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# Reverse engineering of novel industrial fatty acids in seed oils

**Allan Green, Surinder Singh, Xue-Rong Zhou**
CSIRO Plant Industry, PO Box 1600, Canberra ACT 2601, Australia, allan.green@csiro.au

**Abstract**
Oilseeds such as safflower have the potential to provide renewable, cost-competitive and environmentally friendly sources of industrial oils as alternatives to those currently derived mainly from non-renewable and increasingly costly petroleum sources. In contrast to edible oilseeds that typically contain only a small number of fatty acids, there is enormous genetic diversity in fatty acid structure in nature, with many molecules having considerable industrial potential if they could be produced in large volume at low cost. Significant progress has been made in cloning genes for key enzymes responsible for the introduction of a range of new functionalities (such as acetylenic and conjugated bond systems, hydroxyl groups, and epoxy bridges) into conventional fatty acids. However, transgenic expression of these enzymes in oilseeds has consistently resulted in disappointingly low accumulation (< 20%) of the functionalised fatty acid compared to the very high levels (> 80%) typical of the species where the genes were sourced from. Biochemical analysis has revealed that the major impediment to high accumulation of unusual fatty acids (UFAs) in these transgenic oilseeds is the inefficient transfer of the UFAs from the site of synthesis on phospholipids to the principal oil storage molecule, triacylglycerol (TAG). This paper will present progress in a reverse engineering strategy to develop oilseeds that accumulate high levels of industrially useful UFAs by determining the specialised TAG assembly routes used by wild plants to achieve this, and then transgenically expressing the appropriate sets of specialised TAG assembly enzymes in oilseeds.

**Key words:** Vernolic acid – Δ12-epoxygenase – *Bernardia pulchella* – DGAT – triglyceride assembly

## Introduction

Oilseeds such as soybean, rapeseed, sunflower, cottonseed and safflower are important sources of oils used in both food and industrial applications. Because of the predominance of their food use, these oils have been selected to contain only a limited number of edible fatty acids, principally the saturated palmitic acid (C16:0) and stearic acid (C18:0), the monounsaturated oleic acid (C18:1$^{\Delta 9}$) and the polyunsaturated linoleic acid (C18:2$^{\Delta 9,12}$) and α-linolenic acid (C18:3$^{\Delta 9,12,15}$), with only minor proportions of other fatty acids, such as medium chain saturates and long-chain monounsaturates.

In contrast to this uniformity, wild plants and other organisms display an enormous range in the types of fatty acids that they synthesise and accumulate. These include variations in their carbon bond types – such as carbon triple bonds (acetylenics) or *trans* orientation double bonds rather than the normal *cis* double bonds – or the inclusion of side chains, closed carbon ring structures, oxygenated functional groups (e.g. hydroxy or epoxy) or other functionalities (e.g. halogenated). Many of these unusual fatty acids (UFAs) have interesting physical and chemical properties that make them potential alternatives to petrochemicals for a range of industrial applications.

In view of the increasingly urgent need to move away from finite petroleum-based resources, there is now considerable interest in expressing UFAs in oilseed crops to provide renewable raw materials for industry. This “crop biofactory” concept is being actively explored in Australia by CSIRO in partnership with the Grains Research and Development Corporation (GRDC) under their Crop Biofactories Initiative (CBI). CBI has cloned several genes involved in the synthesis of UFAs with either unique positional desaturation or acetylenation, or having epoxy or hydroxy functional groups at various positions in the chain, and is now developing technologies to

engineer high level synthesis and accumulation of these UFAs in transgenic oilseeds, including safflower.

**Synthesising epoxy fatty acids in seed oils**

Our initial work focussed on the synthesis of vernolic acid, an epoxy fatty acid which, although not found in oilseed crops, is known to accumulate to very high levels in seeds of several wild plants, including *Crepis palaestina*, *Vernonia galamensis*, *Stokesia laevis, Euphorbia lagascae* and *Bernardia pulchella*. Vernolic acid is synthesised by a Δ12-epoxygenase acting on linoleic acid esterified to phosphatidylcholine (PC). A gene encoding a Δ12-epoxygenase enzyme was cloned from developing seeds of *Crepis palaestina* (Lee *et al*, 1998) and studied by transgenic expression initially in *Arabidopsis thaliana* ecotype C24. Seed specific expression under the control of the napin promoter resulted in synthesis of around 6% vernolic acid in the seed oil (Singh *et al*, 2001), much lower than the 60% levels typically found in *Crepis palaestina* seed oil. In order to boost the level of linoleic acid substrate available for the epoxygenase and remove competing enzymatic reactions, a *Fad3*/*Fae1* double mutant line (MC49) was developed and used to express the *Cpal2* gene, along with a copy of the *Crepis palaestina* Δ12-desaturase gene (CpDes) to further raise linoleic substrate level (Zhou *et al*, 2006). This resulted in vernolic acid synthesis reaching to over 15% of total fatty acids (Table 1), a significant increase but still well below the levels found in *Crepis palaestina* and other vernolic-rich seed oils.

**Accumulation of UFA in seed oils**

The inability to synthesise large amounts of UFAs through transgenic expression of their catalytic enzymes is an obstacle that has also been encountered with other members of the FAD2 family of enzymes, including the Δ12-hydroxylase from castor bean, the Δ12-acetylenase from *Crepis alpina*, and the Δ12-conjugase from tung (Dyer *et al*., 2008). For example, in a similar study in Arabidopsis seeds, transgenic expression of the castor bean Δ12-hydroxylase gene produced a maximum of 19% hydroxy fatty acids (mainly ricinoleic) in the seed oil, compared to > 85% in castor bean oil (Smith *et al*., 2003).

Mounting biochemical evidence suggests that the likely barrier to high-level accumulation is the inefficient removal of the Δ12-modified fatty acids from their site of synthesis on PC and consequent poor transfer to the triacylglycerol (TAG) storage oils (Cahoon *et al*., 2006). A number of alternative metabolic routes are known for the channelling of normal fatty acids from PC to TAG. One route involves fatty acids being transferred from PC to the acyl-CoA pool by either phospholipase (PLA) or lysophosphatidylcholine acyltransferase (LPCAT) enzymes. From the acyl-CoA pool they are available for incorporation into TAG via the sequential action of the glycerol-3-phosphate acyltransferase (GPAT), lyso-phosphatidic acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT) enzymes of the Kennedy pathway. A second route involves the direct conversion of PC into a DAG molecule, by the action of enzymes such as cytidine-diphosphate-choline:1,2-diacylglycerol cholinephosphotransferase (CPT). A third route involves the direct exchange of fatty acids from the *sn*-2 position of PC to the *sn*-3 position on TAG mediated by a phosphatidylcholine-diacylglycerol acyltransferase (PDAT).

It is reasonable to speculate that the TAG assembly enzyme systems present in Arabidopsis and other oilseeds, although able to handle normal fatty acids efficiently, may not be able to work as well with the different fatty acid structures of the Δ12-modified UFAs. In contrast, the wild species that are naturally rich in these UFAs must have TAG assembly enzymes capable of handling them efficiently. To test this hypothesis, we undertook sequencing of an extensive EST library derived from developing seeds of *Bernardia pulchella*, a species that accumulates extremely high levels of vernolic acid (> 90%) in its seed oil. The aim was to clone genes for all of the key TAG assembly enzymes operating in *Bernardia* seeds and determine if they are able to increase the accumulation of vernolic acid when introduced into plants expressing the Δ12-epoxygenase transgene. Promising results were obtained when one of these transgenes, *BpDGAT2*, was transformed into the Arabidopsis line already carrying the *Cpal2*/*CpDes*

transgene combination (Table 1). Thirty independent T1 plants were established and these displayed vernolic acid contents ranging from 15% to 30%, almost double that of the parental line. As these T1 plants are expected to be hemizygous for the *BpDGAT2* gene there may be scope for further increases in the homozygous progeny.

It is interesting to note that no increase in vernolic acid was observed when the *BpDGAT1* gene was introduced (data not shown), suggesting that specialisation exists amongst the *Bernardia* DGAT enzyme family for ability to handle vernolic acid. This would be consistent with biochemical evidence showing that the DGAT1 and DGAT2 enzymes from tung tree (*Vernicia fordii*) have different acyl specificities and apparently specialised roles in TAG assembly (Shockey et al, 2006), with DGAT2 being expressed seed-specifically and having an enhanced capability for synthesis of trieleostearin, compared to the constitutively-expressed DGAT1 form. Furthermore, the tung DGAT1 and DGAT2 enzymes appear to be located in distinct, dynamic regions of the endoplasmic reticulum (ER) that do not overlap, suggesting that a specialised spatial organisation may exist for enzymes involved in synthesis of TAG containing UFAs.

**Table 1:** Fatty acid composition (% of total fatty acids) of seed oil from *Crepis palaestina*, *Bernardia pulchella*, Arabidopsis C24 ecotype and its *fad3/fae1* mutant (MC49), and T1 derivatives carrying transgenes encoding Δ12-desaturase (*CpDes*) and Δ12-epoxygenase (*Cpal2*) from *Crepis palaestina* and diacylglycerol acyltransferase (*BpDGAT2*) from *Bernardia pulchella*.

| | Fatty acid composition (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:1 | Vernolic |
| *Crepis palaestina* | 4.0 | 2.0 | 11.2 | 23.8 | tr | - | 59.0 |
| *Bernardia pulchella* | 1.3 | 0.5 | 0.3 | 3.9 | 0.4 | 0.2 | 91.2 |
| *Arabidopsis thaliana:-* | | | | | | | |
| C24 | 8.6 | 4.3 | 18.6 | 27.3 | 19.9 | 15.8 | - |
| C24+*Cpal2* | 8.2 | 5.1 | 41.5 | 4.7 | 6.6 | 23.1 | 6.2 |
| MC49 | 8.5 | 2.5 | 37.8 | 48.3 | 1.5 | 0.4 | - |
| MC49+*Cpal2*+*CpDes* | 6.6 | 4.4 | 20.0 | 49.6 | 1.4 | 0.5 | 15.4 |
| MC49+*Cpal2*+*CpDes*+*BpDGAT2* | 5.8 | 2.5 | 16.7 | 44.8 | 0.1 | - | 29.8 |

**Conclusion**

It is becoming increasingly apparent that specialised enzymes for the synthesis of UFAs and their accumulation in TAG exist in wild plants that accumulate these fatty acids. It is also clear that wild plants have found ways to combine and coordinate these enzyme systems into highly efficient biosynthetic pathways that not only enable their high level synthesis and accumulation in seed storage oils, but also ensure their exclusion from the functional cellular membrane lipids where they would be deleterious. Transgenic expression of specialised TAG assembly genes offers a promising route to reverse engineering of novel fatty acid synthesis in oilseeds. In the current study, a gene encoding the DGAT2 enzyme, responsible for the final step in TAG synthesis, cloned from a species rich in vernolic acid was demonstrated to double the accumulation of vernolic acid in seed oil when introduced into Arabidopsis already expressing the Δ12-epoxygenase enzyme.

The achievement of 30% accumulation of a UFA in Arabidopsis seed oil is a significant step along the way towards achieving high-level accumulation in oilseeds. As the basic metabolic machinery of cultivated oilseeds is essentially the same as that of oil-bearing seeds of Arabidopsis and other wild plants, the prospects appear excellent for eventual success in engineering oilseeds, such as safflower, to accumulate very high levels of industrially useful

UFAs. This will open the way for expanded and cost-competitive production of industrial fatty acids as renewable raw materials for industry, and provide new high-value cropping opportunities for grain growers.

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