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ORIGINAL PAPER

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Level of accumulation of epoxy fatty acid in *Arabidopsis thaliana* expressing a linoleic acid Δ12-epoxygenase is influenced by the availability of the substrate linoleic acid

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Abstract *Arabidopsis thaliana* (L.) Heynh. expressing the *Crepis palaestina* (L.) linoleic acid Δ12-epoxygenase in its developing seeds typically accumulates low levels of vernolic acid (12,13-epoxy-octadec-*cis*-9-enoic acid) in comparison to levels found in seeds of the native *C. palaestina*. In order to determine some of the factors limiting the accumulation of this unusual fatty acid, we have examined the effects of increasing the availability of linoleic acid (9*cis*, 12*cis*-octadecadienoic acid), the substrate of the Δ12-epoxygenase, on the quantity of epoxy fatty acids accumulating in transgenic *A. thaliana*. The addition of linoleic acid to liquid cultures of transgenic plants expressing the Δ12-epoxygenase under the control of the cauliflower mosaic virus 35S promoter increased the amount of vernolic acid in vegetative tissues by 2.8-fold. In contrast, the addition to these cultures of linoelaidic acid (9*trans*, 12*trans*-octadecadienoic acid), which is not a substrate of the Δ12-epoxygenase, resulted in a slight decrease in vernolic acid accumulation. Expression of the Δ12-epoxygenase under the control of the napin promoter in the *A. thaliana* triple mutant *fad3/fad7-1/fad8*, which is deficient in the synthesis of tri-unsaturated fatty acids and has a 60% higher level of linoleic acid than the wild type, was found to increase the average vernolic acid content of the seeds by 55% compared to the expression of the Δ12-epoxygenase in a wild-type background. Together, these results reveal that the availability of linoleic acid is an important factor affecting the synthesis of epoxy fatty acid in transgenic plants.

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Introduction

While the membranes of plant cells are composed primarily of five "common" fatty acids—namely, stearic, palmitic, oleic, linoleic and linolenic acids—a very large diversity of fatty acids exists in the reserve triacylglycerols (TAG) of seeds, and nearly 300 naturally occurring fatty acids have been described there to date (Badami and Patil 1981; van de Loo et al. 1993). The structures of these latter fatty acids vary in a number of features, including the length of the acyl chains, the number, position and nature of the unsaturated bonds and the presence of functional groups, such as the hydroxy, epoxy and acetylenic groups. They are collectively referred as "unusual" fatty acids since their structures are different from those of the common fatty acids found in membranes.

Several genes have recently been identified that code for enzymes involved in the synthesis of fatty acids containing unusual groups (for a recent review see Jaworski and Cahoon 2003). In the majority of cases, these enzymes were found to be variants of enzymes involved in the synthesis of the common fatty acids, such as variants of the oleic acid Δ12-desaturase (FAD2) or of the soluble stearoyl-acyl carrier protein (ACP) desaturase (Cahoon et al. 1992; van de Loo et al. 1995). Novel enzymes have also been identified that are thought to play an important role in directing unusual fatty acids towards the storage TAG and away from membrane lipids (Dahlqvist et al. 2000).

Many of the unusual fatty acids have properties that are valuable as renewable feedstocks for the chemical industry. For example, vernolic acid (12,13-epoxy-octadec-*cis*-9-enoic acid) can be used as a plasticizer of polyvinyl chloride, as a precursor in the manufacture of nylon or as a component of adhesives and paints (Budziszewski et al. 1996). Although several native

plants, such as *Crepis palaestina* or *Euphorbia lagascae*, naturally accumulate between 50% and 90% of their seed fatty acids as vernolic acid, they have undesired agronomical features which make them unsuitable as agricultural crops. Efforts have thus been made to transfer the biosynthetic pathway for vernolic acid, as well as for other unusual fatty acids, into present-day oil crop species, such as rapeseed and the model oilseed plant *Arabidopsis thaliana*. However, in contrast to the native plants, the majority of transgenic plants expressing genes involved in the synthesis of unusual fatty acids, including vernolic acid, accumulate only low amounts (less than 20%) of these fatty acids in their seed oil (Jaworski and Cahoon 2003; Voelker and Kinney 2001). Further progress will depend on identifying the factors limiting the accumulation of unusual fatty acids in oilseed crops and on coordinating the expression of several transgenes capable of correcting these limitations.

The accumulation of unusual fatty acid in TAG can be divided into two major steps: first, the insertion of the functional group into the acyl chain and second, the stable incorporation of the unusual fatty acids into TAG. Failure to accumulate unusual fatty acids into oil seeds of transgenic plants could be caused by a number of factors, including low gene expression, poor activity of the fatty acid-modifying enzyme, inappropriate amount or access of the substrate or co-factors to the enzyme or preferential degradation of the newly synthesized unusual fatty acid.

The purpose of the investigation reported here was to analyze the potential role of substrate limitation on the synthesis of vernolic in transgenic *A. thaliana* expressing the *C. palaestina* linoleic acid Δ12-epoxygenase in developing seeds. The *C. palaestina* Δ12-epoxygenase is a desaturase-type enzyme that converts the Δ12 double bond of linoleic acid into an epoxy group (Lee et al. 1998). We performed experiments to examine how modulation of the quantity of linoleic acid influences the synthesis of vernolic acid in transgenic plants.

Materials and methods

DNA constructs

The *Crepis palaestina* linoleic acid Δ12-epoxygenase cDNA (Y16283) (kindly provided by S. Stymne, Swedish University of Agricultural Sciences, Alnarp, Sweden) was cloned as a *Bam*HI fragment either in the pART7 vector, thereby placing the gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter, or in the variant pART7-napin vector, putting the gene under the control of the 1.1-kbp *Brassica napus* napin promoter (Gleave 1992; Poirier et al. 1999). The cDNA with the upstream and downstream regulatory elements were then sub-cloned as a *Sac*I-*Pst*I fragment in the pCAMBIA 1300 vector (http://www.cambia.org.au) containing the hygromycin resistance gene under the control of CaMV 35S promoter. All binary vectors were transferred into *Agrobacterium tumefaciens* strain pGV3101 by electroporation.

Bacterial and plant material

Wild-type *Arabidopsis thaliana*, the *fad3/fad7-1/fad8* mutant (McConn and Browse 1996) and the transgenic *A. thaliana* line PHA10.3 (formerly described as N-PHA-10.3) expressing the *Pseudomonas aeruginosa* PHAC1 synthase modified for peroxisome targeting under the control of the 1.1-kbp *Brassica napus* napin promoter (Poirier et al. 1999), were transformed with the pCAMBIA 1300 vector or constructs containing the Δ12-epoxygenase gene by the floral dip method (Clough and Bent 1998). T_1 transformants were isolated by plating seeds on medium containing half-strength MS salts (Murashige and Skoog 1962), 1% (w/v) sucrose, 0.7% (w/v) agar and 30 μg/ml hygromycin. Hygromycin-resistant plants were subsequently transferred to soil and grown under continuous fluorescent light at 19°C.

For feeding experiments, axenic plants were first grown under constant agitation (100 rpm) for 9 days in liquid media containing half-strength MS salts and 2% sucrose before linoleic acid or linoelaidic acid was added at a concentration of 0.1% (v/v) for 12 h. The plants were then collected, washed extensively with water and freeze-dried. Linoleic acid and linoelaidic acid were purchased from Nu-Chek-Prep (Minnesota, USA).

Fatty acid analysis

Seed or whole-plant fatty acid methyl-esters were prepared by acid-catalysed (1 *N* HCL in methanol, 2 h, 80°C) or base-catalysed (0.1 *M* sodium methoxide in methanol, 1 h, 60°C) *trans*-esterification. Following the reaction, the fatty acid methyl-esters were extracted with hexane and water and the organic phase transferred to vials. Analysis was performed using a gas chromatograph equipped with a glass capillary column (model SP230, Supelco, Bellefonte, Pa.) and a flame ionization detector.

Results and discussion

External supply of linoleic acid to cultures of transgenic plants increases the amount of epoxy fatty acid in vegetative tissues

The presence of epoxy fatty acids in the lipids of transgenic *A. thaliana* expressing the *C. palaestina* Δ12-epoxygenase under the control of the constitutive CaMV 35S promoter is typically undetectable in adult rosettes of soil-grown plants. Similar results have been observed for transgenic plants expressing either the California bay lauroyl-ACP thioesterase or the *Ricinus communis* oleic acid Δ12-hydroxylase in vegetative tissues (Broun et al. 1998; Eccleston et al. 1996). In contrast, epoxy fatty acids are detectable in transgenic lines expressing the *C. palaestina* Δ12-epoxygenase under control of the CaMV 35S promoter when the lipids of 8- to 10-day-old seedlings grown in liquid culture containing sucrose are examined (data not shown). Consequently we used just such a transgenic line (WTSE2.6), which contained approximately 2.8 mol% vernolic acid and 1.8 mol% 12,13-epoxy-octadeca-9*cis*, 15*cis*-dienoic acid (hereafter referred as 12-epoxy-18:2) in its lipid fraction when grown for 9 days in liquid culture, as a first approach to examine the effect of linoleic acid availability on the accumulation of epoxy fatty acids. A

transgenic line transformed with the empty binary vector was used as a control (WTPC7.3).

External free or esterified fatty acids added to either cell cultures or plant tissues have been shown to be readily metabolized and incorporated into a wide range of lipids, including phospholipids, glycolipids and TAG (Stumpf and Weber 1977; Terzaghi 1986a, b, 1989). We have used a similar system to manipulate the level of linoleic acid in plant cells. Transgenic plants were grown for 9 days in media without external fatty acids, followed by the addition of either linoleic acid or linoelaidic acid (9*trans*, 12*trans*-octadecadienoic acid) at a final concentration of 0.1% (v/v) for 12 h. Linoelaidic acid was used as a control because this fatty acid is not a substrate for the Δ12-epoxygenase and cannot be converted to linoleic acid. Since the external linoleic and linoelaidic acid cannot be effectively removed from the plant material before fatty acid analysis (data not shown), the proportion of epoxy fatty acids was expressed as a fraction of the total fatty acids composed of palmitic acid, oleic acid, linolenic acid, vernolic acid and 12-epoxy-18:2 (Table 1).

Addition of linoleic acid was found to increase the level of vernolic acid in line WTSE2.6 from 3.2 mol% to 7.4 mol%, while the amount of 12-epoxy-18:2 increased from 1.8 mol% to 2.3 mol%. In contrast, addition of linoelaidic acid had little impact on the amount epoxy fatty acids, with vernolic acid decreasing slightly but significantly from 3.2 mol% to 2.5 mol% while 12-epoxy-18:2 remained unchanged at 1.7–1.8 mol%. In addition to the synthesis of epoxy fatty acid, expression of the Δ12-epoxygenase in line WTSE2.6 resulted in an approximate twofold increase in the proportion of oleic acid under all treatments. Similar changes in oleic acid contents have been previously observed in transgenic plants producing vernolic acid as a result of the expression of the *C. palaestina* Δ12-epoxygenase or the *E. lagascae* cytochrome P450 epoxygenase as well as in plants expressing other types of divergent oleic acid Δ12-desaturases involved in the synthesis of hydroxylated or conjugated unsaturated fatty acid (Broun and Somerville 1997; Broun et al. 1998; Cahoon et al. 1999, 2002; Singh et al. 2001; Smith et al. 2003). The increase in the proportion of oleic acid resulting from the expression of the epoxygenase was found to be unaffected by the addition of either linoleic acid or linoelaidic acid. Addition of either linoleic or linoelaidic acid also did not lead to significant changes in the proportion of palmitic, oleic and linolenic acid in the control line WTPC7.3. Together, these data indicate that the increase in epoxy fatty acid observed after the addition of linoleic acid to cultures of plants expressing the Δ12-epoxygenase is specific for this fatty acid and that the addition of fatty acids to the growth media for 12 h does not result in an unspecific perturbation of the overall profile of common fatty acids.

Accumulation of epoxy fatty acids in seeds of mutant plants with an increased level of linoleic acid

Genetic engineering of plants aimed at the production of unusual fatty acids primarily targets manipulation of the lipid biosynthetic pathway in developing seeds. The possibility that the availability of linoleic acid may be also influencing the synthesis of epoxy fatty acids in developing seeds was thus examined in transgenic plants expressing the *C. palaestina* Δ12-epoxygenase under the control of the *B. napus* napin promoter. The *A. thaliana* triple mutant *fad3/fad7-1/fad8* has been shown to be strongly deficient in linoleic acid desaturase activity, leading to a strong reduction in the accumulation of trienoic acids and a significant increase in dienoic acids in leaves (McConn and Browse 1996). Analysis of the fatty acid profile in mature seeds revealed a 60% increase of the proportion of linoleic acid, from 29 mol% in the wild type up to 46 mol% in the triple mutant (Table 2). The amount of epoxy fatty acid (vernolic acid + 12-epoxy-18:2) was determined for seeds of plants obtained from the transformation of the same napin-epoxygenase construct into three different lines, namely the *fad3/fad7-1/fad8* mutant, a transgenic line expressing a bacterial polyhydroxyalkanoate (PHA) synthase in the peroxisome and wild-type plants, all in the Columbia background. Expression of the bacterial PHA synthase in the peroxisome leads to polyhydroxyalkanoate synthesis from the intermediate of

Table 1 Effects of the addition of linoleic acid on the accumulation of epoxy fatty acids

Plant line	Treatment	Fatty acids (mol%)[a]				
		16:0	18:1	18:3	Epoxy 1	Epoxy 2
WTPC7.3	No f.a.	15±1	4.0±0.2	81±1	ND	ND
WTSE2.6	No f.a.	16±1	8.7±0.2	71±1	3.2±0.5	1.8±0.1
WTPC7.3	18:2c	15±1	3.9±0.1	81±1	ND	ND
WTSE2.6	18:2c	15±1	8±1	68±2	7.4±0.4	2.3±0.1
WTPC7.3	18:2t	14±1	3.7±0.5	82±1	ND	ND
WTSE2.6	18:2t	15±1	7.3±0.8	74±2	2.5±0.2	1.7±0.1

WTPC7.3 control plants transformned with the empty binary vector, *WTSE2.6* transgenic plants expressing the Δ12-epoxygenase under the CaMV35S promoter
No f.a. no addition of fatty acids, *18:2c* linoleic acid, *18:2t* linoelaidic acid
ND not detected
[a]Values are derived from two independent experiments, mean ± SD

ß-oxidation and has previously been used to study the flux of fatty acids through the peroxisomal ß-oxidation cycle (Mittendorf et al. 1999; Poirier 2002). Plants expressing both the *C. palaestina* Δ12-epoxygenase and the peroxisomal PHA synthase in developing seeds have recently been used to reveal the presence of a futile cycle of epoxy fatty acid degradation through the peroxisomal ß-oxidation pathway (Moire et al. 2004). Since the expression of the PHA synthase in the peroxisome had no impact on seed fatty acid composition (data not shown), we regarded plants expressing both the PHA synthase and Δ12-epoxygenase as controls to the same extent as wild-type plants expressing only the Δ12-epoxygenase.

The amount of unusual fatty acids was determined for a pool of approximately 100 seeds harvested from each primary transgenic plants obtained from the transformation of the napin-epoxygenase construct into either wild-type (lines WTNE), the *fad3/fad7-1/fad8* mutant (lines *fad*NE) or transgenic plants expressing the peroxisomal PHA synthase (lines PHANE) (Fig. 1). The amount of epoxy fatty acids present in mature seeds of 16 independent lines expressing the Δ12-epoxygenase in a wild-type background with and without the napin-PHA synthase varied between 0.5 mol% and 2.9 mol%, (average = 2.2 mol%) with no statistically significant differences between the two groups. In contrast, expression of the napin-epoxygenase in the *fad3/fad7-1/fad8* mutant led to a statistically significant increase in the amount of epoxy fatty acids in seeds, with a range of 2.3–4.6 mol% (average = 3.4 mol%) epoxy fatty acids among 34 independent lines (Fig. 1). Comparison of the fatty acid profile in two *fad3/fad7-1/fad8* mutant lines (*fad*NE1 and *fad*NE35) producing 2.9 mol% and 3.5 mol% vernolic acid, respectively, with the non-transgenic *fad3/fad7-1/fad8* parental line revealed that expression of the Δ12-epoxygenase decreased the level of linoleic acid from 46 mol% to 19–24 mol% and increased the level of oleic acid from 17 mol% to 37–45 mol%. These levels of change in oleic and linoleic acid are similar to the ones observed when a vector-transformed line in a wild-type background (WTPC2.1) was compared with a line that expressed the Δ12-epoxygenase in the wild-type background and accumulated 2.1 mol% epoxy fatty acids (line WTNE7.7) (Table 2). Furthermore, in contrast to the expression of the Δ12-epoxygenase in wild-type *A. thaliana*, expression of the Δ12-epoxygenase in the *fad3/fad7-1/fad8* mutant led to the synthesis of only vernolic acid and not of 12-epoxy-18:2 (Table 2). These results are in accordance with the implication that omega-3 desaturases are involved in the accumulation of 12-epoxy-18:2 either through the Δ15-desaturation of vernolic acid or the Δ12-epoxygenation of linolenic acid (Singh et al. 2001).

Levels of linoleic acid as an important factor for the synthesis of epoxy fatty acids in transgenic plants

Expression of the *C. palaestina* linoleic acid Δ12-epoxygenase in *A thaliana* has been found to lead to a marked increase in oleic acid and decrease in linoleic and linolenic acids (Table 2) (Singh et al. 2001). Similar results have been obtained for transgenic plants expressing a *R. communis* or *Lesquerella fendleri* oleic acid Δ12-hydroxylase, or a *Momordica charantia* linoleic acid Δ12-conjugase, all members of the Δ12 oleic acid desaturase family, as well as in transgenic plants expressing a cytochrome P450 from *E. lagascae* involved in the synthesis of vernolic acid (Broun and Somerville 1997; Cahoon et al. 1999; Cahoon et al. 2002; Smith et al. 2003). One proposed hypothesis to explain this phenotype is that the accumulation of unusual fatty acids on phospholipids

Table 2 Fatty acid composition of mature seeds of transgenic lines expressing the epoxygenase gene

Fatty acids	Fatty acid content[a] (mol%)				
	WTPC2.1[b]	WTNE7.7[c]	*fad3/7/8*[d]	*fad*NE1[e]	*fad*NE35[e]
16:0	7.8±0.1	8.0±0.1	7.1±0.1	6.4±0.1	6.82±0.05
18:0	3.2±0.1	4.1±0.1	2.8±0.1	2.59±0.02	2.57±0.04
18:1	14.9±0.3	31.9±0.5	17.3±0.2	37±2	44.7±0.7
18:2	28.5±0.2	15.2±0.3	46.2±0.1	24±2	19.3±0.9
18:3	19.4±0.1	10.1±0.3	ND	ND	ND
20:0	1.4±0.1	1.84±0.08	1.70±0.03	1.47±0.05	1.27±0.02
20:1	21.3±0.1	24.6±0.1	20.5±0.1	22.7±0.3	23.0±0.3
20:2	1.96±0.03	0.93±0.03	2.8±0.1	1.1±0.2	0.69±0.05
22:1	1.56±0.04	1.38±0.03	1.75±0.02	1.40±0.07	1.15±0.04
Vernolic acid	ND	1.80±0.05	ND	2.9±0.4	3.5±0.2
12-epoxy-18:2	ND	0.3±0.1	ND	ND	ND

[a]Mean ± standard deviation (n=3–5)
[b]Wild-type plants transformed with the control vector
[c]Wild-type plants transformed with the napin-epoxygenase construct
[d]*fad3/fad7-1/fad8* triple mutant
[e]Two independent lines from the *fad3/fad7-1/fad8* triple mutant transformed with the napin-epoxygenase construct
ND not detected

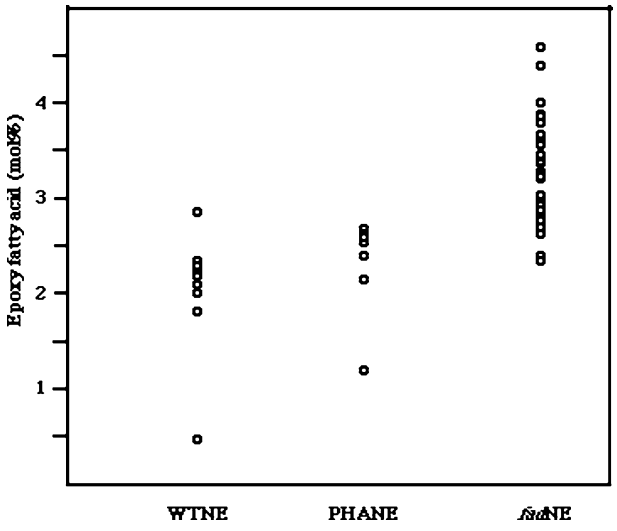

Fig. 1 Analysis of epoxy fatty acids produced in wild-type *Aradidopsis thaliana* and a mutant showing accumulation of linoleic acid. The napin-epoxygenase construct was transformed into either wild-type *A. thaliana* (*WTNE*), wild-type *A. thaliana* expressing a peroxisomal PHA synthase (*PHANE*) or the *fad3/fad7-1/fad8* mutant (*fadNE*). The amount (in mol%) of epoxy fatty acids (vernolic acid + 12-epoxy-18:2) measured in a pool of approximately 100 seeds obtained from each primary transgenic plant is indicated by a *circle*. Statistical analysis (ANOVA) revealed no significant differences between WTNE and PHANE (P=0.35), while highly significant differences were found between *fad* NE and either WTNE or PHANE (P<0.001)

inhibits the activity of the native oleic acid Δ12-desaturase, thereby blocking the conversion of oleic acid to linoleic acid (Smith et al. 2003). Since linoleic acid is the substrate for the *C. palaestina* linoleic acid Δ12-epoxygenase, the reduced level of linoleic acid may be limiting the accumulation of epoxy fatty acids. It has been shown that overexpression of a *C. palaestina* Δ12-oleic acid desaturase along with the *C. palaestina* Δ12-epoxygenase in *A. thaliana* leads to increased levels of both linoleic acid and epoxy fatty acids (Singh et al. 2001). It is however unclear from this work if the increase in epoxy fatty acids was dependant solely on the increased level of linoleic acid or was rather the result of the co-expression of two enzymes from the native *C. palaestina* plant that could potentially interact to more efficiently convert oleic acid into vernolic acid. The presence of such functional multi-component enzyme association has been suggested to be important for the synthesis of unusual monoenoic acid fatty acids in transgenic Arabidopsis expressing a Δ4 16:0-ACP desaturase (Suh et al. 2002).

In this investigation we used two independent methods of manipulating the level of available linoleic acid, and both led to increased synthesis of epoxy fatty acids in transgenic *A. thaliana* expressing the *C. palaestina* Δ12-epoxygenase. First, the growth of the transgenic plants in media containing linoleic acid showed a 2.8-fold increase in the amount of vernolic acid, whereas the addition of the non-substrate linoelaidic acid led to a small decrease in vernolic acid. Second, expression of the *C. palaestina* Δ12-epoxygenase in the *fad3/fad7-1/fad8* mutant, which is deficient in the synthesis of trienoic acid and shows a 60% increase in linoleic acid relative to the wild type, led to an approximately 55% increase in the amount of epoxy fatty acids. This level of increase in epoxy fatty acids is similar to the one observed in transgenic *A. thaliana* co-expressing the *C. palaestina* Δ12-epoxygenase and Δ12-desaturase (Singh et al. 2002). Together, these results indicate that higher levels of epoxy fatty acid production are mediated by the increased availability of the substrate linoleic acid.

The higher accumulation of the fatty acids 16:1Δ4 and 18:1Δ6 through the expression of the *Coriandrum sativum* Δ4 16:0-ACP desaturase in the *fab1* mutant of *A. thaliana*, which is deficient in the elongation of 16:0-ACP to 18:0-ACP, indicated that greater availability of the substrate 16:0-ACP to the *C. sativum* desaturase could also improve the accumulation of unusual monoenoic fatty acids (Suh et al. 2002). However, expression of the *Thunbergia alata* Δ6 16:0- ACP desaturase in the same *fab1* mutant did not lead to significant changes in the accumulation of unusual monoenoic acids relative to the expression of the transgene in wild-type plants (Suh et al. 2002). Smith et al. (2003) recently examined the effects of manipulating the levels of oleic acid on the accumulation of hydroxy fatty acids in seeds of *A. thaliana* expressing the *R. communis* Δ12-oleic acid hydroxylase. Expression of the hydroxylase in a *fae1/fad2* mutant accumulating 440% more oleic acid than a control line only led to a modest 30% increase in the average hydroxy fatty acid content and a 18% increase in the maximum hydroxy fatty acid content in the *fae1/fad2* mutant compared to control (Smith et al. 2003). In contrast, the accumulation of epoxy fatty acids in seeds of *A. thaliana* expressing the *C. palaestina* Δ12-epoxygenase appears to be more responsive to the level of linoleic acid, since a modest increase of 60% in linoleic acid level was accompanied by a 55% increase in the average epoxy fatty acid content and a 60% increase in the maximum epoxy fatty acid content. Thus, improvement in the production of unusual fatty acids through an increased availability of the substrate may not apply to all transgenic plants and must, therefore, be evaluated case by case.

Despite the clear improvement in the accumulation of vernolic acid through the increased availability of linoleic acid, the absolute level of epoxy fatty acids in transgenic *A. thaliana* remained low (lower than 8 mol% in vegetative tissues and lower than 5 mol% in seeds) compared to that observed in the native *C. palaestina* plants (approximately 60 mol% in seed lipids). Clearly, other factors are still limiting epoxy fatty acid accumulation in *A. thaliana*. Singh et al. (2001) reported the synthesis of epoxy fatty acids up to 6.2% in transgenic Arabidopsis expressing the *C. palaestina* linoleic acid Δ12-epoxygenase and up to 9.9% in plants co-expressing the *C. palaestina* linoleic acid Δ12-epoxygenase and Δ12-desaturase. The reasons for the lower amount of epoxy fatty acids in the transgenic plants obtained in this study are unknown but may be due to a combination of factors, including differences in the promoter (0.5-kbp vs.

1.1-kbp napin promoter), binary vector (pBI121 vs. pCAMBIA 1300), ecotype (C24 vs. Columbia) and transformation method (hypocotyl or root transformation vs. floral dip).

The *C. palaestina* linoleic acid Δ12-epoxygenase acts on fatty acids present in phospholipids on the endoplasmic reticulum (Lee et al. 1998). The newly synthesized unusual fatty acids are then transferred from phospholipids to be mobilized into TAG via a number of potential routes, thereby implying the action of either specific phospholipases or acyltransferases, including the phosphatidylcholine:diacylglycerol acyltransferase (Dahlqvist et al. 2000; Ståhl et al. 1995). Potential other factors limiting epoxy fatty acids synthesis in transgenic plants could be the timing and absolute level of expression of the Δ12-epoxygenase, the availability of co-factors required for the epoxygenation reaction as well as the specificity of the enzymes involved in the stable incorporation of epoxy fatty acids into TAG. It is thus clear that in addition to increasing the availability of linoleic acid, further modification of plant metabolism through the use of mutants or the expression of additional transgenes will be required for high-level accumulation of epoxy fatty acids in transgenic plants.

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