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Fatty acids and pharmamolecules

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Introduction

Metabolic engineering of biosynthetic pathways has significant potential for tailoring the chemical composition of our food plants to better match human nutritional requirements, especially as our understanding of the complex interplay between dietary constituents and human nutrition continues to increase. An early example of this was the successful engineering of large increases in β-carotene (provitamin A) in rice to aid in alleviating blindness associated with vitamin A deficiency in many under-developed countries (Ye *et al.* 2000; Paine *et al.* 2005). This so-called 'Golden Rice' product is now approaching commercial introduction, and a similar approach has now been adopted to develop high provitamin A bananas (Paul *et al.* 2017) and other foods. In recent years the genetic understanding of more complex biosynthetic pathways has rapidly increased and, in parallel, our capability for genetic modification has become more and more sophisticated and precise. This has opened up avenues for genetic enhancement of other important plant constituents associated with human health and wellbeing. This chapter provides impressive illustrations of recent complex manipulations of fatty acid and alkaloid biosynthetic pathways as examples of enhancements to both primary and secondary plant metabolites to create new and improved nutritional and pharmaceutical products.

Plant oil composition

Plant oil is composed mostly (>95%) of triacylglycerols (TAGs) that are synthesised and deposited during seed development. TAG molecules consist of three fatty acids esterified to a glycerol backbone and represent one of the most reduced forms of carbons found in nature and hence represent an excellent source of energy. In fact, plant oils (also known as vegetable oils) are an important source of dietary fat for humans, representing ~25% of caloric intake in developed countries (Broun *et al.* 1999). In oilseeds, TAG is stored in oil bodies known as oleosomes that consist of a TAG core surrounded by phospholipid monolayer and an outer layer of oleosin proteins. The plant oils commonly used for human consumption are composed of fatty acids. Plant fatty acids are made up of chains of carbon atoms that may contain no double bonds between the carbon atoms ('saturated' fatty acids) or one or more double bonds in the chain ('unsaturated'). The major plant oil components are the saturated

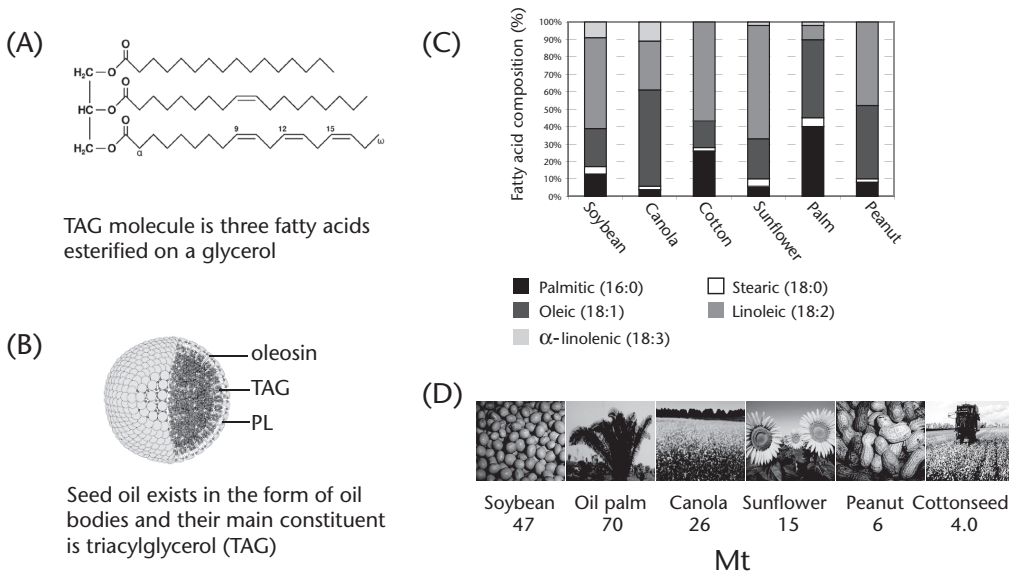

Fig. 7.1. **(A)** Triacylglycerol (TAG) structure. **(B)** Oil body structure (PL = Phospholipid). **(C)** Fatty acid composition of major plant oils. **(D)** Production volumes of six major plant oils (Mt = million tons).

fatty acids, palmitic acid (C16:0) and stearic acid (C18:0), the monounsaturated fatty acid, oleic acid (C18:1) and the polyunsaturated fatty acids (PUFA), linoleic acid (C18:2) and α-linolenic acid (C18:3). World production of plant oils is currently around 180 million tons per year, of which 86% is used for human consumption. Almost all of these oils are obtained from oilseed crops such as soybean, canola, safflower, sunflower, cottonseed and groundnut, or plantation trees such as palm, olive and coconut (Oil World 2017; Fig. 7.1).

Nutritional and functional attributes of plant oils

The nutritional quality of oils depends on the relative content of both saturated, monounsaturated and polyunsaturated fatty acids. The impact of high levels of some saturated fatty acids in the diet, particularly palmitic acid, on increased blood cholesterol and more particularly increased low density lipoprotein (LDL), is well established. Elevated LDL in the blood has been associated with an enhanced risk of cardiovascular disease in humans. Unsaturated fatty acids, both monounsaturated and polyunsaturated, on the other hand generally lower LDL levels (Sacks *et al.* 2017). In addition, *trans*-fatty acids can be generated during the process of hydrogenation. *Trans*-fatty acids are those where the two hydrogen atoms attached to the carbon atoms of a carbon double bond are located on the opposite sides of the bond rather than on the same side as is the case in the normal *cis* configuration; these can also elevate LDL cholesterol in a manner similar to palmitic acid. However, not all saturated fatty acids are associated with elevated cholesterol levels. For example, stearic acid is reported to have neutral effects on blood cholesterol (Woollett and Dietschy 1994). In this respect, the high melting temperature of stearic acid (~70°C) also makes it particularly suitable in solid fat applications. Accordingly, because of its neutral effects on blood cholesterol levels, a high stearic acid-containing oil is a desirable substitute for partially hydrogenated plant oils currently used in margarine production. The number

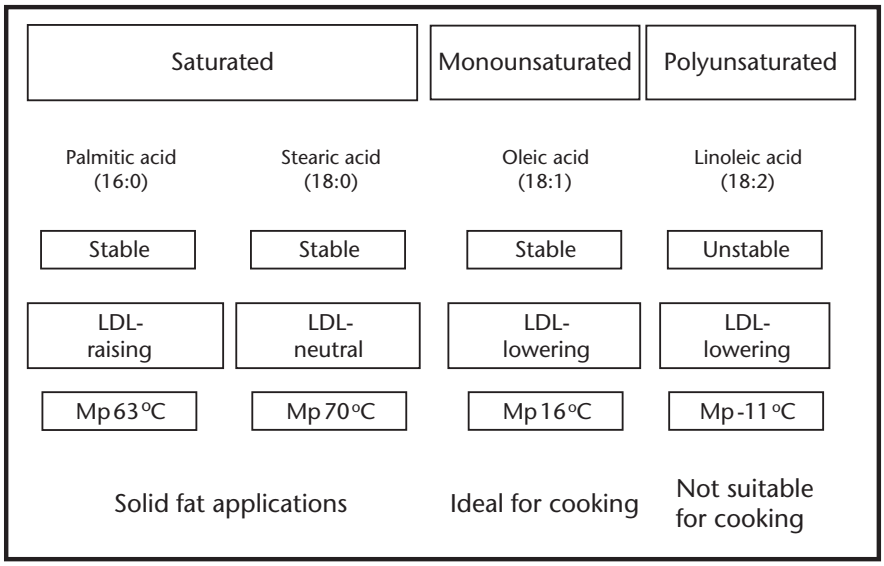

Fig. 7.2. Functional and nutritional attributes of fatty acids in plant oils (LDL = low density lipoprotein; Mp = melting point).

of carbon-carbon double bonds in the fatty acid present in TAG of the oil influences its physical properties such as melting temperature and other chemical properties, as well as its nutritional value, and the applications to which it may be put, particularly in the food industry. For example, the presence of a carbon double bond in a monounsaturated fatty acid or polyunsaturated fatty acid lowers its melting temperature, compared with the melting temperature of a saturated fatty acid of the same carbon chain length, such that the C18 unsaturated fatty acids, oleic acid, linoleic acid and linolenic acid, are all liquid at ambient temperature. Additionally, the susceptibility of a fatty acid to oxidation (rancidity) increases proportionately with the number of carbon double bonds present in the fatty acid molecule, greatly reducing the suitability of oils containing polyunsaturated fatty acids to applications involving the use of prolonged heat in the presence of oxygen, such as cooking and other food service applications, or in non-food applications such as use in the production of cosmetics, pharmaceuticals and candles. For applications that require solid fat components such as in solid cooking fats, shortenings or margarines, it is necessary to have moderately high levels of saturated fatty acids, or the functionally equivalent *trans*-fatty acids (Liu *et al*. 2002a; Fig. 7.2).

Reconfiguring plant oils

Genetic engineering provides a rapid and direct method for manipulating fatty acid composition in oilseeds and other oil-bearing plant tissues. Recent advances in understanding of the biochemical, cellular and molecular mechanisms of plant oil biosynthesis, coupled with the cloning of many of the genes involved in this process, have facilitated the production of designer plant oils with improved nutritional benefits and enhanced functional properties. These novel oils present an exciting opportunity to use genetic engineering to provide sustainable sources of healthier plant oils, as well as specialised industrial oils that

can serve as replacements for fossil oils and assist in mitigating the effects of climate change (Vanhercke *et al.* 2013; Haslam *et al.* 2016). Genetic engineering strategies have been used to create designer plant oils and have mainly focused on: (1) modifying existing plant oils via gene silencing; or (2) creating novel oils via the insertion of new pathways. The next section describes the most significant achievements in genetically modified plant oils using both approaches.

High oleic seed oils: a gene silencing example

Interest in the good health properties of monounsaturated fats has stemmed from the observation that people in Mediterranean countries having high fat diets, made up primarily of high oleic acid, also show a low incidence of coronary heart disease (CHD). This observed phenomenon was supported by research (Mensink and Katan 1992) that showed that diets high in oleic acid can reduce low density lipoprotein cholesterol (LDL) while having no effect on, or may even increase, high density lipoprotein cholesterol (HDL). HDL is protective against CHD and LDL is associated with higher incidence of CHD. Due to their functional, health and sensory attributes, high oleic oils provide a real opportunity to substitute existing products used in high-stability applications. These oils may be used as substitutes for oils with high saturated fatty acid levels such as palm, tallow, dairy and hydrogenated vegetable oils. In addition, oil high in oleic acid also has many industrial uses such as, lubricants (often in the form of fatty acid esters), biofuels, raw materials for fatty alcohols, plasticisers, waxes, metal stearates, emulsifiers, personal care products, soaps and detergents, surfactants, pharmaceuticals, metal working additives, raw material for fabric softeners, inks, transparent soaps, PVC stabiliser, alkyd resins, and intermediates for many other types of downstream oleo chemical derivatives (Vanhercke *et al.* 2013).

Gene technology has been successfully applied to develop a range of plant oils enriched for the desirable oleic acid, principally using gene inactivation approaches such as post transcriptional gene silencing (PTGS) to inactivate fatty acid desaturase and thioesterase genes (Table 7.1). For example, soybean lines with 80% oleic acid in their seed oil were created by co-suppression of the *Fad2* encoded microsomal Δ12-desaturase (Kinney 1996). This reduced the level of Δ12-desaturation and resulted in accumulation of high amounts of oleic acid. Using a similar approach, co-suppression based silencing of the *Fad2* gene was used to raise oleic acid levels in *Brassica napus* and *B. juncea* where oleic acid levels of 89% and 73% were achieved in T1 single seed, respectively (Stoutjesdijk *et al.* 2000). RNAi-mediated gene silencing techniques developed by the CSIRO have also been successfully employed to develop oilseeds with nutritionally improved plant oils. For example, in cottonseed, transgenic expression of a hairpin RNA (hpRNA) gene silencing construct targeted against gh*Fad2*-1 (a seed-specific member of cotton *Fad2* gene family) resulted in the increase of oleic acid from normal levels of 15% up to 77% of total fatty acids in the oil. This increase was mainly at the expense of linoleic acid, which was reduced from normal levels of 60% down to as low as 4%. In addition, further seed-specific targeting of the *FatB* fatty acid thioesterase gene in cotton, resulted in marked reduction of saturated fatty acid palmitic acid: a nutritionally undesirable fatty acid (Liu *et al.* 2002b).

Although these 75–85% oleic acid levels in the genetically improved oils are ideal for their food use, the significant residual levels of polyunsaturates can cause problems in industrial and oleochemical applications. However, the recent development of super-high-oleic safflower (SHO safflower) has now demonstrated the potential to metabolically engineer oilseeds to achieve high oleochemical purity levels. RNAi-mediated gene

Table 7.1. High oleic (HO) plant oils developed through post transcriptional gene silencing (WT = wild type).

Note that oleic acid levels shown for *Brassica napus* and *B. juncea* are T1 single seed data.

Crop	Major fatty acids in seed oil (%)				
	16:0	18:0	18:1	18:2	18:3
WT Soybean	10	3	15	61	9
HO Soybean	6	3	84	1	2
WT *B. napus*	5	4	58	17	10
HO *B. napus*	2	2	89	1	4
WT *B. juncea*	5	2	43	25	13
HO *B. juncea*	2	2	73	5	9
WT Cotton	25	2	15	57	Trace
HO Cotton	15	2	78	4	Trace
WT Safflower	6	3	11	80	0
Super HO Safflower	3	2	93	2	0

silencing targeted against the seed-expressed *FatB* fatty acid thioesterase and *Fad2* desaturase genes has resulted in the development of safflower oil containing up to 94% oleic acid, with only 2% linoleic acid remaining and with no change in fatty acid content elsewhere in the plant and no apparent adverse effects on agronomic performance (Wood *et al*. 2018). This achievement using seed-specific gene silencing contrasts noticeably with previous efforts to raise oleic acid in safflower using mutagenesis approaches that, although successful in reaching levels around 90%, have generally been associated with significantly reduced yield (Skoric *et al*. 2008). The imbalance in fatty acid production was from mutated *Fad2* genes expressed constitutively across the entire plant. Yield reduction was probably caused by reduction in PUFA content in cell membranes in all vegetative tissues (Clemente and Cahoon 2009; Wood *et al*. 2018). The development of safflower with extremely high concentrations of oleic acid only in the seed should open the way for greater industrial application of oleic acid as a chemical feedstock and also provide an additional improvement in stability for direct industrial applications, such as in lubricants and in transformer fluids. Importantly, this achievement now serves as a landmark in demonstrating the potential to metabolically engineer high levels of specific fatty acids in plant oils for oleochemical use. This points a way forward in harnessing the power of genetic engineering and GMOs to mitigate climate change via providing an alternative source for chemicals currently sourced from non-renewable fossil resources. CSIRO has licenced the commercialisation of SHO safflower to an Australian company, Go Resources, and the crop is now entering into the commercialisation phase, with an expected commercial release in 2018.

Long chain ω-3 polyunsaturated plant oils: an insertion of new pathway example

Polyunsaturated fatty acids (PUFA) contain more than one double bond and can be divided into the ω-3 and ω-6 families. The ω-3 PUFA family is marked by the presence of a double bond three carbons away from the CH_3 or ω end while the ω-6 family is

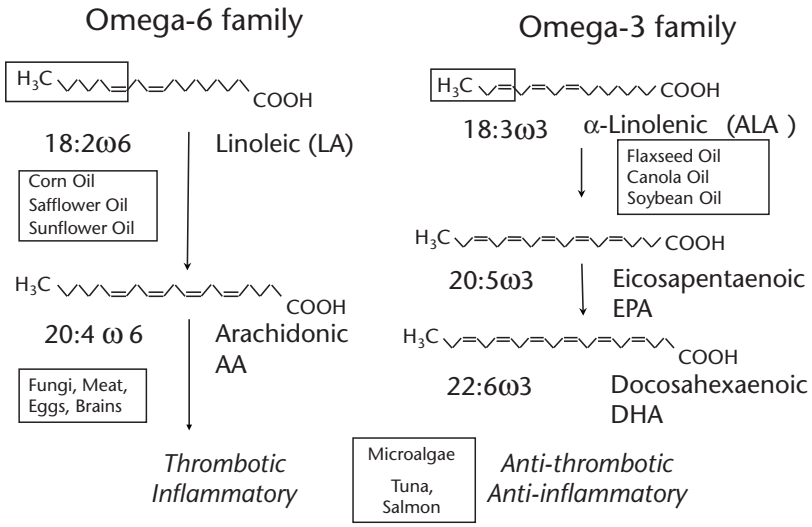

Fig. 7.3. Omega(ω)-3 and ω-6 polyunsaturated fatty acid families. For designating the position of the double bond, methyl end is known as the ω end while the carboxyl end is known as the delta (Δ).

represented by the presence of the first double bond six carbons away from the CH_3 or ω end (Fig. 7.3). Omega-3 PUFAs that are of significance to human physiology and nutrition include the α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA, 22:6) all of which have anti-thrombotic and anti-inflammatory properties. Although physiologically important, ω-6 PUFAs such as linoleic acid (LA, 18:2) and arachidonic acid (AA, 20:4), promote thrombosis and inflammation in humans. ALA and LA are generally described as 'essential fatty acids' because humans and mammals in general are unable to synthesise them *de novo* and therefore must acquire them from the diet. These essential fatty acids can be obtained from plant-based dietary sources such as flaxseed, canola, sunflower and safflower oil. Long chain PUFA (LC-PUFA), defined as containing 20 or more carbon atoms, such as EPA, DHA, AA, are not considered as essential fatty acids, because they can be synthesised from ALA and LA via a series of desaturation and elongation processes. However, it is important to note that this LC-PUFA synthesis from LA and ALA is inefficient. For example, only 7.9% of ALA is converted to EPA, and 3.8% ALA is converted to DHA in humans. Recommended dietary intakes of EPA and DHA can only be achieved through ingestion of marine-derived and algal lipids: in particular oily fish such as salmon and tuna (Plourde and Cunnane 2007; Fig. 7.3). Dietary sources of AA include meat and eggs.

Solutions for meeting the EPA and DHA demand

Omega-3 LC-PUFA, EPA and DHA, have been the focus of a great deal of interest and research. This is due to the many positive benefits to human health associated with adequate dietary intake of these fatty acids. 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 (Galli and Calder 2009;

Giles *et al*. 2013). EPA- and DHA-rich oils are also important in infant nutrition and are present in high concentrations in brain and retina and are important in the development, health and correct functioning of these organs (Simon *et al*. 2011; Ryan *et al*. 2013; Weichselbaum *et al*. 2013). They are also nutritionally important for the survival, growth and general health of aquaculture species particularly at the larval stage. Thus it is not surprising that consumer awareness of the health benefits of EPA and DHA is growing due in part to the consumer engagement efforts of organisations such as the Omega-3 Centre (www.omega-3centre.com) and GOED (www.goedomega3.com).

The main source of dietary EPA and DHA is wild-harvested marine fish whose numbers are generally recognised to be in decline. One widely cited paper describes the amount of large predatory fish in the oceans as being at only 10% of pre-industrial times (Myers and Worm 2003). One of the authors claimed in a later publication that the state of the oceans, climate change and the effect of this on fish stocks were such that 'all commercial fish and seafood may collapse' by 2048 (Worm *et al*. 2006). It is projected by GOED that the increasing demand for EPA and DHA will be difficult to meet from current sources of wild-caught fish. Aquaculture is a growth industry and has the potential to meet the growing demand for these fatty acids. However, it is important to note that fish themselves do not produce EPA and DHA but must, like humans, obtain them through their diet. This means that fish farming requires substantial inputs of EPA and DHA, currently sourced largely from wild-caught fish. It is also noteworthy that microalgae, the major primary producers of EPA and DHA, are also becoming an important direct source of these fatty acids, with microbial EPA and DHA oils now widely available in the market. However, this production tends to supply relatively niche applications such as infant formula and nutraceuticals due to high production costs and is unlikely to scale up adequately for large volume applications such as aquaculture.

Oilseed crops, with their production capacity and relatively low cost, would be an excellent and sustainable source of EPA and DHA oils. For example, calculations can be made to estimate global consumer needs for these oils. Using a daily 500 mg requirement, as is recommended by many national health bodies, a global population reaching 8 billion by 2025, and assuming fish contain 0.2–3.5 g of EPA and DHA per 100 g of oil, it was estimated that the current global fish harvest (93 Mt per annum) will fall well short of meeting this requirement (GOED; www.goedomega3.com). On the other hand, 2.5 million hectares of an oilseed crop (~2% of total world acreage under cultivation to major oilseed crops) containing 10–15% DHA and EPA in its oil could equal all the fish oil currently being used globally. Oilseed crop plants do not naturally synthesise these fatty acids, meaning that a genetic engineering solution would be required before this potential source could be developed. An oilseed crop with EPA and DHA production capability would provide an excellent alternative to marine-sourced oils. Such an EPA- and DHA-rich plant oil could be used in many applications: (1) as an ingredient for aquaculture feeds to provide aquaculture species with EPA and DHA both for their own developmental requirements and to meet consumer demands for EPA and DHA content in fish; (2) as an animal feed to produce EPA- and DHA-enriched meat, eggs and milk; (3) as an ingredient in fortified foods (e.g. bread or milk) when added in an oxidation-protected form; (4) directly, as a nutraceutical supplement similar to the way in which fish oil capsules are currently used. Additionally, an oilseed source of EPA and DHA would be vegetarian and thus could satisfy a requirement for an important sector of the global market (Petrie and Singh 2011).

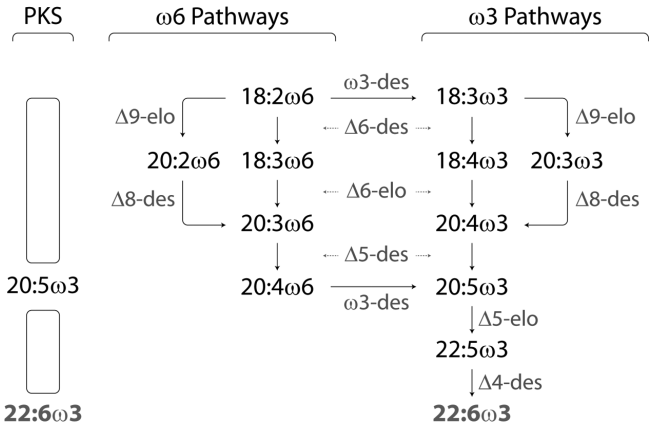

Fig. 7.4. Long chain poly unsaturated fatty acid synthesis pathways (PKS = polyketide synthase).

LC-PUFA biosynthesis pathways

Biosynthesis of LC-PUFA from LA and ALA in organisms such as microalgae, mosses and fungi can occur by a series of alternating oxygen-dependent desaturations and elongation reactions (Fig. 7.4). In one pathway the desaturation reactions are catalysed by Δ6, Δ5 and Δ4 desaturases, each of which adds an additional double bond into the fatty acid carbon chain, while the two Δ6 and a Δ5 elongase reactions each adds a two-carbon unit to lengthen the chain. In total, the conversion of ALA to DHA in these organisms therefore requires three desaturations and two elongations. Genes encoding the enzymes required for the production of LC-PUFA in this aerobic pathway have been cloned from various microorganisms and lower plants, including microalgae, mosses and fungi (Petrie and Singh 2011). In addition, alternative routes have been shown to exist for two sections of the LA and ALA to LC-PUFA pathway in some groups of organisms. For example, the conversion of ALA to eicosatetraenoic acid (ETA) may be carried out by a combination of a Δ9 elongase and a Δ8 desaturase (the so-called Δ8 desaturation route) in certain protists and thraustochytrids (Wallis and Browse 1999; Qi *et al.* 2002). Besides these desaturase/elongase systems, EPA and DHA can also be synthesised through an anaerobic pathway in several organisms such as *Shewanella, Mortiella* and *Schizochytrium*. The operons encoding these polyketide synthase (PKS) enzyme complexes have been cloned from some bacteria and thraustochytrids (Fig. 7.4; Metz *et al.* 2001).

Development of oilseed crops with fish oil-like levels of EPA and DHA

The last decade has seen a great deal of research focused on transforming the fatty acid synthesising capability of oilseed crops to synthesise EPA and DHA. This effort can be seen as the pinnacle in plant metabolic engineering and has required the transfer and seed-specific expression of up to seven transgenes. The critical breakthroughs in optimising the accumulation of EPA and DHA were contributed to by several research groups around the world. These include (1) the use of ω-3 specific desaturases, which prevented the accumulation of unwanted ω-6 fatty acids; and (2) the use of acyl-CoA-dependent desaturases for the first committed (D6) step on the pathway, breaking the substrate-dichotomy bottleneck and reducing the accumulation of GLA. As a consequence, it is now technically possible to accumulate fish oil-like levels of EPA and DHA in seed oils of oilseed crops such as canola and camelina (Petrie *et al.* 2014; Haslam *et al.* 2016; Martin and Li 2017; Sprague *et al.* 2017).

The two oilseed crops that have been worked on as potential platforms for engineering ω-3 LC-PUFA are camelina (*Camelina sativa*) and canola (*Brassica napus*) with research primarily being led by CSIRO (Australia), the Agricultural Science Research Institute, Rothamsted Research (UK), BASF (Germany) and Dow Agrosciences (USA). The successful accumulation of EPA and DHA into plant hosts has been primarily based upon work performed in the model species *Arabidopsis* (Petrie *et al*. 2012; Ruiz-Lopez *et al*. 2013). This technology was subsequently transferred to camelina as a host species with constructs consisting of five or seven marine microalgal genes for the purpose of engineering either an EPA-only, or an EPA + DHA oil (Ruiz-Lopez *et al*. 2014). The authors reported an EPA content of 24% of total fatty acids in the EPA only oil, whereas the EPA + DHA oil product had EPA and DHA contents of 11% and 8%, respectively. Similarly CSIRO scientists, using a different set of seven yeast and microalgal transgenes, demonstrated fish oil-like levels of DHA production, of up to 12.4% of total fatty acids, in oil from transgenic camelina and an EPA content of 0.8–3.3% (Petrie *et al*. 2014). In addition, these EPA and DHA levels have also been replicated outside laboratory conditions when grown in the field, representing a viable alternative to fish oil use in aquafeeds (Usher *et al*. 2015). In addition, BASF Plant Sciences has also been active in developing an EPA and DHA canola crop and have reported EPA and DHA contents of 8.5% and 2.5%, respectively, in field grown canola (WO 2016075327 A2). The alternative anaerobic PKS pathway has also been engineered into canola to produce an oil with EPA and DHA contents of 0.7 and 3.7% of total fatty acids, respectively (Walsh *et al*. 2016).

CSIRO ω-3 LC-PUFA canola crop

A collaboration was formed in 2011 between CSIRO, the Grains Research and Development Corporation (GRDC), and Nuseed, a wholly owned subsidiary of Nufarm Ltd, aimed at developing a genetically modified canola that produces long-chain ω-3 oil at levels equal to that of wild fish. Canola was selected as the target oilseed crop because it is an established major crop associated with high quality and healthy oil and high productivity. In addition, canola is also widely grown in many regions, with more than 10 million ha under cultivation in Australia, Canada and the USA. Strategies described in Petrie *et al*. (2012, 2014) have been successfully translated to canola. Nuseed has completed preparation for regulatory approvals of long-chain ω-3 canola, which included the conduct of field trials both in Australia and the USA. It has been estimated that one hectare of this DHA canola crop will produce a DHA yield equivalent to 10 000 fish of 1 kg. In 2017, Australian, US and Canadian regulatory papers were filed and pending regulatory approvals, commercialisation is expected to commence in 2019 or 2020. Nuseed has also announced commercial brands for the resulting oil products, specific to key end-use markets: Aquaterra™ for aquaculture feed uses and Nutriterra™ for human nutrition applications (http://www.nuseed.com/au/innovation/omega-3).

Pharmamolecules: modifying the morphine pathway in poppies

Papaver species, notably *P. somniferum* (opium poppy), are unique in synthesising and accumulating opioid alkaloid analgesics, codeine and morphine. Opium poppy is one of the oldest known cultivated plants for its use in pain control (Schiff 2002). Of the morphinan alkaloids, codeine and morphine are the most abundant. However, the earlier intermediate thebaine is now valuable as a scaffold for the subsequent synthesis of powerful and

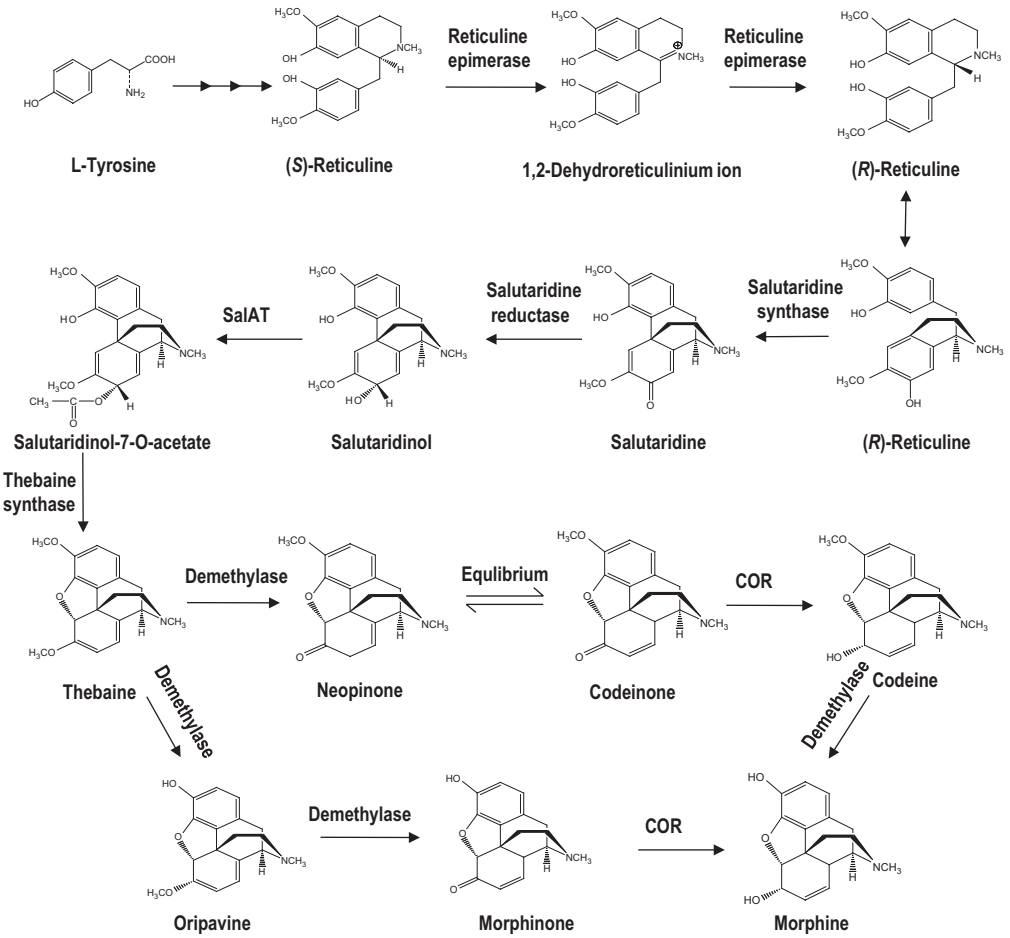

Fig. 7.5. Morphinan alkaloids pathways.

versatile analgesics such as buprenorphine, naltrexone and oxycodone. With five centres of chirality, the structures of morphinan alkaloids present a complexity that renders commercial synthesis uneconomic (leaving poppy cultivation the most effective means to produce opiate analgesics (Gerardy and Zenk 1992).

Not surprisingly, with the advent of molecular biology in recent decades, there has been much interest to elucidate the enzymology and molecular genetics of the pathway and to use that knowledge to manipulate the pathway to achieve greater efficiency of production or cause desirable alternative intermediates to accumulate. The pathway has been elucidated (Fig. 7.5) through the efforts of several laboratories (Unterlinner *et al.* 1999; Millgate *et al.* 2004; Hagel and Facchini 2010; Beaudoin and Facchini 2014; Farrow *et al.* 2015; Winzer *et al.* 2015). Additionally endogenous miRNAs have been identified and implicated in control of the pathway (Boke *et al.* 2015).

Manipulation of the pathway for commercial purposes was first achieved with a mutation developed for the Tasmanian poppy industry, called *thebaine oripavine poppy 1* (*top1*) and named locally as Norman (Fist *et al.* 1998). There is a bifurcated pathway

between thebaine and morphine (Fig. 7.5; Brochmann-Hanssen 1984). *Top1* is a knock-down mutation of the thebaine O-demethylase, which acts on the conversion of thebaine to neopinone, and oripavine to morphinone. The *top1* mutation appears to reduce demethylation of the enol ether in the same position in thebaine and oripavine (Millgate *et al.* 2004). As a consequence, oripavine and thebaine accumulate at the expense of morphine and codeine. The high yield of thebaine in these cultivars has generated a major industrial opportunity for value-added product and efficient production of important semi-synthetic analgesics including oxycodone and buprenorphine. Oripavine may be used for the synthesis of 'nal' compounds, such as naloxone and naltrexone, used in the treatment of opiate addictions.

More recently a double mutation has allowed the development of poppies that accumulate either only thebaine or mainly codeine, the most widely used opioid. The codeine poppy, known locally as Tasman, contains little or no oripavine, morphine or thebaine. In these lines, the *top1* mutation appears to be combined with another mutation affecting the bifurcated morphinan branch. Previously codeine was synthesised by methylation of morphine: a process using solvents with health risks and environmental burdens, and that generates undesirable side products that are difficult to remove. The codeine poppies are now being grown commercially.

The changes described above are the result of induced mutations, and as such could be deployed with little extra regulatory restriction. It has also been possible to make changes to the pathway using genetic engineering. Allen *et al.* (2004) produced transgenic poppy expressing a chimeric hairpin RNA construct designed to silence all members of the codeinone reductase multigene family through RNA interference (RNAi). These poppies accumulated mainly (*S*)-reticuline and its methylated derivatives at the expense of morphine, codeine, oripavine and thebaine. (*S*)-reticuline is seven enzymatic steps upstream of codeinone. The unexpected accumulation of (*S*)-reticuline suggests a feedback mechanism preventing benzylisoquinoline intermediates entering the morphine-specific branch. The accumulation of the non-narcotic alkaloid reticuline is potentially valuable as feedstock for the manufacture of various compounds that have shown anti-malarial activity (Rasoanaivo *et al.* 1998; Angerhofer *et al.* 1999; Camacho *et al.* 2002; Tshibangu *et al.* 2003; Zahari *et al.* 2014) or anticancer activity (Chen *et al.* 2002; Seifert *et al.* 1996). Reticuline itself stimulates hair growth (Nakaoji *et al.* 1997). Similarly RNAi induced silencing of salutaridinol 7-*O*-acetyltransferase (*SalAT*) resulted in the accumulation of the alkaloid salutaridine at up to 23% of total alkaloid; this alkaloid is not detectable in the parental genotype (Allen *et al.* 2008).

Genetic engineering has also achieved increases in alkaloid content. Overexpression of *SalAT* resulted in an increase in capsule morphine, codeine and thebaine on a dry weight basis. The transgenic line with the highest alkaloid content had 41%, 37% and 42% greater total alkaloids than the control in three independent trials over 3 years (Allen *et al.* 2008). Likewise opium poppy transgenically overexpressing either *P. somniferum* codeinone reductase (*PsCor1.1*) or *Eschscholzia californica* P450 reductase (*EcCpr*) had significant increases in alkaloid content in repeated greenhouse trials and a replicated field trial. The increases in total morphinan alkaloid content were between 15% and 60% over control high-yielding genotypes and control non-transgenic segregants (Larkin *et al.* 2007). None of these transgenic advances have been deployed or exploited. The reasons include the fact that the Tasmanian state government, where the Australian poppy industry is located, have imposed a moratorium on genetically modified crops. Now that the industry has expanded into the state of Victoria, where other transgenic crops are already grown, there

may be opportunity to revisit some of these innovations. Given that Australia produces 50% of the world's licit trade in opiates, and given the sometimes unmet demand for these effective analgesic drugs, commercial introduction of these improved products could resolve the unmet need in opiates for pharmacological uses.

Conclusions and perspectives

The examples discussed in this chapter serve to illustrate the present power of biotechnology to create compositional changes in agricultural products that are designed for societal, industrial and environmental benefit. Other chapters in this book will add weight to this assertion. We use the term biotechnology to encompass any genetic modifications informed by deep understanding of metabolic pathways and the impacts and applications metabolic end products can have. At one end of the spectrum, mutations of specific metabolic steps can be profoundly valuable and with the advantage of relative ease of deployment without burdensome regulatory constraint. Newer biotechnology tools are expanding the power of mutations: TILLING allows high-throughput search and recovery of mutations in specific known genes; and gene editing allows the targeted generation of mutations to specific known genes. Gene editing technology is developing rapidly and will increasingly enable specific base changes as well as gene knockouts.

RNAi or artificial micro RNA (amiRNA) knockdown of genes continues to offer some advantages over mutational knockout. In particular RNAi suppression of gene activity affords tissue-specific or developmental stage-specific knockdown, which is an advantage when global loss of gene function might be detrimental to the plant. RNAi can also be employed to knockdown multiple genes, such as a gene family, from one inserted construct when it might be difficult or time consuming to find multiple mutations and assemble them in one plant.

The insertion of new genes, including RNAi or amiRNA genes, to achieve novel products or increased production, requires regulatory approval before release for commercial use. It must also be considered that regulatory approval is required not only in the jurisdiction of production but also in the jurisdictions to which the products will be exported. This necessarily imposes substantial financial and resource burdens that have 'shipwrecked' many potential products. Wherever possible, researchers and companies will opt to develop products using biotechnology where the regulatory burden and cost is less. Sometimes it is not possible to conceive non-GM options. Inserting the entire long chain PUFA pathway into canola is an excellent example of this, and also highlights that the benefits to society and environment can justify the investment to take such innovations to market.

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