

# A non-canonical caleosin from *Arabidopsis* efficiently epoxidizes physiological unsaturated fatty acids with complete stereoselectivity

Elizabeth Blée<sup>1</sup>, Martine Flenet<sup>1</sup>, Benoît Boachon<sup>1</sup> and Marie-Laure Fauconnier<sup>2</sup>

<sup>1</sup> Institut de Biologie Moléculaire des Plantes, Université de Strasbourg, France

<sup>2</sup> Plant Biology Unit, Gembloux Agro-Bio Tech, Université de Liège, Belgium

## Keywords

AtClO4; caleosin; epoxygenase; fatty acid epoxide; peroxygenase

## Correspondence

E. Blée, Institut de Biologie Moléculaire des Plantes, 28 Rue Goethe, 67083 Strasbourg Cedex, France

Fax: +33 3 68 85 19 21

Tel: +33 3 68 85 24 36

E-mail: Elizabeth.Blee@ibmp-cnrs@unistra.fr

(Received 7 June 2012, revised 15 August 2012, accepted 16 August 2012)

doi:10.1111/j.1742-4658.2012.08757.x

In plants, epoxygenated fatty acids (EFAs) are constituents of oil seeds as well as defence molecules and components of biopolymers (cutin, suberin). While the pleiotropic biological activities of mammalian EFAs have been well documented, there is a paucity of information on the physiological relevance of plant EFAs and their biosynthesis. Potential candidates for EFA formation are caleosin-type peroxygenases which catalyze the epoxidation of unsaturated fatty acids in the presence of hydroperoxides as co-oxidants. However, the caleosins characterized so far, which are mostly localized in seeds, are poor epoxidases. In sharp contrast, quantitative RT-PCR analysis revealed that *PXG4*, a class II caleosin gene, is expressed in roots, stems, leaves and flowers of *Arabidopsis*. Expressed in yeast, *PXG4* encodes a calcium-dependent membrane-associated hemoprotein able to catalyze typical peroxygenase reactions. Moreover, we show here that purified recombinant *PXG4* is an efficient fatty acid epoxygenase, catalyzing the oxidation of *cis* double bonds of unsaturated fatty acids. Physiological linoleic and linolenic acids proved to be the preferred substrates for *PXG4*; they are oxidized into the different positional isomers of the monoepoxides and into diepoxides. An important regioselectivity was observed; the C-12,13 double bond of these unsaturated fatty acids being the least favored unsaturation epoxidized by *PXG4*, linolenic acid preferentially yielded the 9,10-15,16-diepoxide. Remarkably, *PXG4* catalyzes exclusively the formation of (*R*),(*S*)-epoxide enantiomers, which is the absolute stereochemistry of the epoxides found *in planta*. These findings pave the way for the study of the functional role of EFAs and caleosins in plants.

## Introduction

Among mammalian oxylipins, epoxy fatty acids function as important biomediators in inflammation, blood pressure regulation, angiogenesis and numerous physiological and pathophysiological processes in the central nervous system (reviewed recently in [1–3]). The biosynthesis of these metabolites involves various cytochrome P450 isoforms that oxidize unsaturated

fatty acids into a multitude of bioactive epoxygenated fatty acid (EFA) regioisomers [4–7].

In comparison, very little is known about the biosynthesis and the biological roles of EFAs in plants. These oxylipins are classically found in seed oils as constituents of triglycerides that accumulate in lipid droplets (LDs). Despite the fact that the different

## Abbreviations

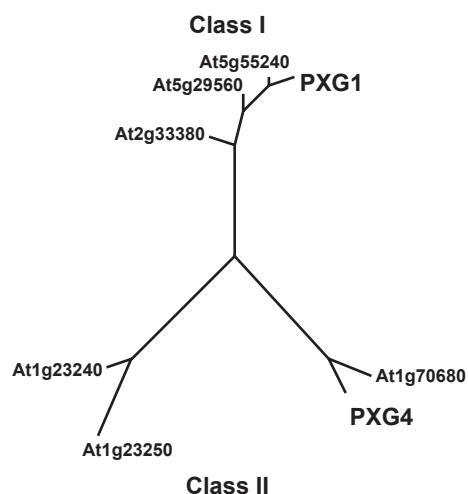
13 HOT, 13-hydroxy-9,11,15-octadecatrienoic acid; 13 HPOD, 13-hydroperoxy-9,11-octadecadienoic acid; 13 HPOT, 13-hydroperoxy-9,11,15-octadecatrienoic acid; EFA, epoxygenated fatty acid; EH, epoxide hydrolase; LD, lipid droplet.

positional isomers of EFAs deriving from unsaturated fatty acids such as 9,10-, 12,13- and 15,16-epoxy derivatives are present in such oils [8], the main focus remained on the formation of vernolic acid, a particular epoxide deriving from linoleic acid. Found at high amounts in the seeds of some exotic plants, vernolic acid (12,13-epoxy-*cis*-9-octadecenoic acid) has several potential industrial applications. It is used, for example, in the production of plastics, adhesives, nylon and paints [9,10]. Considerable effort has therefore been devoted to the identification and expression of the genes controlling its biosynthesis in commercial oil crops. Depending on the species two unrelated epoxygenases have been identified: a delta 12 non-heme diiron protein in the genus *Crepis* [11–13] and a cytochrome P450 in the genus *Euphorbia* [14,15]. However, because of the high regioselectivity of these two enzymes which is restricted to the oxidation of the C-12,13 double bond of linole(n)ic acid, the identity of the biosynthetic enzymes responsible for the occurrence of the numerous other regioisomer and stereoisomer epoxides present in the triglycerides of seed LDs remain to be established.

EFAs are not confined to the seeds. They are also components of cutin, the scaffold of cuticles that cover all the vegetative parts of plants [16]. Cutin monomers were as well found to act as endogenous stress-related signal molecules [17] inducing, for example, resistance of barley against *Erysiphe graminis* [18]. Although most cutin monomers originate from saturated or mono-unsaturated fatty acids, oxygenated derivatives of linoleic and linolenic acids such as 9,10-, 12,13- and 15,16-epoxides have also been reported, albeit more rarely. For example they have been detected in rice species resistant to *Magnaporthe grisea* and have been found to accumulate as defence compounds in sensitive rice species infected by this fungus [19,20]. Generally, EFAs do not accumulate in cells due to their toxicity and their rapid hydrolysis by efficient epoxide hydrolases (EHs). Selective inhibition of EHs in mammalian systems has allowed the modulation of EFA levels and the unravelling of their biological functions (recently reviewed in 21,22). Several inhibitors of mammalian EHs were also shown to inhibit plant EHs *in vitro* [23–25]; however, their selectivity, mode of administration and bioavailability have not yet been studied *in planta*. Alternatively, to assess the biological significance of EFAs in plants the modulation of their cellular levels by altering the gene expression of the epoxidases responsible for their biosynthesis might also be envisaged. Unfortunately, this genetic approach is currently hampered by the lack of genes known to encode such epoxygenases.

In rice, it was shown that epoxy derivatives of linoleic and linolenic acids arise from a distinct branch of the lipoxygenase pathway encompassing an uncharacterized lipid-hydroperoxide-decomposing activity [26,27]. In parallel, *in vitro* studies have revealed the presence, in various seeds and young seedlings, of hydroperoxide-dependent peroxygenase activities that potentially catalyze the epoxidation of unsaturated fatty acids [28,29]. Such peroxygenases were also suggested to be involved in the biosynthesis of C18 cutin monomers [30,31]. Peroxygenase activities were recently demonstrated to be associated with caleosins, a small family of proteins produced by two classes of genes in plants [32]. Class I genes encode for the few caleosins that have been biochemically characterized so far. But these caleosins when heterologously expressed showed only marginal epoxidase activity under standard conditions [32,33]. Therefore class I caleosins can hardly account for an efficient fatty acid epoxidation of unsaturated fatty acids in green plants.

At present, no class II caleosins, named AtC1O4 to AtC1O7 (Fig. 1) in *Arabidopsis* [34], have been characterized biochemically. AtC1O5 (At1g23240) was detected in pollen coat and according to data from the public microarray database its gene is exclusively expressed in flowers, suggesting a role for this caleosin in flowering [35]. In sharp contrast, *AtC1O4*, though present in flowers, is also expressed in roots, leaves and stems, suggesting that *AtC1O4* might be functionally active not only in reproductive systems but also in germination and in vegetative processes [36]. That class II caleosins are peroxygenases, or even enzymes, remains, however, to be demonstrated.



**Fig. 1.** Phylogenetic tree of caleosins putatively present in *A. thaliana* with At4g26740 as PXG1 and At1g70670 as PXG4.

Currently, we do not know which particular enzymes are responsible for the biosynthesis of plant EFAs. We expect candidates for this type of activity to be part of the caleosin-type peroxygenases which were shown to catalyze epoxidation reactions in various seedlings. Here we report that *AtC1O4* encodes a non-seed-specific caleosin able to efficiently catalyze the epoxidation of unsaturated fatty acid double bonds. We found that this epoxidation represents, relative to other types of oxidation, the major reaction catalyzed by *AtC1O4* (renamed here PXG4). Importantly these epoxidations, which are characterized by a very high stereoselectivity, give access to the stereoisomers found *in planta*. We therefore suggest that *AtC1O4* is a gene implicated in fatty acid epoxidation in *Arabidopsis thaliana*.

## Results

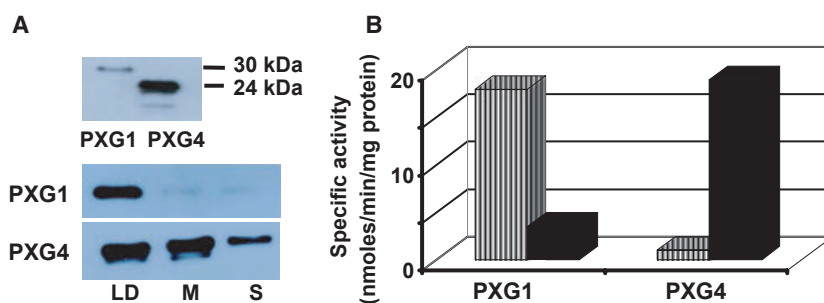
### Characterization of PXG4 as a membrane-bound fatty acid epoxygenase

Class II caleosins lack the canonical central hydrophobic domain that is present in class I caleosins and is presumably responsible for their LD targeting/docking [37]. To determine whether class II members are still membrane-bound proteins, we expressed *PXG1* (At4g26740) and *PXG4* (At1g70670) in yeast as representative of classes I and II respectively. We then assessed the ability of the recombinant proteins to be located on LDs. Western blot analysis of crude extracts of the transformed yeast revealed that, compared with *PXG1* expression, *PXG4* was far more substantially expressed (about fourfold, Fig. 2A). Both isoforms were actively targeted to LDs. The excess of overexpressed *PXG4* was apparently retained in the microsomal fraction containing the endoplasmic reticulum (Fig. 2A). A thinner band of *PXG4* was present

in the western blot analysis of the soluble extract (Fig. 2A) but was no longer visible after its filtration on a 0.75  $\mu\text{m}$  diameter filter or its centrifugation at high speed for 2 h. Therefore, *PXG4* and class I caleosins are mostly membrane-bound proteins that can be recruited to LDs.

We recently reported that *PXG1* like other class I caleosins supports peroxygenase activity, i.e. it catalyzed co-oxidation reactions, such as sulfoxidation, hydroxylation or epoxidation, in the presence of hydroperoxides as co-oxidants [32]. Crude extracts of yeast harboring *PXG1* efficiently oxidized aniline but very poorly epoxidized unsaturated fatty acids such as oleic acid (Fig. 2B). Routinely, we measured a ratio of 1 : 0.3 between the specific activity of aniline hydroxylation and oleic acid epoxidation respectively. In contrast, [ $^{14}\text{C}$ ]oleic acid was rapidly metabolized by *PXG4* in a strictly hydroperoxide-dependent manner. Up to 19  $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  protein of *cis*-9,10-epoxystearate was formed; this was about 20-fold more than the production of hydroxylated aniline by the same yeast crude extract (Fig. 2B). Importantly, radio-labeled epoxy derivative was the sole metabolic product detectable during the oxidation of [ $^{14}\text{C}$ ]oleic acid by *PXG4* in the presence of cumene hydroperoxide. Notably, no [ $^{14}\text{C}$ ]dihydroxy-octadecanoic acid could be detected confirming that the yeast used for expressing caleosins had no EH activity [38].

Oleic acid exhibits a *cis* double bond at position 9,10. Keeping the unsaturation at the same position while shortening the length of the alkyl chain did not noticeably affect the epoxidation rates (Table 1). In contrast, shifting the unsaturation of octadecenoic acid to position 6 or 11 favored the epoxidation of the double bond closest to the carboxylate (Table 1). In addition, *PXG4* exhibited a low selectivity with respect to the configuration of the double bond. For example, *cis* and *trans* vaccenic acids or oleic and elaidic acids



**Fig. 2.** (A) Western blot analysis of crude yeast extracts expressing *PXG1* and *PXG4* using anti-His<sub>6</sub> mouse monoclonal IgG: LD, lipid droplet; M, microsomes; S, supernatant. (B) Specific activities of *PXG1* and *PXG4* measured as aniline hydroxylases (black striped) and oleic acid epoxygenases (black). Enzymatic activities were corrected by the respective expression levels of the two caleosins.

**Table 1.** Substrate specificity of PXG4. Mono-unsaturated and polyunsaturated fatty acids (200  $\mu\text{M}$ ) were incubated in the presence of purified PXG4 (2  $\mu\text{g}$ ) and cumene hydroperoxide (200  $\mu\text{M}$ ) for 30 min at 27 °C. The resulting epoxides and remaining substrates were extracted with ether, methylated with ethereal diazomethane and analyzed by gas chromatography. The given results represent mean values  $\pm$  SE of three biological replicates.

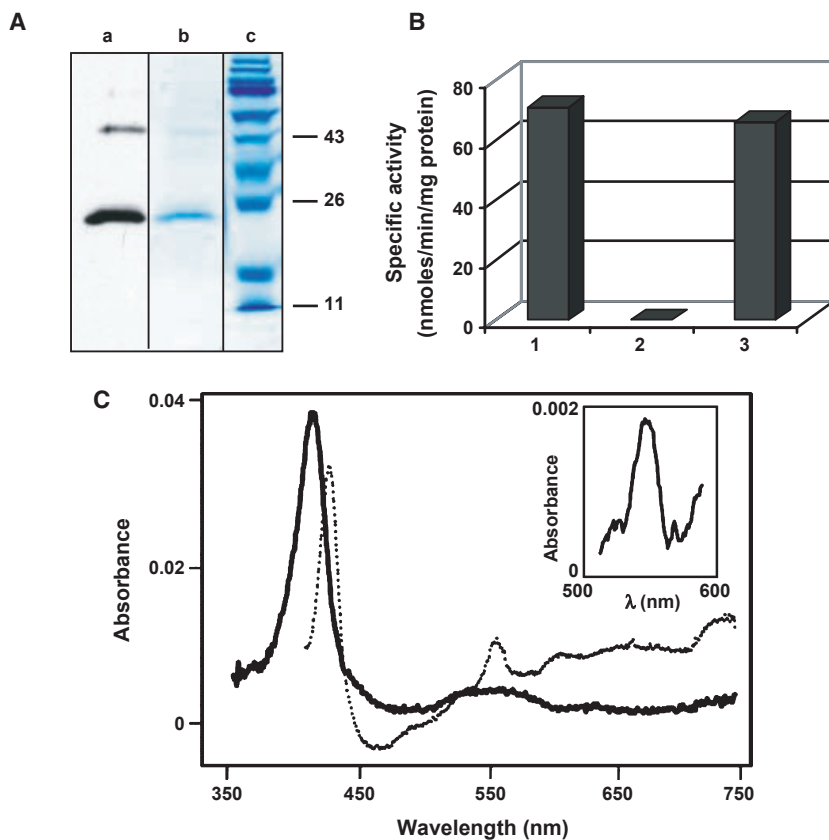
Unsaturated fatty acids	Reaction progress (%)
C14:1-9 <i>cis</i>	23 $\pm$ 1
C16:1-9 <i>cis</i>	36 $\pm$ 2
C18:1-9 <i>cis</i>	32 $\pm$ 2
C18:1-9 <i>trans</i>	15 $\pm$ 1
C20:1-11 <i>cis</i>	22 $\pm$ 1
C18:1-6 <i>cis</i>	53 $\pm$ 2
C18:1-11 <i>cis</i>	31 $\pm$ 2
C18:1-11 <i>trans</i>	12 $\pm$ 1
C18:2-9,12 <i>cis</i>	64 $\pm$ 3
C18:3-9,12,15 <i>cis</i>	74 $\pm$ 4
C18:1-9 <i>cis</i> methyl ester	11 $\pm$ 1

were both epoxidized by this enzyme, albeit at a lower rate for the *trans*- compared with the *cis*-isomer (Table 1). It should be noted here that the methyl ester of oleic acid was a poor substrate for PXG4 (Table 1)

indicating that a free carboxylic group seems necessary for the positioning of the substrate within the active site. Overall these results indicate that, in contrast to the class I caleosins characterized to date, PXG4 is an effective hydroperoxide-dependent fatty acid epoxidase.

### Purification and characterization of recombinant PXG4

To further characterize the catalytic activity of PXG4 we purified a His<sub>6</sub>-tagged version of the recombinant enzyme on an Ni<sup>2+</sup> column. The purified protein gave a single band on SDS/PAGE at 24  $\pm$  2 kDa as revealed by Coomassie Blue staining and western blot analysis using an anti-His-tag IgG (Fig. 3A). In addition, a minor protein band with a molecular mass of about 48 kDa was apparent, suggesting that PXG4 might also exist as a dimer, like the class I caleosins. The term caleosin partly originates from the presence of a strictly conserved EF-hand calcium binding motif that was shown, at least for PXG1, to be crucial for its catalytic activity [32]. Because the EF-hand motif present in PXG4 typifies it as a caleosin, we verified



**Fig. 3.** (A) Analysis of purified recombinant PXG4 by western blot using (a) anti-His-tag IgG, (b) Coomassie Blue staining and (c) molecular weight standards. (B) Purified PXG4 actively catalyzed aniline hydroxylation in the presence of cumene hydroperoxide (1). Extensive dialysis against EDTA completely abolished this activity (2), which could be restored (3) by addition of 2 mM CaCl<sub>2</sub>. These experiments were repeated twice with different batches of purified PXG4 with similar results. (C) Absolute absorption spectra (solid line) and difference spectrum (dithionite reduced minus oxidized, dashed line) of PXG4. Inset, difference spectrum (dithionite reduced minus oxidized) in the presence of 20% pyridine (v/v) in 0.1 M NaOH.

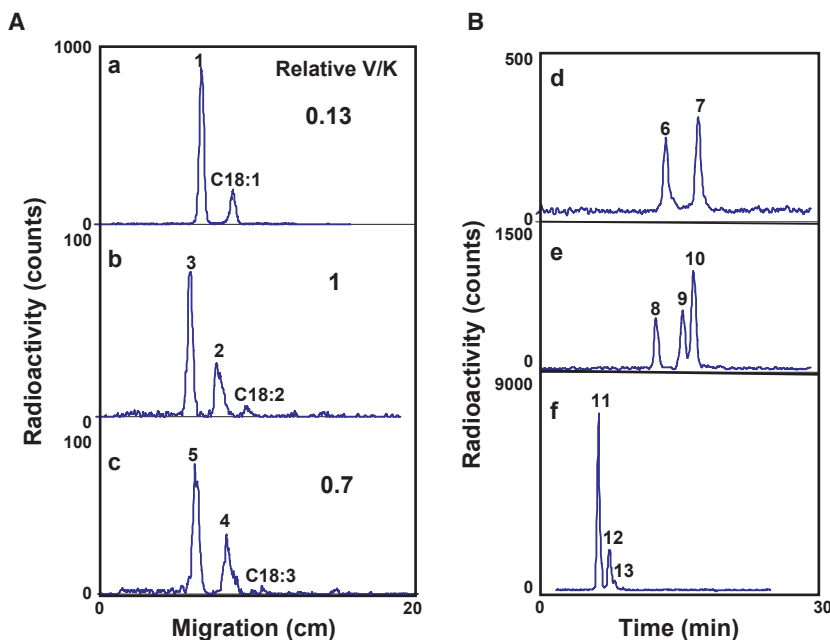
whether the activity of this protein was also calcium-dependent. Extensive dialysis of purified PXG4 against EDTA totally abolished the capacity of this fraction to oxidize aniline in the presence of cumene hydroperoxide as co-oxidant. This enzymatic activity could be restored, however, by addition of  $\text{CaCl}_2$  (Fig. 3B). These results indicate that calcium was also required for the structure/activity of PXG4.

Both the absolute spectrum of oxidized versus dithionite reduced PXG4 (Fig. 3C) and the formation of a pyridine-ferrohemoprotein complex in the presence of pyridine and NaOH (Fig. 3C, inset) at 556 nm suggested that PXG4 contained a protoheme of type b (0.6 nmol of heme per mg protein has been estimated). Indeed, the electronic absorption spectrum of PXG4 (Fig. 3C) is characteristic of a high-spin ferric heme site, with the Soret band at 407 nm and a charge transfer band at 635 nm (Fig. 3C). Such a spectrum is very similar to that of soybean peroxygenase [28] and PXG1 [32]. The EPR spectrum of PXG1 showed that the heme site is in the ferric high-spin oxidation state that is pentacoordinated with histidine as an axial ligand [32]. Hence, the heme site in resting (as isolated) PXG4 is concluded to be probably a pentacoordinated heme in the ferric oxidation state. Such configuration is favorable for the epoxidation reaction with hydroperoxides as oxidants (to form the oxoferryl intermediate state) and lipids as substrates. The prosthetic heme is most probably not covalently bound to the protein because dialysis of purified PXG4 against a mildly acidic buffer (pH 5.5) led to its progressive loss, as monitored by the severe decrease in intensity of the Soret band at 407 nm, yielding finally the apoprotein devoid of catalytic activity. Site-directed mutagenesis has indicated His70 and His133 as the putative heme proximal and distal ligands in PXG1 [32]. Similarly to PXG1, replacing the putative axial ligand (His90 in PXG4 and His70 in PXG1) resulted in complete impairment of the enzymatic activity of PXG4, indicating that this residue is probably required for the structure and/or activity of this protein. Considering the quite favorable yield of expression of the membrane-bound PXG4 in yeast [5–7 (mg protein)·L<sup>-1</sup> of culture], we shall try to crystallize this LD protein. Indeed, X-ray determination should help to rationalize site-directed mutagenesis approaches to identify the active site residues of PXG4.

### PXG4 is a regioselective peroxygenase

PXG4 catalyzed the epoxidation of mono-unsaturated fatty acids, such as oleic acid. From a physiological point of view polyunsaturated fatty acids such as

linoleate and linolenate are of great interest, since their oxidized derivatives have been found to be involved in plant defence mechanisms or in jasmonate production [19,39]. Increasing the desaturation of C18 fatty acids seemed to greatly facilitate their epoxidation (Table 1). We have confirmed this result by incubating [<sup>14</sup>C]linoleic and [<sup>14</sup>C]linolenic acids in the presence of cumene hydroperoxide and PXG4. Epoxidation occurred readily, i.e. under similar experimental conditions the rate of substrate disappearance (expressed as  $V/K_m$ ) was about sevenfold to tenfold higher for linolenic and linoleic acids respectively than for oleic acid (Fig. 4A). Two groups of compounds were formed: the monoepoxides (peaks 2 and 4 in Fig. 4A) and the diepoxides (peaks 3 and 5 in Fig. 4A). The rate of formation of the monoepoxides from C18:2 by PXG4 was about two orders of magnitude faster than the formation of the diepoxide. Curiously, no triepoxide arising from linolenic acid oxidation was detected (Fig. 4Ac). In addition, when the monoepoxides deriving from C18:3 oxidation (Fig. 4A, peak 4) were purified from the TLC plate and further incubated with PXG4 in the presence of cumene hydroperoxide, only diepoxides accumulated (not shown). To determine whether such results stem from a possible regioselectivity of PXG4, we separated the different monoepoxide and diepoxide isomers by straight phase (SP) HPLC using a silica column under isocratic conditions. The two monoepoxides of C18:2 were baseline separated (Fig. 4Bd) and identified by their fragmentation pattern using MS. A slight but constant preference for the formation of 9,10-epoxy-12-octadecenoic acid (60% ± 2%; Fig. 4B, peak 7) was observed during the linoleic acid epoxidation by PXG4. Notably, the ratio between the two regioisomers did not change until the reaction was completed. In addition, the oxidation of the *cis* double bond at position 9,10 prevailed over that at positions 12,13 and 15,16 when linolenic acid was used as the substrate, i.e. 9,10-epoxy-12,15-octadecadienoic acid (Fig. 4B, peak 10) represented one-third of the monoepoxides formed whereas similar amounts of 15,16-epoxy-9,12-octadecadienoic acid (26%; Fig. 4B, peak 9) and 12,13-epoxy-9,15-octadecadienoic acid (24%; Fig. 4B, peak 8) accumulated. The regioselectivity of PXG4 appeared far more pronounced in diepoxide formation. A major peak of 9,10-15,16-diepoxo-12-octadecenoic acid (76%; Fig. 4B, peak 11) eluted initially on the silica column of SP-HPLC followed by a small peak of 9,10-12,13 diepoxo-15-octadecenoate (22%; Fig. 4B, peak 12) and a very small peak of 12,13-15,16-diepoxo-9-octadecenoate (2%; Fig. 4B, peak 13). Taken together, these data indicate that PXG4 is a regioselective enzyme. Epoxidation of



**Fig. 4.** (A) Radio-TLC analysis of the products resulting from the incubation of (a) oleic acid (C18:1), (b) linoleic acid (C18:2) and (c) linolenic acid (C18:3) by PXG4 in the presence of cumene hydroperoxide. Peak 1, 9,10-epoxy-stearic acid; peak 2, 9,10-12,13-diepoxy-stearic acid; peak 3, mixture of 9,10-epoxy and 12,13-epoxy oleic acids; peak 4, mixture of 9,10-epoxy, 12,13-epoxy and 15-16-epoxy linoleic acids; peak 5, mixture of 9,10,12,13-diepoxy, 9,10,15,16-diepoxy and 12,13,15,16-diepoxy oleic acids. (B) Radio-SP-HPLC analysis of the monoepoxides resulting from incubation of C18:2 (d) and the monoepoxides (e) and diepoxides (f) resulting from incubation of C18:3 with PXG4 in the presence of cumene hydroperoxide. Peak 6, 12,13-epoxy-9-octadecenoic acid; peak 7, 9,10-epoxy-12-octadecenoic acid; peak 8, 12,13-epoxy-9,15-octadecadienoic acid; peak 9, 15,16-epoxy-9,12-octadecadienoic acid; peak 10, 9,10-epoxy-12,15-octadecadienoic acid; peak 11, 9,10-15,16-diepoxy-12-octadecenoic acid; peak 12: 9,10-12,13-diepoxy-15-octadecenoic acid; peak 13, 12,13-15,16-diepoxy-9-octadecenoic acid.

polyunsaturated fatty acids was taking place preferentially at position 9,10, then at 15,16 and lastly at 12,13.

#### PXG4 exhibits a complete stereoselectivity forming only (*R*),(*S*) enantiomers

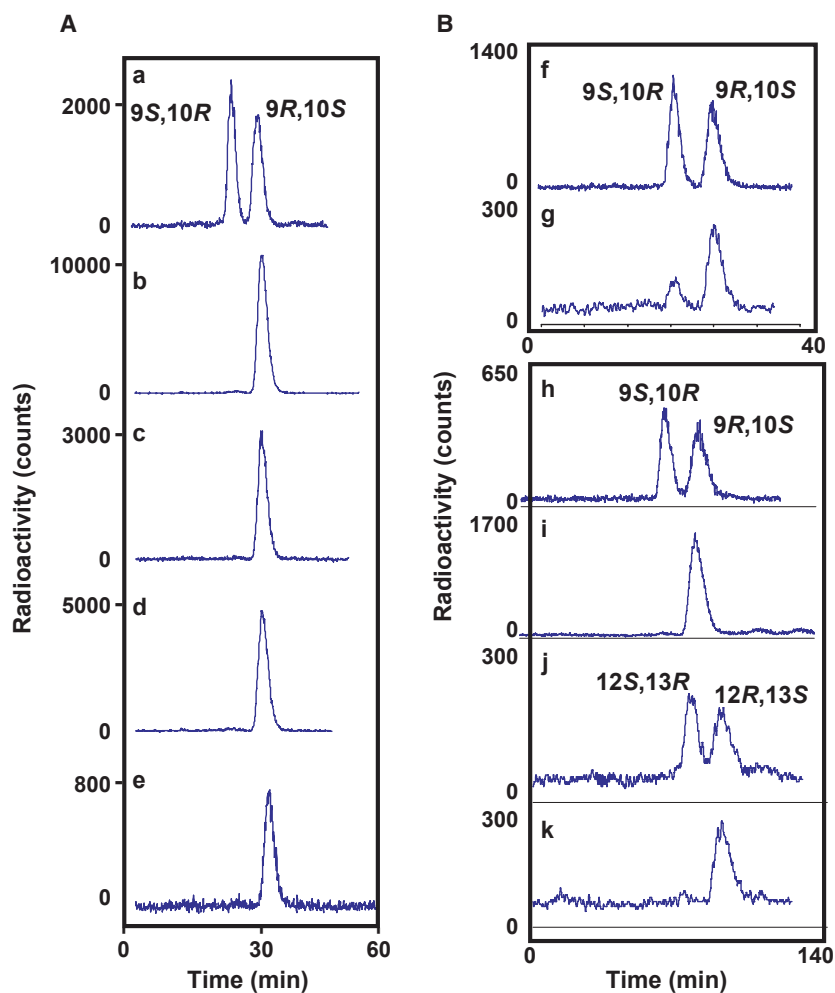
We have previously reported that PXG1 catalyzed asymmetric oxidation [32]. For example, when oleic acid was used as the substrate, PXG1 produced the *cis*-9,10-epoxy-stearic acid with the preferential formation of 9(*R*),10(*S*) enantiomer (40% ee). To evaluate whether PXG4 was also a stereospecific enzyme, we incubated [<sup>14</sup>C]oleic acid in the presence of PXG4 and cumene hydroperoxide, H<sub>2</sub>O<sub>2</sub>, 13-hydroperoxy-9,11-octadecadienoic acid (13 HPOD) or 13-hydroperoxy-9,11,15-octadecatrienoic acid (13 HPOT) as co-oxidants. After the reaction was completed, the enantiomeric composition of the resulting epoxides was determined by chiral phase HPLC. Remarkably, only one epoxide enantiomer (*cis*-9(*R*),10(*S*)-epoxy-stearic acid) was formed (> 99% ee), whatever hydroperoxides had been used as co-oxidant (Fig. 5Aa–e).

In addition, when linoleic acid was used as the substrate instead of oleic acid, PXG4 produced only the enantiomer (*R*),(*S*) of each regioisomer. Therefore, and in sharp contrast with all the peroxygenases studied to date [28,29,40], PXG4 formed only the 9(*R*),10(*S*)-epoxy (Fig. 5Bhi) and the 12(*R*),13(*S*)-epoxy derivatives of linoleic acid (Fig. 5Bjk). We can conclude from these experiments that PXG4 catalyzed the total asymmetric epoxidation of unsaturated fatty acids. It is worth noting, however, that when the methyl ester of oleic acid was used instead of the free fatty acid, the enantiomeric excess of the resulting epoxide dropped from > 99% to 40% (Fig. 5Bfg).

#### PXG4 catalyzed intermolecular and intramolecular transfer of oxygen

Using [<sup>14</sup>C]13 HPOT as mechanistic probe [41], we have previously demonstrated that soybean peroxygenase catalyzes exclusively the heterolytic cleavage of the hydroperoxide bond yielding 13-hydroxy-9,11,15-octadecatrienoic acid (13 HOT), the corresponding alcohol and an oxoferryl intermediate state of the enzyme.



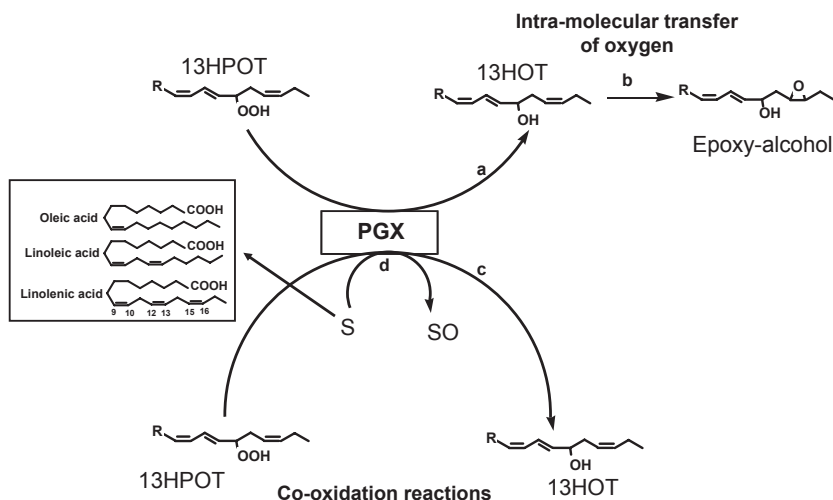


**Fig. 5.** (A) Separation by radio-chiral-phase HPLC of the two enantiomers of racemic methyl 9,10-epoxystearate (a) and of the epoxides resulting from the incubation of C18:1 with PXG4 in the presence of cumene hydroperoxide (b), H<sub>2</sub>O<sub>2</sub> (c), 13 HPOD (d) and 13 HPOT (e). (B) Analysis of radio-chiral-phase HPLC of the epoxide resulting from the incubation of PXG4 with the [<sup>14</sup>C]methyl ester derivative of oleic acid (g). (f) Analysis of racemic 9,10-epoxystearate. (h), (i), (j), (k) Analysis of the enantiomers of the two monoepoxides resulting from the incubation of C18:2 with PXG4 in the presence of cumene hydroperoxide: (h) racemic 9,10-epoxy-12-octadecenoate; (i) 9(*R*),10(*S*)-epoxy-12-octadecenoate formed through PXG4 catalysis; (j) racemic 12,13-epoxy-9-octadecenoate; (k) 12(*R*),13(*S*)-epoxy-9-octadecenoate catalyzed by PXG4.

This reactive intermediate is then able to epoxidize the double bonds of 13 HOT before this reaction product diffuses out of the active site (caged reaction, intramolecular oxygen transfer mechanism). Alternatively, the oxoferryl intermediate can also catalyze co-oxidation which involves an intermolecular oxygen transfer mechanism; this includes the formation of epoxy alcohols deriving from 13 HOT after its diffusion out of the active site and rebinding (Fig. 6). It is of note that during fatty acid epoxidation such as oleic acid by the soybean peroxygenase, epoxy alcohol was formed only as a minor product [28]. As PXG4 actively catalyzed co-oxidation reactions such as the epoxidation of mono-unsaturated fatty acids, we investigated whether this enzyme was able to catalyze the formation of epoxy alcohols from fatty acid hydroperoxides via intramolecular or intermolecular transfers of oxygen.

To study the formation of epoxy alcohol in the absence of any other oxidizable substrate, we incubated purified PXG4 only in the presence of [<sup>14</sup>C]13

HPOT. Two radioactive compounds could be extracted from the reaction medium and separated by RP-HPLC (Fig. 7Aa). The first compound had the same retention time as the standard 13 HOT (12.7 min) and both presented identical mass spectra when analyzed by GC-MS. The second compound had a higher polarity (elution time 5.4 min). GC-MS analysis revealed that it consisted of two epoxy alcohol isomers characterized by their distinct fragmentation patterns. The mass spectrum of the major peak (96%) (i.e. the methyl ester trimethylsilyl ether derivative of 15,16-epoxy-13-hydroxy-9,11-octadecenoic acid) was characterized by a molecular ion at *m/z* 396 and a peak base at *m/z* 73 (trimethylsilyl). Prominent ions were also seen at *m/z* (relative intensity) 311 (45%) (M-CH<sub>3</sub>CH<sub>2</sub>CHCOHCH<sub>2</sub>), 381 (1.2%) (M-15), 367 (2.1%) (M-CH<sub>3</sub>CH<sub>2</sub>), 337 (6.2%) (M-COOCH<sub>3</sub>), 239 (7%) and 157 (8.5%) (cleavage between C9 and C10) and 221 (3.9%) (239-O). Mass spectra of the minor peak (4%) (i.e. the methyl ester trimethylsilyl ether



**Fig. 6.** Simplified scheme for the reactions catalyzed by peroxygenase. In an initial step peroxygenase catalyzes the reduction of 13 HPOT to its corresponding alcohol (13 HOT) (pathway a). This process is followed by oxidation steps (pathways b, c and d). The reaction product can be further transformed by oxidation into epoxy alcohol according to two pathways in competition: (i) an intramolecular oxygen transfer mechanism (pathway b) involving a 13 HOT molecule which remained bound to the active site after step a, and (ii) an intermolecular oxygen transfer mechanism (co-oxidation reaction) involving the rebinding of 13 HOT that has diffused out of the active site (pathway c). S stands for substrate (see inset for the structures of the fatty acids used in this work), SO for oxidized substrate.

derivative of 9,10-epoxy-13-hydroxy-11,15-octadecenoic acid) showed a similar molecular ion at  $m/z$  396 and base peaks at 73 (trimethylsilyl). Other ions could be detected at  $m/z$  (relative intensity) 171 (84%) ( $\text{CH}_3\text{CH}_2\text{CH}=\text{CHCH}_2\text{CH}$ ), 380 (0.3%) (M-O) and 365 (0.6%) (M-COOCH<sub>3</sub>), 282 (20%) (M-O-171-73), 311 (5%), 129 (17.5%) and 103 (14%).

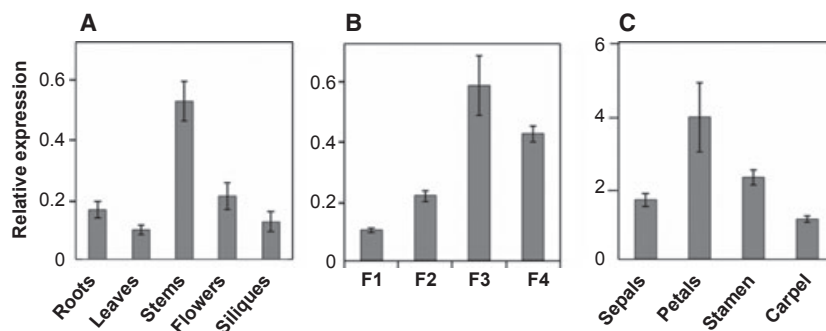
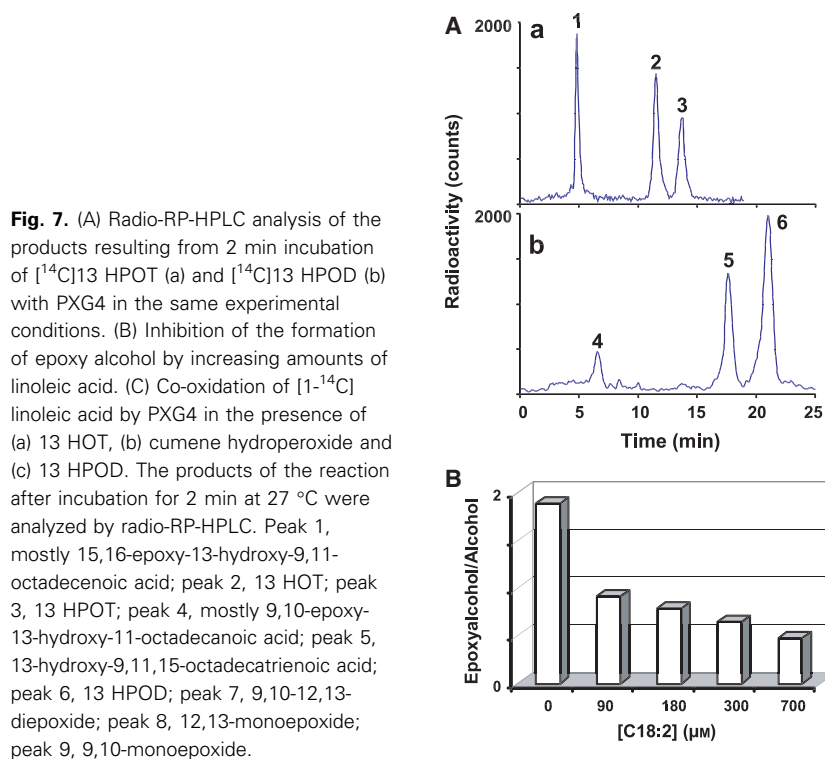
Using [<sup>14</sup>C]13 HPOD as substrate, instead of [<sup>14</sup>C]13 HPOT, considerably slowed PXG4 activity (Fig. 7Ab). The epoxy alcohol represented only 4% of the reaction products when 13 HPOD was incubated in the presence of PXG4 (Fig. 7Ab). This contrasts with the accumulation of 15,16-epoxy alcohol (36% of the reaction products) observed, under the same experimental conditions, in the epoxidation of 13 HOT (Fig. 7Aa).

The reaction mechanism of the soybean peroxygenase predicts that the formation of epoxy alcohol by epoxidation of the alcohol (formed after the heterolytic cleavage of the peroxy O–O bond) could be in competition with the oxidation of other extraneous substrates (Fig. 6, pathways c and d). To test this point, increasing amounts of linoleic acid were incubated with PXG4 in the presence of fixed concentrations of [<sup>14</sup>C] 13 HPOT. As shown in Fig. 7B, increasing concentrations of the free fatty acid resulted in a decreased formation of the epoxy alcohol deriving from 13 HOT. However, even at high concentrations, linoleic acid could not totally abolish the formation of the epoxy alcohol. Conversely, the highest yield of epoxy

alcohol (about 67% of the reaction products) was observed when PXG4 acted on 13 HPOT in the absence of competing substrates (Fig. 7A,B). These data suggested that the oxygen atom originating from the hydroperoxide can be transferred to 13 HOT according to two distinct pathways, i.e. intramolecular and intermolecular, in competition.

Epoxidation of linoleic acid into diepoxides (peak 7), 12,13-monoepoxide (peak 8) and 9,10-monoepoxide (peak 9) (Fig. 7Ca) occurred during the formation of the epoxy alcohol and competed with the epoxidation of 13 HOT (Fig. 8B). In order to evaluate the influence of hydroperoxides on the epoxidation rate, we incubated [<sup>14</sup>C]linoleic acid in the presence of PXG4 and the same concentration (200 μM) of cumene hydroperoxide, 13 HPOD and 13 HPOT as co-oxidants. Analysis of the products showed that the formation of the final product (i.e. the diepoxide) is faster in the presence of cumene hydroperoxide (Fig. 7Cb) than fatty acid hydroperoxides such as 13 HPOD (Fig. 7Cc) or 13 HPOT (Fig. 7Ca). This result indicates that hydroperoxides that cannot be further oxidized by PXG4 after their reduction into alcohols (e.g. H<sub>2</sub>O<sub>2</sub>, cumene hydroperoxide) are strong co-oxidants for epoxidation reactions. In addition, such results strongly suggest that in the presence of other suitable substrates (e.g. in the context of plant cells) co-oxidation reactions such as fatty acid epoxidations might be predominantly catalyzed by PXG4.





### Expression of *PXG4* in plant tissues

In contrast with the seed-specific *PXG1*, class II caleosin gene was found expressed in non-oil storage tissues [32] and *PXG4* particularly in flowers [34]. As well, quantitative RT-PCR confirmed *PXG4* expression among all the tissues of *Arabidopsis*, including flowers (Fig. 8A). This expression was especially important in the petals of fully open flowers (Fig. 8B,C) and to a lesser extent in the inflorescence stems.

### Discussion

Most of the genes encoding the oxylipin biosynthetic enzymes have been identified in plants, with the notable exception of those responsible for the formation of EFAs. Our present work indicates that recombinant class II caleosin *PXG4*, expressed in yeast, catalyzes efficient hydroperoxide-dependent fatty acid epoxygenation reactions and identifies *PXG4* as a new *Arabidopsis* peroxygenase-type caleosin [32]. Several

lines of evidence are also in favor of the participation of PXG4 in fatty acid epoxidation *in vivo* in *Arabidopsis*.

First, *PXG4* is expressed in most organs of *A. thaliana* [36] (Fig. 8). So far, peroxygenases (recently classified as [EC 1.11.2.3](#)) were only found in seeds and young seedlings, leading to the conclusion that these enzymes are seed-specific. Thus, peroxygenase activity has been shown to decrease during germination in soybean and oat [42]. The caleosins identified up to now to be endowed with peroxygenase activity, i.e. *Arabidopsis* PXG1 and PXG2, oat AsPXG1 and rice EFA27, are also mostly present in seeds and seedlings [32,33,43]. Moreover, all our attempts to measure peroxygenase activity in green tissues have failed. This could be due either to a lack of expression of this enzyme in such tissues or to its inhibition by endogenous inhibitors or/and competing substrates such as carotenoids [44], released in the plant extracts. The presence of *PXG4* transcripts in stems, roots and flowers is thus a strong indication that peroxygenase exists and can function in organs other than seeds.

Second, in contrast to oat class I caleosin AsPXG1 for which oleic acid is the preferred lipid substrate [33], PXG4 shows a high catalytic efficiency with physiological polyunsaturated fatty acids such as linoleic and linolenic acids, which are oxidized into their corresponding monoepoxides and diepoxides *in vitro*. Thus, epoxidation of linoleic acid by recombinant PXG4 in the presence of cumene hydroperoxide (which yields preponderantly the diepoxide) proceeds via a very fast initial formation of the monoepoxide, which is further oxidized into the diepoxide but at a much reduced rate (about two orders of magnitude slower than the rate of monoepoxidation). *In vivo*, besides being a slower process, the formation of the diepoxide will also be in competition with the hydrolysis of the monoepoxide by EHs. Both parameters could explain why diepoxides are rarely detected *in planta* whereas PXG4 is able to catalyze their formation *in vitro*. Strikingly, the epoxidation activity of PXG4 exhibited regioselectivities and stereoselectivities that are distinct and complementary to those of the other known plant epoxidases which oxidize mainly the C-12,13 double bond of unsaturated fatty acids. For example, only vernolic acid accumulates in *Arabidopsis* seeds expressing *Crepis*  $\Delta$ 12-epoxygenase [11,45]. Similarly, tobacco callus and somatic soybean embryos expressing the *Euphorbiaceae* CYP726A1 [15] and recombinant *Arabidopsis* CYP77A4 mainly formed the 12(*S*),13(*R*)-epoxy derivative of linoleic acid, i.e. (+)-vernolic acid [46]. In sharp contrast, the C-12,13 double bond of unsaturated fatty acids is the least favored unsaturation

epoxidized by PXG4 (Fig. 4B), leading in the case of linoleic acid to the formation of the opposite stereoisomer, i.e. 12(*R*),13(*S*)-epoxy-9(*Z*)-octadecenoic acid or (–)-vernolic acid. This suggests that plants possess evolutionarily divergent sets of enzymes facilitating the formation of distinct fatty acid epoxides that could play pivotal roles in different stages of development. In this context, it is interesting to note that a peroxygenase and a cytochrome P450 were reported to coexist in *Euphorbia lagascae* seeds, but only peroxygenase activity was detectable in seedlings 2 days after germination [47].

Third, PXG4 differs from all the other peroxygenases by a stereospecificity that has never been described to date, giving access to the fatty acid epoxide stereoisomers found *in planta*. From a physiological point of view, the fact that polyunsaturated fatty acids are actively epoxidized by PXG4 with a high stereoselectivity indicates that the resulting EFAs could be physiological metabolites. Indeed, PXG4 is able to catalyze the formation of the various anti-fungal compounds originating from linoleic and linolenic acids which have been found in rice, i.e. the five epoxy derivatives of linoleic and linolenic acids and the four allyl alcohols originating from the reduction of 9- and 13-hydroperoxides deriving from these two unsaturated fatty acids. Importantly, the rice epoxides have been determined by Kato *et al.* [19] to have the (*R*), (*S*) configuration, i.e. precisely the one obtained with PXG4 (Fig. 5). Altogether these results suggest that the anti-fungal oxylipins might be produced by a rice peroxygenase orthologous to PXG4 or to another member of the class II caleosins. Indeed, since PXG4 shares a high sequence identity with class II caleosins (81%, 64% and 55% with At1g70670, At1g23240 and At1g23250 respectively), it is tempting to ascribe similar catalytic activity to these proteins. However, the identification of PXG4 as the unique fatty acid epoxidase present in *Arabidopsis* or as the first characterized member of a small family of epoxidases remains to be clarified. Noteworthy, the allyl alcohols isolated from rice plants were found to be mostly racemates [19], indicating that not only the hydroperoxides produced by lipoxygenases but also those formed by nonenzymatic peroxidation might be used as co-oxidants by the rice enzyme. Although hydroxy fatty acids that are formed both enzymatically and non-enzymatically accumulate in *Arabidopsis* upon infection with *Pseudomonas syringae* [48], the involvement of PXG4 in the defence responses of *A. thaliana* to pathogens remains to be determined. Recent gain and loss of function experiments have suggested that PXG4 might be involved in drought and salt tolerance

by acting as a negative regulator of abscisic acid responses, possibly through the formation of at present uncharacterized oxylipins [36]. Actually comprehension of the links between the biological roles and catalytic activities of caleosins remains hampered mainly by the difficulty in uncovering their physiological substrates. Since in this work PXG4 was shown to oxidize unsaturated fatty acids into EFAs, the study of the relevance of these metabolites in *A. thaliana* stress tolerance will be of importance. Recent studies have reported that caleosins perform different functions in plant cells. For example, AtPXG1 is involved in the degradation of oil-body storage lipids during seed germination [49] while RD20 (At2g33380) and PXG4 play a role in respectively a positive and negative regulation of abscisic acid responses [36,50]. The molecular mechanisms underlying such biological functions remain unknown, however, but one can speculate that they might be linked to the enzymatic activity of caleosins and to their ability to synthesize oxylipins. The evidence presented here that PXG1 and PXG4 are distinct oxygenases (Fig. 2), which yield different classes of oxygenated products, sustains this hypothesis. Accordingly, a functional specialization of oxylipins depending on their molecular structure has been suggested recently for the 9-lipoxygenase-derived oxylipins [51].

To sum up, until recently the significance of fatty acid epoxidation in plants was largely impeded by the lack of genes known to encode efficient epoxidases. The present identification of such a gene which encodes a non-seed-specific peroxygenase able to potently catalyze the epoxidation of physiological unsaturated fatty acids will now permit the manipulation of EFA levels in plants to assess their biological functions.

## Materials and methods

### Functional expression of At1g70670 in yeast

Full-length At1g70670 DNA (pda03699 provided by RIKEN BRC) [52,53] was PCR amplified using the following primers: 5'-CGGGATCCATGGCTTCCTCTATTCCAC TGG-3' and 5'-GCTCTAGAGTGGTGGTGGTGGTGG TGTTAGTTATGGATGTTTCTTAGAAGTTTTAGAAG ATC-3'. The amplified product was inserted into the *Bam*H1 and *Xba*I restriction sites of pVT102U plasmid [54] in DH5 $\alpha$  *Escherichia coli* and subcloned in yeast Wa6 after sequencing. Expressions and purifications of At4g26740 and At1g77670 on a nickel-nitrilotriacetic acid Superflow column (Qiagen, Courtaboeuf, France) were performed as previously described [32].

### Subcellular fractionation of yeast

Yeast fractionation was carried out at 4–5 °C essentially as previously described [32] with some modifications. Yeast cells (from 50 mL culture) were collected by centrifugation at 3000 *g* for 5 min. The pelleted cells were resuspended in cold buffer A (100 mM KH<sub>2</sub>PO<sub>4</sub>, pH 8, 300 mM NaCl, and yeast protease inhibitor cocktail (Roche Diagnostics, Boulogne Billencourt, France) at 1 tablet·(50 mL)<sup>-1</sup>). An equal volume of glass beads (0.45 mm diameter) was added and the yeast cells were disrupted by vortexing for 30 s (15-fold) with a lag time of 30 s in ice between each stir. Homogenate was removed and the glass beads were washed three times with buffer A. Homogenate and washings were centrifuged initially at 3000 *g* for 5 min, and then at 100 000 *g* for 1 h. After centrifugation, the three fractions (i.e. LDs at the top, cytosol in the middle fraction and the microsomal pellet) were resuspended in the same volume of buffer, which was used immediately for enzymatic activity measurement or for PXG4 purification, or analyzed by western blot as described below.

### Western blot analysis

Proteins were separated by SDS gel electrophoresis on 14% polyacrylamide gels and transferred to Immobilon FL membranes (Millipore, Molsheim, France) at 20 V for 90 min in a transfer apparatus (Bio-Rad, Marnes-la-Coquette, France) using a buffer consisting of 50 mM Tris/HCl, 50 mM boric acid. The membranes were blocked with 5% skim milk in NaCl/P<sub>i</sub> (137 mM NaCl, 27 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.5) for 60 min, rinsed and then incubated overnight at 4 °C with anti-His<sub>6</sub> mouse monoclonal IgG (Roche, Boulogne-Billencourt, France) diluted 1 : 5000 in 5% milk, 0.1% Tween 20 and NaCl/P<sub>i</sub>. After extensive washing in NaCl/P<sub>i</sub> containing 0.1% Tween 20, anti-mouse antibody conjugated to peroxidase (Roche) was diluted 1 : 5000 and used as a secondary antibody. Blots were developed using the ECL kit from Pierce (Thermo Fisher Scientific, Courtaboeuf, France).

### Enzymatic activity

Peroxygenase activity was measured with aniline as the substrate during the purification procedure [32]. The epoxidation of [<sup>14</sup>C] unsaturated fatty acids was performed as described in an earlier study [28]. The specificity constants (or  $V/K_m$ ) were determined under  $[S]_0 \ll K_m$  experimental conditions. Under these conditions, the integrated rate equation for irreversible single-substrate reactions is simplified into a pseudo-first-order rate equation which yields the ratio  $V/K_m$  [55]. For a typical assay, the enzyme was pre-equilibrated for 1 min at 27 °C in 0.1 M potassium phosphate buffer (pH 7.4) containing cumene hydroperoxide (200  $\mu$ M) in a final volume of 400  $\mu$ L. The reaction was

initiated by adding 2  $\mu\text{M}$  of [ $^{14}\text{C}$ ] mono-unsaturated or poly-unsaturated fatty acids. At given times, 50  $\mu\text{L}$  was withdrawn and the reaction was stopped with 50  $\mu\text{L}$  of acetonitrile. The mixture was then applied to a silica gel TLC plate (60 F<sub>254</sub>; Merck Millipore, Guyancourt, France) developed in *n*-hexane/diethylether/formic acid (50 : 50 : 1). Radioactivity associated with bands corresponding to the epoxides and the residual fatty acids was determined. In the absence of enzymatic activity (boiled enzyme) or of cumene hydroperoxide, no transformation of the unsaturated fatty acids was detectable. Under these conditions the reaction rates were linear with regard to enzyme concentration.

Epoxides resulting from the incubation of C18:1 (with the double bond at a different location in the carbon chain) were methylated with ethereal diazomethane before their identification by GC-MS analysis (see below). The mass spectrum of the epoxide resulting from oxidation of oleic acid was identical (after methylation) to that of authentic 9,10-epoxyoctadecanoate methyl ester, i.e. each spectrum featured a molecular ion at  $m/z$  312 and prominent ions at 155, base peak (M-157, loss of  $(\text{CH}_2)_7\text{COOCH}_3$ ), 199 (M-133, loss of  $(\text{CH}_2)_7\text{CH}_3$ ) and 281 (M-31, loss of  $\text{OCH}_3$ ).

### Analytical procedures

Radioactivity was measured on TLC plates with a radioisotope thin-layer analyzer Rita Star, and peak integration was obtained using the Gina-Star TLC software (Raytest, Straubenhardt, Germany).

HPLC analysis was performed under isocratic conditions using a Shimadzu instrument coupled with a radiomatic 500 TR analyzer (Packard Instrument Co. PerkinElmer, Courtaboeuf, France). Peak integration was obtained using FLO-ONE software. SP-HPLC separation of the monoepoxide positional isomers from [ $^{14}\text{C}$ ]linoleic acid was performed at room temperature on a  $\mu\text{Porasil}$  (5  $\mu\text{m}$ ) column (3.9  $\times$  300 mm; Waters, Darmstadt, Germany) with a silica guard cartridge using a solvent mixture of *n*-hexane/isopropanol/acetic acid (995 : 5 : 0.5 at 1.5 mL $\cdot\text{min}^{-1}$ ). In this system, the elution times for 12,13-epoxy-9-octadecenoic acid and 9,10-epoxy-12-octadecenoic acid were 14.20 and 17.80 min respectively. The same chromatographic conditions also enabled 12,13-epoxy-9,15-octadecadienoate, 15,16-epoxy-9,12-octadecadienoate and 9,10-epoxy-12,15-octadecadienoate to be separated from [ $^{14}\text{C}$ ]linolenic acid at 14.60, 17.80 and 19.20 min respectively. Diepoxides from [ $^{14}\text{C}$ ]linolenic acid were separated by reverse phase on a Lirospher 100 RP-18 (5  $\mu\text{m}$ ) column (8  $\times$  125 mm; Agilent Technologies, Interchim, Montluçon, France) using a solvent mixture of methanol/water/acetic acid (75 : 25 : 0.05 at 1 mL $\cdot\text{min}^{-1}$ ). The resolution of the enantiomers of  $^{14}\text{C}$ -labeled methyl epoxy fatty acid derivatives was performed on a Chiralcel OB column (4.6  $\times$  250 mm; Baker Chemical Co., Interchim, Montluçon, France) with a solvent mixture of *n*-hexane/isopropanol (98.5 : 1.5 at

0.3 mL $\cdot\text{min}^{-1}$ ) for methyl *cis*-9,10-epoxystearate. The enantiomers of the methylated monoepoxides of [ $^{14}\text{C}$ ]linoleic acid were separated on the same column with a solvent mixture of *n*-hexane/isopropanol (100 : 0.04 at 0.8 mL $\cdot\text{min}^{-1}$ ). In this system, the elution times for 9*S*,10*R*-12-octadecenoate, 9*R*,10*S*-12-octadecenoate, 12*S*,13*R*-epoxy-9-octadecenoate and 12*R*,13*S*-9-octadecenoate were 77.6, 95.6, 100.8 and 115.1 min respectively. The identification of all the epoxides and epoxy alcohols was performed using larger incubation volumes and radioactive tracers. Products were extracted and their identity was confirmed by GC-MS [28,46,56,57].

Capillary gas chromatography analysis of the mono-unsaturated and polyunsaturated fatty acid methyl esters and their epoxide derivatives was performed on a Varian Star chromatography workstation equipped with a DB1 coated fused column (250  $\mu\text{m}$  film thickness, 30 m, 0.32  $\mu\text{m}$  internal diameter; J.W. Scientific, Agilent Technologies, Massy, France). Analysis was performed ( $\text{H}_2$ , 2 mL $\cdot\text{min}^{-1}$ ) with the following temperature program: 6  $^\circ\text{C}\cdot\text{min}^{-1}$  from 60 to 120  $^\circ\text{C}$ , followed by 5  $^\circ\text{C}\cdot\text{min}^{-1}$  from 120 to 240  $^\circ\text{C}$  and by 10  $^\circ\text{C}\cdot\text{min}^{-1}$  from 240 to 300  $^\circ\text{C}$ . GC analysis was performed on reaction products resulting from three different biological experiences.

The GC-MS analysis was performed on an Agilent 5973 N apparatus with an ionizing energy of 70 eV. The sample was injected directly in splitless mode (injector temperature 250  $^\circ\text{C}$ ) into a DB-5-coated fused column (30 m, 0.25 mm internal diameter; J.W. Scientific) with a temperature program of 3  $^\circ\text{C}\cdot\text{min}^{-1}$  from 100 to 280  $^\circ\text{C}$ , followed by 10 min at 280  $^\circ\text{C}$  ( $m/z$  = 50–700).

### RNA isolation and quantitative RT-PCR

Fresh material was harvested from at least three different plants and immediately frozen in liquid nitrogen. Total RNA was precipitated and extracted by the classical lithium chloride–phenol protocol, treated with DNase I (Fermantas, Thermo Fisher Scientific, Courtaboeuf, France) according to the manufacturer's instructions and further purified with the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions.

cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen, Life Technologies, Saint Aubin, France) in the presence of Oligo(dT)23 (Sigma-Aldrich Chemie S.a.r.l., Saint-Quentin Fallavier, France) from 2  $\mu\text{g}$  of total RNA. The resulting cDNAs were then 10-fold diluted. The oligonucleotides used for *PXG4* were At1g70670, 5'-TCAAACAACCTCTAAAGGCAAA-3', 5'-CCACTCCGTATACCCTGCAA-3'. The vacuolar protein MON1 (At2g28390, 5'-GGATTTTCAGCTACTCTTCAA GCTA-3', 5'-CTGCCTTGACTAAGTTGACACG-3'), the TIP41-like protein (At4g34270, 5'-GAACTGGCTGACAA TGGAGTG-3', 5'-ATCAACTCTCAGCCAAAATCG-3'), the PP2AA3 (At1g13320, 5'-GACCGGAGCCAACCTAGG

AC-3', 5'-AAAACCTTGGTAACTTTTCCAGCA-3') and the conserved protein UCP022280 (At4g26410, 5'-AGCTGAAGTGGCTTCCATGA-3', 5'-GGTCCGACATACCCATGATCC-3') were taken as reference genes to normalize the expression of *PXG4*.

Quantitative RT-PCR plates were prepared with a Biomek 3000 (Beckman Coulter, Villepinte, France) and run on a LightCycler 480 II (Roche). Each reaction was prepared using 2  $\mu$ L cDNA, 5  $\mu$ L of LightCycler 480 SYBR Green I Master (Roche) and 250 nm of forward and reverse primers in a total volume of 10  $\mu$ L. The amplification profile consisted of 95 °C for 10 min and 45 cycles (95 °C denaturation for 10 s, annealing at 60 °C for 15 s, extension at 72 °C for 15 s), followed by a melting curve analysis from 55 to 95 °C to check the specificity of primer pairs. All reactions were performed in triplicate.

## Acknowledgements

The authors are grateful to Francis Schuber (Faculty of Pharmacy, Strasbourg-Illkirch) for critical reading of the manuscript and to Anabella Ivancich (CNRS UMR 8221, CEA-Saclay, France) for helpful discussions. Many thanks also to Alain Rahier (IBMP-CNRS-UPR2357, Strasbourg) for help with the GC-MS apparatus. This work was supported by FNRS of Belgium (FRFC 2.4.563.08-F) to M.-L.F.

## References

- Pfister SL, Gauthier KM & Campbell WB (2010) Vascular pharmacology of epoxyeicosatrienoic acids. *Adv Pharmacol* **60**, 27–59.
- Morisseau C, Inceoglu B, Schmelzer K, Tsai HJ, Jinks SL, Hegedus CM & Hammock BD (2010) Naturally occurring monoepoxides of eicosapentaenoic acid and docosahexaenoic acid are bioactive antihyperalgesic lipids. *J Lipid Res* **51**, 3481–3490.
- Panigrahy D, Edin ML, Lee CR, Huang S, Bielenberg DR, Butterfield CE, Barnés CM, Mammoto A, Mammoto T, Luria A *et al.* (2012) Epoxyeicosanoids stimulate multiorgan metastasis and tumor dormancy escape in mice. *J Clin Invest* **122**, 178–191.
- Van Rollins MR, Baker RC, Sprecher HW & Murphy RC (1984) Oxidation of docosahexaenoic acid by rat liver microsomes. *J Biol Chem* **259**, 5776–5783.
- Capdevila JH, Falck JR & Harris RC (2000) Cytochrome P450 and arachidonic bioactivation: molecular and functional properties of the arachidonate monooxygenase. *J Lipid Res* **41**, 163–181.
- Zeldin DC (2001) Epoxygenase pathways of arachidonic acid metabolism. *J Biol Chem* **276**, 36059–36062.
- Spector AA (2009) Arachidonic acid cytochrome P450 epoxygenase pathway. *J Lipid Res* **50** (Supplement), S52–S56.
- Krewson CF (1967) Naturally occurring epoxy oils. *J Am Oil Chem Soc* **45**, 250–256.
- Budziszewski GJ, Croft KP & Hildebrand DF (1996) Uses of biotechnology in modifying plant lipids. *Lipids* **31**, 557–569.
- Sharmin E, Ashraf SM & Ahmad S (2007) Synthesis, characterization, antibacterial and corrosion properties of epoxides, epoxy-polyols and epoxy-polyurethane coating from linseed and *Pongamia glabra* seed oils. *Int J Biol Macromol* **40**, 407–422.
- Lee M, Lenman M, Banas A, Bafor M, Singh S, Schweizer M, Nilsson R, Liljenberg C, Dahlqvist A, Gummeson PO *et al.* (1998) Identification of non-heme diiron proteins that catalyze triple bond and epoxy group formation. *Science* **280**, 915–918.
- Singh S, Thomaes S, Lee M, Szymne S & Green A (2001) Transgenic expression of a  $\Delta$ 12-epoxygenase gene in *Arabidopsis* seeds inhibits accumulation of linoleic acid. *Planta* **212**, 872–879.
- Rezzonico E, Moire L, Delessert S & Poirier Y (2004) Level of accumulation of epoxy fatty acid in *Arabidopsis thaliana* expressing a linoleic acid  $\Delta$ 12-epoxygenase is influenced by the availability of the substrate linoleic acid. *Theor Appl Genet* **109**, 1077–1082.
- Bafor M, Smith MA, Jonsson I, Stobart K & Szymne S (1993) Biosynthesis of vernolate (*cis*-12-epoxyoctadeca-*cis*-9-enoate) in microsomes preparations from developing endosperms of *Euphorbia lagascae*. *Arch Biochem Biophys* **303**, 145–151.
- Cahoon EB, Ripp KG, Hall SE & McGonicle B (2002) Transgenic production of epoxy fatty acids by expression of a cytochrome P450 enzyme from *Euphorbia lagascae* seed. *Plant Physiol* **128**, 615–624.
- Kolattukudy PE (1980) Biopolyesters of plants: cutin and suberin. *Science* **208**, 990–1000.
- Schweizer P, Felix G, Buchala A, Müller C & Métraux JP (1996) Perception of free cutin monomers by plant cells. *Plant J* **10**, 331–341.
- Schweizer P, Jeanguenat D, Whitacre D, Métraux JP & Mössinger E (1996) Induction of resistance in barley against *Erysiphe graminis* f.sp. hordei by free cutin monomers. *Physiol Mol Plant Pathol* **49**, 103–120.
- Kato T, Yamaguchi Y, Namai T & Hirukawa T (1993) Oxygenated fatty acids with anti-rice blast fungus activity in rice plants. *Biosci Biotechnol Biochem* **57**, 283–287.
- Namai T, Kato T, Yamaguchi Y & Hirukawa T (1993) Anti-rice blast activity and resistance induction of C-18 oxygenated fatty acids. *Biosci Biotechnol Biochem* **57**, 611–613.
- Wagner K, Inceoglu B, Gill SS & Hammock BD (2011) Epoxygenase fatty acids and soluble epoxide hydrolase inhibition: novel mediators of pain reduction. *Agric Food Chem* **59**, 2816–2824.

- 22 Ingraham RH, Gless RD & Lo HY (2011) Soluble epoxide hydrolase inhibitors and their potential for treatment of multiple pathologic conditions. *Curr Med Chem* **18**, 587–603.
- 23 Kiyosue T, Beetham JK, Pinot F, Hammock BD, Yamaguchishinozaki K & Shinozaki K (1994) Characterization of an Arabidopsis cDNA for a soluble epoxide hydrolase gene that is inducible by auxin and water stress. *Plant J* **6**, 259–269.
- 24 Stapleton A, Beetham JK, Pinot F, Garbarino JE, Rockhold DR, Friedman M, Hammock BD & Belknap WR (1994) Cloning and expression of soluble epoxide hydrolase from potato. *Plant J* **6**, 251–258.
- 25 Pinot F, Bosch H, Salaun JP, Durst F, Mioskowski C & Hammock BD (1997) Epoxide hydrolase activities in the microsomes and the soluble fraction from *Vicia sativa* seedlings. *Plant Physiol Biochem* **35**, 103–110.
- 26 Ohta H, Shida K, Peng YL, Furusawa I, Shishiyama J, Aibara S & Morita Y (1990) The occurrence of lipid-hydroperoxide-decomposing activities in rice and the relationship of such activities to the formation of antifungal substances. *Plant Cell Physiol* **31**, 1117–1122.
- 27 Ohta H, Shida K, Peng YL, Furusawa I, Shishiyama J, Aibara S & Morita Y (1991) A lipoxygenase pathway is activated in rice after infection with the rice blast fungus *Magnaporthe grisea*. *Plant Physiol* **97**, 94–98.
- 28 Blée E & Schuber F (1990) Efficient epoxidation of unsaturated fatty acids by a hydroperoxide-dependent oxygenase. *J Biol Chem* **265**, 12887–12894.
- 29 Hamberg M & Hamberg G (1990) Hydroperoxide-dependent epoxidation of unsaturated fatty acids in the broad bean (*Vicia faba* L.). *Arch Biochem Biophys* **283**, 409–416.
- 30 Blée E & Schuber F (1993) Biosynthesis of cutin monomers – involvement of a lipoxygenase/ peroxygenase pathway. *Plant J* **4**, 113–123.
- 31 Lequeu J, Fauconnier ML, Chammai A, Bronner R & Blée E (2003) Formation of plant cuticle: evidence for the occurrence of the peroxygenase pathway. *Plant J* **36**, 155–164.
- 32 Hanano A, Burcklen M, Flenet M, Ivancich A, Louwagie M, Garin J & Blée E (2006) Plant seed peroxygenase is an original heme-oxygenase with an EF-hand calcium binding motif. *J Biol Chem* **281**, 33140–33151.
- 33 Meesapyodsuk D & Qiu X (2011) A peroxygenase pathway involved in the biosynthesis of epoxy fatty acids in oat (*Avena sativa* L.). *Plant Physiol* **157**, 454–463.
- 34 Naested H, Frandsen GI, Jauh GY, Hernandez-Pinzon I, Nielsen HB, Murphy DJ, Rogers JC & Mundy J (2000) Caleosins: Ca<sup>2+</sup>-binding proteins associated with lipid bodies. *Plant Mol Biol* **44**, 463–476.
- 35 Mayfield JA, Fiebig A, Johnstone SE & Preuss D (2001) Gene families from the *Arabidopsis thaliana* pollen coat proteome. *Science* **292**, 2482–2485.
- 36 Kim YY, Jung KW, Yoo KS, Jeung JU & Shin JS (2011) A stress-responsive caleosin-like protein, AtCLO4, acts as a negative regulator of ABA responses in Arabidopsis. *Plant Cell Physiol* **52**, 874–884.
- 37 Chen JCF, Tsai CCY & Tzen JTC (1999) Cloning and secondary structure analysis of caleosin, a unique calcium-binding protein in oil bodies of plant seeds. *Plant Cell Physiol* **40**, 1079–1086.
- 38 Blée E, Summerer S, Flenet M, Rogniaux H, Van Dorsselaer A & Schuber F (2005) Soybean epoxide hydrolase. Identification of the catalytic residues and probing of the reaction mechanism with secondary kinetic isotope effects. *J Biol Chem* **280**, 6479–6487.
- 39 Ziegler J, Wasternack C & Hamberg M (1999) On the specificity of allene oxide cyclase. *Lipids* **34**, 1005–1015.
- 40 Hamberg M & Fahlstadius P (1992) On the specificity of a fatty acid epoxigenase in broad bean (*Vicia faba* L.). *Plant Physiol* **99**, 987–995.
- 41 Blée E, Wilcox AL, Marnett LJ & Schuber F (1993) Mechanism of reaction of fatty acid hydroperoxides with soybean peroxygenase. *J Biol Chem* **268**, 1708–1715.
- 42 Blée E & Durst F (1987) Hydroperoxide-dependent sulfoxidation catalyzed by soybean microsomes. *Arch Biochem Biophys* **254**, 43–52.
- 43 Frandsen GI, Müller-Uri F, Nielsen M, Mundy J & Skriver K (1996) Novel plant Ca<sup>2+</sup>-binding protein expressed in response to abscisic acid and osmotic stress. *J Biol Chem* **271**, 343–348.
- 44 Ishimaru A & Yamazaki I (1977) Hydroperoxide-dependent hydroxylation involving 'H<sub>2</sub>O<sub>2</sub>-reducible hemoprotein' in microsomes of pea seeds. *J Biol Chem* **252**, 6118–6124.
- 45 Hatanaka T, Shimizu R & Hildebrand D (2004) Expression of a *Stokesia laevis* epoxigenase gene. *Phytochemistry* **65**, 2189–2196.
- 46 Sauveplane V, Kandel S, Kastner PE, Ehling J, Compagnon V, Werck-Reichhart D & Pinot F (2008) Arabidopsis thaliana CYP77A4 is the first cytochrome P450 able to catalyze the epoxidation of free fatty acids in plants. *FEBS J* **276**, 719–735.
- 47 Blée E, Stahl U, Schuber F & Stymne S (1993) Regio- and stereoselectivity of cytochrome P-450 and peroxygenase-dependent formation of *cis*-12,13-epoxy-9 (*Z*)-octadecenoic acid (vernolic acid) in *Euphorbia lagascae*. *Biochem Biophys Res Commun* **197**, 778–784.
- 48 Grun C, Berger S, Matthes D & Mueller MJ (2006) Early accumulation of non-enzymatically synthesised oxylipins in *Arabidopsis thaliana* after infection with *Pseudomonas syringae*. *Funct Plant Biol* **34**, 65–71.
- 49 Poxleitner M, Rogers SW, Samuels AL, Browse J & Rogers J (2006) A role for caleosin in degradation of



- oil-body storage lipid during seed germination. *Plant J* **47**, 917–933.
- 50 Aubert Y, Vile D, Pervent M, Aldon D, Randy B, Simonneau T, Vavasseur A & Galaud JP (2010) RD20, a stress-inducible caleosin, participates in stomatal control, transpiration and drought tolerance in *Arabidopsis thaliana*. *Plant Cell Physiol* **51**, 1975–1987.
- 51 Lopez MA, Vicente J, Kulasekaran S, Vellosillo T, Martinez M, Irigoyen ML, Cascon T, Bannenberg G, Hamberg M & Castresana C (2011) Antagonistic role of 9-lipoxygenase-derived oxylipins and ethylene in the control of oxidative stress, lipid peroxidation and plant defence. *Plant J* **67**, 447–458.
- 52 Seki M, Carninc P, Nishiyama Y, Hayashizaki Y & Shinozaki K (1998) High-efficiency cloning of Arabidopsis full-length cDNA by biotinylated CAP trapper. *Plant J* **15**, 707–720.
- 53 Seki M, Narusaka M, Kamiya A, Ishida J, Satou M, Sakurai T, Nakajima M, Enju A, Akiyama K, Oono Y *et al.* (2002) Functional annotation of a full-length Arabidopsis cDNA collection. *Science* **296**, 141–145.
- 54 Verner T, Dignar D & Thomas DY (1987) A family of yeast expression vectors containing the phage fl intergenic region. *Gene* **52**, 225–233.
- 55 Orsi BA & Tipton KF (1979) Kinetic analysis of progress curves. *Methods Enzymol* **63**, 159–183.
- 56 Blée E & Schuber F (1992) Enantioselectivity of the hydrolysis of linoleic acid monoepoxides catalyzed by soybean fatty acid epoxide hydrolase. *Biochem Biophys Res Commun* **187**, 171–177.
- 57 Orellana-Coca C, Adlercreutz D, Andersson MM, Mattiasson B & Hatti-Kaul R (2005) Analysis of fatty acid epoxidation by high performance liquid chromatography with evaporative light scattering detection and mass spectrometry. *Chem Phys Lipids* **135**, 189–199.
- 58 Czechowski T, Stitt M, Altmann T, Udvardi MK & Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiol* **139**, 5–17.