Manipulation of lipid metabolism in transgenic plants: biotechnological goals and biochemical realities
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Introduction

One of the major objectives of modern plant biotechnology is to manipulate the amount and quality of seed storage lipids, particularly for medium to high-value industrial applications. In biochemical terms, this involves the manipulation of the biosynthetic pathways involved in storage triacylglycerol formation. The conventional approach to doing this has been to clone genes that are believed to be involved in the biosynthesis of novel seed oils from donor plant species and to transfer these genes into existing successful oilseed crop species that are readily transformable, such as rapeseed or soybean. However, it is becoming increasingly evident that our expertise in molecular genetic manipulation sometimes considerably outstrips our knowledge of lipid biosynthetic pathways in plants. Although some modest successes have been achieved in altering seed oil content, particularly in rapeseed and soybean, it is now apparent that some aspects of the metabolic pathways of storage triacylglycerol formation and their regulation are much more complex than was suspected hitherto. This has stimulated an increased emphasis on the use of transgenic plants for the study of lipid biochemistry, a process which has been facilitated by the recent isolation of numerous genes involved in plant fatty acid biosynthesis and modification. In this article, recent progress in the use of transgenic plants in the study of plant lipid metabolism and its manipulation will be briefly surveyed.

Fatty acid biosynthesis and modification

Fatty acid biosynthesis de novo in plants is believed to occur mainly or exclusively via a dissociable multi-subunit, prokaryotic-like fatty acid synthetase located in plants. This normally involves the sequential addition of two-carbon units derived from acetyl-CoA in a series of eight condensation cycles to produce the C16, acyl ester palmitoyl-ACP (ACP, acyl carrier protein), although in a few plant species premature chain termination can result in the formation of C14-C16 short/medium-chain fatty acids, as shown in Figure 1. In the majority of lipid-storing plant tissues, palmitoyl-ACP is elongated to the C18, stearoyl-ACP, followed by desaturation to oleoyl-ACP, with both reactions occurring in the plastid. Oleate can then undergo a number of modification reactions on the endoplasmic reticulum to produce polyunsaturates, epoxides, wax esters, hydroxides, conjugates or very-long-chain fatty acids. The fatty acid composition of a given plant storage oil often depends upon the presence or absence of a small number of these oleate-modifying enzymes, some of which are only found in a very narrow range of plant species. The perceived relative simplicity of fatty acid biosynthesis and modification in plants, coupled with the ever increasing economic importance of many vegetable oil products, means that this has been one of the first areas of eukaryotic metabolism to be subject to widespread attempts at pathway engineering. Some of the recent projects in this field are described below.

Polyhydroxybutyrate (PHB) and acetyl-CoA carboxylase

The precursor for acyl lipid formation in all organisms is acetyl-CoA. In some bacteria, such as Alcaligenes eutrophus, acetyl-CoA can be metabolized to a commercially valuable polymeric storage compound, polyhydroxybutyrate (PHB), rather than more conventional storage lipids such as triacylglycerols. The pathway for PHB formation from acetyl-CoA involves three enzymes, as shown in Figure 2. The transfer of the acetoacetyl-CoA reductase and PHB synthase genes into Arabidopsis resulted in the accumulation of small quantities of PHB granules throughout the cells in leaves of the transgenic plants. Unfortunately, many of the transgenic plants were severely stunted. This may be due to the diversion of acetoacetyl-CoA from mevalonate synthesis to PHB formation and/or adverse effects of PHB granule accumulation in certain parts of the cell or in certain plant tissues. Therefore, it was decided to transfer all three PHB biosynthetic genes (including the 3-ketothiolase) to rapeseed under the control of seed-specific gene promoters and containing plastid targeting sequences, in order to ensure that PHB accumulates only in the plastids of seed tissues. Due to the high levels of storage triacylglycerol for-
Pathways for fatty acid biosynthesis and modification of plants

Many of the genes encoding the enzymes involved in these reactions have recently been cloned from various plant species. In several cases, these genes have then been introduced into oilseed crops (normally rapeseed), either in the sense or antisense orientation, in order to effect changes in seed oil composition. The results of these experiments have been invaluable in elucidating the mechanism and regulation of fatty acid and triacylglycerol formation in plants, as described in the text.

During the past two years, acetyl-CoA carboxylase genes encoding polypeptides of 220-240 kDa have been isolated from a wide variety of plant species including rapeseed [7], alfalfa [8], wheat [6,9] and maize [10,11]. These high-molecular-mass forms of plant acetyl-CoA carboxylase are similar to the 220-224 kDa multifunctional polypeptides found in animals and fungi [3]. In order to down-regulate expression of the acetyl-CoA carboxylase gene in rapeseed plants, a partial cDNA encoding part of this gene could be inserted in the antisense orientation into rapeseed explants [6]. Unfortunately, very recent studies indicate that there may be two completely different forms of acetyl-CoA carboxylase in plants [12]. The high-molecular-mass multifunctional polypeptide described above appears to be a minor form of the enzyme in plants, which accounts for only ~20% of the total acetyl-CoA carboxylase activity in leaves and is located mainly in epidermal tissues [12], where it is presumably involved in providing malonyl-CoA for fatty acid elongation for cuticular wax biosynthesis. The major form of acetyl-CoA carboxylase in plants is a plastid-located (and probably partially plastid-encoded) multisubunit enzyme, more similar to that found in prokaryotes [13]. Therefore, in order to suppress the plastid acetyl-CoA carboxylase, which is the target enzyme for the rapeseed PHB project, it now appears that it may be necessary to down-regulate one or more of
Pathway of formation in the bacterium *Alcaligenes eutrophus*

Only one of these enzymes, 3-ketothiolase (acetyl-CoA acetyltransferase), is found in higher plants, where it is involved in the synthesis of isoprenoids via mevalonate. Genes encoding the remaining two enzymes have been isolated from *A. eutrophus* and introduced into *Arabidopsis* and rapeseed in order to obtain PHB accumulation in plants.

**Medium-chain fatty acids**

In species such as California bay and *Cuphea*, the activity of a tissue-specific medium-chain thioester hydrolase (MCH) leads to the premature fatty acid chain termination and the accumulation of high levels of medium-chain (C_{10}-C_{12}) triacylglycerols in the seeds. The isolation of an MCH gene from California bay and its transfer to rapeseed caused a dramatic alteration in the composition of the seed oil of the transgenic plants from virtually undetectable levels of C_{12} lauric acid to 30–40% of total seed oil [14]. Although the presence of MCH is vital for medium-chain oil formation, it is likely that other enzymes such as acyltransferases or 3-ketoacyl-ACP synthetases also play an important role [15,16]. In order to obtain commercially realistic levels of lauric acid (i.e. > 50%) in transgenic rapeseed, it may therefore be necessary to isolate and transfer these additional genes from California bay or another appropriate donor species.

**Saturated fatty acids**

Saturated fatty acids are relatively uncommon in most plant storage lipids, due to the presence in developing seeds of highly active desaturases. For example, rapeseed oil contains ~95% C_{18} fatty acids, of which only 2–4% is the saturated C_{18} stearic acid. In conventional rapeseed cultivars, stearoyl-ACP is desaturated to oleoyl-ACP by a Δ^9 desaturase. This gene was isolated from rapeseed and a truncated cDNA in the antisense orientation was transferred to the same species, resulting in an increase of stearate levels in the seed oil from <2% to as much as 40% [17]. Like the medium-chain fatty acids described above, the production of transgenic rapeseed with levels of stearate greater than 40% may require the transfer of additional genes. For example, rapeseed acyltransferases, and particularly the lysophosphatidic acid (LPA) acyltransferase, appear to discriminate against saturated fatty acids [17]. Therefore, the transfer of genes encoding alternative acyltransferases that more readily accept stearate, e.g. from cocoa, may be required to obtain the desired high-saturate oil composition in rapeseed.

**Petroselinic acid**

Petroselinic acid is an isomer of oleic acid with its double bond in the Δ^8 rather than the Δ^9 position on the acyl chain. It is a potentially valuable industrial feedstock for the manufacture of adipic acid for polymer production and lauric acid for detergent production [18]. Several years ago, the desaturase gene believed to be involved in the formation of...
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Possible pathways involved in the conversion of palmitoyl-ACP into either oleate or petroselinate and their subsequent incorporation into either storage triacylglycerols or membrane lipids

Oleate is formed by the action of three enzymes: 3-ketoyl-ACP synthase II (KAS-II); \( \Delta^9 \)-stearyl-ACP desaturase (\( \Delta^9 \) DES); and oleoyl-ACP hydrolase (OAH). Petroselinate formation may require the activity of three additional enzymes: \( \Delta^4 \)-palmitoyl-ACP desaturase (\( \Delta^4 \) DES), a novel form of 3-ketoyl-ACP synthetase II' (KAS-II'), and a petroseloyl-ACP hydrolase (PAH) [21]. Oleate and petroselinate are then incorporated onto glycerol backbones by the enzymes glycerol 3-phosphate acyltransferase (G3P-AT); LPA acyltransferase (LPA-AT) and diacylglycerol acyltransferase (DAG-AT).

Petroselanic acid was isolated from coriander seed cDNA libraries in our own laboratory [19] and by Cahoon et al. [20]. It was initially expected that the new coriander desaturase, which was similar to \( \Delta^6 \)-stearyl-ACP desaturases previously isolated from other plants, would use the same substrate but insert the double bond in the \( \Delta^6 \) rather than the \( \Delta^9 \) position. However, the introduction of this desaturase gene, driven by the cauliflower mosaic virus 35S promoter, into tobacco explants resulted in the accumulation of only minute amounts of petroselinc and \( \Delta^4 \)-hexadecenoic acids in callus cultures [20].

More recent biochemical data from Ohlrogge et al. [21–24] indicate that the pathway of petroselinc acid formation may be much more complex than was believed initially. Far from simply having a \( \Delta^6 \)-stearyl-ACP desaturase substituting for a \( \Delta^6 \)-stearyl-ACP desaturase, it now appears that coriander contains a \( \Delta^4 \) desaturase which converts palmitoyl-ACP into \( \Delta^4 \)-hexadecenoyl-ACP plus two additional enzymes required for elongation of the latter to petroseloyl-ACP and its subsequent thiolytic cleavage (Figure 3). Therefore, contrary to the original assumption that petroselinc acid production could be engineered in transgenic plants by the transfer of a single acyl-ACP desaturase gene, it now appears that at least three genes may be required [21]. In addition, it is possible that one or more of the rapeese acyltransferases involved in triacylglycerol assembly may discriminate against petroselinc acid. This may require the transfer of yet more coriander genes in order to obtain the high levels of petroselinc required for commercial use of this fatty acid in the oil of transgenic rapeese plants. To complicate the picture still further, the four or more coriander enzymes transferred to rapeese would have to compete for palmitoyl-ACP with the endogenous rapeese enzymes involved in oleate formation, such as 3-ketoacyl-ACP synthase II' and the \( \Delta^6 \)-stearyl-ACP desaturase. In order to facilitate petroselinate formation, it may therefore be necessary to down-regulate (e.g. using antisense methods) one or more of these endogenous rapeese genes. Clearly, the production of a high-petroselinate rapeese is likely to be no mean achievement!

**Polyunsaturated fatty acids**

Genes encoding the endoplasmic reticulum and plastid isoforms of the \( \Delta^{12} \)-oleate desaturase and \( \omega^3 \) linoleate desaturase responsible for polyunsaturate formation have recently been cloned from several high plants [25–27]. These desaturases are all integral membrane proteins that use phospholipids or galactolipids as substrates. Polyunsaturated fatty acids are believed to be important components of plant cell membranes and may play a role in such physiological processes as chilling tolerance and salt tolerance. In addition, these fatty acids are components of many seed oils where their presence may be detrimental to (e.g. \( \alpha \)-linolenic acid in edible
oils) or enhance (e.g. linoleic acid in some edible oils) the quality of the oil. The recent availability of these desaturase genes has led to preliminary efforts to study the role of desaturases in plant lipid metabolism using transgenic plants. For example, \( \alpha_3 \)-linolenate desaturase genes have been transferred to *Arabidopsis* and carrot plants, resulting in an increase in total \( \alpha_3 \)-desaturase enzyme activity and the accumulation of more \( \alpha_1 \)-linolenate in both membrane and storage lipids [25,26]. These data are consistent with the conclusion from earlier studies with *Arabidopsis* mutants that the \( \alpha_1 \) desaturase reactions are rate-limiting steps in \( \alpha_3 \)-linolenate biosynthesis [28]. At present there are many additional experiments underway involving the transfer of sense or antisense copies of these desaturase genes into plants [1]. The properties of the resulting transgenic plants will doubtless elucidate the metabolism and function of polyunsaturates in the near future.

**Acyltransferases**

Acyltransferases are responsible for the transfer of acyl residues from acylthioesters to glycerol backbones during the assembly of both membrane and storage acyl lipids. There is considerable evidence from *in vitro* and compositional studies that acyltransferases from certain plants exhibit high degrees of substrate specificity. For example, the LPA acyltransferase of rapeseed appears to discriminate strongly against erucoyl-CoA as a substrate, leading to only 2–5% of erucate on the sn-2 position of the triacylglycerol, while the sn-1 and sn-3 positions, which are served by different acyltransferases, can contain as much as 80% erucate. So far, the only acyltransferase gene to have been isolated is the glycerol 3-phosphate acyltransferase (G3P-AT). The transfer of the gene for this enzyme from squash and *Arabidopsis* into tobacco [29], and of the *E. coli* gene into *Arabidopsis* [30], led to compositional changes in the fatty acids of chloroplast lipids. This is consistent with a role for acyltransferases in regulating the acyl composition of plant membrane and storage lipids. The challenge ahead is to determine the extent and importance of the role of acyltransferase, particularly with regard to the modification of seed oil composition. This question can only be addressed conclusively when genes encoding the remaining endoplasmic-reticulum acyltransferases have been isolated and transferred to transgenic plants.

The types of environmental stresses, both natural and anthropomorphic in origin, and physiological stresses that a plant is subjected to throughout its life are manifold. Examples are low temperatures combined with high light intensities, drought conditions, damage caused by pathogens, air- and water-borne pollutants and premature senescence [1,2].

The physiological basis of the damage suffered by a plant during stress can often be explained as perturbations in oxygen metabolism leading to enhanced production of reactive oxygen intermediates (ROIs; hydrogen peroxide, superoxide anion and the hydroxyl radical). Most of the information available concerns the performance of photosynthesis in the leaf under stress [3]. Disruption of the flow of energy from light-harvesting reactions, through electron-transport chains to CO2 fixation, leads to 'leakage' of electrons to oxygen, causing the production of ROIs. If the protective mechanisms of the cell are compromised or overwhelmed, the effects on the plant are termed photo-oxidative damage. recognize that during growth and to remove superoxide and hydrogen peroxide by reducing them to water by the concerted action of superoxide dismutases (SODs; EC 1.15.1.1) and catalases or peroxidases. In the chloroplast, the oxidative stress is to minimize production of ROIs catalysed by peroxidases. In the chloroplast, the role of superoxide dismutases (SODs; EC 1.15.1.1) and catalases or peroxidases. In the chloroplast, the

Abbreviations used: APX, ascorbate peroxidase; CaMV, cauliflower mosaic virus; GR, glutathione reductase; ROI, reactive oxygen intermediate; SOD, superoxide dismutase.

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