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Oxylipin formation in *Nostoc punctiforme* (PCC73102) ☆

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Abstract

The dioxygenation of polyunsaturated fatty acids is mainly catalyzed by members of the lipoxygenase enzyme family in flowering plants and mosses. Lipoxygenase products can be metabolized further 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 mammals, flowering and non-flowering plants, information on the relevance of lipid peroxide metabolism in prokaryotic organisms is scarce. Thus we aimed to isolate and analyze lipoxygenases and oxylipin patterns from cyanobacterial origin. DNA isolated from *Nostoc punctiforme* strain PCC73102 yielded sequences for at least two different lipoxygenases. These have been cloned as cDNAs and named NpLOX1 and NpLOX2. Both proteins were identified as linoleate 13-lipoxygenases by expression in *E. coli*. NpLOX1 was characterized in more detail: It showed a broad pH optimum ranging from pH 4.5 to pH 8.5 with a maximum at pH 8.0 and α-linolenic acid was the preferred substrate. Bacterial extracts contain more 13-lipoxygenase-derived hydroperoxides in wounded than in non-wounded cells with a 30-fold excess of non-esterified over esterified oxylipins. 9-Lipoxygenase-derived derivatives were not detectable. 13-Lipoxygenase-derived hydroperoxides in esterified lipids were present at almost equal amounts compared to non-esterified hydroperoxides in non-wounded cells. These results suggest that 13-lipoxygenases acting on free fatty acids dominate in *N. punctiforme* strain PCC73102 upon wounding.

Keywords: Cyanobacteria; Nostocaceae; Lipid peroxidation; Oxylipin formation

1. Introduction

Lipoxygenases (LOXs) constitute a family of non-heme iron containing fatty acid dioxygenases, which catalyze the regio- and stereospecific dioxygenation of polyunsaturated fatty acids (PUFAs) that contain at least one (1*Z*,4*Z*)-pentadiene system. Thus substrates for LOXs are for example linoleic acid (LA), α-linolenic acid (ALA), or arachidonic acid (AA) (Brash, 1999; Liavonchanka and Feussner, 2006). In mammals, LOXs are classified according to their positional specificity of AA oxygenation (Brash, 1999). Because AA is either not present in flowering plants or is a minor constituent of their cellular lipids, plant LOXs are classified into 9- and 13-LOXs with respect to their positional specificity of LA oxygenation (Liavonchanka and Feussner, 2006). Both hydroperoxide derivatives serve as substrates for numerous metabolic reactions: They may be cleaved to aldehydes and ω-oxo acids (Blée, 2002), and the 13-hydroperoxide of ALA may serve as a precursor for the synthesis of jasmonic acid (Wasternack and Hause, 2002). The metabolism of PUFAs via this LOX-catalyzed step and the subsequent reactions are collectively named

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; CP-HPLC, chiral phase-HPLC; GC, gas chromatography; GLA, γ-linolenic acid; HETE, hydroxy arachidonic acid; HODE, hydroxy octadecadienoic acid; HOTE, hydroxy octadecatrienoic acid; HODEme, hydroxy octadecadienoic acid methyl ester; HOTEme, hydroxy octadecatrienoic acid methyl ester; LA, linoleic acid; LOX, lipoxygenase; PUFA, polyunsaturated fatty acid; RP-HPLC, reversed phase-HPLC; SP-HPLC, straight phase-HPLC.

☆ *Sequence data:* The nucleotide sequences reported in this paper are annotated as putative lipoxygenases in the GenBank/EMBL data bank under the accession numbers ZP_00106490 and ZP_00107030.

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LOX pathway and the metabolites that derive there from are called oxylipins (Blée, 2002; Feussner and Wasternack, 2002).

During recent years it became clear that the family of LOXs seems not to be restricted to the animal and plant kingdom, since a few cDNAs coding for LOXs have also been isolated from other eukaryotic organisms, such as corals (Brash et al., 1996) and fungi (Hörnsten et al., 2002). For a long time being LOXs have been considered to be specific for eukaryotic organisms, since only in flowering plants and mammals metabolic pathways of PUFA oxygenation by LOXs have been extensively studied. However, in microorganisms information about enzymatic lipid peroxidation processes is still scarce. Here, only few reports pointing towards the existence of LOX activities in prokaryotes, like for the gram-negative *Pseudomonas aeruginosa* (Porta and Rocha-Sosa, 2001) and the gram-positive *Thermoactinomyces vulgaris* (Iny et al., 1993) are available. Recently, the first LOX from a prokaryotic organism *P. aeruginosa* has been isolated (Vance et al., 2004).

Prokaryotic and eukaryotic algae are well known as sources of biologically active compounds (Cannell, 1993). Many of these compounds such as alcohols, alkanes, aldehydes, ketones and branched hydrocarbons may derive from primary products of LOX activity (Watson, 2003). The first LOX activity that was characterized in more detail in an eukaryotic algae was described for *Chlorella pyrenoidosa* (Zimmerman and Vick, 1973). For prokaryotic algae (cyanobacteria) LOX activity was described first for *Oscillatoria* spp. (Beneytout et al., 1989). The isolated enzyme was characterized as an unspecific linoleate 9/13-LOX. Another cyanobacterium, *Anabaena flos aquae f. flos aquae*, produces hydroxy fatty acids which may originate as well from the action of a LOX (Murakami et al., 1992).

From an evolutionary point of view prokaryotes are of special interest as they are the ancestors of higher eukaryotic organisms. Even cyanobacteria are of special interest for plant physiology since they are the ancestors of plants plastids. These plastids harbour the 13-LOX pathway that led to formation of the phytohormone jasmonic acid and the green leaf volatile (3*Z*)-hexenal (Schilmiller and Howe, 2005). Today there is less information about how and to which extent the LOX pathway is present in other kingdoms than that of mammals and flowering plants and whether it evolved independently in the different kingdoms. But it seems that in all kingdoms analyzed so far one major role of the LOX pathway is its involvement in developmental processes and in abiotic or biotic stress responses (Kühn, 2005; Müller, 2004; Schilmiller and Howe, 2005).

Due to the increasing sequence information from genome sequencing projects not only biochemical but also molecular tools may be used now for identification and isolation of new LOXs having low sequence similarity to the described LOXs from higher eukaryotes. To gain more information on the evolutionary origin of LOXs we screened available genome sequences of microalgae, including cyanobacteria, in public databases and identified the cyanobacterium *Nostoc punctiforme* PCC 73102 as a potential source for LOX genes. For this prokaryotic organisms the complete genome sequence is online available at http://genome.jgi-psf.org/finished_microbes/nospu/nospu.home.html. A search within this database for LOX sequences revealed two genes with homologies to the LOX from *P. aeruginosa*. Here, we report about the isolation and characterisation of these two LOXs and on the possible involvement of the enzymes within *N. punctiforme* PCC 73102 in oxylipins formation by analysing metabolites of the LOX pathway *in vivo*.

2. Results

2.1. Isolation of two putative LOXs from N. punctiforme PCC73102

Database searches and alignments of available LOX amino acid sequences against the public genome sequence from *N. punctiforme* PCC73102 revealed two genes coding for hypothetical proteins with homologies to LOX proteins named NpLOX1 (Acc. No. ZP_00106490) and NpLOX2 (Acc. No. ZP_00107030). The amino acid sequence of NpLOX1 exhibited highest identity of 37% to the LOX from *P. aeruginosa* (Acc. No. NP_249860) and the corresponding sequence of NpLOX2 showed highest identity with 38% to NpLOX1. To isolate the two putative LOXs, gene specific primers were used to amplify the two fragments from genomic DNA. The obtained fragment of NpLOX1 had a complete open reading frame of 1892 bp encoding a protein of 630 amino acids with a molecular size of 70.9 kDa. The open reading frame of NpLOX2 had a length of 1644 bp encoding a protein of 548 amino acids with a molecular size of 62.9 kDa.

Phylogenetic tree analysis of NpLOX1 and NpLOX2 with selected LOXs from plants, mammals, algae, corals and bacteria showed that both NpLOXs form an own clade with other mainly putative prokaryotic LOXs (Fig. 1). Interestingly NpLOX2 falls into a separate group with *N. europaea*, whereas NpLOX1 is found together with all other prokaryotic sequences. Furthermore, the clade of the bacterial LOXs was closer to that of mammalian and eukaryotic algal LOXs than to the sequences from plants.

2.2. LOX activity tests in vitro

For further biochemical characterisation NpLOX1 and NpLOX2 cDNAs were expressed in *E. coli* BL21(DE3) cells and the crude cell extracts were incubated with several fatty acid substrates for activity tests. First analysis showed that both enzymes, NpLOX1 and NpLOX2, respectively, converted the C18- and C20-PUFAs to the same corresponding hydroperoxides where the oxygen was inserted at the ω-6 position. The analysis of products formed by the conversion of LA into (9*Z*,11*E*,13*S*)-13-hydro(pero)xy-octadeca-9,11-dienoic acid (13-H(P)ODE) that were

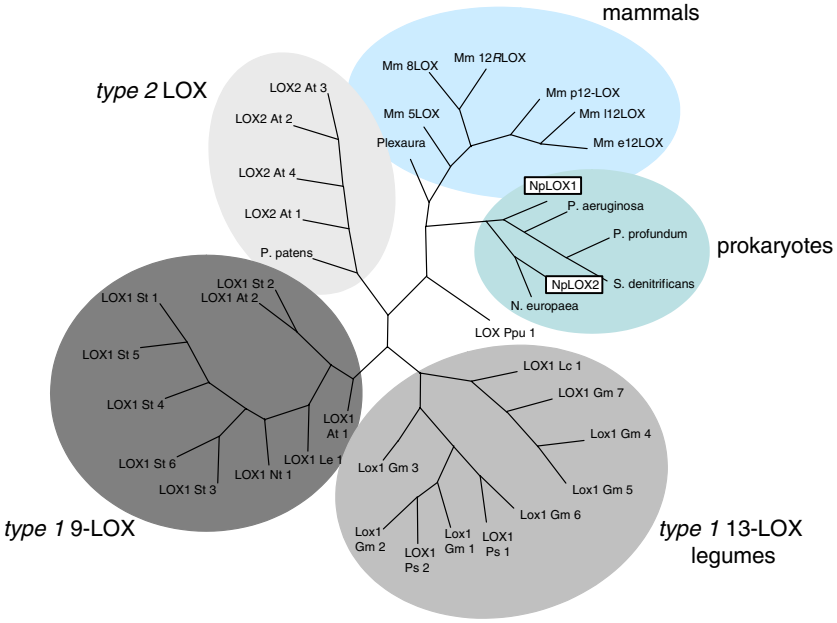

Fig. 1. Phylogenetic tree analysis of NpLOX1 and NpLOX2 amino acid sequences and selected LOXs from mammals, plants, coral, algae and bacteria. Amino acid sequences were aligned with ClustlX and phylogram was constructed with treeview. Accession numbers: *Mus musculus*: 5-LOX AAC37673; 8-LOX CAA75003; 12*R*LOX CAA74714; l12-LOX AAA20658; p12LOX AAA20659; e12LOX NP663717; *Plexaura homomalla*: AOSLOX O16025; *Arabidopsis thaliana type 2* LOXs: At1 Q06327; At2 CAB56692; At3 CAC19364; At4 CAG38328; *Physcomitrella patens*: CAE47464; *Solanum tuberosum*: St1 CAA5572; St2 AAD09202; St3 AAB31252; St4 CAA64766; St5 CAA64765; St6 AAB67860; *A. thaliana type1* 9-LOX: At1 NP175900, At2 NP188879, *Lycopersicon esculentum*: Le1 P38415; *Nicotiana tabacum*: Nt1 CAA58859; *Glycine max*: Gm1 CAA47717; Gm2 P09439; Gm3 CAA31664; Gm4 P38417; Gm5 AAB67732; Gm6 AAA96817; Gm7 AAC49159; *Pisum sativum*: Ps1 AAB71759; Ps2 CAA55318; *Lens culinaris*: Lc1 CAA50483; *Porphyra purpureum*: Ppu AAA61791; *Pseudomonas aeruginosa*: AF479686; *Nitrosomonas europaea*: BX321860; *Shewanella denitrificans*: OS-217:Q3P217; *Photobacterium profundum*: 3TCK: ZP 01218321.

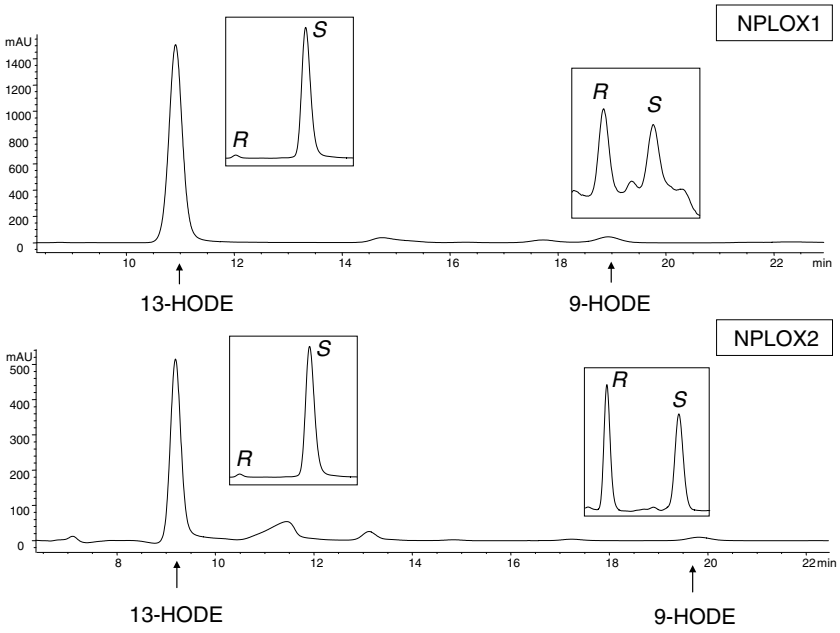

Fig. 2. Analysis of products formed by NpLOX1 and NpLOX2, respectively, with LA at pH 8.0 (here in reduced form for SP-HPLC analysis). Insets show analysis of *R* and *S* enantiomer ratio by CP-HPLC analysis.

detected after reduction as hydroxy fatty acids is shown in Fig. 2 for both LOXs. Due to the similar reaction characteristics, which were determined during these experiments and the same active site determinants in both enzymes, we expected no major differences between both enzymes. Thus only NpLOX1 was analysed in more detail.

The pH-optimum of NpLOX1 was determined by summing up the integrals of all isomeric hydroxy fatty acids

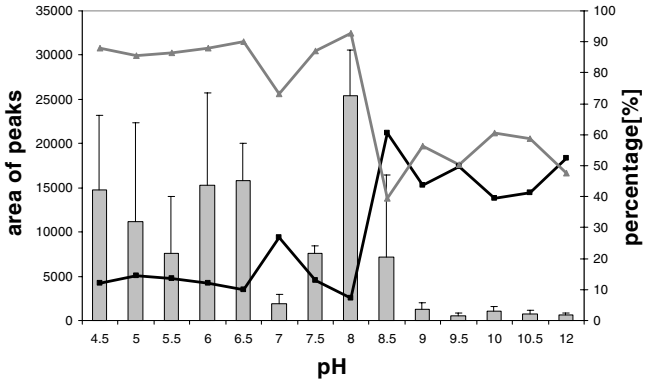

Fig. 3. Enzyme activity and regiospecificity of H(P)ODE production of NpLOX1 depending on different pH-values. The sum of the peak areas determined by HPLC for 9- and 13-H(P)ODE is shown in grey bars as a measure for LOX activity. The regiospecificity is presented by the percentages of 9-H(P)ODE (black curve) and 13-H(P)ODE (grey curve), respectively. Each value represents the average of two independent experiments.

determined by HPLC analysis and formed by incubation with LA at pH values ranging from pH 4.5 to pH 12.0 (Bars in Fig. 3). The pH optimum was rather broad ranging from pH 4.5 to pH 8.5 with a maximum at pH 8.0. 13-H(P)ODE, (9*Z*,11*E*,13*S*,15*Z*)-13-hydro(pero)xy-9,11,15-octadecatrienoic acid (13-H(P)OTE), (6*Z*,9*Z*,11*E*,13*S*)-13-hydro(pero)xy-6,9,11-octadecatrienoic acid (13γ-H(P)-OTE) were preferred products after addition of LA, ALA and γ-linolenic acid (GLA). AA was predominantly converted to its (15*S*)-hydroperoxide (15-H(P)ETE). Chiral phase-HPLC (CP-HPLC) analysis of the enantiomers showed that in all cases formation of the *S*-enantiomer dominated. In Table 1 the relative amounts of the hydro-(pero)xides and the *S*-enantiomers are given in percentages. As for the absolute activity the highest regiospecificity of NpLOX1 was observed at pH 8.0 (92% of 13-H(P)ODE; Fig. 3, lines). However, the highest stereospecificity was measured at pH 6.0, with a relative amount of about 99% of the *S*-enantiomer of 13-H(P)ODE. In summary for 13-H(P)ODE the ratio of *S*/*R* enantiomers was higher than 80% at all pH values tested. Furthermore, these data showed, that 9-H(P)ODE was mainly a product of chemical oxidation at all pH values tested, because its ratio of *S*/*R* enantiomers was almost equal.

The substrate preference of NpLOX1 was determined at pH 8.0 by incubating an equimolar mixture of the four different fatty acids for 30 min with the enzyme extract. Afterwards the reaction was stopped and the consumption of the substrates determined. However, the enzyme showed no substrate preference.

2.3. LOX activity in vivo

To verify the data obtained with the recombinant LOX-enzymes, we aimed to analyze the LOX activity and pathway metabolites within the cyanobacterium *N. punctiforme* PCC73102 next. The specific LOX activity per total *Nostoc* protein was determined by measuring the O_2-consumption with an oxygen electrode using sodium linoleate as substrate at pH 8.0. Therefore the crude extract was separated in a supernatant and pellet fraction by centrifugation at 4500*g* for 10 min. For supernatant LOX activity was under the detection limit, but it was detectable within the pellet fraction with about 909 nmol O_2/min/mg protein.

One possible role of 13-LOXs is their involvement in plants wound response (Schilmiller and Howe, 2005). Thus we compared in wounded and non-wounded cyanobacteria endogenous occurring LOX pathway-products. Therefore half of the harvested cell material was wounded by sonification as described before for diatoms and mosses (Pohnert, 2000; Wichard et al., 2005). In both oxylipin fractions, free and esterified oxylipins, hydro(pero)xy dienoic fatty acids were measured. The amounts of free 13-hydroxy dienoic fatty acids were remarkably higher in samples which have been wounded (13-HODE: 0.13 nmol/g f.w. vs. 7.3 nmol/g f.w.; 13-HOTE: 0.07 nmol/g f.w. vs. 4.29 nmol/g f.w.) whereas no remarkable differences in the amounts of 9-hydroxy dienoic fatty acids could be observed between wounded and non-wounded cells (Fig. 4a). Chiral analysis of the identified substances showed, that only 13-HODE

Table 1
Fatty acid hydroperoxides formed from the reaction of NpLOX1 with different fatty acid substrates at pH 8.0

Substrates	Products				
Arachidonic acid	*15-HETE*	12-HETE	11-HETE	8-HETE	5-HETE
Total hydroperoxides (%)	74 ± 10	1.6 ± 0.2	19.2 ± 14	4.4 ± 3.9	n.d.
S-Enantiomer (%)	97.4 ± 1.2	54.4 ± 9.1	77 ± 12.5	48.1 ± 1.9	
γ-Linolenic acid	*13γ-HOTE*	10γ-HOTE	9γ-HOTE	6γ-HOTE	
Total hydroperoxides (%)	*65* ± 16	4.23 ± 1.2	6.2 ± 1.2	23.5 ± 15	
S-Enantiomer (%)	99.3 ± 0.44	60.5 ± 4.1	62.1 ± 13.9	63.1 ± 6.9	
α-Linolenic acid	16-HOTE	*13-HOTE*	12-HOTE	9-HOTE	
Total hydroperoxides (%)	6.57 ± 5.42	81.2 ± 13.1	5.76 ± 4.63	6.5 ± 3.6	
S-Enantiomer (%)	50 ± 3.5	90.2 ± 12	48 ± 2.2	51 ± 2.8	
Linoleic acid	*13-HODE*	9-HODE			
Total hydroperoxides (%)	94.3 ± 2.7	5.7 ± 2.7			
S-Enantiomer (%)	98.4 ± 0.2	60.2 ± 6.7			

Values represent the mean of two independent experiments, standard deviation is given.

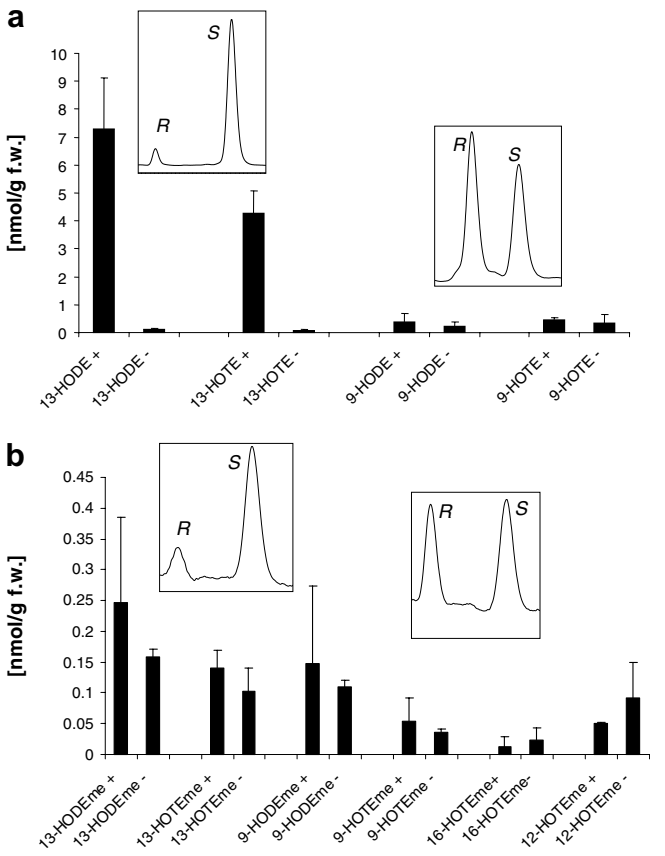

Fig. 4. HPLC-analysis of endogenous (a) non-esterified (HODE) and (b) esterified HODEs (HODEme) of *N. punctiforme* PCC73102. The bars represent the values of detected hydroxy fatty acids from non-wounded (−) or wounded (+) cyanobacteria. Each value represents an average of two independent experiments, standard deviation is given. The insets show the CP-HPLC analyses for 13-HODE and 13-HODEme, respectively. 13-HOTE and 13-HOTEme represent enzymatically produced hydroxy fatty acids whereas 9-HODE, 9-HODEme, 12-HOTEme and 16-HOTEme represent the hydroxy fatty acids produced by chemical oxidation.

and 13-HOTE were products of LOX activity whereas the detected 9-HODE and 9-HOTE originated from autoxidation (insets in Fig. 4a). For the esterified oxylipins the obtained data deviated from that of the free oxylipins. The amounts of esterified hydroxy dienoic fatty acids were close to the detectable limit, especially for the non-wounded cultures, but a tendency was visible. The amounts of esterified oxylipins did only slightly increase after wounding (13-HODEme: 0.16 nmol/g f.w. vs. 0.25 nmol/g f.w.; 13-HOTEme: 0.10 nmol/g f.w. vs. 0.14 nmol/g f.w.) (Fig. 4b). Chirality of the esterified 13-HODE/HOTE (detected as methyl esters: HO(D/T)Eme) were as the corresponding free oxylipins products of LOX activity whereas 9-HODEme/HOTEme as well as 12- and 16-HOTEme seemed to derive from chemical oxidation (insets in Fig. 4b). Further oxylipins like C16- or C20-PUFA-derived hydroxides were not detected, neither in non-wounded nor in wounded cyanobacteria. In addition fatty acid hydroperoxides, jasmonic acid and 12-oxo phytodienoic acid were below the detection limit.

3. Discussion

Oxidation of PUFAs in plants is mainly a controlled process that is catalyzed by enzymes of the LOX enzyme family (Liavonchanka and Feussner, 2006). Products originating from the LOX reaction may be further metabolized in the so-called LOX 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 (Schilmiller and Howe, 2005; Wasternack et al., 2006). Apart from accumulating data in flowering and non-flowering plants, as well as from mammals and eukaryotic algae (Kühn, 2005; Lotzer et al., 2005; Pohnert, 2005), information on the relevance of lipid peroxide metabolism in prokaryotic organisms is scarce (Müller, 2004). Thus we aimed to isolate and analyze LOXs and the corresponding oxylipin patterns from cyanobacterial origin, since these bacteria may be the ancestors for plastids in higher eukaryotes.

Our analysis showed that several cyanobacterial genomes harbour putative open reading frames that may encode for a LOX. *N. punctiforme* strain PCC73102 was chosen as the most promising candidate, since its genome harbours at least two different LOX enzymes. DNA isolated from this bacterium yielded indeed sequences for two different LOXs. They were cloned as cDNAs and named NpLOX1 and NpLOX2, respectively. Interestingly, NpLOX2 seems to be the smallest LOX protein of 584 amino acids isolated so far, since NpLOX1 (this report) and a homologous LOX from the bacterium *P. aeruginosa* (Vance et al., 2004) encode for proteins of 630 and 685 amino acids, respectively. Whereby mammalian LOXs do have about the same size of 660–680 amino acids, plant LOXs constitute normally of more than 860 amino acids (Feussner and Wasternack, 2002). To our knowledge there is only one report describing a LOX of about the same size. It describes a proteolytic fragment harbouring the catalytic domain of soybean LOX1 of about 60 kDa having still LOX activity (Di Venere et al., 2003).

To gain more information on the biochemical features of NpLOX1 and 2 their protein sequences were aligned with other characterized LOXs from plant, moss, coral and *P. aeruginosa*. Diagnostic differences or similarities within the structure are mainly found around the active site residues of the proteins (Liavonchanka and Feussner, 2006). The central histidine-rich region, including three histidines (His-327, His-332, His-510 in NpLOX1) which constitute the iron binding site (Siedow, 1991), are highly conserved in all six LOX-sequences. Two additional amino acids, asparagine (Asn-514 in NpLOX1) and isoleucine (Ile-630 in NpLOX1) which may be also involved in the iron binding are highly conserved in NpLOX1. For NpLOX2 a valine (Val-548) can be found at the C-terminus that corresponds to the position of Ile-630 in NpLOX1. At the position of the three determinants that may be involved in substrate- and regiospecificity (Borngräber

et al., 1996; Hornung et al., 1999; Sloane et al., 1991) both NpLOXs contain different amino acids in comparison to the other four LOX sequences. Particularly, each of the two sequences harbour two leucine residues (Leu-667 and Leu-668) at the SLOANE site instead of methionine (Met-667) and phenylalanine (Phe-668) in *PaLOX13* or glycine (His-667) and phenylalanine (Phe-668) in *AtLOX3*. These amino acids differ remarkably from the determinants described to be involved in determining the positional specificity of plant LOXs (Hornung et al., 1999), making it impossible to predict the possible positional specificity of both LOXs. This is furthermore supported by the observation that the Arg residue as determinant involved in inverse substrate orientation in plant LOXs is missing as well (Hornung et al., 1999). However, the amino acid residue determining the stereospecificity of LOXs (COFFA site) is in both sequences an alanine residue, identifying both LOXs as *S*-specific LOXs (Coffa and Brash, 2004). NpLOX1 and 2 were identified as linoleate 13-LOXs by expression in *E. coli* (Figs. 2 and 3, Table 1).

In higher plants 13-LOXs are not found ubiquitously in the cytosol and, until now, 9-LOXs were only found within this compartment (Feussner and Wasternack, 2002). Thus, the cytosol might be primarily the location of (9*S*)-hydroperoxide-derived oxylipins. In contrast, in the plastid, only 13-LOXs have been detected so far. Therefore, (13*S*)-hydroperoxy PUFAs forming as well as metabolizing activities should be ubiquitously localized in plastids. Taken into consideration that cyanobacteria may be the ancestors of plastids the identification of NpLOX1 and 2 as 13-LOXs is in good agreement with this model. In addition the analysis of endogenous oxylipins in this cyanobacterium (Fig. 4) may indicate that this organism is only capable to form oxylipins that derive directly from the action of 13-LOXs and that additional enzymes metabolizing 13-LOX-derived fatty acid hydroperoxides known from the so-called LOX pathway in higher plants are missing. This is further supported by the observation that we found no other open reading frames in the genome of *N. punctiforme* strain PCC73102 that may encode for other enzymes of the LOX pathway of higher plants. Taken this into account it is tempting to assume that metabolic pathways involved in formation of more complex oxylipins like for instance the jasmonates or green leaf volatiles may have evolved later during evolution.

4. Experimental

4.1. Algae material

N. punctiforme PCC 73102 was cultivated in shaking batch cultures at 120 rpm using 1 l conical flasks at 20 °C in BG 11 medium for cyanobacteria according to *Sammlung für Algenkulturen in Goettingen* (http://www.epsag.uni-goettingen.de/html/sag.html). Cultures were harvested via centrifugation at OD_{600} 1.2.

4.2. Isolation and expression of LOXs

Sequences encoding LOX reported in other species were used to find LOX-like proteins by BLAST searches in *N. punctiforme* database in Joint Genome Institute (http://www.jgi.doe.gov/). Genomic DNA was isolated from approximately 50 mg of frozen cell material using the NucleoSpin® Plant kit (Macherey-Nagel, Düren, Germany). The genes encoding two LOX-like proteins were isolated by PCR using the following primers: *NpLOX40F*, 5′-ATGACTGCTTTATCACCAGATCATTCAATCAGTT-CA-3′; *NpLOX40R*, 5′-TCAGATATTGATGCTCTGAGGAATTTTA-3′ and *NpLOX50F*, 5′-ATGAAACCATACCTCCCTCAGAATGAT-3′; *NpLOX50R*, 5′-TCACACGCTAATACTATTTGACACAAG-3′. The obtained fragments, named LOX40 (NpLOX1) and LOX50 (NpLOX2), were cloned into expression-vector pEXP5-NT/TOPO® (Invitrogen, Karlsruhe, Germany) with N-terminal His-Tag and transformed into *E. coli* BL21(DE3) cells. 30 ml of expression culture was grown until OD_{600} 0.6 at 37 °C, then the culture was induced with 1 M IPTG (1:1000). After culturing overnight at 16 °C, the cells were harvested by centrifugation (4500*g*, 4 °C, 20 min).

4.3. LOX activity assay

For analysis of product specificity of LOX proteins *E. coli* cells of a 30 ml expression culture were sonificated in 2 ml lysis buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 10% glycerol, 0.1% Tween 20). About 500 µl of lysate were incubated in 1.5 ml of 50 mM Tris/HCl (pH 8.0) with 250 µg of fatty acid substrate (LA, ALA, GLA and AA) for 30 min on ice. Reaction was stopped and hydroperoxides were reduced to their corresponding hydroxides by adding 2 ml 50 mM $SnCl_2$ solved in methanol. After acidification to pH 3.0 with glacial acid, the fatty acids were extracted as described (Bligh and Dyer, 1959).

After removing the organic solvent, the residue was reconstituted in 80 µl of methanol/water/acetic acid (85:15:0.1, v/v) and subjected to HPLC analysis. HPLC analysis was performed with an Agilent 1100 HPLC system (Waldbronn, Germany) coupled to a diode array detector. Hydroxy fatty acids were separated from fatty acids by reversed phase HPLC (RP-HPLC; EC250/4 Nucleosil 120-5 C18, Macherey-Nagel, Dueren, Germany) eluted with a solvent system of methanol/water/acetic acid (85:15:0.1, v/v) at a flow rate of 0.2 ml/min. Straight phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (250/4.6, 5 µm particle size, Agilent, Waldbronn, Germany) eluted with a solvent system of hexane/2-propanol/acetic acid (100:5:0.1, v/v) at a flow rate of 0.2 ml/min. CP-HPLC of the hydroxy fatty acids was carried out on a Chiralcel OD-H column (2.1 × 150 mm, 5-µm particle size, Daicel, distributed by VWR, Darmstadt, Germany) with a solvent system of *n*-hexane/2-propanol/acetic acid (100:5:0.1, v/v) and a flow rate of 0.1 ml/min. The absorbance at 234 nm (conjugated

diene system of the hydroxy fatty acids) was recorded simultaneously during all chromatographic steps.

4.4. Determination of LOX activity and LOX products from N. punctiforme cultures

For the measurement of specific LOX activity per total *Nostoc* protein, a cyanobacteria culture of 100 ml was harvested by centrifugation (4500*g* at 4 °C for 10 min). After adding 1 ml of lysis buffer (see Section 4.3) the sample was sonified (2 × 30 s on ice) and afterwards centrifuged for 5 min at 4500*g*. LOX activity was measured in both, pellet and supernatant with an oxygen electrode (Clark). For this 900 µl of 50 mM Tris/HCl, pH 8.0, buffer was mixed with 100 µl of either supernatant or pellet and the reaction was started by adding 10 µl of 10 mM sodium linoleate. Protein was determined by the method of Bradford (Bradford, 1976).

Endogenous LOX product formation was determined using crude extracts from *N. punctiforme* PCC 73102. Two algae cultures of 100 ml were harvested via centrifugation (4500*g* at 4 °C for 10 min) and the pellets resuspended in 1 ml BG11 medium. One culture was wounded by sonification for 40 s on ice (about 20% of cells were lysed by this treatment) and both, non-treated and wounded cultures, were then incubated for 30 min at room temperature. The samples were stored at −20 °C until oxylipin analysis.

Determination of hydroxy and hydroperoxy fatty acids was performed by HPLC-DAD and GC-FID. To about 1.5 g fresh weight (corresponding to 76 mg dry weight) of frozen algal cells 15 ml of extraction medium (*i*-hexane/2-propanol, 3:2 (v/v) with 0.0025% (w/v) butylated hydroxytoluene) and 500 mg glass pearls (0.25–0.5 mm) were added. As an internal standard, (6*Z*,9*Z*,11*E*,13*S*)-13-hydroxy-6,9,11-octadecatrienoic acid was added. The extract was homogenized by shaking for 10 min at 4 °C and then centrifuged at 4500*g* at 4 °C for 10 min. The clear upper phase was collected, and the pellet was extracted three times with 3 ml each of extraction medium. To the combined organic phases, a 6.7% (w/v) solution of potassium sulfate was added to a final volume of 47 ml. After vigorous shaking, the upper hexane rich layer was removed. The upper organic phase containing oxylipins was dried under nitrogen and re-dissolved in 80 µl of methanol/water/acetic acid (85:15:0.1, v/v). At first, oxylipins were purified on RP-HPLC, then SP-HPLC was applied to separate the hydro(pero)xy derivatives of LA and ALA according to Section 4.3.

For the analysis of esterified oxylipins and fatty acids, triricinoleate and triheptadecanoate were used as internal standards, and the extraction was performed as described above. After removing of the solvent, 405 µl of a mixture of toluene and methanol (1:2, v/v) and 150 µl of 0.5 mM sodium methoxide were added. After incubation for 20 min at room temperature, 0.5 ml of 1 M NaCl and 50 µl of HCl (37%, v/v) were added, and the resulting fatty acid methyl esters and their oxidized derivatives were extracted twice each with 0.75 ml of hexane. The combined organic phases were evaporated to dryness under a stream of nitrogen and dissolved in 200 µl of methanol. An aliquot of 20 µl of this solution was dried under nitrogen and dissolved in 10 µl of acetonitrile for the analysis of the fatty acid methyl esters by GC-FID as described before (Hornung et al., 2002). The methyl esters of the oxylipins were analyzed by HPLC-DAD according to the analysis of the free oxylipins (Göbel et al., 2003). Jasmonic acid and 12-oxo phytodienoic acid were determined by GC–MS as described (Stumpe et al., 2005).

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References

Beneytout, J.-L., Andrianarison, R.-H., Rakotoarisoa, Z., Tixier, M., 1989. Properties of a lipoxygenase in green algae (*Oscillatoria* sp.). Plant Physiol. 91, 367–372.

Blée, E., 2002. Impact of phyto-oxylipins in plant defense. Trends Plant Sci. 7, 315–322.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Borngräber, S., Kuban, R.J., Anton, M., Kühn, H., 1996. Phenylalanine 353 is a primary determinant for the positional specificity of mammalian 15-lipoxygenases. J. Mol. Biol. 264, 1145–1153.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brash, A.R., 1999. Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. J. Biol. Chem. 274, 23679–23682.

Brash, A.R., Boeglin, W.E., Chang, M.S., Shieh, B.-H., 1996. Purification and molecular cloning of an 8*R*-lipoxygenase from the coral *Plexaura homomalla* reveal the related primary structures of *R*- and *S*-lipoxygenases. J. Biol. Chem. 271, 20949–20957.

Cannell, R., 1993. Algae as a source of biologically active products. Pestic. Sci. 39, 147–153.

Coffa, G., Brash, A.R., 2004. A single active site residue directs oxygenation stereospecificity in lipoxygenases: stereocontrol is linked to the position of oxygenation. Proc. Natl. Acad. Sci. USA 101, 15579–15584.

Di Venere, A., Salucci, M.L., van Zadelhoff, G., Veldink, G., Mei, G., Rosato, N., Finazzi-Agro, A., Maccarrone, M., 2003. Structure-to-function relationship of mini-lipoxygenase, a 60-kDa fragment of soybean lipoxygenase-1 with lower stability but higher enzymatic activity. J. Biol. Chem. 278, 18281–18288.

Feussner, I., Wasternack, C., 2002. The lipoxygenase pathway. Annu. Rev. Plant Biol. 53, 275–297.

Göbel, C., Feussner, I., Rosahl, S., 2003. Lipid peroxidation during the hypersensitive response in potato in the absence of 9-lipoxygenases. J. Biol. Chem. 278, 52834–52840.

Hörnsten, L., Su, C., Osbourn, A.E., Hellman, U., Oliw, E.H., 2002. Cloning of the manganese lipoxygenase gene reveals homology with the lipoxygenase gene family. Eur. J. Biochem. 269, 2690–2697.

Hornung, E., Walther, M., Kühn, H., Feussner, I., 1999. Conversion of cucumber linoleate 13-lipoxygenase to a 9-lipoxygenating species by

site-directed mutagenesis. Proc. Natl. Acad. Sci. USA 96, 4192–4197.

Hornung, E., Pernstich, C., Feussner, I., 2002. Formation of conjugated $D^{11}D^{13}$-double bonds by D^{12}-linoleic acid (1,4)-acyl-lipid-desaturase in pomegranate seeds. Eur. J. Biochem. 269, 4852–4859.

Iny, D., Grossman, S., Pinsky, A., 1993. Lipoxygenase of the thermophilic bacteria *Thermoactinomyces vulgaris* – properties and study on the active site. Int. J. Biochem. 25, 1325–1330.

Kühn, H., 2005. Biologic relevance of lipoxygenase isoforms in atherogenesis. Expert Rev. Cardiovas. Ther. 3, 1099–1110.

Liavonchanka, A., Feussner, I., 2006. Lipoxygenases: occurrence, functions and catalysis. J. Plant Physiol. 163, 348–357.

Lotzer, K., Funk, C.D., Habenicht, A.J.R., 2005. The 5-lipoxygenase pathway in arterial wall biology and atherosclerosis. Biochim. Biophys. Acta 1736, 30–37.

Müller, M.J., 2004. Archetype signals in plants: the phytoprostanes. Curr. Opin. Plant Biol. 7, 441–448.

Murakami, N., Shirahshi, A.N., Sakakibara, J., 1992. Two unsaturated 9*R*-hydroxy fatty acids from the cyanobacterium *Anabaena flos-aquae f. flos-aquae*. Lipids 27, 776–778.

Pohnert, G., 2000. Wound-activated chemical defense in unicellular planktonic algae. Angew. Chem., Int. Ed. Engl. 39, 4352–4354.

Pohnert, G., 2005. Diatom/copepod interactions in plankton: The indirect chemical defense of unicellular algae. ChemBioChem 6, 1–14.

Porta, H., Rocha-Sosa, M., 2001. Lipoxygenase in bacteria: a horizontal transfer event? Microbiology 147, 3199–3200.

Schilmiller, A.L., Howe, G.A., 2005. Systemic signaling in the wound response. Curr. Opin. Plant Biol. 8, 369–377.

Siedow, J.N., 1991. Plant lipoxygenase – structure and function. Ann. Rev. Plant Physiol. Plant Mol. Biol. 42, 145–188.

Sloane, D.L., Leung, R., Craik, C.S., Sigal, E., 1991. A primary determinant for lipoxygenase positional specificity. Nature 354, 149–152.

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.

Vance, R.E., Hong, S., Gronert, K., Serhan, C.N., Mekalanos, J.J., 2004. The opportunistic pathogen *Pseudomonas aeruginosa* carries a secretable arachidonate 15-lipoxygenase. Proc. Natl. Acad. Sci. USA, 0307308101.

Wasternack, C., Hause, B., 2002. Jasmonates and octadecanoids: signals in plant stress responses and development. Prog. Nucleic Acid Res. Mol. Biol. 72, 165–221.

Wasternack, C., Stenzel, I., Hause, B., Hause, G., Kutter, C., Maucher, H., Neumerkel, J., Feussner, I., Miersch, O., 2006. The wound response in tomato – role of jasmonic acid. J. Plant Physiol. 163, 297–306.

Watson, S.B., 2003. Cyanobacterial and eukaryotic algal odour compounds: signals or by-products? A review of their biological activity. Phycologia 42, 332–350.

Wichard, T., Göbel, C., Feussner, I., Pohnert, G., 2005. Unprecedented lipoxygenase/hydroperoxide lyase pathways in the moss *Physcomitrella patens*. Angew. Chem., Int. Ed. Engl. 44, 158–161.

Zimmerman, D.C., Vick, B.A., 1973. Lipoxygenase in *Chlorella pyrenoidosa*. Lipids 8, 264–266.