The production of vegetable oils with novel properties: Using genomic tools to probe and manipulate plant fatty acid metabolism

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1 Introduction

Before the development of the petroleum industry, plant oils were the main source of aliphatic carbon compounds for industrial purposes. Currently, only about 2% of the plant seed oil produced in the world is used for non-edible purposes [1]. Non-food plant oils are usually derived from specialty crops such as castor or tung, or by the chemical modification of food oils for specific industrial uses such as paints or PVC plasticizers. However, almost all of the aliphatic carbon used for non-food purposes is currently derived from non-renewable carbon resources. With the advent of biotechnology, it has become possible to consider genetically manipulating the fatty acid metabolism in the seeds of crop plants to produce a renewable source of carbon feedstocks for industrial chemicals and polymers. One approach is to genetically alter the relative amounts of the five fatty acids [palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1Δ^{9}), linoleic acid (C18:2$\Delta^{9,12}$), and α-linolenic acid (C18:3$\Delta^{9,12,15}$)] that typically contribute to vegetable oils of temperate crops. For example, the development of oleic acid-rich oils in crops such as soybean has opened up new markets for vegetable oils as oxidatively stable, biodegradable lubricants [2]. An alternative approach, and the focus of this article, is the metabolic engineering of biosynthetic pathways for unusual fatty acids with potential industrial value in seeds of existing crops. The first step in this process, the cloning of the genes responsible for the synthesis of unusual fatty acids, has been greatly facilitated by the advent of modern genomics. The current technical limitation to the production of many industrial oils is the efficient incorporation of these new fatty acids into the triacylglycerols of traditional oilseed crops. It is likely that the use of genomics will also play a major role in overcoming this hurdle and thus make new, non-food crops a reality.

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2 Plant diversity and novel gene sources

The plant kingdom contains a deep reservoir of genes for the production of a wide array of vegetable oils for industrial applications. More than 200 different unusual fatty acids have been identified in plants [3, 4]. These fatty acids can contain more or fewer than the 16 and 18 carbon atoms found in the common fatty acids of vegetable oils [*e.g.*, erucic acid (C22:1Δ^{13}) and lauric acid (C12:0)]. In addition, the double bonds of these fatty acids may be at positions other than the typical Δ^{9}, Δ^{12}, and Δ^{15} sites of unsaturation [*e.g.*, petroselinic acid (C18:1Δ^{6})], and the double bonds may be in the non-typical *trans* configuration [*e.g.*, calendic acid (C18:3$\Delta^{8trans,10trans,12cis}$)]. Furthermore, unusual fatty acids can contain functional groups such as hydroxyl, epoxy, and acetylenic residues [ricinoleic acid (12-hydroxy-C18:1Δ^{9}), vernolic acid (12-epoxy-C18:1Δ^{9}), and crepenynic acid (12-acetylenic-C18:1Δ^{9})]. The occurrence of these fatty acids is primarily restricted to seeds, where they are typically the major components of triacylglycerols. Two exceptional examples of this are the seed oils of castor (*Ricinus communis*), consisting of approximately 90% ricinoleic acid [3, 4], and *Bernardia pulchella*, consisting of approximately 90% vernolic acid [5]. A specific unusual fatty acid can occur widely in a given family. For example, nearly all members of the Apiaceae and Araliaceae families accumulate high levels of petroselinic acid in their seed oils. Similarly, a high content of very long-chain fatty acids ($\geq C_{20}$) is characteristic of seed oils of Brassicaceae species. Alternatively, many different unusual fatty acids can occur within a single family. For example, vernolic acid, calendic acid, crepenynic acid, dimorphecolic acid (9-hydroxy-C18:2$\Delta^{10trans,12trans}$), coronaric acid (9-epoxy-C18:1Δ^{12cis}), and helenyolic acid (9-hydroxy-12-acetylenic-C18:1$\Delta^{10trans}$) are among the fatty acids that occur in seed oils of certain species of the Asteraceae family [3, 4].

The chemical structures of unusual fatty acids often impart useful industrial functionalities to vegetable oils rich in these aliphatic molecules. For example, castor oil rich in hydroxy fatty acids is used as a high-performance lubricant; tung oil rich in conjugated fatty acids is used as

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a drying agent; and *Vernonia* oil rich in epoxy fatty acids has been shown to have potential use in the synthesis of industrial polymers [6].

3 Identifying useful fatty acid metabolism genes

The first challenge to engineering the production of unusual fatty acids in the seeds of existing crops is the identification of genes for the corresponding biosynthetic enzymes. Historically, this was a slow and laborious process that began with radiolabeling studies aimed at elucidating the biosynthetic pathway of a given unusual fatty acid. This was followed by enzyme purification, which was often met with failure due to the membrane-bound nature of many unusual fatty acid biosynthetic enzymes. The advent of high-throughput DNA sequencing, however, has mitigated the need for these two steps. It has become increasingly apparent from published studies that genes associated with unusual fatty acid biosynthesis are moderately to highly expressed in developing seeds (*e.g.* [7]). This property is particularly advantageous for the use of expressed sequence tag (EST) analysis to identify cDNA for unusual fatty acid biosynthetic enzymes. By sequencing the 5' end of as few as 500 randomly chosen cDNA from developing seeds that produce high levels of an unusual fatty acid, it has been possible to obtain a cDNA for the responsible biosynthetic enzyme. This process does not require any prior knowledge of the biosynthetic pathway for the unusual fatty acid. Even when the biosynthetic pathway for an unusual fatty acid is known, EST analysis is often a much more rapid means for identifying cDNA that encode the corresponding biosynthetic enzyme(s). For example, biochemical studies implicated a cytochrome P450 as the epoxygenase that catalyzes vernolic acid synthesis from linoleic acid in *Euphorbia lagascae* seeds [8]. Cytochrome P450 enzymes in plants are membrane associated and thus recalcitrant to purification. In addition, the cytochrome P450 family of enzymes is very large and diverse in plants (*e.g.*, over 250 cytochrome P450 genes occur in *Arabidopsis*) [9]. This makes the isolation of a cytochrome P450 epoxygenase cDNA extremely difficult through the use of PCR amplification with degenerate oligonucleotides. As an alternative approach, the cDNA for the *E. lagascae* epoxygenase was identified as one of two cytochrome P450 classes that occurred in approximately 1,000 EST from developing seeds of this plant [10].

Once a list of expressed sequences has been obtained from a collection of expressed sequences, it can be searched, using appropriate software, for the sequences of genes of interest. Many of the enzymes that catalyze the formation of interesting functional groups are related to the microsomal Δ12 fatty acid desaturases (FAD2 desaturase family) found in oilseed crops [8, 11–17]. The DNA and protein sequences of many of these FAD2 desaturases are known, and distinctive, highly conserved motifs have been identified [18]. Thus, candidate genes that putatively control the formation of hydroxyl, acetylenic, epoxy and other functional groups can be identified based on their homology with FAD2 desaturase genes from soybean, canola, maize, and other plants.

4 Desaturase-related gene families

A phylogenetic tree for many of these FAD2-like desaturases is shown in Fig. 1. The first desaturase-related enzyme to be identified, the hydroxylase from castor, had the substrate and regio-specificites of Δ^{12}-desaturases [7]. That is, the preferred substrate for FAD2 desaturases and Δ^{12}-hydroxylase is oleic acid, and the functional group is inserted at the Δ^{12} position on the carbon chain. The first FAD2-related enzyme identified that had a different substrate specificity was the epoxygenase from *Vernonia galamensis*, whose substrate is linoleic acid [16]. The epoxygenase converts the double bond at carbons 12–13 to an epoxy group; thus, the synthesis of vernolic acid requires the activity of two FAD2-related enzymes, a "classic" desaturase to convert oleic to linoleic acid and a diverged FAD2 to catalyze the formation of the epoxy group at the same position.

A similar modification of linoleic acid by a second FAD2 is found in seeds of *Momordica charantia* which contain α-eleostearic acid [12]. This fatty acid contains the *trans*-Δ^{11} conjugated double bond synthesized by a diverged form of a Δ^{12} oleic acid desaturase known as a fatty acid "conjugase". A similar conjugase in *Impatiens balsamina* [12] catalyzes the conversion of α-linolenic acid to α-parinaric acid by the transformation of the *cis*-Δ^{12} double bond to a *trans*-Δ^{13} conjugated double bond. These were the first FAD2-related enzymes identified that catalyzed the formation of non-methylene-interrupted double bonds. The site of action of these desaturases was, in common with all other members of the FAD2 family identified to this point, the *cis*-Δ^{12} double bond. The activity of FAD2-type enzymes, however, is not limited to the modification of the Δ^{12} position of fatty acids. Calendic acid, the primary fatty acid of *Calendula officinalis* seeds, is a conjugated trienoic fatty acid like α-eleostearic acid, but contains *trans*-Δ^{8}, *trans*-Δ^{10}, and *cis*-Δ^{12} conjugated double bonds. This fatty acid constitutes 50–60% of the seed oil of *Calendula officinalis* and is synthesized by a FAD2-type enzyme that catalyzes the modification of the *cis*-Δ^{9} double bond of linoleic acid to form calendic acid [13].

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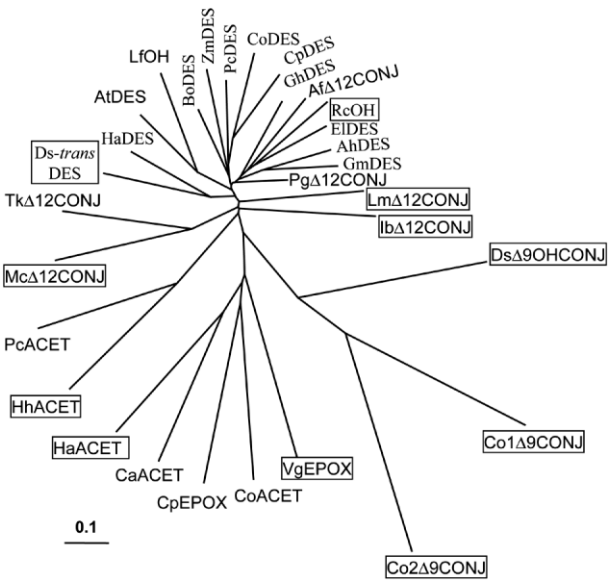

Fig. 1. Phylogenetic comparison of the FAD2 family of enzymes. The phylogenetic tree was generated through analysis of amino acid sequences by use of the neighbor-joining algorithm. The tree includes *cis*-Δ^{12}-oleic acid desaturases (DES), hydroxylases (OH), epoxygenases (EPOX), acetylenases (ACET), Δ^{9}-fatty acid conjugases (Δ^{9}CONJ), Δ^{9}-fatty acid hydroxy conjugases (Δ9OHCONJ), *trans*-Δ^{12}-oleic acid desaturases (trans-DES) and Δ^{12}-fatty acid conjugases (Δ^{12}CONJ). FAD2 polypeptides identified by expressed sequence tag (EST) analyses are boxed. The GenBank accession numbers for the amino acid sequences represented in the phylogenetic tree are: AfΔ^{12}CONJ, AAN87574; AtDES, P46313; AhDES, AAB84262; BoDES, AAC31698; CaACET, CAA76158; CoACET, CAB64256; CoDES, AAK26633; Co1Δ^{9}CONJ, AF310155; Co2Δ^{9}CONJ, AF310156; CpEPOX, CAA76156; CpDES, CAA76157; Ds-transDES (DsFAD2–1), AY494986; DsFAD2–2, AY494985; ElDES, AY486148; GhDES, CAA65744; GmDES, P48630; HaACET, AAO38032; HaDES, T14269; HhACET, AAO38031; IbΔ^{12}CONJ, AAF05915; McΔ^{12}CONJ, AAF05916; LfOH, AAC32755; LmΔ^{12}CONJ, BD224612; PcACET, AAB80697; PgΔ^{12}CONJ, AAO37753; RcOH, T09839; TkΔ^{12}CONJ, AAO37751; and ZmDES, BD235521. Af, *Aleurites fordii*; At, *Arabidopsis thaliana*; Ah, *Arachis hypogea*; Bo, *Borago officinalis*; Co, *Calendula officinalis*; Ca, *Crepis alpina*; Cp, *Crepis palaestina*; Ds, *Dimorphotheca sinuata*; El, *Euphorbia lagascae*; Gm, *Glycine max*; Gh, *Gossypium hirsutum*; Hh, *Hedera helix*; Ha, *Helianthus annuus*; Ib, *Impatiens balsamina*; Lf, *Lesquerella fendleri*; Lm, *Licania michauxii*; Mc, *Momordica charantia*; Pc, *Petroselinum crispum*; Pg, *Punica granatum*; Rc, *Ricinus communis*; Tk, *Trichosanthes kirilowii*; Vg, *Vernonia galamensis*; and Zm, *Zea mays*.

Until the discovery of this enzyme, the involvement of a FAD2-related enzyme in the modification of any double bond other than a *cis*-Δ^{12} bond was unprecedented.

Our understanding of the wide diversity of FAD2-related enzymes was further extended with the discovery of two divergent Δ^{12}-oleic acid desaturases involved in the biosynthesis of an unusual fatty acid, dimorphecolic acid (9-hydroxy-C18:2$\Delta^{10trans,12trans}$) [17]. This fatty acid is the major fatty acid of seeds of *Dimorphotheca* species and contains structural features that are not typically found in plant fatty acids, including a C-9 hydroxyl group, $\Delta^{10,12}$ conjugated double bonds, and a *trans*-Δ^{12} unsaturated bond, which raised the question of how this unusual fatty acid was synthesized. To address this question, a population of EST from developing *Dimorphotheca sinuata* seeds was generated, and from these EST, two divergent forms of the Δ^{12}-oleic acid desaturase, designated DsFAD2–1 and DsFAD2–2, were identified (Fig. 1). Although DsFAD2–1 resembled a classic FAD2-like *cis*-Δ^{12} desaturase, its expression in both *Saccharomyces cerevisiae* and soybean somatic embryos resulted in the accumulation of the *trans*-Δ^{12} isomer of linoleic acid (C18:2$\Delta^{9cis,12trans}$). When DsFAD2–1 was co-expressed with the other desaturase-like EST, DsFAD2–2, in soybean somatic embryos, dimorphecolic acid could be found in the embryo oil, presumably synthesized by the reaction of DsFAD2–2 with the C18:2$\Delta^{9cis,12trans}$ reaction product of DsFAD2–1. These results indicated that DsFAD2–2 displays a substrate preference for the *trans*-Δ^{12}, rather than the *cis*-Δ^{12}, isomer of linoleic acid, and catalyzes the conversion of the *cis*-Δ^{9} double bond of *trans*-Δ^{12} linoleic acid into a C-9 hydroxyl group and a $\Delta^{10trans}$ double bond [17]. As indicated in Fig. 1, such an enzyme can be functionally designated as a "Δ^{9}-hydroxy conjugase".

5 Advantages of EST approaches to cloning fatty acid metabolism genes

The extensive collection of divergent FAD2 polypeptides identified by EST analysis has provided a wealth of primary structural data for comparative analysis of members of this enzyme family. From amino acid sequence alignments, some simple rules have emerged for the rapid identification of FAD2-type polypeptides that catalyze activities other than the typical *cis*-Δ^{12} desaturation of oleic acid. FAD2 polypeptides are diiron-oxo enzymes that contain three histidine motifs or "boxes", which are believed to act in the coordination of active-site iron enzymes [18]. Functionally divergent FAD2 enzymes contain variant amino acid sequences in the vicinity of the first and third histidine boxes. In all typical *cis*-Δ^{12} desaturases, the first histidine box (consensus sequence HECGHH) is preceded by an alanine residue. By contrast, FAD2 sequences that contain a glycine residue in place of this alanine catalyze alternative reactions, including fatty acid hydroxylation and epoxidation. In addition, the third

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histidine box (consensus sequence HVXHH) is followed by the amino acid sequence LFSTMP in all *cis*-Δ^{12} FAD2 desaturases. Based on information gained from EST analyses, it can be said that if any variation in this motif is observed in a new FAD2 sequence, it is very likely that the corresponding enzyme catalyzes a divergent activity.

Although most unusual fatty acids are derived from the activity of one divergent enzyme, examples do exist of biosynthetic pathways for unusual fatty acids that contain two or more novel enzymes. One notable example is the biosynthesis of Δ^{5cis}-eicosenoic acid (C20:1Δ^{5cis}) in seeds of meadowfoam (*Limnanthes* sp.). This fatty acid accounts for more than 60% of the seed oil of meadowfoam, and remarkably, C_{20} and C_{22} fatty acids comprise nearly 95% of this oil [19]. Results from radiolabeling studies of meadowfoam seeds and seed extracts reported by Pollard and Stumpf [19] indicated the involvement of an acyl-CoA desaturase-type enzyme in the formation of the double bond of C20:1Δ^{5cis}, as well as a β-ketoacyl-CoA synthase (KCS) with specifity for palmitoyl (C16:0)-CoA that produces the eicosanoyl (C20:0)-CoA substrate for the Δ^5 desaturase. The use of EST for the identification of genes associated with this pathway proved to be particularly advantageous, given that there were no previous reports of the functional identification of acyl-CoA desaturases in plants [20]. Among 1,145 randomly chosen cDNA from a meadowfoam developing seed library were five sequences for a class of enzyme that was most closely related to acyl-CoA desaturases from yeast and mammalian species. As a proof of function, transgenic expression of a full-length cDNA for the meadowfoam desaturase in soybean somatic embryos was accompanied by the accumulation of novel Δ^5 monounsaturated fatty acids, including Δ^5-hexadecenoic (C16:1Δ^5) and Δ^5-octadecenoic (C18:1ddddd^5) acids. Furthermore, the biosynthetic pathway for C20:1Δ^5 was transferred to soybean somatic embryos by co-expression of the cDNA for the meadowfoam Δ^5 desaturase with a cDNA for a variant KCS that was also identified among EST generated from the meadowfoam developing cDNA seed library [20]. These results demonstrate the further utility of the EST approach for the characterization of more complex biosynthetic pathways. EST analyses can also yield unexpected results. For example, a cDNA for a structurally divergent FAD2 was identified among EST generated from developing seeds of English ivy (*Hedera helix*) [21]. Seeds of this plant, however, do not accumulate any unusual fatty acids that would be expected to arise from a FAD2-type enzyme. Interestingly, the English ivy divergent FAD2 was found to share considerable homology with a functionally uncharacterized, divergent FAD2 from parsley [22]. Expression of the gene for the parsley enzyme, which had been designated ELI12, was previously shown to be strongly induced by fungal elicitation. Similar to the EST analysis of English ivy seeds, a highly expressed cDNA for a divergent FAD2 was also identified among EST from developing seeds of sunflower inflorescences treated with *Sclerotinia sclerotiorum* (white mold) spores [21]. As was the case with English ivy, sunflower seeds were not known to accumulate any FAD2-derived unusual fatty acids. To determine the functions of the English ivy and sunflower divergent FAD2 enzymes, the corresponding cDNA were expressed in soybean somatic embryos. Surprisingly, the expression of these cDNA was accompanied by the accumulation of acetylenic (triple bond-containing) fatty acids, including crepenynic acid. Interestingly, English ivy, sunflower, and parsley do accumulate polyacetylenic secondary compounds with demonstrated pest resistance properties [23]. The initial step in the biosynthetic pathways of these compounds is the production of crepenynic acid, and it is therefore likely that these divergent FAD2 enzymes are associated with the synthesis of polyacetylenes. Such results illustrate the value of EST for providing new and unexpected insights into plant metabolism.

6 Limitations to production of novel oils in transgenic plants

In almost every case where a divergent fatty acid desaturase has been used to manipulate fatty acid metabolism, the resulting novel fatty acid content of the transgenic seed oil has been considerably less than that of the native plant from which the original gene was isolated [7, 11–13]. In addition, the viability and germination of the transgenic seed is often seriously compromised (Kinney and Cahoon, unpublished result). It is still not fully understood why plants such as *Vernonia* and *Calendula* can tolerate a relatively high abundance of unusual fatty acids in their seeds while transgenic *Arabidopsis* or soybeans containing desaturase genes from these species cannot. This difference may be related to the distribution of the unusual fatty acids within the cells of the plant in question. Plants that normally accumulate unusual fatty acids do so almost exclusively in the triacylglycerol fraction of the seed cells. In many transgenic plants containing diverged desaturases, the unusual fatty acids also accumulate in membrane phospholipids, and this may be related to the failure to accumulate significant amounts of the new fatty acids as well as the problems with seed viability [24, 25]. Thus, the target genes for fixing this problem would appear to be those that encode for enzymes that facilitate the exchange of fatty acids between membranes and either the cytoplasm or triacylglycerol. Since large families of acyl-exchange and lipase-like enzymes and their corresponding genes are known, the case for a genomic approach to solve this problem is a strong one [26, 27].

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