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Biotechnology of Oilseeds

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Introduction

Oilseeds are among the most ancient crops domesticated by mankind. One of the first oilseed crops to be cultivated systematically was probably linseed. There is fossil evidence of the selection for agriculture of oil-bearing varieties of linseed from over 8000 years ago. Their first mention in written records is found in ancient Sumerian and Akkadian texts dating from 4000 to 5000 years ago, which refer to oilseeds such as sesame and linseed (Helbaek, 1966). From the beginning of their cultivation, oilseeds were utilized in a variety of edible and non-edible applications. For example, the ancient Persians used sesame oil in cooking, as a body massage, for illumination, in cosmetics and as a lubricant in simple machines.

The relative proportion of oilseed products utilized for non-edible applications began to decline in the early twentieth century, due to the ready availability of large amounts of inexpensive mineral oil derived from fossilized material. More recently, however, the large increases in the price of fossil-derived mineral oils and the recognition that this is a limited resource have focused attention once more on the potential non-food uses of vegetable oils. The market for edible vegetable oils has also increased substantially since the Second World War. This is due to a number of factors, including improvements in agriculture, rising levels of affluence in many consuming countries and the trend away from animal-derived fats for medical or dietary reasons.

The net effect of these various trends has been an enormous increase in the demand for oilseeds and their products, particularly in Europe and the Americas. In the past decade alone, world oilseed production has increased

by more than one-third, from about 160 MT to 215 MT. As much as one-third of the vegetable oil produced is used for non-edible, industrial purposes (Battey, Schmid and Ohlrogge, 1989). The increasing demand for high-quality oilseeds, designed for specific market applications, both edible and non-edible, has coincided with the emergence of new technologies for crop modification. Efforts are now underway in many different countries, aimed at the production of specialized 'designer' oilseed crops. The oil compositions of such crops will be manipulated in order that different crop species or varieties can each be targeted towards a particular commercial sector, for example high oleic edible oils or high linoleic edible oils for edible use, long-chain oils for use as lubricating fluids, short-chain oils for use as detergents, mono-unsaturated oils for use in polymer synthesis, hydroxylated oils for use in lubricants, paints and polymers, and so on.

Four crops—soybean, sunflower, rape and palm—provide over 70% of the world's vegetable oil supplies (Scowcroft, 1990) with cottonseed, coconut and groundnut providing most of the remainder. There are many other sources of vegetable oils, such as olives, grape seed or tomato seed, but these are usually used near the locality where the crops are grown. A detailed and comprehensive account of the cultivation, breeding and uses of all these oilseeds is given in a recent book (Downey, Robbelen and Ashri, 1989). Most vegetable oil is produced for human consumption but about 20–30% is used for non-edible purposes. Oil is not the only economically valuable product of oilseed crops, and most of the other components are sold after processing. The protein meal from the seeds is usually recovered and sold as animal feedstuff. The hulls of the seeds are also used variously in animal feed, as fuel, or in the construction of building or insulation boards. Lecithin is another small but valuable component. In this review the term 'oilseed crops' refers to all crops from which oil is extracted for further use. For some crops, however, oil is not the main product. In soybean, protein is at least as important. For corn (*Zea mays*), starch is the major product for which the crop is grown, mainly for conversion to high-fructose corn syrup. If the oil content of corn rises above just a few per cent, the conversion of the starch is interfered with, so corn varieties with higher oil contents are not grown. Cotton is another example where oil is secondary to the main product of fibre for which cotton is mainly grown.

Some aspects of the genetic engineering of oil quality in oilseeds have been dealt with in the past few years (Knauf, 1987; Battey, Schmid and Ohlrogge, 1989; Somerville and Browse, 1991). In this review, we will consider the application of biotechnology not only to the oil component of oil seeds but also to the protein and other products of potential interest in the future. Lipase is one such product which can be extracted from the seeds of castor bean or from the seedlings of many oilseed plants. This has possible applications in catalysing reactions of lipid substrates dissolved in organic solvents. Oleosins, which surround the oil droplets in the seed and prevent coalescence of the oil in the dry seed, might have uses as emulsifiers.

Uses of vegetable oils

Most vegetable oil is used directly in the human diet as cooking or salad oil and in margarine, or indirectly in processed products such as shortenings and confectionary. There is also a multiplicity of uses of vegetable oils for non-edible purposes, although the amounts involved are often quite small. The major non-edible end use of vegetable oils is in the production of soaps and detergents from palm and coconut oils. Other non-edible uses of vegetable oils are in lubricants, plastics and resins, paints, varnishes and coatings, cosmetics and as precursors to a wide range of chemicals. Three examples of such oils are those derived from linseed, castor and jojoba seeds. Linseed oil, which contains large amounts of α-linolenic acid, is used in coatings and drying agents because the double bonds of α-linolenic acid are very susceptible to oxidation, leading to polymerization, and yielding a solid but soft and flexible product. Castor oil, which contains hydroxylated fatty acids, is used in paints and coatings but also has uses in lubricants, and as a precursor to plastics manufacture. Jojoba oil, which is a liquid wax rather than a triacylglycerol oil, is used in lubricants and cosmetics. It has been reported that it is possible to obtain wax esters with very similar properties to jojoba wax from mustard oil by esterification using a lipase in organic solvents (Mukherjee and Kiewitt, 1988). Such oils can be used directly, or they may be cleaved to partial glycerides for use as emulsifying agents in margarines, or completely cleaved to fatty acids. During the past 10 years, the use of lipases to catalyse such reactions, particularly with higher value oils, has become a reality (Mukherjee, 1990). Using lipases may significantly reduce production costs compared to conventional chemical engineering methods. The oils or fatty acids are often chemically processed by one or more of a wide range of reactions such as hydrogenation, amidation, amination, ozonolysis or epoxidation, depending on the end use. The non-food uses of vegetable oils and the chemical conversions are described in depth elsewhere (Pryde and Rothfus, 1989). It has been estimated that the volume of vegetable oil used for the production of chemicals would double if full use was made of them, rather than using petrochemicals as the raw material (Pryde and Rothfus, 1989).

Changes in the quality of oilseeds sought by users

Changes in oil quality, i.e. the type of fatty acids contained in the triacylglycerols, are desired for both edible and non-edible oils, though it is in the oils for industrial purposes that the most radical alterations are wanted and it is the accomplishment of these changes that will require genetic engineering.

EDIBLE OILS

Polyenoic fatty acids

The double bonds of polyenoic fatty acids such as α-linoleic and linolenic are susceptible to oxidation by molecular oxygen, leading to the formation of

Table 1. Fatty acid nomenclature. Trivial names for fatty acids have been used throughout the manuscript but sometimes the abbreviated designations given below appear. The shorthand designation refers to the number of carbons in the hydrocarbon chain:number of double bonds (see Gunstone, 1986 for more details)

Trivial name	Shorthand designation
lauric	12:0
palmitic	16:0
stearic	18:0
oleic	18:1
linoleic	18:2
linolenic	18:3
gadoleic	20:1
erucic	22:1
nervonic	24:1
ricinoleic	18:1-OH

strongly flavoured compounds which are undesirable in edible oils. The flavour of oxidized α-linolenic acid is particularly strong. Soybean and rape oils contain significant amounts of α-linolenic acid, so decreases in this fatty acid are sought in order to increase the oxidative stability of the oil and thereby improve flavour. The seeds of legumes, such as soybean, also contain lipoxygenases which cause the oxidation of α-linolenate moieties, so breeding and genetic engineering strategies are being devised to eliminate these enzymes. The requirement for hydrogenation could also be reduced, hence minimizing the levels of undesirable *trans* unsaturated fatty acids often present in hydrogenated edible oils. The possible health risks of high levels of dietary *trans* unsaturated fatty acids in some vegetable oil products is currently attracting adverse publicity and any reduction in their amounts would therefore be welcomed by the food industry.

Palmitic acid

A decrease in the levels of palmitic acid in soybean oil is also wanted for health reasons. Excessive dietary intake of this acid has been implicated as a contributory factor in the aetiology of coronary heart disease. At the same time, increases in the levels of stearic acid (which is not believed to have the same attached health risks) are sought. Small increases in the levels of palmitic acid in rape oil are wanted to allow the production of 100% rape oil margarine. High levels of C18 fatty acids in present varieties of rape oil cause crystallization of the oil in the margarine, leading to an unacceptably grainy texture which can only be eliminated by the addition of other types of vegetable oils, e.g. sunflower or soybean, which contain higher levels of palmitate.

Cocoa butter

Cocoa butter substitutes have been sought for use in confectionary since the genuine material is relatively expensive. Substitutes can be made by interest-erification of cheap palm oil with stearic acid by using an *sn* -1,3 specific lipase from *Rhizopus* as catalyst (Macrae and Hammond, 1985). A problem here is

that such substitutes, although they are derived from natural oils and are chemically very similar to cocoa butter triacylglycerols, may only be used as a minor admixture in chocolate products. Hence the use of cocoa butter substitutes is currently limited by labelling regulations, particularly in Europe and N. America.
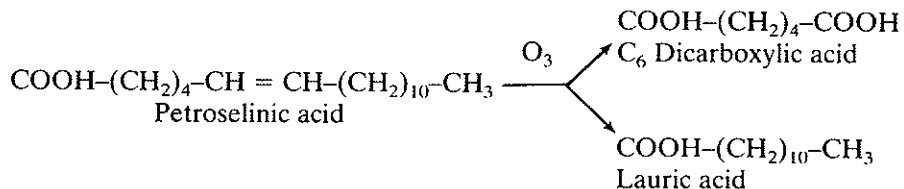
NON-EDIBLE OILS

Lauric acid

Medium-chain-length fatty acids (mainly lauric acid) in seeds of a crop which can be grown in temperate regions are wanted by the detergent industry in Europe and N. America in order to replace or augment the present source of such fatty acids, i.e. imported coconut or palm kernel oils. *Cuphea* oils are also potentially useful in this regard since they contain higher levels of the medium- and short-chain fatty acids than do the tropical palm oils. The production of detergents is the single most important end use of a vegetable oil for non-edible purposes; thus the production of lauric oils within the EEC or US and the development of a domestic source of such oils would save considerable sums of money on the trade balance of these countries. Conversely, of course, this could have a negative effect on those, mainly developing, countries which rely on exporting lauric oils to accrue foreign currency.

Petroselinic acid

An alternative for obtaining lauric acid to that described above is the possibility of growing oilseed crops containing petroselinic acid in the seed oil, since the double bond in the Δ^6 position can be cleaved by ozonolysis to yield C12 and C6 units, as shown below.

$$COOH-(CH_2)_4-CH = CH-(CH_2)_{10}-CH_3 \xrightarrow{O_3} \begin{array}{l} COOH-(CH_2)_4-COOH \\ C_6 \text{ Dicarboxylic acid} \\ \\ COOH-(CH_2)_{10}-CH_3 \\ \text{Lauric acid} \end{array}$$

Petroselinic acid

This reaction yields lauric acid for detergent applications, and also a C6 dicarboxylic acid, which is eminently suitable for use as a monomer in the manufacture of a wide range of industrial polymers, particularly nylons.

Hydroxylated fatty acids

Hydroxylated fatty acids, such as ricinoleic acid, are sought in the seed oils of temperate species since the main source of this acid is castor oil which is

imported from tropical countries where supplies are often uncertain for climatic or other reasons. Ricinoleic acid from castor oil is particularly useful because the OH group in the Δ^{12} position and the double bond in the Δ^9 position allow many chemical conversions to be carried out which are not easily achieved with oils from other plants. Ricinoleic acid is used in lubricants, plasticizers, coatings, surfactants and pharmaceuticals. Derivatives are also used in polyester and other polymer manufacture, in cosmetics, non-drip paints and in greases.

Homogeneous triacylglycerols

Most seed oils contain triacylglycerols with a highly heterogeneous fatty acid composition. Since the end use of many seed oils depends upon only one of these fatty acids, the production of triacylglycerols containing as much of one fatty acid as possible is a major goal of the oilseed biotechnologist. This would make the extraction of relatively pure fatty acids from the oil much easier and more commerically attractive. An already achieved example of this is the breeding of sunflower cultivars that contain greater than 90% oleic acid. A trierucoylglycerol would make processing of erucic acid from high erucic acid rape oil or other sources much more commercially attractive. The main seed oils containing erucic acid, such as rape or mustard, contain this fatty acid almost exclusively in the *sn*–1 and *sn*–3 positions so that a theoretical maximum of less than 70% is achievable at present. Oils containing erucic acid may be employed directly as lubricants, or the erucic acid can be converted by ozonolysis and amidation to monomers, for use in plastic or nylon manufacture.

Substituted fatty acids

The production of seed oils containing any of a wide variety of substituted fatty acids would give savings to industry on the costs of processing simple fatty acids to the more complex ones. Plants have been found whose seed oils contain conjugated polyene, cyclopropene or acetelynic fatty acids which have a wide range of uses in the oleochemicals industry (Gunstone, 1986). It would be beneficial to users if these unusual fatty acids were produced in oilseed crops, since it would decrease some of the chemical processing requirements. An example of such fatty acids are those containing epoxy groups. These are used in epoxy resins and coatings. Vernonia oil contains such fatty acids but since Vernonia has not yet been developed as an oil seed crop, the epoxy fatty acids are currently obtained by epoxidation of soybean oil.

Oil synthesis

In order to set the biotechnological manipulation of oil quality in context, the metabolic pathways involved in oil synthesis will be described briefly. For more detailed description of lipid and storage oil metabolism, the reviews by

Roughan and Slack (1982) and Harwood (1988) give a comprehensive account. Here we shall emphasize the recent research targeted at the molecular genetics of storage lipid synthesis and aspects of the metabolic control of oil production. Most seed storage oils are composed of triacylglycerols, with the notable exception of jojoba, which stores wax esters. The synthesis of triacylglycerols occurs in three main stages; first the synthesis of fatty acids (palmitic, stearic and oleic) in the plastid (*Figure 1*). Oleic acid can then, depending on the plant species, be metabolized to other fatty acids by desaturation, elongation, hydroxylation or other reactions, such as epoxidation, to create modified fatty acids. In the final stage of oil synthesis, the various fatty acids form an acyl-CoA pool, which is thought to be in the endoplasmic reticulum (ER) where they are drawn upon by acyltransferases of the Kennedy pathway to form triacylglycerols (*Figure 2*).

FATTY ACID SYNTHESIS

Fatty acids in plants are synthesized mainly in the plastid (Harwood, 1988). The first committed step of fatty acid biosynthesis is that catalysed by acetyl-CoA carboxylase to create malonyl-CoA (Slabas and Hellyer, 1985). There is some correlative evidence from castor that acetyl-CoA carboxylase is rate-limiting in the synthesis of fatty acids (Simcox *et al.*, 1979). In rape, the measurable activities are low but increase dramatically as oil synthesis begins (Turnham and Northcote, 1983). The notion that acetyl-CoA carboxylase is the major regulatory step of fatty acid synthesis has been strengthened by recent experiments where levels of the intermediates of fatty acid synthesis were measured under conditions of low and high pathway flux (Post-Beittenmiller, Jaworski and Ohlrogge, 1991). These experiments are described in detail on p. 12. Fatty acids are then synthesized by the sequential addition of two carbon units from malonyl-CoA to the acyl chain (*Figure 1*) by a type II fatty acid synthetase. Fatty acid synthetase II is a system consisting of six enzymatic activities, which, although each can be isolated as individual enzymes, is thought to act as a single complex *in vivo*. The lengthening acyl groups are attached to acyl carrier proteins (ACPs) throughout *de novo* fatty acid synthesis.

Acyl carrier proteins

Acyl carrier proteins and their genes are the most thoroughly characterized of the components of the fatty acid and oil synthesis pathways. ACPs are acidic, low molecular weight proteins and are essential co-factors for at least 12 enzymes involved in fatty acid metabolism. Much research effort has been focused on ACP since it is likely that information about the control of expression of ACP in seeds will shed light on the regulation of genes involved with oil synthesis in general during embryo development (Ohlrogge *et al.*, 1987). Immunogold localization studies have shown that ACP is almost exclusively localized to the plastids of rape leaves, where the majority of the ACP was found to be associated with the thylakoid membranes (Slabas and
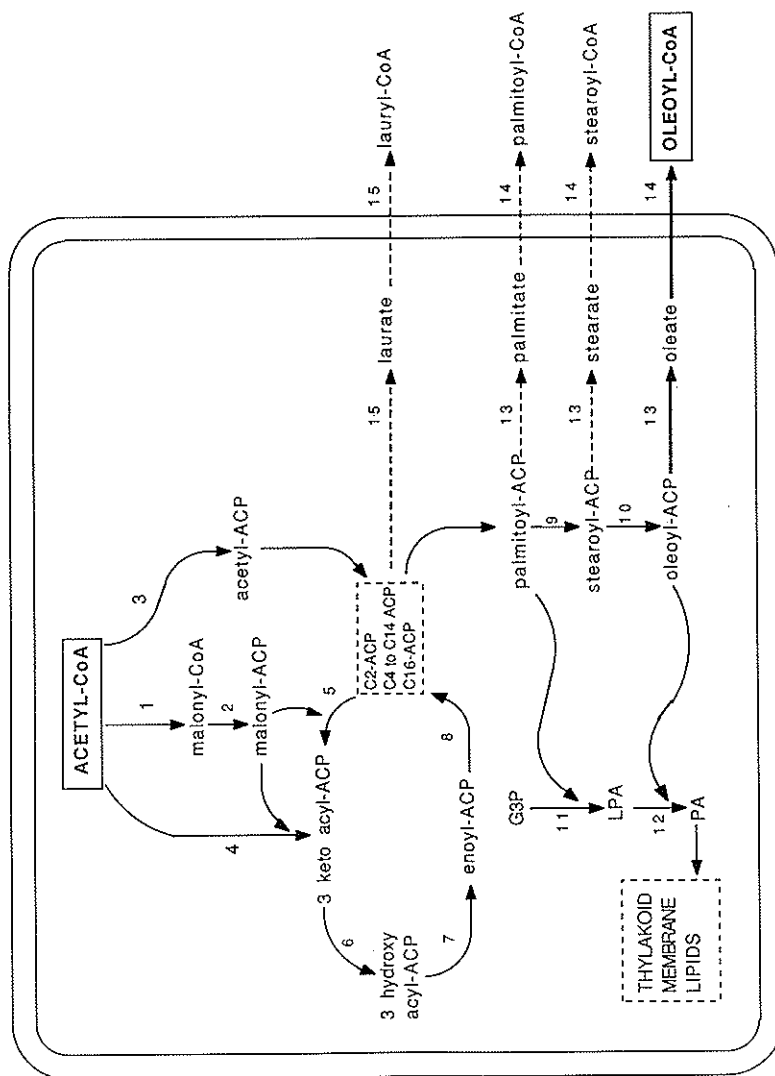
Figure 1. Biochemical pathways involved in the synthesis of fatty acids in the plastid. 1, acetyl-CoA carboxylase; 2, malonyl-CoA:ACP transacylase; acetyl-CoA:ACP transacylase; 3, acetyl-CoA:ACP transacylase; 4, 3-ketoacyl-ACP synthase III; 5, 3-ketoacyl-ACP synthase I; 6, 3-ketoacyl-ACP reductase; 7, 3-hydroxyacyl-ACP dehydratase; 8, enoyl-ACP reductase; 9, palmitoyl-ACP elongase; 10, stearoyl-ACP desaturase; 11, acyl-ACP:glycerol-3-phosphate acyltransferase; 12, acyl-ACP:*lyso*-phosphatidic acid acyltransferase; 13, acyl-ACP thioesterase; 14, fatty acyl-CoA synthetase; 15, medium-chain acyl-ACP thioesterase.
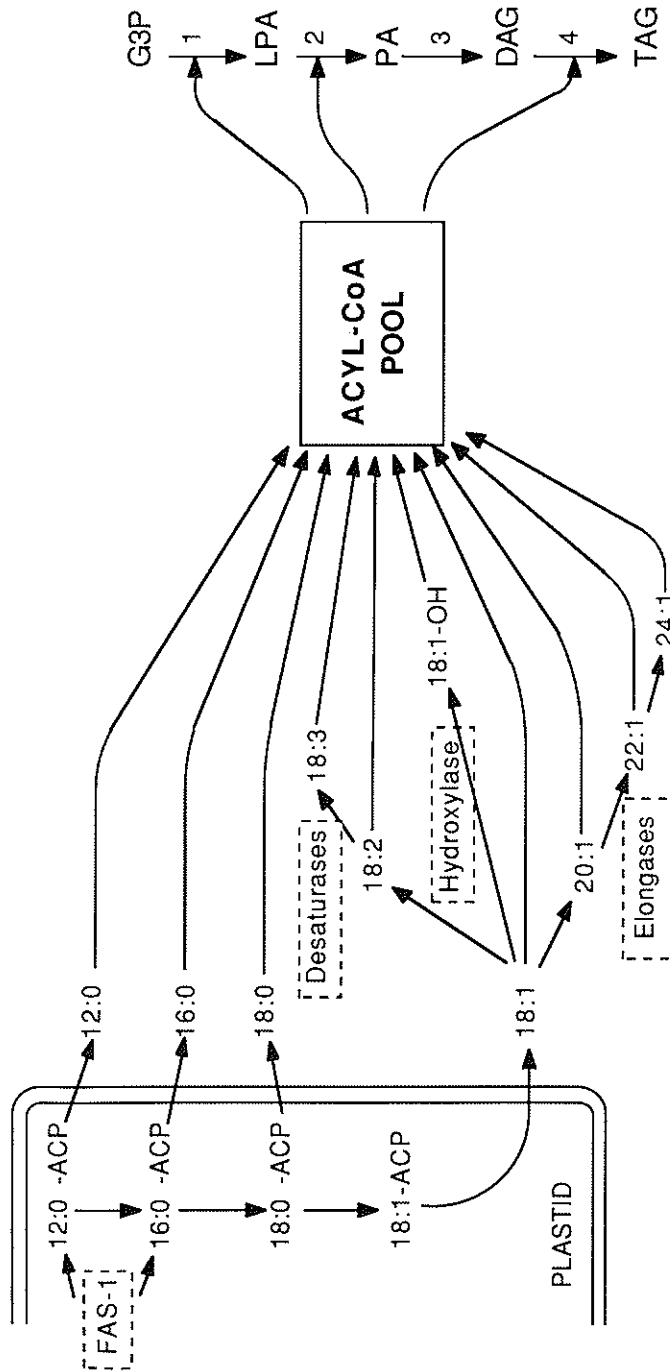
Figure 2. Formation of microsomal acyl-CoA pool from fatty acids generated by the plastid and those converted from oleate (18:1) by desaturation, elongation or hydroxylation. In any particular plant only some of these conversions will occur. The Kennedy pathway draws upon the acyl-CoAs during the synthesis of triacylglycerols. 1, acyl-CoA:glycerol-3-phosphate acyltransferase (G3P-AT); 2, acyl-CoA:lyso-phosphatidic acid acyltransferase (LPA-AT); 3, phosphatidate phosphatase; 4, acyl-CoA:diacylglycerol acyltransferase (DAG-AT).

Smith, 1988). A complete protein sequence of ACP from spinach leaf was reported in 1984 (Kuo and Ohlrogge, 1984) and since then cDNA and genomic clones of ACPs have been analysed from several plant sources (Rose *et al.*, 1987; Scherer and Knauf, 1987; de Silva *et al.*, 1990). Different isoforms of ACP are present in different tissues of the plant (Ohlrogge and Kuo, 1985; Battey and Ohlrogge, 1990; de Silva *et al.*, 1990; Schmid and Ohlrogge, 1990). Two main isoforms of ACP (I and II) have been reported in spinach (Hoj and Svendsen, 1984; Ohlrogge and Kuo, 1985). Acyl-ACP I was preferentially hydrolysed by acyl-ACP thioesterase compared to acyl-ACP II, while acyl groups from acyl-ACP II were preferentially transferred to *sn*-glycerol-3-phosphate (G3P) by acyl-ACP G3P-acyltransferase (Guerra, Ohlrogge and Frentzen, 1986), suggesting a possible role for the two isoforms in regulating the channelling of acyl groups between plastidic and microsomal lipid metabolism. In developing seeds, virtually all the acyl groups synthesized by the plastid are destined for export, to be metabolized further and incorporated into triacylglycerols. In view of this, it might be expected that developing seeds would contain mainly type I ACP (preferentially used by the thioesterase) but it was found that developing seeds of spinach contain only ACP II (Ohlrogge and Kuo, 1985), which is preferentially used by the acyl-ACP:G3P-acyltransferase. The role of the ACP isoforms, and whether or not they are important in regulating the synthesis of acyl groups, is not yet clear. The genome of *Brassica napus* has been shown to contain a large ACP gene family, with as many as 35 genes per haploid genome (de Silva *et al.*, 1990). Several of the genes are expressed in seeds (Safford *et al.*, 1988) in a manner co-ordinated with storage oil synthesis (de Silva *et al.*, 1990).

Fatty acid synthetase

In the first step of chain elongation, 3-ketoacyl-ACP is formed by condensation of acetyl and malonyl groups. For several years it was thought that 3-ketoacyl-ACP synthase I was the only enzyme responsible for the formation of 3-ketoacyl-ACP using acetyl-ACP and malonyl-ACP as substrates (*Figure 1*). Acetyl-ACP and malonyl-ACP are formed from acetyl-CoA or malonyl-CoA and ACP by the action of their respective transacylases (enzymes 2 and 3, *Figure 1*). Acetyl-CoA:ACP transacylase was considered to be a possible rate-limiting step of fatty acid synthesis since it showed the lowest *in vitro* activity of the fatty acid synthetase enzymes (Shimakata and Stumpf, 1983). Recently, however, cerulenin inhibition studies on 3-ketoacyl-ACP synthase I have revealed the presence of 3-ketoacyl-ACP synthase III in plants (Jaworski, Clough and Barnum, 1989). This enzyme uses acetyl-CoA instead of acetyl-ACP, and the activity is five times greater than the acetyl-CoA:ACP transacylase reaction. It is now thought that the 3-ketoacyl-ACP synthase III acts as a by-pass to the relatively slow acetyl-CoA:ACP transacylase, so *in vivo* transacylase activity is probably not important in affecting flux through the fatty acid synthesis pathway. The 3-ketoacyl-ACP synthase III has been found in a wide variety of plant tissues (Walsh, Kloppenstein and Harwood, 1990).

Following the formation of a 3-ketoacyl-ACP, the 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase and *trans*-Δ^2-enoyl-ACP reductase then act sequentially to form the C4 thioester, butanoyl-ACP. This re-enters the cycle of fatty acid synthesis by condensation with an acetate group from malonyl-ACP and undergoes a further six cycles to form the C16 thioester, palmitoyl-ACP. The condensation reactions from butyryl-ACP onwards are believed to be catalysed by 3-ketoacyl-ACP synthase I (KAS I). The 3-ketoacyl-ACP synthase I has recently been purified to homogeneity from developing rape embryos (MacKintosh, Hardie and Slabas, 1989) and it was resolved from 3-ketoacyl-ACP synthase II (KAS II) which was shown to have a slightly higher molecular weight. The KAS II catalyses the elongation of palmitoyl-ACP and is described in the next section. In the native state, both KAS I and KAS II are thought to be homodimers. KAS I has also been purified from developing seeds of soybean (Kinney, Hitz and Yadau, 1990) and from barley (Siggaard-Andersen 1990). The gene for the barley enzyme has also been cloned by Kauppinen (1990). The NADPH-linked β-ketoacyl-ACP reductase has also been purified to homogeneity from spinach (Shimakata and Stumpf, 1982) and avocado mesocarp (Sheldon *et al.*, 1990). Enoyl-ACP reductase occurs in two forms, one requires NADH and the other prefers NADPH as the reductant. The NADH-specific enoyl-ACP reductase has been purified to near homogeneity from developing rape embryos (Slabas *et al.*, 1990) and is thought to contain two closely related polypeptides (Cottingham *et al.*, 1988). The same enzyme was also purified by Kater *et al.* (1991) who also raised antibodies and used these to detect and sequence full-length cDNA clones encoding the enzyme.

Metabolism of palmitoyl-ACP

Palmitoyl-ACP can be elongated to stearoyl-ACP by a further round of fatty acid synthetase activity initiated by 3-ketoacyl-ACP synthase II which is specific in accepting only palmitoyl-ACP as the substrate for elongation. The stearoyl-ACP is then desaturated to oleoyl-ACP by stearoyl-ACP desaturase. This enzyme has been purified to homogeneity from safflower (McKeon and Stumpf, 1982) and more recently from avocado (Shanklin and Somerville, 1991). Others have also recently reported purification of stearoyl-ACP desaturase from soybean (Cheesebrough and Cho, 1990; Kinney, Hitz and Yadau, 1990). Antibodies against the avocado stearoyl-ACP desaturase have been used to isolate cDNA clones from cucumber and castor seeds (Shanklin and Somerville, 1991). The cDNAs have been sequenced and show up to 88% amino acid identity. Comparison of the amino acid sequences with yeast fatty acid synthetase has yielded a region of 48 residues which Shanklin and Somerville suggest might be involved in the binding of ACP. In plastids of developing oil seeds, only a small proportion of acyl groups will be required for plastid membrane lipids, via incorporation by the action of acyl-ACP acyltransferase (Löhden and Frentzen, 1988). The vast majority of acyl groups are destined for export from the plastid for oil synthesis, so acyl-ACP hydrolysis should be the predominant reaction. The proportion of 16:0, 18:0

and 18:1 exported will reflect the activities of the stearoyl-ACP synthetase, stearoyl-ACP desaturase and the selectivity of the acyl-ACP thioesterase(s) (*Figure 1*). The alteration of the relative amounts of these enzymes is a target for genetic manipulation in order to change the levels of these 16:0, 18:0, 18:1 and those fatty acids derived from 18:1 found in storage triacylglycerols. Acyl-ACP thioesterase from *Brassica napus* has been purified and characterized (Hellyer and Slabas, 1990). The enzyme showed a clear preference for oleoyl-ACP which was hydrolysed 8–10 times faster than palmitoyl-ACP or stearoyl-ACP. This mirrors the relative amounts of 16:0 and 18:1, 18:2 and 18:3 found in the seed oil where the 18-carbon fatty acids predominate. Similar results on acyl-chain specificity were also obtained for the acyl-ACP thioesterases from safflower, avocado, coconut and jojoba (McKeon and Stumpf, 1982). In contrast, the acyl-ACP thioesterase from palm, the oil of which contains substantial amounts of palmitic acid, was shown to be more active against palmitoyl-ACP than oleoyl-ACP (Sambanthamurthi and Oo, 1990). The gene encoding the acyl-ACP:glycerol-3-phosphate acyltransferase from *Arabidopsis thaliana* has been cloned and sequenced (Nishida *et al.*, 1990).

It is thought that fatty acids are released from ACP by an acyl-ACP:thioesterase on the inner face of the inner plastid envelope membrane, and, in an as yet undefined manner, the fatty acids then cross the inner envelope membrane and are activated to CoA esters by a fatty acyl-CoA synthetase on its outer face. The outer envelope membrane contains large pores and is not a barrier to the movement of relatively small molecules such as fatty acyl-CoAs. An alternative scheme to the above has been proposed whereby the acyl groups are transferred across the inner envelope membrane attached to a carnitine molecule by an acyl-carnitine translocase. Carnitine long-chain acyltransferase activity has been located in the etioplasts of barley with little detectable contamination from mitochondria (Thomas *et al.*, 1983). This would save the cell ATP required for the re-synthesis of the thioester bond of an acyl-CoA. Carnitine long-chain acyltransferase activity was not, however, detected in seeds of the California Bay plant (Pollard *et al.*, 1991). The mechanism of acyl moiety export from plastids is as yet to be elucidated.

Investigations of the rate-limiting step(s) of fatty acid synthesis which may provide targets for manipulation have already been mentioned above. It was not until earlier this year (1991), however, that *in vivo* evidence of a rate-limiting step of fatty acid synthesis was reported (Post-Beittenmiller, Jaworski and Ohlrogge, 1991). Measurements were made of *in vivo* pools of unacylated ACP and the various forms of acylated ACPs from the intermediate stages of fatty acid synthesis extracted from leaves and seeds. The ACP and acyl-ACPs were separated by gel electrophoresis, immuno-blotted and probed using antibodies raised against ACP. Since the flux through the fatty acid synthesis pathway in leaves is known to decrease in the dark, levels of free and acylated ACP in light and dark were determined, and it was found that the changes in concentrations of the various intermediates could only be accounted for if acetyl-CoA carboxylase provided control over the flux of carbon through the fatty acid biosynthetic pathway. In contrast, the condens-

ing enzymes were found to have little influence over the rate of fatty acid synthesis. These findings are significant but it is recognized that the situation in seeds might well be different from that in leaves.

Formation of short- and medium-chain-length fatty acids

The mechanism of the biosynthesis of the economically important medium-chain-length fatty acids is not yet understood. In plants that synthesize large quantities of medium-chain-length fatty acids, the fatty acid synthetase system is thought to be the same as in other species but chain elongation is terminated early. Several possible mechanisms to explain the formation of medium-chain-length fatty acids have been proposed (Oo and Stumpf, 1979; Harwood, 1988) but there are very few experimental findings that help to confirm any of these ideas. Experiments *in vitro* suggested that increasing the concentration of ACP causes premature acyl-chain termination, resulting in the release of C8–C14 fatty acids (Huang and Stumpf, 1971; Slabas, Roberts and Ormesher, 1982; Singh, Nee and Pollard, 1984). Recently, effects of ACP concentration on chain termination have been tested *in vivo* by expressing spinach ACP in tobacco leaves using the relatively strong promoter from the gene of the small subunit of ribulose bisphosphate carboxylase to drive a higher level of expression of the introduced ACP gene. The levels of ACP in the transgenic tobacco leaves were 3–4 times higher than normal and the heterologous ACP was shown to be used by the tobacco leaves in fatty acid synthesis. However, no significant alteration in the pattern of lipids biosynthesized was observed and this casts doubt on the meaning of the previous *in vitro* experiments (Post-Beittenmiller, Schmind and Ohlrogge, 1989). In another piece of recent work with subcellular fractions from developing embryos of *Cuphea wrightia*, a plant whose seeds contain predominantly medium-chain fatty acids, it was shown that intact plastids synthesized mainly long-chain fatty acids. If a microsomal fraction from the embryo homogenates was included with the plastids, triacylglycerols (TAGs) were synthesized from the newly formed fatty acids. The addition of microsomes also stimulated fatty acid synthesis by the plastids and caused a fourfold increase in the rate of synthesis of lauroyl (C12) moieties (Deerberg *et al.*, 1990), probably by removing fatty acids and hence preventing product inhibition. These authors suggest that a high rate of fatty acid synthesis activity, allowed by an unusually high acetyl-CoA carboxylase activity, may cause early chain termination. However, the inclusion of microsomes in a similar system from coconut, which also normally synthesizes short-chain fatty acids, actually resulted in an increase in longer-chain acyl moieties (Slabas *et al.*, 1984). An alternative and more likely mechanism for early chain termination is the presence of an acyl-ACP thioesterase or a transacylase with a specificity for medium-chain-length acyl groups as shown in *Figure 1*, enzyme 15. Recently, an acyl-ACP thioesterase from seeds of California Bay (whose oil contains 60% laurate and 30% caprate) was found to be almost as active against lauroyl-ACP as against oleoyl-ACP (Pollard *et al.*, 1991). The medium-chain-specific acyl-ACP thioesterase activity was sepa-

rated from long-chain acyl-ACP thioesterase on a heparin–agarose column, demonstrating the presence of separate acyl-ACP thioesterases for different chain lengths of the acyl groups.

FATTY ACID MODIFICATIONS

Desaturation

In developing seeds, the desaturation of oleic acid to linoleic and linolenic is thought to be catalysed by sequential steps. Developing safflower cotyledons have been used as a model system with which it was shown that desaturation of oleoyl moieties occurs when they are esterified to phosphatidylcholine (PC) at either the sn-1 or sn-2 positions (Stymne and Appelqvist, 1978; Slack, Roughan and Browse, 1979; Stobart and Stymne, 1985). Phosphatidylcholine is probably the major substrate for desaturation in most plants but there is evidence that in some cases phosphatidylethanolamine might act as a substrate for desaturation as well (Gennity and Stumpf, 1985). The PC is generated either from sn-1,2 diacylglycerol (DAG), synthesized by the Kennedy pathway by the DAG:choline phosphotransferase reaction, or from lysophosphatidylcholine (LPC) formed by LPC-acyltransferase (*Figure 3*; Slack et al., 1983; Stymne and Stobart, 1984; Stobart and Stymne, 1985). Both of these reactions are reversible so, after desaturation of the oleoyl moiety esterified to PC, either DAG or linoleoyl-CoA are reformed, and in this way the DAG and the acyl-CoA pools are continuously enriched with polyunsaturated fatty acids for incorporation into TAGs. In oilseeds that
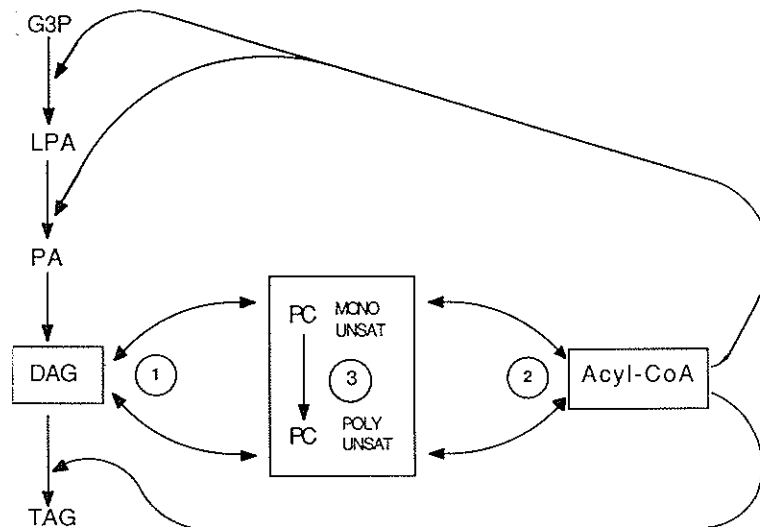

Figure 3.   Desaturation of oleate, and probably linoleate, esterified to phosphatidylcholine in a microsomal compartment. 1, sn-1,2 diacylglycerol:cholinephosphotransferase; 2, acyl-CoA:*lyso*-phosphatidylcholine acyltransferase (LPC-AT); 3, oleate (linoleate) desaturase. The Kennedy pathway of triacylglycerol synthesis shown here is given in more detail in *Figure 2*.

have relatively low levels of polyunsaturated fatty acids, the *lyso-*phosphatidylcholine acyltransferase and DAG:cholinephosphotransferase reactions are found to have much lower activities compared to high polyunsaturate oilseeds (Stobart and Stymne, 1985). Reducing equivalents are transferred to the desaturase from NADH via cytochrome b_5 catalysed by cytochrome b_5 reductase. Cytochrome b_5 has been purified and antibodies raised against it (Kearns, Hugly and Somerville, 1991). The antibodies inhibited desaturation in safflower by 93%, confirming that cytochrome b_5 is the major and possibly sole electron donor for the Δ^{12} desaturase. It is hoped that, ultimately, purified cytochrome b_5 and cytochrome b_5 reductase will be used to allow for the *in vitro* assay of the desaturase, hence permitting its purification.

The desaturation of linoleic to α-linolenic acid has been measured in soybean, safflower and linseed, and is also thought to occur with linoleoyl moieties attached to PC (Slack, Roughan and Browse, 1979; Stymne and Appelqvist, 1980; Stobart and Stymne, 1985). Desaturation of linoleoyl moieties in the Δ^6 position in borage (Griffiths, Stobart and Stymne, 1988) and evening primrose (Mukherjee and Kiewitt, 1987) also occurs on PC to yield γ-linolenic acid. Temperature is known to have an effect on the levels of polyunsaturated fatty acids, and in developing seeds of soybean the activities of the oleoyl and linoleoyl desaturases were shown to decrease dramatically when soybean pods were incubated at higher temperatures (Cheesebrough, 1989). Similarly, rape seeds were shown to synthesize higher proportions of 18:2 at the expense of 18:1 at lower temperatures and lower light intensities (Tremolieres, Dubacq and Drapier, 1982). High oleic sunflower cultivars have been developed by mutagenesis and breeding. It has been shown that the high levels of oleic acid are due to reduced levels of oleate desaturase activity (Garces and Mancha, 1989, 1991; Sperling *et al.*, 1990). Interestingly, in one type of the high-oleic mutants, the developing cotyledons emerge from the hull and become exposed to light. The exposed cotyledons were then able to desaturate oleate to linoleate.

Elongation

The elongation of oleic acid to icosenoic (20:1), erucic (22:1) and sometimes nervonic (24:1) acids has been measured in the developing seeds of several species, including nasturtium (Pollard and Stumpf, 1980a; Murphy and Mukherjee, 1988), meadowfoam (Pollard and Stumpf, 1980b), jojoba (Pollard *et al.*, 1979), *Brassica juncea* (Agrawal and Stumpf, 1985) honesty (Fehling and Mukherjee, 1991) and mustard (Fehling, Murphy and Mukherjee, 1990). Some of the reactions of the elongase system have been studied, although, as with the desaturation systems, none of the enzymes have been purified to homogeneity. The intracellular location of the developing seed elongases has not yet been established, but the 18:0 and 20:0 elongases of leek leaves are believed to reside in the ER and Golgi bodies, respectively (Moreau *et al.*, 1986). The seed elongases are often found in the 15 000 × *g* pellet from homogenates of developing embryos, although the subcellular

origin of this fraction has yet to be determined (Murphy and Mukherjee, 1988). The elongase reaction in honesty (*Lunaria annua*) was shown to occur in a similar manner to the mammalian system, i.e. via the condensation of acyl-CoA and malonyl-CoA to yield β-ketoacyl-CoA which is reduced, dehydrated and then reduced again in an analogous way to the fatty acid synthetase system, to form an elongated acyl chain (Fehling and Mukherjee, 1991). Elongases from honesty (Fehling and Mukherjee, 1991) and jojoba (Pollard *et al.*, 1979) have a broad specificity with regard to the chain length of the acyl-CoA, which would suggest that the pattern of products in the storage oil observed *in vivo* broadly reflects the distribution of acyl groups in the substrate pool of the elongase rather than the substrate selectivity of the elongase.

Hydroxylation

The hydroxylation of oleic acid in the synthesis of ricinoleic acid (*cis*-9-12-hydroxy-octadecaenoic acid; 18:1-OH) has been studied in a microsomal fraction from castor bean (Galliard and Stumpf, 1966; Moreau and Stumpf, 1981). The hydroxylase in microsomes is quite labile and appears to require heat-sensitive factors from the post-microsomal supernatant, including catalase. It was suggested that, like desaturation, the hydroxylation of oleic acid occurs when esterified to PC, although very little ricinoleoyl-PC could be detected (Moreau and Stumpf, 1981). If, indeed, hydroxylation occurs on oleate that is esterified to PC, then the plant must have some mechanism to prevent mixing of the ricinoleoyl-PC with the general membrane phospholipids, since the latter lipids contain very low levels of ricinoleate moieties. The components of the electron transport system involved in oleate hydroxylation have not been established, although it appears that neither P_{450} nor other cytochromes are involved. Hydroxylation reactions in developing seeds of *Wrightia tinctoria* and *W. coccinea* by which isoricinoleic (9-hydroxy-*cis*-12-octadecaenoic) acid is formed have also been investigated (Ahmad, Schiller and Mukherjee, 1986; Ahmad and Mukherjee, 1988).

SYNTHESIS OF TRIACYLGLYCEROLS

In seeds, triacylglycerols are synthesized by the Kennedy pathway in a compartment probably derived from the endoplasmic reticulum, which is found in microsomal fractions of homogenates of developing seeds (*Figure 2*). In recent years, attention has been focused on the acyl-chain specificity of the acyltransferases of the Kennedy pathway, since this plays a major part in the distribution of acyl moieties in the triacylglycerols. The distribution of acyl moieties in the acyl-CoA pool of the microsomal membranes also plays an important role in determining the fatty acid distribution in TAGs but, due to the technical difficulties involved in accurately measuring such pools, very little work has been carried out in this area. The distribution of acyl moieties in the acyl-CoA pool will be a product of the rates of synthesis of each acyl-CoA species from the different pathways outlined above, and the rate of

incorporation of each of these acyl-CoA species into acylglycerols by the acyltransferases (*Figure 2*). In addition to this, it is possible that there will be feedback control from the acyl-CoA pool to the enzymes involved in their synthesis, which will ultimately regulate the concentration of each constituent member of the acyl-CoA pool. In some species the stereospecific pattern of fatty acids esterified to glycerol is not random. For instance, cocoa butter has its distinctive properties due to the large amounts of triacylglycerols with palmitate, oleate and stearate in the *sn*-1,2 and 3 positions, respectively (Padley, Gunstone and Harwood, 1986). In the seeds of borage, palmitate is found mainly in the *sn*-1 position and γ-linolenate is excluded from the *sn*-1 position but is about equally distributed between the *sn*-2 and *sn*-3 positions. Thus it is clear that in some species the selectivity of all three acyltransferases is important in determining the fatty acid distribution in triacylglycerols (Griffiths, Stymne and Stobart, 1988). In studies on all three Kennedy pathway acyltransferases from safflower (Ichihara, 1984; Ichihara, Asalin and Fujii, 1987; Ichihara, Takahashi and Fujii, 1988) and rape (Bernerth and Frentzen, 1990) it was found that the composition of fatty acids at the *sn*-1 position is predominantly affected by the pool sizes of the mixture of acyl-CoAs. The composition of acyl moieties in the *sn*-2 position in both species is governed by the selectivity of the *lyso*-phosphatidic acid acyltransferase (LPA-AT) towards different acyl-CoAs. In high erucic rape, the LPA-AT selects strongly against erucic acid so that the erucic acid in rape oil is esterified almost exclusively to the *sn*-1 and *sn*-3 positions. The composition of the *sn*-3 position, like that of the *sn*-1 position, depends on the concentrations of the constituents of the acyl-CoA pool, although the selectivity of diacylglycerol acyltransferase (DAG-AT) of some varieties of rape might also exert some influence on composition (Bernerth and Frentzen, 1990). The LPA-AT is an important target enzyme for manipulation for those aiming to modify rapeseed oil content. If rape oil containing erucic acid in all three positions is to be produced to improve on the present theoretical limit of 67%, then the selectivity of the LPA-AT must be altered so that it will use erucoyl-CoA. Work on this is already under way in several laboratories. For example, oil from nasturtium contains trierucin, indicating that the LPA-AT can use erucoyl-CoA (Whitfield and Murphy, 1990). In *Limnanthes alba* and *L. douglasii* erucic acid is highly enriched in the *sn*-2 position (Cao, Oo and Huang, 1990; Löhden, Bernerth and Frentzen, 1990), which again indicates that the LPA-AT in these plants can use erucic acid. The ultimate aim of these projects is to isolate the LPA-AT from these species, in order to clone the gene encoding the protein and to express the gene in a high erucic acid oilseed such as rape, in the hope that the developing seeds will then be able to synthesize appreciable quantities of trierucin. To date, none of the microsomal acyltransferases have yet been purified from developing embryos of oilseeds.

The other enzyme of the Kennedy pathway, the phosphatidate phosphatase (*Figure 2*; Ichihara, Norikura and Fujii, 1989) was shown to have very little influence over the fatty acid composition of triacylglycerols in safflower (Ichihara, 1991). It was proposed, however, that the TAG synthesis might be

positively affected by the attachment of the phosphatidate phosphatase to the microsomal membrane or reduced by its detachment (Ichihara, Murota and Fujii, 1990). The process was shown to be controlled by the levels of palmitic, stearic or oleic acids. An increase in concentration of these fatty acids would cause a movement of the enzyme from the cytosol to the microsome, thereby allowing increased flux through to triacylglycerol. It is not clear, however, if the intracellular concentrations of fatty acid *in vivo* would be high enough to cause such an effect, since 0·5–1·0 mM was used in the experiments. Since so little is known about the control of TAG biosynthesis, this observation warrants further investigation.

There are many potential biochemical problems which might occur in the development of genetically engineered rape or sunflower to synthesize oils containing unusual fatty acids. Some of these potential problems were described as a possible scenario for a group trying to insert the gene for a short-chain thioesterase from an animal into an oilseed plant (Knauf, 1987). One area that is likely to be a problem in a transgenic plant which is synthesizing a novel fatty acid is that of the selectivity of the acyltransferases of the Kennedy pathway. It was found that, using pure lauroly-CoA or erucoyl-CoA, microsomes of rape and maize could make LPA but the LPA could not be further metabolized (Sun, Cao and Huang, 1988), again indicating that the glycerol-3-phosphate acyltransferase (G3P-AT) had a much more relaxed specificity for the species of acyl moiety than the LPA-AT. The DAG-AT from rape and maize was shown to use lauroly-CoA and erucoyl-CoA to form TAG (Cao and Huang, 1986, 1987), whereas the LPA-AT has a very strict selectivity, discriminating strongly against lauroyl moieties (Oo and Huang, 1989). Therefore, if rape is genetically engineered to synthesize medium-chain-length fatty acids, for instance, the expression of an LPA-AT that can use medium-chain CoAs as substrate would probably also be required in the developing embryo.

Another potential problem is that of keeping the unusual fatty acids out of the membrane phospholipids, where their presence may adversely affect the structural integrity of the membranes. Studies on lipid synthesis with developing embryos of *Cuphea lanceolata*, which has mainly capric acid (10:0) in the triacylglycerols, showed that the specificities of the LPA-AT, and the CDP-choline:diacylglycerol cholinephosphotransferase, prevent the medium-chain fatty acids from entering PC (Bafor *et al.*, 1990). Thus, the medium-chain fatty acids are directed exclusively to triacylglycerol synthesis in *Cuphea* seeds. In another study, homogenates from developing seeds of safflower were fed lauric and erucic acid, which the TAG synthesis system of the developing embryos would not normally encounter. The lauric and erucic acids were incorporated into triacylglycerols, albeit at much reduced rates compared to palmitic or oleic acids, but the enzymes involved in synthesizing phospholipids clearly excluded them from the membrane lipids (Battey and Ohlrogge, 1989). This implies that the acyltransferases in such seeds are able to incorporate unusual fatty acids into storage triacylglycerols, but such fatty acids are discriminated against by the enzymes responsible for membrane lipid synthesis. It is possible that there are two sets of acyltransferases in the

microsomal compartment of developing embryos, one dedicated to triacyl-glycerol synthesis and the other to membrane lipid synthesis. On the one hand, these studies are encouraging in that they show that developing seeds appear to contain biochemical mechanisms that exclude unusual fatty acids from the membrane lipids. However, they do highlight the need to incorporate genes encoding acyltransferases that will accept unusual fatty acids if the endogenous acyltransferases cannot use them efficiently enough. To this end it is important that the genes for the microsomal acyltransferases are cloned from crop plants and those plants whose seeds contain acyltransferases that have the required characteristics.

Seed storage proteins

Apart from the oil, the other important product of oilseeds is the protein meal left behind after oil extraction. The protein from soybean is used in products for human and animal consumption, although those from other crops tend to be used mainly as fodder supplements for domestic animals. The protein meal, however, is often dietetically poor for several reasons. The levels of sulphur-containing amino acids is low, especially in soybean. The proteins may be poorly digested due to resistance to denaturation, the presence of protease inhibitors in the meal, or the presence of tannins or phytates which precipitate storage proteins (Sgarbieri and Whitaker, 1982). The aims of altering seed protein quality are generally to increase the levels of sulphur-containing amino acids (especially methionine), to improve the digestibility of the proteins and to remove toxins such as glucosinolates and protease inhibitors from the meal. The genetic alteration of storage proteins in legumes has been reviewed by Bliss (1990).

The major storage proteins in seeds occur in two size classes, the 11–12S globulins and the 2S albumins. Soybean also has multi-subunit, glycosylated 7S proteins known as vicilins. The induction of transcription of the genes for seed storage proteins can be caused by abscisic acid (ABA) (Finkelstein *et al.*, 1985; Bewly and Marcus, 1990), although treatment of isolated embryos with either osmoticum, desiccation or jasmonic acid has been shown to induce expression of storage protein genes. Post-transcriptional regulation of storage protein gene expression in soybean has also been demonstrated (Walling, Drews and Goldberg, 1986). Such regulation may be quite strong, since it has been found recently that cultured gametophytic embryos of rape do not accumulate storage proteins, although the genes for the storage proteins are transcribed and the mRNAs accumulate in the presence of ABA (Taylor *et al.*, 1990). The expression of seed storage proteins in developing embryos has received much research attention. Not only are these proteins of economic importance but, since they are synthesized in such large amounts, they offer a relatively simple system for the study of the regulation of gene expression with regard to their organ specificity, their temporal regulation, and also the protein processing and targeting systems of the ER and Golgi. The primary sequences of the storage proteins of many plants' seeds have been determined directly by amino acid sequencing and from genomic and cDNA clones from

rape (Ericson *et al.*, 1986; Schofield and Crouch, 1987; Rodin *et al.*, 1990), sunflower (Allen *et al.*, 1987), soybean (Doyle *et al.*, 1986) and maize (Pederson *et al.*, 1982). These storage proteins are encoded by multi-gene families. Sequence comparisons have shown homology between the genes for distantly related species (Allen *et al.*, 1987). The existence of sequence homologies between the albumin storage proteins from ostrich fern with napin, the 2S albumins from *Brassica* species, shows that the ancestors of these genes were present before the divergence of pteridophytes from the line leading to angiosperms (Templeman, Demaggio and Stein, 1986).

Heterologous expression of a seed storage protein was first demonstrated for phaseolin from *Phaseolus vulgaris* which was transferred to sunflower (Murai *et al.*, 1983). Phaseolin was also expressed in transgenic tobacco and was correctly targeted to the protein bodies of the embryo and endosperm cells (Greenwood and Chrispeels, 1985; Sengupta-Gopolan *et al.*, 1985). Similar work has since been carried out with the 15 kDa zein gene from corn, which was expressed in tobacco under the regulation of the phaseolin gene flanking regions (Hoffman *et al.*, 1987). The transformed tobacco was shown to synthesize the zein in a tissue-specific manner and at the correct time during embryo development. The zein accumulated in the protein bodies of the transgenic tobacco seeds, which was surprising since protein bodies are formed by different mechanisms in monocots and dicots. The gene for a 19 kDa zein from maize was expressed in petunia but, although the gene was efficiently transcribed, very little zein protein accumulated (Williamson *et al.*, 1988). In addition, it was found that low levels of mRNA for the transgenic zein protein accumulated in the leaves, stems and flowers of the transgenic plants (Ueng *et al.*, 1988). The gene for a soybean storage protein has also been expressed in developing tobacco embryos (Barker, Harada and Goldberg, 1988). *In situ* hybridization showed that the mRNA was detectable at highest concentration in the storage parenchyma of the cotyledons, but was not detectable in the leaf or endosperm of the developing seed. It is clear that, like other developmentally regulated genes, the genes for seed storage proteins contain regions in the promoters (*cis*-acting elements) which interact with proteins (*trans*-acting factors) to control the spatial and temporal specificity of their expression. These factors are also highly conserved between plants, allowing faithful expression in heterologous systems (Goldberg, Barker and Perez-Grau, 1989; Bewley and Marcus, 1990).

Seed storage proteins are of considerable dietetic importance and a lot of fundamental work is being carried out to improve their nutritional quality. One of the major aims is to increase the amount of the sulphur-containing amino acids, and especially the levels of methionine in soybean. Several approaches are being taken but until it is possible to regenerate transformed soybean plants which are stable, the final stages of these projects cannot be completed. So far, most work on increasing the methionine content of seed protein has used tobacco as the host plant. Although not a commercially useful source of seed proteins, tobacco offers a facile model system for such molecular genetic studies. Zeins (sulphur-rich prolamines) are rich in methionine and, as mentioned earlier in this section, the 15 kDa zein expressed in

transgenic tobacco seeds reached as much as 1.6% of total seed protein (Hoffman *et al.*, 1987). There were, however, some problems for the plant during early post-germinational growth, since the zein appeared resistant to hydrolysis by the tobacco proteases. In another approach, the phaseolin gene was modified by *in vitro* mutagenesis to increase the number of methionines in the gene product from three to nine. This modified gene was transformed into tobacco (Hoffman, Donaldson and Herman, 1988). As a control, an unmodified phaseolin gene was transformed into separate tobacco plants in parallel experiments. Both the high methionine ('himet') and unmodified phaseolin genes were expressed in the embryos of the transgenic tobacco plants at the correct stage of development. However, although the normal phaseolin accumulated in protein bodies in the transgenic tobacco seeds, the 'himet' phaseolin did not. The 'himet' phaseolin mRNA was translated at a normal rate, and immunogold labelling revealed that the 'himet' protein was synthesized as normal and passed through the ER and Golgi processing systems but then did not accumulate in the protein bodies. It was concluded that the 'himet' protein was hydrolysed in either Golgi vesicles, or soon after it had reached the protein body. This work outlines problems that might be expected when attempting to engineer genetically altered crop proteins.

Other efforts to increase the amount of methionine in seed proteins have involved the expression of the gene for the 2S albumin from brazil nut (*Bertholletica excelsa* HBK), in tobacco seeds (Altenbach *et al.*, 1989). The 2S albumin from brazil nut contains 18% methionine (Ampe *et al.*, 1986; Altenbach *et al.*, 1987; Sun, Altenbach and Leung, 1987) and has a similar structure to the 2S albumins from other plants. The nutritional quality of bean or soy flour was improved in rat and quail feeding experiments when brazil nut meal or purified 2S protein was included (Antunes and Markakis, 1977; Tao *et al.*, 1987). Constructs, again using the phaseolin promoter driving the gene for the brazil nut 2S albumin, were made and tobacco plants transformed. The protein was found to accumulate in tobacco seeds and resulted in a 30% increase in the levels of methionine in the seed (Altenbach *et al.*, 1989).

The 12S and 2S storage proteins of *Arabidopsis* have also been studied and their gene families characterized (Krebbers *et al.*, 1988; Pang, Pruitt and Meyerowitz, 1988). The expression and processing of the *Arabidopsis* 2S albumin in tobacco was reported recently (De Clercq *et al.*, 1990). Recently it has been shown that it might be possible to use oilseed proteins as a vehicle for the biosynthesis of valuable peptides. A construct for the 2S albumin containing a sequence encoding an enkaphalin (a bioactive peptide) was transformed into *Arabidopsis thaliana*. The enkephalin would be synthesized within the structure of the *Arabidopsis* 2S albumin so that it would be synthesized at high rates and be targeted to the storage protein body of the seed, where it would be much less subject to proteolytic degradation than if it remained in the cytoplasm. The sequence of the modified 2S albumin gene also encoded trypsin and carboxypeptidase cleavage sites so the enkephalin could be recovered easily from the mature seeds (Van de Kerckhove *et al.*, 1989). The modified gene was successfully transcribed and translated and the

enkephalin was purified from the storage 2S protein of the seed after treatment with trypsin and carboxypeptidase. This trial using *Arabidopsis* as a model system shows that oilseeds might also be grown for the mass production of a wide range of high-value peptides by including them in the structure of the storage proteins.

Embryogenesis

EARLY EMBRYO DEVELOPMENT

The storage oil and protein bodies of oil seeds are located in either the seed endosperm or in the cotyledons of the embryo. During fertilization, the male and female gametes unite to produce a zygote, which then undergoes cell division and differentiation to form the cotyledons and the axis, which contains the shoot and root meristems. Another fertilization occurs at about the same time as the first, where a male gamete unites with the diploid nucleus of the embryo sac to form a triploid endosperm. In some species, such as oilseed rape or soybean, the endosperm acts as a source of nutrients during embryogenesis but is absorbed before or during seed desiccation, leaving the cotyledons as the sole source of nutrients from stored oil and protein to support early growth of the plant after germination. In other plants, such as castor bean, the endosperm accumulates most of the storage oil and protein and persists through desiccation and dormancy as a major seed tissue which acts as the major source of carbon for seedling growth after germination. In these latter cases, the cotyledons remain relatively small. For a thorough description of seed development from fertilization to dormancy, the reader is directed to a book by Raghavan (1986) and to a review on the regulation of gene expression during embryo development (Goldberg, Barker and Perez-Grau, 1989).

GAMETOPHYTIC EMBRYOS

Although most studies on embryo development have been carried out on zygotic embryos, microspore-derived embryos offer many possibilities as a model system for studying gene expression and storage product synthesis during embryo development. Microspores, precursors of mature pollen, are isolated from anthers at a specific stage of development (Polsoni, Kott and Beversdorf, 1988). Cultivation under appropriate conditions will result in their development through all the stages of embryogenesis, except desiccation, and these embryos may eventually form seedlings which can be grown up into haploid plants. Large quantities of such embryos can be cultured from just one flower bud and therefore might be a good starting material for the purification of embryo-specific enzymes. Microspores and pro-embryos derived from microspores of rape have been transformed by co-cultivation with *Agrobacterium tumefaciens* (Pechan, 1989). Using such methods, it might, therefore, be possible to study the effects of transformation with

seed-specific genes within a few weeks, rather than having to wait months for transformed tissues to regenerate to whole plants, flower and set seed (Polsoni, Kott and Beversdorf, 1988). Studies on the synthesis of storage proteins and oils have already been reported (Taylor *et al.*, 1990; Wilen *et al.*, 1990; Pomeroy *et al.*, 1991). Oil synthesis in gametophytic rape embryos is found to proceed in a similar way to that found in zygotic embryos. In gametophytic embryos cultured on basal media, the major oil-body proteins (oleosins) are synthesized at reduced levels compared to zygotic embryos. The addition of 10 µM abscisic acid (ABA) results in a considerable increase in the levels of oleosin mRNA and protein. In contrast, although transcription of the genes for the storage proteins, napin and cruciferin, can be induced by ABA, and the mRNAs accumulate, very little translation occurs and protein bodies are not formed. This implies that there may be different sets of developmental signals involved in both pre- and post-transcriptional regulation of expression of the genes involved in triacylglycerol formation, napin and cruciferin accumulation, and oleosin synthesis. On a more immediately applicable note, plant-breeding strategies can be accelerated and simplified by using such embryos, and microspore cultivation methods have already been, or are in the process of being, developed for several of the more agriculturally important oilseed species, such as rape (Chuong and Beversdorf, 1985), sunflower (Gürel, Nichterlein and Friedt, 1991) and linseed (Nichterlein *et al.*, 1989).

STORAGE PRODUCT SYNTHESIS

The development of normal zygotic rape embryos both *in planta* and in culture has been studied in some detail and may serve as a useful model system for oilseeds in general (Norton and Harris, 1975; Crouch and Sussex, 1981; Murphy and Cummins, 1989b; Murphy, Cummins and Ryan, 1989). In the early stages of development, the zygote differentiates to form the cotyledons and the embryo axis, passing through the globular, heart and torpedo stages. This period is typified by a great deal of cell division, most of which goes to form the cotyledons. During the next phase, storage oil and proteins are synthesized from imported sucrose and amino acids, and little further cell division occurs. Cell walls also act as a store of complex carbohydrates which can be a significant source of carbon during germination and growth of the seedling. It has been found that the timing of synthesis of the different classes of storage products in oilseed rape is staggered (Murphy and Cummins, 1989b; Murphy, Cummins and Ryan, 1989). The initiation and peak rate of synthesis of triacylglycerols appears to occur some time before that of the storage proteins napin and cruciferin. Oleosins, the proteins which coat oil bodies and prevent coalescence of the oil during desiccation of the seed, are synthesized later still.

The newly synthesized oil accumulates as droplets which do not appear to have a membrane coat and the protein to oil ratio in these immature oil bodies is about 0·1%. The diameter of the droplets is between 1 and 3 µm at this stage. Later in rape embryo development, oleosins are synthesized and

these form an annulus around the oil droplets. During this period, the diameter of the droplet is observed to decrease to a mean of about 0.4 μm. As the oil droplets mature into oil bodies, they accumulate protein, such that in the dry seed the protein to oil ratio of the oil bodies is about 12% (Cummins and Murphy, 1990). During the final stage of embryogenesis, the embryos dehydrate, losing more than 95% of their water content and late-embryo-abundant proteins (LEA) are synthesized. The LEAs are thought to help protect the cell against the potentially damaging effects of dehydration.

GENE EXPRESSION DURING SEED DEVELOPMENT

During embryo development, between 15 000 and 18 000 genes are expressed in soybean and cotton (Galau and Dure, 1981; Goldberg et al., 1981a). The majority of these are constitutive genes, expressed in all tissues of the plant throughout its life cycle. A significant number of such genes are, however, expressed only during embryogenesis (Goldberg, Barker and Perez-Grau, 1989), with many of them being expressed only at specific stages during embryo development. For example, during the mid-maturation phase of embryo development, about 5–10 superabundant mRNAs are synthesized which encode the seed storage proteins. These few RNAs comprise about 50% of the total mRNA mass in soybean (Goldberg et al., 1981b). The study of seed storage protein gene expression has shown that it is temporally regulated, tissue specific and spatially controlled within the embryo. Studies on heterologous expression of storage protein genes in transgenic plants has shown that the genes contain a number of DNA sequence motifs in their non-coding regions which regulate the temporal and spatial specificity of their expression. For instance, the genes encoding a lectin, β-conglycinin and a trypsin inhibitor protein, normally expressed in soybean cotyledons, were all expressed specifically in transgenic tobacco embryos at the correct time (Bray et al., 1987; Barker, Harada and Goldberg, 1988). The genes encoding hordein from barley endosperm and zein from maize endosperm, were expressed specifically in the endosperm of tobacco, again at the correct time (Marris et al., 1988; Schernthaner, Matzke and Matzke, 1988). The accumulation of seed storage proteins is also regulated by post-transcriptional processes through effects on the mRNA, either by controlling its export to the cytoplasm from the nucleus or via differences in its stability (Goldberg et al., 1981a; Chappell and Chrispeels, 1986; Walling, Drews and Goldberg, 1986).

ABSCISIC ACID AND EMBRYO MATURATION

Abscisic acid (ABA) and/or osmotic potential have been found to affect the synthesis of seed storage proteins, to prevent viviparous germination and to confer tolerance to desiccation in developing embryos (Bray and Beachy, 1985; Finkelstein et al., 1985; Finkelstein and Crouch, 1986). It is not clear, however, whether ABA causes the induction of storage protein gene expression directly. Work with rape indicates that osmoticum does not induce an increase in ABA concentration (Finkelstein and Crouch, 1986). When the

osmoticum was not present, however, a higher than normal level of exogenous ABA was required to induce the expression of storage protein genes. Recent work with alfalfa has shown that the effect of osmoticum in inducing the synthesis of embryo-specific storage proteins could not be duplicated by added ABA (Xu, Coulter and Bewley, 1990). Measurements of the levels of ABA in cultured embryos suggest that ABA does not mediate the effects of osmotic agents in the medium (Finkelstein and Crouch, 1986). The mechanisms involved in ABA or osmotic induction of seed storage protein gene expression are not yet clear, but the responsiveness of embryos to a given concentration of ABA changes during embryogenesis and appears to be important in the regulation of seed development (Koornneef *et al.*, 1989). Studies with cultured zygotic embryos of rape (Finkelstein *et al.*, 1985) and also with ABA-synthesis and ABA-responsiveness mutants of *Arabidopsis* (Koornneef *et al.*, 1989) indicate that the primary effect of ABA is to inhibit water uptake and it is the more negative osmotic potential which causes expression of the genes for storage proteins. Further studies on the ABA-insensitive mutants of *Arabidopsis* suggest that there are at least two parallel pathways controlling ABA responses. It has been suggested that apparent 'leakiness' in the ABA mutants might indicate the involvement of other mediators affecting the same secondary messenger pathway as ABA. It was reported recently that jasmonic acid (a plant growth regulator measured in developing rape embryos) also stimulates the expression of the genes for storage proteins in embryos of rape and linseed (Wilen *et al.*, 1991). The picture is further complicated by the findings of studies with gametophytic embryos of rape, which revealed that, although the genes for storage proteins were transcribed in the presence of ABA or osmoticum (Taylor *et al.*, 1990; Wilen *et al.*, 1990), the storage proteins themselves were not accumulated, indicating that either the mRNAs were not translated or the proteins were rapidly turned over. It would thus appear that a maternal influence other than ABA or osmoticum is present which affects translation of storage protein mRNA.

OIL SYNTHESIS DURING EMBRYOGENESIS

The accumulation of oil begins after the period of concerted cell division has ended. On a per embryo basis, the rate of accumulation of oil increases dramatically over a period of just a few days in rape (Murphy and Cummins, 1989b). The activity of the enzyme which catalyses the first committed step of fatty acid biosynthesis (acetyl-CoA carboxylase) increases at the same time as the increase in overall lipid synthesis (Turnham and Northcote., 1983). Similarly, levels of ACP increase in developing soybean seeds (Ohlrogge and Kuo, 1984) and there is a general increase in the activity of the glycolytic pathway in castor bean during the period of lipid synthesis, which would supply the carbon for fatty acid synthesis (Ireland and Dennis, 1981). Indeed, it has been argued that really the induction of oil synthesis does not look nearly as dramatic when compared with other pathways, since much of embryo metabolism increases in activity during this period (Ohlrogge,

Browse and Somerville, 1991). It remains to be seen whether there is a general increase in expression of genes for 'constitutive' lipid synthesis enzymes in line with the increase in metabolism, or whether parallel sets of seed-specific genes are transcribed for lipid synthesis as discussed on pp. 18–19 There is evidence that at least some genes encoding lipid synthesis enzymes may be expressed specifically in seeds, or at very much higher levels than in other tissues. For example, it has been found that specific ACP genes are expressed during embryogenesis in rape (de Silva *et al.*, 1990) and that ACP gene expression in the developing seed was 25 times higher than in leaves. One of the stearoyl ACP desaturase genes was also found to be much more highly expressed in developing seeds of cucumber than in leaves or roots, where expression was barely detectable (Shanklin and Somerville, 1991).

Whereas most of the enzymes involved in lipid synthesis are found in all tissues, DAG-acyltransferase is unique to the synthesis of triacylglycerols and is likely to be induced at mid-maturation in developing seeds. In addition, genes encoding enzymes involved in the synthesis of unusual TAG-specific fatty acids are probably also expressed specifically during the oil synthesis stage of embryogenesis. Examples of such enzymes would include the hydroxylase responsible for the formation of ricinoleic acid in castor bean, elongases catalysing the formation of very-long-chain fatty acids in many cruciferous oilseeds, or the medium-chain acyl-ACP thioesterases responsible for the formation of lauric acid in the seeds of the California Bay tree. It is clear from measurements of product accumulation, in rape at least, that the peaks of rates of synthesis of storage oil, storage protein and oil-body protein are temporally separated (Murphy and Cummins, 1989b), and it was proposed that this was due to temporal specificity of expression of the genes involved (Murphy, 1989). This idea has been supported by recent work in the authors' laboratory on analysis of mRNA levels for napin and oleosin during embryo development of a field-grown rapeseed crop. These studies have shown that, whereas napin mRNA levels reach a peak during mid-maturation and thereafter decline significantly, the levels of oleosin mRNA rise later than those of napin and continue to rise after mid-maturation phase, only reaching a peak when seed maturity is completed (Murphy *et al.*, 1991b). Very recently, Northern blot analysis of mRNAs for enoyl-ACP reductase, ACP and napin at various stages of rape embryo development showed that the peak level of mRNA for enoyl-ACP reductase occurred before that of the mRNA for napin (Kater *et al.*, 1991). The authors suggest that the activity of enoyl-ACP reductase is regulated at the level of transcription. The pattern of levels of mRNAs for the enoyl-ACP reductase, ACP and napin observed supports the notion that the temporal pattern of storage product accumulation previously observed in developing rape embryos (Murphy and Cummins, 1989b) is controlled directly by temporal differences in expression of the genes involved.

Oil synthesis in gametophytic embryos of rape did not require the addition of ABA or the presence of osmoticum (Taylor *et al.*, 1990; Pomeroy *et al.*, 1991), and the pattern of fatty acids in the triacylglycerols was similar to that

in vivo in both high and low erucic acid varieties. On the other hand, zygotic embryos of rape cultured in the absence of added ABA or osmoticum synthesized TAGs containing less 20:1 and 22:1 and more 18:2 and 18:3 than grown embryos *in vivo*. The inclusion of ABA or osmoticum caused an increase in the levels of 20:1 and 22:1 but the 18:2 and 18:3 levels remained high compared to the situation *in vivo*, leaving a relatively depleted level of 18:1 (Finkelstein and Somerville, 1990). In contrast, an increase in sucrose concentration caused a decrease in the linoleic and linolenic acid levels in embryos of *Theobroma cacao* and a corresponding increase in stearic and oleic acids (Pence, Hasegawa and Janick, 1981). These results imply that, while exogenous ABA or osmotica do not significantly affect the timing or magnitude of storage oil accumulation in embryos, they may modulate to some extent the quality (i.e. the fatty acid composition) of such embryos.

Methods for obtaining seed oils with required fatty acid composition

In attempting to obtain oils with novel fatty acid profiles, there is a choice of either domesticating a new crop with a desirable oil composition, traditional breeding and selection in an empirical manner to amplify or delete gene functions, or using genetic engineering to manipulate an already successful crop (e.g. rapeseed or sunflower) to produce novel and useful types of seed oil.

DOMESTICATION OF PLANTS WITH USEFUL SEED OILS

A project run by the US Department of Agriculture has investigated the possibility of domesticating plants whose seeds contain industrially useful fatty acids. Thousands of species of plant were screened and numerous species of interest were found to produce seed containing useful fatty acids in the storage oil. Some of these plants are now grown as oilseed crops, as discussed by Princen (1989) and as outlined below. Crambe and meadowfoam provide very-long-chain fatty acids; jojoba produces long-chain wax esters instead of triacylgylcerols. *Lesquerella* has been tried as an alternative to castor bean as a source of hydroxy fatty acids. Many other possible candidates for development have been described (Princen, 1983). The most important aspect of these projects is the development of agronomic features which will ensure that the crops can be grown commercially for the seed oil. For example, several members of the genus *Cuphea*, have economically valuable short- and medium-chain-length fatty acids in their storage triacylglycerols. Despite intensive efforts over many years, however, it has not been possible to develop convenient agronomic features in *Cuphea* to allow its cultivation as an oilseed crop. This has mainly been due to problems of pod shattering, seed stickiness and a long flowering period (Hirsinger, 1985).

BREEDING AND MUTAGENESIS–SELECTION PROGRAMMES

Most of the advances in changing oilseed crops to date have been gained by conventional breeding programmes. Novel genetic material is incorporated either from other cultivars of the same species or from closely related sexually compatible species which have the required trait. A notable success in the breeding of an oilseed with a novel oil composition was the development of low erucic acid cultivars of rape from plants which had practically no erucic acid (Stefansson, 1983). This changed rape oil from a product grown for non-edible purposes to one for human consumption. Interestingly, there is now a series of breeding programmes aimed at increasing the erucic acid levels still further in high erucic acid rape in order to increase the value of the oil as an industrial feedstock as an alternative to high erucic oil from *Crambe*. In another breeding strategy known as wide crossing, traits are transferred from plants of another genus (Goodman *et al.*, 1987). This method usually requires embryo-rescue techniques whereby embryos are removed from the plant and grown in culture media since the seeds often fail to develop correctly *in planta* (Krauter, Steinmetz and Friedt, 1991).

A common method for obtaining novel mixtures of fatty acids in seed oils is by mutagenesis and screening seed oil of the progeny for alteration in the pattern of fatty acids (Downey, 1987). The quality of soybean oil has been improved by mutagenesis and selection programmes which have resulted in the reduction of the linolenic acid to about 4% (Hammond and Fehr, 1983). High stearic acid mutants have also been bred (Bubeck, Fehr and Hammond, 1989) in order to improve the quality of margarine made from soybean oil. Sunflower oils containing more than 90% oleic acid have been developed (Fick, 1989). Such oils are potentially useful as industrial feedstocks, since pure oleic acid can be extracted from them fairly easily. High-oleic safflower, a superior cooking oil to normal high-linoleic safflower oil, has also been bred (Ladd and Knowles, 1971). Another notable success in the use of mutagenesis has been in the development of a double mutant of linolenate synthesis in linseed. Normal linseed oil contains greater than 50% linolenic acid and is unsuitable for human consumption but is used for a wide range of non-edible purposes. The double mutant of linseed cannot efficiently desaturate linoleic acid so the oil has very low levels of linolenic acid and is suitable for use in a variety of edible products (Green and Marshall, 1984; Green, 1986). Other mutagenesis/breeding programmes to reduce the linolenic acid content in rape oil have yielded lines with between 3 and 5% linolenic acid (Rakow, 1973; Robbelen and Nitsch, 1975).

GENETIC ENGINEERING OF SEED OILS

If it is hoped to alter the quality of seed oil by means of genetic engineering, it will be necessary to up- or down-regulate certain important gene functions and, in some cases, add or delete selected genes. To this end, the genes encoding the enzymes of interest must first be cloned. Intensive efforts to

clone lipid-related genes from oilseeds are now under way in numerous research institutes and industrial laboratories.

The plastidic enzymes catalysing the reactions of fatty acid biosynthesis are soluble, and some have already been purified and their genes or cDNAs encoding them have been cloned. The enzymes involved in the latter, extra-plastidic, steps of lipid metabolism are microsomally bound and have proved very difficult to isolate by normal membrane protein purification methods. The cloning of genes for the acyltransferases and desaturases may therefore require other approaches. Several strategies to achieve this are being used and are listed below.

Arabidopsis *as a tool for molecular genetics*

A genetic approach to cloning the genes for enzymes that have proved to be intractable to conventional protein purification is being used with *Arabidopsis thaliana*. This plant is a good one to use for many reasons, not least because it is the target of a world-wide collaboration to map and sequence its complete genome during the next decade. The benefits of using *Arabidopsis* as a model organism have been expounded recently by Meyerowitz (1987). Mutants of *Arabidopsis* have been created by both EMS (ethyl methane sulphonate) mutagenesis (James and Dooner, 1990; Lemieux *et al.*, 1990) and transposon mutagenesis (Browse, Miquel and Somerville, 1990). Screening of large numbers of seeds has revealed mutants deficient in elongases, oleate and linoleate desaturases, as well as others with elevated palmitic and stearic acids. Although it will require a lot of further research, the genes mutated by the EMS mutagenesis can be isolated using chromosome-walking methods once they have been genetically mapped (Meyerowitz, 1987; Browse, Miquel and Somerville, 1990). In the transposon mutagenesis studies (Browse, Miquel and Somerville, 1990), those genes mutated by insertion of a transposon may be cloned by transposon tagging methods (Balcells, Swinburne and Coupland, 1991). It has been pointed out, however, that given the low number of T-DNA inserts in the mutant lines reported from the transposon mutagenesis studies, background mutation might account for some of the mutations observed, and hence these mutants may not necessarily be tagged and therefore may not be useful in the cloning of these genes (Browse, Miquel and Somerville, 1990). Once the genes for the elongases and desaturases have been cloned from *Arabidopsis* it is hoped that the genes encoding these enzymes in economically important oilseed plants can be cloned using heterologous probes derived from the *Arabidopsis* sequences. This strategy is particularly attractive for cloning genes from oilseed rape because both *Arabidopsis* and rape are closely related members of the Cruciferae and hence the likelihood of using heterologous gene probes is much higher than for more distantly related species of oilseed.

Isolation of genes using heterologous probes

Antibodies raised against (or cDNAs encoding for) soluble enzymes of plastidic lipid metabolism have been used to probe for genes or their proteins which catalyse similar reactions in microsomal membranes. Such an approach is being used in our laboratory to try and isolate genes for the microsomal G3P- and LPA-acyltransferases (Fawcett *et al.*, 1990; Slocombe and Murphy, 1990). Antibodies raised against the plastidic acyl-ACP:G3P-acyltransferase in pea (Douady, Passaquet and Dubacq, 1990) have been found to cross-react with a protein in carbonate-washed microsomes from rape, indicating that a membrane-bound acyltransferase shows some homology with the soluble enzyme from pea chloroplasts. In addition, screening of a genomic library from rape with an oligonucleotide probe made from a cucumber cDNA sequence for the soluble G3P-acyltransferase (Ishizaki *et al.*, 1988) has revealed three classes of clones based on their restriction pattern (Fawcett *et al.*, 1990). These putative clones are now being analysed further in our laboratory.

Complementation of mutants

The gene for a Δ^{12} oleate desaturase has been cloned by complementation from a mutant of the cyanobacterium *Synechocystis* (Wada, Gombos and Murata, 1990) using an *in situ* transformation method. The gene has been sequenced and the authors state that other genes from *Synechocystis* have now been cloned which are homologous to the Δ^{12} desaturase (Wada, Sakamoto and Murata, 1990). This desaturase is a plastid type which inserts a double bond into the Δ^{12} position of an oleic acid residue esterified to galactolipid. It remains to be seen if enough DNA sequence similarity exists between the Δ^{12} oleate desaturase of *Synechocystis* and Δ^{12} desaturases from higher plants for it to be possible to use such a strategy to clone the latter genes as well. Provided that enough DNA sequence homology exists, it might be possible to clone desaturases from higher plants by making probes using these prokaryotic sequences.

Future prospects for genetic engineering

Once the genes encoding the lipid-related enzymes of interest have been cloned, the prospects for transforming oilseed rape or sunflower to express these genes are very good. A variety of methods is available for transforming plants, including *Agrobacterium*-mediated DNA transfer to explants of tissue (Everett, Robinson and Mascarenhas, 1987; Fry, Barnason and Horsch, 1987; Moloney, Walker and Sharmer, 1989), microinjection of DNA directly into cells (Neuhaus *et al.*, 1987), DNA uptake into protoplasts mediated by electroporation (Potrykus *et al.*, 1985; Guerche *et al.*, 1987; Weber *et al.*, 1989) or even the delivery of DNA-tipped projectiles into tissues via a ballistics method (Klein *et al.*, 1988). Soybean has been transformed using a particle gun to deliver DNA (McCabe *et al.*, 1988). However, the transforma-

tion of soybean is not yet routine, with the regeneration of stable transformants remaining a problem. The development of such transformation systems for other legumes, such as alfalfa (D'Halluin, Botterman and de Greef, 1990) and pea (T.J. Higgins, personal communication), give grounds for optimism in this regard.

Expression of heterologous genes in a tissue-specific manner has already been demonstrated for genes encoding seed storage proteins as described on pp. 20–21. It appears that as little as 200 bp of the 5' promotor element is required to obtain seed-specific expression of a storage protein gene (Goldberg, Barker and Perez-Grau, 1989). This work also showed that promotors can often be recognized, albeit at attenuated levels, in heterologous systems. If a novel gene is introduced into a transgenic plant, it is also important to have the correct temporal specificity of expression, since during the development of rape embryos at least, the peak rates of storage oil, storage protein and oleosin synthesis are temporally separated (Murphy and Cummins, 1989b). The expression of the genes encoding storage proteins and oleosins all appear to be regulated to some extent by ABA or by another factor using the same secondary messenger system (Wilen *et al.*, 1991). In contrast, the onset of oil synthesis does not appear to be influenced by ABA and the factor(s) that control the timing of its expression have yet to be established.

Other aspects of oilseed biotechnology which will present problems have already been alluded to. For example, it may not be possible simply to add another gene for a novel activity and expect necessarily to obtain elevated levels of the desired end-product, since other enzymes in the seed using the same substrate will probably have to be down-regulated at the same time. The selectivity of the acyltransferases may not be wide enough to take a novel fatty acid, so that genes for the acyltransferases which can utilize the introduced fatty acid must also be inserted into the transgenic oilseed. Some functions such as desaturation or hydroxylation also require electron transport systems to transfer reducing equivalents to the enzyme–substrate complex and these too might need to be transferred to the transgenic oilseed.

In conclusion, it should be said that, at the moment, the advances in molecular biology are outstripping those of biochemistry. In the future many biotechnological possibilities will exist with regard to the manipulation of the quality of oil in seeds, but for a knowledge-based approach, more resources must first be invested in research on the fundamental biochemistry of lipid metabolism if we are to realize current aspirations aimed at producing a wide variety of genetically engineered 'designer' oilseed crops.

Other oilseed products of biotechnological interest

Oilseeds offer other products which may also be of biotechnological interest. Oilseeds are a source of lipases which can be used as catalysts for bioconversions in industry or in the laboratory. Oilseeds also contain oleosins, proteins that surround the storage oil in the dry seed. These proteins are natural emulsifiers and may well be useful in the preparation of emulsions and foams in the food industry.

LIPASES

Enzymes, such as lipases, have been found to catalyse reactions in organic solvents, provided that a small amount of water is present in the system (Zaks and Klibanov, 1988). Reverse reactions to hydrolysis are possible under such conditions, allowing useful reactions to be catalysed. Since lipids are soluble in organic solvents, the use of lipases in catalysing the interconversion of triacylglycerols and other lipids has received considerable interest in recent years (Macrae and Hammond, 1985; Mukherjee, 1990). The possibility of using plant lipases was already being investigated early this century (Jalander, 1911). Lipases have been used to catalyse esterification of fatty acids and alcohols or acylglycerols and in interesterification of triacylglycerols. Examples of this are the interesterification of cheap vegetable oils to produce cocoa butter substitutes for use in food and cosmetics (Yokozeki *et al.*, 1982; Wisdom *et al.*, 1984). In light of the inherent fatty acid and stereo-selectivity shown by lipases (Sonnet, 1988) they are also used in the kinetic resolution of enantiomers in chiral synthesis, allowing the separation of products that would otherwise be extremely difficult to separate by conventional physio-chemical methods (Kirchner, Scollar and Klibanov, 1985). An example of this is a much cheaper and simpler method for the production of optically pure stereoisomers of β-blocker drugs which reduce side-effects caused by the other isomer (Kloosterman *et al.*, 1988). The immobilized lipases used in these systems are derived from fungi, but another source of lipases recently considered is from the reserve tissues of young oilseed plants, since they contain lipase with high activity (Hills and Mukherjee, 1990). Lipase was isolated from rapeseed cotyledons and precipitated onto Celite, which acted as an inert support, allowing removal of the lipase after completion of the reaction. The rape lipase was found to have novel specificity towards fatty acids in esterification reactions, specifically discriminating against those fatty acids with *cis*-4 or *cis*-6 double bonds, such as petroselinic or γ-linolenic acids (Hills, Kiewitt and Mukherjee, 1990b). This interesting specificity was utilized to produce concentrates of γ-linolenic acid from evening primrose oil by kinetic resolution. All the other fatty acids from the oil except γ-linolenic acid were esterified to butanol using the rape lipase as catalyst, leaving the γ-linolenic acid unesterified. The concentration of γ-linolenic acid was increased from less than 10% to over 80% in one step (Hills, Kiewitt and Mukherjee, 1989). Docosahexaenoic acid from cod liver oil was concentrated in a similar fashion (Hills, Kiewitt and Mukherjee, 1990a). It has been suggested that lipases from other oilseed plants might have different specific-ities which might prove useful in such reactions (Huang, Lin and Wang, 1988).

OLEOSINS

Oleosins are a novel class of protein from oilseeds (Murphy *et al.*, 1991). They are associated specifically with the oil bodies containing the storage triacyl-glycerols (Herman, 1987; Vance and Huang, 1987; Murphy and Cummins,

1989a; Murphy, Cummins and Kang, 1989). Oleosins have been purified from several species, and cDNA or amino-acid sequences have been determined from rape and radish (Murphy *et al.*, 1991a), maize (Vance and Huang, 1987; Qu and Huang, 1990), carrot (Hatzopoulos *et al.*, 1990) and soy (E.M. Herman, personal communication). All oleosins have similar amino-acid sequences, with relatively hydrophilic N- and C-terminal regions but with a stretch of 70 non-polar amino acids in the middle of the protein. The central hydrophobic domain exhibits considerable amino-acid sequence similarity between species. It is likely that the oleosins act as emulsifiers by inserting their hydrophobic domains into the triacylglycerol, with the hydrophilic domains protruding into the aqueous environment. The oleosins probably prevent coalescence of oil droplets during desiccation of the seed and may also contain a lipase-binding site to allow the lipase access to the TAG during early growth of the plant after germination (Murphy *et al.*, 1991a). The non-viability of some stored seeds has been shown to be due to the coalescence of oil during storage, which prevented access of lipases (van Standen, Gilliland and Brown, 1975). Since the oleosins act so efficiently in preventing oil coalescence even under the large pressure present in the dry seeds, they may have applications in the food and other industries as emulsifying agents.

Conclusions and future prospects

In this review, we have described the major classes of useful products derived from oilseeds and have summarized current knowledge concerning the mechanisms of their formation during seed development. With the continued expansion of the market for oilseed products, there is an increasing recognition of the need to develop new varieties of oilseed crops for both current and novel edible and non-edible applications. This need will be fulfilled by combining the tried and tested methods of conventional plant breeding with the new technologies for molecular genetic manipulation. For the oilseed biotechnologist, it is particularly fortunate that the species selected as the major model system for much of the new plant developmental and molecular genetic research, *Arabidopsis thaliana*, is itself an oilseed which is, moreover, closely related to the major oilseed crop, rapeseed.

The new molecular technologies will complement existing expertise in genetics and breeding to provide formidable tools for genetic engineering. Probably the major bottleneck in engineering new oilseed varieties is not at the level of molecular genetics, but is rather in the area of biochemistry. Recognition of this problem has been shown recently by the implementation of a number of research programmes in Europe and in the Americas, aimed at elucidating the fundamental biochemical methods which underlie the formation of oilseed storage products, such as oils and proteins. This encouraging development gives ground for optimism that by the turn of the century oilseed biotechnology will indeed have come of age.

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