

## Identification and Functional Analyses of Two cDNAs That Encode Fatty Acid 9-/13-Hydroperoxide Lyase (CYP74C) in Rice

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**Fatty acid hydroperoxide lyase (HPL), a member of cytochrome P450 (CYP74), produces aldehydes and oxo-acids involved in plant defensive reactions. In monocots, HPL that cleaves 13-hydroperoxides of fatty acids has been reported, but HPL that cleaves 9-hydroperoxides is still unknown. To find this type of HPL, *in silico* screening of candidate cDNA clones and subsequent functional analyses of recombinant proteins were performed. We found that AK105964 and AK107161 (Genbank accession numbers), cDNAs previously annotated as allene oxide synthase (AOS) in rice, are distinctively grouped from AOS and 13-HPL. Recombinant proteins of these cDNAs produced in *Escherichia coli* cleaved both 9- and 13-hydroperoxide of linoleic and linolenic into aldehydes, while having only a trace level of AOS activity and no divinyl ether synthase activity. Hence we designated AK105964 and AK107161 OshHPL1 and OshHPL2 respectively. They are the first CYP74C family cDNAs to be found in monocots.**

**Key words:** fatty acid hydroperoxide lyase; allene oxide synthase; CYP74; *Oryza sativa*; cytochrome P450

Phytooxylipins, which are synthesized from linoleic and linolenic acids by cascade reactions through lipoxygenase, cytochrome P450s, cyclooxygenase, *etc.*, are thought to play an important role in plant defensive reactions and plant development.<sup>1)</sup> CYP74 is a cytochrome P450 that transforms fatty acid hydroperoxides into various derivatives.<sup>2)</sup> CYP74 enzymes are unique in that they do not require molecular oxygen or NAD(P)H-dependent cytochrome P450-reductase for their catalyses. Phytooxylipins produced by CYP74 include 12,13-epoxy-octadecatrienoic acid (allene oxide), a first intermediate for the plant hormone jasmonic acid, produced by allene oxide synthase (AOS, CYP74A);<sup>3–5)</sup> aldehydes and oxo-acids related to plant defensive reactions produced by fatty acid hydroperoxide lyases

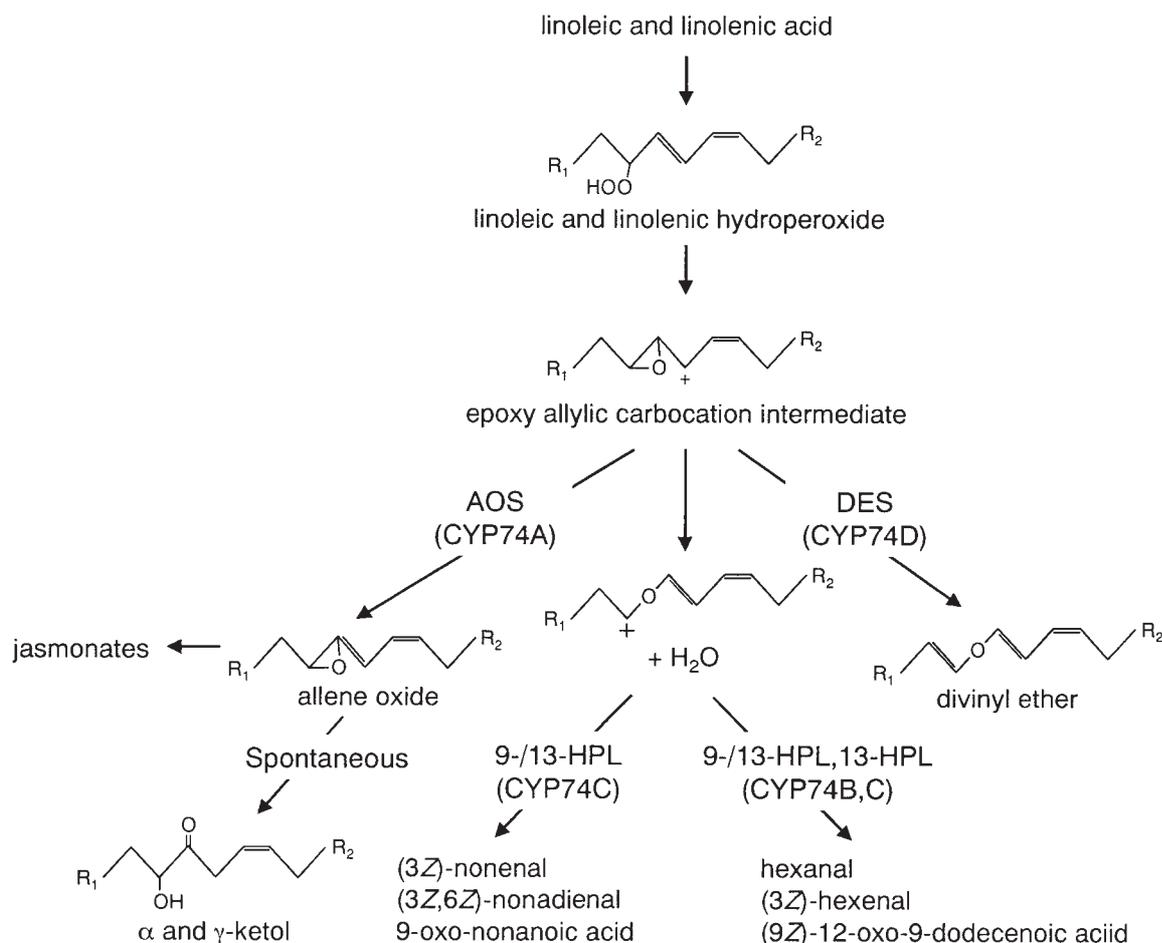
(HPL, CYP74B,C); and colneleic and colnelenic acid as plant defensive compounds produced by divinyl ether synthase (DES, CYP74D).<sup>6,7)</sup> Enzymes belonging to the CYP74 family are closely related in protein primary structure and enzymatic function, where they share common substrate and reaction intermediate called epoxy allylic carbocation (Fig. 1).

HPLs, widely found in the plant kingdom, are well characterized in dicots and are divided into two groups by their substrate specificities (Fig. 1), *viz.*, 13-HPL (CYP74B) and 9-/13-HPL (CYP74C). 13-HPL preferentially cleaves (9Z,11E,13S)-13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD, 13-hydroperoxide of linoleic acid) and (9Z,11E,13S,15Z)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT, 13-hydroperoxide of linolenic acid) into hexanal and (3Z)-hexenal respectively,<sup>8,9)</sup> while 9-/13-HPL has both 13-HPL activity and activity toward (9S,10E,12Z)-9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD, 9-hydroperoxide of linoleic acid) and (9S,10E,12Z,15Z)-9-hydroperoxy-10,12,15-octadecatrienoic acid (9-HPOT, 9-hydroperoxide of linolenic acid).<sup>10)</sup> 9-/13-HPL cleaves 9-HPOD into (3Z)-nonenal and 9-oxo-nonanoic acid, and cleaves 9-HPOT into (3Z,6Z)-nonadienal and 9-oxo-nonanoic acid. (3Z)-Hexenal, (3Z)-nonenal and (3Z,6Z)-nonadienal are enzymatically or spontaneously isomerized into (2E)-hexenal, (2E)-nonenal and (2E,6Z)-nonadienal respectively. In monocots, only 13-HPL is reported in barley,<sup>9)</sup> and no presence of 9-/13-HPL has been reported.

Aldehydes produced by HPL function as defensive compounds in some herbivores.<sup>11,12)</sup> They are not only important for plant defensive reactions, but are also known as negative and positive flavors that determine the quality of food and beverages.<sup>13–16)</sup> For example, in brewing, (2E)-nonenal is considered the major contributor to the cardboard flavor that arises in aged beer.<sup>17–19)</sup> (2E)-Nonenal is also one of the off-flavors of aged milled rice.<sup>20)</sup> During investigation of the formation of

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**Abbreviations:** AOS, allene oxide synthase; DES, divinyl ether synthase; HPL, fatty acid hydroperoxide lyase; 9-HPOD, (9S,10E,12Z)-9-hydroperoxy-10,12-octadecadienoic acid; 13-HPOD, (9Z,11E,13S)-13-hydroperoxy-9,11-octadecadienoic acid; 9-HPOT, (9S,10E,12Z,15Z)-9-hydroperoxy-10,12,15-octadecatrienoic acid; 13-HPOT, (9Z,11E,13S,15Z)-13-hydroperoxy-9,11,15-octadecatrienoic acid



**Fig. 1.** Similarities among Reaction Mechanisms of the Production of Allene Oxide, Aldehydes and Oxo-Acids, and Divinyln Ether by CYP74.

AOS, DES, and HPL share the intermediate epoxy allylic carbocation formed from the acyl hydroperoxide (9-, 13-HPOT, 9-, 13-HPOT). AOS and DES deprotonate the carbocation at different positions, which leads to allene oxide and divinyln ether (derived from 9-HPOT and 9-HPOT) respectively. HPL catalyzes rearrangement and subsequent fragmentation, which leads to the formation of aldehydes and oxo-acids. Allene oxide derived from 13-HPOT is the precursor of jasmonic acid. Allene oxide is unstable, and without downstream enzymes it spontaneously transforms into  $\alpha$ - and  $\gamma$ -ketol. R<sub>1</sub> and R<sub>2</sub> represent C<sub>4</sub>C<sub>7</sub> and (CH<sub>2</sub>)<sub>6</sub>COOH (13-HPOT), C<sub>4</sub>C<sub>9</sub> and (CH<sub>2</sub>)<sub>6</sub>COOH (13-HPOT), (CH<sub>2</sub>)<sub>6</sub>COOH and C<sub>4</sub>C<sub>7</sub> (9-HPOT), and (CH<sub>2</sub>)<sub>6</sub>COOH and C<sub>4</sub>C<sub>9</sub> (9-HPOT) respectively. Symbols: AOS, allene oxide synthase; DES, divinyln ether synthase; HPL, fatty acid hydroperoxide lyase; 13-HPL, fatty acid 13-hydroperoxide lyase; 9-/13-HPL, fatty acid 9-/13-hydroperoxide lyase.

(2*E*)-nonenal in the brewing process, we found that (2*E*)-nonenal was produced by barley lipoxygenase-1 and fatty acid 9-hydroperoxide lyase-like activity.<sup>21)</sup> The fatty acid 9-hydroperoxide lyase-like activity is likely to be an enzyme because it is sensitive to proteinase-K treatment and the activity is terminated by boiling. This finding led us to search for HPL that can cleave 9-HPOT in monocots including rice; however, despite intensive analysis of full-length cDNA in rice, cDNAs that encode 9-/13-HPL (CYP 74C) have never been found. We thought that they have not been identified because of the similarity of their structure to AOS, 13-HPL and DES, and that they are incorrectly annotated. Hence we attempted *in silico* screening of candidate cDNA clones and tested their recombinant proteins as 9-/13-HPL. Here we show that AK105964 and AK107161, previously designated AOS, are 9-/13-HPL. AK105964 and AK107161 designated OsHPL1 and OsHPL2 respec-

tively, are the first CYP74C members to be found in monocots.

## Materials and Methods

**Chemicals.** (2*E*)-Nonenal, hexanal, 13-HPOT, 13-HPOT and 2-hydroxydecanoic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). 9-HPOT was produced through the reaction of recombinant barley LOX-1<sup>22)</sup> and linoleic acid, and purified with a silica-gel column (silica-gel 60, Merck, U.S.A.). (3*Z*)-Nonenal, colneleic acid, 9-HPOT, 13-HPOT, (9*Z*,15*Z*)-13-hydroxy-12-oxo-9,15-octadecadienoic acid, (10*E*,15*Z*)-9-hydroxy-12-oxo-10,15-octadecadienoic acid, (10*E*)-9-hydroxy-12-oxo-10-octadecenoic acid and 9-oxo-nonanoic acid were purchased from Larodan Fine Chemicals (Malmö, Sweden). (2*E*,6*Z*)-nonadienal was purchased from Sigma-Aldrich (St. Louis, MO).

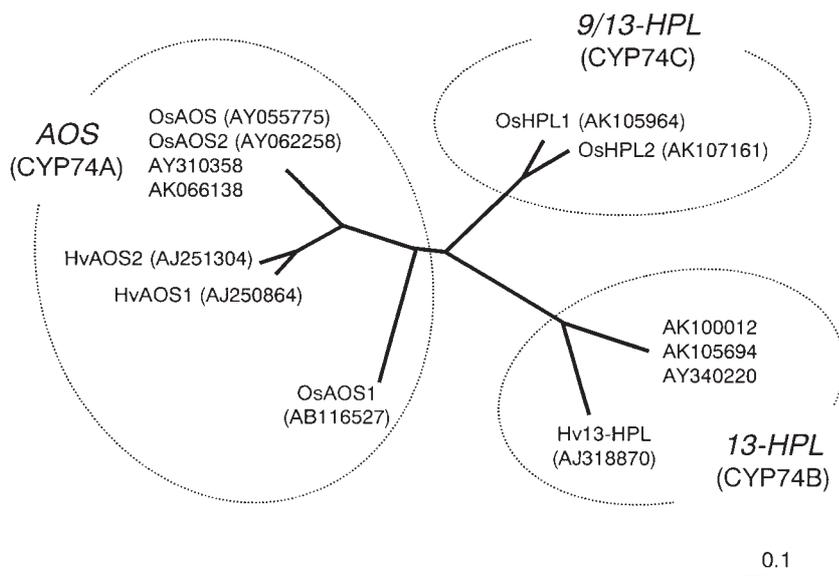
In silico screening, molecular cloning, and *E. coli* expression of *OsHPL1* and *OsHPL2* cDNAs. Candidate cDNAs, which have high homology to functionally assigned barley AOS1 and AOS2 (designated HvAOS1 and HvAOS2 in this paper; Genbank accession nos. AJ250864 and AJ251304 respectively),<sup>23)</sup> barley 13-HPL (designated Hv13-HPL in this paper; Genbank accession no. AJ318870)<sup>9)</sup> and rice AOS (*OsaOS*; Genbank accession no. AY055775)<sup>24)</sup> were extracted from the Knowledge-based *Oryza* Molecular Biological Encyclopedia (KOME) database and the Expert Protein Analysis System (Swiss-Prot and TrEMBL), using the protein-protein BLAST program (threshold, E-value =  $e^{-10}$ ). Phylogenetical analysis was conducted with the DNASIS Pro program (Hitachi Software Engineering, Tokyo, Japan).

PolyA<sup>+</sup>RNA was isolated from etiolated shoots from rice (*Oryza sativa*, cv. Nipponbare) using Tripure isolating reagent (Roche Diagnostics, Tokyo, Japan) and Oligotex dT30 super (Takara, Kyoto, Japan). Single-stranded cDNA was synthesized with Superscript II and oligo(dT)<sub>20</sub> primer (Invitrogen, Carlsbad, CA). cDNAs corresponding to the longest ORF of AK105964 and AK107161 were produced by polymerase chain reaction using primers 5'-gcggatccatggcgcgcccgagccaa-3' and 5'-gcgaattcccgaccaaacactcggcgtc-3' for AK105964, and primers 5'-gcggatccatggcgcgccaccgcagc-3' and 5'-gccgaattccgcaattaaaacatccaccaact-3' for AK107161, with KOD-Plus- DNA polymerase (Toyobo, Tokyo, Japan). The PCR program was 94 °C for 2 min, 40 cycles of 94 °C for 15 sec and 68 °C for 4 min, and 68 °C for 10 min. Amplified fragments were blunt-ended and cloned into the *HincII* site of pUC118, and subsequently subcloned into the *BamHI*-*EcoRI* site of *E. coli* expression vector, pRSETA (Invitrogen). These plasmids (designated pRSETA-*OsHPL1* and pRSETA-*OsHPL2* for AK105964 and AK107161 respectively) are designed to express a recombinant protein with a tag peptide containing a vector sequence and a hexahistidine metal binding domain for affinity-purification (His-tagged protein) consisting of MRGSHHHHHH-GMASMTGGQQMGRDLYDDDDKDRWGS (36 residues). After confirming that the DNA sequences were identical to the data present in the KOME database, expression vectors (pRSETA, pRSETA-*OsHPL1* and pRSETA-*OsHPL2*) were transformed into BL21 (DE3) pLysS. These strains were cultured separately in 100 ml of L-broth at 16 °C until the cell density reached OD<sub>600</sub> = 0.7, and further cultured at 16 °C for 16 h after the addition of isopropyl- $\beta$ -thiogalactopyranoside at a final concentration of 2 mM. Bacterial cells were recovered by centrifugation and suspended in 10 ml of 10 mM Tris-HCl (pH 7.2), 0.1% Tween 20, and 1 mM  $\beta$ -mercaptoethanol. After freezing and thawing, the sample was incubated with 100 U of DNase I (Nippon Gene, Tokyo, Japan) at 25 °C for 10 min, and then centrifuged at 10,000  $\times g$  to obtain *E. coli* lysate. The protein content of *E. coli* lysate was determined by Bio-Rad

protein assay (Bio-Rad, Hercules, CA, U.S.A.). An aliquot (18 mg) of *E. coli* lysate was subjected to Ni-NTA superflow (bed volume 0.25 ml, Qiagen, Hilden, Germany). After washing the resin with 10 ml of 10 mM Tris-HCl (pH 7.2), 0.1% Tween 20, 1 mM  $\beta$ -mercaptoethanol, 10 mM imidazole, and 0.3 M NaCl, His-tagged protein was eluted with 0.75 ml of 10 mM Tris-HCl (pH 7.2), 0.1% Tween 20, 1 mM  $\beta$ -mercaptoethanol, 250 mM imidazole, 0.3 M NaCl. The eluate was concentrated with Centricon 5000 (Waters, Massachusetts, U.S.A.) at a final volume of 100  $\mu$ l to obtain an affinity-purified fraction. An aliquot of the affinity-purified fraction (2  $\mu$ l) was analyzed by SDS-PAGE (5–20% gradient gel).

*Enzyme assays and product analysis.* GC-MS analysis of aldehydes produced by *OsHPL1* and *OsHPL2* proteins was as follows: *E. coli* lysate (100  $\mu$ g) was diluted to 1 ml of 0.1 M Tris-HCl (pH 7.0) in a GC vial and mixed with either 9-, 13-HPOD or 9-, 13-HPOT at a final concentration of 0.1 mM. After capping, a 100  $\mu$ m membrane-thick polydimethylsiloxane solid phase micro extraction fiber probe (Supelco, Bellefonte, PA) was inserted into the headspace of the vial, and further incubated at 40 °C for 5 min. The probe was then introduced into the injection port and thermally desorbed for 5 min at 260 °C onto a DB-1 column (liquid phase = dimethylpolysiloxane, 30 m  $\times$  0.25 mm, film thickness = 1  $\mu$ m, J&W Scientific, CA, U.S.A.). MS analysis was performed with the Hewlett Packard HP6890/MSD system in scan mode. Products were identified by mass-spectrum analysis and the coincidence of retention time with authentic compound except for (3Z,6Z)-nonadienal.

Production of 9-oxo-nonanoic acid from 9-HPOD and 9-HPOT, colneleic acid from 9-HPOD,  $\alpha$ - and  $\gamma$ -ketol from 9-, 13-HPOD and 9-, 13-HPOT by *E. coli* lysate of *OsHPL1* and *OsHPL2* was measured by LC-MS as follows. *E. coli* lysate (10  $\mu$ g) was diluted to 0.1 ml of 0.1 M Tris-HCl (pH 7.0) and incubated with either 9-, 13-HPOD or 9-, 13-HPOT at a final concentration of 0.1 mM at 30 °C for 20 min. The reaction was terminated by adding 0.1 ml of ethanol containing 10 ppm of 2-hydroxydecanoic acid as an internal standard. The sample was filtered with a Centricon 5000 (Waters), and the eluate (5  $\mu$ l) was subjected to HPLC (Hewlett Packard series 1100, Agilent Technologies, CA, U.S.A.) equipped with C<sub>18</sub> reverse-phase HPLC column (Asymmetry, 3.5  $\mu$ m, 2.1  $\times$  150 mm, Waters) at 50 °C. The mobile phase consisted of solvent A (0.5% acetic acid) and solvent B (acetonitrile). The HPLC run program was a linear gradient of 100% A to 100% B (0–10 min), following 100% B (10–16 min); the flow rate was 0.3 ml/min. MS analysis was performed by Micromass ZQ (Waters) in negative electrospray ionization mode (capillary voltage -3.2 kV, source and desolvation temperature 120 °C and 350 °C respectively). Products were measured in select-ion mode using 2-hydroxyde-



**Fig. 2.** Phylogenetical Analysis (unrooted tree) of Candidate cDNAs in Rice Having High Homology to Rice AOS, Barley AOS and Barley 13-HPL.

The databases used were the Knowledge-based Oryza Molecular Biological Encyclopedia (KOME) database (<http://cdna01.dna.affrc.go.jp/cDNA/>) and the Expert Protein Analysis System (Swiss-Prot and TrEMBL, <http://us.expasy.org/>). cDNAs are designated as gene names and/or Genbank accession numbers.

canoic acid as an internal standard. The pseudo-molecular ions monitored were  $m/z$  of 187.1 (2-hydroxy-decanoic acid), 171.1 (9-oxo-nonanoic acid), 309.2 ( $\alpha$ - and  $\gamma$ -ketol from 9- and 13-HPOT), 311.2 ( $\alpha$ - and  $\gamma$ -ketol from 9- and 13-HPOD), and 293.2 (colneleic acid). We also monitored  $m/z$  of 211.2 for detection of (9Z)-12-oxo-9-dodecenoic acid from 13-HPOD and 13-HPOT, though the amount was not determined due to the unavailability of authentic compound.

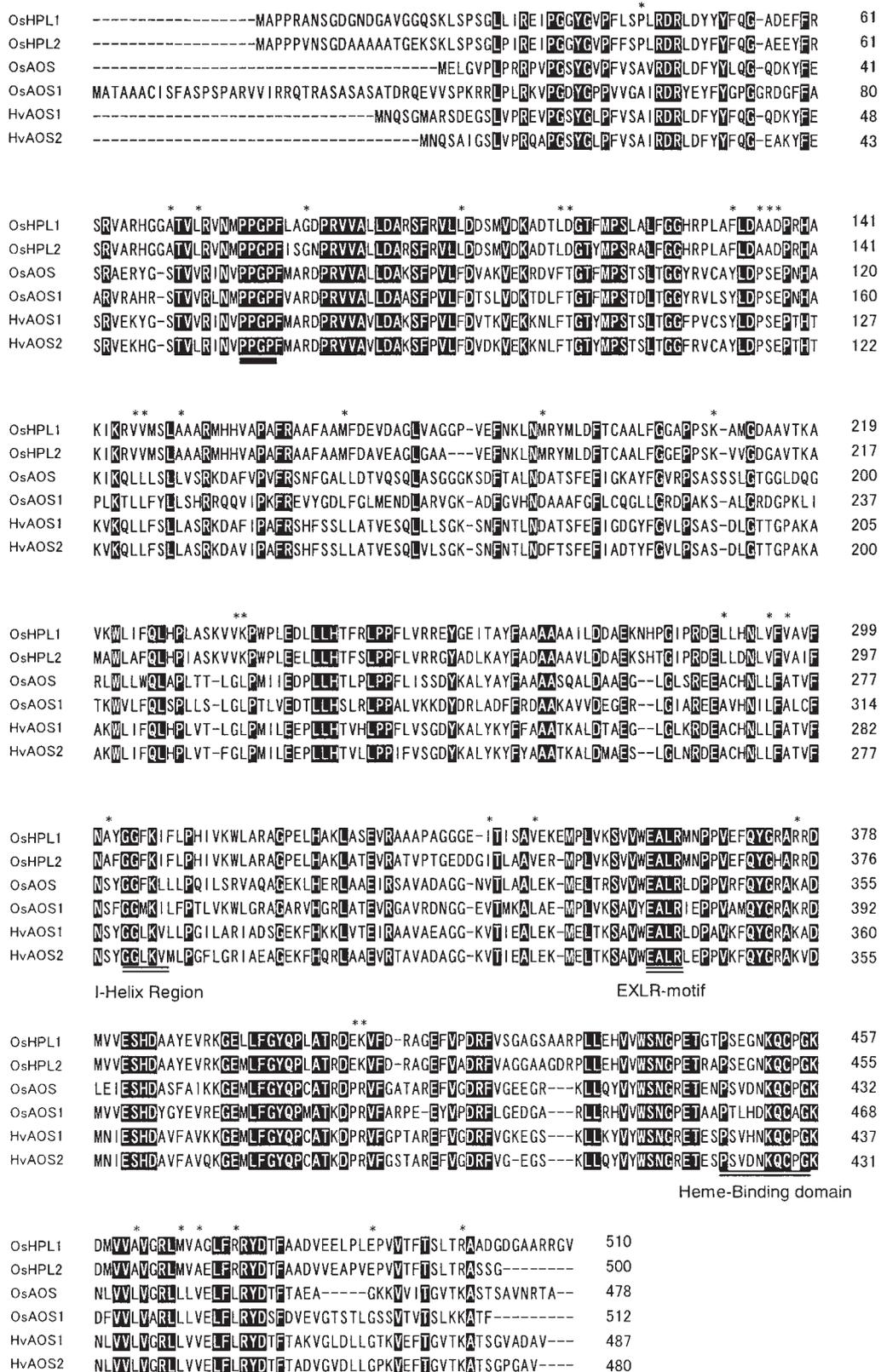
The substrate specificities of OsHPL1 and OsHPL2 were assayed spectrophotometrically by monitoring decreases in the substrates as follows: 9-, 13-HPOD or 9-, 13-HPOD was diluted to 1 ml of 0.1 M Tris-HCl (pH 7.0) in a glass cell at a final concentration of 40  $\mu$ M. An aliquot of the affinity-purified fraction (2  $\mu$ l) was mixed in, and the absorbance at 234 nm was monitored at room temperature. Activity was determined as the rate of consumption of conjugated diene chromophore ( $\epsilon = 25,000$ ).

## Results

### *In silico screening, molecular cloning, and E. coli expression of OsHPL1 and OsHPL2 cDNAs*

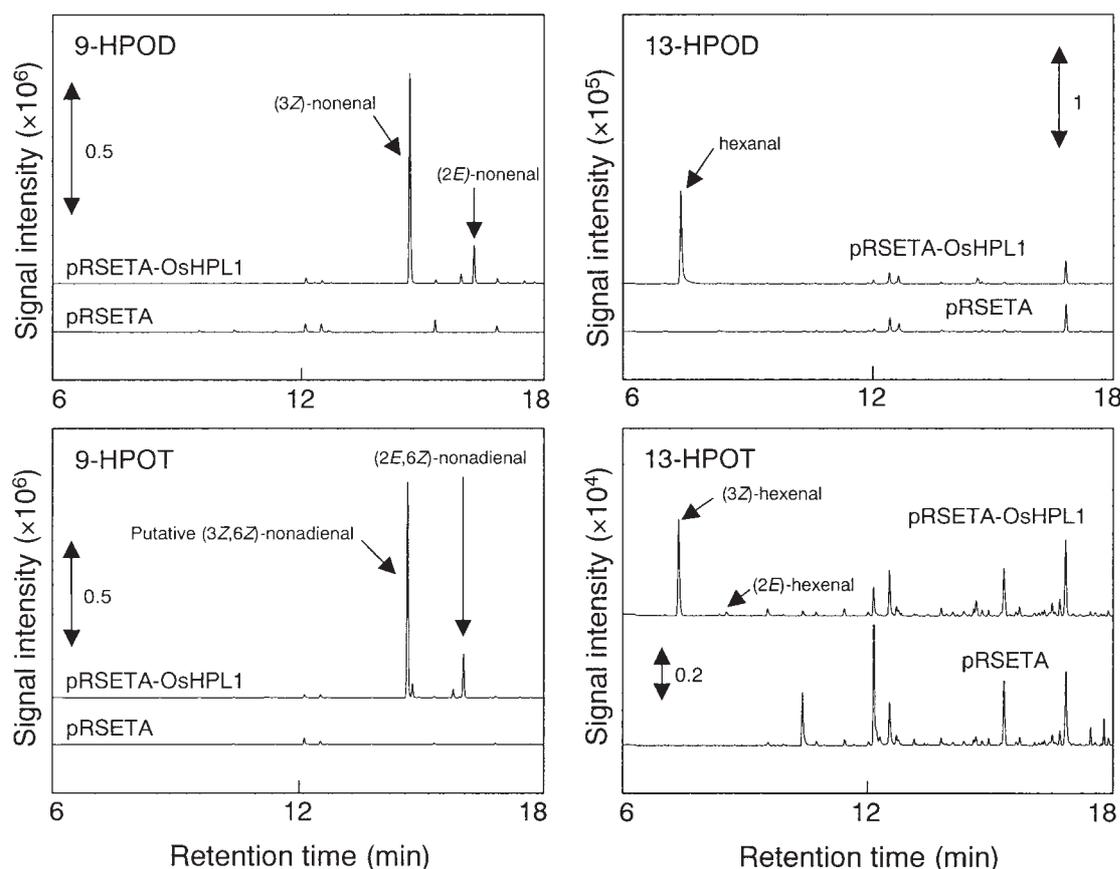
*In silico* screening for rice proteins that have a high homology to functionally assigned barley AOS1 and AOS2 (designated HVAOS1 and HVAOS2 in this paper; Genbank accession no. AJ250864 and AJ251304 respectively),<sup>23</sup> barley 13-HPL (designated as Hv13-HPL in this paper; Genbank accession No., AJ318870)<sup>9</sup> and rice AOS (designated as OsAOS in this paper; Genbank accession No., AY055775),<sup>24</sup> following phylogenetical analysis revealed the presence of two cDNAs, desig-

nated AK105964 and AK107161, which were distinctively grouped from 13-HPL and AOS (Fig. 2). AK105964 and AK107161 were previously designated OsAOS4 and OsAOS3 respectively, but the catalytic activities of the recombinant proteins of these cDNAs were not clarified.<sup>25</sup> AK105964 and AK107161 were also annotated as putative AOS on the KOME database, but we thought that they might have a catalytic activity of 9-/13-HPL. To confirm this, we cloned cDNAs corresponding to the longest open reading frame of AK105964 and AK107161 by RT-PCR from polyA<sup>+</sup>-RNA extracted from etiolated shoots. The nucleotide sequences of cloned AK105964 and AK107161 were identical to the data presented on the KOME database. For simplicity, from this point we temporarily designate AK105964 and AK107161 OsHPL1 and OsHPL2 respectively. The deduced amino acids of OsHPL1 and OsHPL2 corresponding to the longest open reading frames are shown in Fig. 3. They have calculated molecular masses of 55.3 kDa and 54.4 kDa, comprising of 510 and 500 amino acid residues respectively. The longest open reading frames of OsHPL1 and OsHPL2 were subcloned to pRSETA expression vector, and we designated them pRSETA-OsHPL1 and pRSETA-OsHPL2 respectively. These vectors are designed to express recombinant protein of either OsHPL1 or OsHPL2 protein with a N-terminal tag peptide (MRGS-HHHHHHGMASMTGGQMQGRDLYDDDDKDRWGS, 36 residues), which contains amino acid residues derived from the vector sequence and hexa-histidine metal binding domain for affinity-purification (the His-tagged protein).



**Fig. 3.** Comparison of the Protein Sequences of OsHPL1, OsHPL2, Barley AOS, and Rice AOS.

The amino acid sequences of OsHPL1, OsHPL2, OsAOS, OsAOS1, HvAOS1 and HvAOS2 were aligned using the ClustalW 1.8 program (<http://clustalw.genome.jp>). Black boxes indicate amino acid residues that are conserved between all sequences. Subfamily-specific amino acid residues are indicated with an asterisk. The I-helix GXXX(F/L), EXLR motif and PXVXNKQCPG for heme-binding domain are double-underlined. The PPGP tetrapeptide motif for catalytic stability of the cytochrome P450 family is underlined by black bars. The fusion proteins of OsHPL1 and OsHPL2 contain 36 residues of a N-terminal tag-sequence (MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGS) in front of the methionine of the indicated sequence.



**Fig. 4.** GC-MS Analysis of Aldehydes Produced by the Incubation of *E. coli* Lysate of pRSETA (vector control) and pRSETA-OsHPL1 with Either 9-, 13-HPOD or 9-, 13-HPOT.

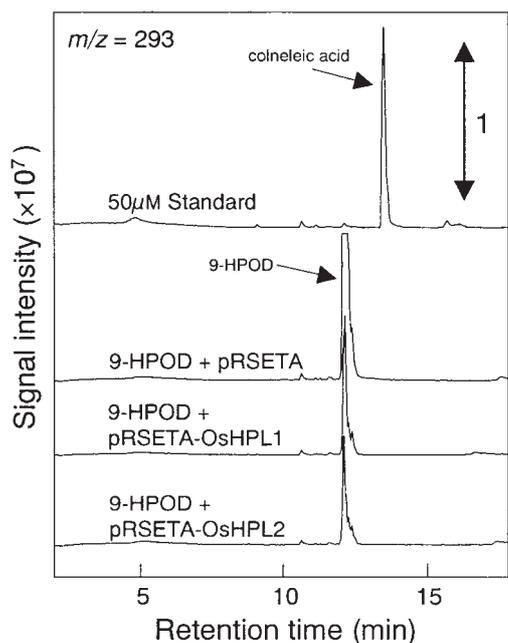
Total ion chromatographs are presented. The peaks indicated by arrows were identified as respective compounds by coincidence of the retention time of authentic compounds and analysis of mass spectra, except for (3Z,6Z)-nonadienal. Double-headed arrows represent a scale for signal intensity. Similar results were obtained for pRSETA-OsHPL2.

#### GC-MS and LC-MS analysis of the products formed by OsHPL1 and OsHPL2 with various fatty acid hydroperoxides

To identify the catalytic property of OsHPL1 and OsHPL2 as HPL, we firstly performed a qualitative analysis with GC-MS, whether OsHPL1 and OsHPL2 produce aldehydes with incubation of various fatty acid hydroperoxides. Since similar results were obtained for OsHPL1 and OsHPL2, here we show only the result obtained for OsHPL1. Figure 4 summarizes the total-ion chromatographs of the aldehydes produced by the incubation of *E. coli* lysate of pRSETA-OsHPL1 with 9-, 13-HPOD, or 9-, 13-HPOT. *E. coli* lysate of pRSETA-OsHPL1 cleaved 9-HPOD into a main product of (3Z)-nonenal ( $m/z = 122, 111, 96, 84, 69, 55, 41$ ) and a minor product of (2E)-nonenal ( $m/z = 122, 111, 96, 83, 70, 55, 41$ ). Part of the production of (2E)-nonenal was due to the spontaneous isomerization of (3Z)-nonenal, as was confirmed by GC-MS analysis of an authentic compound. Incubation of 9-HPOT with the lysate of pRSETA-OsHPL1 resulted in the formation of a major product with a minor product of (2E,6Z)-nonadienal [ $m/z = 138, 123, 109, 70, 41$ ]. The major peak, which had a parent ion of 138 and fragment ions

of 123 ( $M^+ - CH_3$ ), 109 ( $M^+ - CHO$ ), 95, 79, 67, 55 ( $C_4H_7^+$ ), was an isomer of nonadienal, putatively identified as (3Z,6Z)-nonadienal. Incubation of 13-HPOD with the lysate of pRSETA-OsHPL1 resulted in the production of hexanal ( $m/z = 82, 72, 56, 44$ ). Incubation of 9-HPOD, 9-HPOT and 13-HPOD with the lysate of pRSETA-OsHPL1 resulted in the formation of other trace peaks, which were also observed in pRSETA (insert-free vector control), and were from the host or non-enzymatic transformation of substrates. Incubation of 13-HPOT with the lysate of pRSETA-OsHPL1, but not with that of pRSETA, resulted in the formation of a peak identified as (3Z)-hexenal ( $m/z = 98, 83, 69, 55, 41$ ) and a trace peak of (2E)-hexenal ( $m/z = 98, 83, 69, 55, 41$ ). The other signals appeared at retention times of 8–18 min in pRSETA-OsHPL1, which signals were also observed for pRSETA, and were again from the host or non-enzymatic transformation of 13-HPOT.

Secondly, to determine the main catalytic activity of OsHPL1 and OsHPL2 as HPL, AOS or DES, the metabolites produced from either 9-, 13-HPOD, or 9-, 13-HPOT were analyzed by LC-MS. To test for OsHPL1 or OsHPL2 as DES activity, *E. coli* lysates of pRSETA-OsHPL1 and pRSETA-OsHPL2 were incubated with 9-



**Fig. 5.** LC-MS Analysis of the Products Generated by Incubation of 9-HPOD and *E. coli* Lysates of pRSETA, pRSETA-OsHPL1 or pRSETA-OsHPL2.

*E. coli* lysate was incubated with 9-HPOD at a final concentration of 0.1 mM, and the reaction was terminated by adding an equal volume of ethanol containing an internal standard. Colneleic acid was detected by monitoring  $m/z$  of 293.2. The double-headed arrow represents a scale for signal intensity.

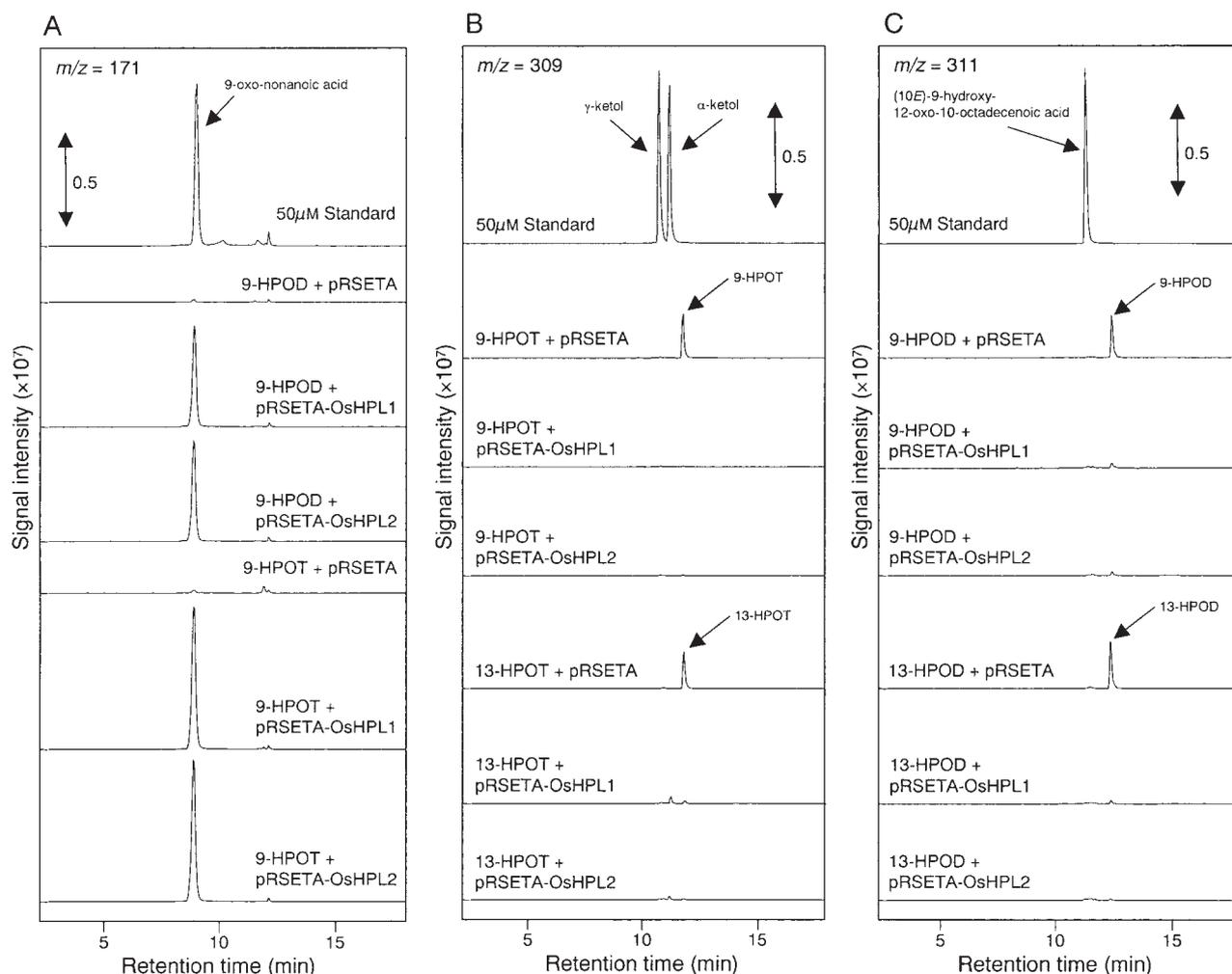
HPOD. We did not detect any colneleic acid, a divinyl ether converted by DES from 9-HPOD, indicating that neither OsHPL1 nor OsHPL2 has DES activity (Fig. 5). In plant cells, AOS catalyzes the transformation of 13-HPOT into (9*Z*,11*E*,15*Z*,12*R*,13*S*)-12,13-epoxy-9,11,15-octadecaric acid (allene oxide), which is subsequently metabolized into *cis*-(+)-12-oxo-phytodienoic acid by allene oxide cyclase, leading to jasmonate metabolism. In the absence of allene oxide cyclase, this allene oxide is chemically hydrolyzed into (9*Z*,15*Z*)-13-hydroxy-12-oxo-9,15-octadecadienoic acid ( $\alpha$ -ketol, about 80%), (10*E*,15*Z*)-9-hydroxy-12-oxo-10,15-octadecadienoic acid ( $\gamma$ -ketol, about 10%) and racemic *cis*-12-oxo-phytodienoic acid. 13-HPOD is also metabolized by AOS, and in addition to 13-hydroperoxides, it has been shown that two barley AOSs (HvAOS1 and HvAOS2) are able to catalyze 9-HPOD and 9-HPOT.<sup>23</sup> To test for OsHPL1 and OsHPL2 as AOS, we incubated 9-, 13-HPOD, and 9-, 13-HPOT with *E. coli* lysate of pRSETA-OsHPL1 and pRSETA-OsHPL2, and monitored for  $m/z$  of 309 ( $\alpha$ - and  $\gamma$ -ketol from 9- or 13-HPOT) and  $m/z$  of 311 ( $\alpha$ - and  $\gamma$ -ketol from 9- or 13-HPOD). Incubation of 13-HPOT resulted in the formation of (9*Z*,15*Z*)-13-hydroxy-12-oxo-9,15-octadecadienoic acid ( $\alpha$ -ketol, Fig. 6B), but the transformation rates observed were only 1.3% and 2.5% for OsHPL1 and OsHPL2 respectively (Table 1). But, we did not detect any  $\alpha$ - or  $\gamma$ -ketol by incubation of 9-, 13-HPOD or 9-HPOT (Fig. 6B, C). This indicates that AOS is not the major activity of OsHPL1 or OsHPL2. In

contrast, OsHPL1 and OsHPL2 efficiently transformed 9-HPOD and 9-HPOT into 9-oxo-nonanoic acid (Fig. 5A). We monitored  $m/z$  of 171 to quantify the production of 9-oxo-nonanoic acid, and found that the transformation rate was 72–80% (Table 1). When 13-HPOD or 13-HPOT was incubated with *E. coli* lysate of pRSETA-OsHPL1 and pRSETA-OsHPL2, we detected a single peak at  $m/z$  of 211 at a retention time of 10.5 min, which corresponds to (9*Z*)-12-oxo-9-dodecenoic acid (data not shown). Although we could not quantify the amount of (9*Z*)-12-oxo-9-dodecenoic acid due to the unavailability of the authentic compound, the signal intensity was similar to that of 9-oxo-nonanoic acid, suggesting that the same level of (9*Z*)-12-oxo-9-dodecenoic acid was produced as of 9-oxo-nonanoic acid. Correctly, the results of GC-MS and LC-MS analyses indicate that the major activity of OsHPL1 and OsHPL2 is HPL. Thus, OsHPL1 (AK105964) and OsHPL2 (AK107161) cDNAs previously annotated as allene oxide synthase in rice have 9-/13-fatty acid hydroperoxide lyase activity. OsHPL1 and OsHPL2 are the first CYP74C family enzymes to appear in monocots.

#### Substrate specificity of OsHPL1 and OsHPL2

Finally, to compare the catalytic activities of OsHPL1 and OsHPL2, we attempted to purify recombinant proteins from *E. coli* lysates of pRSETA-OsHPL1 and pRSETA-OsHPL2. The condition of recombinant protein was pre-culture at 16°C and induction at 16°C. A preliminary experiment revealed that no visible recombinant protein was observable in the affinity-purified sample purified from *E. coli* pre-cultured at 37°C and induction at 16°C by SDS-PAGE analysis. Both pRSETA-OsHPL1 and pRSETA-OsHPL2, but not pRSETA, produced a single protein with an apparent molecular weight of 54 kDa on SDS-PAGE, which was good agreement with the calculated molecular weights of 59.6 kDa and 58.7 kDa of the His-tagged fusion protein of OsHPL1 and OsHPL2 respectively. However, the expression levels of fusion proteins of OsHPL1 and OsHPL2 were very low, so that affinity-purified fractions contained co-purified host proteins that bound to the Ni-NTA affinity column.

Using these affinity-purified fractions, we compared the substrate specificity of OsHPL1 and OsHPL2. The relative activity toward 9-HPOT, 9-HPOD, 13-HPOT and 13-HPOD was 100:66:20:61 for OsHPL1, and 100:93:85:59 for OsHPL2, respectively. This was in sharp contrast to 13-HPL (CYP74B) found in barley that specifically metabolizes 13-hydroperoxides of linoleic and linolenic acid.<sup>9</sup> Both OsHPL1 and OsHPL2 showed highest activity toward 9-HPOT, and less activity toward 9-HPOD and 13-HPOD. The remarkable difference in substrate specificity between OsHPL1 and OsHPL2 was the activity toward 13-HPOT. OsHPL2 showed similar levels of activity toward 9-HPOT and 13-HPOT, while the activity of OsHPL1 toward 13-HPOT was only 20% of that toward 9-HPOT.



**Fig. 6.** LC-MS Analysis of the Products Generated by Incubation of Various Fatty Acid Hydroperoxides and *E. coli* Lysates of pRSETA, pRSETA-OsHPL1 and pRSETA-OsHPL2.

Each *E. coli* lysate was incubated with the indicated fatty acid hydroperoxide at a final concentration of 0.1 mM, and the reaction was terminated by adding an equal volume of ethanol containing an internal standard. Production of 9-oxo-nonanoic acid from 9-HPOD and 9-HPOT (A),  $\alpha$ - and  $\gamma$ -ketol from 9-HPOT and 13-HPOT (B), and  $\alpha$ - and  $\gamma$ -ketol from 9-HPOD or 13-HPOD (C) was detected by monitoring  $m/z$  of 171.1, 309.2, and 311.2, respectively. Double-headed arrows represent scales for signal intensity. Symbols:  $\alpha$ -ketol, (9Z,15Z)-13-hydroxy-12-oxo-9,15-octadecadienoic acid;  $\gamma$ -ketol, (10E,15Z)-9-hydroxy-12-oxo-10,15-octadecadienoic acid.

**Table 1.** Quantification of the Products Formed by OsHPL1 or OsHPL2 with Various Fatty Acid Hydroperoxides

Enzyme	Substrates	Products (nmol)		
		9-oxo-nonanoic acid	$\alpha$ -ketol	$\gamma$ -ketol
OsHPL1	9-HPOD	7.2	ND	ND
	13-HPOD	—	ND	ND
	9-HPOT	7.2	ND	ND
	13-HPOT	—	0.25	ND
OsHPL2	9-HPOD	8.0	ND	ND
	13-HPOD	—	ND	ND
	9-HPOT	7.7	ND	ND
	13-HPOT	—	0.13	ND

Ten nmol of fatty acid hydroperoxides was incubated with *E. coli* lysates. Values are the means of duplicate measurements. —, not tested. ND, not detected.

## Discussion

We have shown that AK105964 and AK107161, cDNAs previously annotated as AOS,<sup>25)</sup> had a catalytic activity of 9-/13-HPL, but had no DES activity and only a trace level of AOS activity. We designated AK105964 and AK107161 OsHPL1 and OsHPL2 respectively, and these are the first members of CYP74C found in monocots. As previously described, to date seven cDNAs have been annotated as AOS in rice. This study indicates that two of them are 9-/13-HPL. AOS is a key enzyme for the biosynthesis of jasmonates, important plant hormones that regulate the expression of stress proteins and pathogen-defense related genes, growth, pollen maturation and release, whereas 9-/13-HPL produces completely different metabolites, aldehydes and oxo-acids, for plant-microbe or plant-insect interactions. From this point of view, this study clarifies the

classification of the members of the CYP74 family in rice, and thus would help in understanding the physiological roles of AOS and 9-/13-HPL genes in rice.

As shown in Fig. 2, phylogenetical analysis indicated that OsHPL1 and OsHPL2 are more closely related to CYP74A than to CYP74B, and this might be one of the reasons for the incorrect annotation of OsHPL1 and OsHPL2 as AOS (Fig. 1). The primary structures of 9-/13-HPL and AOS of rice are well resembled. Comparisons of the amino acid sequences of OsHPL1, OsHPL2, OsAOS, OsAOS1 (Genbank accession no. AB116527),<sup>25)</sup> HvAOS1 and HvAOS2 revealed 165 positions of conserved amino acids (Fig. 3). These include the I-helix GXXX(F/L) and EXLR motifs involved in the O<sub>2</sub> binding reported in dicotyledoneous species,<sup>26)</sup> PPGP tetrapeptide motif for the catalytic stability of the cytochrome P450 family,<sup>27)</sup> and PXVXNKQCPG for the heme-binding domain.<sup>23)</sup> On the other hand, we observed 34 subfamily-specific amino acids that might reflect the differences in catalytic activity between AOS and 9-/13-HPL. In dicots, some subfamily-specific amino acids were found between HPL and AOS, and these are proposed for the differences in catalytic activity. For example, the nine amino acid residues upstream of the cysteine involved in heme binding is always glutamic acid in AOS, while that of HPL is a neutral amino acid.<sup>10,26)</sup> However, the corresponding amino acid residues in OsAOS and OsAOS1 are glutamic acid and alanine respectively, and in OsHPL1 and OsHPL2 they are glycine and arginine respectively. Further study is needed to elucidate the structure-function relationship that determines AOS and HPL activity. It is intriguing that OsAOS1, whose catalytic activity has not yet been reported, is more closely related to OsHPL1 and OsHPL2. The catalytic activity of OsAOS1, which is characterized as the gene for *cpm-1* that controls the process of anthesis and phytochrome-mediated inhibition of coleoptile growth,<sup>25)</sup> is of interest.

The primary structures of OsHPL1 and OsHPL2 are very similar; they share 85% of their amino acid residues. It has been shown that OsHPL1 and OsHPL2 have no chloroplast transit peptide,<sup>25)</sup> but the localization of OsHPL1 and OsHPL2 into chloroplast is not precluded, because immunochemical analysis has revealed that HvAOS1 and HvAOS2 that lack chloroplast transit peptides are localized within chloroplasts.<sup>23)</sup> The expression levels of OsHPL1 and OsHPL2 proteins in *E. coli* showed differently. The level of OsHPL1 was much lower than that of OsHPL2 (data not shown). This might reflect the different toxicity or stability in *E. coli*. The remarkable difference between OsHPL1 and OsHPL2 is the catalytic activity toward 13-HPOT, but the biological meaning of this is unclear. To speculate on the difference in the physiological roles of OsHPL1 and OsHPL2, it is interesting that Haga *et al.* found that the expression of OsHPL1 (described as OsAOS4), but not of OsHPL2 (described as OsAOS3) is regulated by

phytochrome. OsHPL1 is up-regulated by red and far-red light in seedling shoots. On the other hand, the transcript of OsHPL2 was too low to determine.<sup>25)</sup> To date, there is no evidence that HPL is controlled by phytochrome, and this sheds a new light on 9-/13-HPL as related to light regulation of gene expression. OsHPL1 and OsHPL2 are very closely located at 31.7 cM on the chromosome 2 (locus name, AP004996; KOME database). From sequence analysis of PAC clone P0027A02, the coding regions of OsHPL1 and OsHPL2 are located at 71520–73052 bp and 61930–63432 bp respectively. Between OsHPL1 and OsHPL2, a hypothetical ORF, which codes for a peptide with 111 amino acid residues predicted at 64835–65170 bp (Protein ID no. BAD17183.1) has been identified. The regulation and evolution of OsHPL1 and OsHPL2 gene is for further work.

In contrast to C<sub>6</sub> aldehydes, the roles of C<sub>9</sub> aldehydes in plant defensive reaction are unclear. Identification of OsHPL1 and OsHPL2 enabled a transgenic approach to analyze C<sub>9</sub> aldehydes in plant defensive reactions. In addition, since (2*E*)-nonenal is considered to be the aged flavor in cereal products, including rice and beer,<sup>20,21)</sup> rice germplasms that lack OsHPL1 and/or OsHPL2 might contribute to the development of a new rice cultivar with improved food quality.

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