Abstract

Seed-specific expression in Arabidopsis thaliana of oleate hydroxylase enzymes from castor bean and Lesquerella fendleri resulted in the accumulation of hydroxy fatty acids in the seed oil. By using various Arabidopsis mutant lines it was shown that the endoplasmic reticulum (ER) Δ12-3 desaturase and the FAE1 condensing enzyme are involved in the synthesis of polyunsaturated and very-long-chain hydroxy fatty acids, respectively. In Arabidopsis plants with an active ER Δ12-oleate desaturase the presence of hydroxy fatty acids corresponded to an increase in the levels of 18:1 and a decrease in 18:2 levels. Expression in yeast indicates that the castor hydroxylase also has a low level of desaturase activity.

Production of hydroxy fatty acids in the seeds of Arabidopsis thaliana

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Introduction

Hydroxy fatty acids accumulate in the seeds of a small number of plant species. Of these, castor bean (Ricinus communis) is the most well known, producing a seed oil containing 85–90% ricinoleic acid (18:1-OH). This fatty acid is a valuable raw material and is used in a variety of applications ranging from the manufacture of Nylon and speciality lubricants to paints and cosmetics. Castor oil is the only significant commercial source of hydroxy fatty acids but is less than ideal as the seeds contain potent toxins and allergens. There is considerable interest in finding an alternative supply of hydroxy fatty acids, either from another species of plant (such as members of the Lesquerella genus), or by the genetic engineering of an existing crop species.

In castor, 18:1-OH is formed by the direct Δ12-hydroxylation of oleic acid (18:1) esterified to position sn-2 of phosphatidylcholine [1]. The reaction is catalysed by a protein almost identical to an endoplasmic reticulum (ER) Δ12-desaturase. A cDNA encoding the castor Δ12-oleate hydroxylase has been isolated [2,3] and a similar sequence,

References


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also encoding a A12-oleate hydroxylase, was recently isolated from Lesquerella fendleri [4]. Expression of these genes in Arabidopsis thaliana resulted in the accumulation of up to four different hydroxy fatty acids in the seed oil [3,4] and indicated that the enzyme from Lesquerella was bifunctional with both A12-oleate hydroxylase and A12-oleate desaturase activities [4]. This paper describes some of our observations on the accumulation of fatty acids in Arabidopsis plants expressing the two hydroxylase enzymes.

Experimental
Sequences encoding the castor bean [2] and L. fendleri [4] A12-oleate hydroxylase and the Lesquerella hydroxylase promoter [4] were obtained from Professor C. Somerville (Carnegie Institution of Washington, Stanford, CA, U.S.A.). The napin promoter was from A. thaliana. Arabidopsis plants were transformed using the floral dip procedure [5] and transformed seeds were selected on kanamycin (50 μg/ml). Plants were grown to maturity at 20 °C under constant light. For seed lipid analysis around 40–50 seeds from each individual transformed plant were directly methylated by refluxing at 80 °C for 90 min in 1 M HCl in methanol. Fatty acid methyl esters (FAMEs) were extracted with hexane and separated by GC. For the yeast expression analysis the castor and Lesquerella sequences were cloned separately into the vector pESC-TRP (Stratagene, La Jolla, CA, U.S.A.) downstream of the GAL1 promoter. The constructs also contained a sequence encoding tobacco cytochrome b5 downstream of the GAL1 promoter. Transformed yeast (Saccharomyces cerevisiae strain YPH499) were grown on selective media plates containing 2% galactose at 30 °C for 5 days. Cells were scraped and directly methylated as outlined above. Control yeast was transformed

Figure 1
Gas chromatograms of FAMEs from seed lipids of A. thaliana plants
Wild-type, fad2/fae1 double-mutant and fad3 mutant plants were transformed with the Lesquerella oleate hydroxylase gene downstream of the napin promoter (wild-type plants) or the Lesquerella hydroxylase promoter (fad2/fae1 and fad3 plants). Hydroxy fatty acid nomenclature: 18:1-OH is ricinoleic acid (12-hydroxyoctadeca-9-enoic acid); 18:2-OH is densipolic acid (12-hydroxyoctadeca-9,15-dienoic acid); 20:1-OH is lesquerolic acid (14-hydroxyeicosa-11-enoic acid); 20:2-OH is auricolic acid (14-hydroxyeicosa-11,17-dienoic acid).

Untransformed

Wild type

Transformed with Lesquerella oleate hydroxylase

fad2/fae1

fad3
with the vector containing the cytochrome $b$, but without a hydroxylase gene.

**Results and discussion**

*One of the aims of this work was to maximize production of hydroxy fatty acids in *Arabidopsis* seed oil. In initial experiments the castor and *Lesquerella* oleate hydroxylases were expressed separately in an *Arabidopsis fad2/fae1* double mutant line. These plants have lesions in the genes encoding the ER Δ12-desaturase (*FAD2*) and the condensing enzyme involved in the elongation of 18:1 (*FAE1*) and produce a seed oil containing over 80% 18:1. For seed-specific expression the hydroxylase genes were placed downstream of either the *Arabidopsis* napin promoter or the *Lesquerella* hydroxylase promoter. Transformed plants produced two different hydroxy fatty acids in their seed oil, 18:1-OH and densipolic acid (18:2-OH; Figure 1). Levels were generally below 20% of total seed fatty acids although the maximum observed was 42%. There was a linear relationship between the levels of 18:1-OH and 18:2-OH. The presence of 18:2-OH is considered to be due to the activity of the ER $n-3$ desaturase (*FAD3*), which can desaturate 18:1-OH at the Δ15 position [6]. When the *Lesquerella* hydroxylase was expressed in an *Arabidopsis fad3* mutant line, which contains an inactive ER $n-3$ desaturase, no $n-3$ unsaturated hydroxy fatty acids were observed (Figure 1). This is further evidence that *FAD3* is involved in the *in vivo* synthesis of*

**Figure 2**

Gas chromatograms of FAMEs prepared from yeast cells expressing the castor and *Lesquerella* oleate hydroxylases.

Details are given in the text. The four major fatty acids of yeast are labelled.
polyunsaturated hydroxy fatty acids. Expression of the Lesquerella hydroxylase in wild-type Arabidopsis (Figure 1) resulted in the production of the four hydroxy fatty acids described previously [3]. The hydroxy fatty acid profiles of seed in the mutant and wild-type plants also demonstrate that the condensing enzyme FAE1 is involved in the synthesis of C_{18} hydroxy fatty acids.

In the transformed Arabidopsis fad2/fael plants, increasing levels of hydroxy fatty acids were correlated with a significant decrease in the levels of 18:1 accumulating. Levels of other fatty acids showed only minor changes, with the exception of 18:2, which increased with increasing hydroxy fatty acid levels. In the case of the Lesquerella hydroxylase this increase is likely to be due to the 18:1 desaturase activity of the bifunctional enzyme. The results for the castor enzyme suggest that it may also be acting as a desaturase. In these plants the decrease in 18:1 levels roughly corresponded to the combined increase in the levels of 18:2 and 18:1-OH.

Expression of the hydroxylases in a plant with an active FAD2 desaturase resulted in a dramatic decrease in 18:2 content and an increase in 18:1 levels. These changes do not appear to correspond simply to competition for substrate between the endogenous oleate desaturase and the oleate hydroxylase. It has been suggested previously that the hydroxylase enzyme may have some direct inhibitory effect on the FAD2 desaturase [3].

Levels of 20:1-OH (lesquerolic acid) in plants containing a FAE1 condensing enzyme were correlated with the amounts of 18:1-OH produced. However, the higher levels of 18:1 in plants producing hydroxy fatty acids did not lead to an increase in the production of 20:1.

To assess the ability of the castor oleate hydroxylase to act as a desaturase the gene was expressed in yeast. Transformed yeast accumulated low levels of 18:1-OH (Figure 2) but also accumulated 16:1-OH and two diunsaturated fatty acids 16:2 and 18:2. These results confirm that the castor enzyme has desaturase activity, although this is very much lower than that of the Lesquerella hydroxylase (Figure 2).

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References

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Anaerobic lipoxygenase activity from Chlorella pyrenoidosa

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Abstract
The micro-alga Chlorella pyrenoidosa expresses an enzymatic activity that cleaves the 13-hydroperoxy derivatives of linoleic acid [13-hydroperoxy-9(Z),11(E)-octadecadienoic acid, 13-HPOD] and linolenic acid [13-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid, 13-HPOT] into volatile C_{18} and non-volatile C_{19} oxo-products. This enzymatic activity initially was attributed to a hydroperoxide lyase enzyme; however, subsequent studies showed that this cleavage activity is the result of lipoxygenase activity under anaerobic conditions. Headspace analysis of the volatile products by GC/MS showed the formation of pentane when the substrate was 13-HPOD, whereas a more complex mixture of hydrocarbons was formed when 13-HPOT was the substrate. Analysis of the non-volatile cleavage products from 13-HPOD by liquid chromatography/MS indicated the formation of 13-oxo-