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Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates

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Abstract

Allene oxide synthase, an enzyme of the octadecanoid pathway to jasmonates, was cloned from *Arabidopsis thaliana* as a full-length cDNA encoding a polypeptide of 517 amino acids with a calculated molecular mass of 58705 Da. From the sequence, an N-terminal transit peptide of 21 amino acids resembling chloroplast transit peptides was deduced. Three out of four invariant amino acid residues of cytochrome P450 heme-binding domains are conserved and properly positioned in the enzyme coding region, including the heme-accepting cysteine (Cys-470). Southern analysis indicated in *A. thaliana* only one allene oxide synthase gene to be present. While transcript levels were rapidly and transiently induced after wounding of the leaves, allene oxide synthase activity remained nearly constant at a low level of ca. 0.8 nkat per mg of protein. The cDNA encoding *A. thaliana* allene oxide synthase was highly expressed in bacteria giving rise to a polypeptide of the calculated molecular mass. The protein was enzymatically active, and verification of the reaction products by GC-MS showed that it was capable of utilizing not only 13-hydroperoxylinolenic acid (13-hydroperoxy-9(Z), 11(E), 15(Z)-octadecatrienoic acid), but also 13-hydroperoxylinoleic acid (13-hydroperoxy-9(Z), 11(E)-octadecadienoic acid) as substrate. The data suggest parallel pathways to jasmonates from linolenic acid or linoleic acid in *A. thaliana*.

Introduction

Recently, it has been realized that octadecanoid and/or dodecanoid metabolites (jasmonates) derived from α-linolenic acid [27, 32] and also from linoleic acid [4] serve as signalling molecules to regulate diverse aspects of plant life such as senescence [17, 24], herbivore defense [6], pathogen defence [8] and mechanotransduction [5].

The first enzyme in the biosynthesis of jasmonic

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X92510.

acid [27] is allene oxide synthase (EC 4.2.1.92, hydroperoxide dehydratase), an enzyme that converts fatty acid hydroperoxides to unstable allene epoxides [22] which either spontaneously, or through action of an allene oxide cyclase [9, 10], cyclize to form cyclopentenone acids. 13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid (13-HPOT), produced from α-linolenic acid through lipoxygenase catalysis, is converted by this pathway to the jasmonate precursor 12-oxo-phytodienoic acid (12-OPDA).

The recent elucidation of the mode of action of the *Pseudomonas* toxin coronatine [31] has shown that 12-OPDA, or an octadecanoid compound derived from it, rather than jasmonic acid, is a signal transducer in mechanotransduction associated with tendril coiling. Blechert *et al.* [4] have, by using the β-oxa-tetrahomo analogues of jasmonic acid, demonstrated that β-oxidation of 12-OPDA may not be required for physiological activity in inducing low-molecular-weight defence compounds in various plant cell cultures. Thus, the enzymatic formation of cyclic octadecanoids from fatty acid hydroperoxides catalysed by allene oxide synthase has to be regarded the crucial step in biosynthesis of this novel group of plant signalling molecules.

Flax seed allene oxide synthase has been characterized, purified [22, 25] and cloned [23] and shown to represent a novel class of low-carbon-monoxide-affinity cytochrome P450 enzymes now designated as CYP74. Its physiological role in the seed is, however, unclear, even more since total enzyme activity is very high here compared to vegetative tissue. A second CYP74, showing 85% similarity to the flax enzyme, has recently been identified as the major rubber particle protein, RPP [16], and shown to catalyse the conversion of 13(S)-hydroperoxy-9(Z),11(E)-octadecadienoic acid to the corresponding ketol fatty acid. We therefore set out to clone allene oxide synthase from leaves of *Arabidopsis thaliana* as a prerequisite for functional analysis of the enzyme *in vitro* and *in vitro*. When wounded, *A. thaliana* produces relatively large amounts of jasmonic acid (*vide infra*). This species therefore should be particularly useful for the molecular analysis of signaling *via* the octadecanoid pathway. We report here the cloning, molecular characterization and functional expression of a cDNA encoding the *A. thaliana* enzyme. We further show that *A. thaliana* allene oxide synthase, when expressed from its cDNA in a bacterial host, gives rise to 12-OPDA from 13-HPOT and the corresponding pentyl analogue from 13-hydroperoxy-9(Z),11(E)-octadecadienoic acid (13-HPOD), suggesting that parallel pathways from lipoxygenase products of 1,4-diene-type fatty acids to cyclic oxoenoic acids are initiated by this enzyme.

Material and methods

Plant materials

Seeds from *Linum usitatissimum* were obtained from local distributors and grown for two weeks at 60% relative humidity with an 8 h photoperiod, 200 $\mu E\ m^{-2}\ s^{-1}$ photosynthetically active radiation, at 20 °C. *A. thaliana* (L.) Heynh., ecotype *Columbia*, was cultivated in controlled chambers at 70% relative humidity with a daily schedule of 10 h light (210 $\mu E\ m^{-2}\ s^{-1}$ photosynthetically active radiation) at 20 °C and 14 h darkness at 17 °C. Plants used for RNA and DNA isolation and wounding experiments were 4 weeks old. Leaves of the rosette were wounded by crushing across the midvein with a hemostat.

Purification and sequencing of flax allene oxide synthase

Allene oxide synthase was isolated from acetone powder from flax seed basically following the procedure of Song and Brash [22], with some modifications. The anion exchange column applied was Q-Sepharose fast flow (Pharmacia), and eluting fractions containing activity were concentrated on a second, 1 ml, column of Q-Sepharose. Chromatofocusing on a Mono-P column was omitted. Purified allene oxide synthase (15 μg) was sequenced from the N-terminus after SDS-PAGE and electrophoretical transfer onto glass fibre membranes [14].

Assays for allene oxide synthase activity

Enzyme activity was measured spectrophotometrically at 235 nm as described [7], using 13-HPOT, 13-HPOD and 15(S)-hydroperoxy-11(Z),13(E),17(Z)-eicosatrienoic acid (15-HPET) as substrates. These substrates were prepared from the corresponding fatty acids using established protocols [7, 26, 28, 29] and purified to homogeneity as described [7]. Verification of allene oxide synthase reaction products was done as follows: 4 μmol substrate were added to 0.7 mg of protein (flax extract or bacterial protein) in 50 ml 50 mM phosphate buffer, 5 mM Mega-9, pH 7 at room temperature and incubated for 10 min. The reaction was stopped by acidifying to pH 3 with HCl. Products were extracted with peroxide-free diethyl ether, which was subsequently evaporated. Products of flax reference allene oxide synthase preparations were redissolved in *n*-hexane and purified by high-performance liquid chromatography (HPLC) on a Zorbax BG-Y-370 column (Knauer, 250 mm × 4 mm, 5 μm inner diameter) in *n*-hexane/2-propanol/acetic acid (100:1.62:0. 11, v/v/v) at a flow rate of 1.5 ml/min. Retention times were: 16 min (3-oxo-2-(2′-pentenyl)cyclopent-4-eneoctanoic acid, 12-oxo-phytodienoic acid, 12-OPDA), 14 minutes (3-oxo-2-(2′-pentyl)cyclopent-4-eneoctanoic acid), 16 min (3-oxo-2-(2′-pentenyl)cyclopent-4-enedecanoic acid), and 14.5 min for the 13-HPOD-derived α-ketol, 13-hydroxy-12-oxo-9(Z)-octadecaenoic acid, respectively. Fractions containing the products were dried, dissolved in methanol, treated with ethereal diazomethane, dried again and redissolved in chloroform. Methoxyamine derivatives of ketol methyl esters were prepared according to Vick and Zimmerman [29]. Bacterial reactions were treated the same except that the HPLC step was omitted to allow a complete GC-MS survey of all reaction products. All samples were finally subjected to capillary gas chromatography/mass spectroscopy using a Finnigan (San Jose, CA) Magnum ion trap mass spectrometer run in electron impact (70eV) mode or in chemical ionization mode with methanol as reactand gas. Products were separated on a DB-17 WCOT fused silica capillary column (J&W Scientific, Folsom, CA) (30 m × 0.25 mm, 0.25 μm coat) using helium as carrier gas. Temperature program: 80 °C for 1 min, then linear gradient to 200 °C at a rate of 30 °C/min followed by a rate of 5 °C/min up to 250 °C.

Determination of jasmonic acid

Extraction of jasmonic acid from plant material, methylation and quantitation with an enzyme-linked immunosorbent assay (ELISA) was as described [1].

RNA extraction and analysis

Total RNA was isolated by standard procedures (2). Poly(A)$^+$ mRNA was isolated using Oligotex-dT (Qiagen). RNA was separated on 1.5% (w/v) agarose gels under denaturing conditions (formaldehyde) and transferred onto Hybond-N nylon membranes (Amersham) with 20× SSPE as transfer buffer using standard procedures [21]. For hybridization, probes were labelled with (α-^{32}P)-dATP and the Klenow reaction. Hybridization with the PCR fragment L1 as well as with the cDNA clone A2 was done at 65 °C, 6× SSPE, 7.5× Denhardt's reagent, 200 μg/ml salmon sperm DNA and 1×10^6 dpm/ml of labeled probe for 18 h. Filters were placed on Kodak X-Omat film.

PCR cloning

First-strand cDNA was synthesized from 200 ng of poly(A)$^+$ mRNA using 300 ng of oligo (dT) as a primer with Superscript reverse transcriptase according to the protocol of the manufacturer (Gibco/BRL). From this cDNA, 2% were used for PCR reactions (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% (v/v) Triton X-100, 2 mM $MgCl_2$, 0.2 mM dNTP each, 25 pmol of primers, 2.5 units *Taq*-polymerase). The temperature program used was: 1 min 94 °C, 2 min 37 °C, 3 min 72 °C, 29 cycles; 5 min 72 °C, 16 h 4 °C, 1 cycle.

The primers used were: 5′-GGGATCTCGCGG-GACGAAGCTT-3′ and 5′-TCTCGAGCGT-CTCCGTCTCCGGCCCG-3′. The PCR mixture was run on an 1.5% agarose gel, and the 0.47 kb band was extracted following the procedure of Benson and Zagrsky [3]. The purified DNA (designated fragment L1) was cloned into the vector pBluescript II SK (Stratagene) and electrotransfected into *Escherichia coli* strain XL 1-blue. Sequencing confirmed its identity and position within the flax allene oxide synthase-coding sequence.

Screening of the cDNA library and DNA sequencing

A cDNA library maintained in λZAP and prepared from leaves of 3-week old *A. thaliana* cv. Columbia, was kindly provided by Dr Rüdiger Hell, Bochum. The cDNA library was screened with the 463 base PCR fragment L1 from flax (65 °C, 6× SSPE, 7.5× Denhardt's reagent, 200 μg/ml salmon sperm DNA, 1×10^6 dpm/ml of labelled probe, 18 h). Positive clones were converted into phagemids by *in vivo* excision. The inserts and the PCR product L1 were sequenced on both strands from multiple, overlapping partial clones using the Sequenase kit, version 2.0 (USB) and (^{35}S)-αdATP (Amersham).

Isolation of genomic DNA and Southern blot analysis

Total DNA was isolated from 2 g of leaves as described [18]. For Southern blot analysis, 20 μg of DNA were digested with restriction enzymes as indicated in Results (10 U/μg DNA), subsequently separated on a 0.6% (w/v) agarose gel and transferred onto Hybond-N nylon membranes (Amersham) with 10× SSPE. Hybridization conditions with the full-length cDNA clone A1 were as mentioned above.

Expression of allene oxide synthase cDNA in a bacterial host

For overexpression, cDNA clone A1 was restricted with the enzymes *Bam*HI and *Kpn*I. The fragment was cloned into the expression vector pQE30 (Qiagen) and transfected into *E. coli* strain M15. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to log-phase bacteria up to a concentration of 2 mM for 4–5 h. Bacteria were lysed by sonication after a freeze/thaw-cycle and centrifuged for 10000 × *g* for 20 min. The activity was found exclusively in the pellet. This fraction was used to perform SDS-PAGE (10% separating gel) [12] and for measurement of allene oxide synthase activity. Inclusion body protein was prepared as described [11].

Results and discussion

N-terminal sequencing of allene oxide synthase from flax and generation of PCR fragments

Flax allene oxide synthase was purified to apparent homogeneity from seeds according to Song and Brash [22] with slight modifications as described in Materials and methods. The preparation consisted of a prominent 55 kDa polypeptide with a minor component at 53 kDa. Both were enzymatically active (data not shown). On two-dimensional electropherograms, microheterogeneity of both polypeptides was evident. Sequencing of the N-terminus of the 55 kDa allene oxide synthase yielded the following sequence: G/SLFGESPIKIT/P. The first and last amino acid could not be determined unambiguously. The first residue is either a glycine or a serine and the last residue either a threonine or a proline. The sequence SLFGESPIKIT is identical with amino acids 46–56 of the published flax allene oxide synthase deduced from the nucleotide sequence [23], except for position 5, where an aspartic acid is substituted by a glutamic acid. Interestingly, this sequence lies within the signal peptide, deduced by Song *et al.* [23] based on the aminoterminal sequence determined for their enzyme preparation (*vide infra*). Degenerate oligonucleotides were derived from the sequence FGESP, end-labelled with (γ-^{32}P)-ATP and hybridized to total RNA from *L. usitatissimum* and *A. thaliana*

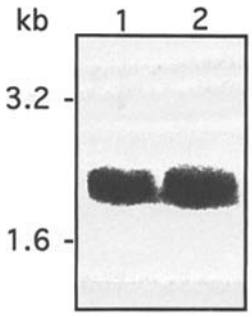

Fig. 1. Northern blot analysis of *Arabidopsis* and *Linum* allene oxide synthase RNA. Total RNA (20 μg) from *L. usitatissimum* (lane 1) and *A. thaliana* (lane 2) were separated electrophoretically on an 1.5% formaldehyde agarose gel, transferred onto Hybond-N nylon membranes and hybridized with the ^{32}P-labelled PCR fragment L1 generated from the flax allene oxide synthase.

in northern blot analyses. In both species, the probe hybridized to transcripts of a size of 2.2 kb (data not shown), which corresponded to the published size of the flax allene oxidase synthase mRNA [23]. A PCR fragment (L1) was then generated from flax seed cDNA using oligonucleotides designed from the sequence. The fragment had a size of 464 bases corresponding to nucleotides 1229–1692 of the flax sequence. Using fragment L1, northern analysis of total RNA from *L. usitatissimum* and *A. thaliana* in both cases revealed, under stringent conditions, a transcript of the expected size of 2.2 kb (Fig. 1), showing that the PCR fragment could be used to screen for the *A. thaliana* allene oxide synthase homologue.

cDNA cloning, characterization of the primary sequence and structure of the heme-binding domain

A cDNA library from vegetative rosettes of *A. thaliana* was screened with fragment L1, and 7 positive clones could be isolated. The largest clone, A1, had a size of 1799 bases. Sequence analysis showed that this clone contained the complete coding region for allene oxide synthase as well as non-coding 3′ and 5′ sequences including poly(A) (Fig. 2). A second clone, A2, used for northern hybridizations was a C-terminal partial clone and had a size of 886 bases encompassing nucleotides 912–1798 of the sequence shown in Fig. 2. Comparison with the published sequence of flax showed an overall similarity of 62.5% based on identical nucleotides and 54.3% based on identical amino acids. The deduced mature enzyme of 496 amino acids (56556 Da calculated molecular mass) showed a degree of similarity of 58.5% (identical amino acids) and 73.1% (identical plus similar amino acids) to the corresponding flax sequence. The primary sequence proved clearly homologous to the one from flax, but with some notable differences (cf. Fig. 3). The flax enzyme was reported to contain a 58 amino acid long N-terminal transit peptide reminiscent of chloroplast or mitochondrial leader sequences. Close inspection of the *A. thaliana* sequence revealed the presence of a much shorter leader peptide. By applying established criteria [30], the leader encompasses only the first 21 amino-terminal amino acids (underlined in Figs. 2 and 3) with the −3 to −1 sequence SKA (asterisks in Fig. 3) preceding the proposed cleavage site. This transit peptide fulfils many of the criteria valid for chloroplast leaders: (a) D, Y and E are absent, (b) R and L are not enriched (contrary to mitochondrial transit peptide (mtTP) sequences), (c) the sequence starts with MA which is typical for chloroplast leader sequences but uncommon in mtTPs, and the aminoterminal half does not form an amphipathic helix (which is typically found in mtTPs) and carries no charged residues, (d) the sequence contains 20% S and bears a net positive charge clustered at the C-terminal half of the leader sequence, (e) a β-sheet precedes the cleavage site which contains an A residue and (f) the −2 and −10 arginines, typical for mtTPs, are absent. Thus, the transit peptide is predicted to target the enzyme to the chloroplast, which is in accordance with biochemical data [13, 29]. Re-inspection of the much longer transit peptide proposed for the flax enzyme ([23]; cf. Fig. 3) which was based on results from N-terminal amino acid sequencing of the mature protein revealed some problems. The proposed cleavage site and its upstream environment violate several criteria for chloroplast transit peptides. N-terminal sequencing of mature flax allene oxide

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GGCACGAGCTCAATCACAACACTCGCCACTGTTTCGAATAGATGGCTTCTATTTCAGCCCCTTTTCCGATTTCT    74
                                        *  M  A  S  I  S  A  P  F  P  I  S     11

CTCCACCCAAAAACCGTACGATCAAAGGCGTTGAAATTCCGAGTTTTGACCCGTCCGATCAAAGCTTCCGGGTCA   149
L  H  P  K  T  V  R  S  K  A  L  K  F  R  V  L  T  R  P  I  K  A  S  G  S     36

GAAACTCCTGATCTAACCGTAGCGACACGAACCGGATCCAAAGATCTCCCGATCCGAAACATACCGGGAAACTAC   224
E  T  P  D  L  T  V  A  T  R  T  G  S  K  D  L  P  I  R  N  I  P  G  N  Y     61

GGTTTACCAATCGTAGGACCAATCAAAGACCGTTGGGATTACTTTTACGACCAAGGAGCTGAAGAGTTCTTCAAA   299
G  L  P  I  V  G  P  I  K  D  R  W  D  Y  F  Y  D  Q  G  A  E  E  F  F  K     86

TCACGAATCCGTAAATACAACTCCACGGTGTACAGAGTCAACATGCCACCGGGAGCTTTTATCGCCGAGAATCCA   374
S  R  I  R  K  Y  N  S  T  V  Y  R  V  N  M  P  P  G  A  F  I  A  E  N  P    111

CAAGTCGTGGCTTTACTCGACGGTAAAAGCTTCCCGGTTTTATTCGATGTCGATAAAGTCGAAAAGAAAGATCTT   449
Q  V  V  A  L  L  D  G  K  S  F  P  V  L  F  D  V  D  K  V  E  K  K  D  L    136

TTCACCGGTACTTACATGCCGTCAACGGAACTAACCGGAGGCTACCGTATCCTCTCGTACCTCGATCCATCGGAG   524
F  T  G  T  Y  M  P  S  T  E  L  T  G  G  Y  R  I  L  S  Y  L  D  P  S  E    161

CCTAAACACGAAAAGCTCAAAAATCTCCTTTTCTTCCTCCTCAAGTCATCTCGAAACCGGATCTTCCCTGAGTTT   599
P  K  H  E  K  L  K  N  L  L  F  F  L  L  K  S  S  R  N  R  I  F  P  E  F    186

CAAGCTACTTACTCCGAGCTTTTCGATTCTTTGGAGAAAGAGGCTTTCCCTTTAAGGGAAAGCGGATTTCGGCGG   674
Q  A  T  Y  S  E  L  F  D  S  L  E  K  E  A  F  P  L  R  E  S  G  F  R  R    211

TTCCAGCGACGGAACCGCCTTTTATTTCTTGGCTCGAGCTTTCTACGGGACGAATCCCGCAGATACAAGCTCAAA   749
F  Q  R  R  N  R  L  L  F  L  G  S  S  F  L  R  D  E  S  R  R  Y  K  L  K    236

GCCGACGCTCCGGGTTTGATCACTAAATGGGTTTTATTCAATCTCCATCCATTACTCTCTATTGGTTTACCGAGA   824
A  D  A  P  G  L  I  T  K  W  V  L  F  N  L  H  P  L  L  S  I  G  L  P  R    261

GTTATAGAAGAACCTCTCATCCATACATTTAGTCTACCACCGGCGTTAGTCAAATCTGATTACCAGAGACTCTAC   899
V  I  E  E  P  L  I  H  T  F  S  L  P  P  A  L  V  K  S  D  Y  Q  R  L  Y    286

GAGTTTCTTAGAATCCGCGGTGAGATTCTCGTTGAAGCCGATAAATTGGGTATCTCACGAGAAGAAGCTACTCAC   974
E  F  L  R  I  R  G  E  I  L  V  E  A  D  K  L  G  I  S  R  E  E  A  T  H    311

AATCTTCTCTTCGCCACGAGCTTCAACACGTGGGGTGGGATGAAGATTTTGTTTCCGAATATGGTTAAACGTATC  1049
N  L  L  F  A  T  S  F  N  T  W  G  G  M  K  I  L  F  P  N  M  V  K  R  I    336

GGGCCGGCGGTCATCAAGCTTCATAACCGCTTAGCGGAGGAGATTAGATCTGTGATTAAATCCAACGGCGGAGAA  1124
G  P  A  V  I  K  L  H  N  R  L  A  E  E  I  R  S  V  I  K  S  N  G  G  E    361

CTCACGATGGGAGCGATTGAGGGAAATGAGTTAACCAAATCAGTGGTTTACGAATGTCTCCGGTTTGAACCACCG  1199
L  T  M  G  A  I  E  G  N  E  L  T  K  S  V  V  Y  E  C  L  R  F  E  P  P    386

GTTACGGCTCAATACGGTAGAGCGAAGAAGGATCTGGTTATCGAAAGCCACGACGCGGCGTTTAAAGTCAAAGCC  1274
V  T  A  Q  Y  G  R  A  K  K  D  L  V  I  E  S  H  D  A  A  F  K  V  K  A    411

GGTGAGATGCTTTACGGTTATCAACCGTTGGCGACGCGAGATCCGAAGATTTTTGATCGGGCGGATGAGTTTGTG  1349
G  E  M  L  Y  G  Y  Q  P  L  A  T  R  D  P  K  I  F  D  R  A  D  E  F  V    436

CCGGAGAGATTCGTCGGAGAAGAAGGAGAGAAGCTTTTGAGGCATGTGTTGTGGTCGAATGGACCGGAGACGGAG  1424
P  E  R  F  V  G  E  E  G  E  K  L  L  R  H  V  L  W  S  N  G  P  E  T  E    461

ACTCCGACGGTGGGGAATAAACAATGCGCCGGTAAGGATTTTGTTGTTTTGGTGGCGAGGTTGTTTGTGATTGAG  1499
T  P  T  V  G  N  K  Q  C  A  G  K  D  F  V  V  L  V  A  R  L  F  V  I  E    486

ATTTTCCGGCGATATGATTCGTTTGATATTGAGGCCGGTACGTCGCCGTTAGGAAGCTCCGTTAATTTCTCGTCG  1574
I  F  R  R  Y  D  S  F  D  I  E  A  G  T  S  P  L  G  S  S  V  N  F  S  S    511

TTAAGGAAAGCTAGCTTTTAGGAGCCAAGGGTAAATTTGTAATTCTATATGTTCGCACCTGTGTAGTCCATCTGT  1649
L  R  K  A  S  F  *                                                           517

TTTCTTGTTCCAGTTTCGTGAATTTTGAGAAAATGTTGATAATCTGTTGCTGCTCTACATGTTCGTTTTTTTGTC  1724
ATAATATTAAAGTTCAAGCTGGTAATAAGTATTTACAAATCTGTGAGATAATTTCAAAAAAAAAAAAAAAAAAAA  1799
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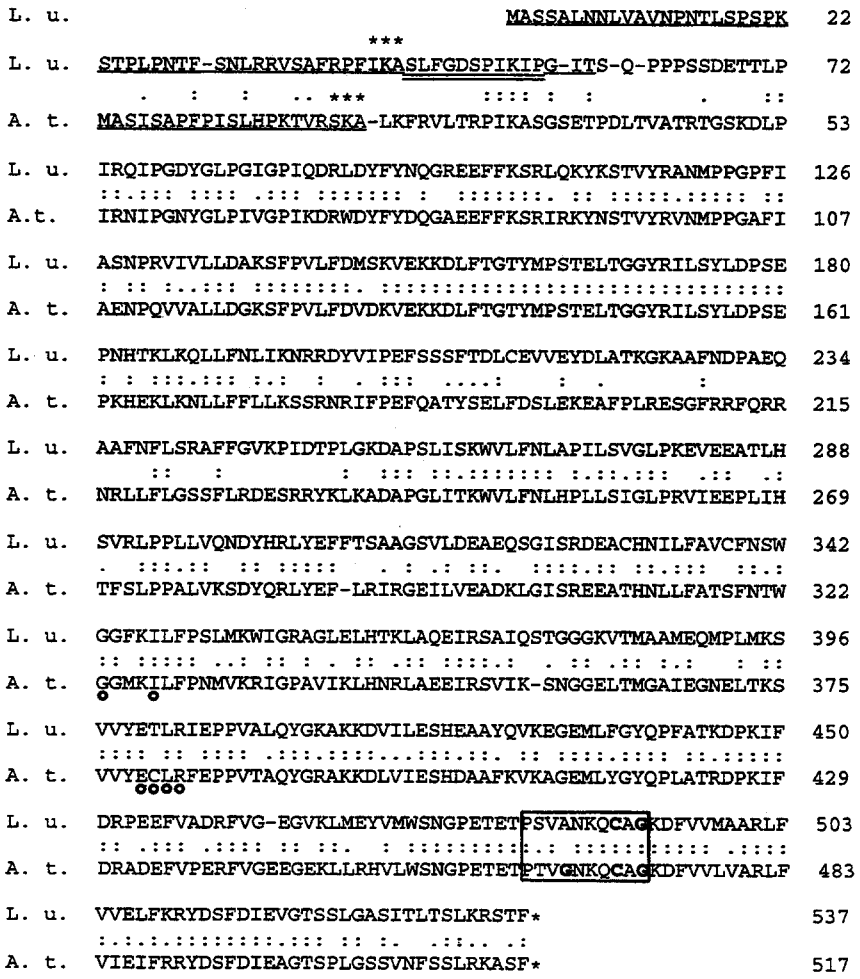

Fig. 3. Comparison of amino acid sequences of the flax (L.u.) and *Arabidopsis* (A.t.) allene oxide synthases (flax sequence according to [23]). The proposed transit peptides are underlined. Doubly underlined is that part of the published flax sequence to which the N-terminal sequence determined in this study is homologous. Note that it lies within the proposed [23] transit peptide sequence and in an area of significant similarity between the two sequences. ***(−1 to −3) region of the most likely signal peptidase cleavage sites of flax and *A. thaliana* allene oxide synthase proposed in this study. Boxed is the heme-binding domain characteristic of cytochrome P450 enzymes. A consensus sequence of PsVsNKQCAG (s = similar residue) for the two now known plant allene oxide synthases is derived for this motif, which is embedded in stretches of highly conserved amino acids. Identical residues (:), similar residues (.), S, T; D, E; N, Q; R, K; I, L, M, A, V; F, Y, W.

synthase in our study, then, gave a different sequence (doubly underlined in Fig. 3) located *within* the proposed leader and starting at Ser-46. Importantly, the −3 to −1 motif preceding Ser-46, IKA, is homologous to the SKA proposed to precede the processing site of the *A. thaliana* en-

Fig. 2. Nucleotide and deduced amino acid sequence of allene oxide synthase cDNA from *A. thaliana*. The putative transit peptide is underlined. The arrow indicates the start of the truncated coding sequence used for bacterial expression of enzymatically active allene oxide synthase. Doubly underlined is the heme-binding consensus domain with the heme-attaching cysteine shown in bold face. Asterisks denote stop codons.

zyme. Thus, we find Ser-46, rather than Ser-60, to represent the start of the mature flax enzyme. On this basis, it is also easier to understand the strong sequence similarity around amino acid residues 30 to 38 (51 to 58 in the flax sequence) which would be difficult to understand, if part of a transit peptide.

Flax allene oxide synthase activity has been shown to be largely associated with a chloroplast membrane fraction with little enzyme appearing soluble [29]. From the membrane, the enzyme can be solubilized with mild detergents [22]. The N-terminus of the mature *A. thaliana* allene oxide synthase is predicted to form a short, amphiphilic helix with a net positive charge (also conserved in the flax enzyme). This would be a candidate site of membrane attachment of the enzyme. However, is must be noted that the bacterially expressed allene oxide synthase, which lacks this portion of the N-terminus, remained associated with bacterial membranes after lysis of the cells (*vide infra*). Collectively, our, as well as the biochemical data [29] suggest allene oxide synthase to be a chloroplast enzyme. Since there is no evidence for a composite transit peptide, at least in the short leader sequence of the *A. thaliana* enzyme, it can be expected that allene oxide synthase remains associated with the envelope after import into the chloroplast.

Whereas in flax, the common motif for the heme-binding domain of cytochrome P450 enzymes, FXXGXXXCXG, was maintained only in the last two conserved amino acids (C and G), the allene oxide synthase from *A. thaliana* showed three out of four invariant amino acids (Gly-466, Cys-470 and Gly-472) with only the first amino acid of the motif, phenylalanine, replaced by proline (Pro-463) as in the flax enzyme (Fig. 3, boxed sequence element). The heme-binding domain of the *A. thaliana* enzyme is thus more similar to the general cytochrome P450 consensus sequence than the flax domain which is very divergent. The corresponding sequence of guayule RPP [16] is PXXEXXXCXG. A common feature of the three allene oxide synthases now known appears to be the proline which replaces the phenylalanine as the first conserved amino acid of the heme-binding domain of typical cytochrome P450s (Pro-463 in the *A. thaliana* sequence). This residue is a logical target for structure-function experiments on the heme-binding pocket of allene oxide synthases.

Deviations could also be expected for the oxygen-binding pocket which is another highly conserved feature in the I helix region located in the C-terminal third of cytochrome P450s, notably the sequence GxxxT, in which G and T establish close contact with oxygen [19], starting 56 residues upstream of a highly conserved ETLR motif. This motif is present as ECLR (amino acids 379 to 382) in the *A. thaliana* sequence, but the GxxxT motif is changed to GxxxI (open circles in Fig. 3), just as in the flax [23] and guayule RPP [16] sequences, suggesting a second site for

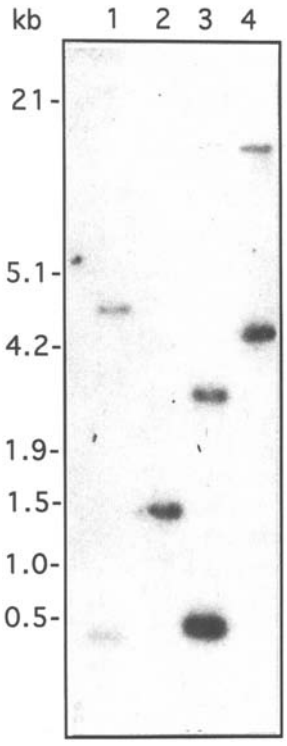

Fig. 4. Southern analysis of DNA from *A. thaliana* probed with allene oxide synthase cDNA. Equal amounts (20 μg) of DNA from *A. thaliana* leaves were digested with the restriction enzymes *Hin*dIII, *Bam*HI, *Xho*I and *Eco*RI (lanes 1–4), separated by electrophoresis, transferred onto-Hybond-N nylon membranes and probed with ^{32}P-labelled cDNA (clone A1). The film was exposed for 3 days.

structure-activity experiments that would lend itself to probe the evolution of the peroxide dehydrase vs. oxygenase activities of cytochrome P450 enzymes. As more sequences become known, further structural elements will emerge that help to an understanding of the molecular basis of the allene oxide synthase reaction.

Southern analysis of DNA extracted from *A. thaliana*, restricted with *Bam*HI and hybridized to the ^{32}P-labelled cDNA (clone A1), revealed only one band, while restriction with the enzymes *Hin*dIII, *Xho*I and *Eco*RI gave two hybridization signals (Fig. 4), reflecting the presence of internal restriction sites for *Hin*dIII and *Xho*I and a potential *Eco*RI site within the gene. These results suggest that there is likely only a single gene for allene oxide synthase in the genome of *A. thaliana*.

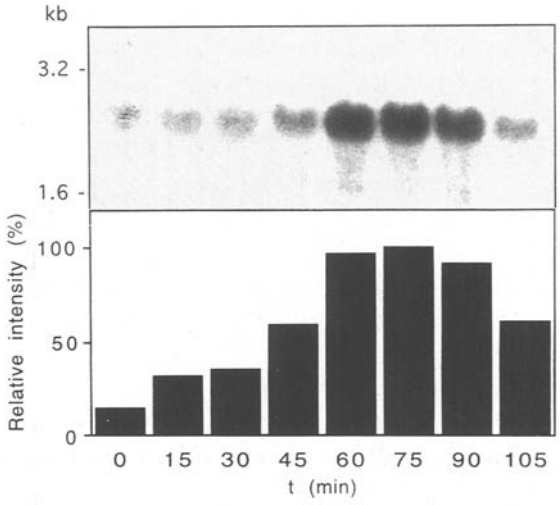

Fig. 5. Northern analysis of RNA from wounded *Arabidopsis* leaves probed with an allene oxide synthase cDNA clone. Leaves were wounded and RNA extracted at different time points. In each lane 15 μg of total RNA have been loaded and separated by gel electrophoresis. After transfer to Hybond-N nylon membranes the RNA was probed with ^{32}P-labelled allene oxide clone A2. The film was exposed for 3 days. Shown are an autoradiogram and the corresponding densitogram.

Levels of allene oxide synthase mRNA, enzyme activity and accumulation of jasmonic acid after wounding

When levels of allene oxide synthase transcript were determined in *A. thaliana* after the leaves had been wounded with a hemostat, a rapid, transient increase was observed (Fig. 5). Detailed kinetics of the reaction revealed first accumulation of the transcript already 15–30 min after wounding, with a maximum at 75 min (ca. 13-fold increase over levels of unwounded controls) and a decrease to 45% of the maximum value within the next 30 min. Levels of free jasmonic acid followed similar kinetics with a delay of the maximum of ca. 15 min, increasing from 0.55 to 3.64 pmol per g fresh mass (6.6-fold increase) (Fig. 6B). The enzymatic activity of allene oxide synthase in leaf tissue, however, remained very low and was close to the limits of detectable levels. Within these limits, no significant increase of activity (average: 0.76 ± 0.16 nkat per mg protein) could be measured after wounding (Fig. 6A). The increase in jasmonic acid level is thus not due to a generally increased amount of allene oxide synthase enzyme and probably reflects increased substrate flow through the pathway. Nevertheless, the clear increase in allene oxide synthase mRNA level may indicate a local gene activation around the wound site, not readily detectable at the enzymatic level in the whole leaf extracts used in this study. It is thus conceivable that allene oxide synthase levels, in addition to substrate availability, regulate output through the octadecanoid pathway. Further experiments with transgenic sense and antisense plants will resolve this issue.

Bacterial overexpression of allene oxide synthase and identification of reaction products

The cDNA clone A1 was restricted with *Bam*HI and *Kpn*I and subcloned into the histidin-tagged expression vector pQE30 to give pQE30-AOS. This strategy removed the putative transit peptide as well as the first 27 amino acids of the mature enzyme (cf. Fig. 2, arrow) and added the sequence MRGSHHHHHH N-terminal to the residual sequence (MRGS representing the vector's ATG plus part of the multiple cloning site followed by

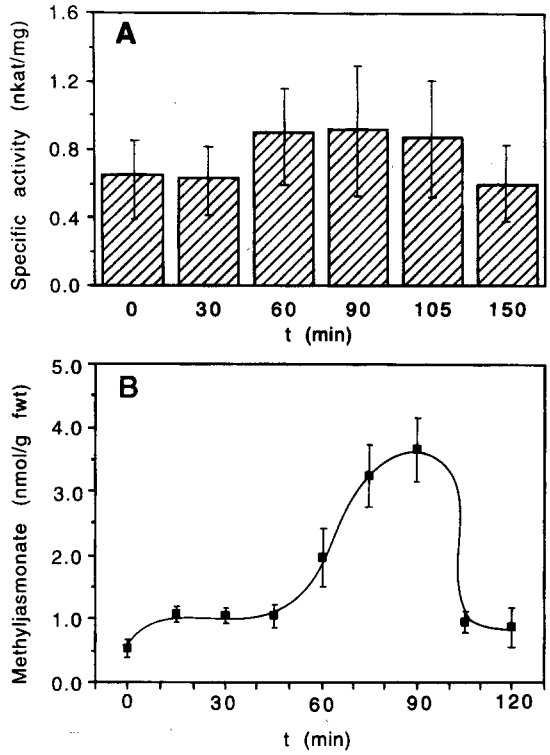

Fig. 6. Allene oxide synthase activity (A) and jasmonic acid level (B) in leaves of *A. thaliana* at different times after wounding. A. Enzymatic activity representing means ± s.d. of $n = 3$ independent experiments. B. Jasmonic acid levels in unwounded control and in wounded leaves of *A. thaliana*. The data are means ± s.d. of $n = 3$ independent experiments.

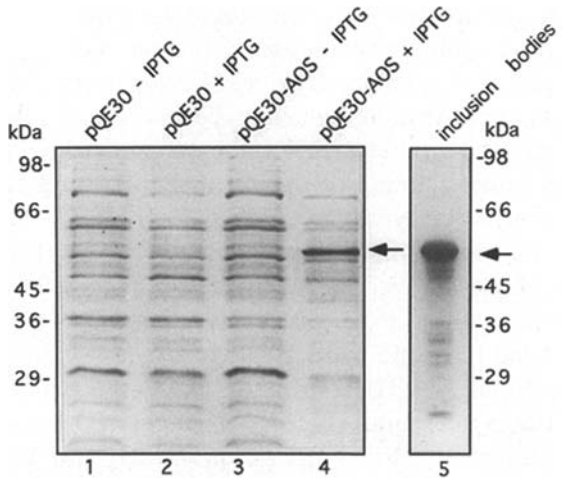

Fig. 7. Bacterial overexpression of allene oxide synthase. The cDNA clone A1 was subcloned into the vector pQE30 and transfected into *E. coli* strain M15. Protein patterns of bacteria containing the vector alone (lanes 1, 2) or with the insert (lanes 3, 4, 5) are shown in the absence (lanes 1, 3) or in the presence (lanes 2, 4, 5) of IPTG. The arrows mark the position of the overexpressed protein. Lanes 1–4, total cellular protein (10 μg per lane); lane 5, protein solubilized from inclusion bodies (10 μg per lane).

the hexa-histidine tag). After transfection of *E. coli* with the vector pQE30-AOS by electroporation and induction with IPTG, an additional, prominent band of the expected size of 55 kDa (calculated size 54869 Da) could be seen when protein patterns of bacterial extracts were compared in SDS-PAGE (Fig. 7). Most of the polypeptide was found in inclusion bodies (Fig. 7, lane 5).

No activity of allene oxide synthase could be measured in bacteria alone, nor after transfection with the vector pQE30, using any of three substrates (*vide infra*) known to be converted by flax allene oxide synthase [4, 22, 28]. However, bacteria transfected with pQE30-AOS showed clearly detectable enzymatic activity in the fraction of soluble proteins with all three substrates, 13-HPOT, 13-HPOD and 15-HPET (Table 1) after induction of transcription with IPTG. Substrate conversion initially was tested spectrophotometrically and compared to the reference reactions obtained with flax enzyme preparations (Table 1). All three substrates were converted by the cloned *A. thaliana* enzyme at comparable specific activities.

Product analysis in each case was carried out

Table 1. Enzymatic activity of *A. thaliana* allene oxide synthase expressed from its cDNA in *E. coli*.

Substrate	Specific activity (nkat per mg protein)		
	pQE30-AOS[a]	pQE30[b]	flax-AOS[b]
13-HPOT	7.3	0	1.9
13-HPOD	7.0	0	2.5
15-HPET	9.2	0	1.7

[a] Extracts were made from IPTG-induced *E. coli* M15 transfected with the vector pQE30-AOS containing the coding sequence of the *A. thaliana* enzyme (pQE30-AOS).

[b] Controls: extracts from bacteria transfected with the expression vector lacking the cDNA insert (pQE30); extracts from flax seed (flax-AOS).

using capillary gas chromatography coupled to ion trap mass spectrometry in electron impact (EI) as well as chemical ionization (CI) mode. Due to self-protonation reactions, electron impact mass spectra of amphiphilic lipids measured in ion trap spectrometers are slightly different from spectra obtained in quadrupol or sector field instruments. It was therefore necessary to obtain spectra of the products of reference reactions using flax enzyme preparations. The product composition obtained from the flax enzyme reaction in each case was very similar to that obtained from the overexpressed, cloned enzyme, and mass spectra obtained for corresponding reaction products were identical. For clarity, only the data for the cloned enzyme are presented (Fig. 8A–D). When 13-HPOT or 15-HPET were added as substrates, the dominant reaction products were the α-ketols and the cyclization products, 12-oxophytodienoic acid (or 3-oxo-2-(2′-pentenyl)cyclopent-4-eneoctanoid acid, from 13-HPOT, cf. Fig. 8A) or 3-oxo-2-(2′-pentenyl)cyclopent-4-enedecanoic acid, the dihomo-analogue of 12-OPDA, from 15-HPET (cf. Fig. 8C). Both were identified unambiguously as their methyl esters by their retention times and EI spectra in comparison with the flax reference compounds and the literature spectra [28]. The $[M+H]^+$ ion, rather than M^+ was observed in the case of 3-oxo-2-(2′-pentenyl)cyclopent-4-enedecanoic acid (cf. Fig. 8C). In addition, the CI spectrum for 12-OPDA methyl ester gave *m/z* 307, representing the expected $[M+H]^+$ ion and *m/z* 275 (307-CH_3OH) as expected for the methyl ester. The CI spectrum of the methyl ester of 3-oxo-2-(2′-pentenyl)cyclopent-4-enedecanoic ester gave,

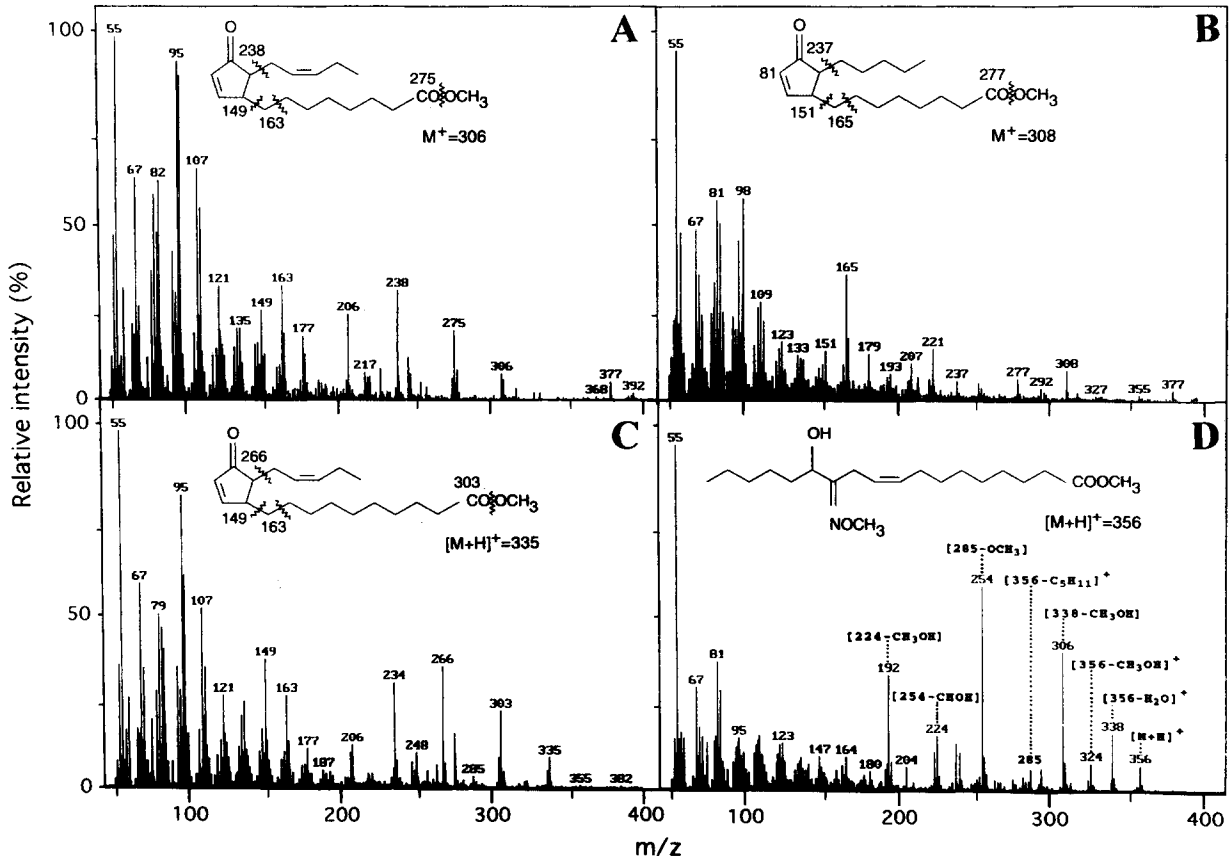

Fig. 8. Mass spectral analysis of reaction products obtained from cloned *A. thaliana* allene oxide synthase expressed in *E. coli* M15: full-scan EI (70 eV) spectra are shown. A. Substrate 13-HPOT. B, D. Substrate 13-HPOD. C. Substrate 15-HPET.

correspondingly, m/z 335 ($[M+H]^+$) and m/z 303 (335-CH_3OH). When 13-HPOD was used as substrate, the cyclization product was less prominent than with the trienoic substrates, and the α-ketol was by far the most abundant metabolite. Nevertheless, 3-oxo-2-(2′-pentyl)cyclopent-4-eneoctanoic acid was unambiguously identified as the methyl ester (Fig. 8B) by retention time, EI and CI spectrum in accord with the flax reference reaction and the literature [4]. The CI spectrum gave m/z 309 ($[M+H]^+$) and m/z 278 (309-CH_3OH). Allene oxide formation from 13-HPOD was further demonstrated by the occurrence of the α-ketol which was analyzed as the methyl ester methoxime derivative [29] (Fig. 8D). Due to self protonation, $[M+H]^+$ rather than M^+ was observed as the molecular ion. This was validated through the CI spectrum which also gave m/z 356 (the expected protonated molecular ion) as well as m/z 338 (loss of water) and m/z 307 (338-CH_3OH), in accord with the proposed structure. The observed fractionation (cf. Fig. 8D) is homologous to that reported for the methyl ester methoxime derivative of 13-hydroxy-12-oxo-*cis*,*cis*-9, 15-octadecadienoic acid, the α-ketol formed from allene oxide synthase action on 13-HPOT [29].

These results prove that the ability to convert linolenic acid-as well as linoleic acid-derived 13-hydroperoxides to allene oxides is an intrinsic property of allene oxide synthase. Cyclization of these allene oxides gives 12-OPDA or the analogous pentyl derivative, which, by reduction and β-oxidation would yield either jasmonic acid or dihydrojasmonic acid, respectively. Dihydrojasmonic acid is a metabolite known to occur naturally [15] and to have biological activity [20]. Thus, a linoleic acid route to jasmonates in addition to the well known linolenic acid route [27] must be postulated.

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