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ScienceDirect
Phytochemistry 67 (2006) 2030–2040
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Lipoxygenases during *Brassica napus* seed germination ☆

Nina Terp [a,b], Cornelia Göbel [b,c], Anders Brandt [a], Ivo Feussner [b,c,*]

[a] *Carlsberg Laboratory, Department of Physiology, Gamle Carlsberg Vej 10, DK-2500 Copenhagen, Denmark*
[b] *Institute of Plant Genetics and Crop Plant Research (IPK), Department of Molecular Cell Biology, Correnssstr. 3, D-06466 Gatersleben, Germany*
[c] *Georg-August-University of Göttingen, Albrecht-von-Haller-Institute of Plant Sciences, Department of Plant Biochemistry, Justus-von-Liebig-Weg 11, D-37085 Göttingen, Germany*

Received 19 April 2006; received in revised form 18 June 2006
Available online 1 August 2006

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.

* Corresponding author. Address: Department of Plant Biochemistry, Albrecht-von-Haller-Institute for Plant Sciences, George-August-University Goettingen, Justus-von-Liebig-Weg 11, D-37077 Goettingen, Germany. Tel.: +49 551 395743; fax: +49 551 395749.
E-mail address: ifeussn@gwdg.de (I. Feussner).

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doi:10.1016/j.phytochem.2006.06.023

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