RESEARCH PAPER

Divinyl ether synthesis in garlic bulbs

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Abstract

Formation of 13-lipoxygenase-derived divinyl ethers has been described in garlic bulbs. Here, the identification of a cDNA from garlic is described, which encodes for an enzyme that corresponds to divinyl ether synthases (DES). The recombinant protein was expressed in Escherichia coli and shown to metabolize 13-hydroperoxy as well as 9-hydroperoxy linole(n)ic acid to etherole(n)ic and colnele(n)ic acid, respectively. This biochemical feature classifies it as a member of the CYP74C subfamily of cytochrome P-450 enzymes. Product analysis after incubation of purified recombinant enzyme and fatty acid hydroperoxides revealed the formation of a mixture of different cis/trans isomers with one isomer often dominant. RNA blot analyses showed a constitutive expression of DES transcripts predominant in below-ground organs of garlic. By exogenous application of salicylic acid and sorbitol, but not by methyljasmonate, the transcript was also induced in leaves. Whereas the prominent divinyl ether in garlic was the 13-lipoxygenase-derived etheroleic acid, analysis of transgenic Arabidopsis expressing garlic DES showed that 9-lipoxygenase-derived colnelenic acid dominated 24 h after wounding. These data indicate that the product pattern of this DES from garlic depends on the substrate availability and that the enzyme is the first member in the group of 9/13-DES.

Key words: Allium sativum, cytochrome P-450, oxylipin metabolism, product specificity, substrate specificity.

Introduction

Divinyl ethers are a group of oxidized fatty acid derivatives containing ether oxygen within their hydrocarbon chains (Blée, 1998). In plants they may be produced by sequential action of a lipoxygenase (LOX) and a divinyl ether synthase (DES) (Grechkin, 1998). In higher plants it was shown that linoleic (LA) and α-linolenic acid (LnA) are the main precursors of such compounds (Feussner and Wasternack, 2002). Incubation of LA or LnA with potato tuber or tomato root protein extracts led to the formation of fatty acid 9-hydroperoxides which are further metabolized to divinyl ethers, such as colneleic (CA) or colnelenic acid (CnA), respectively (Grechkin, 1998). The DES responsible for the CA and CnA formation in potato and tomato have been cloned and characterized (Itoh and Howe, 2001; Stumpe et al., 2001). The analysis of their sequences led to classification of the enzymes within the group of cytochrome P450 enzymes, specifically in the CYP74D subfamily. The CYP74 enzyme family also includes allene oxide synthases (AOS, CYP74A, C) and hydroperoxide lyases (HPL, CYP74B, C) (Feussner and Wasternack, 2002), enzymes that also use fatty acid hydroperoxides derived from LOX activity as substrates. In contrast to most P450-containing enzymes, proteins from the CYP74 subfamily require neither molecular oxygen nor NADPH (Noordermeer et al., 2001) as cosubstrates. AOS catalyse the conversion of fatty acid hydroperoxides to allene oxides which are rapidly hydrolysed to their corresponding ketols or undergo non-enzymatic cyclization to racemic cyclopentenone derivatives. In addition, AOS is part of the pathway leading to the formation of jasmonic acid (JA), a plant hormone involved in plant development and stress responses (Wasternack and Hause, 2002). HPL cleave hydroperoxide substrates into short chain aldehydes and α-oxo fatty acids.

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Abbreviations: AOS, allene oxide synthase; C(n)A, colnele(n)ic acid; DES, divinyl ether synthase; E(n)A, etherole(n)ic acid; HPL, hydroperoxide lyase; HPOD, hydroperoxy linoleic acid; HPOT, hydroperoxy linolenic acid; JA, jasmonic acid; L(n)A, linole(n)ic acid; LOX, lipoxygenase.

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Materials and methods

Plant material and treatments

Garlic (Allium sativum) bulbs were purchased from the local market and grown under a 16 h light/8 h dark regime at 22 °C. For chemical treatment, leaves were separated from bulbs using a razor blade and put into solutions containing either 50 μM salicylic acid, 50 μM methyljasmonate, or 1 M sorbitol. Pots with the bulbs and roots were left in the respective solution for the indicated time.

Arabidopsis thaliana (Col-0) wild-type and transgenic plants were grown under short-day conditions [8 h of light (~100 μE) at 22 °C/16 h of dark at 20 °C]. Six-to-eight-week-old plants were mechanically wounded using tweezers.

To generate transgenic Arabidopsis plants expressing AsDES the coding region was cloned into pCAMBIA3300, using Gateway technology (Invitrogen, Karlsruhe, Germany), and transformed into Agrobacterium tumefaciens. Arabidopsis (Col-0) plants were transformed by the floral dipping procedure (Clough and Bent, 1998). BASTA-resistant transformants (T1) were allowed to self-pollinate and the resulting BASTA-resistant T2-plants were used for further analysis.

Isolation and recombinant expression of DES from garlic

Based on an incomplete sequence of an expressed sequence tag with similarity to cDNA sequences encoding CYP74 enzymes in an onion EST database (EST682106), primers were designed to amplify the corresponding sequence from garlic root first-strand cDNA (sense primer 5’-GGC GTT TAC GAA GTC TAA CGA GGC GCG-3’ and antisense primer 5’-GCT CAG TCA CCA GCT TCT TGC CGT C-3’). RNA from garlic roots was isolated with Trizol (Gibco-Invitrogen, Karlsruhe, Germany) according to the manufacturer’s recommendations. First-strand cDNA was generated by reverse transcription with SuperScriptIII (Gibco BRL, Egggenstein, Germany) and used as a template for PCR-based cloning. The sequence of the resulting amplified cDNA was determined and used to generate gene-specific primers for a 5’- and 3’-RACE-PCR approach. The RACE-PCR was carried out with the SMART RACE-PCR amplification Kit (Clontech, Heidelberg, Germany). For recombinant expression, primers were designed to amplify the entire coding sequence (AsDES) that introduced BamHI and PstI restriction sites (5’-GGA TCC ATG TCC ACC TCC AAC GGC AGC AC-3’ and 5’-CTG CAG TCA GAC TGG CTT GGT CAG CTC-3’). The restriction sites were used for in-frame cloning into the pQE30 expression vector which allowed the production of the AsDES protein with an N-terminal His tag. The resulting plasmid was transformed into E. coli host strain SG 13009 (Qiagen, Hilden, Germany). The recombinant enzyme was expressed and purified as described before (Stumpe et al., 2001).

Enzyme activity tests, product analysis, and oxylipin profiling

The 9- and 13-hydroperoxy fatty acid substrates were prepared from LA and LnA (Cayman Chemicals, Ann Arbor, MI, USA) using soybean 13-LOX (Sigma, Deisenhofen, Germany) and recombinant potato tuber 9-LOX (Geerts et al., 1994). Divinyl ether fatty acids used as standards were obtained from Larodan (Malmo¨, Sweden) or prepared by incubation of fatty acid hydroperoxides with either protein extract of garlic bulb (Grechkin et al., 1997) or recombinant DES of Solanum tuberosum (Stumpe et al., 2001).

For product analysis, 100 nmol of substrate was incubated with recombinant AsDES in 100 mM sodium phosphate buffer, pH 7.0. After 10 min incubation time, reaction products were extracted according to Bligh and Dyer (1959). The organic phases were evaporated under a nitrogen stream and analysed by reversed phase HPLC with a methanol:water/acetic acid gradient on an Agilent 1100 HPLC system (Waldbronn, Germany) as previously described (Stumpe et al., 2001). For detection of divinyl ethers, the absorbance at 252 nm and 268 nm was recorded (Gobel et al., 2002). For further verification, divinyl ether-containing fractions obtained by reversed-phase HPLC were methylated with (trimethylsilyl)-diazomethane and analysed by GC/MS as described (Stumpe et al., 2001). To determine cis/trans double-bond configuration, methylated divinyl ether was analysed on two normal phase columns of Zorbax SIL (4.6×250; Agilent, Waldbronn, Germany).
connected in series and eluted by hexanecetyl acetate (98.5:1.5, v/v) according to (Grechkin et al., 1997). In addition, the assignment was done in comparison with authentic standards (Laroden, Malmö, Sweden).

The activity of the recombinant enzyme was determined in 100 mM sodium phosphate buffer, pH 5.5, containing the substrate in a concentration between 5 μM and 50 μM by measuring the decrease of the absorbance at 234 nm. Kinetic parameters were calculated by nonlinear regression to Michaelis–Menten using SIGMAPLOT.

For oxylipin profiling plant material was extracted and analysed as described previously (Stumpe et al., 2006).

**Northern blot analysis**

RNA extracted from garlic leaves, roots, and bulbs was denatured using glyoxal/DMSO, separated on a phosphate gel, and transferred to a nylon plus membrane (Roth, Karlsruhe, Germany). A full-length PCR fragment of the gene labelled with the HexaLabel DNA labelling kit (Fermentas, St heon-Rot, Germany) was used as a probe. Blots were pre-hybridized, hybridized, and washed at 65 °C according to standard protocols (Ausubel et al., 1993).

**Results**

**Isolation of a CYP74 enzyme from garlic**

Up to now only DES with high substrate specificity for 9-fatty acid hydroperoxides have been cloned and characterized (Feussner and Wasternack, 2002). From garlic and a number of Ranunculaceae species it is known that these plants contain DES activities capable of metabolizing 13-hydroperoxides (Grechkin et al., 1995; Hamberg, 2002). In order to characterize the enzymes which are responsible for these activities, the intention was to isolate and clone cDNAs that may encode a 13-DES. By screening public EST databases an incomplete sequence (EST682106) from onion with similarity to known CYP74 enzymes was identified. Based on this information, RACE-PCR was performed to obtain the full-length clone using onion as well as garlic cDNAs as templates. One complete cDNA was obtained from the garlic cDNA library. The deduced protein was tentatively named AsDES and had a length of 472 amino acids and a calculated molecular mass of 52.8 kDa. The activity of several CYP74 enzymes has been described to be located in plastids (Stumpe and Feussner, 2006). Analysis of amino acid sequences of different cloned members of the CYP74 family revealed the presence of plastidic transit peptides (Laudert et al., 1996; Howe et al., 2000; Sivasankar et al., 2000). The deduced amino acid sequence of AsDES lacks such a typical transit peptide.

The novel CYP74 enzyme described here is most similar to the amino acid sequence of the AOS from Arabidopsis (accession number CAA63266; 46% identity). As seen in the phylogenetic tree analysis (Fig. 1), the AsDES does not easily group into an existing CYP74 subfamily due to its comparably low sequence identity to other CYP74 enzymes. However, the protein shares typical sequence motives known from other CYP74 enzymes, and its biochemical features (see below) classify it as a member of the CYP74C subfamily.

**Functional expression in E. coli and biochemical properties of recombinant AsDES**

Based on the sequence information and the low sequence identity to known CYP74 it is not possible to predict its enzymatic activity. To characterize the associated activity, the full-length coding sequence of AsDES was cloned into the plasmid pQE30 and heterologously expressed in E. coli. When assayed in vitro, the recombinant enzyme exhibited high activity at different pH values ranging from 5 to 7.5, with the highest activity observed at about 5.5. Using 13-HPOT as substrate and performing an HPLC analysis for product analysis, a reaction product with a retention time of 32 min that has a UV absorption maximum at 252 nm was observed (Fig. 2A). The methylated derivative of this product was analysed using GC/MS, and the associated mass spectrum exhibited a dominant ion at m/z 308 which is the molecular mass of EA (Fig. 2B). The fragmentation pattern is in agreement with the mass spectrum of authentic EA. Similar results were observed using the other hydroperoxides as substrates: 9-HPOT was metabolized to CA, 9-HPOT to CnA, and 13-HPOT to EA by recombinant AsDES protein confirmed by GC/MS. In order to analyse the substrate specificity, the activity of AsDES was tested spectrophotometrically against mentioned fatty acid hydroperoxides. AsDES was most active against 13-HPOT in respect to turnover rate (kcat ~ 550 s⁻¹) and catalytic activity (kcat/Km = 4.4 × 10⁶ M⁻¹ s⁻¹). The turnover rates using other fatty acid hydroperoxides were less than one-fifth of 13-HPOT (9-HPOT, ~ 70 s⁻¹; 13-HPOT, ~ 40 s⁻¹; 9-HPOT, ~ 10 s⁻¹). Nevertheless the Km values against these substrates (9-HPOT, ~ 30 μM; 9-HPOT, ~ 20 μM; 13-HPOT, ~ 10 μM) were much less than Km against 13-HPOT (>100 μM). Therefore AsDES was assigned as non-specific 9/13-DES.

When garlic protein extracts were used to characterize DES activity, the formation of different cis/trans double-bond isomers of EA and EnA was observed (Grechkin et al., 1997). HPLC analysis of methylated divinyl ether formed by recombinant AsDES showed, with all tested substrates, at least three different divinyl ethers. However, using 13-HPOT, 9-HPOT, and 9-HPOT, one major product was found (about 90% of the sum of the three products; Table 1). By comparison to available standards these divinyl ethers could be identified as 9Z,11E,10E-EA (derived from 13-HPOT), 8E,9E,10E,12Z-EA (derived from 9-HPOT), and 8E,9E,10E,12Z-EcA (derived from 9-HPOT). Based on publications from Grechkin and Hamberg on cis/trans isomers of divinyl ethers formed by protein extracts, the other products were tentatively assigned as the 9Z- and 11Z-isomers of EA and, in the case
of C(n)A, as the respective ω6E- and ω8Z-isomers by their retention time and UV absorption maxima (Grechkin et al., 1995; Grechkin and Hamberg, 1996; Hamberg, 2004). Incubation of AsDES with substrate at different pH values (5.5–8.5) did not alter the ratio of the three different isomers. Using 13-HPOT, the recombinant enzyme was rather unspecific, here two major products were formed: about 60% 9Z,11E,ω3Z,ω5E-EnA and about 30% its 9E-isomer (Table 1). Protein extract of garlic bulbs was also incubated with the four different substrates and the composition of the divinyl ether isomers was analysed. No significant differences to the isomer ratio obtained with the purified recombinant protein were observed.

Analysis of AsDES transcript accumulation in garlic

Based on the sequence information of AsDES, it was now possible to analyse the expression of the transcript in different garlic organs. Analysis of the organ-specific expression of AsDES revealed that the corresponding mRNA was present mainly in bulbs and roots and hardly detectable in leaves (Fig. 3).

Exogenous application of plant signal molecules like methyljasmonate or salicylic acid, as well as abiotic stresses like wounding or osmotic stress, have been shown to regulate enzymes involved in the LOX pathway. To determine whether the expression of AsDES in garlic is regulated by such treatments, garlic leaves were separated from bulbs and put into solutions containing 50 μM salicylic acid, 50 μM methyljasmonate, or 1 M sorbitol to mimic osmotic stress. In the control leaves treated with water, there was a transient transcript accumulation in leaves after 24 h. By using salicylic acid it was possible to induce transcripts after 6 h that accumulated further up to 48 h (Fig. 4A, SA). Sorbitol treatment led to an accumulation after 24 h similar to the control but the signal stayed up to 48 h (Fig. 4A, SO). Methyljasmonate had no effect on the expression of AsDES (data not shown).

Fig. 1. An unrooted phylogenetic tree of CYP74 enzymes from various plants. Phylogenetic analysis was performed using the programs ClustalX and Phylip. The analysis is based on the following accession numbers: MsHPL1, CAB54847; MsHPL2, CAB54848; MsHPL3, CAB54849; AtHPL, AAC69871; LeHPL, AAF67142; CaHPL, AAA97465; StHPL, CAC44040; NaHPL, CAC91565; HvHPL, CAC82980; MaHPL, CAB39331; ZmHPL, AAS47027; CsHPL1, AAF64041; PgHPL, AAK15070; SiDES, CAC28152; LeDES, AAG42261; NdDES, AAL40900; HvAOS1, CAB86384; HvAOS2, CAB86383; OsAOS, AAL38184; PaAOS1, CAA55025; LeAOS1, CABB8032; LeAOS2, AAF67141; StAOS1, CAD29735; StAOS2, CAD29736; AtAOS, CAA63266; CmAOS, AAM66138; LuAOS, AAA03353; MtAOS, CAC86897; CmHPL, AAK54282; CsHPL2, AF229812; MtHPL1, CAC86898; MtHPL2, CAC86899; StAOS3, CAI30876; LeAOS3, AAN76867; AsDES, AJ867809.
shown). Similar results had been observed in roots but were less pronounced (Fig. 4B).

Oxylipin profiling of garlic organs and transgenic AsDES-expressing Arabidopsis

To get to know more about the LOX pathway in garlic, the content of different oxylipins was measured in leaves, bulbs, and roots. Whereas under standard conditions oxylipins in leaves were hardly detectable, bulbs and roots could be analysed. In both organs, oxylipins of the 13-LOX pathway were most prominent. The precursors of these oxylipins in garlic are LA and to a lesser extent LnA: in roots the amount of non-esterified LA reached 6 nmol g$^{-1}$ f.w. whereas LnA was below 1 nmol g$^{-1}$ f.w. In bulbs the ratio between LA and LnA was similar but the amount of LA was only about 1 nmol g$^{-1}$ f.w. (Fig. 5A). Free fatty acids were metabolized by LOX to fatty acid hydroperoxides, which are highly reactive compounds. They serve as substrate for different enzymes leading to the formation of keto and hydroxy fatty acids and, in garlic, also to divinyl ether fatty acids. When analysing the amount of such oxylipins in roots, the majority, namely 13-HOD, 13-KOD, and EA, were derived from 13-HPOD. The amounts of these compounds were about in the same range (0.3–0.6 nmol g$^{-1}$ f.w. Fig. 5B, C). Oxylipins derived from other fatty acid hydroperoxides were found only in smaller quantities (often 10 times less). These data indicate that AsDES in garlic preferentially used 13-HPOD as substrate since only EA...

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Table 1. Analysis of AsDES reaction products

Purified recombinant AsDES was incubated with 9- and 13-hydroperoxides, respectively. The resulting divinyl ethers were methylated and their cis/trans isomer composition was determined by HPLC. EA, Etheroleic acid (R$_1$, C$_{7}$H$_{14}$COOH; R$_2$, C$_{4}$H$_{9}$); EnA, etherolenic acid (R$_3$, C$_{7}$H$_{14}$COOH; R$_2$, C$_{4}$H$_{9}$); CA, colneleic acid (R$_1$, C$_{5}$H$_{11}$; R$_2$, C$_{6}$H$_{12}$COOH); CnA, colnelenic acid (R$_1$, C$_{5}$H$_{9}$; R$_2$, C$_{6}$H$_{12}$COOH). Each value represents an average of four independent analyses; the standard deviation is given.

Fig. 2. RP-HPLC analysis of products formed from 13-HPOD by AsDES in vitro. (A) 13-HPOD was incubated with recombinant DES from garlic. The resulting products were analysed by RP-HPLC-DAD. The chromatogram recorded at 252 nm is shown. The inset shows the UV spectrum of the substance eluting at 32 min. (B) The reaction product was purified by HPLC and analysed as methyl ester derivative by GC/MS. The mass spectrum of the product is shown. The experiment is representative of two independent experiments yielding similar results.

Fig. 3. RNA gel blot hybridization of garlic tissues using AsDES cDNA as a probe. Ten micrograms of RNA from different organs were loaded. The autoradiograph of the blot is shown together with a photograph of an ethidium bromide stained gel. L, Leaves; B, bulbs; R, roots. The experiment is representative of three independent experiments yielding similar results.
as product was detected, although other fatty acid hydroperoxides were present in the tissue.

DES activity detected in garlic bulbs showed a specificity against 13-HPOD whereas the recombinant protein seemed to be rather unspecific. In order to test whether this difference in substrate specificity was due to lack of availability of 9-LOX-derived metabolites in garlic, AsDES was expressed in *Arabidopsis thaliana* that harbours almost equal amounts of 9- and 13-LOX-derived fatty acid hydroperoxides. Transformation of *Arabidopsis* with pCAMBIA vector containing the full-length cDNA of AsDES under the control of the CaMV 35S promoter and mediating resistance to BASTA yielded 11 transgenic lines. However, only one line in the T2 generation showed expression of the transgene. This line (B4), an empty vector control (EV45), and wild type (Col-0) were analysed regarding their oxylipin pattern. Since wounding activates the LOX pathway in *Arabidopsis*, oxylipins were measured 6 h and 24 h after wounding. Fatty acid hydro(pero)xides accumulate up to 7 nmol g\(^{-1}\) f.w. [13-H(P)OT], whereas 9-KOT was the prominent keto fatty acid that reached 140 nmol g\(^{-1}\) f.w. JA increased up to 20 nmol g\(^{-1}\) f.w. (Fig. 6, wild-type data not shown). In the same range as these oxylipins, divinyl ether accumulation was observed in the transgenic line B4: CnA at about 15 nmol g\(^{-1}\) f.w. and EnA at about 3.5 nmol g\(^{-1}\) f.w. (Fig. 6G). CA and EA could not be separated under the conditions used for oxylipin profiling and were measured together. This mixture accumulated up to 10 nmol g\(^{-1}\) f.w. 24 h after wounding.

**Discussion**

Here the isolation and characterization of a novel CYP74 enzyme from garlic is presented. As far as is known it is a new DES which uses predominantly 13-HPOD as substrate. This feature distinguishes this enzyme from other cloned DES enzymes from Solanaceae species (Itoh and Howe, 2001; Stumpe *et al.*, 2001; Fammartino *et al.*, 2007). The conversion of LA into the divinyl ether CA was first reported in the early 1970s by Galliard and co-workers (Galliard and Phillips, 1972). Related activities leading to formation of EA and EnA were described from garlic and different Ranunculaceae species and a number of algae (Grechkin *et al.*, 1995; Hamberg, 2002, 2004).

In general, DES activity appears to be characteristic for certain genera of plants, whereas AOS and HPL are widely distributed within the plant kingdom (Stumpe and Feussner, 2006). From the phylogenetic analysis (Fig. 1) one may assume that DES may have evolved from AOS by gene duplication in certain plant families. The low sequence similarity of AsDES to known CYP74 enzymes can be explained, firstly, by the phylogenetic distance between garlic and the plant species where the other
CYP74s come from and, secondly, by its novel enzymatic activity.

The divinyl ethers produced in various plants may differ in their cis/trans double-bond configuration (Hamberg, 2005). Whereas incubation of protein extract of garlic bulbs with 13-HPOD led to the predominant formation of 9Z,11E,ω5E-EA (Grechkin et al., 1997), using different *Ranunculus* species 9Z,11E,ω5Z-EA was formed (Hamberg, 2002). This observation is explained by the stereo-specific abstraction of different protons during DES reaction. Beside such isomers, the minor formation of the 9E and 11Z isomer was reported using garlic bulbs. The origin for such isomers could be due to some rotation freedom of bonds in the epoxy allylic cation intermediate during the DES reaction (Grechkin et al., 1997) or the presence of an isomerase in the garlic extract. Using purified recombinant AsDES it was possible to show that the isomer mixture of formed divinyl ether is similar to that found by incubation with garlic protein extract (Table 1). That might exclude an isomerase reaction and favour the inefficiency of tight binding of the intermediate.

As was shown for DES from Solanaceae (Itoh and Howe, 2001; Stumpe et al., 2001; Fammartino et al., 2007), the AsDES is also preferentially expressed in below-ground organs, such as bulbs and roots (Fig. 3). Since the DES of potato can be induced in leaves by pathogens like *Phytophthora infestans* and *Pseudomonas syringae* (Stumpe et al., 2001), the regulation of AsDES expression in garlic leaves was analysed by plant signal compounds (salicylic acid and methyljasmonate) and sorbitol-induced osmotic stress. Salicylic acid and methyljasmonate were shown to play a critical role in the activation of a subset of defence gene expression and induction of phytoalexin synthesis (Rosahl and Feussner, 2005). The ASDES transcript is induced in garlic leaves by salicylic acid after 6 h but not by methyljasmonate. This might indicate that AsDES is involved in pathogen defence reactions that depend on salicylic acid since divinyl ether can inhibit pathogen growth (Prost et al., 2005).

In potato and garlic, divinyl ethers are predominantly found in subterranean organs (Stumpe et al., 2006; Fig. 5). However, in contrast to potato, 13-H(P)OD is the major LOX product in roots of garlic resulting in preferential formation of 13-LOX-derived divinyl ethers. In bulbs the amount of 13-H(P)OD as well as of EA is much less than in roots. Nevertheless in both below-ground organs, the analysis of endogenous oxylipins indicates that the specific accumulation of 13-hydroperoxides may determine the specificity of the DES reaction, although the isolated AsDES uses 9- and 13-H(P)OD similarly. This assumption was supported by expression of AsDES in *Arabidopsis*, a plant that synthesized 13- and 9-hydroperoxides in comparable amounts after wounding. Indeed this metabolic situation leads to the accumulation of a different set of divinyl ethers in wounded rosette leaves (Fig. 6). Interestingly, the major divinyl ether formed was CnA, the reaction product of 9-HPOT. This might be explained as a result of different intracellular pools of the substrates in *Arabidopsis* leaves. Although upon wounding an almost equal increase in 13- and 9-hydroperoxides was observed, 13-hydroperoxides were only synthesized in chloroplasts since all four 13-LOX in *Arabidopsis* contain a putative plastidic transit peptide (Feussner and Wasternack, 2002). However, 9-hydroperoxides were solely formed in the cytosol. Since AsDES is lacking a plastidic transit peptide it is most likely localized in the cytosol and therefore it has only limited access to 13-hydroperoxides. Together these data indicate that the product pattern of this divinyl ether synthase from garlic depends on the substrate availability and that the enzyme is the first member in the group of 9/13-DES.

**Fig. 5.** Oxylipin profile of bulbs and roots of garlic. Oxylipins were extracted from various garlic organs and quantified as described in the Materials and methods. (A) Free fatty acids; (B) hydro(per)oxy fatty acids; (C) divinyl ethers. Each value represents an average of three independent analyses and the standard deviation is given.
Fig. 6. Oxylipin profile of wounded transgenic AsDES-expressing Arabidopsis plants compared with empty vector controls. Rosette leaves were wounded and harvested after the indicated time points. Oxylipins were extracted and quantified as described in the Materials and methods. (A, B) Hydroxy fatty acids; (C, D) keto fatty acids; (E, F) jasmonic acid and 12-oxophytodienoic acid (OPDA); (G, H) divinyl ethers.

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