# Oilseed Crops with Fish Oil-like Levels ω3 LC-PUFA

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Omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (ω3 LC-PUFA) play a critical physiological role in health and are nutritionally important for both humans and animals. The abundance of marine-derived resources of the health-benefitting ω3 LC-PUFA is either static or in some cases declining. Alternative source of ω3 LC-PUFA is required to meet the increasing demand. Oilseed crops containing fish oil-levels of ω3 LC-PUFA and importantly also containing a high ω3/ω6 ratio have been developed.

Keywords: DHA ; DPA ; EPA ; ω3 LC-PUFA ; oilseed crops

## 1. Introduction

Omega-3 long-chain polyunsaturated fatty acids (ω3 LC-PUFA, defined as containing 20 or more carbon atoms), including eicosapentaenoic acid (EPA, 20:5ω3), docosapentaenoic acid (DPA, 22:5ω3), and docosahexaenoic acid (DHA, 22:6ω3), are beneficial to human health throughout the whole lifespan [1]. They are essential components of cell membranes important for cell function as well as precursors for biologically active signalling molecules in mammals.

DHA is one of the most important ω3 LC-PUFA. Sub-optimal levels of DHA in the human body are associated with an increased risk of several diseases [2]. Ghasemi Fard et al. [3] provided a comprehensive collection of evidence and a critical summary of the documented physiological effects of high DHA fish oils on human health. The positive effects of EPA and DHA have been reported across a range of degenerative and inflammatory disorders such as heart disease, stroke, rheumatoid arthritis, asthma and some cancers, diabetes mellitus, multiple sclerosis, dementia, and clinical depression [2][4][5]. EPA- and in particular DHA-rich oils are also important in infant nutrition, with DHA present in high concentrations in the brain and retina, and these two key LC-PUFA are important in the development, health, and enhanced functioning of these and other organs [6][7][8].

Similar to DHA and EPA, ω3 DPA is gaining increasing recognition and importance because of its unique properties. ω3 DPA is the precursor of many lipid mediators involved in the pro-resolution of inflammation with specific effects compared to other ω3 LC-PUFA [9]. The presence of ω3 LC-PUFA in human tissues and its relative abundance in human milk have long served as clues to its importance in human health. It is increasingly recognized as an important part of our diet. Numerous trials have demonstrated a clear link between ω3 DPA intake and better health, while multiple in vitro and in vivo studies have shown direct effects of ω3 DPA on inflammation, improved plasma lipid profile, and cognitive function [10][11]. Morin et al. [12] reported that ω3 LC-PUFA monoacylglycerides (MAG) were found to be better absorbed in cultured human colorectal cancer cells compared to the corresponding free fatty acids. Furthermore, that study demonstrated that ω3 DPA-MAG had increased anti-proliferative and pro-apoptotic effects, decreased cell proliferation and induced apoptosis, when compared to DHA-MAG and EPA-MAG. Recently, Ghasemi Fard et al. [13] summarised the physiological effect, delivery, fatty acid metabolism, and bioavailability of ω3 DPA.

ω3 LC-PUFA are also essential for fish development [14]. They are nutritionally important for the survival, growth, and general health of aquaculture species, particularly at the larval stage. Reduced accumulation of ω3 LC-PUFA in farmed fish also decreases the nutritional value of the final product [15][16].

The current principal sources of ω3 LC-PUFA for human consumption are wild-caught marine fish species, krill, and some algae. The increasing demand for these fatty acids has contributed in some regions to overfishing of many source species, generating a huge negative environmental impact [17]. In addition, global warming leading to an increase in water temperature, depending on the climate scenario and location, could result in a 10 to 58% loss of globally available DHA by 2100 [18]. The ω3 LC-PUFA in these fish species are accumulated up the food web, primarily originating from microalgae. While aquaculture is an alternative way to replace the wild fish stocks for human consumption of ω3 LC-PUFA, farmed fish need sustainable sources of ω3 LC-PUFA in their diet for their development and growth. This requirement constrains

the impact that aquaculture per se can have on mitigating the decline in wild fish stocks, including in some cases due to unsustainable harvesting of wild fisheries.

Fermentation of microalgae containing ω3 LC-PUFA has also been seen as a potential solution in this area. However, growing microalgae heterotrophically has its own challenges, including energy consumption, the high capital investment required for large-scale fermentation facilities, reproducibility and consistency of production, efficiency of cell breaking, high production cost, and other factors.

## 2. Development of Oilseed Crops with Fish Oil-like Levels of ω3 LC-PUFA

The above challenges have led to the exploration of alternative and sustainable approaches. Metabolic engineering of land-based oilseed crops to produce fish oil levels of ω3 LC-PUFA is one of the most striking and ambitious examples of such a strategy [19]. High oil yield and relatively low production costs of oilseed crops can provide an economic and sustainable production platform for oil containing ω3 LC-PUFA. For example, canola (*Brassica napus* L.) picks itself as a potential oil platform for EPA and DHA production. Canola seed yields have been reported up to 4 T/ha with 40–45% seed oil content. It has broad agronomic and geographic adaptation, considerable genetic resources, and substantially developed germplasms. These aspects make canola the second largest oilseed crop (behind soybean), producing 84.8 million metric tons (MMT) globally in 2022/23 [20] and representing an ideal vehicle for producing ω3 LC-PUFA.

LC-PUFA can be synthesised by two distinct pathways: the aerobic pathway utilizing fatty acid desaturases and elongases, and the anaerobic polyketide synthase (PKS) pathway [21]. The aerobic pathway uses sequential oxygen-dependent desaturation and elongation steps coupled with electron flow. The same set of desaturases and elongases can synthesise ω6 DPA or ω3 DHA from the ω6 substrate linoleic acid (LA, 18:2ω6) or the ω3 substrate α-linolenic acid (ALA, 18:3ω3), respectively. The PKS pathway synthesises LC-PUFA directly from malonyl-CoA and acetyl-CoA without the need for oxygen for desaturation [22][23]. Such a complex pathway makes it challenging to engineer for achieving the desired high levels of specific end products. In the last two decades, these two distinct pathways have been introduced into oilseeds to produce ω3 LC-PUFA including EPA, ω3 DPA, and DHA [24][25][26]. The introduced aerobic pathway required genes for the five desaturation and/or elongation steps from ALA to DHA, while the introduced PKS pathway contained several genes for multiple domains. Most recently, production of ω3 docosatrienoic acid (DTA, 22:3ω3) in *B. carinata* was achieved by introducing a minimal single elongase from the plant *Eranthis hyemalis* that can elongate a wide range of PUFA, thus converting plant endogenous ALA to ω3 eicosatrienoic acid (ETA, 20:3ω3) then further converting ETA to DTA [27]. Generally, the production of ω3 LC-PUFA with the PKS pathway has resulted in only low levels of products [24]; however, efforts with the aerobic pathway have produced ω3 LC-PUFA at the same levels as found in wild fish oils [19][28].

Since the earlier demonstration of successful metabolic engineering of EPA or DHA production at low levels in yeast or seed oils [22][29][30][31], development of commercially sustainable oilseed crops with fish oil-like levels of ω3 LC-PUFA has been one of the main targets by a range of researchers and companies in the last two decades and longer in some cases. Previous reviews of the research and development on oilseed sources of ω3 LC-PUFA are available in [19][32][33][34][35].

Several of the research efforts have ceased, and/or not achieved what were seen as very difficult aims. The collaboration between CSIRO, the Grains Research and Development Corporation (GRDC), and Nuseed has continued and has successfully developed a genetically engineered canola, event NS-B5ØØ27-4, that produces ω3 LC-PUFA containing oil with levels of 9.7% DHA, 1% DPA, and 0.5% EPA [26]. An ongoing breeding program aims to further increase ω3 LC-PUFA levels in the oil. The project was initiated by CSIRO researchers in 1997, although it took several years to build momentum. The research team comprised: marine microalgae researchers, plant geneticists, plant breeders, marine oil chemists, food technologists, and other specialists. One key aspect was that the CSIRO research team accessed a unique selection of microalgae from the CSIRO-based Australian National Algae Culture Collection (http://www.csiro.au/ANACC (accessed on 1 June 2023)). The algae collection had been established at CSIRO for strategic research on algal chlorophyll and carotenoid pigments as applied in biological oceanographic research.

Another collaboration between BASF and Cargill has also generated a transgenic canola, event LBFLFK, that produces 0.3% DHA, 2% DPA, and 4% EPA in refined, bleached, and deodorized oil [36]. Key desaturase and elongase enzymes identified, validated (in yeast and plant models), and developed and used in the CSIRO-Nuseed project are listed in **Table 1**.

**Table 1.** DHA biosynthesis enzymes. In the isolation of an efficient synthesis pathway, key desaturase and elongase enzymes were isolated from strains held in the CSIRO-based Australian National Algae Culture Collection

(http://www.csiro.au/ANACC (accessed on 1 June 2023)).

| Enzyme | Conversion | Comment | References |
|---|---|---|---|
| ***Micromonas persilla* Δ6-desaturase** | **18:3ω3 to 18:4ω3 (ALA to SDA)** | **Use of a marine microalgae Δ6-desaturase with a higher preference for ω3 substrate than ω6 substrate** | [37] |
| ***Pyraminomas cordata* Δ6-elongase** | **18:4ω3 to 20:4ω3 (SDA to ETA)** | **High conversion efficiency of SDA to ETA via Δ6-elongase** | [38] |
| ***Pavlova salina* Δ5-desaturase** | **20:4ω3 to 20:5ω3 (ETA to EPA)** | **Demonstrated the acyl-CoA desaturation ability** | [38][39] |
| ***Pyraminomas cordata* Δ5-elongase** | **20:5ω3 to 22:5ω3 (EPA to ω3 DPA)** | **Highly efficient Δ5-elongase targeted to maximise the elongation from EPA to ω3 DPA** | [38] |
| ***Pavlova salina* Δ4-desaturase** | **22:5ω3 to 22:6ω3 (ω3 DPA to DHA)** | **Demonstrated the acyl-CoA desaturation ability** | [39] |
| **ω3 desaturases from various sources** | **conversion of ω6 PUFA and ω6 LC-PUFA to ω3 PUFA and ω3 LC-PUFA** | **Results in very low amounts of ω6 fatty acids and contributed to the high ω3/ω6 ratio** | [40] |

Multiple attempts have been made to achieve the fish oil-like levels of ω3 LC-PUFA for commercialisation. The first consideration was to enhance the fatty acid flux from oleic acid (OA, 18:1ω9) to ω3 ALA by introducing a yeast Δ12-desaturase and ω3-desaturase, in addition to the endogenous Δ12-desaturase and Δ15-desaturase. ω3-Desaturases can convert a range of ω6 fatty acids including LA, γ-linolenic acid (GLA, 18:3ω6), dihomo-γ-linolenic acid (DGLA, 20:3ω6), arachidonic acid (ARA, 20:4ω6), docosatetraenoic acid (DTA, 22:4ω6), and docosapentaenoic acid (ω6 DPA, 22:5ω6) into the corresponding ω3 fatty acids with different substrate preferences [40]. The introduced ω3-desaturase maximally converts ω6 LA to ω3 ALA, thus making higher levels of ω3 substrate available for the biosynthesis pathway. The remaining low amount of LA is used for synthesis of the downstream ω6 LC-PUFA, which can also be converted to their ω3 counterparts by ω3-desaturase. This resulted in very low amounts of ω6 fatty acids and contributed to the high ω3/ω6 ratio [26] that is desired for both human and fish health.

The second consideration was to use a marine microalgae *Micromonas pusilla* Δ6-desaturase with a higher preference for the ω3 substrate than the ω6 substrate [37]. The combined effect of the enhanced fatty acid flux from OA to ALA, and the ω3 substrate preference of the Δ6-desaturase, led to the elevated production of ω3 fatty acids at the early steps of the biosynthetic pathway.

The third consideration was to use acyl CoA desaturases for subsequent steps in the pathway rather than phosphatidylcholine (PC) type desaturases to avoid excessive acyl shuffling between acyl-CoA and acyl-PC pools, as the fatty acid elongation occurs in acyl-CoA pools. Phylogenetic analysis of amino acid sequences showed that the *M. pusilla* Δ6-desaturase, *Pavlova salina* Δ5- and Δ4-desaturases used in the ω3 LC-PUFA containing canola event, NS-B5ØØ27-4, clustered with other demonstrated acyl-CoA desaturases. *M. pusilla* Δ6-desaturase has been demonstrated to have acyl-CoA desaturation ability [37]. *P. lutheri* Δ4-desaturase, from a very closed related species to *P. salina*, has also been shown to desaturate acyl-CoA substrates [41].

The fourth consideration was to use a highly efficient Δ5-elongase from the microalga *Pyramimonas cordata* to maximise the elongation from EPA to ω3 DPA. Earlier proof of concept work had expressed the DHA biosynthetic pathway containing *P. salina* Δ5-elongase in *Arabidopsis* and successfully produced DHA in seed oil, but only at low levels (<1%). The conversion rate of the Δ5-elongation step was the major bottleneck, with the efficiency lower than 20% [30]. The *P. cordata* Δ5-elongase showed much higher efficiency for elongating EPA to ω3 DPA in yeast cells [38] than the *P. salina* Δ5-elongase [42]. The superior conversion efficiency of *P. cordata* Δ5-elongase was confirmed to be as high as 90% in *Arabidopsis* seeds [43].

In addition, the large T-DNA vector consisting of seven genes in the DHA biosynthetic pathway plus a selection marker had been carefully designed with multiple seed-specific promoters. The promoter expression timing was an important factor to reduce accumulation of intermediate fatty acids. The direction of gene expression cassettes and the inclusion of non-coding spacers between cassettes was another consideration designed to maximise the gene expression levels. Finally, thousands of lines were created having stable inserts with relatively low copies of T-DNA. The selected elite canola event, NS-B5ØØ27-4, contains a multi-copy of the full transgene construct at one locus plus an extra partial T-DNA insertion at another locus effectively acting as a ‘booster’ for the full pathway with increased gene dosage.

In the case of canola event LBFLFK, gene dosage with multiple copies of alternate genes for the same enzymatic activity was a contributing factor [19]. The transgene cassette had a total of 12 genes for ω3 LC-PUFA biosynthesis, with two copies inserted in the canola chromosome. Increased gene dosage has also been applied in engineering EPA production in the yeast *Yarrowia lipolytica* by integrating five to seven copies of each desaturase or elongase gene, with a total number of 24 desaturase/elongase genes for maximised EPA accumulation [44]. These approaches collectively provide successful strategies for metabolic engineering that utilise complex multiple gene pathways.

In addition to canola, other oilseed crops such as *Camelina sativa* have been engineered for the production of ω3 LC-PUFA oil using a similar approach. A transgene cassette expressing five genes or seven genes for ω3 LC-PUFA biosynthesis from OA in *C. sativa* resulted in 24% EPA or 11% EPA and 8% DHA in seed oil, respectively [45]. Petrie et al. [46] describe the production of fish oil-like levels (>12%) of DHA in *C. sativa* seed oil and achieving a high ω3/ω6 ratio. The same T-DNA vector for producing fish oil-like levels of DHA in canola [26] was used to engineer DHA production in *B. juncea*, with up to 17% DHA produced in the $T_4$ seed oil of some *B. juncea* lines. Interestingly, some lines with a truncation of the T-DNA insert that eliminated the Δ4-desaturase activity stably accumulated 12% of ω3 DPA [47]. This was the first example of land plant-based oil seed ω3 DPA production, and was 2–3 times higher than most other natural sources. An exception for the occurrence of ω3 DPA is the abalone, a group of small to very large marine gastropod molluscs in the family Haliotidae, which can contain elevated ω3 DPA, e.g., 13–14% [48]. The distribution of abalone globally is restricted to a limited number of countries, with only a comparatively small harvest available. Abalone would not be able to serve as a sustainable large-scale source of ω3 DPA.

The development of a new and sustainable source of ω3 DPA further demonstrated the capability of engineering a complex metabolic pathway in different oilseed crops. This demonstration also offered a sustainable source of ω3 DPA, which is currently only available from wild oceanic species or seals with limited quantities for commercial use. Studies have also shown that DPA is much more effective at reducing the risk of cardiovascular disease, and that DPA is more effective than EPA at promoting endothelial migration [10]. A 1:1 ratio (*w*/*w*) of DHA to DPA in *B*. *juncea* seed oil has also been achieved [47]. The combination of DHA and DPA may be an excellent future dietary means for promoting cardiovascular health. Other attempts for producing stearidonic acid (SDA, 18:4ω3), a medium chain length ω3 PUFA, were also reported in linseed [49] and soybean [50]; the latter has been deregulated in the USA.

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