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Production of Polyunsaturated Fatty Acids in Transgenic Plants

PATRICIA VRINTEN[1], GUOHAI WU[1], MARTIN TRUKSA[1] AND XIAO QIU[2]*

[1]Bioriginal Food and Science Corporation, 102 Melville Street, Saskatoon, Saskatchewan, S7J 0R1, Canada and [2]Department of Applied Microbiology and Food Science, University of Saskatchewan, 51 Campus Drive, Saskatoon, Saskatchewan, S7N 5A2, Canada

Introduction

An ever-increasing body of knowledge points to the importance of very long chain ω3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA; 22:6Δ4,7,10,13,16,19) and eicosapentaenoic acid (EPA; 20:5Δ5,8,11,14,17) in human health. Over the last century, the dietary ratio of ω3/ω6 fatty acids has decreased dramatically in western countries and this change is believed to have contributed to higher levels of cardiovascular and inflammatory disease (Sontrop and Campbell, 2006). Low blood lipid concentrations of ω3 PUFAs are also thought to increase susceptibility to depression, and the benefit of treatment with EPA or EPA plus DHA for depression or bipolar disorder has been demonstrated (reviewed in Sontrop and Campbell, 2006).

A high intake of PUFAs, particularly ω3 PUFAs, may reduce the risk of developing Alzheimer's disease, and feeding with DHA reduced the accumulation of amyloid beta-peptide in brains of transgenic mice (Oksman *et al.*, 2006). DHA in particular is thought to improve learning ability and contribute to brain development in infants and normal brain function in adults (Horrocks and Yeo, 1999). With preterm infants, adding ω3 PUFAs to formula appears to improve both visual acuity and cognitive development (Fleith and Clandinin, 2005).

*To whom correspondence may be addressed (xiao.qiu@usask.ca)

Abbreviations: ACP, acyl carrier protein; ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EDA, eicosadienoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid; PUFAs, polyunsaturated fatty acids; SDA, stearidonic acid; VLCPUFA, very long chain polyunsaturated fatty acid.

The clearest positive effect of EPA and DHA, however, is in protection against heart disease, the leading cause of death in western nations. The American Heart Association Nutrition Committee has recommended consuming fish, particularly oily fish, at least twice a week, while individuals with hypertriglyceridemia should take supplements containing EPA plus DHA (Lichtenstein *et al.*, 2006); such recommendations are based largely on the results from randomized control trials demonstrating the protective effects of consuming these ω3 PUFAs (Breslow, 2006). Supplementation with fish oil (DHA plus EPA) appears to improve cardiovascular outcomes through reductions in total mortality, coronary heart disease death, and sudden death (Harper and Jacobson, 2005).

Although humans can synthesize both DHA and EPA from the precursor essential fatty acid α-linolenic acid (ALA; 18:3Δ9,12,15), direct uptake appears to be significantly more effective (Horrocks and Yeo, 1999), and the high amounts of DHA in neural and retinal tissues combined with the limited storage of ω3 PUFAs in adipose tissues suggest that a relatively constant dietary supply of this fatty acid is required (Arterburn *et al.*, 2006). Presently, the main dietary sources of both EPA and DHA are fish and fish oil. However, consumption of fish is a major source of human exposure to environmental contaminants, including carcinogenic contaminants such as DDT and non-carcinogenic contaminants such as methyl mercury (Sidhu, 2003); while these risks are often deemed acceptable, this depends on the source and species of fish consumed (Foran *et al.*, 2005), and risk levels may be of particular concern to sensitive populations such as women of child-bearing age and young children (Park and Johnson, 2006; Foran *et al*., 2005). This, combined with decreasing fish stocks throughout the world, raises questions regarding the sustainability of this source of very long chain PUFAs, and has resulted in a strong interest in the production of long-chain polyunsaturated fatty acids in plants.

Fatty acid biosynthesis: plants versus very long-chain PUFA producing organisms

De novo fatty acid synthesis in plants occurs in plastids and is catalyzed by multisubunit fatty acid synthase (FAS) complexes. The final products of FAS are 16:0- and 18:0-acyl carrier protein (ACP); commonly, 18:0-ACP undergoes desaturation by a soluble stearoyl-ACP desaturase to form 18:1Δ9-ACP (Voelker and Kinney, 2001). At this stage acyltransferases may transfer acyls from ACP to glycerol, leading to plastid glycerolipid assembly, but the majority of fatty acids are hydrolyzed from ACP by acyl-ACP thioesterase, leave the plastid, and are esterified to coenzyme A (CoA) to form acyl-CoA (reviewed in Ohlrogge and Jaworski, 1997). Through a series of reactions, the acyl moieties can become esterified to phosphatidylcholine (PC) and can then undergo desaturation by Δ12- and Δ15-desaturases to form linoleic acid (LA; 18:2Δ9,12) and ALA.

Since mammals lack the desaturases required for forming LA and ALA, these are considered essential fatty acids that must be supplied in the diet. The synthesis of very long chain PUFAs in humans and many other eukaryotes starts with Δ6-desaturation, which introduces a double bond between carbons six and seven of LA and ALA, forming γ–linolenic acid (GLA; 18:3Δ6,9,12) and stearidonic acid (SDA; 18:4Δ6,9,12,15). A series of alternating elongation and desaturation reactions result

in the synthesis of arachidonic acid (ARA, C20:4Δ5,8,11,14) and EPA (*Figure 1*). Unlike the mammalian enzymes, most "front-end" desaturases from lower eukaryotes are believed to act on fatty acids linked to phosphatidylcholine (PC). Since fatty acid elongases use acyl-CoA substrates, the alternating elongation/desaturation pathway requires the exchange of fatty acid between these two pools (Domergue *et al.*, 2003; Abbadi *et al.*, 2004).

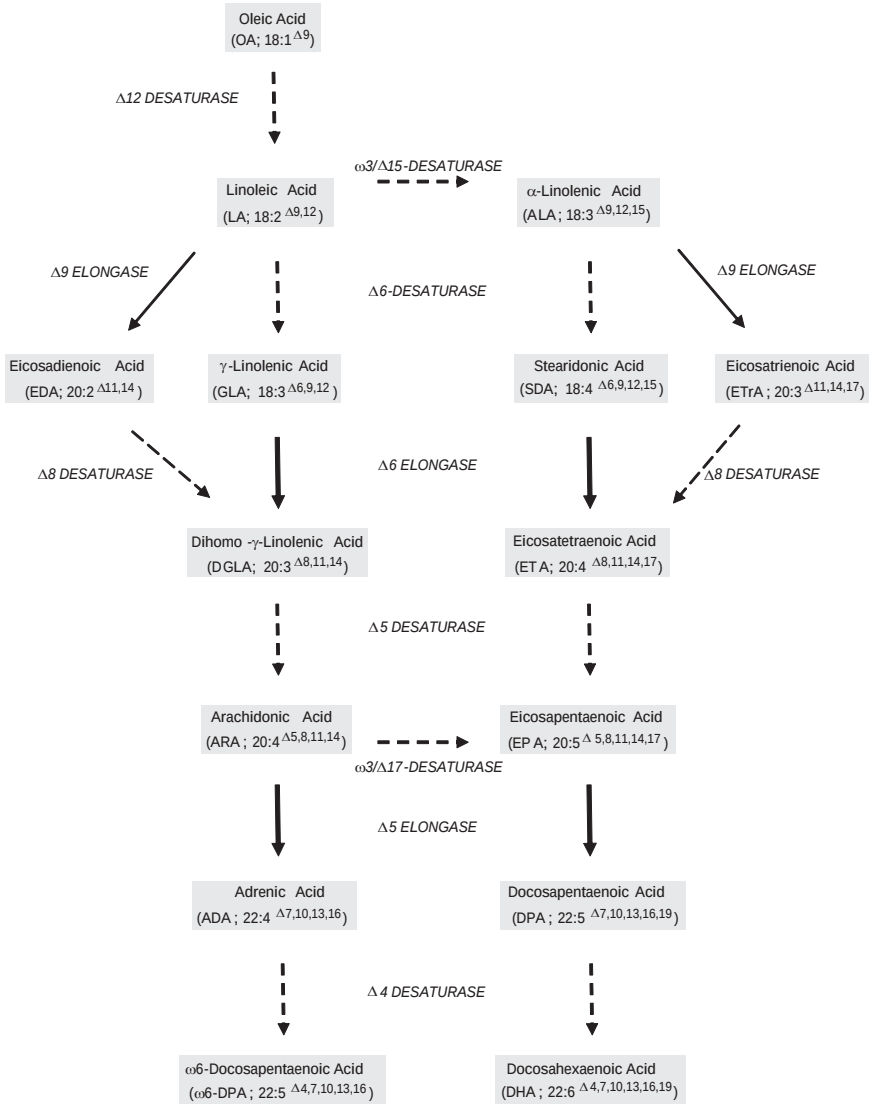

Figure 1. Pathways of long-chain polyunsaturated fatty acid biosynthesis. The conventional Δ6-desaturase/Δ6-elongase and the alternative Δ9-elongase pathways are shown starting from LA and ALA. In mammals, biosynthesis of DHA from EPA proceeds through the production of 24:6n-3, followed by ß-oxidation, whereas certain microorganisms produce EPA or DHA through a polyketide synthase-like system (not shown).

The final steps of the pathway vary between organisms (reviewed in Qiu, 2003); in certain lower eukaryotes Δ4-desaturation of docosapentaenoic acid (DPA; 22:5Δ7,10,13,16,19) produces DHA, whereas in mammals, EPA is subjected to two rounds of elongation, followed by Δ6-desaturation, to produce the C24 fatty acid tetracosahexaenoic acid (THA; 24:6Δ6,9,12,15,18,21), which is converted to DHA via β-oxidation.

Plants contain relatively small amounts of monounsaturated or saturated very long chain fatty acids with 20 or more carbons (reviewed in Voelker and Kinney, 2001). While the elongation complex involved in synthesizing these fatty acids consists of four components, namely the condensing enzyme/elongase (3-ketoacyl-CoA synthase), 3-ketoacyl-CoA reductase, 3-hydroxyacyl-CoA dehydratase, and enoyl-CoA reductase, it is primarily the condensing enzyme that determines substrate specificity (Millar and Kunst, 1997). In plants, very long chain condensing enzymes are encoded by a family of related genes, the fatty acid elongase-like (*FAE*) genes. The very long chain fatty condensing enzymes in yeast, which are encoded by the ELO (elongase) genes *ELO2* and *ELO3*, are unrelated in sequence to the plant FAE proteins. In both animals and lower eukaryotes, it appears that the condensing enzymes involved in VLCPUFA biosynthesis are more related to the ELO-like, rather than the FAE-like proteins. However, the remaining three components of the yeast elongase complex seem able to substitute for the corresponding plant factors, and likewise, plant elongase components appear to be able to work with ELO-like condensing enzymes.

Production of γ–linolenic acid and stearidonic acid in transgenic plants

Both GLA and SDA are produced naturally in certain plants; GLA is found in borage and primrose seed, while hemp seed and black current contain both SDA and GLA. However, these plants are difficult to cultivate and are relatively poor yielding, thus there is considerable interest in producing GLA and SDA in oilseed crops. Since oilseeds typically contain appreciable amounts of LA or ALA, production of GLA or SDA requires only the expression of a Δ6-desaturase gene.

The first identified Δ6-desaturase gene was isolated from the cyanobacterium *Synechocystis* by a gain-of-function screening method; a *Synechocystis* cosmid library was conjugated into a cyanobacterium (*Anabaena*) lacking Δ6-desaturase activity and the gene was recovered from transconjugants producing GLA (Reddy *et al.*, 1993). However, when this gene was expressed in tobacco (*Nicotiana tabacum*) plants under the control of a constitutive promoter (Reddy and Thomas, 1996), GLA represented only about 1%, and SDA only 1-3%, of C18 fatty acid. Neither GLA nor SDA was detected in seeds of transgenic plants, probably because the constitutive 35S promoter conditioned the expression of only a low level of Δ6-desaturase mRNA in seed. Sayanova *et al.* (1997) followed a PCR-based strategy using degenerate primers based on conserved regions of related proteins to isolate a borage Δ6-desaturase cDNA. Expression of this cDNA in transgenic tobacco plants under the control of a constitutive promoter led to the accumulation of approximately 13% GLA and 10% SDA in leaf tissue (Sayanova *et al.*, 1997), while a low level of GLA (approximately 2%) also accumulated in mature seed tissue (Sayanova *et al.*, 1999).

The borage Δ6-desaturase was isolated independently by another group using an expressed sequence tag (EST) based strategy (Thomas *et al.*, 1997) and was expressed under the control of both constitutive and seed-specific promoters in plants (Qiu *et al.*, 2002). Expression of the cDNA under the constitutive 35S promoter in flax (*Linum usitatissimum*) led to the accumulation of similar levels of GLA and SDA as were seen in tobacco. Flax has very high endogenous ALA and was thus considered a good potential host for SDA production, and flax plants carrying the borage desaturase under the control of the napin seed-specific promoter did indeed accumulate approximately ten times more SDA than GLA in seed (Qiu *et al.*, 2002). However, total GLA and SDA represented only 0.1-2% of seed fatty acids, likely due to poor performance of the *Brassica* napin promoter in flax. Conversely, use of the napin promoter in *Brassica juncea* led to the accumulation of up to about 10% GLA and 3% SDA in transgenic seeds. Results with a Δ6 desaturase isolated from the ARA and EPA producing fungus *Pythium irregulare* appeared to be much more successful; when this gene was expressed under the control of the napin promoter in *B. juncea*, levels of GLA reached as high as 40% of total seed fatty acids (Hong *et al.*, 2002). This was somewhat surprising, as we would expect the codon usage pattern of the borage desaturase gene to be more suitable; however, the fungal desaturase may not be as tightly regulated in plants as are plant counterparts, or the *P. irregulare* desaturase may simply be more active (Hong *et al.*, 2002). Expression of a Δ6-desaturase gene isolated from the fungus *Mortierella alpina* (Huang *et al.*, 1999; Sakuradani *et al.*, 1999) in *Brassica napus* (Liu *et al.*, 2001) led to the production of significant amounts of GLA (13.2%), but the levels did not reach those in *B. juncea*, likely due to lower substrate availability. While the untransformed *B. juncea* host line had approximately 40% LA (Hong *et al.*, 2002) the *B. napus* host had only 19.9% (Lui *et al.*, 2001). By simultaneously expressing the *M. alpina* Δ6-desaturase and an *M. alpina* Δ12-desaturase, Lui *et al.* (2001) were able to increase the level of GLA to 44.7% and eliminate the side product 18:2Δ6,9 which accumulated to significant levels (4.5%) when the Δ6-desaturase was expressed alone. When lines containing the *M. alpina* Δ6- and Δ12- desaturases were hybridized with a transgenic line carrying a *B. napus* Δ15-desaturase transgene, the ω3 fatty acid SDA accumulated to 23% of total fatty acids; interestingly, when all three genes were combined on one vector, SDA levels reached only 16% (Ursin *et al.*, 2003).

In soybean (*Glycine max*), LA and ALA represent about 55% and 10% of total seed storage lipids, suggesting that in this crop, like *B. juncea*, the use of a single Δ6-desaturase would allow the production of significant amounts of GLA (Sato *et al.*, 2004). Seventeen lines carrying the borage Δ6-desaturase under the control of the seed-specific ß-conglycin promoter were analyzed, and GLA levels in the T_1 generation averaged from 3.4 to 28.7% while SDA levels ranged from 0.6 to 4.2% (Sato *et al.*, 2004). These levels were considerably better than results observed with the same gene in *B. juncea* (Qiu *et al.*, 2002), again emphasizing that not only the desaturase itself but also the level of endogenous substrate in the host plant and the particular promoter used will affect the amount of product obtained. Furthermore, the wide range of average GLA and SDA levels observed in positive lines by Sato *et al.* (2004) indicates the effect of factors such as number and location of T-DNA insertions in creating variation between plants carrying the same construct.

Eckert *et al.* (2006) describe the production of SDA in the high LA plant soybean

through the seed-specific expression of the borage Δ6-desaturase and the *Arabidopsis* Δ15-desaturase. Expression of the Δ15-desaturase alone was capable of raising the ALA level in seed from approximately 10% to as high as 53%, and co-expression of both desaturases lead to average SDA levels as high as 29.5%. High SDA-containing seed appeared normal and germinated well, which was attributed to an increased relative level of palmitic acid in phospholipids.

Production of very long chain polyunsaturated fatty acids

Although it appeared possible to produce highly unsaturated 18-carbon (C18) fatty acids in plants at levels that could be considered economically feasible for commercial production, producing longer chain fatty acids was more complicated, and necessitated adding one or more elongases to the pathway.

The first step in the production of ARA or EPA from GLA or SDA requires the activity of an elongase that is active with Δ6-desaturated fatty acids. Such activity was first demonstrated by the heterologous expression in yeast of open reading frames or cDNAs from *Caenorhabditis elegans* (Beaudoin *et al.*, 2000), *Homo sapiens* (Leonard *et al.*, 2000) and the fungus *M. alpina* (Parker-Barnes *et al.*, 2000). The worm and human enzymes were identified based on similarity to yeast ELO sequences, while the *M. alpina* cDNA was isolated by transforming yeast cells with a *M. alpina* cDNA expression library and screening individual yeast clones for GLA-elongating activity (Beaudoin *et al.*, 2000). The *M. alpina* elongase was highly specific for C18 fatty acids, and acted on both ω3 and ω6 fatty acids, with a higher percent conversion of the substrate SDA (73%) than GLA (62%). Conversely, an elongase from the moss *Physcomitrella patens* (Zank *et al.*, 2002), which was identified through the analysis of ESTs, showed somewhat higher activity with GLA (51%) than with SDA (45%). This elongase also showed a significant level of activity (36%) with the C16 fatty acid 16:3Δ7,10,13, suggesting that it has no strict chain length restrictions, although it was not capable of elongating any of the C20 fatty acids tested. Importantly, both the moss and the fungal elongase sequences had more similarity to yeast ELO-like rather than plant FAE-like proteins, making the future identification of PUFA elongases much more straightforward.

Once elongases capable of elongating SDA or GLA to dihomo-γ-linolenic (DGLA; 20:3Δ8,11,14) or eicosatetraenoic acid (ETA; 20:4Δ8,11,14,17) had been identified, the next step in the pathway required the activity of a Δ5-desaturase to produce ARA or EPA (*Figure 1*). The first such desaturase was isolated from *M. alpina* (Knutzon *et al.*, 1998; Michaelson *et al.*, 1998) using PCR-based methodology. When expressed in yeast, this enzyme was capable of producing ARA in the presence of exogenously supplied DGLA (Knutzon *et al.*, 1998; Michaelson *et al.*, 1998). When this desaturase was expressed in canola (Knutzon *et al.*, 1998), new peaks corresponding to taxoleic acid (18:2Δ5,9) and pinolenic acid (18:3Δ5,9,12) were identified; these fatty acids are the Δ5-desaturation products of the endogenous canola fatty acids oleic acid (OA; 18:1Δ9) and LA. Co-expression of the *M. alpina* elongase and Δ5-desaturase genes in yeast fed SDA or GLA allowed the production of EPA or ARA (Parker-Barnes *et al.*, 2000).

Seed-specific expression of the *M. alpina* Δ6-desaturase, Δ5-desaturase and Δ6-elongase genes in soybean combined with the down-regulation of the endogenous

Δ15-desaturase led to the production of 2.1% ARA in transgenic embryos, although this level dropped in T1 (0.8%) and T2 (0.5%) seeds (Chen *et al.*, 2005). Since the same promoter was used to control all genes, four copies of this promoter were present in the construct, and the authors suggested that methylation in the promoter region increased in successive generations. Interestingly, a patent application by Kinney *et al.* (2004) reports the use of the same *M. alpina* Δ5- and Δ-6 desaturases and *M. alpina* elongase achieving EPA levels of 9.3% in somatic soybean embryos. However, in this case individual genes were under the control of different seed-specific promoters, and both an ω3/Δ17-desaturase from *Saprolegnia diclina* and an ω3/Δ15-desaturase from *Arabidopsis* were included in constructs to convert ω6 to ω3 fatty acids. Replacing the *M. alpina* Δ6-desaturase with a *S. diclina* Δ6-desaturase appeared to increase EPA levels in embryos slightly (Kinney *et al.*, 2004), and plants derived from some of these embryos produced seed having EPA levels reaching up to almost 20%. Surprisingly, transgenic embryos also contained up to about 4% DPA, the elongation product of EPA, indicating that the *M. alpina* elongase had some activity on C20 fatty acids when expressed in plants.

A very comprehensive study by Abbadi *et al.* (2004) compared four constructs containing various combinations of Δ6-desaturases from *P. patens*, borage and the diatom *Phaeodactylum tricornutum*, Δ6-elongases from *P. patens* and *C. elegans*, and Δ5-desaturases from *M. alpina* and *P. tricornutum*, under the control of various combinations of different seed-specific promoters. These constructs were used to transform both the high LA plant tobacco and the high ALA plant flax. Only constructs carrying the *P. tricornutum* Δ5- and Δ6-desaturases and the *P. patens* elongase were successful when expressed in plants, and a construct with each of these genes under the control of the USP promoter from *Vicia faba* was used for further testing. Seed-specific expression of these genes in transgenic plants led to the high accumulation of Δ6-desaturated fatty acids (~30%), but elongation appeared to be relatively poor, as C20 PUFAs totaled only around 5%. However, the Δ5-desaturase appeared to be quite efficient, as up to 2% ARA was produced in tobacco, while up to 1.5% ARA and 1% EPA accumulated in flax. The "bottleneck" at the Δ6-elongation step was attributed to inefficient movement between lipid pools; after desaturation on phosphatidylcholine the desaturated products were postulated to be immediately channeled to triacylglycerols, thereby limiting acyl-CoA dependent elongation.

Once the synthesis of EPA had been achieved, the activities of both a Δ5-elongase and a Δ4-desaturase were required to produce DHA. Until relatively recently, it was not certain that the Δ4-desaturation step outlined in *Figure 1* occurred in nature; in mammals at least, it appears that DHA is synthesized from EPA through the Sprecher pathway, involving two elongations and a ß-oxidation step (reviewed in Qiu, 2003). However, the identification of a Δ4-desaturase from *Thraustochytrium* sp. (Qiu *et al.*, 2001) indicated that at least in some lower eukaryotes, EPA is elongated to ω3 DPA, which is then desaturated to produce DHA, as outlined in *Figure 1*. When expressed in yeast, this Δ4-desaturase was capable of acting on both the ω3 fatty acid DPA and the ω6 fatty acid adrenic acid (ADA; 22:4Δ7,10,13,16). The ability of this gene to function in plants was demonstrated by expressing it in *B. juncea* plants and supplying transgenic plants with exogenous DPA, which led to the production of 3-6% DHA in leaf tissue.

Genes encoding enzymes capable of elongating EPA were first identified in

mammals; these elongases are capable of elongating both C18 and C20 PUFAs (Leonard *et al.*, 2000). However, such mammalian genes are less than optimal for DHA production in transgenic plants, both because of their lack of substrate specificity, and because of potential problems with public acceptance. Meyer *et al.* (2004) describe the isolation and characterization of elongases acting on EPA from a number of species including the fish *Oncorhynchus mykiss*, the frog *Xenopus laevis*, the sea squirt *Ciona intestinalis* and the algae *Ostreococcus tauri* and *Thalassiosira pseudonana*; elongase sequences were identified by searching genomic and EST databases and candidates were tested by expressing open reading frames in yeast. While elongases from *O. mykiss*, *X. laevis* and *C. intestinalis* resembled mammalian elongases in that they were capable of acting on both C18 and C20 fatty acids, the algae species each contained two types of elongase, one type specific for C18 PUFAs and showing highest activity with Δ6-desaturated fatty acids (GLA and SDA) and the other type specific for C20 PUFAs and showing highest activity with EPA. Similarly, Pereira *et al.* (2004) isolated an elongase from the alga *Pavlova* that was capable of elongating both EPA and ARA, but was not active on C18 or C22 fatty acids.

Several studies describing attempts to synthesize DHA in plants have been published recently. In the patent application of Kinney *et al.* (2004), transgenic soybean somatic embryos accumulating up to 3.3% of total fatty acid as DHA were produced through the expression of Δ5- and Δ6-desaturases and a Δ6-elongase from *M. alpina*, plus a *Pavlova* sp. Δ5-elongase, a *Schizochytrium aggregatum* Δ4-desaturase and a *S. diclina* Δ17-desaturase. However, the combined levels of EPA, DPA and DHA in these embryos appeared to be considerably lower than the nearly 20% EPA achieved in soybean seeds in the same study (Kinney *et al.*, 2004).

Wu *et al.* (2005) investigated the synthesis of DHA in transgenic *B. juncea* plants via the stepwise addition of one or a few genes from the VLCPUFA pathway to transformation constructs, allowing individual steps of the pathway to be observed. The first construct contained only the minimal set of genes required to produce ARA and EPA, namely a Δ5-desaturase from *Thraustochytrium* sp., a Δ6-desaturase from *P. irregulare*, and a Δ6-elongase from *P. patens*. High levels of Δ6-desaturated fatty acids, particularly GLA, were produced, while the Δ6-elongase functioned less efficiently, as indicated by relatively low levels of C20 PUFAs. However, the conversion efficiency of the Δ5-desaturase was very high (94.2%), resulting in the production of 7.3% ARA and 0.8% EPA. The addition of a *Calendula officinalis* Δ12-desaturase to increase the amount of the initial substrate, LA, led to substantial increases in both ARA, which reached as high as 17.7%, and EPA, which averaged 1.3%. Addition of a second elongase from *Thraustochytrium* sp. led to small but significant increases in both EPA and ARA, while adding a *Phytophtora infestans* ω3/Δ17-desaturase led to further substantial increases in EPA, which reached an average of 8.1%, with a concurrent drop in ARA. Finally, an *O. mykiss* Δ6/Δ5-elongase plus both a Δ4-desaturase and a putative lysophophatidic acid acyl transferase from *Thraustochytrium* sp. were added, leading to the production of EPA levels up to 15% and DHA levels up to 1.5% in seeds. Importantly, the VLCPUFAs in transgenic *B. juncea* seeds were almost exclusively present as triacylglycerols, and seed appeared morphologically normal and showed no obvious germination problems. It was also noted that elongation steps tended to be much less efficient than desaturation steps and elongation of EPA to DPA appeared to be particularly inefficient. In this study,

the same strong seed specific promoter was used in front of each gene, which would be expected to result in a high and uniform expression of all genes. However, much like what was observed by Chen *et al.* (2005) levels of fatty acids dropped in subsequent generations (Qiu *et al.*, unpublished data), possibly indicating silencing due to the presence of many copies of the promoter sequence.

Robert *et al.* (2005) attempted to avoid the "elongation bottleneck" by the use of a Δ5/Δ6 desaturase from the fish *Danio rerio* that is thought to act on acyl-CoA substrates, as are the elongases. By combining this desaturase with the *C. elegans* Δ6-elongase, they were able to obtain ARA levels of 0.2-1.4% and EPA levels of 0.4-2.3% in transgenic seed of the model plant *Arabidopsis*. Plants carrying the EPA construct were transformed with a second construct carrying a Δ4-desaturase and Δ5-elongase from *Pavlova salina*, and up to 0.5% DHA was detected in seed of T1 plants. The authors suggest that the use of the acyl-CoA dependant desaturase did in fact lead to a more efficient synthesis of C20 PUFA than the acyl-PC pathway. However, although 67% of SDA was elongated, only 17% of EPA was converted to DPA, suggesting that the availability of an acyl-CoA linked substrate alone will not overcome problems with elongation efficiencies.

Alternative pathway

In some organisms, such as the soil amoeba *Acanthamoeba* and euglenoid species, ARA or EPA are produced by an alternative pathway, where LA or ALA are first elongated by a C18-Δ9 elongase, producing eicosadienoic acid (EDA, C20:2Δ11,14) or eicosatrienoic acid (ETrA, C20:3Δ11,14,17). These C20 products then undergo sequential desaturation by Δ8- and Δ5-desaturases, yielding ARA and EPA (*Figure 1*). This pathway may also occur in certain tissues in mammals, although this is still the subject of some debate (Chen *et al.*, 2000). The first Δ8-desaturase was isolated by Wallis and Browse (1999) from the species *Euglena gracilis*, and showed high activity with EDA and ETrA, as well as a lower level of activity with 20:1Δ11. More recently, a Δ8-desaturase has been isolated from *Acanthamoeba castellanii* (Sayanova *et al.*, 2006a), and while it remains unclear whether the alternative pathway occurs in mammalian cells, the identification of the *A. castellanii* gene indicates this pathway does occur in non-photosynthetic organisms.

The first C18-Δ9 PUFA-specific elongase encoding cDNA was isolated from the DHA producing microalga *Isochrysis galbana* (Qi *et al.*, 2002). When this cDNA was expressed in yeast fed LA or ALA, the conversion to EDA or ETrA was approximately 45%; conversely, GLA was not elongated, nor was 18:1Δ9. Expression of this gene in *Arabidopsis* under the control of a constitutive promoter resulted in accumulation of EDA and ETrA in all tissues examined (Fraser *et al.*, 2004).

By expressing a C18-Δ9 elongase from *I. galbana*, a Δ8-desaturase from *E. gracilis*, and a Δ5-desaturase from *M. alpina* under the control of constitutive promoters, Qi *et al.* (2004) were able to produce *Arabidopsis* plants with 6.6 mol% of total leaf fatty acid as ARA and 3.0 mol% as EPA. These levels are considerably higher than levels obtained in seeds of tobacco or flax using the conventional pathway (Abbadi *et al.*, 2004). Qi *et al.* (2004) suggest that the reversed order of the elongation and desaturation steps in the alternative pathway may circumvent to some degree the problem with movement of substrate from the site of desaturation to the acyl-CoA

pool, since *Arabidopsis* leaves appear to contain abundant cytoplasmic pools of LA-CoA and ALA-CoA. However, Sayanova *et al.* (2006a) noted that transgenic plants carrying a Δ9-elongase accumulated substantial elongated C20 fatty acids in the acyl-CoA pool whether or not a Δ8-desaturase was present, suggesting that movement of these fatty acids from the acyl-CoA pool to phospholipids represented a bottleneck in this system, although the desaturation of C20 fatty acids appeared to be quite efficient.

When the alternative pathway was reconstituted in plants (Qi *et al.*, 2004) the undesirable side products 20:3Δ5,11,14 and 20:4Δ5,11,14,17 accumulated to significant levels, likely via Δ5-desaturation of EDA and ETrA. These side-products are not found in *I. galbana* (Qi *et al.*, 2002) or *E. gracilis* (Korn, 1964) suggesting that the timing of expression of the Δ8- and Δ5-desaturases may be strictly regulated in these species, or that the Δ5-desaturases from these organisms may have different substrate specificities and do not modify EDA or ETrA. Furthermore, fatty acid synthesis in leaves is very different from that in seeds, and to date, a high level of VLCPUFA production in seeds has not been demonstrated using the alternative pathway.

Polyketide synthase-like pathway

The first indication of the existence of a novel pathway for very long chain PUFA biosynthesis in marine bacteria was attained when the transfer of a 38 kb genomic fragment isolated from the EPA-producing marine bacteria *Shewanella* to *E. coli* or *Synechococcus* resulted in the production of EPA (Yazawa, 1996; Takeyama *et al.* 1997). This genomic fragment contained eight open reading frames (ORFs), three of which had homology to genes related to *de novo* fatty acid synthesis or to chain elongation, and it was speculated that the remaining five ORFs included desaturase genes. Related genes were also found in eukaryotic marine microorganisms such as the DHA producing organism *Schizochytrium*, and it was observed that certain domains in the PUFA synthesizing ORFs appeared to be more closely related to polyketide synthases (PKS) rather than to desaturases or elongases (Metz *et al.*, 2001). Although the exact sequence of reactions in the PKS-like pathway has not been determined, a primer molecule is thought to go through a number of reiterative rounds of reactions where condensation is followed by complete or partial cycles of ketoreduction, dehydration, and enoyl reduction, with double bonds possibly being inserted through the activity of a bifunctional dehydratase/isomerase (reviewed in Wallis *et al.*, 2002). The fatty acyl chain, which is esterified to an acyl carrier protein, is thus elongated by two carbons per round. This pathway is envisioned to have several potential advantages over the Δ6-desaturation/elongation pathway or the alternative pathway for the production of VLCPUFAs in plants. Only five ORFs from *Shewanella* appear to be necessary and sufficient for EPA production (Orikasa *et al.*, 2004), while the *Schizochytrium* DHA PUFA synthase consists of 3 subunits. In both *E. coli* and *Synechococcus*, expression of the EPA synthesis cluster led to the production of EPA with few other differences in fatty acid composition observed (Takeyama, 1997). The ability to produce high levels of EPA or DPA in plants with low levels of intermediates or byproducts would provide important advantages for commercialization. However, successful expression of PKS-like PUFA genes in plants has yet to be reported. This pathway differs considerably from endogenous plant

pathways involving endoplasmic reticulum localized fatty acyl chain elongation or desaturation, and in some respects appears to more closely resemble the plastid localized FAS system, suggesting that control of genes and achievement of a high level of enzyme activity in plants may prove somewhat challenging.

Challenges and future directions

The "proof of concept" for the production of PUFAs in plants via transgenic engineering has now been clearly demonstrated, therefore attaining commercially viable levels of these fatty acids, particularly very long chain fatty acids such as DHA and EPA, is the next major goal. Reduction or elimination of unwanted side products or intermediates will also be an important step. Several routes toward achieving these goals are presently being investigated.

Early attempts to isolate PUFA biosynthesis genes were complicated because of a lack of sequence information, and often involved gain-of-function screening methods (Reddy *et al.*, 1993; Parker-Barnes *et al.*, 2000). With the wide range of sequences now available for PUFA synthesis genes and the large amount of data available from genomic or EST sequencing projects, isolating PUFA genes has become considerably simpler, and often involves database searches (Meyer *et al.*, 2004; Domergue *et al.*, 2005a) or the design of degenerate oligonucleotide primers based on known sequences (Sayanova *et al.*, 1997; Qiu *et al.*, 2001). Identifying new desaturase or elongase genes still has a potentially major role in increasing the efficiency of PUFA production; even a small difference in efficiency at one step of the pathway, particularly at an early step, can translate into significant increases in the amount of final product. This, however, assumes that the conversion efficiency for a particular enzyme will remain the same with higher levels of substrate, which might not always be the case. For example, while the *Isochrysis* Δ9 elongase showed similar conversion levels with LA and ALA in yeast (Qi *et al.*, 2002), in *Arabidopsis* a higher proportion of LA was elongated (Qi *et al.*, 2004), perhaps because a lower level of LA than ALA was present in *Arabidopsis* leaf tissue.

With the availability of larger numbers of genes, and consequently exponentially greater numbers of gene combinations, an efficient method of evaluating constructs becomes critical. While testing genes in yeast gives a good indication of the activity of an enzyme with a particular substrate, it does not always perfectly mirror the results in plants. This was demonstrated by the elongation of EPA in plants by both the *M. alpina* (Kinney *et al.*, 2004) and the *C. elegans* (Robert *et al.*, 2005) Δ6-elongases, although such activity was not detected in yeast (Parker-Barnes *et al.*, 2000; Beaudoin *et al.*, 2000). The availability of a rapid, species-specific method to test constructs, such as the soybean somatic embryo system (Kinney *et al.*, 2004) is therefore very advantageous, particularly for testing sets of promoter/gene combinations. However, the final test of a construct remains the production of mature transgenic plants with morphologically normal seeds having high levels of the desired fatty acid.

Higher levels of VLCPUFA synthesis in plants might be achieved through increasing the efficiency of flux between the phospholipid and acyl-CoA pools (Abbadi *et al.*, 2004; Domergue *et al.*, 2005b). Renz *et al.* (2004) have cloned putative acyl-CoA:lyso-phosphatidylcholine acyltransferase genes from several species, in the hope that such

genes could improve Δ6-elongation efficiency (Domergue *et al.*, 2005b). The validity of this approach has yet to be demonstrated in plants; although Wu *et al.* (2005) included a *Thraustochytrium sp.* lysophosphatidic acid acyltransferase in plant constructs, it was not clear what, if any, improvement to the system this gene conditioned. Methods of allowing desaturation and elongation to occur in the same pool have also been investigated. While experiments with algae, worms, fungi and lower plants suggest that Δ6-desatuation occurs mainly on phosphatidylcholine (PC) in these organisms (Domergue *et al.*, 2003), Domergue *et al.* (2005a) found that the Δ6-desaturase from the microalga *O. tauri* appears to be an acyl-CoA desaturase. At least in yeast, this resulted in high efficiency of elongation by the *P. pat*ens Δ6-elongase, since nearly all the desaturated products were elongated. However, it remains to be seen how well this gene will function in plants.

It is possible to increase the total level of end product and at the same time reduce undesirable intermediates by forcing fatty acids toward one branch of the pathway, i.e. convert ω6-fatty acids to ω3-fatty acids, or vice versa. This has been done by including ω3/Δ15- and ω3/Δ17-desaturases in transformation constructs (Wu *et al.*, 2005; Kinney *et al.*, 2004; Eckert *et al.*, 2006), but it may also be useful to identify enzymes that are specific for one branch of the pathway, particularly enzymes that are involved in early steps of the pathway, such as the Δ6-desaturase. Most identified Δ6-desaturases can work on both ω3- and ω6-fatty acids, and activity depends more on substrate availability than specificity. However, certain *Primula* species accumulate mostly ω3 or ω6 fatty acids, and Δ6-desaturases from individual species, while not showing strict substrate specificity, show preference for one or the other type of fatty acid (Sayanova *et al.*, 2006b). In the future, enzymes showing greater specificity might be obtained either through the identification of new genes or via protein engineering.

Finally, several fungal bifunctional Δ12/ω3 fatty acid desaturases have recently been characterized, and show promise for increasing the level of ω3 substrate in high LA plants (Damude *et al.*, 2006). Expression of the Δ12/ω3 desaturase from *Fusarium moniliforme* in soybean somatic embryos resulted in ALA levels of up to 72.4% (Damude *et al.*, 2006), which is considerably higher than the 53% ALA obtained using an *Arabidopsis* ω3/Δ15 desaturase (Eckert *et al.*, 2006). This is reminiscent of the better results obtained in *B. juncea* plants with a fungal Δ6-desaturase (Hong *et al.*, 2002) than with the borage Δ6-desaturase (Qiu *et al.*, 2002), suggesting a reduced level of regulation of fungal enzymes in plant hosts may be in effect.

Conclusions

The increasing realization of the important health benefits of long-chain polyunsaturated fatty acids, combined with uncertainty regarding the sustainability of current sources of these fatty acids, has led to great interest in the engineering of VLCPUFA-producing transgenic plants. However, the biosynthetic pathway leading to production of these fatty acids is complex, and involves the coordinated regulation of a large number of genes. Reconstruction of a number of pathways, including the "conventional" Δ6-desaturase/Δ6-elongase pathway, the "alternative" Δ9-elongase/Δ8-desaturase pathway, and the anaerobic PKS-like pathway, is being pursued in plants. The fundamental genes involved in each step of all three pathways have now

been isolated. To date, *in planta* results from the conventional pathway are the most promising, but this could change quickly. Transgenic plants producing economically significant levels of polyunsaturated C18 fatty acid have already been produced, and the production of DHA in seeds of crop plants, albeit at low levels, has been demonstrated. While there are still challenges to be met, particularly in producing high levels of the important ω3-polyunsaturated fatty acid DHA, it now appears clear that transgenic VLCPUFA crops will be available in the foreseeable future.

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