

RESEARCH PAPER

# The lipoxygenase-dependent oxygenation of lipid body membranes is promoted by a patatin-type phospholipase in cucumber cotyledons

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This paper is dedicated to Professor Dr H. Kindl, Marburg, Germany, on the occasion of his 75th birthday.

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## Abstract

Oilseed germination is characterized by the mobilization of storage lipids as a carbon and energy source for embryonic growth. In addition to storage lipid degradation in germinating oilseeds via the direct action of a triacylglycerol lipase (TGL) on the storage lipids, a second degradation pathway that is dependent on a specific lipid body trilinoleate 13-lipoxygenase (13-LOX) has been proposed in several plant species. The activity of this specific 13-LOX leads first to the formation of ester lipid hydroperoxides. These hydroperoxy fatty acids are then preferentially cleaved off by a TGL and serve as a substrate for glyoxysomal  $\beta$ -oxidation. As a prerequisite for triacylglycerol (TAG) mobilization, a partial degradation of the phospholipid monolayer and/or membrane proteins of the oil body has been discussed. Evidence has now been found for both processes: partial degradation of the proteins caleosin and oleosin was observed and simultaneously a patatin-like protein together with transient phospholipase (PLase) activity could be detected at the oil body membranes during germination. Moreover, *in vitro* experiments with isolated oil bodies from mature seeds revealed that the formation of 13-LOX-derived lipid peroxides in lipid body membranes is increased after incubation with the purified recombinant patatin-like protein. These experiments suggest that *in vivo* the degradation of storage lipids in cucumber cotyledons is promoted by the activity of a specific oil body PLase, which leads to an increased decomposition of the oil body membrane by the 13-LOX and thereby TAGs may be better accessible to LOX and TGL.

**Key words:** Hydroperoxy polyunsaturated fatty acids, oil body degradation, phospholipase function, storage lipid degradation.

## Introduction

The germination process of oilseed plants involves degradation of storage lipids, which have been deposited in specific organelles, the so-called lipid bodies or oil bodies (Huang, 1996). These storage lipids may serve as an energy supply,

Abbreviations: CsLbLOX, *Cucumis sativus* lipid body lipoxygenase; CsPAT, *Cucumis sativus* patatin-like protein; DAG, diacylglycerol; GC, gas chromatography; HPLC high-performance liquid chromatography; HPO(D/T) hydroperoxy octadeca(di/tri)enoic acid; 13-H(P)OD, (9Z,11E,13S)-13-hydro(pero)xy-octadeca-9,11-dienoic acid; LA, linoleic acid; LOX, lipoxygenase; MS, mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLase, phospholipase; PS, phosphatidylserine; TAG, triacylglycerol; TGL, triacylglycerol lipase; TLC, thin-layer chromatography.

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but they mainly provide carbon equivalents for synthetic processes in the growing seedling (Graham, 2008). It is generally accepted that free fatty acids are initially released from the storage triacylglycerols (TAGs) by endogenous lipases and subsequently degraded via  $\beta$ -oxidation in the glyoxysomes (Penfield *et al.*, 2009).

Linoleic acid [LA, 18:2 or (9Z,12Z)-octadeca-9,12-dienoic acid] is a major storage fatty acid in many oilseeds as well as in cucumber cotyledons (Weichert *et al.*, 2002). It may be degraded either directly via  $\beta$ -oxidation or by an alternative pathway of storage lipid mobilization, which is dependent on a specific lipoxygenase (LOX) isoform. Both pathways may occur in parallel in several oilseeds such as cucumber, sunflower, and linseed (Liavonchanka and Feussner, 2006). This LOX isoform, which is expressed at early stages of germination, associates with lipid body membranes and catalyses stereo-specific oxygenation of the linoleate moieties of TAGs to (9Z,11E,13S)-13-hydroperoxy octadeca-9,11-dienoic acid (13-HPOD) (Feussner *et al.*, 1998; Gerhardt *et al.*, 2005). In addition, an enzyme activity responsible for the specific release of these oxygenated fatty acid residues from TAGs has been described (Balkenhohl *et al.*, 1998), and isolated oil bodies release preferentially (9Z,11E,13S)-13-hydroxy octadeca-9,11-dienoic acid (13-HOD) into the medium (Feussner *et al.*, 1995). Large amounts of free 13-HOD have been demonstrated to occur in the cytosolic extract from cucumber cotyledons (Weichert *et al.*, 2002). The expression kinetics of this trilinoleate 13-LOX, its enzymatic characteristics, and its *in vivo* activity have indicated an additional mechanism for the degradation of storage lipids during germination (Feussner *et al.*, 2001). According to this concept, the LA moieties of the storage TAGs are oxygenated to 13-HPOD, which is subsequently released from lipid bodies and reduced to 13-HOD, presumably by the peroxygenase activity of the lipid body protein caleosin (Hanano *et al.*, 2006). 13-HOD is then degraded via glyoxysomal  $\beta$ -oxidation.

Lipid bodies or lipid droplets are lipid storage organelles that are found ubiquitously in eukaryotes (Murphy, 2001; Goodman, 2008). They consist of a hydrophobic matrix of TAGs surrounded by a phospholipid monolayer, associated mainly with proteins called oleosins (Hsieh and Huang, 2004), as well as calcium-binding peroxygenases called caleosins (Chen *et al.*, 1998; Naested *et al.*, 2000; Hanano *et al.*, 2006), and hydroxysteroid dehydrogenases called steroleosins (Lin *et al.*, 2002; Baud *et al.*, 2009) in plants and perilipins in animals (Brasaemle, 2007). Oleosins, the most abundant protein in plant lipid bodies, as are perilipins in those of animals, serve as stabilizing principles and are believed to form a steric barrier to prevent coalescence of lipid bodies when the seed is in a desiccated state (Leprince *et al.*, 1998; Murphy and Vance, 1999). Thus, lipid bodies and lipid droplets are rather stable and, as a first step in the mobilization of storage lipids, the disruption of the integrity of the protein coat and/or the phospholipid monolayer may be required to facilitate or promote access to the TAGs (Feussner *et al.*, 2001). However, knowledge about early events of lipid mobiliza-

tion during germination is still scarce. So far evidence has been found for proteolytic degradation of the structural proteins, the oleosins in plants (Matsui *et al.*, 1999; Vandana and Bhatla, 2006) or perilipins in animals (Brasaemle, 2007). However, data on the degradation of the phospholipid monolayer by a patatin-like phospholipase (PLase) have been reported only for cucumber and sunflower (Noll *et al.*, 2000; Gupta and Bhatla, 2007) whereas lipid bodies from almond may be directly degraded by triacylglycerol lipase (TGL) (Beisson *et al.*, 2001). Nothing is known so far regarding this for yeast and animals.

During early stages of lipid mobilization a patatin-like protein exhibiting PLase activity may be present at lipid bodies of cucumber seedlings (May *et al.*, 1998). The protein, designated CsPAT, is a homologue of the vacuolar storage protein patatin of potato tubers (Andrews *et al.*, 1988; Sonnewald *et al.*, 1989), and immunodetection in subcellular fractions of cucumber seedlings revealed that it may be exclusively confined to lipid bodies (May *et al.*, 1998). At the stage of maximal CsPAT expression, the phospholipid monolayer of cucumber lipid bodies contains holes of  $\sim 80$  nm in width and 2.45 nm in depth which are sufficiently large to provide access to 100 kDa enzymes, such as LOX and TGL (Noll *et al.*, 2000). Recent findings demonstrated that phosphatidylcholine (PC), a constituent of lipid body membranes, was degraded with concomitant accumulation of lyso-PC during germination of dark-grown sunflower seedlings (Gupta and Bhatla, 2007). In addition, a simultaneous enhancement of *in vivo* PLase activity was observed, indicating the involvement of a PLase in causing changes in the lipid body membrane phospholipids and thereby making them susceptible to lipolytic enzymes (Gupta and Bhatla, 2007).

Due to the degradation of oleosins that accompanies the mobilization of storage lipids, a putative proteolytic activity was proposed (Huang, 1992). Partial digestion of lipid body-associated proteins of cucumber seedlings using trypsin led to oxygenation of TAGs by the lipid body LOX (CsLbLOX), thereby LOX activity correlated with the concentration of trypsin (Matsui *et al.*, 1999), indicating that the removal of proteins covering the lipid body surface allows access of the LOX to its substrate. In rapeseed seedlings the partial degradation of oleosin isoforms from 19 kDa to 16 kDa fragments was observed prior to the complete degradation of lipid bodies. The latter does not necessarily lead to the disruption of the integrity of the lipid body phospholipid monolayer, but may increase the accessibility of lipolytic enzymes to the TAGs (Murphy, 2001). Furthermore, in sunflower cotyledons the activity of a probable lipid body-associated thiol-protease has been demonstrated, which is possibly responsible for gradual degradation of oleosins during seedling growth (Vandana and Bhatla, 2006).

Here a temporal correlation is shown between LOX activity, formation of its products, and the transient accumulation of a protein with PLase activity and the occurrence of the patatin-like PLase CsPAT on lipid bodies of cucumber cotyledons during germination. Additionally

immunogold labelling experiments revealed the same intracellular localization of CsPAT as the lipid body-associated LOX at the phospholipid monolayer of lipid bodies. In accordance with the detected PLase activity it could be demonstrated that lipid body-derived PC was specifically degraded with concomitant accumulation of its degradation product lyso-PC, whereas the other phospholipids did not significantly change during germination. *In vitro* experiments with isolated lipid bodies from seedlings revealed that the formation of esterified 13-HPOD by recombinant lipid body-associated LOX was increased in the membrane fraction by concomitant incubation with the recombinant patatin-like CsPAT. These observations suggest that *in vivo* the mobilization of storage lipids may be promoted by the activity of a specific lipid body PLase, which facilitates an increased decomposition of the oil body membrane by the 13-LOX, and thereby the accessibility for the LOX as well as the lipase substrates may increase.

## Materials and methods

### Plant growth and isolation of lipid bodies

Cotyledons of cucumber (*Cucumis sativus*, var. Chinesische Schlangengurken, Erfurt, Germany) seedlings were germinated for various time periods in the dark at 28 °C and 100% humidity. Isolation of lipid bodies was carried out by repeated ultracentrifugation according to Feussner and Kindl (1992). In brief, cotyledons were homogenized at 4 °C with razor blades in grinding medium consisting of 15% (w/v) sucrose, 150 mM TRIS-HCl, pH 7.5, 10 mM KCl, 1.5 mM EDTA, 0.1 mM MgCl<sub>2</sub>, and 5 mM dithiothreitol. After centrifugation for 45 min at 100 000 g the fat layer was removed and suspended in density gradient buffer, consisting of 10% sucrose, 50 mM TRIS-HCl buffer, pH 7.5, 10 mM KCl, 1.5 mM EDTA, and 0.1 mM MgCl<sub>2</sub>. Subsequent flotation, performed as described above, yielded purified lipid bodies.

### Expression and purification of CsLbLOX and CsPAT

For expression of CsLbLOX with an N-terminal His tag the LOXpQE-30 plasmid was used (Feussner *et al.*, 1998). Expression of N-terminal His-tagged CsPAT was performed with pQE11-CS291 as described by May *et al.* (1998). For expression of CsPAT with a C-terminal His tag the partial gene was amplified from pQE11-C291 and subcloned into pQE70. After transformation of expression plasmid into *Escherichia coli* SG13009[pREP4] cells, 200 ml of expression culture was grown until an OD<sub>600</sub> of 0.6 at 37 °C was reached, and then expression was induced with 1 mM isopropyl-β-D-galactopyranoside (IPTG). Culturing was carried out for 10 d at 10 °C. Cells were disrupted by sonification and His-tagged proteins were purified by affinity chromatography.

### Analysis of lipid body-associated proteins

Lipid bodies were homogenized in 0.2 M borate buffer, pH 8.25. After precipitation with acetone/ethanol (4:1, v/v) and washing with 80% ethanol, proteins were analysed by SDS-PAGE. For western blot analyses the following antibody concentrations were used: anti-LOX at 1:1000 (Hause *et al.*, 2000), anti-PAT at 1:2000 (May *et al.*, 1998), anti-caloesin at 1:1000 (anti-f-CLO1, Poxleitner, 2006 #15141), and anti-oleosin at 1:1000 (rapeseed, Tzen, 1990 #7622).

### Determination of enzyme activity

LOX activity was measured polarographically by determining oxygen uptake using an oxygen electrode (Clark) at 20 °C. A volume of 950 µl of 0.2 M borate buffer, pH 8.25, was mixed with 50 µl of lipid body suspension (50 mg ml<sup>-1</sup>) and the reaction was started by adding LA (0.89 mM final concentration). Protein concentration was estimated in Coomassie blue-stained polyacrylamide gels.

For PLase A<sub>2</sub> activity measurements, 10 mg of lipid bodies were homogenized in 300 µl of 50 mM TRIS-HCl, pH 8.25 and mixed with 0.9 µl of 1 M CaCl<sub>2</sub>, and 2 µl of [<sup>14</sup>C]PC (L-α-1-palmitoyl-2-linoleoyl-[linoleoyl-1-<sup>14</sup>C]PC); NEN, Boston, MA, USA). The reaction was carried out for 90 min at room temperature. The lipid bodies were removed by flotation. After addition of 100 µl of acetic acid, two chloroform extractions were performed. Evaporated samples were subjected to thin-layer chromatography (TLC) with chloroform/methanol/H<sub>2</sub>O (65:25:4, v/v/v) as solvent. <sup>14</sup>C-Labeled PC incubated with PLase A<sub>2</sub> (Sigma, Munich, Germany) was used as a control. Phosphor-storage screens exposed to TLC plates were analysed with a Fluorescent Image Analyzer (Fuji Film, Japan) and the optical density of radioactive bands was evaluated with the AIDA/2D Densitometry program.

Lipase activity was determined by incubating 7 mg of lipid bodies suspended in 100 µl of 100 mM TRIS-HCl buffer, pH 8.5, with 50 mM NaCl, 5 mM CaCl<sub>2</sub>, and 0.1 nmol [<sup>14</sup>C]triolein (carboxyl-[<sup>14</sup>C]triolein); NEN, Boston, MA, USA) for 120 min at 37 °C. Reactions were stopped by the addition of 10 µl of acetic acid and 200 µl of hexane. Extracted lipids were analysed by TLC with chloroform/acetone (96:4, v/v) as solvent. The optical density was determined as described above.

For determination of the pH optimum of CsPAT, the affinity-purified C-terminal-tagged CsPAT protein was incubated with PC (2-linoleoyl-PC; Sigma, Munich, Germany) in 50 mM sodium phosphate buffer (pH between 5 and 9) and 25 mM CaCl<sub>2</sub> for 70 min at 30 °C. After acidification to pH 3.0 with glacial acid, the fatty acids were extracted as described (Bligh and Dyer, 1959) and analysed by HPLC with an Agilent 1100 HPLC system (Waldbronn, Germany) coupled to a diode array detector (HPLC-DAD). LA was separated and quantified by reversed-phase HPLC on an EC250/2 Nucleosil 120-5 C18 column (Macherey-Nagel, Dueren, Germany) with a solvent system of methanol/water/acetic acid (95:5:0.1, v/v/v), and a flow rate of 0.2 ml min<sup>-1</sup>. For detection of fatty acids the absorbance at 202 nm was recorded.

To analyse the interaction of CsPAT and CsLbLOX activity, 4 mg of lipid bodies of seeds (soaked in water for 2 h) were re-suspended in 50 mM sodium phosphate buffer, pH 8.0, with 25 mM CaCl<sub>2</sub> and incubated with 400 µl of affinity-purified CsPAT protein solution and 200 µl of purified CsLbLOX (900 µl final volume) for 120 min at 30 °C. The reaction was stopped by adding 100 µl of acetic acid, and esterified hydro(peroxy) fatty acids as well as esterified fatty acids were extracted and trans-methylated as described in the following section. The methyl esters of hydro(peroxy) fatty acids and fatty acids were analysed by reversed-phase HPLC as described above. For detection of hydro(peroxy) fatty acids, the absorbance at 234 nm was recorded, indicating the conjugated diene system. Pre-incubation of resuspended lipid bodies with immobilized proteinase K was carried out for 60 min at 30 °C with shaking. After removal of proteinase K by centrifugation, the lipid body supernatant was incubated with purified CsLbLOX as described above.

### Lipid analysis

Lipid extracts of lipid bodies were prepared according to Bligh and Dyer (1959). For analysis of esterified oxylipins and fatty acids, 20 mg of lipid bodies were used. The evaporated organic phase was dissolved in 2 ml of chloroform. An aliquot of 200 µl of this solution as well as triricinoleate and triheptadecanoate as internal standards was transmethylated with sodium methoxide as

described previously (Lang and Feussner, 2007). The analysis of the corresponding fatty acid methyl esters was performed by gas chromatography with a flame ionization detector as described (Stumpe *et al.*, 2005). The methyl esters of the oxylipins were analysed by HPLC with a diode array detector according to the analysis of the free oxylipins (Göbel *et al.*, 2003). First, oxylipins were purified by reversed-phase HPLC, then straight-phase HPLC was performed for separation of hydroperoxy fatty acids and hydroxy fatty acids.

Lipid body membrane phospholipids were isolated according to Tzen *et al.* (1993). A preparation of 30 mg of lipid bodies homogenized in 500  $\mu$ l of density gradient buffer (see above) was extracted with 1 ml of diethyl ether. After vigorous shaking and being left for 15 min at room temperature the sample was centrifuged at 13 600 *g* for 4 min. The upper ether layer was discarded and the lower aqueous layer and interfacial material were extracted with 1 ml of diethyl ether an additional three times. The lower aqueous phase, together with the interfacial material, was placed under nitrogen for 1 h in order to remove traces of ether. After adding 750  $\mu$ l of chloroform/methanol (2:1, v/v) and gentle shaking, the sample was centrifuged at 13 600 *g* for 4 min. The lower chloroform layer (containing phospholipids) was washed three times with methanol/water (1:1, v/v), followed by centrifugation at 13 600 *g* for 15 min. Evaporated samples were subjected to TLC with methyl acetate/2-propanol/chloroform/methanol/0.25% aqueous potassium chloride (25:25:25:10:9, v/v/v/v) as solvent. After visualization in aqueous 10% (w/w) CuSO<sub>4</sub> containing 8% H<sub>3</sub>PO<sub>4</sub> and subsequent heating to 170 °C, the phospholipids were quantified densitometrically with the AIDA/2D Densitometry program using phospholipid standards (Sigma, Munich, Germany) to establish standard curves.

For analysis of neutral lipids, the lipid extract of 30 mg of lipid bodies was loaded on a solid phase extraction column (Strata SI-1 Silica, 55  $\mu$ m, 70 A, 500 mg per 6 ml; Phenomenex, Torrance, CA, USA) pre-equilibrated with chloroform. The neutral lipids were eluted with chloroform and separated by TLC using petroleum ether/diethyl ether/acetic acid (70:30:0.5, v/v/v) as solvent. Migration of standard lipids (5  $\mu$ g) was used to identify neutral lipids. For the identification of oxygenated neutral lipids, a cell lysate of a 10 ml *E. coli* culture expressing CsLbLOX was incubated with 1.2 mg of trilinolein emulsified in 50  $\mu$ l of 5% (w/v) gum arabic by sonification. The reaction was carried out for 30 min at room temperature. Reaction products were extracted as described (Bligh and Dyer, 1959) and analysed by reversed-phase HPLC according to Feussner *et al.* (1997). Separated triple-, double-, and mono-oxygenated trilinolein were subjected to TLC analysis. Visualization and densitometric quantification of neutral lipids was performed as described above, using trilinolein (Sigma, Munich, Germany) to establish a standard curve.

To investigate oxygenated neutral lipids by mass spectrometry the corresponding lipids were isolated from the TLC plate and extracted from the silica gel with the TLC solvent. After vigorous shaking and centrifugation, the liquid phase was purified with absorbent cotton and evaporated. The samples were analysed by Ultra Performance LC™ (ACQUITY UPLC™ System, Waters Corporation, Milford, USA) combined with a time-of-flight mass spectrometer (LCT Premier™, Waters Corporation). For chromatography, an ACQUITY UPLC™ BEH SHIELD RP18 column (1  $\times$  100 mm, 1.7  $\mu$ m particle size, Waters Corporation) was used at a temperature of 40 °C, a flow rate of 0.2 ml min<sup>-1</sup>, and with the following gradient: 0–0.5 min 40% B, 0.5–5.5 min from 40% B to 100% B, 5.5–10 min 100% B, and 10–12 min 40% B [solvent system A, water/methanol/acetonitrile/formic acid (90:5:5:0.1, v/v/v/v); B, acetonitrile/formic acid (100:0.1, v/v)]. The MS was operated in positive electrospray ionization mode in W optics and with a mass resolution >10 000. Data were acquired by MassLynx software (Waters Corporation) in centroided format over a mass range of *m/z* 500–1000 with a scan duration of 0.5 s and an interscan delay of 0.1 s. The capillary and the cone voltage were

maintained at 2.700 V and 30 V, respectively, and the desolvation and source temperature at 250 °C and 80 °C, respectively. Nitrogen was used as cone (30 l h<sup>-1</sup>) and desolvation gas (600 l h<sup>-1</sup>). For accurate mass measurement of >5 ppm root mean squared, the mass spectrometer was calibrated with phosphoric acid 0.01% (v/v) in acetonitrile/water (50:50, v/v) and the Dynamic Range Enhancement mode was used for data recording. All analyses were monitored by using leucine-enkephalin ([M+H<sup>+</sup>] 556.2771 *m/z*, Sigma-Aldrich, Deisenheim, Germany) as lock spray reference compound at a concentration of 1  $\mu$ g ml<sup>-1</sup> in acetonitrile/water (50:50, v/v) and a flow rate of 20  $\mu$ l min<sup>-1</sup>.

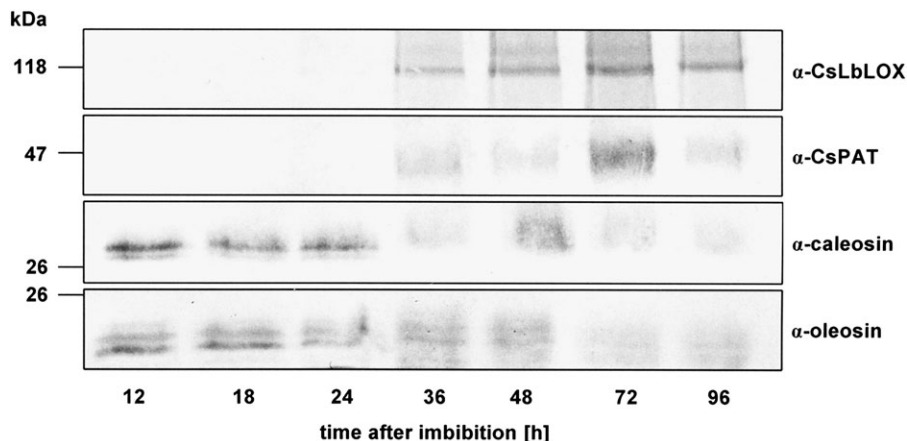
#### *Immunogold electron microscopy*

For the fixation, 1 mm<sup>2</sup> pieces of cotyledons were kept for 3 h at room temperature in 100 mM sodium phosphate buffer (pH 7.2), containing 3.0% (v/v) formaldehyde, 0.25% (v/v) glutaraldehyde, and 0.05% (v/v) Triton, after a vacuum infiltration for a few seconds in the same medium. Samples were washed with 100 mM sodium phosphate buffer for 10 min followed by three washes for 10 min with distilled water. Dehydration of samples was done stepwise by increasing the concentration of ethanol and concomitantly lowering the temperature (progressive lowering of temperature) using an automated freeze substitution unit (AFS, Leica, Benzheim, Germany). The steps of progressive lowering of temperature substitution were performed as follows: 30% (v/v), 40% (v/v), and 50% (v/v) ethanol for 30 min at 4 °C, 60% (v/v) and 75% (v/v) ethanol for 1 h at -15 °C, 90% (v/v), and twice 100% (v/v) ethanol for 1 h at -35 °C each. The samples were infiltrated subsequently with Lowycryl HM20 resin (Plano GmbH, Marburg, Germany) at -35 °C as follows: 33% (v/v) in ethanol overnight, 50% (v/v) and 66% (v/v) HM20 resin in ethanol for 4 h each, and then 100% (v/v) HM20 overnight. Samples were transferred into gelatin capsules, kept there for 6 h in fresh resin, and polymerized at -35 °C for 3 d under indirect UV light. The cured blocks were cut into ultra-thin sections with a thickness of 70–90 nm on an ultramicrotome (Ultra cut F; Reichert) and mounted on copper grids. Then the sections on the grids were blocked on a drop of buffer containing 3% bovine serum albumin for 15 min. Thereafter the grids were directly transferred on to a drop of anti-LOX, anti-patatin, anti-glycolate oxidase (Behrends *et al.*, 1982), and anti-isocitrate lyase antibody (Frevort and Kindl, 1978) at an appropriate dilution and incubated for 1 h at room temperature. In control experiments, the antibodies were replaced with adequate pre-immune sera. Then the specimens were washed five times with washing buffer for 5 min each and incubated with protein A-gold (5 nm) for 45 min. Thereafter the grids were washed three times with washing buffer and twice with distilled water for 5 min each. Finally specimens were stained with uranyl acetate prior to examination in a Zeiss CEM 902A transmission electron microscope at 80 kV.

## Results

### *Lipid body-associated proteins in germinated seedlings*

Previous subfractionation experiments revealed that the patatin-like protein CsPAT was exclusively confined to the lipid body fraction (May *et al.*, 1998). Here, the appearance of CsPAT was determined during germination with respect to other proteins associated with the lipid body membrane. Analyses of lipid body-associated proteins by western blot were carried out using total protein extracted from isolated lipid bodies of cotyledons harvested at seven different time points of germination (Fig. 1). The first occurrence of CsPAT was detectable after 36 h of



**Fig. 1.** Time course of lipid body-associated proteins during germination of cucumber. Proteins were prepared from isolated lipid bodies of cotyledons harvested at the time points indicated, separated by SDS-PAGE, and analysed by western blotting using anti-CsLbLOX, anti-CsPAT, anti-caleosin, and anti-oleosin antiserum.

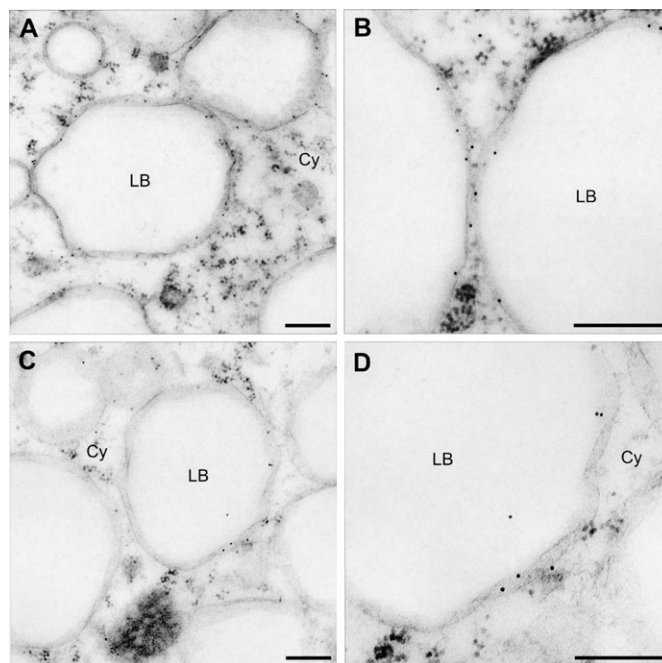
germination. After 72 h of germination the highest amount of CsPAT was found. In comparison, the amount of CsLbLOX was increased continuously, showing a maximum at 72 h of germination as detected for CsPAT. Interestingly, at the same time point of germination when the amounts of CsPAT and CsLbLOX were increasing, from 36 h after imbibition onward, the amount of two other lipid body proteins, caleosin and oleosin, started to decrease, probably via degradation.

Furthermore, to investigate the intracellular localization of CsLbLOX and CsPAT, ultra-thin sections of cotyledons from 72 h germinated etiolated seedlings were incubated with anti-CsLbLOX and anti-CsPAT antiserum, respectively, and immunogold-labelled specimens were analysed by transmission electron microscopy. CsLbLOX was detectable at the membrane of the lipid bodies and also in trace amounts in the cytosol around these organelles (Fig. 2A, B). CsPAT, however, was solely localized at the lipid body membrane (Fig. 2C, D; Supplementary Fig. S1 available at *JXB* online). The smaller dense organelles between the lipid bodies were identified as glyoxysomes with anti-glycolate oxidase and anti-isocitrate lyase antibodies (Supplementary Figs S2, S3).

#### *PLase activity and membrane phospholipid composition of lipid bodies during germination*

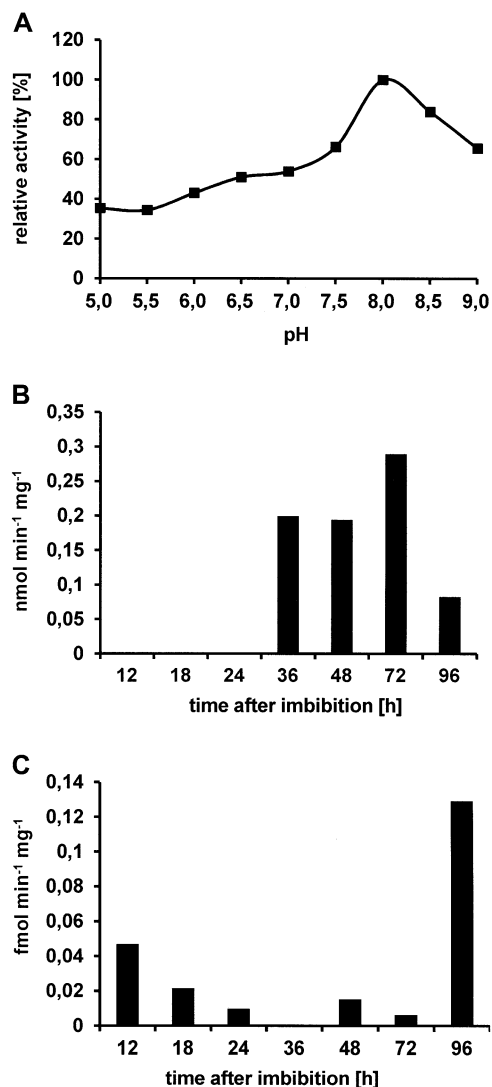
The PLase activity of recombinant patatin-like protein CsPAT was described earlier (May *et al.*, 1998). To establish optimal assay conditions, the pH optimum of recombinant CsPAT was measured first. The C-terminal tagged protein was expressed in *E. coli* cells and purified by affinity chromatography. The pH optimum was tested by incubation of the purified protein with PC at pH values ranging from pH 5 to pH 9 and analysing the release of LA via reversed-phase HPLC. The recombinant CsPAT showed highest activity between pH 8 and 8.5 and thus seemed to prefer slightly alkaline conditions, as did CsLbLOX (Feussner and Kindl, 1994) (Fig. 3A).

With the assumption of the involvement of a PLase in lipid body membrane degradation during lipid mobilization, PLase activity was investigated directly on isolated lipid bodies during germination (Fig. 3B). To this end, lipid bodies were incubated with PC,  $^{14}\text{C}$  labelled at the *sn2* position, at pH 8.25, and extracted lipids were analysed by TLC. Activity was detectable between 36 h and 96 h of germination (Fig. 3B). This result correlates with the occurrence of CsPAT protein during germination (Fig. 1).



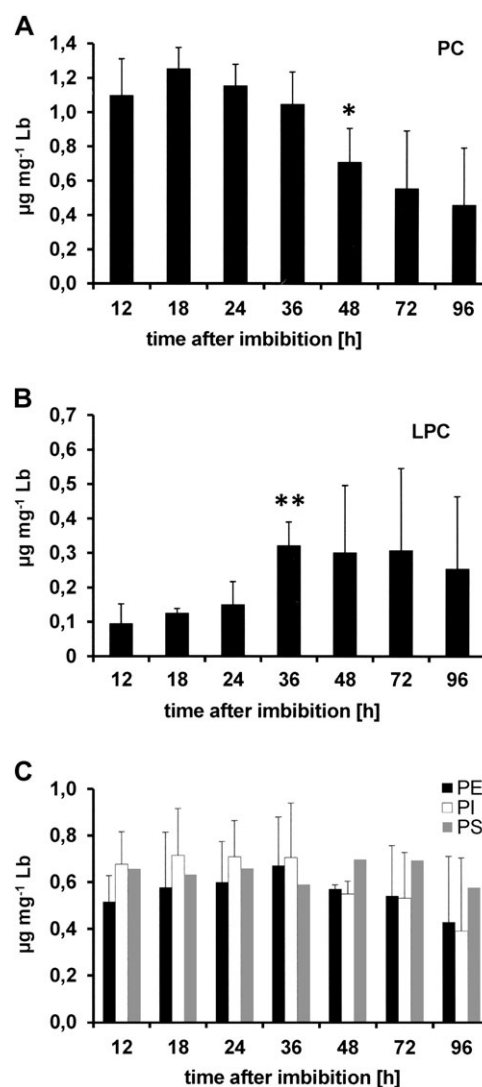
**Fig. 2.** Intracellular localization of CsLbLOX and CsPAT. Transmission electron microscope images of cotyledons from 72 h germinated etiolated seedlings. Immunogold labelling with antibodies against CsLbLOX (A, B) and CsPAT (C, D) with significant localization of the 5 nm gold particles at the membrane of the lipid bodies. Cy, cytoplasm; LB, lipid body. Scale bars represent 200 nm.

In cucumber a TGL catalyses the release of oxygenated fatty acid residues from TAGs (Balkenhohl *et al.*, 1998). To analyse lipid body membrane degradation in the temporal context of overall lipid degradation, TGL activity was determined at isolated lipid bodies during germination using  $^{14}\text{C}$ -labelled triolein as substrate, since radioactive LOX-derived TAGs as substrates were not available. Lipase activity was clearly detectable at 96 h of germination (Fig. 3C), while in contrast PLase activity at the lipid bodies decreased considerably at this time point (Fig. 3B).



**Fig. 3.** Analysis of PLase A<sub>2</sub> and TGL activity. (A) Analysis of the pH optimum of recombinant CsPAT acting on PC. PLase A<sub>2</sub> activity was measured by incubation of affinity-purified CsPAT with 2-linoleoyl-PC at various pH values, followed by determining the release of linoleic acid using reversed-phase HPLC. Relative activity was calculated based on the highest value at pH 8. (B) PLase A<sub>2</sub> activity at isolated lipid bodies incubated with  $^{14}\text{C}$ -labelled PC. (C) TGL activity at isolated lipid bodies incubated with  $^{14}\text{C}$ -labelled triolein. Activity was detected by TLC and quantified by 2D densitometry. Activities are given per mg lipid body fresh weight (B and C). Data represent the mean of two independent experiments.

The degradation of the lipid body membrane could coincide with changes in the phospholipid composition of the membrane. Thus the content of individual phospholipids of the lipid body membrane during germination was investigated. TLC analysis revealed that the amount of PC was depleted continuously as germination proceeded, whereas simultaneously the amount of its degradation product, lyso-PC, increased (Fig. 4A, B). The degradation of PC ( $P=0.098$  by Student's *t*-test for a decrease between 36 h and 48 h) and the accumulation of lyso-PC were increased further after 36 h of germination ( $P=0.037$  by Student's *t*-test for an increase between 24 h and 36 h), concomitant with the time point when PLase activity was primarily detectable (Fig. 3B) and CsPAT protein was



**Fig. 4.** Profile of lipid body membrane phospholipids during germination. Phospholipids were extracted from isolated lipid bodies and subjected to TLC. Individual phospholipids were quantified by 2D densitometry: (A) PC; (B) lyso-PC; (C) PE, PI, and PS. Amounts are given in  $\mu\text{g mg}^{-1}$  lipid body fresh weight. Data shown are the means of three independent experiments. For PS, data from two experiments are available. The standard deviation is given. \* $P=0.098$ ; \*\* $P=0.037$  by Student's *t*-test.

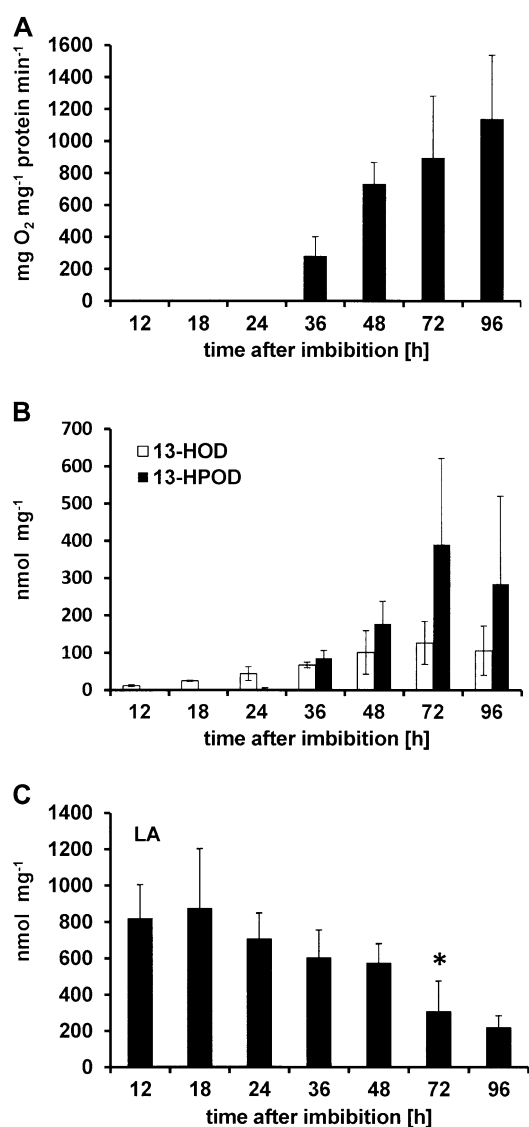
detected (Fig. 1). This indicates a possible role for CsPAT acting as a PLase on PC and therefore partially degrading the lipid body membrane. At later stages of germination (48–96 h) the amount of lyso-PC formed stayed at the level found at 36 h, leading to a continuous decrease in PC at the lipid body membrane. In addition to PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) were identified as constituents of the lipid body membrane (Fig. 4C). These three compounds, however, occurred to nearly the same extent, and each represented ~50% of the amount of PC at early stages of germination (12–24 h, Fig. 4A versus 4C). The PE, PI, and PS content did not change significantly during the analysed period of germination.

#### LOX activity and storage lipid oxygenation during germination

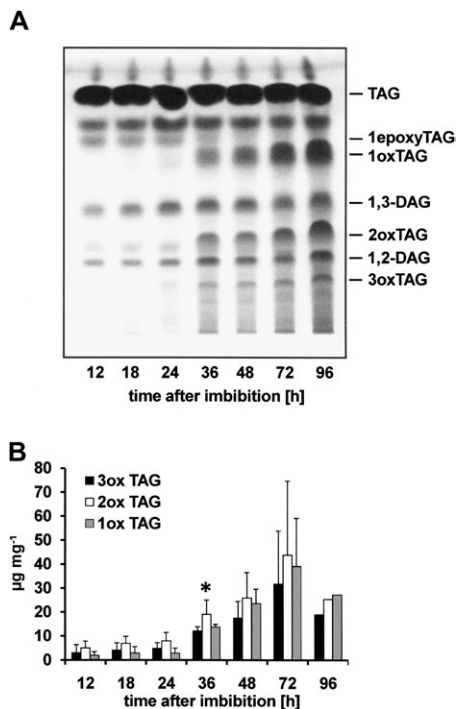
In cucumber a specific lipid body LOX is capable of oxygenating esterified fatty acids, resulting mainly in esterified 13-H(P)OD (Feussner *et al.*, 1995, 1997). Thus the occurrence of LOX activity and its metabolites in lipid body preparations during germination was analysed next. The LOX activity was determined by measuring the O<sub>2</sub> consumption with an oxygen electrode at isolated lipid bodies incubated with LA. The first LOX activity appeared after 36 h and increased as germination proceeded (Fig. 5A). After 96 h the activity was 4-fold higher than after 36 h. The content of LA-derived LOX products [13-H(P)OD] was determined in the TAG fraction of isolated lipid bodies. The primary LOX product 13-HPOD was measurable after 24 h at a low amount, and its content increased up to 48 h of germination (Fig. 5B). Although the measured LOX activity was still high after 96 h, the amount of 13-HPOD reached a plateau after 48 h, which may be a result of PLase and TGL activities (Fig. 3B, C). The reduced LOX product 13-HOD was additionally found at the earlier time points of germination (12–24 h) (Fig. 5B). Analysis of the enantiomer composition (*S/R* ratio) revealed the enzymatic origin of 13-HOD measured from 18 h of germination onwards, indicating the involvement of a LOX in its biosynthesis *in vivo*, although LOX activity was below the detection limit at the early time points (18 h and 24 h). The content of esterified LA, the LOX substrate, decreased from 875 nmol mg<sup>-1</sup> after 18 h to 220 nmol mg<sup>-1</sup> after 96 h, representing a depletion of 75% during the analysed germination period (Fig. 5C). When LOX activity was detectable, a statistically significant decrease between 48 h and 72 h was detectable ( $P=0.037$  by Student's *t*-test) and after 72 h of germination 63% of esterified LA was oxygenated.

The lipid body LOX is capable of oxygenating *in vitro* di- and trilinolein to the corresponding mono-, di-, and trihydroperoxy derivatives (Feussner *et al.*, 1998). Here the composition of neutral lipids in lipid bodies during the time course of germination was investigated in more detail. TLC analysis of neutral lipids extracted from lipid bodies revealed no obvious changes in the quantity of TAG,

1,3-diacylglycerol, and 1,2-diacylglycerol during germination, while for mono-, di-, and trihydro(pero)xy derivatives of TAG strong increases were visible after 36–96 h (Fig. 6A), reflecting the accumulation of esterified 13-HPOD and 13-HOD. Mono-, di-, and trihydro(pero)xy derivatives of TAG occurred to nearly the same extent at the different stages of germination, with a slight preponderance of the dihydro(pero)xy derivative (Fig. 6B).



**Fig. 5.** Analysis of LOX activity, product accumulation, and substrate consumption during germination. (A) LOX activity at isolated lipid bodies measured polarographically with an oxygen electrode. (B) The LA-derived LOX product 13-H(P)OD determined by transmethylation of the lipid extract from isolated lipid bodies, purification via reversed-phase HPLC, and subsequent separation by straight-phase HPLC. (C) LOX substrate LA in TAGs of lipid bodies analysed by transmethylation of lipid extract from isolated lipid bodies and following GC. Amounts are given in nmol mg<sup>-1</sup> lipid body fresh weight (B and C). Data shown are the means of three (A and B) and four independent experiments (C), respectively. The standard deviation is given. \* $P=0.037$  by Student's *t*-test.



**Fig. 6.** TLC analysis of neutral lipids of isolated lipid bodies during germination. Lipid extracts of lipid bodies were fractionated by Strata SI-1 silica columns. Neutral lipids were separated by TLC, visualized in aqueous  $\text{CuSO}_4$  with subsequent heating, and identified by co-migration of standards as indicated in the Materials and methods. Oxygenated TAG species were quantified by 2D densitometry. (A) TLC of neutral lipids. (B) Content of oxygenated TAG species during germination. The amount is given in  $\mu\text{g mg}^{-1}$  lipid body fresh weight. Data shown are the means of three independent experiments. At the 96 h time point, data from one experiment are available. The standard deviation is given. TAG, triacylglycerol; DAG, diacylglycerol; 3oxTAG, trihydro(pero)xy derivative of TAG; 2oxTAG, dihydro(pero)xy derivative of TAG; 1oxTAG, monohydro(pero)xy derivative of TAG; 1epoxyTAG, monoepoxy derivative of TAG. \* $P=0.012$ , 0.049, and 0.001 by Student's *t*-test.

A statistically significant increase in the amount of oxygenated TAG species was detectable at 36 h ( $P=0.012$ , 0.049, and 0.001 by Student's *t*-test), reaching a plateau afterwards that correlates with the time course of esterified 13-HPOD accumulation, LOX activity, and LOX occurrence.

In addition to the identification of the hydro(pero)xy derivatives of TAG by co-migration of appropriate standard lipids in TLC analysis, mono- and dihydro(pero)xy derivatives of TAG could be verified via liquid chromatography–mass spectrometry (LC-MS) analysis. Mono-oxygenated TAG species showed only esterified hydroperoxides, whereas in double oxygenated TAG species those esterified with two hydroperoxides as well as those with one hydroperoxide and one hydroxide were found (Table 1). The composition of fatty acids esterified to the glycerol backbone deduced from the suggested formula of oxygenated and non-oxygenated TAGs reflected a high ratio of

LA. HPLC analysis of transmethylated hydroxides and hydroperoxides revealed predominantly 13-HPOD and 13-HOD as oxygenated fatty acids of the mono- and double oxygenated TAGs. Furthermore, a signal that could not be identified by TLC analysis but whose intensity diminished as germination proceeded was determined via LC-MS analysis to be TAG with one epoxide as the functional group (Fig. 6A, Table 1).

#### Interaction of CsLbLOX and CsPAT in vitro

For analysis of a direct interaction between CsLbLOX and CsPAT activity, an *in vitro* incubation system was used. Lipid bodies of seeds imbibed for two hours were incubated with recombinant CsLbLOX in the presence or absence of recombinant CsPAT. The isolated lipids were then separated into phospho- and neutral lipids, and the ratio of 13-H(P)OD as the product to LA as the substrate of the LOX reaction was determined. In comparison with the control, lipid bodies of mature seeds incubated without the addition of any recombinant enzyme, the 13-H(P)OD/LA ratio only increased in the presence of CsLbLOX alone or together with CsPAT (Fig. 7). The 13-H(P)OD/LA ratio of seed lipid bodies incubated with CsPAT alone did not show significant differences from the control. Interestingly, the 13-H(P)OD/LA ratio of the TAG fraction was about the same when lipid bodies were incubated with CsLbLOX alone or together with CsPAT (Fig. 7, grey bars). However, the 13-H(P)OD/LA ratio of the phospholipid fraction was significantly increased when lipid bodies were incubated with CsLbLOX together with CsPAT in comparison with CsLbLOX alone (Fig. 7, black bars;  $P=0.00009$  by Student's *t*-test). These results suggest a promoting function of CsPAT for CsLbLOX activity against the phospholipid constituents of the lipid body membrane. For analysis of the degradation of the lipid body membrane via digestion of the integral membrane proteins (caleosins and oleosins) the same *in vitro* system was used and seed lipid bodies were pre-incubated with proteinase K. Subsequent incubation with recombinant CsLbLOX revealed no increase in the LOX activity (data not shown).

## Discussion

In oilseed plants such as *Arabidopsis* or cucumber, storage lipids, localized in lipid bodies, are mobilized to provide carbon equivalents for their post-germinative growth (Graham, 2008). The classical pathway of this storage lipid mobilization is initiated by the activity of TGL, which hydrolyses storage TAGs to glycerol and free fatty acids (Quettier and Eastmond, 2009). The released fatty acids may be converted to acyl-CoAs and degraded via  $\beta$ -oxidation to acetyl-CoAs in the glyoxysomes. Finally, the latter may enter the glyoxylate cycle, and the carbon source is transconverted into glucose via gluconeogenesis (Graham, 2008). Due to the detection of a lipid body-specific LOX in seedlings of cucumber and other oilseed plants such as sunflower and



**Table 1.** Identification of TLC signals by LC-MS

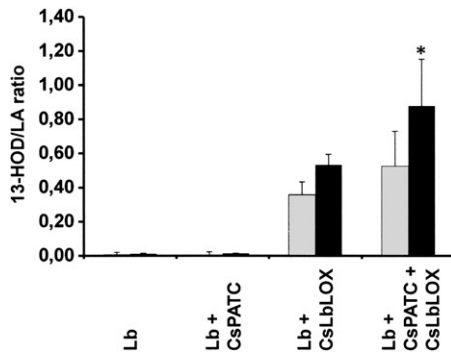
Signal	RT (min)	Experimental mass of (M <sup>+</sup> NH <sub>4</sub> ) <sup>+</sup>	Theoretical mass of (M <sup>+</sup> NH <sub>4</sub> ) <sup>+</sup>	RMS error	Formula	Apparent lipid molecular species; interpretation (TAG with)	Ratio (%)
1epoxyTAG	7.33	910.7487	910.7500	0.0031	C57H100NO7	1×Epoxy-	19
	7.61	912.7658	912.7656	0.0010	C57H102NO7	54:6; 3×18:2	22
	7.76	886.7504	886.7500	0.0017	C55H100NO7	54:5; 2×18:2, 1×18:1	26
	8.16	888.7649	888.7656	0.0018	C55H102NO7	52:4; 1×18:2, 1×18:1, 1×16:1	5
	8.31	888.7626	888.7656	0.0036	C55H102NO7	52:3; 1×18:2, 1×18:1, 1×16:0	3
	8.32	914.7810	914.7813	0.0025	C57H104NO7	52:3; 1×18:2, 1×18:1, 1×16:0	25
1oxTAG	7.2	928.7597	928.7605	0.0026	C57H102NO8	1×HPOD-	13
	7.51	930.7753	930.7762	0.0024	C57H104NO8	54:6; 3×18:2	11
	7.6	904.7603	904.7605	0.0019	C55H102NO8	54:5; 2×18:2, 1×18:1	14
	8.02	906.7751	906.7762	0.0026	C55H104NO8	52:4; 2×18:2, 1×16:0	13
	8.07	932.7913	932.7918	0.0027	C57H106NO8	52:3; 2×18:1, 1×16:1	19
	8.05	880.7585	880.7605	0.0035	C53H102NO8	54:4; 2×18:1, 1×18:2	6
	8.65	934.8076	934.8075	0.0009	C57H108NO8	50:2; 2×16:0, 1×18:2	15
	8.69	908.7909	908.7918	0.0020	C55H106NO8	54:3; 3×18:1	9
	8.69	908.7909	908.7918	0.0020	C55H106NO8	52:2; 2×18:1, 1×16:0	9
2oxTAG	6.57	960.7508	960.7504	0.0010	C57H102NO10	2×HPOD-	10
	6.67	936.7532	936.7504	0.0007	C55H102NO10	54:6; 3×18:2	28
	6.74	962.7684	962.7660	0.0005	C57H104NO10	52:4; 2×18:2, 1×16:0	25
	7.03	964.7834	964.7817	0.0026	C57H106NO10	54:5; 2×18:2, 1×18:1	18
	7.03	964.7834	964.7817	0.0026	C57H106NO10	54:4; 2×18:2, 1×18:0	18
	6.78	944.7529	944.7555	0.0061	C57H102NO9	1×HPOD-, 1×HOD-	6
	6.8	918.7410	918.7398	0.0007	C55H100NO9	54:6; 3×18:2	7
7.05	946.7717	946.7711	0.0009	C57H104NO9	52:5; 2×18:2, 1×16:1	6	
TAG	7.89	902.8182	902.8177	0.0013	C57H108NO6	54:5; 2×18:2, 1×18:1	10
	8.05	894.7523	894.7551	0.0035	C57H100NO6	54:3; 3×18:1	4
	8.07	876.8016	876.8020	0.0009	C55H106NO6	54:7; 2×18:2, 1×18:3	11
	8.61	896.7701	896.7707	0.0016	C57H102NO6	52:2; 2×18:1, 1×16:0	28
	9.48	898.7840	898.7864	0.0035	C57H104NO6	54:6; 3×18:2	21
	9.57	872.7686	872.7707	0.0048	C55H102NO6	54:5; 2×18:2, 1×18:1	26

flax, an alternative pathway of storage lipid mobilization has been proposed (Feussner *et al.*, 2001). Here the degradation of storage lipids may be initiated by the hydroperoxidation of either linoleic or linolenic acid residues of the TAGs mediated by the LOX activity.

The germination-relevant storage organelle, the lipid or oil body, is characterized by a stable amphiphatic surface built up of structural proteins, oleosins, and the phospholipid monolayer, which preserve the integrity of lipid bodies and prevent them from undergoing coalescence (Hsieh and Huang, 2004). Until now it has not been clear how lipid-mobilizing enzymes, such as TGL and LOX, obtain access to their substrates through the stable lipid body membrane. Several studies have indicated that the disruption of the integrity of the lipid body membrane may be facilitated by the proteolytic digestion of structural proteins of the lipid bodies (oleosins) and by the partial degradation of the phospholipid monolayer of this organelle, presumably by a patatin-like PLase (Matsui *et al.*, 1999; Noll *et al.*, 2000; Vandana and Bhatla, 2006; Gupta and Bhatla, 2007). In the phospholipid monolayer of lipid bodies of 60-h-old cucumber seedlings, holes of ~80 nm in width and 2.45 nm in

depth could be found, which would be sufficiently large to facilitate access to the TAG matrix for 100 kDa enzymes (Noll *et al.*, 2000). At the same germination time, maximum expression of the patatin-like PLase CsPAT was detectable, suggesting the involvement of this enzyme in the partial degradation of the lipid body membrane (May *et al.*, 1998; Noll *et al.*, 2000). In this study new evidence was found indicating that the mobilization of storage lipids by LOX activity is promoted by a PLase activity, presumably the patatin-like CsPAT in cucumber seedlings.

Analyses of the temporal correlation of the accumulation of CsPAT and the lipid body LOX, CsLbLOX, showed that both proteins occurred transiently at the same time during germination at the lipid body membrane with a maximum amount after 72 h (Fig. 1). These results confirm previous investigations of the appearance of CsPAT and CsLbLOX proteins during germination (Feussner and Kindl, 1992; Feussner *et al.*, 1996; Höhne *et al.*, 1996; May *et al.*, 1998). At early time points of germination the CsLbLOX is present on lipid bodies but it cannot exert its activity on the TAGs inside the lipid bodies; if it is assumed that CsPAT, acting as a PLase, is responsible for the partial



**Fig. 7.** *In vitro* analysis of the interaction of recombinant CsLbLOX and recombinant CsPAT. Lipid bodies (Lb) of seeds imbibed for two hours were incubated with CsLbLOX and/or C-terminal His-tagged CsPAT (CsPATC), as indicated, for 120 min. Lipids were extracted, separated into phospho- and neutral lipids, lipid hydroperoxides were reduced, transmethylated, and the 13-hydro(pero)xylinoic acid/linoleic acid ratio (13-H(P)OD/LA ratio) was determined by reversed-phase HPLC. Ratios of the TAG fraction are shown as grey bars and ratios of the phospholipid fraction are shown as black bars. Data shown represent the mean of three independent experiments. The standard deviation is given. \* $P=0.00009$  by Student's *t*-test.

degradation of the lipid body phospholipid monolayer, CsLbLOX thus gains access to its substrate. Alternatively, CsLbLOX activity may be regulated by an as yet unknown mechanism at the protein level.

The decline in the protein content of the lipid body-associated proteins oleosin and caleosin that started in parallel with the accumulation of CsPAT after 36 h of germination was conspicuous (Fig. 1). This provides evidence for a proteolytic digestion of these structural proteins in order to facilitate the accessibility of the lipid body for lipid-mobilizing enzymes as was proposed in some other studies. Matsui *et al.* (1999) demonstrated that the lipid body LOX of cucumber could act on neutral lipids in lipid bodies only after lipid body proteins were degraded by trypsin *in vitro*. In addition to the observation of partial degradation of oleosin isoforms from 19 kDa to 16 kDa fragments shortly before the degradation of lipid bodies in rapeseed seedlings, the activity of a probable lipid body-associated thiol-protease could be detected in sunflower seedling cotyledons that occurred simultaneously with the degradation of oleosins during germination (Murphy *et al.*, 2001; Vandana and Bhatla, 2006). However, in the latter study it was not shown that oleosins were the actual substrate of the thiol-protease, nor could the corresponding protein or gene be identified. In addition it should be noted that a similar situation was observed for lipid droplets in animal systems (Brasaemle, 2007). Here, lipid body proteins, for example perilipin, prevent lipase attack until they are destabilized by phosphorylation.

Since the whole surface of lipid bodies is protected by oleosins and other associated proteins, a temporally correlated proteolytic degradation of oleosins and the partial decomposition of the lipid body membrane by the patatin-

like PLase CsPAT seem to be very effective to produce 80 nm holes in the phospholipid monolayer, as has been described (Noll *et al.*, 2000). Using the same type of experiments, evidence is provided here that CsPAT has a promoting function for CsLbLOX activity against the phospholipid constituents of the lipid body membrane, at least *in vitro*. However, it should be noted that all attempts to analyse the function of CsPAT under *in vivo* conditions failed. Expression of the enzyme under the control of a seed-specific promoter in tobacco and *Arabidopsis* never led to plants in which CsPAT accumulated to amounts that were comparable with those observed in seedlings (Hause *et al.*, 2000).

Investigations of the intracellular localization of CsLbLOX and CsPAT by immunogold labelling and transmission electron microscopy could demonstrate primarily the direct localization of both proteins at the lipid body membrane and for LOX in the surrounding cytosol of this organelle (Fig. 2, and Supplementary Figs 1–3 at *JXB* online). So far the localization of the CsPAT only could be shown to be confined to lipid bodies via immunodetection in subcellular fractions of cucumber seedlings (May *et al.*, 1998), whereas a previous analysis revealed an immunocytochemical localization of the CsLbLOX at the membrane of lipid bodies in cotyledons (Feussner *et al.*, 1996). In addition to the similar accumulation of CsPAT and CsLbLOX protein at lipid bodies during germination, the intracellular localization provides further evidence for the involvement of CsPAT in storage lipid mobilization.

In a previous analysis, PLase A<sub>2</sub> activity could be detected for the heterologous expressed patatin-like protein CsPAT and it was shown that the recombinant protein was able to cleave PC with palmitoyl, linoleoyl, and hydroperoxidized linoleoyl residues at the *sn*2 position equally well (May *et al.*, 2000). However, the existence of this enzyme activity on lipid bodies of cucumber seedlings has never been proven. Here PLase activity was analysed on isolated lipid bodies during germination. With the assumption that CsPAT represents the PLase responsible, lipid bodies were incubated with PC <sup>14</sup>C-labelled at the *sn*2 residue. Using this approach PLase activity was detectable, whereas the occurrence and the PLase activity during germination correlated with the occurrence of CsPAT protein (Figs 1, 2, 3B). This result indicates indirectly the partial degradation of the lipid body phospholipid monolayer by a PLase. In protoplasts from sunflower seedlings a PLase A<sub>2</sub> activity could also be demonstrated, which was found *in vivo* from 2 d of germination onward and was enhanced further on the surface of lipid bodies in 4- to 5-d-old cotyledons (Gupta and Bhatla, 2007). Although the present results only provide evidence for a promoting function of CsPAT in the case of CsLbLOX activity against the phospholipid constituents of the lipid body membrane, it is tempting to assume that the potential partial degradation of the lipid body membrane via PLases represents a general mechanism during the early phase of germination in oilseeds to initiate the degradation of storage lipids and to ensure a full supply of carbon and energy equivalents

before an increased mobilization of TAGs during later stages of germination via TGL dominates (Fig. 3B, C).

## Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1.** Intracellular localization of CsPAT. Transmission electron microscope images of cotyledons from 72 h germinated etiolated seedlings. Immunogold labelling with antibodies against CsPAT with significant localization of the 5 nm gold particles at the membrane of the lipid bodies.

**Figure S2.** Intracellular localization of glyoxysomes. Transmission electron microscope images of cotyledons from 72 h germinated etiolated seedlings. Immunogold labelling with antibodies against isocitrate lyase with significant localization of the 5 nm gold particles at the matrix of glyoxysomes.

**Figure S3.** Intracellular localization of glyoxysomes. Transmission electron microscope images of cotyledons from 72 h germinated etiolated seedlings. Immunogold labelling with antibodies against glycolate oxidase with significant localization of the 5 nm gold particles at the matrix of glyoxysomes.

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## References

- Andrews DL, Beames B, Summers MD, Park WD.** 1988. Characterization of the lipid acyl hydrolase activity of the major potato (*Solanum tuberosum*) tuber protein, patatin, by cloning and abundant expression in a baculovirus vector. *Biochemical Journal* **252**, 199–206.
- Balkenhohl T, Kühn H, Wasternack C, Feussner I.** 1998. A lipase specific for esterified oxygenated polyenoic fatty acids in lipid bodies of cucumber cotyledons. In: Sánchez J, Cerdá-Olmedo E, Martínez-Force E, eds. *Advances in plant lipid research*. Sevilla: Secretariado de Publicaciones de la Universidad de Sevilla, 320–322.
- Baud S, Dichow NR, Kelemen Z, et al.** 2009. Regulation of HSD1 in seeds of *Arabidopsis thaliana*. *Plant and Cell Physiology* **50**, 1463–1478.
- Behrends W, Rausch U, Löffler HG, Kindl H.** 1982. Purification of glycolate oxidase from greening cucumber cotyledons. *Planta* **156**, 566–571.
- Beisson F, Ferte N, Bruley S, Vouloury R, Verger R, Arondel V.** 2001. Oil-bodies as substrates for lipolytic enzymes. *Biochimica et Biophysica Acta* **1531**, 47–58.
- Bligh EG, Dyer WJ.** 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917.
- Brasaemle DL.** 2007. Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *Journal of Lipid Research* **48**, 2547–2559.
- Chen ECF, Tai SSK, Peng CC, Tzen JTC.** 1998. Identification of three novel unique proteins in seed oil bodies of sesame. *Plant and Cell Physiology* **39**, 935–941.
- Feussner I, Bachmann A, Höhne M, Kindl H.** 1998. All three acyl moieties of trilinolein are efficiently oxygenated by recombinant His-tagged lipid body lipoxygenase. *in vitro*. *FEBS Letters* **431**, 433–436.
- Feussner I, Balkenhohl TJ, Porzel A, Kühn H, Wasternack C.** 1997. Structural elucidation of oxygenated storage lipids in cucumber cotyledons. Implication of lipid body lipoxygenase in lipid mobilization during germination. *Journal of Biological Chemistry* **272**, 21635–21641.
- Feussner I, Hause B, Nellen A, Wasternack C, Kindl H.** 1996. Lipid-body lipoxygenase is expressed in cotyledons during germination prior to other lipoxygenase forms. *Planta* **198**, 288–293.
- Feussner I, Kindl H.** 1992. A lipoxygenase is the main lipid body protein in cucumber and soybean cotyledons during the stage of triglyceride mobilization. *FEBS Letters* **298**, 223–225.
- Feussner I, Kindl H.** 1994. Particulate and soluble lipoxygenase isoenzymes—comparison of molecular and enzymatic properties. *Planta* **194**, 22–28.
- Feussner I, Kühn H, Wasternack C.** 2001. The lipoxygenase dependent degradation of storage lipids. *Trends in Plant Science* **6**, 268–273.
- Feussner I, Wasternack C, Kindl H, Kühn H.** 1995. Lipoxygenase-catalyzed oxygenation of storage lipids is implicated in lipid mobilization during germination. *Proceedings of the National Academy of Sciences, USA* **92**, 11849–11853.
- Frevert J, Kindl H.** 1978. Plant microbody proteins. Purification and glycoprotein nature of glyoxysomal isocitrate lyase from cucumber cotyledons. *European Journal of Biochemistry* **92**, 35–43.
- Gerhardt B, Fischer K, Balkenhohl TJ, Pohnert G, Kühn H, Wasternack C, Feussner I.** 2005. Lipoxygenase-mediated metabolism of storage lipids in germinating sunflower cotyledons and  $\beta$ -oxidation of (9Z,11E,13S)-13-hydroxy-octadeca-9,11-dienoic acid by the cotyledonary glyoxysomes. *Planta* **220**, 919–930.
- Göbel C, Feussner I, Rosahl S.** 2003. Lipid peroxidation during the hypersensitive response in potato in the absence of 9-lipoxygenases. *Journal of Biological Chemistry* **278**, 52834–52840.
- Goodman JM.** 2008. The gregarious lipid droplet. *Journal of Biological Chemistry* **283**, 28005–28009.
- Graham IA.** 2008. Seed storage oil mobilization. *Annual Review of Plant Biology* **59**, 115–142.
- Gupta A, Bhatla SC.** 2007. Preferential phospholipase A<sub>2</sub> activity on the oil bodies in cotyledons during seed germination in *Helianthus annuus* L. cv. *Morden*. *Plant Science* **172**, 535–543.
- Hanano A, Burcklen M, Flenet M, Ivancich A, Louwagie M, Garin J, Blee E.** 2006. Plant seed peroxygenase is an original

heme-oxygenase with an EF-hand calcium binding motif. *Journal of Biological Chemistry* **281**, 33140–33151.

**Hause B, Weichert H, Höhne M, Kindl H, Feussner I.** 2000. Expression of cucumber lipid body lipoxygenase in transgenic tobacco. Lipid body lipoxygenase is correctly targeted to seed lipid bodies. *Planta* **210**, 708–714.

**Höhne M, Nellen A, Schwennesen K, Kindl H.** 1996. Lipid body lipoxygenase characterized by protein fragmentation, cDNA sequence and very early expression of the enzyme during germination of cucumber seeds. *European Journal of Biochemistry* **241**, 6–11.

**Hsieh K, Huang AHC.** 2004. Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells. *Plant Physiology* **136**, 3427–3434.

**Huang AHC.** 1992. Oil bodies and oleosins in seeds. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**, 177–200.

**Huang AHC.** 1996. Oleosins and oil bodies in seeds and other organs. *Plant Physiology* **110**, 1055–1061.

**Lang I, Feussner I.** 2007. Oxylipin formation in *Nostoc punctiforme* (PCC73102). *Phytochemistry* **68**, 1120–1127.

**Leprince O, Vanaelst AC, Pritchard HW, Murphy DJ.** 1998. Oleosins prevent oil-body coalescence during seed imbibition as suggested by a low-temperature scanning electron microscope study of desiccation-tolerant and -sensitive oilseeds. *Planta* **204**, 109–119.

**Liavonchanka A, Feussner I.** 2006. Lipoxygenases: occurrence, functions and catalysis. *Journal of Plant Physiology* **163**, 348–357.

**Lin LJ, Tai SS, Peng CC, Tzen JT.** 2002. Steroleosin, a sterol-binding dehydrogenase in seed oil bodies. *Plant Physiology* **128**, 1200–1211.

**Matsui K, Hijiya K, Tabuchi Y, Kajiwara T.** 1999. Cucumber cotyledon lipoxygenase during postgerminative growth. Its expression and action on lipid bodies. *Plant Physiology* **119**, 1279–1287.

**May C, Höhne M, Gnau P, Schwennesen K, Kindl H.** 2000. The N-terminal  $\beta$ -barrel structure of lipid body lipoxygenase mediates its binding to liposomes and lipid bodies. *European Journal of Biochemistry* **267**, 1100–1109.

**May C, Preisig-Müller R, Höhne M, Gnau P, Kindl H.** 1998. A phospholipase  $A_2$  is transiently synthesized during seed germination and localized to lipid bodies. *Biochimica et Biophysica Acta* **1393**, 267–276.

**Murphy DJ.** 2001. The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Progress in Lipid Research* **40**, 325–438.

**Murphy DJ, Hernández-Pinzón I, Patel K.** 2001. Role of lipid bodies and lipid-body proteins in seeds and other tissues. *Journal of Plant Physiology* **158**, 471–478.

**Murphy DJ, Vance J.** 1999. Mechanisms of lipid body formation. *Trends in Biochemical Sciences* **24**, 109–115.

**Naested H, Frandsen GI, Jauh GY, Hernandez-Pinzon I, Nielsen HB, Murphy DJ, Rogers JC, Mundy J.** 2000. Caleosins:  $Ca^{2+}$ -binding proteins associated with lipid bodies. *Plant Molecular Biology* **44**, 463–476.

**Noll F, May C, Kindl H.** 2000. Phospholipid monolayer of plant lipid bodies attacked by phospholipase  $A_2$  shows 80 nm holes analyzed by atomic force microscopy. *Biophysical Chemistry* **86**, 29–35.

**Penfield S, Pinfield-Wells HM, Graham IA.** 2009. Storage reserve mobilisation and seedling establishment in Arabidopsis. *The Arabidopsis book. The American Society of Plant Biologists* 1–17.

**Quettier A-L, Eastmond PJ.** 2009. Storage oil hydrolysis during early seedling growth. *Plant Physiology and Biochemistry* **47**, 485–490.

**Sonnewald U, Studer D, Rocha-Sosa M, Willmitzer L.** 1989. Immunocytochemical localization of patatin, the major glycoprotein in potato (*Solanum tuberosum* L.) tubers. *Planta* **178**, 176–183.

**Stumpe M, Carsjens J-G, Stenzel I, Göbel C, Lang I, Pawlowski K, Hause B, Feussner I.** 2005. Lipid metabolism in arbuscular mycorrhizal roots of *Medicago truncatula*. *Phytochemistry* **66**, 781–791.

**Tzen JTC, Cao Y-z, Laurent P, Ratnayake C, Huang AHC.** 1993. Lipids, proteins, and structure of seed oil bodies from diverse species. *Plant Physiology* **101**, 267–276.

**Vandana S, Bhatla SC.** 2006. Evidence for the probable oil body association of a thiol-protease, leading to oleosin degradation in sunflower seedling cotyledons. *Plant Physiology and Biochemistry* **44**, 714–723.

**Weichert H, Kolbe A, Kraus A, Wasternack C, Feussner I.** 2002. Metabolic profiling of oxylipins in germinating cucumber seedlings—lipoxygenase-dependent degradation of triacylglycerols and biosynthesis of volatile aldehydes. *Planta* **215**, 612–619.