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Lipoxygenases during *Brassica napus* seed germination ☆

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Abstract

The peroxidation of polyunsaturated fatty acids is mostly catalyzed by members of the lipoxygenase enzyme family. Lipoxygenase products can be metabolized further in the oxylipin pathway and are known as signalling substances that play a role in plant development as well as in plant responses to wounding and pathogen attack. Apart from accumulating data in model plants like *Arabidopsis*, information on the relevance of lipid peroxide metabolism in the crop plant oilseed rape is scarce. Thus we aimed to analyze lipoxygenases and oxylipin patterns in seedlings of oilseed rape. RNA isolated from 3 day etiolated seedlings contains mRNAs for at least two different lipoxygenases. These have been cloned as cDNAs and named Bn-Lox-1fl and Bn-Lox-2fl. The protein encoded by Bn-Lox-2fl was identified as a 13-lipoxygenase by expression in *Escherichia coli*. The Bn-Lox-1fl yielded an inactive protein when expressed in *E. coli*. Based on Bn-Lox-1fl active site determinants and on sequence homology the Bn-Lox-1fl is most likely a 9-lipoxygenase. Both genes are expressed in light-grown and etiolated cotyledons as well as in leaves. Bn-Lox-2fl protein is more abundant in cotyledons of etiolated seedlings than in cotyledons of green seedlings. Both 13- and 9-lipoxygenase-derived hydroperoxides can be detected during germination. Etiolated seedlings contain more lipoxygenase-derived hydroperoxides in non esterified fatty acids than green seedlings. The 13-lipoxygenase derivatives are 6-8-fold more abundant than the 9-derivatives. Lipoxygenase-derived hydroperoxides in esterified lipids are almost not present during germination. These results suggest that 13-lipoxygenases acting on free fatty acids dominate during *B. napus* seed germination.

Keywords: Brassica napus; *Brassicaceae*, β-Oxidation; Lipid peroxidation; Oil seed rape; Oxylipin formation

1. Introduction

Lipoxygenases (LOXs) are non-heme iron-containing dioxygenases which catalyse the oxidation of polyunsaturated fatty acids containing a *cis*, *cis*-1,4-pentadiene moiety (Brash, 1999). The addition of molecular oxygen at C9 or C13 of the acyl chain leads to formation of either 9- or 13-hydroperoxides derived from linoleic or linolenic acid, the two most common LOX substrates in higher plants (Feussner and Wasternack, 2002). Both hydroperoxide derivatives can be enzymatically cleaved to aldehydes and ω-oxo acids (Blée, 2002), and the 13-hydroperoxide of linolenic acid can serve as a precursor for the synthesis of jasmonic acid (Wasternack and Hause, 2002).

Abbreviations: CP-HPLC, chiral phase-HPLC; GC, gas chromatography; HODE, hydroxy octadecadienoic acid; HOTrE, hydroxy octadecatrienoic acid; HPODE, hydroperoxy octadecadienoic acid; HPOTrE, hydroperoxy octadecatrienoic acid; LOX, lipoxygenase; LBLOX, lipid body lipoxygenase; RP-HPLC, reversed phase-HPLC; SP-HPLC, straight phase-HPLC.

☆ *Sequence data:* The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL data bank with accession numbers AY162142, AY162143.

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While knowledge on the physiological function of plant LOXs is just beginning to accumulate, a huge amount of data exists on the enzymatic reaction mechanism of plant LOXs (Liavonchanka and Feussner, 2006). Moreover, the crystal structure of soybean seed LOX1 has been described in detail (Minor et al., 1996). Recently, amino acid determinants forming the shape of the substrate-binding pocket have been identified in LOX1-type enzymes from cucumber, soybean, potato and pea (Hornung et al., 1999, 2000; Hughes et al., 2001a,b; Ruddat et al., 2004). Thus it seems that a (Ser/Thr)–Val motif at the bottom of the substrate-binding pocket may be indicative for plant 9-LOXs, whereas a (Cys/Ser/Thr)–(Phe/His) motif at the bottom of the substrate-binding pocket may be indicative for plant 13-LOXs (Liavonchanka and Feussner, 2006).

Plant LOXs can be divided into two families based on their structural features. The gene subfamily named LOX2-type-LOXs has an amino terminal plastid targeting peptide, and the gene family named LOX1 is lacking this extension (Feussner and Wasternack, 2002). Several LOXs of the LOX1-subfamily have been identified, e.g. from *Arabidopsis thaliana* (Melan et al., 1993), potato (Geerts et al., 1994), and cucumber (Matsui et al., 2006). The first identified LOX2 was AtLOX2 from *A. thaliana* (Bell et al., 1995), and since then other LOXs of type 2 have been characterized, including two from tomato (Heitz et al., 1997). The AtLOX2 and the two tomato LOXs are targeted to isolated pea chloroplasts *in vitro* (Bell et al., 1995; Heitz et al., 1997). An important function of the chloroplast-localized LOX2-type LOXs is their involvement in catalysing the oxygenation of free linolenic acid as the initial step in jasmonic acid biosynthesis (Wasternack et al., 2006).

Other proposed function for LOXs in plants is their involvement in growth and development, since high amounts of LOX protein are found in rapidly growing tissue (Siedow, 1991). LOXs also play a role in plant–microbe interaction, as their genes are induced in response to pathogen attack and upon wounding (Rosahl and Feussner, 2005). A LOX1-type 13-LOX is associated with the lipid body membrane of germinating cucumber seeds and was shown to accept free polyunsaturated fatty acids as substrates as well as fatty acid esters within the group of storage lipids (Feussner et al., 1997). This finding suggested that at least in some oil seeds the initial step in the degradation of polyunsaturated fatty acids housed within the triacylglycerols via β-oxidation is initiated by a specific lipid body-associated LOX, named LBLOX (Feussner et al., 2001).

To gain more information on the role of LOXs during germination in crop plants we aimed to analyze seedlings of *Brassica napus*. Here, two different LOX cDNAs were identified from germinating seeds. The expression of the corresponding genes and proteins and the accumulation of their metabolites were analysed during germination. The analyses suggest that primarily 13-LOXs are active during *B. napus* seed germination in the dark.

2. Results

2.1. Isolation of two different cDNAs encoding LOXs

Two different LOX cDNAs were obtained from RNA isolated from 3 day-old etiolated cotyledons. One, named *Bn-Lox-1fl*, encodes an 840 amino acid polypeptide. The other cDNA, named *Bn-Lox-2fl*, encodes a polypeptide of 892 amino acids.

The nucleotide sequences of *Bn-Lox-1fl* and *Bn-Lox-2fl* have no significant homology against each other, but the deduced amino acid sequences are 41% identical. The two encoded proteins of *Bn-Lox-1fl* and *Bn-Lox-2fl* are aligned in Fig. 1. The positional specificity of plant LOXs may be determined by specific amino acids at the bottom of the substrate-binding pocket (Liavonchanka and Feussner, 2006). Thus, *Bn-Lox-1fl* may code for a 9-LOX since its sequence contained a Thr–Val motif at the critical position (Fig. 1, asterisks). Its sequence shows highest similarity to the AtLOX1 from *Arabidopsis* (92%) (Melan et al., 1993) and the LOXA from tomato (68%) (Ferrie et al., 1994). *Bn-Lox-2fl* may encode a 13-LOX since its sequence harbours an Arg–Phe motif at the same position (Fig. 1, asterisks). Its sequence shows highest similarity to the AtLOX2 from *Arabidopsis* (75%) (Bell et al., 1995) and the LOXC1 from rice (54%) (Peng et al., 1994). Interestingly, this is the first 13-LOX from higher plants harbouring an Arg residue at this critical position. In all previously analysed plant LOXs, this first residue is a smaller Ser, Thr or Cys (Liavonchanka and Feussner, 2006) except PpLOX1 that was isolated from the non-flowering plant *Physcomitrella patens* that harbours a histidine residue at this position (Senger et al., 2005). Amino acid residues involved in the binding of the iron in the active site (His517, His522, His708 and Ile857 of Bn-Lox-1fl) are highly conserved throughout all LOXs (Siedow, 1991) as they are between the two amino acid sequences (Fig. 1). However, the Bn-Lox-1fl protein has a Ser at position 712 instead of the fifth conserved ligand, an Asn residue, but the Bn-Lox-2fl protein harbours the conserved Asn residue. All known cDNAs coding for plant LOXs have an Asn codon at this position, while all sequences coding for mammalian LOXs have a His codon in this position (Kühn et al., 2005). Since the Ser codon was unexpected, we also sequenced the corresponding region in genomic DNA and found that the AGC codon for the Ser712 of Bn-Lox-1fl is present in the DNA of *B. napus*, *B. oleracea* and *B. rapa* genomes. This suggests that residue Ser712 is not a cloning artefact. The *Bn-lox-2fl* encodes an amino terminal peptide of 34 amino acids which has features of a plastidic transit peptide. The putative cleavage site is between Ser34 and Ala35 and is indicated in Fig. 1 by a vertical arrow.

A. thaliana is the closest relative of oilseed rape of which the genome sequence is available. From its genome 6 different LOX genes have been identified (Feussner and Wasternack, 2002): two LOX1-type enzymes coding for 9-LOXs (AtLOX1 and 5) and four LOX2-type enzymes coding

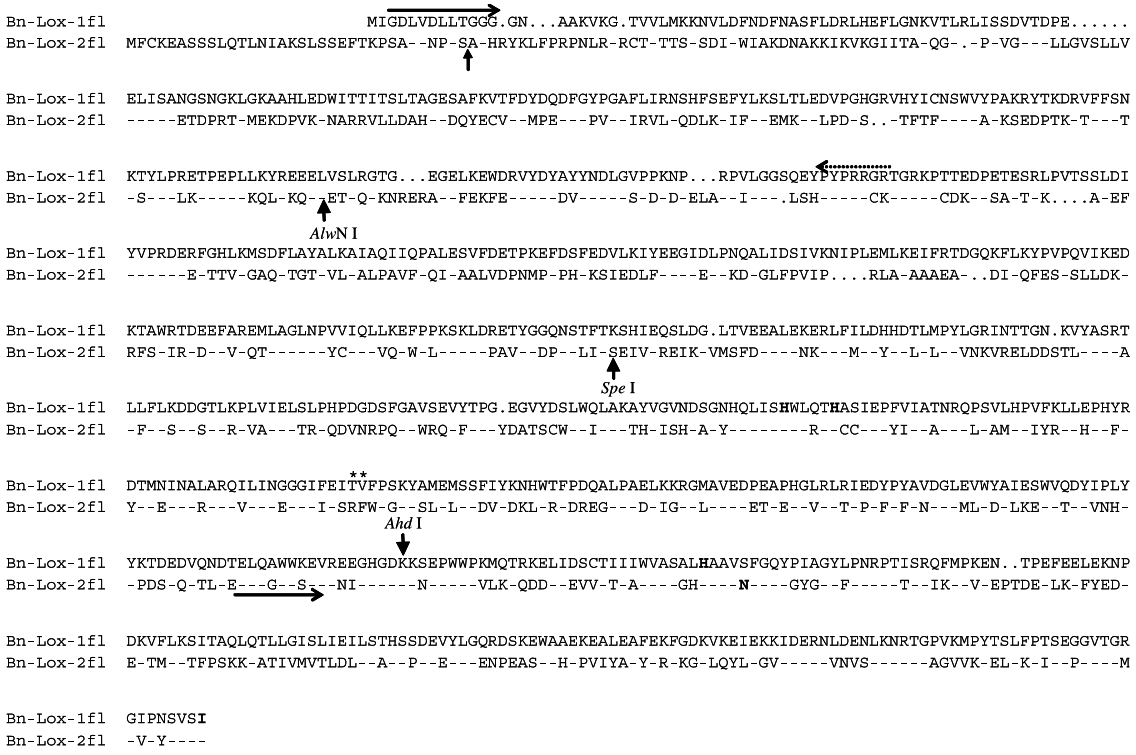

Fig. 1. Sequence analysis of the two LOX cDNAs. Deduced amino acid sequences Bn-Lox-1fl and Bn-Lox-2fl were aligned using CLUSTAL W (Thompson et al., 1994). Identical residues are indicated by a hyphen (-). Dots (·) indicate gaps introduced to maximize the alignment. The putative transit peptide cleavage site in the deduced amino acid sequence of Bn-Lox-2fl is indicated by a vertical arrow. Amino acids thought to be involved in ligand binding of the iron in the active site (His517, His522, His708 and Ile857 of Bn-Lox-1fl and Asn746 of Bn-Lox-2fl) are indicated in bold letters. The residues Thr574-Val575 (Bn-Lox1fl) possibly determining 9-LOX specificity and the corresponding residues Arg/Phe in Bn-Lox-2fl possibly determining 13-LOX activity are indicated by asterisks. The regions from which the primers were derived to obtain the two initial partial clones are indicated by horizontal arrows. *Ahd*I indicates the position of the restriction site used to assemble the Bn-Lox-1 cDNA sequence. *Spe*I and *Alw*NI are the positions of the two restriction sites used to assemble the entire Bn-Lox-2fl cDNA.

for 13-LOXs (AtLOX2, 3, 4 and 6). Therefore we performed phylogenetic tree analysis in order to support the finding that *Bn-lox-1fl* is a close homologue of AtLOX1 and *Bn-lox-2fl* a homologue of AtLOX2. As shown in Fig. 2 the newly identified *Bn-lox-1fl* from oilseed rape groups indeed best with ALOX1 and *Bn-lox-2fl* with AtLOX2 into one subfamily.

2.2. Heterologous expression of LOX protein

The *Bn-Lox-2fl*, the truncated *Bn-Lox-2*, without the putative plastid transit peptide and *Bn-Lox-1fl*, were expressed in *E. coli* M15 cells. Cultures induced at 37 °C yield LOX proteins of the expected sizes, which are deposited in the inclusion body fraction. However, induction followed by growth at 10 °C rendered a portion of the LOX protein soluble. The soluble LOXs were incubated with linoleic acid, and the resulting products were characterized by HPLC. The Bn-Lox-2 protein converted linoleic acid almost exclusively into 13-H(P)ODE (Fig. 3). The formation was due to enzymatic lipid peroxidation since CP-HPLC analysis showed a strong preponderance of the *S*-enantiomer of 13-H(P)ODE (*S*/*R* ratio of 91/9, inset in Fig. 3). The full-sized Bn-Lox-2fl polypeptide, with the putative plastid transit peptide, was inactive, suggesting that the presence of the transit peptide prevents proper folding of the enzyme. The truncated recombinant Bn-Lox-2 protein has a pH optimum of about pH 6 and the enzyme accepts free polyenoic fatty acids as substrates. Conversion of esterified fatty acid derivatives was not observed. α-Linolenic acid was converted completely into 13-H(P)OTrE (>99%), and arachidonic acid into 15-H(P)ETE (>99%). When the endogenous substrates linoleic and α-linolenic acid were applied in equal amounts as a mixture, a preferred formation of 13-HOTrE over 13-HODE (ratio of 77/23) was observed, suggesting that α-linolenic acid is the preferred substrate of Bn-Lox-2. Trilinoleate and phosphatidyl choline did not serve as substrates. Attempts to demonstrate LOX activity of the Bn-lox-1fl-encoded polypeptide at pH 5.0 and 7.5 were not successful. Thus we tested whether the lack of activity may be due to Ser at position 712 and changed the AGC codon to an AAC codon for Asn by site-directed mutagenesis. However, this did not lead to an active enzyme either.

2.3. Expression of Bn-Lox-1fl and Bn-Lox-2fl

Southern blot analyses of *Brassica* DNA suggested that Bn-Lox-1fl is only present in a few copies while Bn-Lox-2fl seemed to be present in multiple copies in the *Brassica*

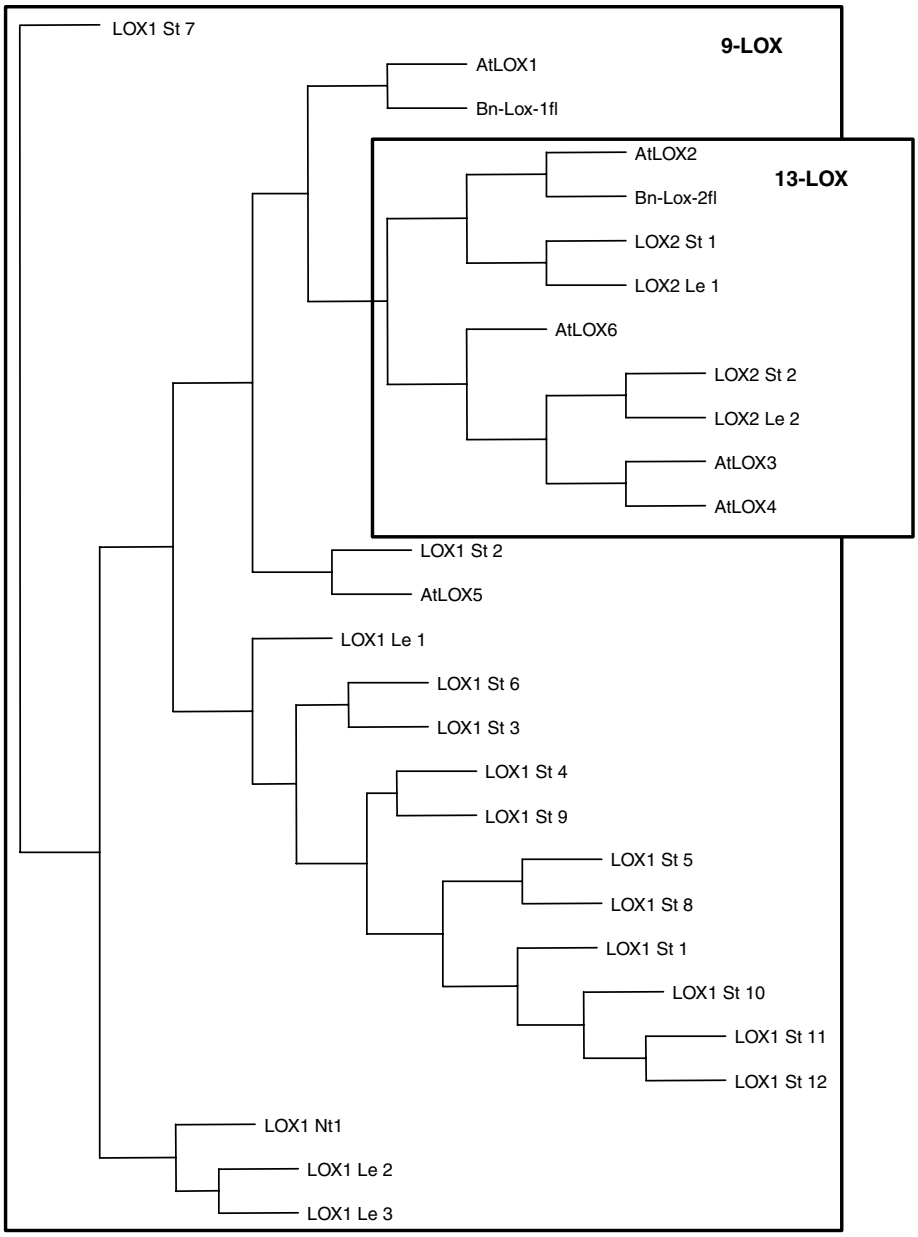

Fig. 2. Phylogenetic tree analysis of certain plant LOXs. The analysis was performed with phylip 3.5, and the proteins mentioned in the tree refer to the corresponding accession numbers in the gene bank. For clarification, within the tree only sequences from distinct plant species have been included and have been partially renamed according the nomenclature of (Feussner and Wasternack, 2002): *Arabidopsis thaliana*: AtLOX1 (Q06327), AtLOX2 (P38418), AtLOX3 (AAF79461), AtLOX4 (AAF21176), AtLOX5 (CAC19365), AtLOX6 (AAG52309); *Lycopersicum esculentum*: LOX1:Le:1 (tomLOXA, P38415), LOX1:Le:2 (tomLOXB, P38416), LOX1:Le:3 (tomLOXtox, AAG21691), LOX2:Le:1 (tomLOXC, AAB65766), LOX2:Le:2 (tomLOXD, AAB65767); *Nicotiana tabacum*: LOX1:Nt:1 (NtLOX, S57964); *Solanum tuberosum*: LOX1:St:1 (SOLTULOX1, S44940), LOX1:St:2 (STLOX, AAD09202), LOX1:St:3 (StLOX1, S73865), LOX1:St:4 (CAA64766), LOX1:St:5 (CAA64765), LOX1:St:6 (POTLX-2, AAB67860), LOX1:St:7 (POTLX-3, AAB67865), LOX1:St:8 (POTLX-1, AAB67858), LOX1:St:9 (AAD04258), LOX1:St:10 (pLOX2, AAB81595), LOX1:St:11 (pLOX1, AAB81594), LOX1:St:12 (CAB65460), LOX2:St:1 (StLOXH1, CAA65268), LOX2:St:2 (St-LOXH3, CAA65269).

genome (results not shown). The expression of these genes was analysed by semi-quantitative RT-PCR with specific primers on RNA isolated from cotyledons during germination in the dark and in the light and from different tissues of the *B. napus* plants (Fig. 4a and b). Transcripts for *Bn-Lox-1fl* and *Bn-Lox-2fl* were detectable in cotyledons from etiolated seedlings and green seedlings throughout the 12-day period (Fig. 4a and b, lanes 1–8). Comparing the bands for *Bn-Lox-1fl* and *Bn-Lox-2fl* the *Bn-Lox-2fl* transcript was detectable from day 2 on throughout the analyzed period both in etiolated and green seedlings. The *Bn-Lox-1fl* transcript was prominent at day 2 but only weakly present in the later part of the period analyzed. It appears that in etiolated seedlings the *Bn-Lox-2fl* transcript is more abundant than *Bn-Lox-1fl* in particular at the later stages of germination. Both transcripts were present in 3-

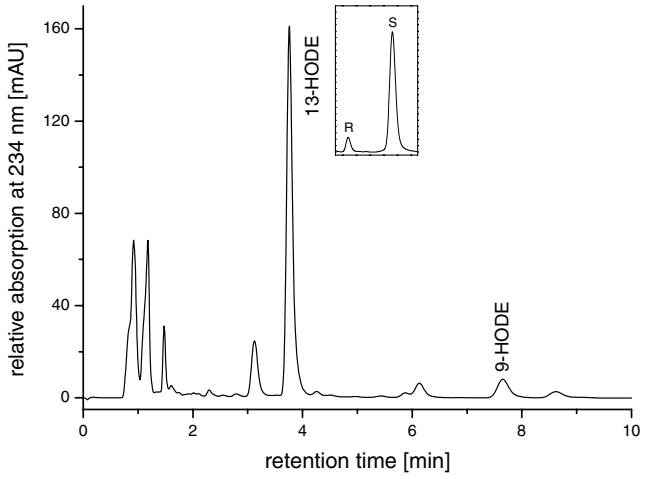

Fig. 3. SP-HPLC analysis of hydro(pero)xy fatty acids formed from linoleic acid by Bn-lox-2 polypeptide expressed in *E. coli*. The soluble protein was incubated with linoleic acid at room temperature at pH 7.5 for 20 min. The formed hydroperoxy fatty acid derivatives were reduced by adding sodium borohydride, and the reaction was stopped by lowering the pH to 4.0 with glacial acid. Fatty acids were isolated by extraction with diethyl ether, and oxygenated linoleic acid derivatives were isolated by RP-HPLC. Positional isomers were separated by SP-HPLC. Analysis of the *S*/*R*-ratio of 13-HODE by CP-HPLC is shown in the inset.

week-old true leaves (Fig. 4a and b, lane 10). In addition, the *Bn-Lox-1fl* was expressed in senescent leaves and in roots (Fig. 4a, lanes 11 and 12) while the *Bn-Lox-2fl* transcript was almost absent from these tissues. The *Bn-Lox-2fl* transcript was detected in the dry seed, indicating that the mRNA is synthesized and stored during seed maturation (Fig. 4a, lane 9).

2.4. Western blot analyses of LOX polypeptides

Two antibodies were generated using an N-terminal and a C-terminal peptide from Bn-Lox-2fl as antigen named A and B, respectively. Both antibodies detected only the Bn-Lox-2fl polypeptide (Fig. 5a, lane 2, part I and II) while an antibody against the cucumber LB LOX recognized both he Bn-lox-1 and the Bn-lox-2 polypeptide (Fig. 5a, lane 1 and 2, part III). In extracts of etiolated cotyledons, the three antibodies recognized a 100 kDa LOX polypeptide during early germination (Fig. 5b, lane 6–10, part I, II and III). The LOX polypeptide was more prominent in etiolated cotyledons than in green cotyledons using antibody B and the LB LOX antibody (Fig. 5b, part II and III), while the LOX polypeptide was not detectable in green cotyledons using antibody A (Fig. 5b, lane 1–5, part I). Since the cucumber LB LOX antibody yielded a polypeptide pattern similar to the pattern recognized by antibody B, the similarity in the patterns suggests that LOX1-type polypeptides in *B. napus* may not be abundant protein during germination. While LOX polypeptide recognized by antibody B barely was detectable in extracts of 2 weeks old green cotyledons it appeared after 3 and 4 weeks of germination (Fig. 5c, lane 1, 2 and 3). It also became detectable after treatment of the 2 weeks old green cotyledons with jasmonic acid (Fig. 5c lane 5 and 6). The protein was absent in senescent cotyledons (Fig. 5c, lane 4) and in senescent leaves (Fig. 5d, lane 8). The LOX polypeptide was also present in true leaves of 10-week-old plants (Fig. 5c, lane 7). Interestingly, two different sized LOX polypeptides were present in extracts of immature flowers, in mature flowers and in immature pods (Fig. 5c lane 9, 10, 11 and 12). In conclusion, these analyses suggest that the LOX2-type proteins in *B. napus* are abundantly present in etiolated cotyledons during germination, in true leaves, immature and mature flowers, as well as immature pods.

2.5. Fatty acids and LOX-derived oxylipins during germination

The so-called "oxylipin signature" comprising LOX-derived metabolites may be diagnostic for the endogenous

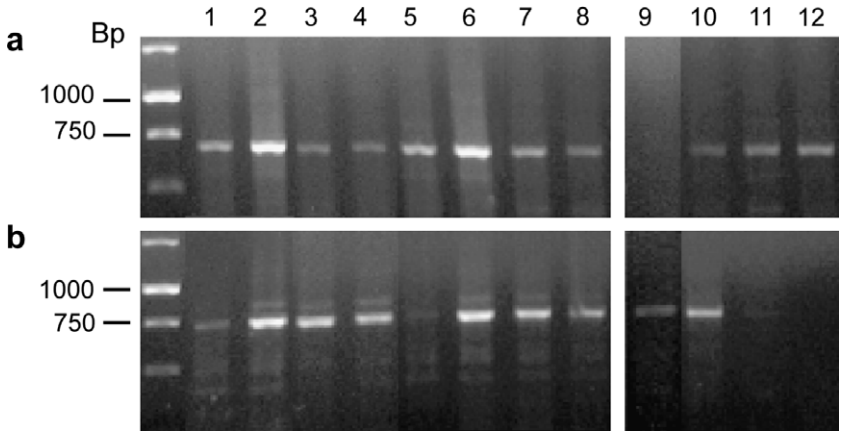

Fig. 4. RT-PCR expression analysis of *Bn-Lox-1* and *Bn-Lox-2*. RT-PCR products were obtained by using primers specific for *Bn-lox-1fl* (a) and *Bn-lox-2fl* (b). Lanes 1, 2, 3 and 4: RNA was isolated from etiolated cotyledons germinated for 1, 2, 5 and 12 days, respectively. Lanes 5, 6, 7 and 8: RNA was isolated from light-grown cotyledons germinated for 1, 2, 5 and 12 days, respectively. Lane 9: RNA was isolated from dry seeds. Lane 10: RNA was isolated from true leaves. Lane 11: RNA was isolated from senescent leaves. Lane 12: RNA was isolated from roots.

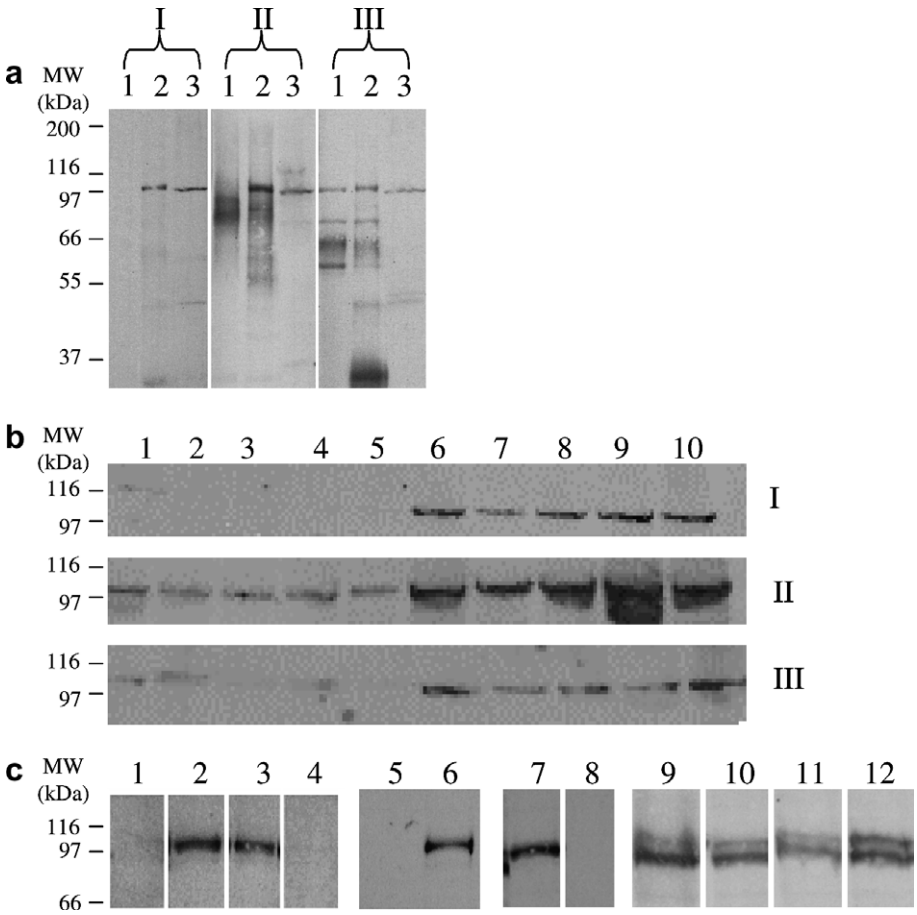

Fig. 5. Western blot analysis. (a) Specificity of the different antibodies used. Proteins were separated by SDS-PAGE, blotted onto nitrocellulose membranes and probed with antibodies raised against the N-terminal peptide of Bn-Lox-2, antibody A (I), the C-terminal peptide of Bn-Lox-2, antibody B (II) or the cucumber LBLOX (III). Lane 1: extract of Bn-Lox-1 protein expressed in *E. coli*. Lane 2: extract of Bn-Lox-2 protein expressed in *E. coli*. Lane 3: extract of 5-day-old etiolated cotyledons. (b) Western blot analysis of germinated cotyledons. Extracts of etiolated and green grown cotyledons were homogenized and proteins were separated by SDS-PAGE and blottet onto a nitrocellulose membrane. The blot was probed with antibody A (I), antibody N (II) and the LBLOX antibody (III). Green seedlings: lane 1–5; etiolated seedlings: lane 6–10. Seedling germinated 3 days (lane 1, 6), 5 days (lane 2, 7), 8 days (lane 3, 8), 11 days (lane 4, 9) and 15 days (lane 5, 10). (c) LOX polypeptides from different developmental stages. Different organs of *B. napus* were homogenized, and proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane and probed with ab-B. Lanes 1–3: extract of cotyledons from plants grown for 2, 3 and 4 weeks, respectively. Lane 4: extract of senescent cotyledons from a 5-week-old plant. Lanes 5–6: Extract of seedlings germinated for 2 weeks in the presence of light. Extracts of untreated (lane 5) and methyl jasmonate-treated (lane 6). Lane 7: extracts of leaves from plants grown for 10 weeks. Lane 8: extracts of senescent leaves from a 10-week-old plant. Lane 9: extract of inflorescences from an 8-week-old plant. Lanes 10 and 11: extracts of flowers from plants grown for 9 or 10 weeks, respectively. Lane 12: extract of immature pods from a 10-week-old plant.

activity of a certain LOX and cooperating downstream enzymes (Feussner and Wasternack, 2002). Therefore we compared the oxylipin profile in etiolated and green cotyledons during the first 2 weeks of germination.

Results of the analysis of esterified hydro(pero)xy fatty acids are shown in Fig. 6a and b. In etiolated cotyledons at day 2, newly formed esterified 13-LOX-derived oxylipins 13-HPODE, 13-HPOTrE and 13-HOTrE became detectable (Fig. 6a). All substances increased in their amounts to about 10–19 nmol/g f.w. till day 3 and stayed at that level till the end of the analysed time period. In green cotyledons, this increase was observed as well, but maximum levels of 13-LOX-derived 13-H(P)OTrE were in this tissue about 15 nmol/g f.w. (Fig. 6b). In dry seeds, 13-HODE was a major esterified oxylipin in amounts of 9.5 nmol/g f.w. But it was derived from non-enzymatic lipid peroxidation. The amount of esterified 13-HODE increased to levels of about 12.5 nmol/g f.w. in etiolated cotyledons (Fig. 6a) and decreased continuously in green cotyledons till day 14 to a level of about 2 nmol/g f.w. (Fig. 6b). However, the *R* to *S* ratio suggested a non-enzymatic origin of these compounds (data not shown).

In dry seeds, 9-HODE was detected at 7.1 nmol/g f.w. and was the other major esterified oxylipin. Unlike 13-HODE, this is derived from non-enzymatic lipid peroxidation since its *R* to *S* ratio was higher than 12 to 88 (data not shown). The enzymatic reaction may have occurred during seed maturation or during seed storage. The amount of esterified 9-HODE stayed at that level in etiolated cotyledons (Fig. 6a) while it decreased continuously in green cotyledons till day 11 to a level of about 1 nmol/g f.w. (Fig. 6b). In both etiolated and green tissue, the *R* to *S*

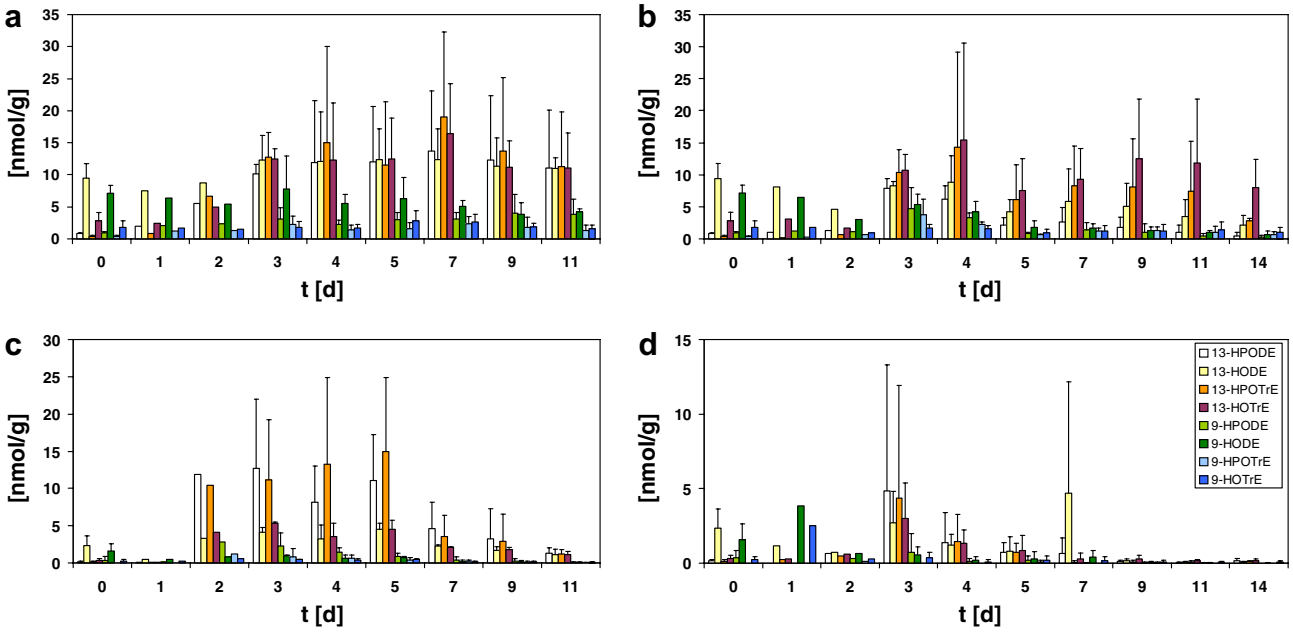

Fig. 6. Oxylipin profiles during germination. *B. napus* seedlings were either grown without light (a, c) or in the presence of light (b, d) for 0–14 days. The cotyledons were harvested and lipids were extracted. The amounts of esterified (a and b) and free (c and d) oxylipins were determined by HPLC. The mean value and the SD of three independent experiments are shown. At days 1 and 2 only the mean values of two independent experiments are shown.

ratio decreased during the time course of germination, suggesting that no further enzymatic synthesis of 9-HODE occurred after the onset of germination. Concerning additional non-enzymatic lipid peroxidation products, a transient accumulation of 12- and 16-HOTrE peaking at day 3 with a level of 2 and 4 nmol/g f.w., respectively, was detected (data not shown).

Results of the analysis of non-esterified oxylipins are shown in Fig. 6c and d. A 3-fold higher accumulation of oxylipins was observed in the etiolated cotyledons in comparison to the light-grown cotyledons (Fig. 6c vs. d). In dry seeds, the 13-HODE and 9-HODE were detected as major non-esterified oxylipins in amounts of 2.2 nmol/g f.w. and 1.5 nmol/g f.w., respectively. But all non-esterified oxylipins detected in dry seeds were derived from non-enzymatic lipid peroxidation, since their *R* to *S* ratio was about 45–55 (data not shown). The major non-esterified oxylipins in etiolated cotyledons (Fig. 6c) were 13-LOX-derived 13-HPODE and 13-HPOTrE. They accumulated transiently between day 2 and 5. The highest level of 13-HPODE was detected at day 3 with 12.7 nmol/g f.w. and of 13-HPOTrE at day 5 with 15 nmol/g f.w. (Fig. 6c). The accumulation of the corresponding hydroxy derivatives was about 3-fold lower in comparison to the hydroperoxides and the *R* to *S* ratio suggested also an enzymatic origin of these compounds (data not shown). In green cotyledons, an increase of 13-LOX-derived 13-HPODE and 13-H(P)OTrE was observed as well, but only at day 3. The maximum levels in this tissue were about 4.5 nmol/g f.w. (Fig. 6d).

As additional oxylipins, two representatives of the allene oxide synthase pathway, 12-oxo phytodienoic acid and jasmonic acid, were measured in one experiment. The 12-oxo phytodienoic acid accumulated transiently with a maximum between day 3 and day 5 to levels of about 0.7 nmol/g f.w. The transient accumulation of this compound was similar in etiolated and green cotyledons. However, a transient accumulation of jasmonic acid was only detectable in green cotyledons, peaking at day 3 with 0.35 nmol/g f.w. Non-esterified 12- and 16-HOTrE derived from non-enzymatic reactions were not detected in these analyses (data not shown).

These analyses of oxylipins suggest that one or more 13-LOXs are active during early stages of seed germination in both etiolated and green seedlings. In addition they seem to act preferentially on free polyunsaturated fatty acids.

3. Discussion

The two LOXs from *B. napus*, Bn-Lox-1fl and BnLox-2fl, described in this paper are the first LOXs identified in *B. napus*. They differ in structure, activity and expression pattern.

The Bn-Lox-2fl protein is 75% identical to the deduced protein of AtLOX2 of Arabidopsis. Both contain a putative amino terminal plastid transit peptide with a predicted cleavage site after Ser35 (Fig. 1). Both contain a Phe residue in the second position described by Hornung and coworkers (Hornung et al., 1999), which in plants may be indicative for 13-LOX activity. This prediction was confirmed by the identification of the recombinant Bn-Lox-2 as linoleate 13-LOX (Fig. 3). AtLOX2 and two tomato LOXs, also with transit peptides, were shown to be

imported into isolated pea chloroplast *in vitro* (Bell et al., 1995; Heitz et al., 1997), suggesting that these LOXs indeed are chloroplast localized. However, the Bn-Lox-2fl polypeptide described in this paper is probably not present in fully developed chloroplasts, since antibody-A, specific for the Bn-Lox-2fl protein, barely recognized LOX polypeptides in extracts of light-grown seedlings or in leaves of mature green plants (Fig. 5). *Both Bn-Lox-1fl* and *Bn-Lox-2fl* transcripts were present in early stages of germination in both dark- and light-grown seedlings (Fig. 4). The appearance of hydroperoxy derivatives of free linoleic and linolenic acid showed that mainly one or more 13-LOXs were active during early growth and development of the seedlings (Fig. 6c and d). Since antibody-A did not recognize polypeptides in light-grown plants it is possible that the RT-PCR amplified *Bn-Lox-2fl* transcripts were derived from highly homologous genes encoding other isoenzymes of the LOX2-type. This is not an unreasonable assumption since *Bn-Lox-2fl* may be encoded by a small multigene family in *B. napus*. Antibody-B, also detected LOX polypeptides in green tissue, suggesting that different isozymes of the LOX2-type indeed are expressed in green and etiolated tissues. The genome of *A. thaliana* contains 4 LOX2-type genes, and two different LOX2-type LOXs have been characterized in tomato and potato (Feussner and Wasternack, 2002). Thus, it is very likely that *B. napus* contains other LOX genes homologous to *Bn-Lox-2fl* which are expressed in light-grown seedlings, and these may be localized in fully developed chloroplasts.

The mobilization of storage lipids is an important feature in germination of oil seed rape. In *C. sativus* a 13-LOX associated with the lipid body membrane oxygenates triacylglycerides possibly as a first step in the degradation of esterified unsaturated fatty acids via β-oxidation (Feussner et al., 2001). However, LOX polypeptides detected in this study in etiolated *B. napus* cotyledons were not enriched in the lipid body fraction (data not shown). The amount of hydroperoxy derivatives of esterified linoleic and linolenic acid was rather low in comparison to the situation found in cucumber and sunflower cotyledons (Feussner et al., 1997; Weichert et al., 2002) (Fig. 6a and b). This absence of a LOX-dependent degradation of triglycerides in *B. napus* may be connected with the fact that the reserve lipids of rape do not contain high amounts of polyunsaturated fatty acids like linoleic and linolenic acid. By contrast, the data in this report on non-esterified oxylipins (Fig. 6c and d) indicate that one or more 13-LOXs indeed are active during germination. The appearance of these 13-LOX-derived products of free fatty acids, indicates that they are part of other biosynthetic activities during germination, producing, e.g. C6-aldehydes, jasmonic acid or other secondary products. This has been shown recently to occur in germinating cucumber seedlings and may protect the seedling from pathogen attack (Weichert et al., 2002).

The high sequence homology of Bn-Lox-1fl to AtLOX1 as well as its characteristic active site amino acid motif TV makes it is very likely that *Bn-Lox-1fl* codes for a LOX1-type 9-LOX (Feussner and Wasternack, 2002). However, the recombinant Bn-Lox-1fl protein was inactive. All plant LOXs identified so far have an Asn residue in the position corresponding to amino acid 694 of soybean LOX-1, and it acts as one of five iron ligands within the active site of this enzyme. Most mammalian LOXs with the exception of arachidonate 5-LOXs have a His residue in that position (Kühn et al., 2005). As shown by site-directed mutagenesis of soybean LOX-3, the Asn713 residue is important for enzyme activity (Kramer et al., 1994). However the mouse 8*S*-LOX (Jisaka et al., 2001) and the homologous human 15*S*-LOX (Brash et al., 1997) with specificity for arachidonic acid contain a Ser residue instead of the Asn or His residue in the position equivalent to that described above. The mouse 8*S*-LOX is catalytically active, and site-directed mutagenesis substituting the Ser in that position with an Asn, His or Ala residue yielded enzymes with equivalent or even higher activity than the wild type enzyme (Jisaka et al., 2001). Bn-Lox-1fl has a Ser instead of the Asn residue in position 712, and it is the first plant LOX with a residue different from Asn or His at that position. Changing the Ser712 to an Asn residue did not yield active enzyme using linoleic acid as a substrate. It is conceivable, although unlikely, that the Bn-lox-1 protein has activity towards another unsaturated fatty acid substrate. On the other side it is well known for mammalian 5-LOXs that these proteins are very unstable as recombinant proteins as well (Kühn et al., 2005).

In conclusion, this study suggests that mainly 13-LOX enzymes are active during *B. napus* seed germination in the dark. The roles of the two LOXs characterized in this paper are different due to their different expression patterns and structures. We expect that additional LOX genes are active during the growth and development of the *B. napus* plant.

4. Experimental

4.1. Plant material

Brassica napus cv. Senator seeds, supplied by Nielsen and Smith A/S, Postbox 140, DK-2600 Glostrup, Denmark, were germinated on Whatman paper (1 and 2 d seedlings) or in vermiculite at 22 °C in the dark or in constant light for up to 14 d. Plants were grown to maturity in a growth cabinet at 22 °C, 16 h light and 18 °C, 8 h dark cycle.

4.2. Isolation of LOX cDNAs

The full-length cDNA of Bn-Lox-1fl was assembled of different fragments made by PCR, 5′race PCR or reverse transcription (RT)-PCR. Primers were derived from the sequence of *A. thaliana* LOX-1 (Melan et al., 1993), *Cucumis sativus* LBLOX (Höhne et al., 1996), or the

Bn-Lox-1 cDNA itself. A cDNA library made of mRNA from 3 d etiolated seedlings was used as a template. Fragments were ligated together to obtain a 2520 bp full-length Bn-Lox-1fl cDNA. The assembled cDNA contained the AtLOX1 nucleotide sequence encoding the 9 highly conserved C-terminal amino acids.

The full-length cDNA of Bn-Lox-2 was also assembled by PCR and RT-PCR, using primers derived from *A. thaliana* LOX-2, *Cucumis sativus* LB-LOX or the Bn-Lox-2 cDNA itself. Fragments were ligated together to obtain the full-length Bn-Lox-2fl cDNA. The truncated cDNA of the Bn-Lox-2fl without the region coding for the putative transit peptide was PCR amplified with the primers 5′-GCAGGACATCGTTACAAACTG-3′ and 5′-GGTGTTCCCTACAGTGT-GTCTATT-3′ (Bn-Lox-2) using the cDNA of Bn-LOX-2fl as template. The cDNA is called Bn-lox-2.

PCR was carried out according to the manufacturer's instructions (Promega, Mannheim, Germany). Multiple independent PCR-derived clones were sequenced to verify the sequence to the two lipoxygenase cDNA. Recombinant plasmids were isolated from single colonies employing a Qiagen (Hilden, Germany) kit and sequenced using Prism BigDye™ terminator technology (PE Biosystems, Naerum, Denmark) and the reactions were analysed on an ABI Prism 310 DNA sequencer (PE Biosystems, Naerum, Denmark).

Reverse transcription-PCR was performed with the reverse transcriptase M-MLV (Promega, Mannheim, Germany). After addition of 200U M-MLV reverse transcriptase, the reaction was left at 50 °C for 1.5 h and then heated to 70 °C for 5 min. The PCR components were added as described above and subcycling PCR (Liu and Sommer, 1998) was performed. 5′ race was carried out according to the manufacturer's manual (Clontech, Heidelberg, Germany).

4.3. Expression of LOX cDNAs in E. coli and generation of antibodies

Bn-Lox-1fl cDNA was ligated into the pQE-30 vector (Qiagen, Hilden, Germany) between the restriction sites *Bam*HI and *Sal*I, and the Bn-Lox-2fl cDNA and its truncated version were inserted between the restriction sites *Sal*I and *Sph*I. Transformed *E. coli* M15 cells were induced with 2 mM of IPTG at 37 °C for 2 h. The expressed LOX proteins were insoluble at this temperature. Induction of the cells at 10 °C for 16 h rendered a portion of the protein soluble. For measuring LOX activity the cells were harvested and resuspended in either 5 ml 50 mM Tris/HCl, pH 7.5, 10% glycerol, 0.5 M NaCl, 0.1% Tween-20 or in 5 ml 50 mM sodium phosphate, pH 5.0%, 10% glycerol, 0.5 M NaCl, 0.1% Tween-20 and sonicated for 5×30 s. Cellular debris were pelleted and the supernatant assayed for LOX activity as described (Feussner et al., 1998) with the following modifications. The respective recombinant LOX was incubated with 1 μl of 250 mg/ml linoleic acid (peroxide free, Cayman, Ann Arbor, MI, USA) for 30 min. The reaction was stopped by adding 100 μl of glacial acetic acid and 10 μl of sodium borohydride in methanol (10 mg/ml) to reduce the formed hydroperoxides. The hydroxides were extracted twice each with 1 ml of diethyl ether and were reconstituted in 25 μl of methanol:H_2O:glacial acetic acid (90:10:0.1 (v/v)) for HPLC analysis. The products were analysed as described under "4.6. Quantitative analysis of plant fatty acids and oxylipins", with the following modifications of the solvent systems, for the RP-HPLC being methanol:H_2O:glacial acetic acid (90:10:0.1 (v/v)) and for the SP-HPLC being *n*-hexane:2-propanol:glacial acetic acid (100:1:0.1 (v/v)).

The 285 bp following the coding region for the transit peptide of Bn-Lox-2fl and the 1485 bp encoding the carboxy terminal of Bn-Lox-2fl were amplified by PCR and ligated into the pET-24a vector (Novagen, Madison, WI, USA) between the *Eco*RI and *Hind*III restriction sites. Transformed *E. coli* BL21 cells were induced with 1 mM IPTG at 37 °C and grown for two more hours. Inclusion bodies were isolated and the recombinant protein purified by nickel-NTA (Qiagen, Hilden, Germany) column chromatography. Antibodies directed against the expressed parts of the protein were made in rabbits according to published standard protocols (Harlow and Lane, 1998). The antiserum directed against the N-terminal peptide was named antibody-A. This antibody recognized the recombinant Bn-Lox-2 polypeptide, as well as a 92 kDa polypeptide present in 5-day-old etiolated cotyledons. As expected, the Bn-Lox-1fl polypeptide was not recognized by antibody-A. An antiserum against a C-terminal peptide was named antibody-B.

The LBLOX antibody was directed against a recombinant LBLOX from cucumber as described (Hause et al., 2000).

4.4. Methyl jasmonate treatment, protein isolation and western blotting

Plants grown for 2 weeks in growth cabinet, were placed under a plastic bag, and treated with 25 μl of a solution of 10 mM methyl jasmonate in ethanol applied on a cotton stick. The non-treated control plants were likewise placed under a plastic bag. Plant material (3.8 g) was harvested and extracted in 10 ml 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 4 mM EDTA, 0.3 M sucrose, 1 mM PMSF, and 1 tablet Complete protease inhibitor cocktail (Roche, Mannheim, Germany) and analyzed on SDS-PAGE and western blot.

4.5. RT-PCR expression analyses

RNA was isolated from seeds, 1, 2, 3, 7 and 12-day-old cotyledons grown in the dark or in the light, 4-week-old true leaves, senescent leaves and 3-week-old roots grown in the light. Two micrograms of RNA were used in each RT-PCR reaction. The specific PCR primers used for

Bn-Lox-1fl analysis were 5′TTGGAGGGTCACAGGAGTATC-3′ and 5′-ATAAACACCACCGGAAACAAGG-3′ and for Bn-Lox-2fl, the two specific primers were 5′-CGTCCCATTTTGGGAGGTCTCT-3′ and 5′GAGAGAGTTGGATGATAGCACCT-3′. A RT-PCR was carried out using both Bn-lox-1fl and Bn-lox-2fl primer sets on RNA from 5 day old etiolated cotyledons for 5–32 PCR cycles. The PCR products were analysed by agarose gel electrophoresis and stained with SYBR green. PCR product formation was linear up to 20 PCR cycles. These conditions were used in subsequent RT-PCR reactions.

Extraction of DNA, RNA, cDNA synthesis, western and Southern blot analyses were performed essentially as described (Geshi and Brandt, 1998).

4.6. Quantitative analysis of plant fatty acids and oxylipins

Lipids were extracted from 0.3 g of etiolated (0–11 d) and light-grown (0–14 d) cotyledons as described (Weichert et al., 2002). The extracted lipids were reconstituted in 1 ml of isopropanol and aliquoted into two fractions, A (405 μl) and B (450 μl). A was analysed directly on RP-HPLC for free oxylipins. B was transmethylated to measure esterified oxylipins by HPLC. Transmethylation procedure: The isopropanol was evaporated, and 333 μl of a methanol:toluene (1:1 (v/v)) mixture and 167 μl of a 0.5 M solution of sodium methoxide were added. After incubation for 20 min at room temperature under constant shaking, the reaction was stopped by the addition of 500 μl of 1 M NaCl, 50 μl of 37% HCl and 750 μl of *n*-hexane. The organic phase was recovered after centrifugation for 2 min in a microcentrifuge. The lower phase was re-extracted with *n*-hexane. The combined organic phases were evaporated and re-dissolved in 100 μl of acetonitrile. The samples were analysed by HPLC as described below.

HPLC analysis was carried out on an Agilent 1100 HPLC system coupled to a diode array detector (Waldbronn, Germany). For analysis of oxylipins, the samples were first purified by RP-HPLC. This was performed on an ET250/2 Nucleosil 120-5C-18 column (Macherey and Nagel, Düren, Germany; 2.1 × 250 mm, 5 m particle size) with a methanol:H_2O:glacial acetic acid (85:15:0.1 (v/v)) solvent system at a flow rate of 0.180 ml/min. Samples were evaporated and re-dissolved in 80 μl of the solvent before injection. Fractions containing either jasmonic acid and 12-oxo phytodienoic acid or hydroperoxy and hydroxy polyenoic fatty acid derivatives were collected. Separation of hydroperoxy and hydroxy fatty acids was performed by SP-HPLC on a Zorbax RX-SIL column (Agilent, Waldbronn, Germany; 2.1 × 150 mm, 5 m particle size) with a *n*-hexane:isopropanol:glacial acetic acid (100:1:0.1 (v/v)) solvent system at a flow rate of 0.200 ml/min. Analysis of enantiomeric configuration of these fatty acid derivatives was carried out by CP-HPLC on a Chiracel OD-H column (Merck, Darmstadt, Germany; 2.1 × 150 mm, 5 m particle size) with a *n*-hexane:isopropanol:glacial acetic acid (100:5:0.1 (v/v)) solvent system and a flow rate of 0.1 ml/min. The conjugated diene system at 234 nm was monitored. An *R* to *S* ratio of 20 to 80 or lower was considered to be indicative for LOX activity. An *R* to *S* ratio higher than 20 to 80 is likely due to non-enzymatic lipid peroxidation of the unsaturated fatty acids.

For the analysis of jasmonic acid and 12-oxo-phytodienoic acid, the corresponding RP-HPLC fraction was derivatized as described (Stumpe et al., 2005). Gas chromatography/mass spectrometry (GC/MS) was performed with a Finigan GCQ GC/MS system equipped with a capillary Rtx-5 column (5% diphenyl–95% polydimethyl siloxane, 30 m × 0.25 mm; 0.25 μm coating thickness; Restek, Germany). Helium was used as carrier gas (40 cm × s^{-1}). An electron energy of 70 eV, an ion source temperature of 140 °C, and a temperature of 275 °C for the transfer line were used. The samples were measured in the NCI mode, using ammonia as reactant gas and the splitless injection mode (opened after 1 min) with an injector temperature of 250 °C. The temperature gradient was 60–180 °C at 25 °C min^{-1}, 180–270 °C at 5 °C min^{-1}, 270 °C for 1 min, 270–300 °C at 10 °C min^{-1}, and 300 °C for 25 min.

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