *Phaeodactylum tricornutum* front-end desaturases involved in eicosapentaenoic acid biosynthesis. Eur J Biochem 2002; 269: 4105-4113.
20. Kang TJ, Yang MS. Rapid and reliable extraction of genomic DNA from various wild-type and transgenic plants. BMC Biotechnol 2004; 4:20. doi: 10.1186/1472-6750-4-20.
21. Shirley BW, Hanley S, Goodman HM. Effects of ionizing radiation on a plant genome: analysis of two *Arabidopsis* transparent testa mutations. Plant Cell 1992; 4: 333-347.
22. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*- mediated transformation of *Arabidopsis thaliana*. Plant J 1998; 16: 735-743.
23. Tonon T, Harvey D, Larson TR, Graham IA. Identification of a very long chain polyunsaturated fatty acid Δ4-desaturase from the microalga *Pavlova lutheri*. FEBS Lett 2003; 553: 440-444.
24. Lightner J, Lark E, James D, Browse J. Novel mutations affecting leaf stearate content and plant size in *Arabidopsis*. Theor Appl Genet 1997; 94: 975-981.
25. Petrie JR, Shrestha P, Belide S, Mansour MP, Liu Q, Horne J, Nichols PD, Singh SP. Transgenic production of arachidonic acid in oilseeds. Transgenic Res 2012; 21: 139-147.
26. Qi BX, Fraser T, Mugford S, Dobson G., Sayanova O, Butler J, Napier JA, Stobart AK, Lazarus CM. Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. Nat. Biotechnol 2004; 22: 739-745.
27. Yongmanitchai W, Ward OP. Growth of and omega-3 fatty acid production by *Phaeodactylum tricornutum* under different culture conditions. Appl. Environ Microbiol 1991; 57: 419-425.

10/30/2012