Specific Adduction of Plant Lipid Transfer Protein by an Allene Oxide Generated by 9-Lipoxygenase and Allene Oxide Synthase

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Lipid transfer proteins (LTPs) are ubiquitous plant lipid-binding proteins that have been associated with multiple developmental and stress responses. Although LTPs typically bind fatty acids and fatty acid derivatives in a non-covalent way, studies on the LTPs of barley seeds have identified an abundantly occurring covalently modified form, LTP1b, the lipid ligand of which has resisted clarification. In the present study, this adduct was identified as the α-ketol 9-hydroxy-10-oxo-12(Z)-octadecenoic acid. Further studies on the formation of LTP1b demonstrated that the ligand was introduced by nucleophilic attack of the free carboxylate group of the Asp-7 residue of the protein at carbon-9 of the allene oxide fatty acid 9(S),10-epoxy-10,12(Z)-octadecadienoic acid. This reactive oxypilin was produced in barley seeds by oxygenation of linoleic acid by 9-lipoxygenase followed by dehydration of the resulting hydroperoxide by allene oxide synthase. The generation of protein-oxypilin adducts represents a new function for plant allene oxide synthase. The biosynthesis of the jasmonate family of plant hormones. Additionally, the LTP-allene oxide synthase interaction opens new perspectives regarding the roles of LTPs in the signaling of plant defense and development.

Lipid transfer proteins (LTPs) are ubiquitous plant lipid-binding proteins. They form a multigenic family composed by two subfamilies, LTP1 and LTP2, whose members have molecular masses around 9,000 and 7,000, respectively. LTP1 and LTP2 display low or no sequence identities except for a common motif of 8 cysteines forming four disulfide bonds, which is now considered as the LTP signature. This signature is related to an α-helical fold in which a large hydrophobic tunnel or cavity allows the binding of different types of lipids and hydrophobic molecules (1, 2). LTPs have been associated with a multitude of developmental and stress responses, and their precise function is still unknown.

Concerning plant development, it has been suggested that LTPs are involved in the mobilization of seed storage lipids (3), in the formation of cuticles (4, 5), in pollen tube adhesion (6, 7), in endosperm programmed cell death (8), and in cell wall loosening (9). Concerning plant defense against biotic and abiotic stress, it has been shown that LTPs are associated with cold and wounding stress (10, 11), are induced by microbial pathogens (12), and can inhibit fungal growth in vitro (13, 14). Recently, it has been shown that LTPs and oomycetous elicitors compete for a plasma membrane receptor involved in the hypersensitive response and systemic acquired resistance (15, 16). Besides, the observation that a mutant of Arabidopsis thaliana affected in an LTP2 gene was unable to develop systemic acquired resistance (17) suggests that, in planta, LTPs are probably involved in the signaling of plant defense mechanisms rather than in a direct inhibitory activity on microbial pathogens.

Most of the putative functions of LTPs are related to their capabilities of binding lipids and other hydrophobic molecules. For example, it has been recently shown that LTPs can induce plant protection when they are loaded with jasmonic acid (but not with linolenic acid) (18) and that the cell wall loosening effect of LTP is modulated by lipid binding (9). Although LTPs typically bind lipids in a non-covalent way, we have previously found that an adduct is covalently bound to wheat LTP1 (19). The adduct with a molecular mass of about 294 Da has been also found in barley LTP1, where a structure has been proposed from NMR and mass spectrometry analysis. It was suggested that the adduct corresponds to a new post-translational modification (20). The proposed
structure was an oxygenated fatty acid derivative with an odd number of carbon atoms (C17) that does not fit with current fatty acid pathways described in plants.

The history of allene oxide synthase (AOS) goes back to 1966, in which year Zimmerman reported the formation of the α-ketol fatty acid 13-hydroxy-12-oxo-9-octadecenoic acid from linoleic acid in a preparation of flaxseed (21). Sequential actions of lipxygenase (LOX) and a new enzyme termed “hydroperoxide isomerase” were implicated in this conversion. The reaction mechanism of hydroperoxide isomerase was studied by Hamberg (22), who demonstrated that the true products are unstable allene oxides, which undergo spontaneous hydrolysis to provide the α-ketols isolated in the earlier studies. In subsequent studies, Brash et al. (23) isolated the methyl esters of two allene oxides and additionally characterized and cloned the hydroperoxide isomerase (24, 25). This turned out to be a cytochrome P-450 protein related to mammalian thromboxane and prostacyclin synthases and was renamed allene oxide synthase. AOS is widely distributed in higher plants, and a catalytically distinct form related to catalases is present in coral (for reviews, see Refs. 26 and 27). The primary function of AOS in higher plants is to provide the linolenic acid-derived allene oxide that, in the presence of allene oxide cyclase (28), undergoes cyclization into 12-oxo-10,15-phytodienoic acid. This cyclopentenone serves as the precursor of the jasmonate subfamily of oxylipins (29).

The present study is concerned with the structure and mode of formation of the covalently modified LTP1. We report that LTP1 traps an allene oxide formed from linoleic acid by sequential action of 9-LOX and AOS.

EXPERIMENTAL PROCEDURES

Chemicals—Linoleic acid, [13C]linoleic acid, and nordihydroguaiaretic acid (NDGA) were obtained from Sigma. The oxylipins used including the furane fatty acid 10,13-epoxy-10,12-octadecadienoic acid and the α-ketol 9-hydroxy-10-oxo-12(Z)-octadecenoic acid were purchased from Larodan Fine Chemicals, Malmö, Sweden. Maize AOSs were prepared as described previously (28).

Plant Material—Barley seeds (Hordeum vulgare cv. Scarlett) were provided by Malteurop (Reims, France). Embryos were dissected from dry barley seeds and grounded with liquid nitrogen. For studies on germination, mature barley seeds were sterilized in a solution of sodium hypochloride as described previously (30) and were allowed to germinate in the dark at 25 °C on moist paper, and about 40 seedlings were collected daily, transferred in liquid nitrogen, and freeze-dried before LTP extraction. To avoid any artificial LTP adduction during the extraction process, NDGA was added in the extraction buffer.

Purification of Barley LTP1 and LTP1b—LTP1 and LTP1b were extracted as described previously (31). The purity of LTPs was checked by SDS-PAGE and electrospray mass ionization spectrometry analyses (ESI-MS), as detailed in a previous work (32).

To isolate the non-adducted LTP1, the corresponding barley powder was defatted successively with CH₂Cl₂-CH₃OH 2:1 (v/v) and pure CH₂Cl₂. After filtration, the defatted barley powder was dried under vacuum for 12 h before extraction.

Cleavage and Chemical Modifications of the Lipid-like Adducts—The adduct-protein bond was cleaved by transesterification of LTP1b, the adducted LTP1 with boron trifluoride (BF₃), in methanol (33). The released methyl ester was extracted with CH₂Cl₂. The organic phase was thoroughly washed with water and dried with anhydrous sodium sulfate. After filtration, CH₂Cl₂ was evaporated under a nitrogen stream. The methyl esters were silylated by adding 100 µl of N,O-bis (trimethylsilyl) trifluoroacetamide (1% trimethylchlorosilane) (Sigma), and the reaction proceeded for 30 min at 70 °C.

LTP1b was also submitted either to methoxamine derivatization (Pierce), as indicated by the supplier, or to NaBH₄ reduction in methanol on ice for 15 min. For NaBH₄ reduction and methoxymethylene derivatization, jasmonate (Sigma) and 13-oxo-9,11-octadecadecenoic acid (Cayman Chemicals, Ann Arbor, MI) were used as positive controls.

Gas Chromatography-Mass Spectrometry—Derivatized extracts were separated and identified on an HP-5890 gas chromatograph equipped with a DB5-MS column (30 m × 0.25 mm, film thickness 0.25 µm) and coupled to an HP-5971A mass detector. The oven temperature was programmed at 100 °C (1 min) and then increased 20 °C min⁻¹ to 230 °C (1 min), 5 °C min⁻¹ to 280 °C (0 min), and finally, to 20 °C min⁻¹ to 310 °C. Electron impact ionization was operated at 70 eV.

High resolution mass spectrometry was performed with an SX 102 mass spectrometer (Jeol, Tokyo, Japan) coupled to an HP-5890A gas chromatograph (Agilent, Massy, France), and a MDN55 (Supelco, Lyon, France) column (30 m × 0.25 mm × 0.25 µm) was used with helium as carrier gas. The column temperature program was: 100 °C (1 min) and then increased 20 °C min⁻¹ to 230 °C (1 min), 5 °C min⁻¹ to 280 °C (0 min), and finally, to 20 °C min⁻¹ to 310 °C. Electron impact ionization was operated at 70 eV. The source was held at 250 °C. High resolution (R > 10,000) selected ion monitoring was used as an acquisition technique using perfluorokerosene for mass calibration and lock mass.

NMR Characterizations—NMR spectra were recorded on a Bruker ARX 400 spectrometer operating at a carbon frequency of 100.62 MHz. The chemical shifts of the carbon were calibrated from internal trimethylsilyl propionic acid with methyl assigned at 0 ppm. The NMR experiments were realized using a single pulse detection of carbon with power-gated 1H decoupling. The 90° carbon pulse was set to 11.25 µs, micro second and a proton decoupling (WALTZ16) of 3 kHz was applied during acquisition (1.25 s) but also during the recycle delay (5 s) to gain signal by nuclear Overhauser enhancement (34). Each NMR experiment was realized at 298 K with 5,000 scans.

Tryptic Digestion of LTP1b—1 mg of adducted LTP1 was reduced with 15 mg of Tris 2-carboxyethylphosphine in a 0.1 M phosphate buffer (pH 5) with 6 µM guanidine HCl for 30 min at 30 °C and then alkylated by adding 15 mg of N-ethylmaleimide at room temperature for 1 h. The protein was purified by HPLC as described above and checked by mass spectrometry. 0.1 mg of reduced and alkylated LTP1b was subjected to trypsin digestion (20:1 by weight) in a 50 mM Tris-HCl (pH 7) for 15 min at 37 °C.
The tryptic fragments were first analyzed by MALDI-MS on a M@LDI-LR instrument (Micromass/Waters, Manchester, UK) equipped with a laser at 337 nm. The peptide mixture was mixed with the matrix preparation (1:1, v/v), consisting of α-cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid, both dissolved at 5 mg/ml in acetonitrile containing 30% 15 mM NH₄H₂PO₄ and 0.1% trifluoroacetic acid. The tryptic fragments of adducted LTP were further measured by tandem mass spectrometry (ESI-MS/MS). Measurements were performed with a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Global, Micromass/Waters, Manchester, UK) equipped with an ESI source. The peptide mixtures were diluted in water and acetonitrile (1:1, v/v) acidified with 0.1% formic acid (1 nmol·ml⁻¹) and were infused at a continuous flow rate of 2 μl·min⁻¹.

Production of Specific Monoclonal Antibody against Adducted LTP1—BALB/c mice received four injections of purified adducted LTP1 emulsified with complete or incomplete Freund’s adjuvant. Hybridoma were produced as described by Kohler and Milstein (35) and were screened for the presence of antibodies against adducted LTP1 or LTP1 by indirect ELISA. We chose an antibody, named anti-LTP1b, which shares a strong reactivity for the adducted protein, whereas it did not recognize the non-adducted LTP1.

Screening of Hybridoma Supernatant—Hybridoma supernatants were incubated for 2 h at 3 7° C. Samples were added to microtiter plates and left overnight at 4 °C with 500 ng/well of adducted LTP1 or LTP1 in 10 mM carbonate-bicarbonate buffer (pH 9.6). Uncoated sites were blocked with 4% defatted skimmed milk in phosphate-buffered saline. Hybridoma supernatants were incubated for 2 h at room temperature. After washes, bound antibodies were detected by incubation with horseradish peroxidase anti-mouse IgG (170-4 °C with 500 ng/well of adducted LTP1 or LTP1 in 10 mM carbonate-bicarbonate buffer (pH 9.6). Uncoated sites were blocked with 4% defatted skimmed milk in phosphate-buffered saline. Hybridoma supernatants were incubated for 2 h at room temperature. After washes, bound antibodies were detected by incubation with horseradish peroxidase anti-mouse IgG (170-

| Substrate | Adducted barley LTP1 |
|-----------|-----------------------|
| WF        | 1.67 ± 0.16           |
| DF        | ND                    |
| EF        | ND                    |
| DF + EF   | 1.76 ± 0.09           |

Adduction of LTP1 with an Allene Oxide

Determination of Adducted LTP1 Content by Sequential Competitive ELISA—LTP1 was extracted by a gentle stirring of 1 g of barley powder for 1 h at 25 °C with 10 ml of water. After centrifugation, 50 μl of supernatant was submitted to ELISA analysis for the quantification of adducted LTP1. Microtiter plates were coated with adducted LTP1 and saturated as described above. Anti-LTP1b antibody at appropriate dilution was preincubated with adducted LTP1 (0.5–20 μg/ml) or with diluted barley extracts for 2 h at 37 °C. Samples where added to the microtiter plate and incubated for 2 h at room temperature. After washes, the remaining antibodies were detected as described for indirect ELISA. Absorbance of the standards was plotted versus concentrations and fitted with log-logit regression. Competitive assay allows a dose-response detection of adducted LTP1 between 0.3 and 5 μg/ml, and no inhibition was observed with non-adducted LTP1 as competitor.

RESULTS

Preliminary Characterization of LTP1s from Barley Powders—LTP1b was isolated as described previously (19). In agreement with the presence of a covalently bound lipid adduct, defatting the material with CH₂Cl₂-CH₂OH did not remove the adduct (data not shown).

Extraction and purification (19) of LTP1s (adducted and non-adducted) starting with defatted or non-defatted barley powders gave about the same yield of materials, i.e. 65 and 70 mg, respectively, from 100 g of barley powder. Almost all LTP1 was adducted when the protein was extracted from non-defatted barley powder. On the contrary, defatted barley powder yielded only non-adducted LTP1, as shown by ELISA (Table 1) and confirmed by mass spectrometry analyses. Requirement of embryo for the formation of adducted LTP1 was highlighted by comparing the adducted LTP1 contents in soluble protein extracts from three sources, i.e. de-embryonated, embryo, and whole barley seed powders (Table 1). Importantly, no adducted LTP1 was revealed by ELISA in the absence of embryo. As a control, adducted LTP1s could not be detected in an aqueous embryo extract. In another experiment, the embryo powder was re-added to the de-embryonated barley powder, a procedure that allowed the recovery of adducted protein. Therefore, barley embryo and lipids were required for the formation of adducted LTP1.

Linoleic Acid Is a Precursor of the Lipid-like Adduct—A 1-ml aqueous dispersion of isolated and defatted barley embryo powder (1 mg) was incubated for 15 min with purified LTP1 (1 mg) in the presence or absence of [13C]linoleic acid (0.2 mM). By coupling reversed phase HPLC and mass spectrometry, we observed that adduction proceeded only when linoleic acid was added in the reaction medium and that 13C was incorporated in the adducted LTP1 (molecular mass, 9999 Da).

The Lipid-like Adduct Is an α-Ketol—The experiments described above suggested that the lipid adduct was an oxylipin formed via
the LOX pathway. In agreement with previous work (19), the adduct was released from LTP1 by alkaline hydrolysis. After methylation and silylation, GC-MS revealed multiple products that increased in number with time of hydrolysis. This confirmed that the adduct was unstable under alkaline conditions, as observed previously (20).

Methods employing non-alkaline conditions or in situ stabilization of the adduct prior to alkaline hydrolysis were next tried. First, direct transmethylation was conducted with BF₃/methanol on the freeze-dried pure adducted protein. After extraction with CH₂Cl₂, the transmethylated adduct (I) was analyzed by GC-MS and GC-high resolution mass spectrometry:

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\text{m/z (ion attribution, elemental composition) (relative intensity percentage):} \\
308 [M+H⁺, C₁₉H₃₂O₃]⁺ (18), 277 [M-OCH₃, C₁₈H₂₉O₂]⁺ (9%), 251 [M-CH₃, C₁₈H₂₇O₃]⁺ (10), 151 [C₁₀H₁₅O]⁺ (100), 95 [C₆H₇O]⁺ (32), 165 (12), 55 (8).
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The MS fragmentation pattern as well as the elemental composition indicated that product (I) (Fig. 1) was identical to methyl 10,13-epoxy-10,12-octadecadienoate, a furan fatty acid methyl ester. The parent ion of m/z 308 is in accordance with methylation of the 294 Da adduct (+14 Da) deduced from the difference between theoretical and experimental masses of the protein. However, when free furan acid was incubated with barley LTP1, no adduction could be observed by LC-MS analysis. Apparently, the furan acid was not present as such in LTP1b but was formed during the BF₃ treatment.

Secondly, treatments of LTP1b with NaBH₄ followed by 0.2 M NaOH released CH₂Cl₂-extractable material that was derivatized by trimethylsilyl. Two major products (II) and (III) with identical MS fragmentation pattern were obtained:

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\text{m/z (ion attribution) (relative intensity percentage) 515 [M-15]+ (2.5), 419 (11), 329 (419-90) (34), 317 (100), 213 (26), 147 (25), 73 (73).}
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This fragmentation was identical to that observed for the authentic
Methoxyamine derivatization of LTP1b gave rise to isomers (IV) and (V), identical to the syn-/anti-isomers of the methoxime derivative of authentic 9-hydroxy-10-oxo-12(Z)-octadecenoic acid. In addition, treatment of LTP1b with sodium borodeuteride followed by alkaline hydrolysis produced 9,10-dihydroxy-12(Z)-octadecenoic acid, which was labeled with one deuterium atom exclusively at C-10, thus showing that this was the original oxo carbon. Furthermore, treatment of the 9-hydroxy-10-oxo-12(Z)-octadecenoic acid (9,10-ketol) standard with BF₃/methanol yielded methyl 10,13-epoxy-10,12-octadecadienoate (I), thus confirming the origin of the furan ester formed on transmethylation of LTP1b.

All together, these results clearly indicated that the lipid adduct from barley LTP1b is 9,10-ketol. This oxylipin is formed from linoleic acid. Despite the presence of a 13C-enriched adduct, two-dimensional spectra aimed at collecting correlations between directly bound carbons were unsuccessful due to the long acquisition times required. Correlations between protons and carbons were also difficult to acquire as the major protons signals were provided by the protein. The structure was deduced from chemical shifts as well as their hyperfine structure induced by directly bound carbons J-couplings (Fig. 2 and supplemental Table 1). Three carbon signals were detected in the region between 170 and 220 ppm corresponding to the carboxylic acid and carbonyl functions. The COO group (C1) could be distinguished from the carbonyl ones (C10) by a doublet (scalar-coupled with C7) at a higher field (181 ppm). The olefinic carbons (C12 and C13) showed multiplets between 120 and 140 ppm, whereas the C9 carbon resonated at 82.8 ppm. The aliphatic carbons could be observed in the 0–40-ppm region with a net distinction for the methyl group C18 at 16.4 ppm that exhibited a doublet by coupling with the neighboring C17 methane. The structure deduced from the 13C spectrum fitted well with the above mentioned 9-hydroxy-10-oxo-12(Z)-octadecenoic acid structure. The narrow width of NMR signals suggested that the adduct has a high mobility despite being covalently attached to the protein.

To identify the amino acid residue involved in the adduction, LTP1b was reduced and alkylated under acidic conditions to prevent any release or degradation of the adduct. Trypsin hydrolysates of non-adducted and adducted barley LTP1 were compared. Comparison of the mass profiles by MALDI-MS highlighted the probable modification of peptide T1 (MH⁺/H₁₁₀₀₁ 1088.5 Da) in adducted LTP since this T1 fragment peptide is observed with a MH⁺/H₁₁₀₀₁ of 1382.5 (data not shown). To locate the modification within the polypeptide chain of fragment T1, the tryptic mixtures of LTP1s were further examined by tandem mass spectrometry (ESI-MS/MS). According to the abundant y₂ and y₃ fragment ions in the MS-MS spectra from LTP1 and LTP1b, Asp-7 was unambiguously identified as involved in the adduction (Fig. 3).

Barley LTP1 Specifically Traps the Unstable Allene Oxide 9(S)-Epoxy-10,12(Z)-octadecadienoic Acid—When the 9,10-ketol standard was incubated with barley LTP1, no adduction occurred, as indicated by LC-MS analysis (result not shown). α-Ketols are formed by hydrolysis of unstable allene oxides gen-

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**FIGURE 4. Barley LTP1 traps a reactive allene oxide produced by AOS.** A, reactions were performed in a 2-ml suspension of maize AOS in the presence of 300 μM of 9-HPOD as substrate (except controls 1 and 2 [ctrl1 and ctrl2] preincubated for 5 min with 100 μM NDGA (except for control 2). Barley LTP1 (50 μM) was added at different times of incubation (0 s–10 min). Incubations were conducted at 0 °C and were stopped 5 min after the addition of LTP1 by freezing in an ethanol-dry ice mixture. Incubates were stored at −80 °C until LC-MS analysis. AddLTP, adducted LTP. B, plot of the percentage of adduction versus molar ratios of 9,10-allene oxide/LTP1.
Adduction of LTP1 with an Allene Oxide

Adduction of LTP1 with a lipid-like molecule is a more complex and exciting phenomenon than expected from previous preliminary studies (19, 20). Adduction involves three main collaborative partners, i.e. lipids, LTP1, and embryo.

The LOX/AOS pathway is involved in LTP1 adduction. This was confirmed (i) by determination of the structure of the adduct, i.e. an α-ketol, and (ii) by reproducing adduction by exposing LTP1 to either linoleic acid and an aqueous suspension of isolated defatted barley embryo or 9-HPOD and maize AOS. 9-LOX and AOS are specifically located in the embryo (40, 41), in agreement with the fact that LTP1 adduction requires the presence of the embryo.

It should be noted that 9-LOX is the major LOX in the embryo of quiescent barley seeds (42, 43) and that AOS is the major enzyme metabolizing lipoxygenase-generated hydroperoxides in embryos of quiescent barley grains (41). The very efficient trapping of 9,10-allene oxide by 50 μM LTP1 in an aqueous environment (about 55 M water) (Fig. 4) suggests affinity binding of allene oxide to LTP1. MS sequencing of tryptic digest of reduced and alkylated adducted LTP1 shows unambiguously that Asp-7 is involved in the adduction. Interestingly, an aspartate is also involved in the epoxy ring opening of epoxyhydrolases (44). In both cases, a nucleophilic attack of the carboxylate leads to the opening of the epoxy ring and formation of the ester bond between the acyl chain and the protein (Fig. 6).

Adducted LTP1 Is Formed during Germination—Cereal seeds are rich in lipoxygenase activity and in secondary hydroperoxide-metabolizing enzymes, and the activity of these enzymes generally increases during germination (37). Barley LTPs were extracted from germinating seeds in the presence of 10 mM NDGA, a LOX inhibitor. We had previously observed that this concentration of NDGA prevents any LTP adduction (data not shown). An increase of adducted LTP1 content was observed by ELISA from the second day after germination (Fig. 5). Barley LTP1 is synthesized in the aleurone (38), whereas lipids and enzymes are located in the embryo (37). During germination, a cell decompartmentalization occurs, controlled by programmed cell death (39), that allows LTP1 adduction.

DISCUSSION

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What is strikingly pointed out by these results is the relative specificity of the adduct-protein complex, and therefore, of the allene oxide for LTP1. This specificity is highlighted by the fact that full adduction is observed even in a complex aqueous dispersion of barley powder where many different recognized lipid partners of LTP1 are present. This specificity should be related to a good positioning of the allene oxide in an active site allowing a rapid catalytic process. The structure of the barley LTP1 complexed with three molecules of 1-α-lysophosphatidylcholine lauroyl (Protein Data Bank code 1MID) revealed a third cavity with the Asp-7 at the entrance and where only the 9-carbon methyl-terminal part of the alkyl chain is buried in the hydrophobic part of this cavity (Fig. 7). Actually, looking at the position of the epoxide group and Asp-7, only the C9 to C18 part of the allene oxide should enter the cavity. Lys-11 located in the entrance of this binding site could interact with the carboxylate group of the allene oxide to improve its anchoring in the protein. This insertion of the allene oxide suggests that a large portion of the allene oxide is exposed on the protein surface. In agreement with such a model, the monoclonal antibody highly specific for the adducted LTP1 and not for the non-adducted protein shows that the α-ketol composed a large part of the epitopic site. Furthermore, the α-ketol is highly mobile since it gives rise to NMR signals with thin bandwidths. This anchoring of the aliphatic chain is reduced for the 13-allene oxide that should lead to a lower affinity of the 13-isomer. This explains the weak adduction of LTP1 in the presence of the 13-allene oxide.

The high specificity of LTP adduction as well as the natural occurrence of LTPb during seed germination implies a significant biological role of the LTP/oxylipin complex. In the case of plants, reactive oxygen species are generated upon germination-induced programmed cell death (45) together with expression of LTP genes (8). The products of the LOX pathway display cell toxicity (46–48), and sequestration of such molecules by proteins could prevent cell injury. In this sense, animal cells have evolved strategies for rapid trapping of toxic oxylipins. For example, the cyclopentenone prostaglandin 15dPGJ2 is adducted to intracellular thioredoxin, and overexpression of thioredoxin suppressed the 15dPGJ2-induced cell injury (49).

In conclusion, as observed in animal cells (49–51), oxylipin adduction to protein also occurs in plant. Since oxylipin bursts as well as LTPs have been associated with cell injury occurring on development or in response to biotic and abiotic stress (1, 27, 52), we have opened a new way to explore the biological function of the multigenic and very diverse family of plant LTPs as well as the biological function of AOS metabolites. In this sense, the easy production, in vitro, of adducted LTPs with aqueous suspensions of embryos or isolated enzymes and lipid substrates will provide a useful tool for further studies on the biological role of oxylipin-adducted LTPs.

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