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Expression of a *Stokesia laevis* epoxygenase gene

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Abstract

Epoxy fatty acids have a number of important uses and there is interest in enzymes catalyzing their synthesis from renewable sources. Both cytochrome P450 monooxygenases and divergent forms of di-iron desaturases are known to produce epoxy fatty acids in plants. Degenerate primers based on conserved sequences of Δ^{12} desaturase-like genes led to the isolation of an epoxygenase gene from *Stokesia laevis*. The cDNA is 1.4 kb and it encodes 378 amino acids. The similarities of this gene at the amino acid sequence level with epoxygenases of *Vernonia* and *Crepis*, and the Δ^{12} desaturases of soybean, *FAD2-1* and *FAD2-2*, are 84%, 69%, 49%, and 55%, respectively. When the vector, pYES2, was used to transform yeast, epoxy fatty acid formation was observed in the cells. The effects of electron donors in the yeast expression system were tested but cytochrome b_5 and cytochrome b_5 reductase genes from *Arabidopsis thaliana* co-expressed with the epoxygenase had little effect on vernolic acid accumulation in the yeast. Finally, this gene, driven by a seed-specific phaseolin promoter, was cloned into a TDNA-vector and transferred into *Arabidopsis* plants. The results showed that T_2 seeds of transgenic *Arabidopsis* expressing the *Stokesia* gene accumulated vernolic acid but no vernolic acid was detected in control plants. Northern blot analysis indicates this *S. laevis* epoxygenase gene is expressed mainly in developing seeds and no transcript was detected in leaves or roots.

Keywords: *Stokesia laevis*; Asteraceae; Epoxygenase; Vernolic acid

1. Introduction

Epoxy fatty acids have many industrial applications such as in drying oils. Currently, epoxidized soybean and linseed oils e.g., linoleic acids, are produced by introducing an epoxy group across the double bonds of polyunsaturated fatty acids. This is a costly process and it would likely be more economical if the biosynthetic reactions in oilseed themselves converted the polyunsaturated fatty acids into epoxy fatty acids. There was no known way to produce a commercial oilseed that accumulates epoxy fatty acids by conventional breeding and genetics. However, certain genotypes of several plant species accumulate high levels of epoxy fatty acids in the seed oil. Epoxy fatty acids, like vernolic (E-12,13-epoxyoctadeca-E-9-enoic) **2** and coronaric (E-9,10-epoxyoctadeca-E-12-enoic) acids, have been found as a component of the seed oil of species represented by a number of plant families, such as Asteraceae, Euphorbiacea, Pnagraceae, Dipsacaceae, and Valerianacea (Smith, 1970). One of the highest known natural accumulators of vernolic acid **2** is *Vernonia galamensis* in which it can constitute 80%, of triglyceride fatty acids (Perdue, 1989, Pascal and Correal, 1992, Bafor et al., 1993, Thompson et al., 1994). *Stokesia laevis* another Asteraceae species native to the southeastern US has seed oil containing 60–70% vernolic acid **2**.

Many plants are known to possess enzymes that transform unsaturated fatty acids into epoxy fatty acids (Blee, 1998, Gardner, 1991). The process by which the seeds of certain species of *Vernonia*, *Stokesia* and

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Euphorbia synthesize the epoxy fatty acid, vernolic acid **2**, appears to be due to an enzyme not present in major commercial oilseeds. Biochemical studies by Bafor et al. (1993) indicate that developing seeds of these plants contain an enzyme known as an epoxygenase which converts linoleic acid **1** into vernolic acid **2** in a one step reaction (Fig. 1). Although many plants including soybeans have enzymes such as lipoxygenase and peroxygenase that can produce epoxy fatty acids in some disease resistance reactions (Blee, 1998), they do not have mechanisms for accumulation of epoxytriglycerides in their seeds unlike epoxy triglyceride accumulators such as *Vernonia* and *Stokesia* mentioned above (Bafor et al., 1993, Hildebrand et al., 2002).

The original studies on epoxy fatty acid synthesis and accumulation in oilseeds by Bafor et al. (1993) indicated that the epoxy fatty acid that accumulates in seeds of *Euphorbia lagascae*, vernolic acid **2**, is synthesized by an epoxygenase enzyme which is a P450 monooxygenase enzyme. It was assumed that other epoxy fatty acid accumulators such as *Vernonia* similarly synthesized epoxy fatty acids by P450 monooxygenase enzymes. However, studies by our group (10–12) indicated that Δ^{12} fatty acid desaturase-like enzymes are responsible for vernolic acid **2** biosynthesis in epoxy fatty acid accumulators of the Asteraceae such as *Vernonia*, *Crepis* and *Stokesia* unlike the Euphorbiacea epoxide accumulator, *E. lagascae*, which our studies confirmed relied on a P450 monooxygenase (Seither, 1996, Seither et al., 1996, 1997). Lee et al. (1998) and Hitz (1998) confirmed this with cloning cDNAs encoding epoxygenases from *Crepis palaestina* and *V. galamensis* that they found to be members of a growing family of Δ^{12} fatty acid desaturase-like analogs that also includes hydroxylases, acetylenases and conjugases (Cahoon et al., 2001). Cahoon et al. (2002) cloned and characterized the epoxygenase from *E. lagascae* showing that it is indeed a cytochrome P450 monooxygenase.

Kinney et al. (1998) developed a transgenic soybean expressing *Vernonia* epoxygenase, but the content of epoxy fatty acid in seed oil was only about 8%. This suggests that high concentrations of epoxy fatty acids in membrane lipids might be toxic and additional enzymes are needed for selective accumulation of epoxy fatty acids in triacylglyceride.

We here report cloning an epoxygenase gene from *S. laevis* and the expression of this gene in yeast and plants.

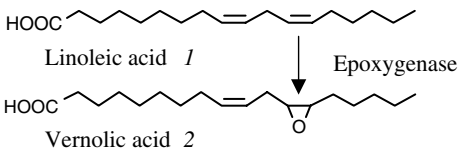

Fig. 1. Vernolic acid **2** synthesis from linoleic acid **1**.

2. Results and discussion

2.1. cDNA cloning

The genes of the plant Δ^{12} desaturase family are highly homologous. To design degenerate primers, conserved regions in epoxygenases and an acetylenase but different from desaturases were chosen. As a result of RT-PCR and RACE, the epoxygenase cDNA cloned from *S. laevis* was fully sequenced. It is 1.4 kb, the ORF is 1134 bp and encodes 378 amino acids. The GenBank Accession Number is AY462108. The deduced amino acid sequences of these genes have high similarities to Δ^{12} desaturases of many other plant species. The amino acid similarities of the protein encoded by this gene with epoxygenase of *V. galamensis* and *Crepis*, Δ^{12} desaturase of soybean, FAD 2-1 and FAD 2-2 are 84%, 68%, 49% and 55%, respectively.

Lenman et al. (1998) reported a partial sequence of an epoxygenase-like gene of *V. galamensis*. It is quite different from *Vernonia* epoxygenase cloned by Hitz (1998). We also cloned an epoxygenase gene from *V. galamensis*. As the results of sequencing, this cDNA was slightly different from the gene cloned by Hitz (1998). They have a six amino acid difference, one gap and the similarity is 98.4%. These differences are mainly at the N-terminal region. It is suggested that *V. galamensis* has more than one isozyme of epoxygenase.

The phylogenetic tree analysis of the amino acid sequences of the proteins encoded (Fig. 2) suggests the plant Δ^{12} desaturase gene family includes epoxygenase, hydroxygenase, acetylenase and conjugase. *Stokesia* epoxygenase is closer to *Vernonia* epoxygenase than *Crepis* epoxygenase. Although they all belong Asteraceae family, *Crepis* is in a different tribe from the one which *Vernonia* and *Stokesia* are in. This fact may explain their genetic distance of epoxygenase genes.

2.2. Gene testing in yeast

The cDNA from *S. laevis* was first tested in a yeast, *Saccharomyces cerevisiae*, expression system. However no vernolic acid **2** was detected by our first approach. In a control culture without linoleic acid **1** feeding, linoleic acid **1** was also not detected. It is known that yeast cells accumulate oleic acids and this system works for Δ^{12} desaturases testing. Therefore, it was suggested that this gene was not a Δ^{12} desaturase. We tried to modify the yeast expression system using *Vernonia* epoxygenase gene as a positive control, by administering ^{14}C-labeled linoleic acid **1** as a substrate. The transformed yeast also did not produce any vernolic acid **1**. Assays of microsomes isolated from the transgenic yeast were likewise negative. It seemed very difficult to achieve epoxygenase expression in yeast despite the effective interaction with the electron donors, cytochrome b_5 and cytochrome b_5

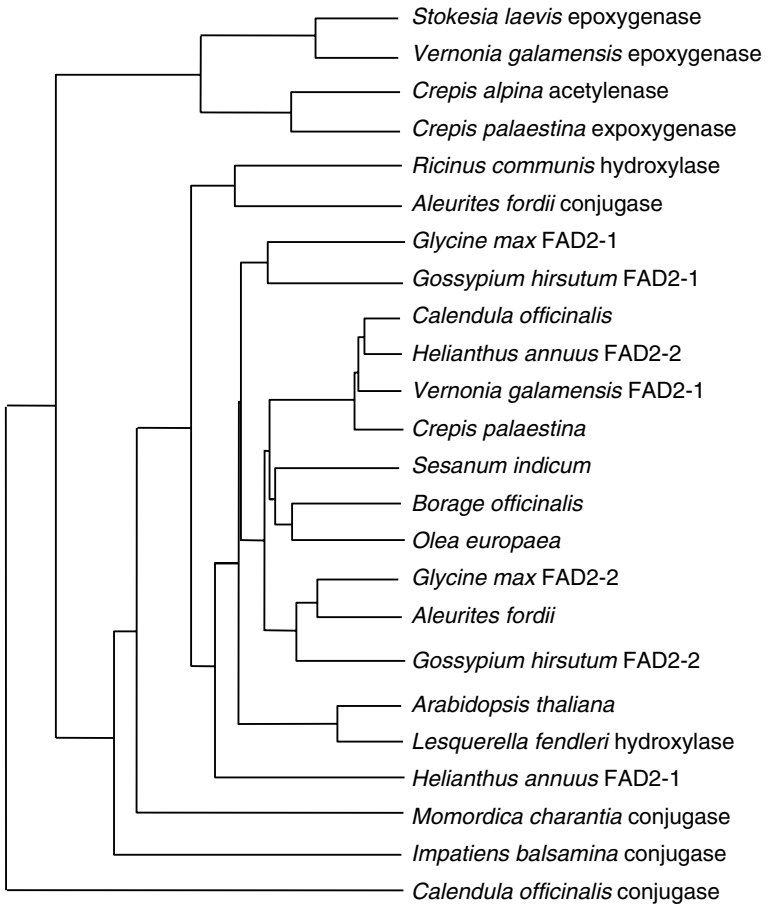

Fig. 2. Phylogenetic tree analysis of plant microsomal Δ^{12} desaturases, epoxygenases, hydroxygenases, conjugases and an acetylenase. The phylogenetic tree represents results from the unweighted pair-group method of the arithmetic average (UPGMA) clustering analysis of amino acid sequences obtained using Kimura distance method (Kimura, 1983). The entries with plant names only are microsomal Δ^{12} desaturases. The GenBank accession numbers used for the analysis were: *Crepis alpina* acetylanase, Y16285; *Crepis palaestina* epoxygenase, Y16283; *Ricinus communis* hydroxylase, U22378; *Aleurites fordii* conjugase, AF525535; *Glycine max* FAD2-1, L43920; *Gossypium hirsutum* 1, X97016; *Calendula officinalis*, AF343065; *Heliantus annuus* FAD2-2, AF251842; *Vernonia galamensis* desaturase FAD2-1, AF188263; *Crepis palaestina*, Y16284; *Sesamum indicum*, AF192486; *Borage officinalis*, AF074324; *Olea europea*, AY083163; *Glycine max* FAD2-2, L43921; *Aleurites fordii*, AF525534; *Gossypium hirsutm* 2, Y10112; *Arabidopsis thaliana*, L26296; *Lesquerella fendleri* hydroxylase, AF016103; *Helianthus annuus* FAD2-1, U91341; *Momordica charantia* conjugase, AF182521; *Impatiens balsamina* conjugase, AF182520; *Calendula officinalis* conjugase, AF343064; and *Vernonia galamensis* epoxygenase, United States Patent: 5,846,784.

reductase with various homologous Δ^{12} desaturases tested with the same vector in yeast (Hage et al., 2000; data not shown).

To clarify the effects of electron donors in a yeast expression system, cytochrome b_5 and cytochrome b_5 reductase genes from *A. thaliana* were introduced into yeast as a second approach. Unexpectedly, we detected vernolic acid **2** and epoxystearic acid in both yeast lines transformed with the *Stokesia* epoxygenase gene with (0.64%) or without (0.55%) *A. thaliana* cytochrome b_5 and cytochrome b_5 reductase genes, but not in control yeast cells (Fig. 3) in media supplemented with linoleic acid. An average of 0.64% of vernolic acid **2** in yeast expressing *Stokesia* epoxygenase gene together with *A. thaliana* cytochrome b_5 and cytochrome b_5 reductase genes is slightly higher than in yeast expressing the *Stokesia* epoxygenase gene only. However, this difference is not significant. Dyer et al. (2002) reported conjugated fatty acids formation in yeast transformed with tung conjugase gene also using the pYES vector. These facts suggest that the vector used, pYES2, led to successful production of these unusual fatty acids in yeast cells.

2.3. Gene testing in plants

To verify the enzymatic activity of the gene product in plants, the protein was expressed in seeds of *A. thaliana*. In seeds of transgenic *Arabidopsis*, vernolic acid was detected with GC–MS, but not in control plants transformed with the empty vector (Fig. 4). Sixteen individual T_2 seeds were tested by GC. The contents of vernolic acid **2** were 1.3–10.6 μg/mg DW, the average was 5.8 μg/mg DW, and their percentages of vernolic

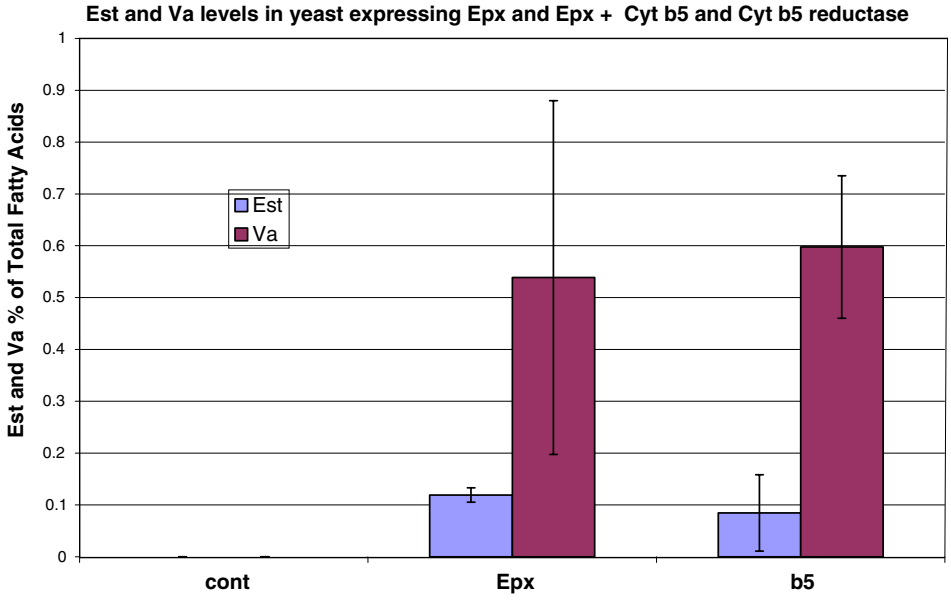

Fig. 3. GC analysis of epoxy stearic (Est) and vernolic (Va) acids in yeast expressing *S. laevis* epoxygenase (EPX) or *S. laevis* EPX + *Arabidopsis* Cytochrome b_5 and Cytochrome b_5 reductase (EPX + b_5, $b_5$red). The numbers in the figure showed % of epoxy stearic and vernolic acids of total fatty acids. Yeast were transformed with the expression vector pYES2 and pESC lacking a cDNA insert for the vector control (CTR), pYES-St EPX together with pESC (EPX) and pYES-St EPX together with pESC-At Cyt b_5 and Cyt b_5 reductase (EPX + b_5, $b_5$red). The means represent five replications and the detection limit for Est and Va were ~0.05% of total fatty acids in this experiment.

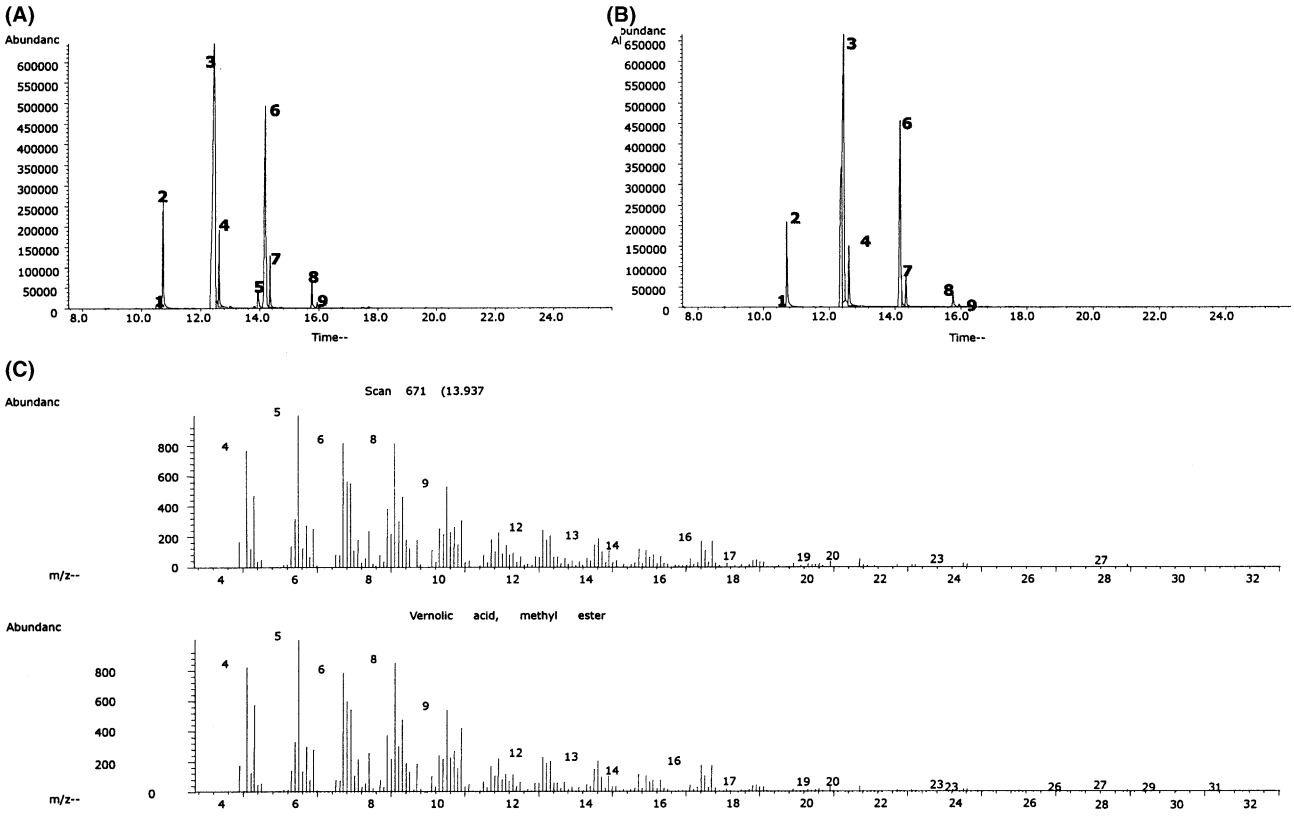

Fig. 4. GC–MS analysis of fatty acid derivatives from transgenic *Arabidopsis* seeds. (A) Chromatograms from *Arabidopsis* transformed with pCAMBIA1201 inserted with the cDNA from *S. laevis*. (B) Chromatograms from *Arabidopsis* transformed with empty vector, pCAMBIA1201 as a control. (C) Mass spectrum of the compound giving rise to peak 5 at 13.94 min in chromatogram (A), and a standard vernolic acid **2**. *m*/*z*, mass-to-charge ratio. Peak 1, 7-hexadecenoic acid; 2, hexadecanoic acid; 3, 9-octadecenoic acid; 4, octadecanoic acid; 6, 11-eicosenoic acid; 7, eicosanoic acid; 8, docosenoic acid; 9, docosanoic acid.

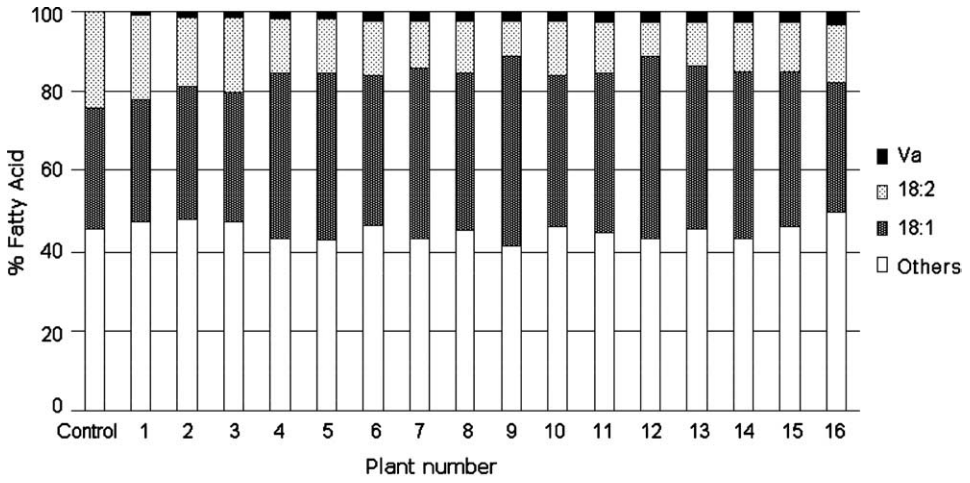

Fig. 5. Seed oil fatty acid profile for transgenic *Arabidopsis thaliana* carrying the *S. laevis* epoxygenase. Control (CTRL) seeds of vector transformed T_2 *Arabidopsis* seeds = the average of two replications. Plants 1–16 are T_2 seed analyses of different epoxygenase transgenic plants. *Va = vernolic acid.

acid **2** in the total fatty acids were 1.1–3.1%, the average value was 2.4%.

Singh et al. (2001) reported that a very marked increase in oleic acid (18:1) and decrease in linoleic (18:2) and linolenic (18:3) acids in *Arabidopsis* plants transformed with the *C. palaestina* epoxygenase gene. The same trend is found in our results (Fig. 5), this indicates endogenous Δ^{12} desaturation was reduced in these transgenic plants.

2.4. Analysis of epoxygenase mRNA expression

The presence of the epoxygenase gene transcripts in various *S. laevis* tissues was analyzed by RNA blot analysis with the cloned cDNA as a probe (Fig. 6). The transcript expressed strongly in developing embryos and slightly in mature embryos but it was not detected in leaves, or roots. This is not surprising since epoxy fatty acids only accumulate in oil of developing seeds but this is the first confirmation of this fact of desaturase-like epoxygenases. Previously Cahoon et al. (2002) reported that the gene encoding a cytochrome P450 epoxygenase enzyme from *E. lagascae*, CYP726A1, is expressed in developing seeds but is not detectable in leaves. The presence of epoxygenase transcript in mature seeds is likely due to incompletely turned-over RNA remaining from expression earlier in seed development.

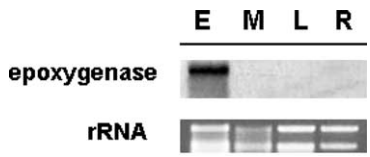

Fig. 6. RNA blot analysis of the *Stokesia* epoxygenase gene in different *Stokesia* plant tissues. A DIG-labeled probe corresponding to the full length *Stokesia* epoxygenase gene was hybridized to 5.5 μg of total RNA from developing embryos (E), mature seeds (M), leaves (L) and roots (R).

3. Experimental

3.1. cDNA cloning

A partial *S. laevis* epoxygenase cDNA fragment was obtained from RNA of developing embryos of *S. laevis* using an Access RT-PCR System (Promega Co.). The PCR mixtures contained 1 μg of total RNA template, 0.2 mM dNTPs, 2.5 U of AMV reverse transcriptase, 2.5 U of *Tfl* polymerase and 1 μM each of two degenerate primers described below. Reaction mixtures were incubated in a thermocycler (Perkin–Elmer, Model 2400) for 45 min at 48 °C, followed by 2 min at 94 °C and 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C. The PCR primers used (5epoxy, 5′-GGICAY-GARTGYGGNCAYCAYGC-3′ and 3epoxy, 5′-AC-RTGIGTRTGNGTNACRTCRTG-3′) represent two peptide sequences, [GHECGHHA] and [HDVTHTHV], which are the conserved regions in amino acid sequences of desaturase-like epoxygenases of *Crepis palaestina* and *V. galamensis*. The amplified products of ~S620 bp were fractionated on a 1% agarose gel, extracted from the gel using Quiaquick Gel Extraction Kit (Qiagen Inc.) and subcloned into the pGEM-T Easy vector (Promega Co.). The DNA inserted was sequenced in both directions.

For determination of the full-length cDNA sequence, a Rapid Amplification of cDNA Ends (RACE) strategy was applied to obtain the 5′ and 3′ ends beyond the internal fragment cloned with the internal primers above. A cDNA was synthesized from poly(A) + RNA

of developing seeds of *S. laevis* using a Marathon cDNA Amplification Kit (BD Biosciences Clontech). We then designed the following two primers from the sequence information of the partial cDNA fragment of *S. laevis* epoxygenase; 5′-ST: 5′-CGCAACCTGGATTCGCTCACGCTCGG-3′, and 3′-ST: 5′-CCCAGCTCAGGACTTACTCCACATACG-3′. The 5′-half and 3′-half of the cDNAs were amplified using the PCR conditions described in the user manual of the kit. Fractionation of the amplified fragments, cloning and sequencing were carried out as described above.

The deduced amino acid sequence similarities were determined by a software, Lasergene (DNASTAR Inc.). A phylogenic tree was drawn using 'SEQ Web, version 1.1' (Genetics Computer Group Inc.).

3.2. Gene testing in yeast

For expression in yeast, two different approaches were applied. At first, a *Bam*HI site was introduced at the first ATG and *Bsm*BI and *Eco*RI sites were introduced at the end of the open reading frame or coding sequence (ORF) of the cDNA by PCR mutagenesis. The ORF sequence of the cDNA was amplified using two primers (StexpF1: 5′-ACGCGGATCCATGTCGGATTCATATGATG-3′, StexpR1: 5′-GACGCGTCTC*GAATTC*TACATTTTATGGTACCAATATG-3′, *Bam*HI and *Bsm*BI sites are underlined, *Eco*RI site is in italic), and cloned into the pGEM-T Easy vector and verified by DNA sequencing. The *Bam*HI–*Bsm*BI fragment covering the entire ORF of the cDNA was cut out from pGEM-R Easy vector and ultimately cloned into the respective sites of the expression vector, pYeDP60. The yeast strain INVSc1 was transformed with the vector, harboring either no insert or the cDNA. The transformed yeasts were cultured in expression media with or without 1 μM linoleic acid for two days and the cells were collected. Their lipids were extracted with chloroform:methanol (2:1), methylated with diazomethane and sodium methoxide, and the methyl ester fatty acids were analyzed with gas chromatography–mass spectrometry.

For the second approach, the different plasmid vectors pYES2 (Invitrogen, Carlsbad, CA) and pESC (Stratagene, La Jolla, CA), were used to transform the yeast strain INVSc1. Both plasmids contain an *Escherichia coli* replication origin, a yeast 2m plasmid replication origin, an *E. coli* ampicillin-resistance gene and the yeast *URA3* gene in pYES2 and *TRP1* gene in pESC. The pYES2 carries the promoter and enhancer sequence from the *GAL1* gene for regulated expression. On the other hand, the pESC contains the *GAL1* and *GAL10* yeast promoters in opposing orientation.

To study the *Stokesia* epoxygenase gene function in yeast, a full-length cDNA fragment was cloned into pYES2 to give St EPX-pYES2. pYES2 together with pESC as a vector control (CTR), St EPX-pYES2 together with pESC (EPX) were transformed into yeast cell and selected on yeast minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose).

The total lipid composition of yeast was determined from cells harvested from a 3 ml liquid culture according to slightly modified the method described by Cahoon et al. (2002). Yeast CTR and EPX, bred colonies containing the pYES2 and pESC expression plasmids with or without St EPX cDNA were grown for three days at 30 °C in media (Cahoon et al., 2002) lacking uracil and tryptophan, and were supplemented with glycerol and glucose to final concentration of 5% (v/v) and 0.5% (w/v), respectively. Cells were washed twice in fresh media (Cahoon et al., 2002). The washed cells were then diluted to $OD_{600} = 0.4$ in media consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) galactose, 0.01% (w/v) adenine, and 0.2% (w/v) Tergitol NP-40. The media was supplemented with linoleic acid **1** at a final concentration of 470 nM. The cultures were incubated with shaking at 250 rpm at 20 °C and grown to $OD_{600} = 12$. Cells were collected by centrifugation and washed with sterilized water and pelleted cells were freeze-dried. Fatty acid methyl esters were prepared by transesterification of the dried cell pellet in 1% (w/v) sodium methoxide in methanol and analyzed using gas chromatography.

3.3. Gene testing in plants

For the *Arabidopsis thaliana* expression system, the TDNA-vector, pCAMBIA 1201 was utilized. A *Bsm*BI site was introduced at the first ATG and an *Sma*I site was introduced at the end of the ORF of the cDNA by PCR mutagenesis. The ORF sequence of the cDNA was amplified using two primers, (StexpF2: 5′-GACGCGTCTCCCATGTCGGATTCATATGATG- 3′, StexpR2: 5′-GACGCCCGGGTTACATTTTATGGTACCAATATGTCCC- 3′, *Bsm*BI and *Sma*I sites are underlined), and cloned into the pGEM-T Easy vector and verified by DNA sequencing. The *Bsm*BI–*Sma*I fragment covering the entire open reading frame of the cDNA was cut out from pGEM-T Easy vector and ultimately cloned into the respective sites of a vector, which contains a phaseolin promoter cassette (Kawagoe et al., 1994; Bustos et al., 1998). The *Pst*I fragment including the cDNA with the phaseolin cassette was cut out from the pPHI4752 vector and cloned into the respective multi-cloning site of pCAMBIA1201, T-DNA vector (CAMBIA, 2003).

This construct, pCAMBIA-ST was transformed into the *Agrobacterium tumefaciens* strain C58 harboring GV3850 vector by a triparental matings method. The original pCAMBIA1201 was also transformed into *Agrobacterium* as a control.

A. thaliana ecotype Columbia plants were transformed with the *Agrobacterium* carrying pCAMBIA-ST

or pCAMBIA1201 using a simplified dipping method (Clough and Bent, 1998). T_1 seeds were collected and cultured on selection media (MS salts, B5 vitamins, 1% sucrose, 25 mg/l hygromycin, 500 mg/l cefotaxime and 0.8% Phytagar, pH 5.8). Surviving plantlets were transferred into soil and T2 seeds were collected. For lipid extraction, seeds were ground in chloroform–methanol (2:1). The extracts were brought to dryness under N_2 gas stream. The lipid residues were immediately dissolved in a few drops of diazomethane and 0.5 ml 1% (w/v) sodium methoxide solution and shaken for 45 min at room temperature. The methyl ester fatty acids were extracted in hexane. The samples were analyzed using GC–MS or GC (Dahmer et al., 1989).

3.4. Analysis of epoxygenase mRNA expression in different tissues

RNA was isolated from each tissue such as developing seeds, leaves and roots using the Trizol reagent as described by the manufacturer (Invitrogen) and from mature seeds as described by Naito et al. (1994). RNA was separated on a 1% denaturing formaldehyde gel and transferred onto a Zeta-Probe Blotting Membrane (Bio-Rad Laboratories) according to Chomczynski (1992). Equalized loading of RNA was checked by ethidium bromide staining of rRNAs. The entire coding region of the epoxygenase gene from *S. laevis* was labeled with digoxigenin (DIG)-UTP by a PCR DIG Probe Synthesis Kit (Roche Applied Science). The hybridization was performed at 65 °C overnight in 1 mM EDTA, 0.5 M Na_2HPO_4 (pH 7.2) and 7% SDS. The final washing step were performed at 65 °C in 1 mM EDTA, 40 mM Na_2HPO_4 and 2% SDS. Hybridized mRNAs were detected with alkaline phosphatase conjugated anti-DIG antibody (Roche Applied Science) and its chemiluminescent substrate, CDP-Star (Roche Applied Science)

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