**PLANT SEED PEROXYGENASE IS AN ORIGINAL HEME-OXYGENASE WITH A EF-HAND CALCIUM-BINDING MOTIF**

Abdulsamie Hanano‡, Michel Burcklen‡, Martine Flenet‡, Anabella Ivancich‡, Mathilde Louwagie¶, Jérôme Garin¶, and Elizabeth Blée‡

From the‡Laboratoire des Phytooxylipines, IBMP-CNRS-UPR 2357, 28, Rue Goethe, 67083-Strasbourg Cedex, France ; § Service de Bioénergétique, CNRS-URA 2096, DBJC, CEA Saclay, 91191- Gif-sur-Yvette, France ; ¶ Laboratoire de Chimie des Protéines, ERM-0201 INSERM/CEA, 17, rue des Martyrs, 38054 Grenoble Cedex 9 France

Running Title: Plant peroxygenase as calcium-dependent enzyme

Address correspondence to: Elizabeth Blée, Laboratoire des Phytooxylipines, IBMP-CNRS-UPR 2357, 28, Rue Goethe, 67083-Strasbourg Cedex, France, Tel. +33 390 241 836; Fax: +33 390 241 921; E-mail: elizabeth.blee@ibmp-ulp.u-strasbg.fr

A growing body of evidence indicates that phytooxylipins play important roles in plant defense responses. However, many enzymes involved in the biosynthesis of these metabolites are still elusive. We have purified one of these enzymes, the peroxygenase (PXG), from oat microsomes and lipid droplets. It is an integral membrane protein, requiring detergent for its solubilization. Proteinase K digestion showed that PXG is probably deeply buried in lipid droplets or microsomes with only about 2 kDa at the C-terminal region accessible to proteolytic digestion. Sequencing of the N-terminus of the purified protein showed that PXG had no sequence similarity with either a peroxidase, or a cytochrome P450, but rather with caleosins, i.e. calcium-binding proteins. In agreement with this finding, we demonstrated that recombinant thale cress and rice caleosins, expressed in yeast, catalyze hydroperoxide-dependent mono-oxygenation reactions that are characteristic of PXG. Calcium was also found to be crucial for peroxygenase activity whereas phosphorylation of the protein had no impact on catalysis. Site-directed mutagenesis studies revealed that PXG catalytic activity is dependent on two highly conserved histidines, the 9 GHzEPR spectrum being consistent with a high-spin pentacoordinated ferric heme.

A large body of evidence indicates that A growing body of evidence indicates that phytoxylipins play important roles in plant defense responses. However, many enzymes involved in the biosynthesis of these metabolites are still elusive. We have purified one of these enzymes, the peroxygenase (PXG), from oat microsomes and lipid droplets. It is an integral membrane protein, requiring detergent for its solubilization. Proteinase K digestion showed that PXG is probably deeply buried in lipid droplets or microsomes with only about 2 kDa at the C-terminal region accessible to proteolytic digestion. Sequencing of the N-terminus of the purified protein showed that PXG had no sequence similarity with either a peroxidase, or a cytochrome P450, but rather with caleosins, i.e. calcium-binding proteins. In agreement with this finding, we demonstrated that recombinant thale cress and rice caleosins, expressed in yeast, catalyze hydroperoxide-dependent mono-oxygenation reactions that are characteristic of PXG. Calcium was also found to be crucial for peroxygenase activity whereas phosphorylation of the protein had no impact on catalysis. Site-directed mutagenesis studies revealed that PXG catalytic activity is dependent on two highly conserved histidines, the 9 GHzEPR spectrum being consistent with a high-spin pentacoordinated ferric heme.
among which allene oxide synthase and fatty acid hydroperoxide lyase have been best characterized (13). The cloning of genes encoding some of these hydroperoxide transforming enzymes, has revealed that they so far all belong to a new family of unusual cytochromes P450s referred to as CYP74. They differ from the classical P450s by their exclusive reaction with fatty acid hydroperoxides instead of using molecular oxygen and reductants. To date, research in the oxylipins field has largely focused on two specific branches of the LOX pathway, namely those involving CYP74A (allene oxide synthase) and CYP74B (hydroperoxide lyase), which result in the production of jasmonates and volatile aldehydes, respectively. In contrast, little is known at the molecular level on the enzymes involved in the other branches of the LOX pathway. Although the molecular mechanism of the peroxygenase is now better defined, the gene encoding this protein and the nature of the gene product are still unknown. Thus one primary goal of the present investigation was to identify the PXG gene and to compare the encoded protein with other oxygenases such as cytochromes P450 and peroxidases. Although PXG activities have been detected in a wide variety of plants, previous efforts to purify this enzyme to homogeneity or to clone its encoding gene were unsuccessful. PXG shares some features with CYP74s, i.e. they are both membrane-bound hemoproteins that accept fatty acid hydroperoxides as substrates. Therefore, we have first assumed that PXG was a new member of the CYP74 family. But all of our attempts to clone the gene encoding the peroxygenase by sequence similarity with CYP74 members failed. Therefore, we purified the peroxygenase from oat seeds, sequenced the N-terminus, identified Arabidopsis homologues by data-base screening, expressed the corresponding genes in yeast and demonstrated that PXG was a distinct and atypical oxygenase. In contrast to all other oxygenases involved in the oxylipin pathway, it belongs to a small family of proteins, known as “caleosins”(14). These proteins contain a Ca$^{2+}$-binding motif and several phosphorylation sites that could be important for activity and regulation of PXG. This enzyme was present in the endoplasmic reticulum, but also in lipid bodies that we will here refer to as lipid droplets (LDs) in accordance with a recently suggested nomenclature (15). They are small vesicles composed of a core of lipids surrounded by a half-unit membrane of phospholipids and some proteins embedded therein (16). The apparent dual localization of PXG raised the question of the mode of integration of intrinsic membrane proteins into bi- or monolayer phospholipid structures. Importantly, the identification of PXG as a caleosin also opens new perspectives for possible physiological roles of this type of proteins which had no function identified to date.

**EXPERIMENTAL PROCEDURES**

**Materials**– Commonly used chemicals and reagents were of the highest purity available. Purified oligonucleotides were provided either by Life Technologies, Eurogentec or by Sigma Genosys. [1-14C] oleic acid (52 Ci/mole) and [1-14C] linoleic acid (55 Ci/mole) were purchased from DuPont, NEN Research Products. Rice seeds (Oryza sativa, var. Indica, cv. IR64), soaked for 2 h in water, were germinated on two layers of cloth saturated with water under a photoperiod of 16h. Plants were harvested, seven days later, frozen in liquid nitrogen and kept at -80°C until use.

**Preparation of oat subcellular fractions**– Isolation of microsomal fraction, from oat seeds soaked overnight in water, was performed essentially as described previously for soybean seedlings (3). During this procedure (after the second centrifugation step at 100 000 x g) a floating layer consisting of lipid droplets was collected from the top of the tubes with a pipette. The crude lipid droplet fraction was carefully washed with 100mM potassium pyrophosphate buffer that contained 0.1M sucrose (pH 7.4). After centrifugation at 100 000 x g for 45 min the lipid droplet fraction was then resuspended in 10mM potassium phosphate buffer containing 0.1M sucrose (pH 7.4) and centrifuged at 100 000 x g for 45 min. LDs were finally resuspended in 10 mM Tris-HCl buffer containing 10% glycerol (pH 8).

**Purification of oat peroxygenase**– All the subsequent steps were performed at 4°C. Washed microsomes or LDs (30mg protein), resuspended in 5ml of a 10mM Tris-HCl buffer, pH 8, containing 10% glycerol (buffer A), were treated with emulphogene (polyoxyethylene 10 tridecyl ether from Sigma, final concentration 0.2% v/v) for 45 min. The mixture was then centrifuged at 100 000 x g for 45 min. The supernatant was applied to a 1x 2 cm column of DEAE-Trisacryl.
M (BioSepra) equilibrated with buffer B (buffer A containing 0.2% emulphogene (v/v)). After the column was washed with buffer B to eliminate the first protein peak, peroxygenase activity was eluted with a linear NaCl gradient (0→1M) in buffer B (2x30mL). The flow rate was 0.5mL/min and 5 mL fractions were collected. The fractions containing the peak of peroxygenase were pooled and dialyzed overnight against 2 x 2L 10mM sodium acetate pH 5.5 containing 10% glycerol and 0.2% (v/v) emulphogene (buffer C). The dialyzed fraction was applied on a 1x 10 cm column of CM-Sepharose CL-6B (Pharmacia) equilibrated with buffer C. After the column was washed with buffer C, peroxygenase activity was eluted with a linear NaCl gradient (0→1M) in buffer C (2x30mL). The flow rate was 0.5mL/min and 5 mL fractions were collected. This purification protocol was repeated 3 times to isolate sufficient amount of protein. The fractions containing the peak of peroxygenase activity were pooled and dialyzed overnight against buffer C and applied on a column of CM-Sepharose CL-6B. Peroxygenase activity was eluted as described above. The total amount of proteins was measured after each purification step by the Bradford assay (Bio-Rad) using bovine serum albumin as a standard.

Subcloning of AtPXG1, AtPXG2, and OsPXG- Full-length AtPXG1 (At4g26740) and AtPXG2 (At5g55240) were amplified from an Arabidopsis cDNA library by PCR using primers AtPXG1F and AtPXG1R and AtPXG2F and AtPXG2R respectively (Table I). To facilitated cloning, BamHI or XbaI restriction sites were attached to the primers. For the further purification of AtPXG1, PCR was performed using primers AtPXG1NHis and AtPXG1CHis to add a His- tag at either N-or C-terminal ends of the PXG gene. We used also PCR for adding the FLAG-epitope at the C-terminus of AtPXG1. The amplified products were first cloned into the pCR®2.1-TOPO vector (TOPO TA cloning® kit, Invitrogen) and after sequencing, subcloned into the yeast constitutive expression vector pVT102U (17) using the BamHI/XbaI site.

mRNAs were isolated from rice seedlings (0.5g) with the “QuickPrep Micro mRNA purification kit”(Pharmacia biotech.). First strand cDNA was synthesized by incubation of mRNA (3 µg) with 5U of Moloney murine leukemia virus reverse transcriptase (Promega) and oligo (dT)24 at 37°C for 2 h in a 50 µl reaction volume. The cDNA (5µl) was subsequently amplified by PCR in a 50 µl reaction volume using 25 µl HIFI PCR Master (Roche) and 400 nM gene-specific primers OsPXGF and OsPXGR. The amplicon was subcloned as described above.

Expression and purification of recombinant PXGs – Addition of His-or Flag-tags either at the N-or C-terminus did not modify the catalytic activity of the resulting enzyme. Therefore we used recombinant enzymes with tags added to their C-terminus. His-tagged, or Flag-tagged AtPXG1, AtPXG2 and OsPXG were expressed in Saccharomyces cerevisae Wa6 (ade, his7-2 leu2-3 leu2-112 ura3-52) (18). Expression of the recombinant PXGs in transformed yeast cells was carried out as followed: 6 ml of S medium (7g/l yeast nitrogen base, 1g/l casamino acids, 20g/l glucose supplemented with 50 µg/ml histidine, 200 µg/ml adenine and 50 µg/ml leucine) was inoculated with recombinant yeast and grown for 2 days with shaking at 30°C. Then 1 ml portions were used to inoculate six 250 ml cultures of S medium. After 2 days, the resulting cultures were pelleted by centrifugation at 5000 x g and the cells were washed with 200 ml of buffer D (50mM Tris-HCl, pH 7.5). The pellet was resuspended in 100ml of buffer D containing 0.6M sorbitol and the yeast cells were disrupted with glass beads. The resulting lysate was centrifuged once at 10 000 x g for 15 min. The supernatant was recovered and centrifuged at 100 000 x g for 90 min. The pellet was finally resuspended in 10mM potassium phosphate, pH 8 containing 10% glycerol (v/v) and treated with emulphogene (final concentration: 0.2 %) for 45 min at 4°C. The mixture was centrifuged at 100 000 x g for 2 h. His-tagged PXG, or Flag-tagged PXGs present in the supernatant were purified on a Ni-NTA Superflow column (Qiagen), or on a Flag affinity gel (Sigma), respectively, using procedures recommended by the manufacturer. Such purifications were carried out at 4°C in presence of 10% glycerol and 0.2% emulphogene (v/v). The purity of the samples was confirmed by SDS-PAGE followed by silver staining.

Enzymatic activities- Peroxygenase activity was routinely measured (e.g, during the purification procedure) with aniline as substrate (2). Sulfoxidase activity was assayed by using either methyl p-tolyl sulfoxide or thiobenzamide as substrates, as previously described (19, 20).
Epoxidation of [1-14C] oleic and linoleic acids was performed according to (3). The metabolism of [1-14C]13-hydroperoxy-octadecadienoic acid was studied as described before (5).

Site-directed mutagenesis- Site-directed mutagenesis was performed using the QuickChange™ site directed mutagenesis kit of Stratagene with the sense mutations primers described in the PCR primers sections (modified codons underlined and the nucleotide changes indicated in bold in Table I).

Heme content determination- The heme staining procedure was carried according to (21). Hemin (from Sigma) was used as standard for the quantification of heme at 370 nm.

Oat antibodies and Western Blot Analysis- The production of rabbit polyclonal antibodies was performed using standard immunization protocols. Proteins were fractionated by 15% SDS-PAGE and electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a mini-transblot transfer cell apparatus (Bio-Rad). High precision Protein™ Standards (Bio-rad) were used as molecular weight markers. For detection of AtPXG:His, a mouse monoclonal anti-His antibody and an antimouse antibody conjugated to peroxidase were used at 1:500 and 1:5000 dilutions, respectively. Blots were developed using the ECL kit from Pierce.

N-terminus sequencing of PXGs- After separation by SDS-PAGE using a 12.5% polyacrylamide gel, the proteins were transferred to a PVDF membrane. Edman degradation was performed with an automated sequenator (Applied Biosystems 492 Procise).

Proteolysis – Microsomes were washed in 50 mM Tris/HCl buffer (pH 8) containing 10 mM CaCl2 and resuspended in an equal volume of this buffer. Aliquots of microsomes (100 µl containing 1.5 mg proteins) were incubated with increased quantities of proteinase K (from 15 µg to 750 µg) in a total volume of 175 µl at 37°C overnight. Aliquots of 50 µl were removed for immediate measure of PXG residual activity. The proteinase was then inhibited by the addition of 1mM PMSF. A similar protocol was used for proteinase K treatment of LDs.

Phosphorylation experiments- Proteinase-treated microsomes were incubated in the presence of 0.1 unit of casein kinase II and 4 µCi [γ-35S] ATP in 100 mM KH2PO4 buffer (pH 7.5) containing 8 mM MgCl2 for 4 h at 27°C. Proteins and peptides separated by SDS/PAGE were transferred to a PVDF membrane for immunodetection. Similar protocol was used for the phosphorylation of purified AtPXG1 except that this fraction was incubated for 1 hour at 4°C to preserve enzymatic activity.

Synthesis of radiolabelled substrates-Racemic [1-14C] 9,10-epoxyoctadec-9(Z)-11(E)-dienoic acid were synthesized as previously described (3). Electron Paramagnetic Resonance (EPR) spectroscopy- Conventional 9-GHz EPR measurements were performed using a Bruker ER 300 spectrometer with a standard TE102 cavity equipped with a liquid helium cryostat (Oxford Instrument) and a microwave frequency counter (Hewlett Packard 5350B). The spectra of frozen samples of oat peroxygenase (0.1 mM concentration, 50 mM buffer pH 5.0) and horseradish peroxidase (0.5 mM, pH 7.0 10mM MOPS buffer) were recorded at 4K.

Analytical Procedures- Radioactivity was measured on TLC plates with a Berthold TLC linear detector LB 2821, and peak integration was obtained by using the program CHROMA 1D (Packard Instrument Company). Radioactivity was also determined in a liquid scintillation spectrometer (LS 9000, Beckman). Chiral-phase HPLC was performed, under isocratic conditions, on a Shimadzu instrument coupled with a radiomatic 500TR analyzer (Packard Instrument Co.). Peak integration was obtained using Flo-one software.

The resolution of the enantiomers of [14C]-labeled methyl cis-9,10-epoxyoctadecanoic acid was performed on a Chiralcel OB column (4.6 x 250 mm; Baker Chemical Co.) with a solvent mixture of n-hexane/isopropanol (98:2:1 at 0.3 ml/min). The separation of the R and S enantiomers of methyl p-tolyl sulfoxide was achieved on the same chiral column eluted with a solvent mixture of n-hexane-isopropanol (80:20 at 0.6 ml/min). Products obtained after reaction of [1-14C]13 HPOD with recombinant AtPXG1 were separated by RP-HPLC on a Lichrospher (Agilent technologies)100 RP-18(5 µm) column with a solvent mixture of...
Identification of the trihydroxy-derivative of linoleic acid was achieved by GC-MS analysis after methylation of the acid function with ethereal diazomethane and silylation of the hydroxyl groups with N-methyl-N-trimethylsilyl-trifluoroacetamine (Pierce). GC-MS analysis was performed on an Agilent 5973 N apparatus with ionizing energy of 70 eV. The sample was injected directly into a DB-5 coated fused capillary column (30m; 0.25 mm internal diameter; J. W. Scientific) with a temperature program of 10°C/min from 100°C to 280°C followed by 10 min at 280°C.

RESULTS

Purification of PXG from oat seed microsomes– Peroxygenase activity was purified from oat seeds and was found to be localized to microsomal fractions. The membrane-bound peroxygenase could not be released by treatment with 3M KCl confirming that the enzyme is an integral membrane protein (19). We therefore tested various detergents (CHAPS, BIGCHAP, octylglucoside, Triton X100, emulphogene) for their ability to solubilize the enzyme. Emulphogene (0.2%) was among the most effective. The solubilized membrane extract was purified according to the protocol described in the “Experimental Procedures” chapter. The instability of the peroxygenase caused severe problems especially during dialysis and concentration of the pooled fractions but addition of glycerol (10%) contributed to some extent to stabilize PXG (Table II). Further purification using strong cationic exchanger (Mono-S) or hydrophobic column (alkyl-Superose) resulted in a loss of enzyme activity. It is unclear whether this loss of activity is the result of inactivation or irreversible binding of the enzyme to the columns. Analysis on SDS-PAGE of the final fractions containing PXG activity showed a bulk of proteins of low molecular weight near the migration front but also a band around 27 kDa, whose intensity was correlated with peroxygenase activity (Fig. 2). The N-terminus sequence of this protein (AVVVSDAMSSVAKGAPVTAQ) exhibited similarity with ATS1 (39% identity) encoding an embryo-specific gene in Arabidopsis (22) and with abscisic acid-induced EFA 27 in rice (35%), a membrane-bound protein having a mass of 27 kDa (23). These proteins belong to a small family called caleosin (24) because they contain a calcium-binding domain and seem to possess similar structural features with oleosins, which are found in lipid droplets. This apparent localization of caleosin prompted us to examine if lipid droplets display PXG activity.

Purification of PXG from oat seed lipid droplets– Purified lipid droplets isolated from oat seeds were able to perform co-oxidation reactions known to be catalyzed by peroxygenases. For example, they actively oxidized thiobenzamide to its sulfoxide (65.8 nmoles/min/mg protein) and oleic acid into 9,10-epoxystearate (51.4 nmoles/min/mg protein). It should be noted that these activities were about 4 times higher than those determined in microsomal fractions. Consequently, we performed the purification of PXG from lipid droplets following the same protocol used for the microsomal fraction. Detergent was required to solubilize the peroxygenase activity from lipid droplets suggesting that the protein was buried into the phospholipids monolayer or lipid core of these organelles. Silver nitrate staining of the purified enzyme fraction separated by SDS-PAGE showed two major bands at about 40 kDa and 27 kDa. But only the intensity of the latter was correlated with the activity of the peroxygenase. The N-terminus sequence of this 27 kDa protein (AVVVSDAMSSVAKGAPVTAQRSxxD) was identical to that of the protein isolated from oat seeds microsomes (but extended the sequence by some amino acids) and thus also showed homologies with caleosins (48% and 43% with ATS1 and EF27, respectively). Considering that most of caleosins identified so far have a molecular weight around 25-29 kDa, we hypothesized that the purified 27kDa protein might be a caleosin supporting peroxygenase activity.

Expression of caleosins in yeast and identification as plant peroxygenases - In order to validate that PXG was indeed a caleosin, we have expressed, in yeast, the first caleosin identified in Arabidopsis (At4g26740, also named ATS1 or AtClo1, (22, 25)). Crude extracts of yeast expressing the recombinant protein catalyzed co-oxidation reactions typical of peroxygenase such as sulfoxidation of thiobenzamide (5.8 nmoles/min/mg protein), hydroxylation of aniline (4.5 nmoles/min/mg protein) or epoxidation of oleic acid (1.5 nmoles/min/mg protein). Importantly, all these
activities were strictly hydroperoxide dependent. Yeast crude extracts were then subfractionated by differential centrifugations into 100 000g supernatant, microsomes and lipid droplets. Whereas microsomes and lipid droplets actively catalyzed co-oxidation reactions (for example, 62 nmoles/min/mg protein and 124 nmoles/min/mg protein of thiobenzamide sulfoxide formed respectively), the soluble fraction was found inactive. Neither extract from wild type WA6 nor yeast transformed with an empty vector showed any catalytic activity. Based on these experiments we annotate At4g26740 encoding peroxygenase as AtPXG1.

To evaluate if such oxidative capacities of AtPXG1 are common to other caleosins, we have studied two other members of this family of proteins: Atclo2, recently renamed ATS2 which is believed to be associated with dormancy or germination of Arabidopsis seeds (26) and EFA27 from rice (23). The corresponding genes (At5g55240 and X89891, respectively) were expressed in yeast. Fig. 3 shows that both microsomal and oleosomal fractions prepared from the transformed yeasts catalyzed the oxidation of thiobenzamide, aniline and oleic acid with similar efficiency as AtPXG1. Taken together, these results show that plant peroxygenases are caleosins.

Characterization of purified AtPXG1-
To further characterize AtPXG1 in regard to PXG activity, a His6-tagged version of AtPXG1 was expressed in yeast and was used for purification by affinity chromatography on a Ni²⁺ column. SDS-PAGE of the purified enzyme showed one single band at 32 ± 4 kDa as revealed by silver nitrate staining and western-blot analysis using an anti-His-tag antibody. The purity of AtPXG1 was 98% as assessed by scanning densitometry. The recombinant enzyme is somewhat larger than predicted suggesting that it might be covalently modified, as it was previously reported for this protein but also for an oleosin (22, 27). To tentatively identify this modification, we used site-directed mutagenesis. First we replaced threonine residues at positions 15 and 116 and the asparagine at position 109 by a valine. These positions were predicted to be possible sites of O- and N-glycosylation by the programs NetOgly 1.0, YinOyan and Net-N-Glyc respectively. These mutations did not affect the molecular weight of the recombinant proteins.

Another posttranslational modification is SUMOylation of proteins resulting in particular in an enhancement of their stability (28). Analysis of AtPXG1 sequence revealed that it possessed a high sumoylation potentiality at the position K196 (SUMOplot™ Prediction: http://www.abgent.com/doc/sumoplot). However, replacement of K196 with a valine resulted in a protein with the same mass as the wild type enzyme. Together, it seems that glycosylation or sumoylation processes were not responsible of the modification of the mass of the recombinant protein. To ensure that we have purified a peroxygenase rather than another oxidase, we used the purified AtPXG1 to provide additional proof of the identity of the 32kDa recombinant protein as a peroxygenase.

- AtPXG1 is a hemoprotein:
We had previously demonstrated that the soybean peroxygenase contains an iron protoporphyrin IX (heme) that supports catalytic activity (3). Thus we looked for the presence of a heme in AtPXG1 although there is no report on the presence of a prosthetic group in caleosins. First, a peak at 407 nm, representative of the Soret band of hemoproteins, could be detected in the light absorbance spectra of the purified fraction of AtPXG1. Second, addition of cumene hydroperoxide (Fig. 4, inset) resulted in a gradual decrease of this Soret band. As found for the soybean peroxygenase (3), such an effect was correlated with a decline of hydroperoxide supported oxidations by AtPXG1. Fig.4 shows the clear correlation between these two phenomena. Both the decrease in the absorbance at 407 nm and the enzyme inactivation follow pseudo-first order kinetics with similar half-life (about 3.5 min). Together, these results confirmed that AtPXG1 contains a heme responsible of its enzymatic activity.

- AtPXG1 catalyzed intramolecular oxygen transfer:
We next verified that purified AtPXG1 was capable to catalyze the intramolecular transfer of oxygen in fatty acid hydroperoxide in the absence of other oxidizable compound as we found previously for the partially purified soybean peroxygenase (5). For this purpose, purified AtPXG1 was incubated in the sole presence of [1³C]13-HPOD (13-hydroperoxy-octadeca-9,11-dienoic acid) and the products of the reaction were analyzed by radio-HPLC (Fig. 5). After 1 hour of incubation at 26°C, about
80% of the fatty acid hydroperoxide was transformed into a polar compound (peak 1: elution time: 3.3 min). The mass spectra of its methyl ester-trimethylsilyl ether derivative was identical to those of Me3Si derivative of methyl 9,10,11-trihydroxyoctadecanoate (29). This compound likely derived from the spontaneous chemical hydrolysis of 9,10-epoxy-11-octadecenoic acid which was found unstable at acidic pH. Peak 2 (elution time: 31.3 min) was 13-HOD (13-hydroxy-octadeca-9,11-dienoic acid), the methyl ester-trimethylsilyl ether derivative of this compound co-chromatographed with authentic standard and mass spectra of the two compounds are identical. Peak 3 represented the residual substrate. Thus AtPXG1, like the soybean peroxygenase, was capable to catalyze the reduction of fatty acid hydroperoxide with the concomitant formation of an epoxyalcohol.

-AtPXG1 catalyzed stereospecific oxidation reactions:

Another characteristic of the soybean peroxygenase is the high stereospecificity of the catalyzed alkylaryl sulfide oxidation and unsaturated fatty acid epoxidation (3, 20). We found that when AtPXG1 was incubated in the presence of methyl p-tolyl sulfide, and cumene hydroperoxide as an oxygen donor, the (S)- sulfoxide was formed with about 60% enantiomeric excess. If oleic acid was used as substrate instead of sulfide, AtPXG1 produced cis-9,10-epoxysearic acid, which consisted largely in the 9(R),10(S)-enantiomer (70%). Thus AtPXG1 catalyzed asymmetric co-oxidation reactions.

-Inhibition of AtPXG1:

Characterization of the soybean peroxygenase has revealed that it is sensitive to some inhibitors. Of these, we have tested β-mercaptoethanol (19) and the organo-phosphorus terbufos (7). The sulfoxidation of thio benzamide, catalyzed by AtPXG1, was completely abolished in the presence of 1 mM of β-mercaptoethanol, which probably acts as a competitive inhibitor (Blée, unpublished results). In contrast, terbufos is a suicide substrate for plant peroxygenases and at a concentration of 3 mM it effectively inactivated the activity of AtPXG1.

Together, these results conclusively demonstrate that AtPXG1 is a peroxygenase.

Characterization of the heme microenvironment using EPR spectroscopy and site-directed mutagenesis- The way heme proteins interact with their prosthetic group largely determines their catalytic activity and biological functions (30). In cytochrome P450, a cysteine thiolate was identified to be the heme axial ligand whereas a histidine is coordinated to the heme in horseradish peroxidase. The 9 GHz EPR spectrum of peroxidases reflects structural features of the heme active site and correlates to the oxidation and spin states of the heme iron, as well as the coordination number of the iron (for a review see (31)). The EPR signal with two main resonances at $g_{||} \approx 6$ and $g_{\perp} \approx 2$ are characteristic of ferric heme iron (S=5/2) in the high spin state. In particular, horseradish peroxidase (HRP) shows a rhombically distorted EPR signal with $g_e = 6.44$, $g_y = 5.05$ and $g_{Bz} = 1.96$ (Fig. 6). In agreement, the crystal structure of this enzyme showed that the heme iron is pentacoordinated, with His 170 being the axial ligand and His 42 being the catalytically relevant residue in the distal heme side (32). We have used low temperature EPR spectroscopy to characterize the heme site in the oat peroxygenase. The spectrum (Fig. 6) agrees well with those reported for pentacoordinated high-spin heme enzymes, with resonances at $g_e = 6.22$, $g_y = 5.53$ and $g_{Bz} = 1.98$. The small but significant difference in rhombicity of the EPR signal of peroxygenase as compared to HRP is not surprising since the distal side residues (and/or their relative position to the heme iron) are not necessarily the same in these enzymes. It was shown that the ferric 9-GHz EPR spectrum is very sensitive to changes in the distal heme side in mono- and bifunctional peroxidases (33, 34). Moreover, even among peroxidases such differences in the rhombicity of the signal was observed. For example, one of the isoforms of turnip peroxidases showed an EPR signal (see Fig. 1 in (33)) very similar to that of the peroxygenase in Fig. 6. The EPR spectrum of peroxygenase in Fig. 6 also showed that there is no low-spin ferric heme signal characteristic of the coordination of the distal histidine as the sixth ligand to the heme iron as in the case of cytochromes (see (35)and references therein).

In order to assess the identity of the axial ligand in plant peroxygenases, we have used site-directed mutagenesis on AtPXG1. First we have ruled out any implication of cysteine in heme coordination by mutating Cys21 and Cys236, the only two Cys residues present in the primary sequence of AtPXG1. Their point mutation to glycine did not modify the enzymatic activities
of the mutated proteins indicating strongly that these residues were not involved in the coordination of the heme in AtPXG1. Sequence alignment of plant caleosins shows that a certain number of histidines are conserved, including those corresponding to His52, His59, His70, His137, His134 and His138 of AtPXG1. To investigate the role of these histidines, we have constructed mutants in which each of these residues was replaced by valine. In addition, to avoid any interference, a Flag peptide (DYKDDDDK) was inserted for purification instead of a His-tag (36).

Replacement of histidine at positions 52, 59, 131, 134 and 138 gave mutants that could be expressed in yeast in quantities similar to the wild-type enzyme. In sharp contrast, replacement of His70 by a valine severely affected the expression of the corresponding mutant protein as revealed by western blot analysis using anti-flag antibodies (not shown). Only two of these mutations affected the hydroxylase activity of the resulting proteins after their purification by affinity chromatography. Mutations of the highly conserved His70 and His138 resulted in a complete loss of detectable peroxygenase activity. Such an effect could result from a deficit of the prosthetic group in these mutants. We have thus measured the heme content in the purified fractions of H70V and H138V and indeed found that H138V possesses only 4% of the heme content of AtPXG1 and it was barely measurable in H70V (less than 1%). Therefore, we have tentatively assigned His70 to the heme axial ligand in AtPXG1 presuming that the mutant H70V, devoid of its prosthetic group and probably misfolded, was more sensitive to destruction by the yeast proteasome. Obviously, the exact role(s) of His70 and His138 need further clarification.

**Ca2+ is required for AtPXG1 activity-** In contrast to cytochrome P450, it is well known that a calcium ion is present in many peroxidases, where it seemed to play a structural role for these enzymes (37). It was also suggested that a functional calcium-binding site was essential for the biosynthesis of active animal peroxidases (38). Initial data base searches indicated that caleosins contain a conserved Ca2+-binding, EF-hand motif (23, 24, 39), while a recent analysis of EF-hand-containing proteins in Arabidopsis did not identify any caleosin (40). However, careful inspection of an alignment of AtPXG1 with the sequences of two known EF-hand Ca2+-binding proteins (the allograft inflammatory factor-1 AIF-1 and the tescalin Tsc) revealed probable remnants of ancestral EF-hand domains within AtPXG1 that may coordinate Ca2+ ions (Fig. 7, A). Accordingly, the caleosin EFA27 was found to do bind calcium in rice (23). To define a possible role of calcium in the structure and/or activity of AtPXG1, we have modified the EF-hand domain of AtPGX1 by site-directed mutagenesis. It was shown that mutation of the first amino acid of the EF-hand loop, an Asp coordinating Ca2+, abrogates calcium binding by AIF-1 and Tsc (41, 42). On this basis, we replaced the Asp at position X of the EF-hand with Val as shown by the arrow in Fig. 7, A. This point mutation did not alter the peroxygenase activity (Fig. 7, B) possibly suggesting that calcium had only an insignificant role in the structure/activity of plant peroxygenases. To substantiate this point, we have extensively dialyzed AtPXG1 against chelating agent (EDTA) to remove any trace of metal in the protein. This treatment completely abolished co-oxidation properties of AtPXG1, that could be restored (up to 70%) by adding 1 mM CaCl2 to the medium (Fig. 7, B). From these experiments, it appears that Ca2+ ions are present in AtPXG1 and are required for its structural integrity but that mutation of the first aspartate in the EF-hand motive is not sufficient to abolish function of AtPXG1.

**Phosphorylation of AtPXG1 does not modify its catalytic activity-** Although AtPXG1 has several putative phosphorylation sites, it is unclear whether it binds phosphate, and if so, how it affects the enzyme activity. We verified the phosphate-binding potential of AtPXG1 by incubating the protein in the presence of casein kinase and [35S]-ATP. A similar reaction without casein kinase served as a negative control. Fig. 8 shows that AtPXG1 does bind phosphate and that casein phosphorylation sites are responsible for this binding. However phosphorylation did not result in any change in the sulfoxidation of thiobenzamide catalyzed by AtPXG1. This indicated that phosphorylation is probably not a required modification for catalytic activity.

**Association of PGX with membranes -** PXG can only be extracted from oat membranes (lipid droplets or microsomes) with detergents, raising the question of its mode of association with lipid mono- or bilayers. It has been speculated that caleosins might adopt different tertiary structures depending whether they are binding to ER or LDs (43). Caleosins binding to ER were
suggested to adopt a type I orientation, i.e. with the N-terminal domain on the lumen side and the C-terminus facing the cytosol. According to this hypothesis, the active site of PXG would face the ER lumen whereas the phosphorylation sites would be in contact with the cytosol. When bound to lipid droplets, it was suggested for thermodynamic reasons that caleosins expose both their polar N- and C-terminal domains to the cytosol (43, 44). To experimentally test these hypotheses, we used digestions with a protease to identify the fragments of PXG which are proteolytically protected and hence presumably buried within the ER lumen or in the core of the LDs. We have first used a proteinase K vs. microsomes (or lipid droplets) ratio of 0.04 because such conditions were previously shown to completely degrade caleosins incorporated in artificial lipid droplets within 10 min (45). However under the given conditions this treatment did not alter PXG activity in microsomes or LDs. Therefore we have increased the incubation time. After 16 h at 37°C, microsomes treated with protease (PK/microsomes=0.01) still exhibited 60% of residual PXG activity, whereas lipid droplets treated with a higher amount of protease (PK/LD =0.2) retained only 27% of the enzymatic activity. After proteolytic treatment, the proteins were separated by SDS-PAGE and analyzed by western-blot using an anti-oat PXG antibody. Surprisingly, very little degradation of PXG had occurred during proteolysis (Fig. 9, a). A major band of about 25kDa could be observed both in microsomal and lipid droplet fractions, suggesting that only a small fragment of about 2 kDa was accessible to proteolytic action. This fragment is presumably located at the C-terminus of PXG for the following reasons: i) the 25 kDa truncated PXG can no more be phosphorylated ii) a putative phosphorylation site for casein kinase II (“SLFE”), which is strongly conserved among caleosins, is located at position 225-230 in AtPXG1 iii) the molecular mass of the peptide corresponding to the S225-Cter sequence in AtPXG1 is about 2.4 kDa (Fig. 9, b and c). The fact that the truncated 25kDa protein cannot be further degraded even in the presence of high amounts of proteinase K (Fig. 9, a-b) indicates that most of PXG is inaccessible because it is presumably deeply buried within membranes, in the ER lumen or in the core of lipid droplets. A protein band with a MW of approximately 54 kDa was also revealed by western-blot both in microsomes and lipid droplets (Fig. 9, a). This value corresponds closely to that expected for a dimeric form of PXG. Intriguingly, the disappearance of this band upon proteolytic treatment was correlated with the loss of PXG activity. These results may thus indicate that PXG is catalytically active as a dimer only.

DISCUSSION

PXG is a unique oxygenase- Our data identify a plant peroxygenase as a caleosin and consequently ascribe an enzymatic function to this class of proteins. This study revealed also that caleosins are hemoproteins. Such results were unforeseen because “caleosins” were mostly typified by the presence of a single EF-hand calcium binding domain that is particularly conserved in all plant and fungal species described to date (25). No function has been attributed to these proteins, although it has been speculated that they might play a role in membrane fusion and lipid trafficking through Ca2+-mediated processes (46). We have demonstrated here that calcium was indispensable for the enzymatic activity of PXG. Because this activity was not modulated in vitro by addition of calcium (not shown), our results are instead consistent with a role for this ion in preserving a catalytically active conformation of the protein. Such a function in maintaining the structure in the heme environment has already been shown for one of the archetypal hem-containing peroxidases: the horseradish peroxidase (HRP), where only one Ca2+ is essential for the protein structure (47, 48). PXG does not share any significant sequence similarity with peroxidases, neither with any member of the large cytochrome-P450 family. However, these three classes of hemoproteins share similarities in their catalytic mechanisms. For example, all three are thought to use an oxoferryl intermediate in their reactions that is depending on the architecture of their active sites (20, 49, 50). Therefore and because heme binding imposes strong constraints to the structure of a protein (51) it was important to identify the heme ligands in PXG. Site-directed mutagenesis experiments ruled out any contribution of a thiolate ligand, but indicated that two histidine residues were implicated in heme attachment and enzyme activity. It seems unlikely that both these histidines ligand the iron heme in PXG, as known for cytochrome b, since the EPR spectrum clearly showed the g-value of...
a high-spin pentacoordinated heme iron, with no g-component of low-spin hexacoordinated species (52). Most probably, PXG contains imidazole as its proximal ligand, and a second histidine at its distal site. Presently, it is unclear why the mutation of the proposed distal-site histidine resulted in heme loss and inactivation of PXG. One explanation might be that the distal histidine mutation leads to a modification of the structural environment of the heme. Besides axial ligation, heme might be held by the pressure of a peptide on one pyrrole ring as it was already shown for a cytochrome P450 (30).

Concerning this point, we have observed an extreme lability of PXG enzymatic activity, probably due to the loss of the prosthetic group as a result of conformational change during freezing, for example. The fact that PXG has a histidine as the axial ligand, but is a “pure” peroxygenase raises some intriguing questions concerning its mechanism. The distal machinery of both peroxidases and cytochrome P450s is thought to be critical in determining whether these enzymes act as oxygenases or peroxidases (53-56). Therefore, the further identification of structural elements and crucial residues of the active site of PXG should help in better understanding the mechanistic features of this atypical hemoprotein.

**Structural considerations** – The name, caleosin, originates from the putative oleosin-like association of these proteins with lipid droplets (24, 25). From a structural point of view, oleosins are characterized by a long hydrophobic central domain, which adopts a hairpin conformation and which, after crossing the outer phospholipid monolayer, is embedded into the neutral lipid core of the lipid droplet. In contrast, the amphipathic N- and C-terminal domains of the oleosins are located at the surface of the LDs where they interact with polar head groups of the phospholipids (57). Caleosins also possess these three domains, but two of them are characterized by additional key features: the N-terminal region contains a calcium-binding EF-hand motif and the C-terminal region has putative protein kinase phosphorylation sites (24, 43). Accordingly, we could localize the active site of the Ca²⁺-dependent PXG within the N-terminus and also establish that the enzyme could be phosphorylated by casein kinase II. Concerning the hydrophobic domain, *in silico* analysis of the hydropathy profile of twenty caleosins revealed that they can be divided into three classes depending on the location of this domain in the primary structure of these proteins (Fig. 10, b). While located central in class I, the hydrophobic region is shifted to the N-terminus in class II and appears randomly located in fungal caleosins (Fig. 10, b). Strikingly, these three sub-families perfectly match the three clades determined by constructing a phylogenetic tree with the full-length sequences of the caleosins (Fig. 10, a).

Class I groups most of plant caleosins exhibiting the key structural domains of oleosins. These caleosins are present in seeds but also in vegetative tissues, stems or siliques (14, 23, 39). Some of them are inducible by abiotic stress or abscisic acid (23, 39). On the other hand, very little is known on plant caleosins from Class II. Their hydrophobic domain is closer to the N-terminal region, but this does not necessarily imply that these proteins are not membrane-bound since AtPXG5 was recently found in the pollen coat (58). The gene encoding this latter caleosin was placed in tandem with At1g23250 on chromosome 1 probably resulting from a fairly recent duplication. A similar duplication might also have occurred to give rise to AtPXG4 and At1g70680 on the same chromosome 1. Finally the last class comprises yet uncharacterized caleosins from fungi. Although the significance of such a classification remains to be elucidated, it raises intriguing possibilities related to PXG specificities and functions. All classes of caleosins share the two histidine residues and the calcium binding site required for PXG activity, except At1g 23250 from Class II. We have demonstrated that three members of Class I act as peroxygenases. Catalytic activity as PXG of fungal caleosins and members of Class II remains to be confirmed. The possibility, that fungal caleosin could catalyze the formation of anti-fungal epoxy- and hydroxy-fatty acid derivatives, raises the question of the physiological relevance of such compounds in fungi and of the regulation of their biosynthesis during plant-pathogen interaction.

The present identification of the gene encoding plant peroxygenase should give a firm basis for forthcoming studies on the physiological importance of this enzyme.
REFERENCES

43. Murphy, D. J. (2001). *Prog. Lipid Res.* **40**, 325-438
61. Felsenstein, J. (2005). PHYLP (Phylogeny Inference Package) version 3.6 (Distributed by the author). Department of Genetics, University of Washington, Seattle, USA

**FOOTNOTES**

§ Present address: UMR Phytopharmacie et Biochimie des Interactions Cellulaires- BP 86510- 21065 Dijon cedex- France.

*We thank Jérôme Giraudat and Cerasem-Semences de France for the gift of Arabidopsis cDNA library and oat seeds respectively, Frederic Duval and Jürgen Ehling for assistance in art graphic and phylogenetic tree reconstruction, Francis Schuber and Luis Ruzo for critical review of the manuscript.*
**FIGURE LEGENDS**

Fig.1. Reactions catalyzed by plant peroxynase in the presence of hydroperoxides. This enzyme mediates the exclusive heterolytic cleavage of the hydroperoxide, yielding the corresponding alcohol and a ferryl-oxo complex. Then the oxygen atom of this intermediate can be transferred to an acceptor molecule that can bind the active site following an intermolecular oxygen transfer (A). Following this mechanism, peroxynase mediates co-oxidative reactions such as sulfoxidation, hydroxylation or epoxidation (A). When starting with fatty acid hydroperoxide, the ferryl-oxo complex intermediate epoxidizes the more reactive nonconjugated double bond (generally in configuration cis) either before it diffuses out of the active site (intramolecular mechanism) (B) or after its reassociation with the active site through an intermolecular mechanism (A).

Fig.2. Analysis of purified peroxynase from microsomal fraction of oat seeds by SDS-PAGE. The three final fractions (A,B and C) containing peroxynase activity (measured as sulfoxidation of thiobenzamide) were analyzed under denaturing conditions on a 15% (w/v) polyacrylamide gel stained with silver nitrate (a). The intensity of the 27 kDa band followed the capability of transformation of oleic acid (peak 2) into 9,10-epoxy stearic acid (peak 1) by peroxynase present in fractions A, B and C (b).

Fig.3. Co-oxidation reactions catalyzed by AtPXG1 (1), AtPXG2 (2) and Os PXG (3). Sulfoxidation of thiobenzamide (white bars), hydroxylation of aniline (grey bars) and epoxidation of oleic acid (black bars) were measured in microsomes and lipid droplets fractions from transformed yeasts. Results are means ± standard deviation (n=3).

Fig.4. Inactivation of peroxynase activity (□) and decrease of the Soret band (♦) as a function of time at 26°C. The reaction mixture contained purified AtPXG1 (0.7 mg of protein in 1 ml of 10 mM potassium buffer, pH 8, containing 10% glycerol and 0.2% emulphogene) and cumene hydroperoxide (5 mM). The decay of the Soret band is expressed as the absorbance decrease at 407 nm compared to the total decreased in A 407, i.e. (A t−A ∞)/A 0−A ∞ with A 0 and A ∞ the initial and final (end point) absorbances respectively. Inset, repetitive scanning of the absolute spectrum of AtPXG1 obtained on addition of cumene hydroperoxide.

Fig.5. RP-HPLC-analysis of the products formed after incubation of 13-HPOD in presence of purified AtPXG1. 13-HPOD (60 µM, 50x 10^4 cpm) was incubated in presence of purified AtPXG1 (100 µg of protein) in 300 µl acetate buffer (0.1M, pH 5.5) during 1 hour. Products of the reaction were detected by radioactivity (a, b) and at 234 nm (c). Peak 1: 9,10,13-trihydroxy-11-octadecenoic acid; peak 2:13-HOD and peak 3: 13-HPOD.

Fig.6. 9-GHz EPR spectra of oat peroxynase (top) and horseradish peroxidase (bottom) in the resting (ferric) state. Experimental conditions: temperature, 4.2 K ; microwave frequency, 9.42 GHz; modulation amplitude, 4 G; modulation frequency, 100 kHz; microwave power, 2 mW.

Fig.7. Requirement of calcium for PXG activity. A) Conservation of the EF-hand loop in AtPXG1, AtPXG2, EFA 27, tescalin and allograft inflammatory factor-1. The consensus EF-hand domain consists of a long helix-loop-helix array. Only the EF-hand loop were aligned for AtPXG1 (A. thaliana, At4g26740), AtPXG2 (A. thaliana, At5g55240), EFA 27 (O. sativa, X 89891), Tsc (Mus. Musculus, AAH19492) and AIF-1 (H. sapiens U49392). The most common amino acids that bind Ca^{2+} at the X, Y and Z positions include aspartate (D) and asparagine (N). The residue at position Y donates a carbonyl oxygen, whereas a glutamate (E) or an aspartate (D) is present at position Z. The glycine at position G permits a bend in the EF-hand loop, and the residue at position I is hydrophobic (isoleucine, valine) (59). The arrow indicates the aspartate at position X of AtPXG1 that was mutated to valine to obtain a putative Ca^{2+}-binding-deficient mutant (D75V). B) Ca^{2+}-dependence of AtPXG1 activity. Co-oxidations activities: thiobenzamide sulfoxidation (black points) aniline hydroxylation (black points) aniline hydroxylation (
black striped) and oleic acid epoxidation (grey) were measured for AtPXG1 (1), for AtPXG1 after extensive dialysis of the fraction containing the purified enzyme (2), for AtPXG1 after dialysis followed by the addition of 1 mM CaCl₂ in the medium (3) and for the mutant D75V (4).

Fig.8. Phosphorylation of AtPXG1. AtPXG1 (150 µg of protein) was incubated in the presence of ATP₃⁵S, (10 μCi), MgCl₂ (8 mM) and casein kinase (1U) in 300 µl of 10 mM potassium buffer, pH 8 containing 20% glycerol and 0.2% emulphogene for 4 hours at 4°C. An aliquot (50 µl) was analyzed in SDS-PAGE (b, AtPXG1 at left, molecular weight markers at right). Radioactivity on dry gel was read with a TLC radiodetector (a).

Fig.9. Proteolysis and phosphorylation of peroxigenase present in microsomes and lipid droplets. (a) Western blot analysis of peroxigenase in oat microsomes and lipid droplets treated (+) or untreated (-) by proteinase K. (b) western-blot analysis of peroxigenase present in microsomes treated by increasing amounts of proteinase K (band 1: untreated, band 2: PK/PXG=0.01, band 3: PK/PXG=0.02 and band 4: PK/PXG=0.1) (c) Residual activity of oleic acid oxidation (♦) and phosphorylation (□) performed by microsomes treated by increasing amounts of proteinase K.

Fig.10. Classification of caleosins according to their evolutionary relationship (A) and their hydropathy (B). Phylogenetic tree was constructed using an alignment of full length protein sequences generated using Dialign (60) and were used for a maximum likelihood analysis using the program PHYLIP (61). Bootstrap values from 100 replicates are given at the nodes. Protein sequences are from AtPXG1 (A. thaliana, At4g26740), AtPXG2 (A. thaliana, At5G55240), AtPXG3 (A. thaliana, At2g33380), ATPXG4 (A. thaliana, At1g70670), AtPXG5 (A. thaliana, At1g23240), SiPXG (S. indicum, AAF13743), GmPXG (G. max, AAB71227), OsPXG (O. sativa,CAA61986), HvPXG (H. vulgare, AYO43288), FsPXG (F. silvatica, CAE 51349), CdPXG (C. dactylon, AAS48644), bci4 (H. vulgare, AJ25283), AnPXG (A. nidulans, XP_681860), NcPXG (N. crassa, Q7S2T2), CgPXG (C. globosum, EAQ92068), MgPXG (M. grisea, XP_365887), UmPXG (U. maydis, Q4PAW0).
TABLE I

Summary of primers:

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtPXG1F</td>
<td>CGGGATCCATGGGGTCAAAGACGGAGAT</td>
</tr>
<tr>
<td>AtPXG1R</td>
<td>GCTCTAAGATTAGTAGTGCTGTCTTTGTC</td>
</tr>
<tr>
<td>AtPXG2F</td>
<td>CGGGATCCATGACGTCGATGGAGAGGAT</td>
</tr>
<tr>
<td>AtPXG2R</td>
<td>CGGGATCCATGGCAGGAGAGGCAGAGGC</td>
</tr>
<tr>
<td>AtPXG1NH</td>
<td>GGGATCCATGCACCACCACCACCACCATGGGGTCAAAGACGGAGAT</td>
</tr>
<tr>
<td>AtPXG1CH</td>
<td>GCTCTAGATTAGTGTCGATGGTGCTGTCTTTGTC</td>
</tr>
<tr>
<td>AtPXG1NF</td>
<td>CGGGATCCATGGGGTCAAAGACGGAG</td>
</tr>
<tr>
<td>AtPXG1CF</td>
<td>GCTCTAGATTATTATCATCATCATCTTTTATAATCGTAGATGCTGTCTTTGTC</td>
</tr>
<tr>
<td>OsPXGF</td>
<td>CGGGATCCATGGCGGAGGAGGCGGCGCTAGC</td>
</tr>
<tr>
<td>OsPXGR</td>
<td>GCTCTAGACCTACTCTTGCTCTCTCATGTGC</td>
</tr>
<tr>
<td>T15VF</td>
<td>GGAGAGAGACGCAATGGGTCTGTGTGGCTCCCTATGCGCCG</td>
</tr>
<tr>
<td>H52V</td>
<td>GCAAGCACCAGACAGAAGAAGGTCTACGGAACTCCAGGC</td>
</tr>
<tr>
<td>H59V</td>
<td>GGAAATCAGGACGGGTAAAGATTACGG</td>
</tr>
<tr>
<td>H70V</td>
<td>CTCTCAGTTCCCTCAAAGACGGGTCTCCCTCTGCTATCG</td>
</tr>
<tr>
<td>T116VF</td>
<td>CCTAGCTCTACTATAGCCTGGGCTCCCTCTCTGCTGTCTACG</td>
</tr>
<tr>
<td>H131V</td>
<td>CTTTCTTCCCTATATACATAGTTAACATACACAAGCTCAAAGG</td>
</tr>
<tr>
<td>H134V</td>
<td>CCGATATACATACACACATAGGTAAATGCAAAGCATGG</td>
</tr>
<tr>
<td>H138V</td>
<td>CACAGATCCAAAGGGTGGGAAGTGC</td>
</tr>
<tr>
<td>K196VF</td>
<td>GGATGGATCGCAGGCCTAGAATAGGCTGGGACTG</td>
</tr>
<tr>
<td>C221G</td>
<td>GCTATGCGCGGGGTTGCTGATGGAGC</td>
</tr>
<tr>
<td>C230G</td>
<td>GCTTGTTCAGTACGGTGCAAAATCTACGC</td>
</tr>
</tbody>
</table>
TABLE II

Representative purification of membrane-bound peroxigenase from oat seedlings

The enzyme was purified starting from 30g dry weight of oat seeds. The activity was assayed with 1 mM thiobenzamide in presence of 1 mM cumene hydroperoxide.

<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Total Activity nmoles/min</th>
<th>Specific Activity (nmoles/min/mg)</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>464</td>
<td>15.4</td>
<td>1</td>
</tr>
<tr>
<td>Solubilization</td>
<td>291</td>
<td>63.7</td>
<td>4.2</td>
</tr>
<tr>
<td>DEAE-Trisacryl</td>
<td>300</td>
<td>150.1</td>
<td>9.8</td>
</tr>
<tr>
<td>Dialysis</td>
<td>273</td>
<td>147.9</td>
<td>9.6</td>
</tr>
<tr>
<td>CM-Sepharose</td>
<td>256</td>
<td>365.5</td>
<td>24.7</td>
</tr>
</tbody>
</table>
A) Intermolecular oxygen transfer (Co-oxidation reactions)

B) Intramolecular oxygen transfer

Fig. 1
Fig. 2

A BC

Migration (cm)

Radioactivity (cpm)

27 kDa

a

b

1

2

A B C

Radioactivity (cpm)

Migration (cm)

Fig. 2
Fig. 3

Specific Activity (nmole/min/mg protein)

Lipid droplets

Microsomes

1
2
3

1
2
3

0 20 40 60 80 100 120 140 160
Fig. 5

Absorbance (234nm) | Radioactivity (arbitrary units)

- Time (min)
- 10 20 30 40 50

Lines and data points indicating trends over time for absorbance and radioactivity.
Fig. 6
A)

\[ \begin{array}{cccccc}
1 & 2 & 3 & 4 & 5 & 6 \\
\text{nX*Y*Z} & \text{G} & \text{Y} & \text{I} & \text{X*Z} & \text{n}
\end{array} \]

- AtPXG1: F D I D D N G I I Y P W E T 87
- AtPXG2: F D L D N N G I I Y P F E T 86
- EFA 27: F D L D G D G I V Y P W E T 89
- Tsc: Y D S D S D G R I T L E E Y 70
- AIF-1: F D L N G N G D I D I M S L 135

B)

**Fig. 7**

```
<table>
<thead>
<tr>
<th>Specific Activity (mnoles/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000</td>
</tr>
<tr>
<td>2500</td>
</tr>
<tr>
<td>2000</td>
</tr>
<tr>
<td>1500</td>
</tr>
<tr>
<td>1000</td>
</tr>
<tr>
<td>500</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>
```

```
<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

```
I X * * Z
n
```

```
X * Y * Z
G Y I X * Z n
```
Fig. 8
Fig. 9
Fig. 10
Plant seed peroxynenase is an original heme-oxygenase with an EF-hand calcium-binding motif
Abdulsamie Hanano, Michel Burcklen, Martine Flenet, Anabella Ivancich, Mathilde Louwagie, Jérôme Garin and Elizabeth Blée

J. Biol. Chem. published online September 6, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M605395200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2006/09/06/jbc.M605395200.citation.full.html#ref-list-1