THESIS ON NATURAL AND EXACT SCIENCES B183

Allene Oxide Synthase-lipoxygenase Pathway in Coral Stress Response

HELIKE LÕHELAID

TUT PRESS

TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Science
Department of Chemistry

This dissertation was accepted for the defense of the degree of Doctor of Philosophy in Chemistry on November 17, 2014.

Supervisor: **Professor Nigulas Samel**
Department of Chemistry, Tallinn University of Technology, Estonia

Reviewed by: **Dr. Vello Tõugu**
Department of Gene Technology, Tallinn University of Technology, Estonia

Opponents: **Professor Andrew F. Rowley**
Centre for Sustainable Aquaculture Research, Department of Pure & Applied Ecology, School of the Environment & Society Swansea University, United Kingdom

Professor Tanel Tenson
Institute of Technology, University of Tartu, Estonia

Defense of the thesis: January 28, 2015

Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any other academic degree.

/Helike Lõhelaid/

Copyright: Helike Lõhelaid, 2015
ISSN 1406-4723
ISBN 978-9949-23-724-1 (publication)
ISBN 978-9949-23-725-8 (PDF)

LOODUS- JA TÄPPISTEADUSED B183

Alleenoksiidi süntaas-lipoksügenaasi rada korallide stressivastuses

HELIKE LÕHELAID

TTÜ KIRJASTUS

CONTENTS

LIST OF PUBLICATIONS

This dissertation is based on the following publications, which will be referred to in the text by their Roman numerals.

I Lõhelaid, H., Järving, R., Valmsen, K., Varvas, K., Kreen, M., Järving, I., Samel, N. (2008). "Identification of a functional allene oxide synthase-lipoxygenase fusion protein in the soft coral *Gersemia fruticosa* suggests the generality of this pathway in octocorals." Biochim. Biophys. Acta 1780(2): 315-321

II Lõhelaid, H., Teder, T., Tõldsepp, K., Ekins, M., Samel, N. (2014). "Up-regulated expression of AOS-LOXa and increased eicosanoid synthesis in response to coral wounding." PLoS ONE 9(2): e89215

III Lõhelaid, H., Teder, T., Samel, N. (2014). "Lipoxygenase-allene oxide synthase pathway in octocoral thermal stress response." Coral Reefs DOI: 10.1007/s00338-014-1238-y

INTRODUCTION

Marine ecosystems are living multi-species systems that are challenged and adapt to environmental changes. Corals are integral species in major marine ecosystems worldwide and are endangered due to biotic and abiotic stressors. Understanding the interplay between stress elicitors and early responses generated would improve the predictive power and measures for preventing the loss or decline in coral reefs. Because corals are some of the simplest animal species, they further represent valuable model organisms to study stress and adaptation. Thus, the basic results of oxylipin pathways obtained in corals are likely applicable to higher animals, which, due to their complexity and restrictions, are difficult to study *in vivo*.

Oxylipins are well-established stress mediators in vertebrates and plants, synthesized mainly by lipoxygenase (LOX) and cyclooxygenase (COX), and LOX and allene oxide synthase (AOS) pathways, respectively. In corals, besides COX and LOX enzymes, the initial oxidation of arachidonic acid (AA) is catalyzed by a natural fusion protein, comprised of a catalase related peroxidase and a lipoxygenase (AOS-LOX). The product of the C-terminal LOX domain (8*R*- hydroperoxide) is sequentially converted by the N-terminal AOS to an unstable allene oxide (AO), which decomposes in water into 8-hydroxy, 9-keto-5*Z*,11*Z*,14*Z*-trienoic acid (α-ketol) and 9-oxo-prosta-5*Z*,10,14*Z*-trienoic acid (cyclopentenone/ preclavulone A). Although AOS-LOX and derived eicosanoids have been detected in two soft coral species, the biological significance of AOS-LOX metabolites in corals has not been established.

The biosynthesis of eicosanoids varies among corals. For example, in addition to metabolites of LOX and AOS-LOX pathways, large amount of COX products, endogenous prostaglandins (PGs), are present in *Plexaura homomalla*, but no COX activity is detected *in vitro*. However, conclusive evidence shows that COX enzymes are responsible for PG synthesis in *P. homomalla.* Whereas both endogenous PGs, and COX activity *in vitro* are detected in *Gersemia fruticosa,* in some corals neither PGs nor COX are found. Based on the genome of *Acropora digitifera,* stony corals are devoid of the COX gene. At the same time, the AOS-LOX gene is present in both soft and stony corals. The biological function of this widely distributed AOS-LOX pathway in corals is unknown.

According to previous studies conducted by our group, *G. fruticosa* contains all of the aforementioned AA pathways. Unfortunately, due to its extreme natural environment, the coral is unsuitable for *in vivo* experiments. In order to find a more convenient model for stress experiments, the octocorals *Capnella imbricata, Sarcophyton sp., Lobophyton sp*. and *Xenia sp.* were analyzed for their fatty acid content. Eventually, due to its high AA content, easily cultivated and fast growing *C. imbricata* was chosen for further studies of the oxylipin metabolism.

In this thesis the biosynthetic AOS-LOX pathway, which converts AA to allene oxide, was established for the Arctic *G. fruticosa* and Indo-Pacific *C.*

imbricata, and identified as a fast and sensitive pathway induced in response to mechanical and thermal stress in *C. imbricata in vivo*. Observed changes suggest the importance of the AOS-LOX pathway in mediating the early stress response of coral.

ABBREVIATIONS

8*R*-HETE	8*R*-hydroxy-(5*Z*, 9*E*, 11*Z*, 14*Z*)-eicosatetraenoic acid
8*R*-HpETE	8*R*-hydroperoxy-(5*Z*, 9*E*, 11*Z*, 14*Z*)-eicosatetraenoic acid
α-ketol	8-hydroxy, 9-keto-(5*Z*, 11*Z*, 14*Z*)-eicosatrienoic acid
AA	arachidonic acid, (5*Z*, 8*Z*, 11*Z*, 14*Z*)-eicosatetraenoic acid
ALA	α- linolenic acid, (9*Z*, 12*Z*, 15*Z*)-octadecatrienoic acid
AO	allene oxide; 8, 9- epoxy-(5*Z*, 9*E*, 11*Z*, 14*Z*)-eicosatetraenoic acid
AOC	allene oxide cyclase
AOS	allene oxide synthase
AOS-LOX	allene oxide synthase-8*R*-lipoxygenase fusion protein
COX	cyclooxygenase
Cyclopentenone	9-oxo-prosta-(5*Z*, 10, 14*Z*)-trienoic acid
CYP	cytochrome P450 family proteins
dnOPDA	dinor-oxo-phytodienoic acid; 10-oxo-(8, 13*Z*)-phytodienoic acid
DES	divinyl ether synthase
EPA	(5*Z*, 8*Z*, 11*Z*, 14*Z*, 17*Z*)-eicosapentaenoic acid
Grp78/BiP	glucose-regulated protein 78/binding immunoglobulin protein
HPL	hydroperoxide lyase
HETE	hydroxyeicosatetranoic acid
HpETE	hydroperoxyeicosatetranoic acid
HSF	heat shock factors
HSR	heat shock response
Hsp(70)	heat shock protein (70 kDa)
JA	jasmonic acid; (+)-7- iso-jasmonic acid
LA	linoleic acid, (9*Z*, 12*Z*)-octadecadienoic acid
LOX	lipoxygenase
OPDA	oxo-phytodienoic acid; (+)-12-oxo-(10, 15*Z*)-phytodienoic acid
PUFA	polyunsaturated fatty acid
qPCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
RP-HPLC	reverse phase-high performance liquid chromatography
RT-PCR	reverse transcription polymerase chain reaction
TXA_2	thromboxane A_2
UPC	unidentified polar compounds
UPR	unfolded protein response

1. REVIEW OF THE LITERATURE

1.1. Oxylipin pathways

Oxylipins are commonly found in aerobic organisms (plants, animals and fungi) (Blee 1998,2002; Rowley et al. 2005; Wasternack 2007; Serhan et al. 2008a; Wasternack and Hause 2013a). They are formed from polyunsaturated fatty acids (PUFA) by pathways involving at least one mono- or dioxygen-dependent oxidation (Gerwick et al. 1991). The main precursors of oxylipins are C16 and C18 PUFAs in plants (Wasternack and Hause 2013a) and C20 (C22) PUFAs in animals (Wymann and Schneiter 2008). PUFAs are converted to corresponding oxylipins by the activity of lipoxygenase (LOX), cytochrome P450 (CYP) and alpha-dioxygenases (DOX) in plants (Fig. 1a) and LOX, cyclooxygenase (COX) and CYP enzymes in animals (Fig. 1b) (Blee 2002). It should be noted that while plants lack COXs, some CYP families (e.g. CYP74) are not present in higher animals (Nelson et al. 2013). Due to the labile nature of the compounds, oxylipins are messengers acting in an auto-paracrine manner, and mostly are not stored in tissues, but rather are formed in response to various stimuli (Serhan et al. 1996; Blee 1998; Serhan et al. 2008a). At the same time, some oxylipins (e.g. phytoprostanes) are also formed non- enzymatically under conditions of oxidative stress via the action of reactive oxygen species (ROS) (Mueller 2004; Mueller and Berger 2009), generating highly complex oxylipin patterns.

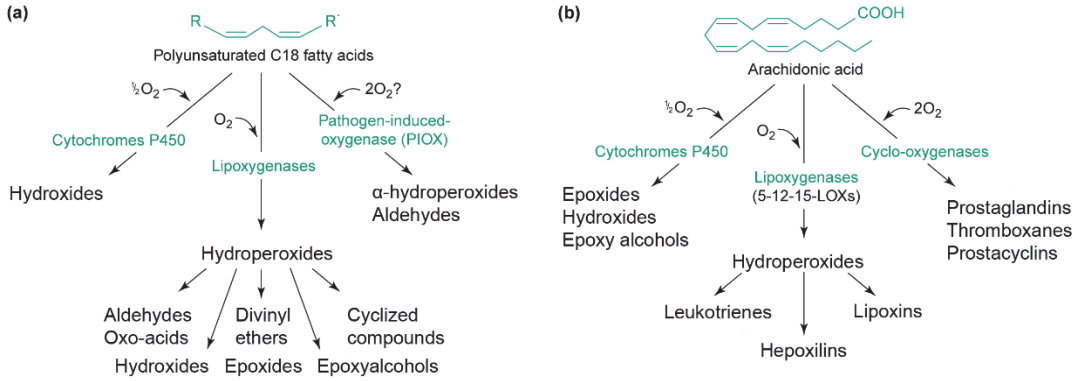


Figure 1. Oxidative metabolism of PUFAs in plants (a) and mammals (b) (adapted from (Blee 2002)).

1.1.1. Plant-derived oxylipins

Different classes of oxylipins are formed from hexatrienoic (C16:3), linoleic (LA, C18:2) or α-linolenic (ALA, C18:3) acids by at least six different enzyme families (Fig. 1a) (Blee 2002; Feussner and Wasternack 2002; Mosblech et al. 2009;

Wasternack and Kombrink 2010; Wasternack and Hause 2013a). LOXs and DOXs conduct the formation of regio- and stereospecific fatty acid hydroperoxides (Hamberg et al. 2003; Wasternack and Hause 2013a). Fatty acid hydroperoxides formed by LOXs are substrates for downstream CYP family proteins, including allene oxide synthase (AOS), hydroperoxide lyase (HPL) and divinyl ether synthase (DES), leading to the formation of unstable allene oxides (AO), short chain aldehydes and divinyl ether containing PUFAs, respectively (Fig. 2) (Howe and Schilmiller 2002; Lee et al. 2008). The relative levels of jasmonic acid (JA), oxo-phytodienoic acid (OPDA) and C16 derived dinor-oxo-phytodienoic acid (dnOPDA) are oxylipin signatures of plants. It is notable that under normal conditions the oxylipin signatures in different plant species are similar, but not identical (e.g. potatoes *versus Arabidopsis thaliana*) (Weber et al. 1997). In general, oxylipin synthesis is induced by wounding, and the metabolites formed up-regulate its synthesis pathway (Wasternack 2007).

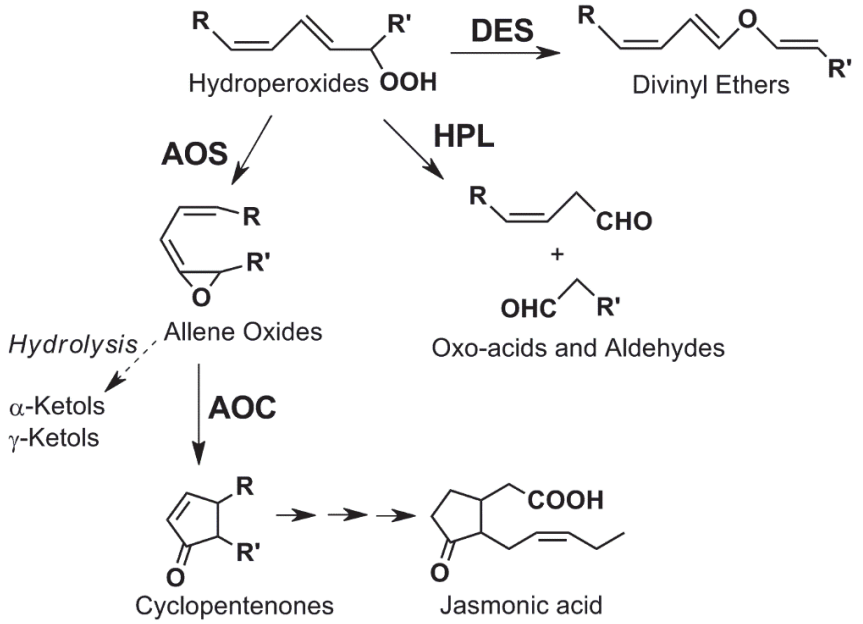


Figure 2. Plant oxylipin pathways. LOXs convert LA and ALA to corresponding 9- and 13-hydroperoxides. AOS converts fatty acid hydroperoxide to allene oxide, HPL into volatile compounds and DES into divinyl ether containing fatty acids. R and R' vary according to the substrate.

As previously described, a great number of enzymes are responsible for the metabolism of fatty acids. Here the focus is on the biosynthesis of oxylipins initiated by the LOX and followed by either AOS or HPL.

The best-characterized pathway of plant oxylipins is the formation of the stress mediator JA via its precursor OPDA (Wasternack and Kombrink 2010).

Accordingly, the biosynthesis of JA is catalyzed from ALA by sequential activities of LOX, AOS and allene oxide cyclase (AOC) (Blee 2002; Block et al. 2005). While the non-enzymatic cyclization of AO leads to a racemic mixture of OPDA, AOC catalyzes the stereospecific formation of 9*S*, 13*S* enantiomer essential in the formation of JA (Ziegler et al. 1997). The first steps of JA synthesis (up to OPDA formation) take place in the plastids, while the shortening of the carboxylic acid side-chain and the formation of JA are conducted in peroxisomes (Fig. 3) (Wasternack 2007). JA is elicited in response to injury, pathogenesis and herbivory (Farmer et al. 2003; Schilmiller and Howe 2005). Jasmonates, which are comprised of JA, methyl ester- and amino acid conjugates of JA, are involved in growth and development, and are essential in the local and systemic signaling of defense (Wasternack and Kombrink 2010; Wasternack and Hause 2013a). However, OPDA, the precursor of JA, is a signal mediator in tendril coiling in the climbing plant *Bryonia dioica* (Blechert et al. 1999), which has been found to be a potent regulator of the wound response in *A. thaliana* (Stintzi et al. 2001). Importantly, OPDA and JA regulate a distinct set of genes in response to wounding (Taki et al. 2005).

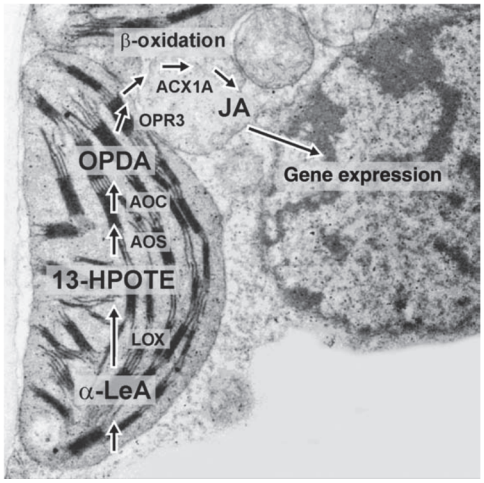


Figure 3. Localization of the lipoxygenase pathway in a plant cell (Wasternack 2007).

In parallel with the AOS route, the HPL pathway mediates the conversion of 13*S*- fatty acid hydroperoxide to non-volatile traumatin (C12 oxo-acid) and volatile C6 aldehydes (Fig. 2) (Zimmerman and Coudron 1979; Vick and Zimmerman 1987; Bate et al. 1998; Howe et al. 2000; Grechkin and Hamberg 2004; Halitschke et al. 2004). The volatile compounds are instantly synthesized at the chloroplast membranes in response to wounding, e.g. the unstable (*Z*)-3-hexenal generated is detected 1-2 s after injury (Fall et al. 1999), stimulating the healing of the wound and inducing the expression of the prosystemin gene, which in turn acts as a positive feedback loop enhancing wound signal. Metabolites of the unstable (*Z*)-3-hexenal are (*E*)-2-hexenal, hexenols and hexenyl acetates.

Hexanals and hexenals have antibacterial (Croft et al. 1993) and antifungal properties (Major et al. 1960; Gueldner et al. 1985; Sivasankar et al. 2000). In addition, they function as messengers in plant-to-plant signaling (Bate and Rothstein 1998) and "attract" or "repel" insects (Croft et al. 1993; Turlings et al. 1995). The volatile aldehydes have a compound specific aroma, also detectable by humans. For example, the leaf aldehyde (*E*)-2-hexenal in a 1:1 mix with hexenol emits a "fresh green" scent of newly mown grass. Hexanal or (*E*)-3-hexanal, the result of 13- hydroperoxide break-down, emits a beany-grassy odor. (*E*)-3-noneal or (*E*)-3, (*E*)-6 nonadienal, produced from 9-hydroperoxide, emit a cucumber-pear-like odor (Noordermeer et al. 2001b). For humans, the "green odor" has anti-stress properties (Oka et al. 2008). Accordingly, the volatiles (including JA) are used as scents in fragrances.

Traumatin and traumatic acid are formed from the carboxyl end of the fatty acid hydroperoxides; they are wound hormones that promote growth (Zimmerman and Coudron 1979). For example, 9-OH traumatin is involved in the fast wound response, produced non-enzymatically and enzymatically within the first hour, and is not stored (Kallenbach et al. 2011).

1.1.2. Animal-derived oxylipins

Eicosanoids are the main branch of oxylipins synthesized in animals from arachidonic acid (AA, C20:4ω6) and other C20 polyenoic acids by fatty acid di- and monooxygenases, LOX, COX and cytochrome P450 epoxygenases, respectively (Fig. 1b and Appendix 1) (Brash 1999; Rouzer and Marnett 2003; Nelson et al. 2013). In addition to AA, other PUFAs, such as eicosapentaenoic acid and docosahexaenoic acid (DHA, C22:6ω3), are the precursors for important PUFA derivatives: resolvins and protectins (Serhan et al. 2002; Serhan et al. 2008a), which mediate the resolution of inflammation.

Vertebrate eicosanoids

Animal 5-, 8-, 11-, 12- and 15-LOXs (E.C. 1.13.11.-) catalyze the conversion of AA into corresponding 5-, 8-, 11-, 12- and 15- hydroperoxyeicosatetraenoic acids (HpETEs) (Brash 1999) (Appendix 1, b). Depending on the species and cell-type specific expression of enzymes, the content and distribution of eicosanoids vary. For example, six LOXs with 5-, 12-, 15- specificity are expressed in human (Horn et al. 2013), while additional 8-LOX is expressed in mouse skin (Jisaka et al. 2000). Thus far, the LOX with 11*R*-specificity has been identified only in marine invertebrates, such as hydra (Di Marzo et al. 1993), sea urchins (Hawkins and Brash 1987) and corals (DiMarzo et al. 1996; Varvas et al. 1999; Mortimer et al. 2006). In terrestrial organisms, the prevalent stereoconfiguration of LOX products is *S*, while *R* stereospecificity is more pronounced in marine invertebrates.

HpETEs are involved in cell migration (chemotactic) and are potent pro- and anti-tumorigenic agents. For example, 5- and 12-HETEs formed by 5- and 12-LOX, and 13-HODE formed by 15-LOX are involved in the proliferation and inhibition of apoptosis, angiogenesis, cancer invasion and metastasis, while 15- and 8-HETE formed by 15-LOX2 and 8-LOX are involved in the differentiation, growth arrest and induction of apoptosis (Pidgeon et al. 2007; Moreno 2009). In addition, they mediate atherosclerosis (15-HETE) and allergic inflammation (Duroudier et al. 2009). Downstream LOX cascades produce leukotrienes and lipoxins, which promote inflammation and are involved in the initiation and resolution of inflammation, respectively (Funk 2001; Serhan and Chiang 2002; Haeggström and Funk 2011).

Another oxygenation route converting AA to prostaglandins (PGs) is catalyzed by COXs, also known as prostaglandin endoperoxide synthases (E.C. 1.14. 99.1). All vertebrates have two COX isozymes, a constitutive COX-1 and an inducible COX-2 (Funk 2001). Both COXs catalyze the formation of PGG_2 (cyclooxygenase activity) and its reduction to PGH_2 (peroxidase activity) (Rouzer and Marnett 2003; Schneider et al. 2007). The main differences between COX-1 and COX-2 are their genetic regulation and function (Rouzer and Marnett 2005; Blobaum and Marnett 2007). The formation of PGH_2 by COXs is a rate-limiting step in its downstream conversion to prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, and PGD_2, as well as the conversion to thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) by corresponding isomerases or synthases (Fig. 4 and Appendix 1, a) (Rouzer and Marnett 2011).

Inhibition of COX reduces inflammation, pain and fever (Flower 2006). The anti-inflammatory effect of non-steroidal anti-inflammatory drugs (NSAIDs) is related to COX-2 inhibition (Vane and Botting 1998). COX-2 is also involved in the promotion and progression of cancer, although, the role is tumor specific (Krishnamoorthy and Honn 2011; Moga 2013). In conferring their biological function, e.g. evoking an inflammatory response after injury, PGs have opposite effects. For example, depending on the timing and course of inflammation, they can either induce vasoconstriction ($PGF_{2\alpha}$, TXA_2, TXB_2) or vasodilation (PGE_1, PGE_2, PGI_2), inhibition of platelet aggregation (PGD_2, TXA_1, PGE_1, PGI_2) (Murakami 2011; Ricciotti and FitzGerald 2011) or aggregation of platelets (PGE_2) (Howie et al. 1973; Kobzar et al. 1997). In addition, elevated levels of PGE_2 sensitize spinal neurons (inducing the sense of pain) (Grace et al. 2014), act on the hypothalamus to cause fever (Coceani and Akarsu 1998), and are involved in the complex process of labor (e.g. cervical ripening and contractions) (Kelly et al. 2009).

Animal CYPs catalyze the biotransformation-oxygenation of drugs and xenobiotic toxicants, as well as endogenous PUFAs (Capdevila et al. 2000; Nelson et al. 2013). For example, CYP1B, CYP2A, CYP2B, CYP2C, CYP2E, CYP2J, CYP4A and CYP11 monooxygenases are present in the human heart, and all require O_2, CYP reductase and NADPH for catalysis (Seubert et al. 2007).

Using AA as a substrate, they produce various hydroxy fatty acids, e.g. 16- and 20-HETE, and four regioisomers of epoxyeicosatrienoic acid (EETs). For example, the epoxygenation of AA leads to 5,6-, 8,9-, 11,12-, and 14,15-EETs in humans (Appendix 1, c) (Rouzer and Marnett 2011). EETs are further converted to corresponding dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolase, e.g. 14, 15-DHET is formed from 14, 15-EET (Zeldin et al. 1993). In vertebrates, the representatives of CYP family converting fatty acid peroxides are CYP5A1 (thromboxane synthase) and CYP8A1 (prostacyclin synthase) (Brash 2009).

The bioactivity of EETs depends on the specific regioisomer and the cellular context. EETs regulate ion transport and blood pressure (vasodilators) (Larsen et al. 2006; Capdevila 2007), sodium excretion (Capdevila and Falck 2002; Capdevila et al. 2014) and angiogenesis (Wang 2005). EETs are involved in cardio-protection after injury (Seubert et al. 2007). Endothelial-derived EETs promote tissue growth, e.g. liver regeneration and compensatory growth of the kidney and lung *in vitro* (Panigrahy et al. 2013). 5,6- and 8,9-EETs promote endothelial cell migration and angiogenesis (Pozzi et al. 2005), while 11,12-EET is anti-inflammatory (Node et al. 1999) and anti-migratory for vascular smooth muscle cells (Sun et al. 2002; Spector and Norris 2007). 20-HETE produced by ω-hydroxylases is a vasoconstrictor inhibiting Ca^{2+}- sensitive K^{+} channels (Seubert et al. 2007), and promotes endothelial cell proliferation (Guo et al. 2007).

Coral eicosanoids

Corals are invertebrate animals (Kingdom *Animalia*; phylum *Cnidaria*; class *Anthozoa*) (Hyman 1940) that are divided into two major subclasses: reef-building *Hexacorallia* and soft corals *Octocorallia* (Zhang 2011), both comprised of azooxanthellate or zooxanthellate, the latter living in symbiosis with unicellular algae, *Symbiodinium sp.* species.

Coral oxylipin studies started with the detection of large quantities of PG and PG-esters (2-3% of dry weight) in the soft coral *Plexaura homomalla* (Weinheimer and Spraggins 1969). Thereafter, a plethora of eicosanoids have been discovered, which vary depending on the species and location (Corey et al. 1973; Brash et al. 1987; Corey et al. 1987; Corey et al. 1988; Varvas et al. 1993; Varvas et al. 1999). In soft corals, AA is an abundant fatty acid (10-25%), being the primary precursor of eicosanoids (Imbs et al. 2006; Imbs and Yakovleva 2011). To a lesser degree (3-10%) AA also contributes to the fatty acid content of stony corals (Latyshev et al. 1991; Figueiredo et al. 2012; Voolstra et al. 2012). Free AA is metabolized by COX (Varvas et al. 1994a; Koljak et al. 2001; Valmsen et al. 2001) or LOX (Brash et al. 1996; Mortimer et al. 2006) into PGs or H(p)ETEs, respectively (Fig. 4). In addition to 11*R*-LOX (Mortimer et al. 2006; Eek et al. 2012; Jarving et al. 2012), a unique allene oxide synthase- 8*R*-lipoxygenase (AOS-LOX) fusion protein pathway was discovered in corals (Koljak et al. 1997). In this naturally occurring protein, a LOX (with 8*R*-stereospecificity) is fused to an AOS and thus

mediates two sequential catalytic steps: AA oxidation into 8*R*-hydroperoxyeicosatetraenoic acid (8*R*-HpETE) and the conversion of hydroperoxide to labile allene epoxide (Fig. 4) (Koljak et al. 1997; Boutaud and Brash 1999).

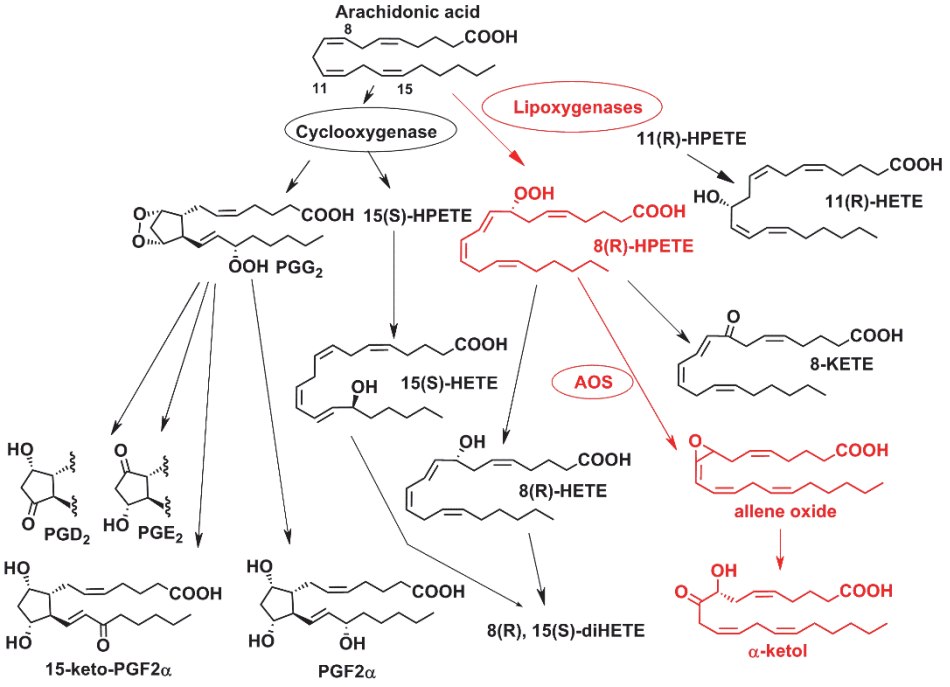


Figure 4. Endogenous eicosanoids identified in the soft coral G. fruticosa (Varvas et al. 1993; Varvas et al. 1999). AOS-LOX pathway is depicted in red.

In essence, the coral AOS-LOX pathway is similar to the plant JA pathway (Fig. 5), except for the separately expressed LOX and AOS proteins in plants (Wasternack 2007), and the non-enzymatic decomposition of AO into a mixture of α-ketol and cyclopentenone in corals (Fig. 5) (Koljak et al. 1997). Initially, the cyclopentenone was thought to be the precursor of coral PGs, but the cloning and characterization of functional coral COXs indicated the existence of parallel oxygenation routes (Koljak et al. 2001; Valmsen et al. 2001).

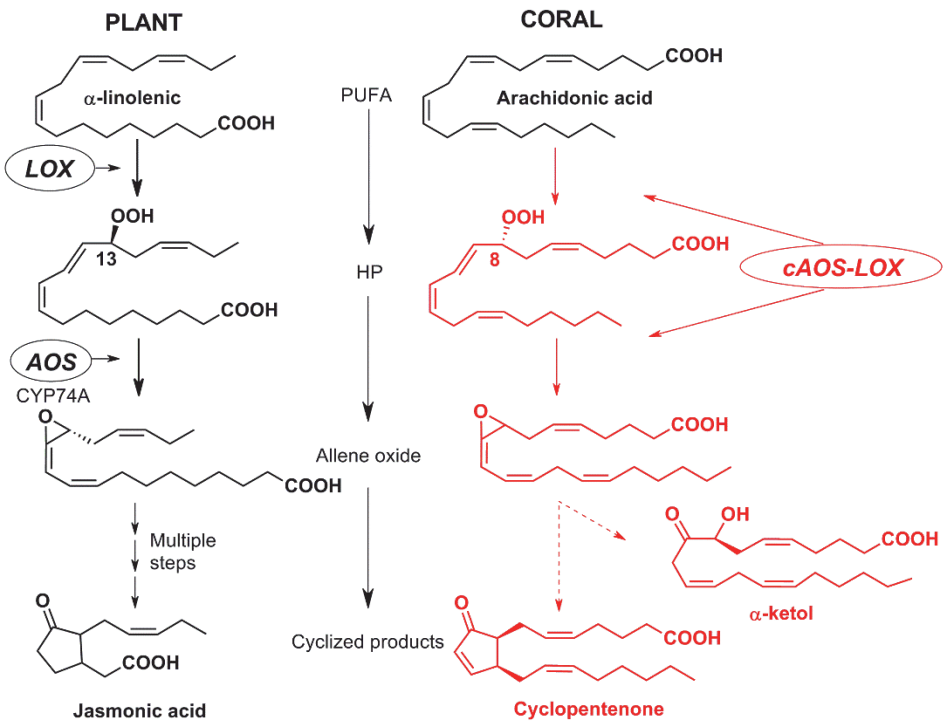


Figure 5. Formation of oxylipins containing the cyclopentenone moiety by corals and plants, (adapted from (Koljak et al. 1997)). PUFA- polyunsaturated fatty acid substrate; HP- fatty acid hydroperoxide. AOS-LOX pathway is depicted in red.

Interestingly, while *P. homomalla* contains a considerable amount of PGs, during biosynthesis with exogenous AA no PGs are formed (Corey et al. 1973; Corey et al. 1988). On the contrary, besides the PGs detected as free acids in coral extracts of *G. fruticosa*, the coral homogenate biosynthesizes optically active PGs *in vitro* (Varvas et al. 1993; Varvas et al. 1999). However, *Clavularia viridis* converts AA via 8*R*-HpETE to clavulones (preclavulone A) (Corey et al. 1987), bromovulones and iodovulones (Fig. 6) (Honda et al. 1987; Watanabe et al. 2001). Altogether, this data is indicative of species-specific eicosanoid biosynthesis. Although AOS-LOXs are not involved in the biosynthesis of coral PGs, they still might be involved in the synthesis of clavulone-like derivatives.

Current literature on coral eicosanoids contains data on the identification of naturally occurring compounds (Corey et al. 1973; Corey et al. 1985; Varvas et al. 1993; Varvas et al. 1994b), the elucidation of metabolic pathways involved in their biosynthesis (Brash et al. 1987; Corey et al. 1987; Koljak et al. 1997; Varvas et al. 1999; Koljak et al. 2001), and the effects of lipid extracts or isolated compounds on other systems (Hashimoto et al. 2003). But to date, only the role of prostaglandins in the chemical defense of the coral *P. homomalla* has been proposed (Pawlik et al. 1987; Gerhart 1991; O'Neal and Pawlik 2002; Whalen et

al. 2010). In spite of the wide occurrence of different oxylipins (hydroxy fatty acids, prostaglandins and their derivatives, chlorovulones, punaglandis etc.) in invertebrates (Brash et al. 1987; Rowley et al. 2005), their exact functions in those organisms are unclear.

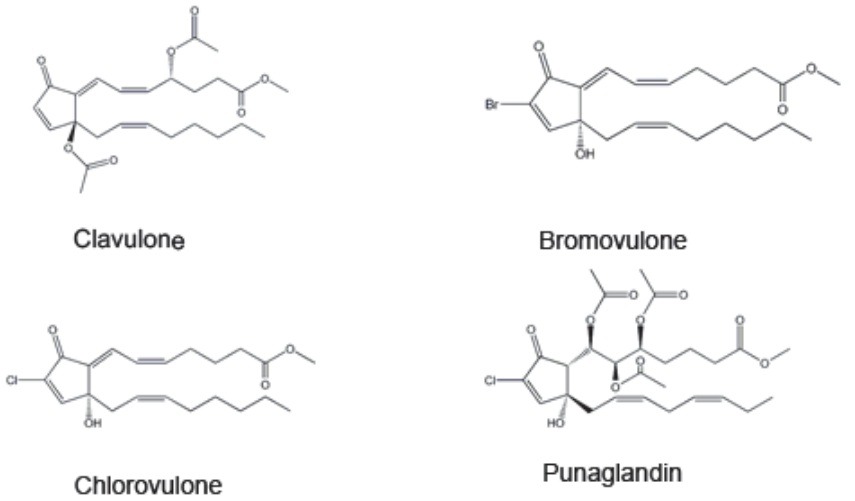


Figure 6. Atypical eicosanoids isolated from C. viridis.

1.2. Key enzymes of the Lipoxygenase pathway

1.2.1. Lipoxygenases (EC 1.13.11.-)

LOXs are non-heme iron containing dioxygenases that catalyze the stereo-specific peroxidation of PUFAs containing at least one 1-cis, 4-cis-pentadiene system to form biologically active mediators (Fig. 1) (Brash 1999). LOXs are classified in terms of their positional specificity. Plant LOXs are comprised of linoleate 9- and 13-LOXs (Feussner and Wasternack 2002) while animal LOXs are arachidonate 5-, 8-, 11-, 12- and 15-LOXs (Brash 1999; Brash et al. 1999). For example, plant 9- and 13-LOXs catalyze the oxidation of ALA at the carbon C9 or C13 and the formation of 9*S*- and 13*S*-hydroperoxy fatty acids, respectively (Fig. 2), and animal 5- and 15-LOXs catalyze the oxidation of carbon C5 or C15 of AA and the formation of 5*S*- and 15*S*-hydroperoxy fatty acids, respectively (Brash et al. 1999). Although not containing substrate PUFAs, LOXs are also isolated from gram-negative bacteria (Hansen et al. 2013). In addition, not only AA, EPA and DHA but also LA and ALA are oxygenated by animal LOXs, e.g. 15-LOX converts LA to 13-hydroperoxy- octadecadienoic acid in human epidermis (Ziboh et al. 2000).

Mechanism of catalysis

Fatty acid oxygenation by LOXs contains four steps: 1) stereoselective hydrogen abstraction from the bis-allylic methylene group, either from the carbon 7 (C7), C10 or C13 of AA, 2) radical rearrangement, resulting in delocalized carbocation, 3) regioselective oxygen addition to the antarafacial plane (an antarafacial relationship between the initial hydrogen-abstraction and sequential oxygenation), and 4) reduction of a peroxy radical to form the fatty acid hydroperoxide, resulting in either *R*- or *S*-stereoconfiguration of the peroxyl group formed (Fig. 7) (Brash 1999; Schneider et al. 2007). Altogether, twelve possibilities for AA oxygenation exist (the hydroperoxide in *R*- or *S*-stereoconfiguration is formed at C5, C8, C9, C11, C12 or C15) (Schneider et al. 2007).

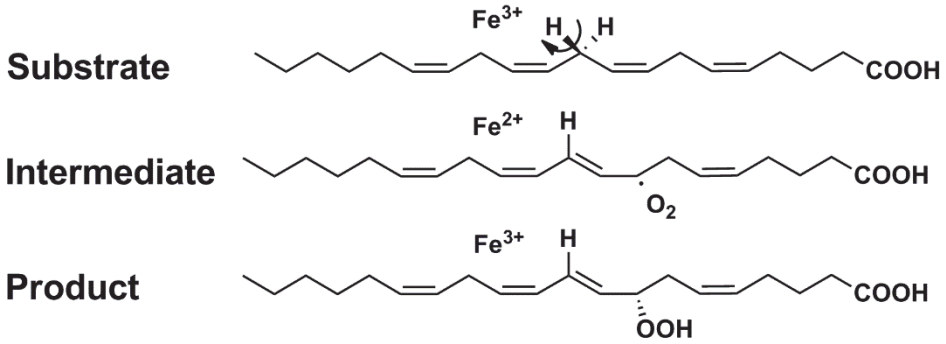


Figure 7. Lipoxygenase-catalyzed reaction. Hydrogen abstraction and antarafacial insertion of oxygen into AA by 8R-LOX.

Structure

Overall, plant LOXs are larger (94-104 kDa) than their animal counterparts (75-80 kDa) (Brash 1999; Kuhn et al. 2005). To date, the crystal structures of soybean LOX-1 and -3 (Minor et al. 1996; Skrzypczak-Jankun et al. 1997), rabbit reticulocyte 15-LOX-1 (Gillmor et al. 1997), human 12-LOX (Xu et al. 2012), coral 8*R*-LOX of AOS-LOX fusion protein (Neau et al. 2009) and 11*R*-LOX (Eek et al. 2012) have been defined. All LOX monomers are comprised of the N-terminal β-barrel and C-terminal catalytic domain.

The regulatory β-barrel is known as the PLAT (Polycystin-1, LOX, Alpha-Toxin), LH2 (LOX homology) or C2-like domain (similar to the calcium-dependent membrane-binding domain of phospholipases and kinases), and is involved in binding Ca^{2+} and interactions with membranes (Hammarberg et al. 2000; Kulkarni et al. 2002; Walther et al. 2004; Oldham et al. 2005b; Gilbert et al. 2008). At the same time, it is not essential for the catalysis *per se* (Walther et al. 2011). Still, the interactions between the β-barrel and the catalytic domain ensure the stability and full activity of the whole protein (Ivanov et al. 2011).

Based on sequence alignments, the catalytic center is conserved throughout the LOX gene family. Accordingly, structures of their active sites, where LOXs incorporate a non-hem iron, are almost superimposable (Hammarberg et al. 2000; Walther et al. 2004; Kuhn et al. 2005; Oldham et al. 2005b). Atypically, Mg^{2+} can be incorporated within LOXs as a co-factor (Oliw et al. 2004). The *R- or S-* stereospecificity of LOXs is defined by the sequence determinant located near the substrate entry site, Ala in *S-* or Gly in *R*-LOXs (Coffa et al. 2005).

Mostly LOXs are dependent on free fatty acid substrates, but some of them require their substrate directly from membranes (Takahashi et al. 1993; Maccarrone et al. 1994; Upston et al. 1997). Upon membrane binding by the PLAT-domain, intramolecular conformational changes occur, activating and enhancing the activity of LOX (Ivanov et al. 2010; Ivanov et al. 2011; Jarving et al. 2012; Di Venere et al. 2014). LOX insertion into membranes is additionally facilitated by surface amino acids of the catalytic domain (Walther et al. 2004; Jarving et al. 2012).

1.2.2. CYP74s of Cytochrome P450

Plant atypical heme-dependent fatty acid hydroperoxide-metabolizing enzymes belong to the CYP74 subfamily (EC 4.2.1.92) of the large and variant cytochrome P450 monooxygenase superfamily (P450/CYP). A study conducted on plant P450 revealed the presence of the CYP74 subfamily in all six plant genomes currently available (Nelson et al. 2008). The CYP74 subfamily is comprised of AOS (CYP74A) (Song and Brash 1991b; Laudert et al. 1996; Howe et al. 2000), 13- HPL (CYP74B), 9 and 13-HPL (CYP74C) (Vick and Zimmerman 1987; Tijet et al. 2001; Grechkin and Hamberg 2004) and DES (CYP74D) (Itoh 2000), which however are not equally present in all plant species. *A. thaliana* encodes one AOS and HPL per genome, while tomatoes have six CYP74s: AOS1, AOS2; HPL, CYP74C3, CYP74C4 and DES (Nelson et al. 2008). In plants, either 9*S*- or 13*S*-hydroperoxy fatty acids are the substrate for specific 9- or 13-AOSs. The subcellular location is an important switch defining the "selection" between the alternative pathways. 9-LOX is cytosolic, while 13-LOX and 13-AOS are located in plastids (Fig. 3) (Feussner and Wasternack 2002; Farmaki et al. 2006).

Mechanism of catalysis

CYP74s are specialized for the metabolism of fatty acid hydroperoxides (Tijet and Brash 2002). AOS dehydrates the hydroperoxy fatty acids into unstable AOs, which in water hydrolyze into α- and γ-ketols or are cyclized by a sequential allene oxide cyclase step (Feussner and Wasternack 2002). In contrast to other monooxygenase P450s, they use their hydroperoxide substrates as a source of oxygen donor and for reducing equivalents (Fig. 8) (Howe and Schilmiller 2002; Brash 2009). Two other P450s that also share those catalytic features, but do not

belong to the CYP74 family, are prostacyclin synthase and thromboxane synthase (Brash 2009).

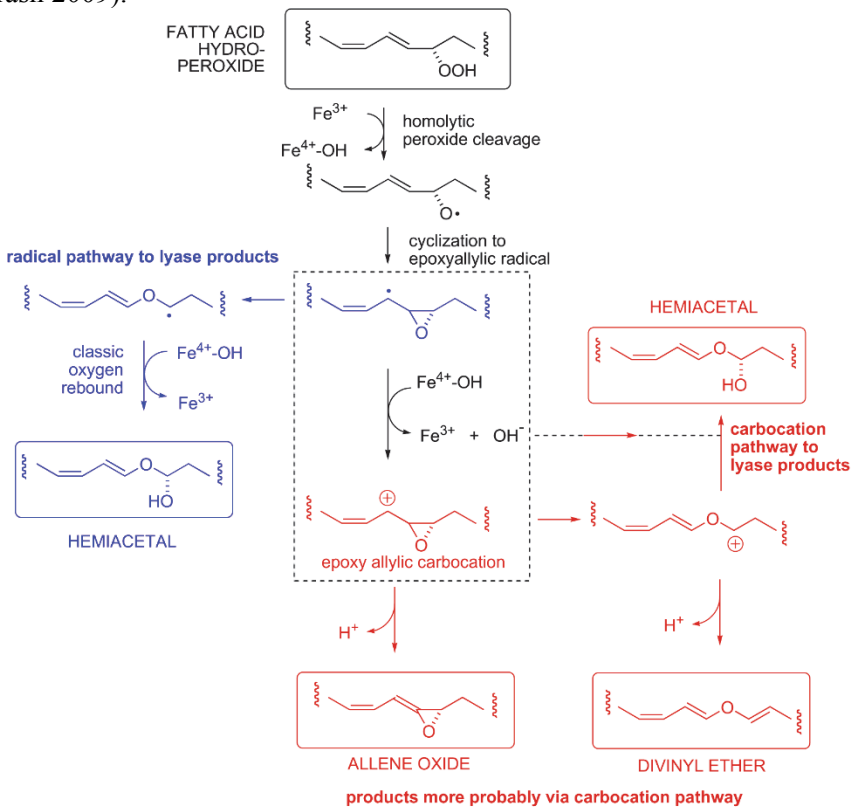


Figure 8. Proposed radical and ionic pathways to CYP74 products catalyzed by AOS, HPL and DES (Brash 2009).

Plant HPLs cleave the C-C bond adjacent to the hydroperoxy group of fatty acid hydroperoxide substrates. Consequently, oxo-acids and volatile aldehydes are formed (Fig. 2) (Bate et al. 1998; Tijet et al. 2001; Brash 2009). The 3*Z*-aldehydes formed can isomerize to their 2*E*-isomers and be reduced by alcohol dehydrogenase to corresponding alcohols (Bate and Rothstein 1998; Noordermeer et al. 2001b).

Structure

According to the crystal structures of plant CYP74s, 13-AOSs from *A. thaliana* and guayule *Parthenium argentatum* (Chang et al. 2008; Lee et al. 2008), they share a common fold with other P450s. However, all CYP74 sequences are devoid of conserved motifs (e.g. I-helix) of P450s and contain a unique nine amino acid insert at the heme-binding region responsible for the specific reactions catalyzed (Nelson et al. 2008). AOS and HPL proteins are both located in

membranes (Froehlich et al. 2001) and their interaction with membranes is mediated via the large hydrophobic surface area (Lee et al. 2008). Accordingly, the presence of membranes regulates the activity of HPL *in vitro* (Noordermeer et al. 2001a).
The structure-reaction coupling within the CYP74 family was further enhanced by a study with the AOS of tomatoes, where a single amino acid mutation (either Phe295Ile or Ser297Ala) converted the AOS activity to HPL (Toporkova et al. 2008). The same conversion was indicated by the *A. thaliana* AOS Phe137Leu mutant, whereas HPL activity was further enhanced by an additional mutation, Ser155Ala (Lee et al. 2008).

In order to study the direct effect of specific genes and their products, targeted gene knock-out studies were conducted. In animals, knock-out mice deficient in 5-LOX are less sensitive to asthma (Chen et al. 1994), and 12-LOX knock-out mice die after birth due to dehydration via skin (Epp et al. 2007), while the disruption of 12/15-LOX protects against atherosclerosis (Cyrus et al. 1999).

In plants, LOX-3, -4 mutants (devoid of JA) are male sterile (Caldelari et al. 2010). As previously noted, *A. thaliana* has only one *AOS* and one *HPL* gene per genome (Nelson et al. 2008). Similarly to plants lacking LOX, knock-out plants devoid of AOS are not able to synthesize JA; consequently, they have invalid wound response and are male sterile (Park et al. 2002). At the same time, normal growth and development are not affected by the lack of active HPL (Duan 2005). Both being located in thylakoid membranes in potatoes, AOS and HPL compete for the same substrate (Farmaki et al. 2006). Accordingly, silencing the activity of HPL in *Nicotiana attenuata* reduces the release of volatiles and amplified JA production and accumulation, and *vice versa* (Halitschke et al. 2004).

1.2.3. Coral allene oxide synthase-lipoxygenase fusion proteins

One coral specific branch of the arachidonate metabolism is catalyzed by the unique fusion protein comprised of N-terminal AOS and C-terminal 8*R*-LOX domains. Coral and plant AOS catalyze identical reactions (Fig. 5). Based on the structural determinants, the coral AOS is related to bovine catalase, although their amino acid sequence identity is only ~11% (Koljak et al. 1997; Oldham et al. 2005a). Also, the 8*R*-LOX domain of coral AOS-LOX fusion protein is more similar in size, sequence and substrate specificity to mammalian than to plant LOXs (Boutaud and Brash 1999; Neau et al. 2009). The structure of the separately expressed AOS and LOX domains (Oldham et al. 2005b,a; Neau et al. 2009), as well as the structure of the whole coral AOS-LOX fusion protein have been resolved (Fig. 9) (Gilbert et al. 2008). The crystal structure reveals a close interaction between AOS and LOX domains, which might directly influence the catalytic activities of the fusion protein. The covalent link between AOS and LOX domains assures the correct location of both proteins within a cell, as well as the timing of the products formed (spatial and temporal regulation). When expressed

separately, Ca^{2+} induces membrane association of the 8*R*-LOX domain *in vitro*, but AOS is not bound to membranes unless covalently linked to 8*R*-LOX and expressed as a fusion protein (Gilbert et al. 2008).

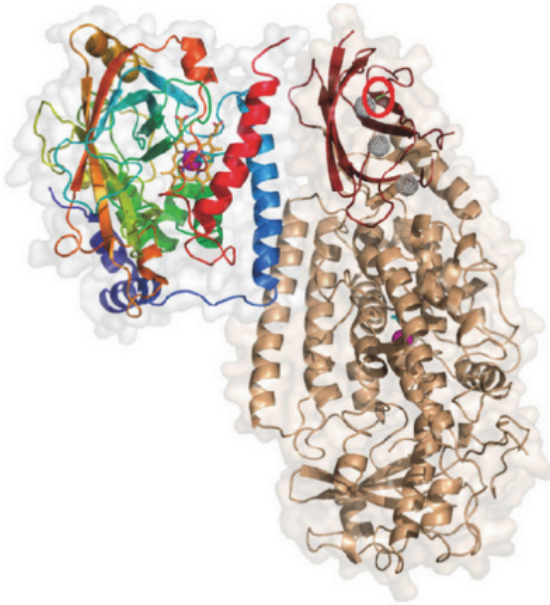


Figure 9. The structure of P. homomalla AOS-8R-LOX fusion protein. N-terminal AOS domain on the left side (multi-colored) and C-terminal LOX on the right side (golden). Red circle, β-barrel domain of LOX with bound Ca^{2+} (Gilbert et al. 2008).

As in other LOXs, the interaction of the LOX domain with Ca^{2+} is mediated by its N-terminal C2-like β-barrel (PLAT) domain (Oldham et al. 2005b). Another calcium-related feature is that LOX binds to membranes only in the presence of Ca^{2+}. The amino acids involved in membrane binding are Trp41, Phe42 and Trp77; the numbering of the LOX domain, designated as A2, starts from A374 of the fusion protein. The catalytic non-heme iron of LOX domain is coordinated by His385, His390, His571, Asn575, and Ile694. The substrate binding channel of LOX is lined with Leu386 and Leu628, assuring the right substrate position in the active site, while Gly428 determines the *R*-stereoconfiguration of the 8-HpETE formed (Oldham et al. 2005b).

The U- shape substrate binding channel of the AOS domain is well suited for fatty acid hydroperoxide substrates. The formation of AO is mediated by the His67 and Asn137 of the AOS domain (Oldham et al. 2005a). Although, there is enough space for H_2O_2 to access catalytic heme, AOS does not confer the catalase reaction. It was proposed that instead of a heterolytic bond cleavage, as in catalase reaction, homolytic cleavage takes place by coral AOS, similar to the reaction catalyzed by plant CYPs (Oldham et al. 2005a).

Although many details are known about the structure and catalytic properties of the AOS-LOX fusion protein and the up-regulation of AOS-LOX transcript in response to white band disease (Söderhäll et al. 2013), elevated UV radiation (Aranda et al. 2011) and temperature have been detected recently (Polato et al. 2013), there are no data available about the produced eicosanoids or their function in corals.

1.3. Biological functions of lipid mediators

In all species studied to date, lipid mediators mediate important adaptation responses to cellular stress. Organisms continuously sense and respond to environmental conditions to maintain their homeostasis under changing conditions and survive, at whatever cost, to produce offspring and maintain the species. Still, the cellular resources are often, if not always, limited. For example, the amount of energy available for growth or reproduction under normal conditions does not change while under stress. Rather, the same resources are re-evaluated and "spent" on alternating the metabolism to adequately respond, tolerate/sustain and overcome the stress (Fig. 10). This results in decline in other possible routes, e.g. decreased metabolism and growth (Spriggs et al. 2010). For example, the detection systems plants use to perceive herbivore attack are intermingled with the hormone pathways that reprogram the plant; this also applies to the detection of other stressors, followed by sequential reprogramming (Mittler et al. 2012).

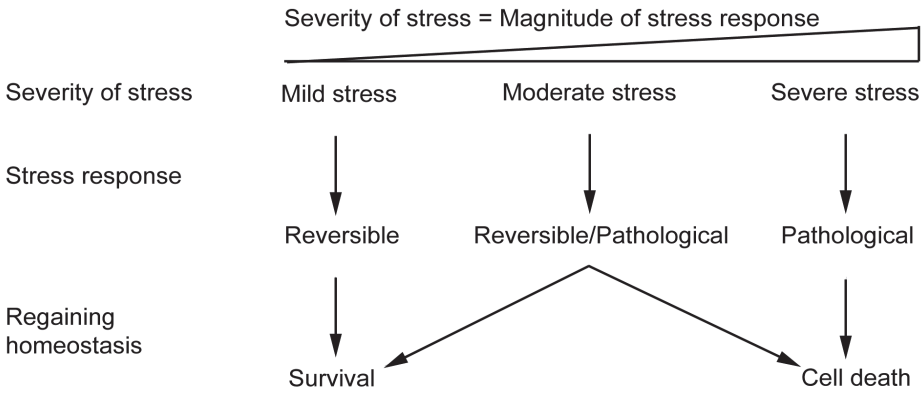


Figure 10. Simplified illustration of relationships between applied stress and the stress response generated with possible outcomes.

In physical terms, stress is defined as mechanical force (tension, compression or shear) per unit area applied to an object, resulting in mechanical deformation (Park and Lakes 2007). Due to the complexity of a biological system, it is difficult

to define stress in biological terms. In general, stress is defined as a condition that disturbs the normal function of the biological system or a condition that decreases a fitness (Bijlsma and Loeschcke 2005). The most practical definition of biological stress is: an adverse force or condition which inhibits the normal functioning and well-being of a biological system (Jones et al. 1989). At the same time, a condition which may be stressful for one organism may be the optimum for another organism. Thus, the definition of stress is true only for an organism experimentally tested within a certain, specified setting. Overall, external stressors may be biotic (pathogens, herbivores and carnivores) or physical (temperature, salinity, water, nutrient deprivation in soil, chemicals and pollutants, oxidative stress, mechanical stress and radiation).

1.3.1. Response to wounding

The initial wound response in animals and plants aims for a rapid and efficient isolation of the wound to minimize both the loss of vital fluids and environmental challenges (Maffei et al. 2007; Proksch et al. 2008; Rodriguez et al. 2008; Palmer et al. 2011; Ariel and Timor 2013).

Plant wound response

The wound response in plants includes the instant release of Ca^{2+}, ROS, leaf volatiles and traumatin (Leon et al. 2001; Maffei et al. 2007), accompanied by the rapid synthesis and accumulation of the stress hormone JA via a lipoxygenase pathway, involving LOX, AOS and AOC steps (Reymond et al. 2000; Kombrink 2012; Wasternack and Hause 2013b). After the wounding, the AOS route leads to the formation of JA (Song et al. 1993; Laudert et al. 1996), while volatile C6 aldehydes and non-volatile oxylipins are instantly generated by HPL (Table 1) (Vick and Zimmerman 1987; Bate et al. 1998; Howe et al. 2000; Halitschke et al. 2004). To induce the synthesis of JA, only one of four *A. thaliana* 13(*S*)-LOXs is up-regulated an hour upon wounding (Bell et al. 1995; Schommer et al. 2008; Glauser et al. 2009). Matching the same time window, the sequential step catalyzed by AOS is also up-regulated at the transcriptional (Laudert et al. 1996; Bate et al. 1998) and translational level (Gfeller et al. 2011), and is further enhanced by positive feedback regulation by JA and OPDA. A similar principle is used in jasmonate, auxin, gibberellin and ethylene perception and signaling (Wasternack and Hause 2013a).

Table 1. Up-regulation of the lipoxygenase pathway in the plant wound response. JA: jasmonic acid, dnOPDA: dinor-oxo-phytodienoic acid. Systemic- systemic response.

Transcript/protein/oxylipins	Species	Regulation	Time	Reference
LOX mRNA (induced by wound, MeJA)	*Arabidopsis thaliana*	up	at 6h	(Bell and Mullet 1993)
JA and MeJA	*Glycine max*	up	8h	(Creelman et al. 1992)
LOX mRNA/JA	*A. thaliana*	up	at 4h	(Bell et al. 1995)
JA	*Vicia faba*	up	45’	(Blechert et al. 1995)
AOS mRNA/JA	*A. thaliana*	up	1-1,5h and 1-1,5h	(Laudert et al. 1996)
AOS mRNA/protein/oxylipins (MeJA)	*A. thaliana*	up/systemic	6h/24h/24-48h	(Laudert and Weiler 1998)
AOS mRNA	*Linum usitatissimum*	up	0-26h, (max 6h)	(Harms et al. 1998)
AOS mRNA/protein	*Hordeum vulgare*	up	4-6h	(Maucher et al. 2000)
AOS mRNA	*Solanum lycopersicum*	up	1-20h, max 4h	(Sivasankar et al. 2000)
Transcripts/oxylipins	*A. thaliana*	up	15’ - 24h, JA (max 2h)	(Reymond et al. 2000)
AOS wt vs knock-out, mRNA/JA	*A. thaliana*	up	2-8h/no expr.; JA 1h/no JA	(Park et al. 2002)
LOX, AOS, HPL, AOC proteins	*Solanum tuberosum*	up	2-24h	(Farmaki et al. 2006)
JA	*A. thaliana*	up/systemic	1.5h, in systemic leaves JA up at 180s	(Glauser et al. 2009)
Wt vs AOS mutant. LOX, AOS, AOC proteins;	*A. thaliana*	up		(Gfeller et al. 2011)
LOX, AOS, AOC mRNA/JA	Several plants in parallel	up	Max at 20’(tomato)-40’(others)	(Wu et al. 2012)
JA in LOX mutants	*A. thaliana*	up	90s, long distance sign.	(Chauvin et al. 2013)
JA, dnOPDA, OPDA	*A. thaliana and S. tuberosum*	up	at 1.5h and at 4h	(Weber et al. 1997)
OPDA, JA	*H. vulgare*	up	JA 12-16h, 4h before OPDA	(Kramell et al. 2000)
OPDA, dnOPDA, JA. Wt/Opr3mut	*A. thaliana*	up	Wt max 3h	(Stintzi et al. 2001)
OPDA, JA	*S. lycopersicum*	up	120’, 40’	(Stenzel et al. 2003)
HPL mRNA	*A. thaliana*	up	30-60’	(Bate et al. 1998)
HPL leaves, flower; AOS stem	*S. lycopersicum*	up/herbivory	4h/0.5-12h, max 4h//syst., both 8h	(Howe et al. 2000)
HPLs mRNA/volatiles	*Oryza sativa*	up	at 2h, only in leaves	(Chehab et al. 2006)
Volatiles	*Poplus tremula*	up	5-100’	(Fall et al. 1999)
C6-volatiles and JA	*A. thaliana*	up/herbivory	2-60’, max 30’	(Howlett et al. 2008)

Animal wound response

The initial wound response in animals, including corals, aims for rapid and efficient provisional plugging of the wound to minimize both the loss of vital fluids and environmental challenges (e.g. bacterial contamination) (Proksch et al. 2008; Rodriguez et al. 2008; Palmer et al. 2011; Ariel and Timor 2013). In vertebrate animals, the immediate release of cell-derived damage signals, including Ca^{2+}, ATP and ROS, defines the wound area and severity of damage within the first minutes after injury (Cordeiro and Jacinto 2013). On a cellular level, the wound repair in vertebrates has four phases: 1) hemostasis/coagulation, 2) inflammation, 3) proliferation and 4) remodeling (Singer and Clark 1999; Maderna and Godson 2009; Schultz et al. 2011). The same wound repair phases are observed in cnidarians (Olano and Bigger 2000; Palmer et al. 2008; Reitzel et al. 2008). Coral wound response includes the recruitment of granular amoebocytes (Mydlarz et al. 2008; Palmer et al. 2008), which are important in pathogen clearance.

Acting cooperatively, eicosanoids mediate the initial stages of wound response (e.g. vasoconstriction and clot formation in higher animals) and the onset of the secondary inflammatory phase. For example, TXA_2 induces platelet aggregation (Ricciotti and FitzGerald 2011), while leukotriene C4 (LTC_4) and leukotriene D_4 (LTD4) are involved in increasing blood vessel permeability, and LTB_4 recruits neutrophils to the site of injury (Serhan et al. 2008b; Haeggström and Funk 2011). Additionally, HETEs are involved in the onset and end of the inflammatory phase of wound repair, promoting cell migration and modulating the central signal pathways involved in cell cycle control (Moreno 2009). The aforementioned eicosanoids are pro-inflammatory and chemotactic to innate immune system cells (Serhan et al. 2008b; Moreno 2009), while the formation of lipoxins indicates the resolution of the inflammatory phase (Serhan et al. 2007; Maderna and Godson 2009).

Although oxylipin pathways have been thoroughly studied in plants and higher animals, their role in corals remains unknown.

1.3.2. Thermal stress response

Temperature impacts all levels of life. Sessile terrestrial organisms are well adapted to temperature changes in large amplitudes, whereas sessile marine organisms, such as corals, have evolved in more stable environments. Due to global climate warming corals have reached their upper tolerance threshold values (27-28°C), the exceeding of which results in bleaching events, the loss of symbiotic algae *(Symbiodinium sp.)* (Jokiel and Coles 1990). The average temperature change depends on the location and depth. The annual sea surface temperature (SST) of tropical waters varies between 25.5-30°C (Hoegh-Guldberg

1999). In comparison, acute heat exposures up to 5–8 degrees have been observed in shallow waters within a few hours (Jokiel 2004; Matz et al. 2012).

The molecular events involved in coral thermal stress response have been well-studied in reef-building corals (Table 2 and the references therein) and the sea anemone *Nematostella vectensis* (Goldstone 2008), revealing a conserved response to elevated temperature via heat shock proteins and antioxidant enzyme systems (Lesser and Farrell 2004; DeSalvo et al. 2008; Fitt et al. 2009).

To be able to respond quickly and adequately, and to induce a heat shock response (HSR) on time, the cell's sensory system must effectively integrate various stress signals (lipid mediators, ROS and unfolded proteins, as well as changes in the RNA and DNA topology). One hallmark of thermal stress is the unfolding of proteins and the exposure of their hydrophobic interiors. Stress-induced accumulation of unfolded proteins is detected by Hsps. Under normal conditions Hsps are bound with heat shock factors (HSFs). When Hsps interact with unfolded proteins, HSF transcription factors are released, which in turn initiate the transcription of Hsp genes through binding to heat shock elements, altogether being the essence of the unfolded protein response (UPR) that directs the survival and apoptotic signaling pathways.

Highly conserved heat shock proteins (Hsps) are present in bacteria, plants and animals, including marine invertebrates (Lindquist 1986). In addition to heat, Hsps are also up-regulated in response to oxidative stress, chemicals and heavy metals (Søørensen et al. 2003). Although they are recognized as molecular chaperones, it is now clear that most HSPs are not chaperones, and *vice versa*, most chaperones are not HSPs (plant *versus* human chaperone network (Finka et al. 2011)). Hsps are grouped based on their molecular weight into small, 60, 70 and 90 kDa Hsps. 70 kDa Heat Shock Proteins (Hsp70) have important functions in protein biogenesis and stress tolerance (Lindquist 1986). *Hsp70* up-regulation is widely used as a stress marker for estimating coral well-being (reviewed in (van Oppen and Gates 2006)). Although similarly affected by climate change and temperature, the heat stress response in soft corals remains poorly studied.

Table 2. Conducted genomic and transcriptomic studies on corals. Asterisk: the up-regulation of AOS-LOX is mentioned. Abbreviations: Develop.: development; Loc.: the impact of location; Imm.: immune response; WBD: white band disease; Allorec.: allorecognition.

Species		**Condition**	**Reference**
Acropora digitifera	genome	Normal	(Shinzato et al. 2011)
A. millepora	transcriptome	Development	(Grasso et al. 2008)
A. millepora	transcriptome	Normal (larva)	(Meyer et al. 2009)
A. millepora	transcriptome	Heat (larva)	(Rodriguez-Lanetty et al. 2009)
A. millepora	transcriptome	Loc.-Turbidity	(Bay et al. 2009)
A. millepora	qPCR	Heat	(Seneca et al. 2010)
A. millepora	qPCR	Heat	(Souter et al. 2011)
A. millepora	transcriptome	Heat	(Ferse et al. 2012)
A. millepora	transcriptome	Acidification	(Moya et al. 2012)
A. millepora	qPCR	Immune response	(Duffield et al. 2012)
A. millepora	qPCR	Heat	(Voolstra et al. 2013)
A. hyacinthus	transcriptome	Heat	(Barshis et al. 2013)
A. palmata	transcriptome	Normal	(Polato et al. 2011)
*A. palmata**	transcriptome	Heat	(Polato et al. 2013)
A. palmata	transcriptome	Heat, develop.	(Portune et al. 2010)
*A. cervicornis**	transcriptome	WBD	(Söderhäll et al. 2013)
Favia sp.	transcriptome	Normal	(Pooyaei Mehr et al. 2013)
Monastrea faveolata	transcriptome	Heat	(DeSalvo et al. 2008)
M. faveolata	transcriptome	Heat (embryo)	(Voolstra et al. 2009a)
M. faveolata	transcriptome	Symbiosis	(Voolstra et al. 2009b)
M. faveolata	transcriptome	Development	(Reyes-Bermudez et al. 2009)
M. faveolata	transcriptome	Heat	(Polato et al. 2010)
M. faveolata	transcriptome	Darkness	(DeSalvo et al. 2011)
*M. faveolata**	transcriptome	UV	(Aranda et al. 2011)
Platygyra carnosus	transcriptome	Normal/tumor	(Sun et al. 2012)
Pocillopora damicornis	transcriptome	Location	(Traylor-Knowles et al. 2011)

(Table 2. continued)

P. damicornis	transcriptome	pH	(Vidal-Dupiol et al. 2013)
Porites asteroides	qPCR	Heat-Light	(Kenkel et al. 2011)
P. asteroides	transcriptome	Loc.-Heat	(Kenkel et al. 2013)
P. asteroids	qPCR	Heat	(Kenkel et al. 2014)
P. australiensis	transcriptome	Normal	(Liu et al. 2014)
Stylophora pistillata	transcr./qPCR	Imm. and allorecognition	(Oren et al. 2010)
S. pistillata	transcriptome	Heat, pH, light, loc.	(Voolstra et al. 2014)
Soft corals			
Gorgonia ventalina	transcriptome	Immune response	(Burge et al. 2013)
Sinularia polydactyla and S. maxima	transcriptome	Develop. in diff. species	(Hoover et al. 2007)

Alteration in lipids in response to heat

When cells are exposed to elevated temperatures, AA is released (Calderwood et al. 1989). As membranes are the dynamic source of AA, the eicosanoid repertoire generated is highly dependent on membrane composition and cellular context. Cell membranes are not static but dynamic and, as such, they can act as sensors for cell stress and activators of HSR (Nagy et al. 2007). Thus, when estimating stress response, the membrane-associated heat-sensitive proteins and their role in membrane quality control and downstream signaling should also be considered (Horvath et al. 2008). The fluidity of the membrane is dependent on the relative proportion of unsaturated fatty acids in the membrane (Steponkus 1984). For example, higher proportions of saturated fatty acids are detected in chilling-sensitive plants; therefore, they have a higher transition temperature. In contrast, chilling-resistant species contain a higher proportion of unsaturated fatty acids and have a lower transition temperature (Webb et al. 1994). While acclimation to low temperatures is associated with increases in membrane fluidity, the increase in heat stress resistance depends on increases in membrane rigidity (Iba 2002; Upchurch 2008). Accordingly, the content of membrane PUFAs is regulated in response to elevated temperatures in corals (Imbs and Yakovleva 2011).

Overall, cellular fatty acid signaling is a complicated network of interactions, in which effective combinations vary depending on location and timing, all together forming a finely tuned "concert", assuring the correct signal pattern generated in response to a stressful event. Unfortunately, different metabolic-signaling routes have mostly been investigated in isolation. In order to gain insight into these complex relationships, studies combining different "-omics" (transcriptomics, proteomics and metabolomics) are needed.

2. AIMS OF THE STUDY

The main overall aim of this study was to investigate whether the AOS-LOX fusion protein pathway, responsible for AO biosynthesis is common among octocorals, and whether it contributes biologically to coral homeostasis *in vivo*.

Consequently, the specific aims were to:

- Find a suitable model for elucidating the biological role of the AOS-LOX pathway and, as a result to identify the endogenous metabolites of AA and the *in vitro* activity of AOS-LOX in various octocorals
- Clone, express and characterize the enzymes involved in AO synthesis
- Evaluate changes in the gene expression and metabolite synthesis of coral response to mechanical injury
- Test the sensitivity of the coral AOS-LOX pathway to thermal stress

3. MATERIALS AND METHODS

All procedures were performed according to the manufacturer's instructions or according to the standard protocols described in detail in corresponding publications:

Publication I

RNA extraction
PCR cloning (Reverse Transcription-PCR and 5'-3' RACE)
Protein expression
RP-HPLC coupled with *ion trap* MSMS or Radio flow detector
Normal-phase high-performance liquid chromatography
Chiral-HPLC

Publication II

Coral cultivation and propagation in a laboratory reef aquarium
RNA extraction
PCR cloning (Reverse Transcription-PCR and 5'-3' RACE)
Artificial wounding experiments
Quantitative real-time PCR (qPCR)
Protein expression
In vitro incubations of the radio-labeled substrate with recombinant protein or coral tissue preparations
RP-HPLC coupled with ESI Q-TOF MSMS or Radio flow detector

Publication III

Coral cultivation and propagation in the laboratory reef aquarium
RNA extraction
RT-PCR
Thermal stress experiments
qPCR
In vitro incubations of the radio-labeled substrate with coral tissue preparations
Extraction of endogenous eicosanoids
RP-HPLC coupled with ESI Q-TOF MSMS or Radio flow detector

cDNA sequences obtained and uploaded to the GeneBank with accession numbers:
AOS/8R-LOX of *G. fruticosa* (EU082210.1)
AOS-LOXa and *AOS-LOXb* of *C. imbricata* (KF000373.1 and KF000374.1, respectively)
Hsp70s of *C. imbricata* (KJ452159 and KJ452160)

4. RESULTS

Publication I

- AOS-LOX fusion protein was cloned from the Arctic octocoral *G. fruticosa* (Fig. 11), sharing the amino acid identity of 84% and 87% with AOS-LOX fusion proteins of *P. homomalla* and *C. viridis,* respectively.
- The fusion protein was expressed as an active fusion enzyme, as well as AOS and LOX domains separately in the bacterial expression system. The fusion protein converted [1-^{14}C] AA into labile AO via 8*R*-HpETE intermediate, detected by the formation of stable end products: α-ketol (8-hydroxy, 9-keto-5*Z*, 11*Z*, 14*Z*-trienoic acid) and cyclopentenone (9-oxo-prosta-(5*Z*, 10, 14*Z*)-trienoic acid). The product patterns formed by AOS-LOXs of *G. fruticosa* and *P. homomalla* were identical.

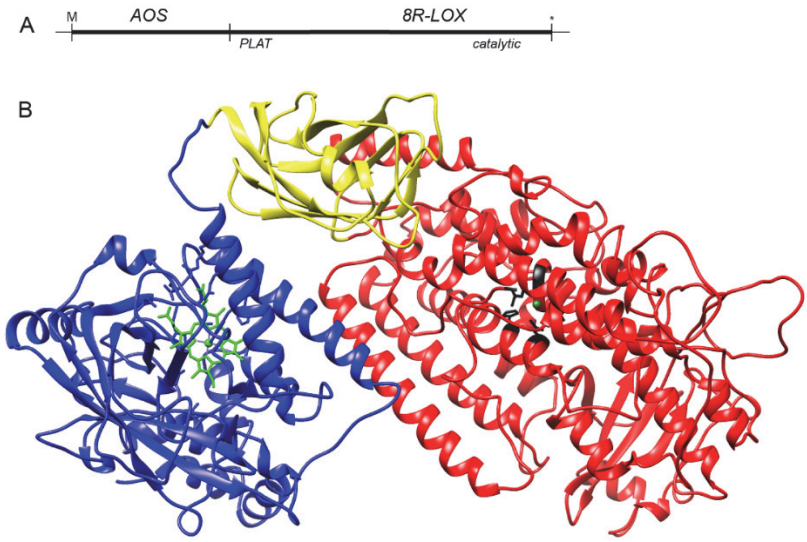


*Figure 11. The structure of the AOS-LOX fusion protein of G. fruticosa, A) coding sequence, M- methionine, *- stop; B) the structure model of the AOS-LOX fusion protein: N- terminal AOS domain (amino acids 1-373, blue) with hem (light green); C- terminal LOX domain, PLAT-domain (374-489, yellow) and catalytic domain (490-1066, red), non-hem iron (dark-green) with coordinating amino acids (black). The model was created with CPH 3.0., using P. homomalla AOS-LOX (Protein Data Bank code: 3DY5) as a template.*

Publication II

- In the Indo-Pacific octocoral *C. imbricata* (Fig. 12), AA is an abundant fatty acid. Exogenously added [1-^{14}C] AA is metabolized by the crude

coral homogenate mainly into 8-HETE and by the diluted homogenate into unidentified polar compounds (UPC), α-ketol and cyclopentenone.

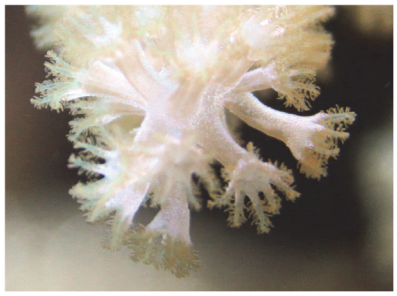
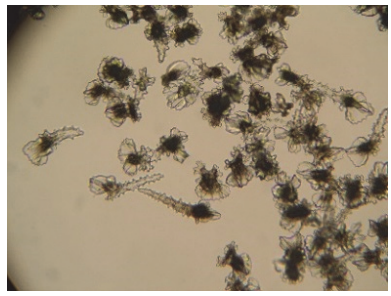
a)  b) 

Figure 12. Soft coral C. imbricata, a) polyps and b) sclerites. Photos by Lõhelaid, H. (a) and Ekins, M. (b).

- Besides the metabolites of the AOS-8*R*-LOX route, several HETEs (LOX products), but no PGs (COX products) were detected (Fig. 13).
- Two AOS-LOX isoforms, designated as *AOS-LOXa* and *AOS-LOXb,* were cloned from *C. imbricata*. The isoforms shared 88% amino acid identities, whereas identities with *G. fruticosa AOS-LOX* were 82% and 81%, respectively).
- Both fusion proteins were expressed in a bacterial expression system. AOS-LOXa converted [1-^{14}C] AA into previously known AOS-LOX metabolites: α-ketol and cyclopentenone (70% and 14% of total radioactivity, respectively), while AOS-LOXb products were volatile short-chain UPC (90% of total radioactivity). Trapping the intermediate product 8-H(p)ETE as 8-HETE by a mild reducing agent indicated that the initial oxidation of [1-^{14}C] AA by LOX domains of both fusion proteins was identical (Fig. 13).
- From the two isoforms cloned from *C. imbricata*, only *AOS-LOXa* transcript was up-regulated in response to incision wounding, accompanied by enhanced levels of AOS-LOXa metabolites. Concurrently, *AOS-LOXb* expression remained stable, and its product level decreased in response to stress (Fig. 13).

Publication III

- The transcript and metabolite levels of AOS-LOXs were recorded in response to modest (28°C) and severe (32°C) thermal stress within 24h. In response to thermal stress, only *AOS-LOXa* transcript was up-regulated, whereas higher up-regulation folds were detected in response to modest temperature elevation.
- Two heat shock proteins, Hsp70 and Grp78/Bip used as positive stress indicators, were up-regulated in response to elevated water temperature.

In both cases, higher transcript levels were detected in response to severe temperature change.

- Similarly to wounding, in response to elevated temperature the level of α-ketol formed from [1-^{14}C] AA by AOS-LOXa increased while UPC synthesized by AOS-LOXb decreased (Fig. 13). The conversion of AA to α-ketol was higher in response to severe temperature elevation.
- To gain further insight into the endogenous metabolites formed, the level of eicosanoids was determined in ethyl acetate (EtOAc) extracts of freshly homogenized coral. Under normal conditions, cyclopentenone and α-ketol were abundant with UPC as a minor metabolite detected.
- An increase in endogenous α-ketol levels was also detected in response to thermal stress, corresponding to the rise in α-ketol formation from the exogenous AA. However, in both cases, the detected levels of α-ketol were higher in response to the severe heat stress.
- In summary, a model indicating the possible place for AOS-LOX in the coral thermal stress was proposed (Fig 14).

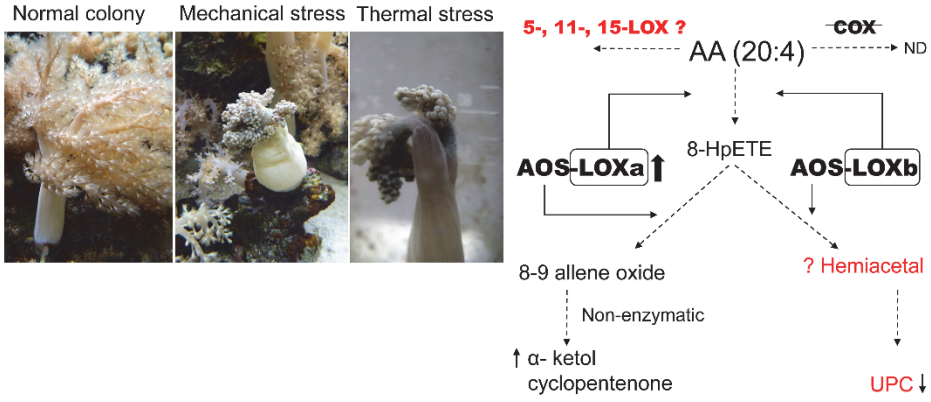


Figure 13. AA metabolism in normal and stressed C. imbricata (II and III). Stress induces the gene expression (thick black arrow) and metabolite levels of AOS-LOXa (α-ketol,↑). However, the gene expression of AOS-LOXb level remains stable and the product level decreases (UPC,↓). Products formed from [1-^{14}C]AA by the G. fruticosa AOS-LOX and C. imbricata AOS-LOXa fusion proteins are identical (I and II). Photos are intended to indicate the difference between normal and stressed coral colonies (by Lõhelaid, H.).

5. DISCUSSION

Most of the stress studies have been conducted on stony corals, the model of soft corals used here is a complementary system for *in vivo* analysis of the lipid mediator biochemistry and physiology of organisms. The work of this thesis identifies the presence of an active AOS-LOX fusion protein pathway in different octocoral (soft coral) species and its functional significance in their homeostasis and stress response. The findings further identify novel products synthesized by the fusion proteins.

In short, the results of this thesis are as follows. (I) Two AOS-LOXs with identical product profiles to that of the AOS-LOX fusion protein of *P. homomalla* were cloned from soft corals *G. fruticosa* and *C. imbricata*. The data suggest that AOS-LOX is a general eicosanoid metabolic pathway widely distributed in soft corals. (II) The fast-growing soft coral *C. imbricata* was found to be a suitable model to study the eicosanoid metabolism *in vivo*. (III) As a proof of the concept, the coral AOS-LOX fusion protein was found to be involved in the early stress response to various stressors. In corals, as in other organisms, eicosanoids act as potential early stress mediators. (IV) *AOS-LOXa* or its metabolites could be used as biomarkers to detect the stress response in corals. (V) The second AOS-LOX isozyme of *C. imbricata,* AOS-LOXb, converts exogenous AA to unidentified compounds: UPCs. In essence, UPCs are coral analogs of the products formed by plant HPLs, suggesting a novel HPL-like activity in corals.

The soft corals studied are found in a wide range of different living habitats, including Caribbean, Arctic and Indo-Pacific waters. The data for eicosanoid content now reveal that different AA metabolic routes are used in combination, even within corals belonging to the same phylogenetic taxa. While COX, LOX and AOS-LOX pathways (transcripts, as well as metabolites) were present in Arctic *G. fruticosa* and Caribbean *P. homomalla* (Varvas et al. 1993; Koljak et al. 1997; Varvas et al. 1999; Koljak et al. 2001; Valmsen et al. 2001), only the transcripts and metabolites of LOX and AOS-LOX were detected in the Indo-Pacific *C. imbricata* (Fig. 13). This finding is in accordance with a previous octocoral study reported for *Pseudoplexaura porosa* which contains only abundant levels of 8*R*-HETE, less 15-HETE (90% and 10% of total HETE content, respectively) and no PGs (Bundy et al. 1986). This supports the fact that eicosanoid profiles are species-specific and can vary even within the same species. For example, depending on the location, 15*R*- and *15S*- PGs are formed in *P. homomalla* (Valmsen et al. 2004). Another principal finding of this study was that several AOS-LOX fusion proteins are expressed and produced different metabolites in parallel within a single organism. In plants, LOX, AOS and HPL are all expressed separately. LOX generates the hydroperoxy fatty acid precursors for sequential AOS and HPL catalytic steps. Thus, both routes compete for the same substrate, resulting in different oxylipin profiles necessary for the induction of the appropriate stress response (Kallenbach et al. 2011). From the evolutionary standpoint, the act of fusion of two enzymes is related to catalyzing consecutive

steps within a certain pathway (Marcotte et al. 1999). The functional coupling of both alternative plant pathways in coral further indicates the importance of the coupling of both 8*R*-HpETE conversion to AO or volatiles and their control over time and space. Although the distinct roles of these metabolites in coral remain unknown, the alternative-competitive pathways could be needed for the temporal regulation assuring the correct metabolite profile generated in response to stress. As both fusion proteins of *C. imbricata* use AA as a substrate, it would be of interest to examine their locations within intact cells.

Although plethora of eicosanoids have been detected in soft corals (Corey et al. 1973; Corey et al. 1985; Brash et al. 1987; Corey et al. 1988; Brash 1989; Song and Brash 1991a), the biological significance of those compounds in coral has remained unknown. According to different lines of evidence provided here, including the up-regulation of transcripts and metabolites in response to various abiotic stressors, the involvement of an AOS-LOX pathway in the general stress response of coral has been confirmed. As we detected, AOS-LOXa is up-regulated at early stages of the stress response (within the first 24 hours). Thus, the gene expression profile generated by *C. imbricata* AOS-LOXa matches the immediate wave pattern (Wenemoser et al. 2012). Similar expression patterns were observed for plant LOX in potatoes (Farmaki et al. 2006) and AOS in *A. thaliana*, flax and tomatoes (Harms et al. 1998; Laudert and Weiler 1998; Howe et al. 2000) in response to wounding.

The AOS-LOX role in stress response

Depending on different aspects, such as growth conditions, nutrition, health etc., challenging environmental conditions generate multiple signals that induce several cellular pathways in parallel (Kultz 2005; Finka et al. 2011). All of those signals converge at cellular checkpoints. Sequentially, a specific - highly context dependent - response is generated, which ensures the adequate response to overcome stress under defined conditions. Furthermore, ensuring that the "new" balance falls into the correct homeostasis (survival) window, two parallel higher order control systems are present. A cellular stress response adjusts the system to sustain, tolerate and eventually overcome stressful conditions. A cellular homeostasis response is a set of measures that helps to restore normal homeostasis, similar to the state prior to stress occurrence. Here we focus on the early cellular stress response.

According to our data, literature and annotated databases, we have proposed a mechanism of stress in corals (Fig. 14). To sum up, AOS-LOXa metabolites are part of a general stress response acting at early stages of stress. Hsp70 is indicative of heat and oxidative stress, accumulation of unfolded proteins and ROS in the cytoplasm. Hsp70 mediates the induction of HSR and UPR via HSF responsive genes, whereas the up-regulation of Grp78 indicates ER stress and mediates the UPR via PERK, ATF6 and IRE1 (Fig. 14).

In response to heat or injury, various signals from different pathways merge and regulate the downstream cascades leading either to cell survival or apoptosis. In both cases, the first cellular signals generated are Ca^{2+}, ATP and ROS (Leon et al. 2001; Maffei et al. 2007; Mittler et al. 2012; Wenemoser et al. 2012). Their gradients initiate downstream cascades (either the heat shock response or the wound response) in plants and animals (Sung et al. 2003; Maffei et al. 2007; Finka et al. 2011; Moreno and Orellana 2011; Mittler et al. 2012; Balogh et al. 2013). The eicosanoid cascades are additionally regulated by stress-induced protein kinases: JNK, PKC, ERK1/2 and p38MAPK (Fig. 14) (Berenbaum et al. 2003; Balboa and Balsinde 2006; Radmark and Samuelsson 2009; Haeggstrom and Funk 2011). In the case of thermal stress, survival or apoptosis is determined by the overall balance between ERK and SAPK/JNK/p38MAPK-activated cascades (Park et al. 2005).

Oxidative stress is closely connected with thermal stress (DeSalvo et al. 2008; Voolstra et al. 2009a). The thermal stress-induced accumulation of unfolded proteins and oxidative stress is detected by Hsp70s (Fig. 14) (van Oppen and Gates 2006). In different coral species, elevated levels of ROS have been detected in response to heat stress (Lesser 2006; Mydlarz and Jacobs 2006). For instance, the direct sensing of hydrogen peroxide by HSFs leads to the induction of heat shock response (Fig. 14) (Zhong et al. 1998).

Under normal conditions, the ER-specific glucose-regulated protein 78 (Grp78 or BiP) is constitutively expressed. Its levels are induced against protein denaturation, glucose deprivation and perturbed calcium levels (Resendez et al. 1988), as well as being increased by elevated temperature, indicative of ER stress (Lee 2005; Luan et al. 2009). In corals, the up-regulation of Grp78 has also been detected in response to UV radiation (Aranda et al. 2011). Similar to other Hsp70s, the induction of Grp78 is regulated at the transcriptional level by transcription factor complexes (including ATF6) attached to the conserved stress response element (ERSE) (Fig. 14) (Parker et al. 2001). Grp78 with other Ca^{2+}-buffering chaperones affects the Ca^{2+} level within the ER and the release into the cytosol (Lievremont et al. 1997; Coe and Michalak 2009) (Fig. 14). Moreover, Grp78 is the key regulator of ER transmembrane proteins (Ire1, Atf6 and PERK and their homologs) (Fig. 14), which are the main initiators of the UPR throughout *Metazoa* (Schroder and Kaufman 2005; Hollien 2013). As the UPR directs survival and apoptotic signaling pathways, it is an important checkpoint measuring the severity of stress, which eventually determines the cell's fate, either survival or apoptosis.

Although the up-regulation of HSPs in response to elevated temperature is a general feature in the living organisms, the direct mechanism of action of the AOS-LOX pathway in coral stress response remains elusive.

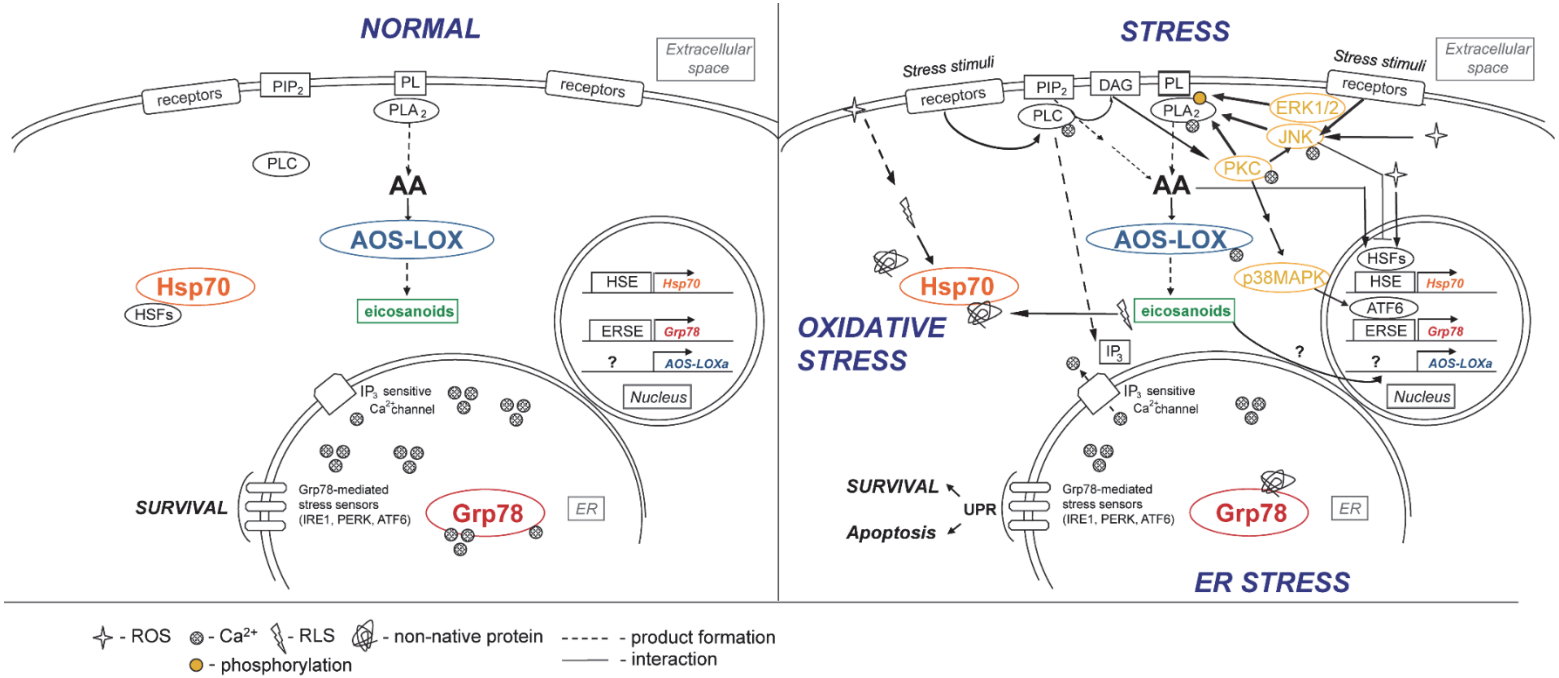


Figure 14. A proposed model of coral stress response. In the presence of stress, AOS-LOX activity is enhanced by elevated levels of Ca^{2+}. In parallel, the availability of substrate AA is increased by the activation of PLs by Ca^{2+} and corresponding kinases. The effect is further amplified by the up-regulated expression of AOS-LOX. Hsp70 up-regulation indicates oxidative stress and the presence of unfolded proteins, while the regulation of Grp78 indicates ER stress. Abbreviations include: AA – arachidonic acid, AOS-LOX – allene oxide synthase-lipoxygenase fusion protein, ATF6 – activating transcription factor 6, DAG –diacylglycerol, ER – endoplasmic reticulum, ERK1/2 – extracellular signal-regulated kinase, ERSE – endoplasmic reticulum stress element, Grp78 – glucose-regulated protein 78 kDa, HSE – heat shock element, HSFs – heat shock factors, Hsp70 – heat shock protein 70 kDa, IP_3 – inositol 1,4,5-triphosphate, IRE1 – inositol-requiring enzyme 1, JNK – c-Jun N-terminal kinase, p38MAPK – p38 mitogen-activated protein kinase, PERK – protein kinase RNA-like endoplasmic reticulum kinase, PIP_2 – phosphatidylinositol 4,5-bisphosphate, PL – phospholipid, PKC – protein kinase C, PLA_2 – phospholipase A_2, PLC – phospholipase C, RLS – rapid lipid species, ROS – reactive oxygen species, UPR – unfolded protein response. Oval shapes – enzymes.

The cyclopentenone formed by AOS-LOXa possesses a high resemblance to stress mediators in plants (Stintzi et al. 2001) and other compounds with cyclopentenone moiety (Amici et al. 1992). Due to their electrophilic properties (*α, β*- unsaturated carbonyl groups), cyclopentenones have the potential to promote the transcription of Hsp70s through the release of HSF1 (Fig 14) (Amici et al. 1992; Elia 1996; Jacobs and Marnett 2007; Higdon et al. 2012; West et al. 2012). Still, despite the electrophilic moiety of cyclopentenone, the AO as a potential mediator should not be overlooked, especially when only the level of α-ketol increases during the coral stress response and the level of cyclopentenone remains stable. This contradicts of the assumed non- enzymatic degradation of AO, by which the ratio of α-ketol and cyclopentenone as degradation products should remain the same, even in the case of elevated formation of AO. There are other enzymes that either prevent the formation of cyclopentenone or direct the formation of α-ketol. Nevertheless, elevated levels of *AOS-LOXa* transcript and α-ketol univocally indicate the presence of the elevated level of stress in *C. imbricata.*

The production of eicosanoids is up-regulated in response to various stressors, but transcript levels, as well as protein levels, are not the sole determinants of enzyme activity. For example, 5-LOX activity can be modulated by many factors, including the availability of substrate, serine phosphorylation and translocation (from the cytosol to the nuclear envelope) (Radmark and Samuelsson 2008). Upstream regulation of AA release occurs predominantly via phospholipase A_2 (PLA_2) (Serhan et al. 1996; Balsinde et al. 2002; Schaloske and Dennis 2006) and to a lesser extent by phospholipase C (PLC) (Harden and Sondek 2006). Phospholipases are activated via different receptors (e.g. G-protein coupled receptors) in response to a range of stimuli (Harden and Sondek 2006), including Ca^{2+} (Evans et al. 2001), lipid peroxides (Ermak and Davies 2002) and heat (Jurivich et al. 1996). Consistently high activities of PLA_2 are detected across *Cnidaria* (Nevalainen et al. 2004; Romero et al. 2010), indicating a conserved pathway. Indicative of the crosstalk between Ca^{2+} and eicosanoid formation in corals, the LOX domain of the AOS-LOX fusion protein binds membrane in a Ca^{2+}-dependent manner (Gilbert et al. 2008), and the enzymatic activity of AOS-LOX *in vitro* is enhanced by Ca^{2+} (Boutaud and Brash 1999).

Besides Ca^{2+} and H_2O_2 gradients formed at the wounding site, eicosanoids could also serve as chemotactic agents in corals. The migration-invasion of coral immune cells (phagocytic granular amoebocytes, phagocytic cells and melanin-containing granular cells) from the surrounding tissues to the site of injury is a well-established phenomenon (Olano and Bigger 2000; Vargas-Ángel et al. 2007; Mydlarz et al. 2008; Palmer et al. 2008; Palmer et al. 2011). However, currently it is not established which coral cells contribute to the synthesis and release of eicosanoids *in vivo*. Thus, the next challenge is to determine the exact location of the eicosanoid synthesis and to estimate the changes in protein profiles in response to stress.

Implications and outlook

Due to the continuously evolving climate warming, corals in several habitats are endangered (Hoegh-Guldberg and Bruno 2010). Accordingly, field studies are strictly regulated by protective laws. Collecting coral specimens for the laboratory is just as complicated. Another hurdle is the labile nature of oxylipins, which must be taken into account in every step of collection, transportation, sampling and analysis. In this light, the *in vivo* results of the current thesis are even more notable. For the first time, oxylipins were detected and elevated from fresh coral homogenate preparations. This minimized artifacts which otherwise compromise biochemical analysis of lipid mediators from corals after harvest. In addition, the workflow was optimized for speed and quality of extraction.

Coral eicosanoid pathways emerge as conserved regulators of cell stress responses, with potential as biomarkers to detect stress in both soft and stony corals. Although a part of the fauna of the endangered Great Barrier Reef, *C. imbricata* itself is not on the list of protected species. We suggest the use of *C. imbricata* as an indicator species to detect the severity of stress.

Besides the other cnidarian genomes: *Nematostella* (Putnam et al. 2007) and *Hydra* (Zacharias et al. 2004; Chapman et al. 2010), the only coral genome available belongs to *Acropora digitifera* (Shinzato et al. 2011). Serving as a convenient object of research, most of the stony coral stress-studies are conducted on *A. millepora* (Table 2, and the related references). Based on the data available, *AOS-LOX* is also present in stony (reef-building) corals (Lee et al. 2008), while *COXs* are not (unpublished data). Although the up-regulation of *AOS-LOX* transcripts in response to white band disease (Söderhäll et al. 2013), elevated UV radiation (Aranda et al. 2011) and temperature (Polato et al. 2013) have been detected by transcriptome studies, the eicosanoid profiles generated by stony corals are still not known. The detection of oxylipins and functional characterization of the AOS-LOX pathway in stony corals will be the subject of future studies.

CONCLUSIONS

This is the first study of the biological role of eicosanoids in coral homeostasis. Our data suggests that:

- The AOS-LOX fusion protein pathway of AA metabolism is common in octocorals.
- Despite the high sequence identity, the AOS-LOXa and -b isoforms of *C. imbricata* convert AA to different products and undergo differential regulation during the stress response in corals.
- *C. imbricata* is a suitable model organism for estimating stress response in soft corals via the AOS-LOX pathway.
- In *C. imbricata,* AOS-LOXa is involved in the mediating early response to wound and temperature stress *in vivo*.

Therefore, AOS-LOX emerges as part of the general early stress response in *Cnidaria*.

REFERENCES

Amici C, Sistonen L, Santoro MG, Morimoto RI (1992) Antiproliferative prostaglandins activate heat shock transcription factor. Proceedings of the National Academy of Sciences of the United States of America 89:6227-6231

Aranda M, Banaszak AT, Bayer T, Luyten JR, Medina M, Voolstra CR (2011) Differential sensitivity of coral larvae to natural levels of ultraviolet radiation during the onset of larval competence. Molecular ecology 20:2955-2972

Ariel A, Timor O (2013) Hanging in the balance: endogenous anti-inflammatory mechanisms in tissue repair and fibrosis. The Journal of pathology 229:250-263

Balboa MA, Balsinde J (2006) Oxidative stress and arachidonic acid mobilization. Biochimica et biophysica acta 1761:385-391

Balogh G, Péter M, Glatz A, Gombos I, Török Z, Horváth I, Harwood JL, Vígh L (2013) Key role of lipids in heat stress management. FEBS letters 587:1970-1980

Balsinde J, Winstead MV, Dennis EA (2002) Phospholipase A(2) regulation of arachidonic acid mobilization. FEBS letters 531:2-6

Barshis DJ, Ladner JT, Oliver TA, Seneca FO, Traylor-Knowles N, Palumbi SR (2013) Genomic basis for coral resilience to climate change. Proceedings of the National Academy of Sciences of the United States of America 110:1387-1392

Bate NJ, Rothstein SJ (1998) C6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. The Plant journal : for cell and molecular biology 16:561-569

Bate NJ, Sivasankar S, Moxon C, Riley JM, Thompson JE, Rothstein SJ (1998) Molecular characterization of an Arabidopsis gene encoding hydroperoxide lyase, a cytochrome P-450 that is wound inducible. Plant Physiol 117:1393-1400

Bay LK, Ulstrup KE, Nielsen HB, Jarmer H, Goffard N, Willis BL, Miller DJ, Van Oppen MJ (2009) Microarray analysis reveals transcriptional plasticity in the reef building coral Acropora millepora. Molecular ecology 18:3062-3075

Bell E, Mullet JE (1993) Characterization of an Arabidopsis lipoxygenase gene responsive to methyl jasmonate and wounding. Plant Physiol 103:1133-1137

Bell E, Creelman RA, Mullet JE (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 92:8675-8679

Berenbaum F, Humbert L, Bereziat G, Thirion S (2003) Concomitant recruitment of ERK1/2 and p38 MAPK signalling pathway is required

for activation of cytoplasmic phospholipase A2 via ATP in articular chondrocytes. The Journal of biological chemistry 278:13680-13687
Bijlsma R, Loeschcke V (2005) Environmental stress, adaptation and evolution: an overview. Journal of Evolutionary Biology 18:744-749
Blechert S, Bockelmann C, Fusslein M, Von Schrader T, Stelmach B, Niesel U, Weiler EW (1999) Structure-activity analyses reveal the existence of two separate groups of active octadecanoids in elicitation of the tendril-coiling response of Bryonia dioica Jacq. Planta 207:470-479
Blechert S, Brodschelm W, Holder S, Kammerer L, Kutchan TM, Mueller MJ, Xia ZQ, Zenk MH (1995) The octadecanoic pathway: signal molecules for the regulation of secondary pathways. Proceedings of the National Academy of Sciences of the United States of America 92:4099-4105
Blee E (1998) Phytooxylipins and plant defense reactions. Progress in lipid research 37:33-72
Blee E (2002) Impact of phyto-oxylipins in plant defense. Trends in plant science 7:315-322
Blobaum AL, Marnett LJ (2007) Structural and Functional Basis of Cyclooxygenase Inhibition. Journal of medicinal chemistry 50:1425-1441
Block A, Schmelz E, Jones JB, Klee HJ (2005) Coronatine and salicylic acid: the battle between Arabidopsis andPseudomonasfor phytohormone control. Molecular Plant Pathology 6:79-83
Boutaud O, Brash AR (1999) Purification and catalytic activities of the two domains of the allene oxide synthase-lipoxygenase fusion protein of the coral Plexaura homomalla. The Journal of biological chemistry 274:33764-33770
Brash AR (1989) Formation of an Allene Oxide from (8r)-8-Hydroperoxyeicosatetraenoic Acid in the Coral Plexaura-Homomalla. Journal of the American Chemical Society 111:1891-1892
Brash AR (1999) Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. The Journal of biological chemistry 274:23679-23682
Brash AR (2009) Mechanistic aspects of CYP74 allene oxide synthases and related cytochrome P450 enzymes. Phytochemistry 70:1522-1531
Brash AR, Baertschi SW, Ingram CD, Harris TM (1987) On non-cyclooxygenase prostaglandin synthesis in the sea whip coral, Plexaura homomalla: an 8(R)-lipoxygenase pathway leads to formation of an alpha-ketol and a Racemic prostanoid. The Journal of biological chemistry 262:15829-15839
Brash AR, Boeglin WE, Chang MS, Shieh BH (1996) Purification and molecular cloning of an 8R-lipoxygenase from the coral Plexaura homomalla reveal the related primary structures of R- and S-lipoxygenases. The Journal of biological chemistry 271:20949-20957

Brash AR, Jisaka M, Boeglin WE, Chang MS (1999) Molecular cloning of a second human 15S-lipoxygenase and its murine homologue, an 8S-lipoxygenase. Their relationship to other mammalian lipoxygenases. Advances in experimental medicine and biology 447:29-36

Bundy GL, Nidy EG, Epps DE, Mizsak SA, Wnuk RJ (1986) Discovery of an arachidonic acid C-8 lipoxygenase in the gorgonian coral Pseudoplexaura porosa. The Journal of biological chemistry 261:747-751

Burge CA, Mouchka ME, Harvell CD, Roberts S (2013) Immune response of the Caribbean sea fan, Gorgonia ventalina, exposed to an Aplanochytrium parasite as revealed by transcriptome sequencing. Frontiers in Physiology 4

Caldelari D, Wang G, Farmer EE, Dong X (2010) Arabidopsis lox3 lox4 double mutants are male sterile and defective in global proliferative arrest. Plant Molecular Biology 75:25-33

Calderwood SK, Bornstein B, Farnum EK, Stevenson MA (1989) Heat shock stimulates the release of arachidonic acid and the synthesis of prostaglandins and leukotriene B4 in mammalian cells. Journal of cellular physiology 141:325-333

Capdevila JH (2007) Regulation of ion transport and blood pressure by cytochrome p450 monooxygenases. Current opinion in nephrology and hypertension 16:465-470

Capdevila JH, Falck JR (2002) Biochemical and molecular properties of the cytochrome P450 arachidonic acid monooxygenases. Prostaglandins & other lipid mediators 68-69:325-344

Capdevila JH, Falck JR, Harris RC (2000) Cytochrome P450 and arachidonic acid bioactivation. Molecular and functional properties of the arachidonate monooxygenase. Journal of lipid research 41:163-181

Capdevila JH, Pidkovka N, Mei S, Gong Y, Falck JR, Imig JD, Harris RC, Wang W (2014) The Cyp2c44 epoxygenase regulates epithelial sodium channel activity and the blood pressure responses to increased dietary salt. The Journal of biological chemistry 289:4377-4386

Chang Z, Li L, Pan Z, Wang X (2008) Crystallization and preliminary X-ray analysis of allene oxide synthase, cytochrome P450 CYP74A2, from Parthenium argentatum. Acta crystallographica Section F, Structural biology and crystallization communications 64:668-670

Chapman JA, Kirkness EF, Simakov O, Hampson SE, Mitros T, Weinmaier T, Rattei T, Balasubramanian PG, Borman J, Busam D, Disbennett K, Pfannkoch C, Sumin N, Sutton GG, Viswanathan LD, Walenz B, Goodstein DM, Hellsten U, Kawashima T, Prochnik SE, Putnam NH, Shu S, Blumberg B, Dana CE, Gee L, Kibler DF, Law L, Lindgens D, Martinez DE, Peng J, Wigge PA, Bertulat B, Guder C, Nakamura Y, Ozbek S, Watanabe H, Khalturin K, Hemmrich G, Franke A, Augustin R, Fraune S, Hayakawa E, Hayakawa S, Hirose M, Hwang JS, Ikeo K,

Nishimiya-Fujisawa C, Ogura A, Takahashi T, Steinmetz PRH, Zhang X, Aufschnaiter R, Eder M-K, Gorny A-K, Salvenmoser W, Heimberg AM, Wheeler BM, Peterson KJ, Böttger A, Tischler P, Wolf A, Gojobori T, Remington KA, Strausberg RL, Venter JC, Technau U, Hobmayer B, Bosch TCG, Holstein TW, Fujisawa T, Bode HR, David CN, Rokhsar DS, Steele RE (2010) The dynamic genome of Hydra. Nature 464:592-596

Chauvin A, Caldelari D, Wolfender J-L, Farmer EE (2013) Four 13-lipoxygenases contribute to rapid jasmonate synthesis in wounded Arabidopsis thaliana leaves: a role for lipoxygenase 6 in responses to long-distance wound signals. New Phytologist 197:566-575

Chehab EW, Raman G, Walley JW, Perea JV, Banu G, Theg S, Dehesh K (2006) Rice HYDROPEROXIDE LYASES with unique expression patterns generate distinct aldehyde signatures in Arabidopsis. Plant Physiol 141:121-134

Chen XS, Sheller JR, Johnson EN, Funk CD (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372:179-182

Coceani F, Akarsu ES (1998) Prostaglandin E2 in the Pathogenesis of Fever: An Updatea. Annals of the New York Academy of Sciences 856:76-82

Coe H, Michalak M (2009) Calcium binding chaperones of the endoplasmic reticulum. General physiology and biophysics 28 Spec No Focus:F96-F103

Coffa G, Schneider C, Brash AR (2005) A comprehensive model of positional and stereo control in lipoxygenases. Biochemical and biophysical research communications 338:87-92

Cordeiro JV, Jacinto A (2013) The role of transcription-independent damage signals in the initiation of epithelial wound healing. Nature Reviews Molecular Cell Biology 14:249-262

Corey EJ, Washburn WN, Chen JC (1973) Studies on the prostaglandin A 2 synthetase complex from Plexaura homomalla. Journal of the American Chemical Society 95:2054-2055

Corey EJ, Lansbury PT, Yamada Y (1985) Identification of a New Eicosanoid from Invitro Biosynthetic Experiments with Clavularia-Viridis - Implications for the Biosynthesis of Clavulones. Tetrahedron Letters 26:4171-4174

Corey EJ, Matsuda SPT, Nagata R, Cleaver MB (1988) Biosynthesis of 8-R-Hpete and Preclavulone-a from Arachidonate in Several Species of Caribbean Coral - a Widespread Route to Marine Prostanoids. Tetrahedron Letters 29:2555-2558

Corey EJ, Dalarcao M, Matsuda SPT, Lansbury PT, Yamada Y (1987) Intermediacy of 8-(R)-Hpete in the Conversion of Arachidonic-Acid to Pre-Clavulone-a by Clavularia-Viridis - Implications for the

Biosynthesis of Marine Prostanoids. Journal of the American Chemical Society 109:289-290
Creelman RA, Tierney ML, Mullet JE (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proceedings of the National Academy of Sciences of the United States of America 89:4938-4941
Croft K, Juttner F, Slusarenko AJ (1993) Volatile Products of the Lipoxygenase Pathway Evolved from Phaseolus vulgaris (L.) Leaves Inoculated with Pseudomonas syringae pv phaseolicola. Plant Physiol 101:13-24
Cyrus T, Witztum JL, Rader DJ, Tangirala R, Fazio S, Linton MF, Funk CD (1999) Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. The Journal of clinical investigation 103:1597-1604
DeSalvo MK, Estrada A, Sunagawa S, Medina M (2011) Transcriptomic responses to darkness stress point to common coral bleaching mechanisms. Coral Reefs 31:215-228
DeSalvo MK, Voolstra CR, Sunagawa S, Schwarz JA, Stillman JH, Coffroth MA, Szmant AM, Medina M (2008) Differential gene expression during thermal stress and bleaching in the Caribbean coral Montastraea faveolata. Molecular ecology 17:3952-3971
Di Marzo V, De Petrocellis L, Gianfrani C, Cimino G (1993) Biosynthesis, structure and biological activity of hydroxyeicosatetraenoic acids in Hydra vulgaris. The Biochemical journal 295 (Pt 1):23-29
Di Venere A, Nicolai E, Ivanov I, Dainese E, Adel S, Angelucci BC, Kuhn H, Maccarrone M, Mei G (2014) Probing conformational changes in lipoxygenases upon membrane binding: Fine-tuning by the active site inhibitor ETYA. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1841:1-10
DiMarzo V, Ventriglia M, Mollo E, Mosca M, Cimino G (1996) Occurrence and biosynthesis of 11(R)-hydroxy-eicosatetraenoic acid (11-R-HETE) in the Caribbean soft coral Plexaurella dichotoma. Experientia 52:834-838
Duan H (2005) Variations in CYP74B2 (Hydroperoxide Lyase) Gene Expression Differentially Affect Hexenal Signaling in the Columbia and Landsberg erecta Ecotypes of Arabidopsis. Plant Physiology 139:1529-1544
Duffield JS, Puill-Stephan E, Seneca FO, Miller DJ, van Oppen MJH, Willis BL (2012) Expression of Putative Immune Response Genes during Early Ontogeny in the Coral Acropora millepora. PloS ONE 7:e39099
Duroudier NP, Tulah AS, Sayers I (2009) Leukotriene pathway genetics and pharmacogenetics in allergy. Allergy 64:823-839
Eek P, Jarving R, Jarving I, Gilbert NC, Newcomer ME, Samel N (2012) Structure of a Calcium-dependent 11R-Lipoxygenase Suggests a

Mechanism for Ca2+ Regulation. Journal of Biological Chemistry 287:22377-22386
Elia G (1996) 2-Cyclopenten-1-one, a New Inducer of Heat Shock Protein 70with Antiviral Activity. Journal of Biological Chemistry 271:32192-32196
Epp N, Furstenberger G, Muller K, de Juanes S, Leitges M, Hausser I, Thieme F, Liebisch G, Schmitz G, Krieg P (2007) 12R-lipoxygenase deficiency disrupts epidermal barrier function. The Journal of cell biology 177:173-182
Ermak G, Davies KJ (2002) Calcium and oxidative stress: from cell signaling to cell death. Molecular immunology 38:713-721
Evans JH, Spencer DM, Zweifach A, Leslie CC (2001) Intracellular calcium signals regulating cytosolic phospholipase A2 translocation to internal membranes. The Journal of biological chemistry 276:30150-30160
Fall R, Karl T, Hansel A, Jordan A, Lindinger W (1999) Volatile organic compounds emitted after leaf wounding: On-line analysis by proton-transfer-reaction mass spectrometry. J Geophys Res-Atmos 104:15963-15974
Farmaki T, Sanmartin M, Jimenez P, Paneque M, Sanz C, Vancanneyt G, Leon J, Sanchez-Serrano JJ (2006) Differential distribution of the lipoxygenase pathway enzymes within potato chloroplasts. Journal of experimental botany 58:555-568
Farmer EE, Alméras E, Krishnamurthy V (2003) Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Current Opinion in Plant Biology 6:372-378
Ferse SCA, Bellantuono AJ, Granados-Cifuentes C, Miller DJ, Hoegh-Guldberg O, Rodriguez-Lanetty M (2012) Coral Thermal Tolerance: Tuning Gene Expression to Resist Thermal Stress. PloS ONE 7:e50685
Feussner I, Wasternack C (2002) Thelipoxygenasepathway. Annual Review of Plant Biology 53:275-297
Figueiredo J, Baird AH, Cohen MF, Flot JF, Kamiki T, Meziane T, Tsuchiya M, Yamasaki H (2012) Ontogenetic change in the lipid and fatty acid composition of scleractinian coral larvae. Coral Reefs 31:613-619
Finka A, Mattoo RU, Goloubinoff P (2011) Meta-analysis of heat- and chemically upregulated chaperone genes in plant and human cells. Cell stress & chaperones 16:15-31
Fitt WK, Gates RD, Hoegh-Guldberg O, Bythell JC, Jatkar A, Grottoli AG, Gomez M, Fisher P, Lajuenesse TC, Pantos O, Iglesias-Prieto R, Franklin DJ, Rodrigues LJ, Torregiani JM, van Woesik R, Lesser MP (2009) Response of two species of Indo-Pacific corals, Porites cylindrica and Stylophora pistillata, to short-term thermal stress: The host does matter in determining the tolerance of corals to bleaching. Journal of Experimental Marine Biology and Ecology 373:102-110

Flower RJ (2006) Prostaglandins, bioassay and inflammation. British journal of pharmacology 147:S182-S192
Froehlich JE, Itoh A, Howe GA (2001) Tomato allene oxide synthase and fatty acid hydroperoxide lyase, two cytochrome P450s involved in oxylipin metabolism, are targeted to different membranes of chloroplast envelope. Plant Physiol 125:306-317
Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294:1871-1875
Gerhart DJ (1991) Emesis, Learned Aversion, and Chemical Defense in Octocorals - a Central Role for Prostaglandins. American Journal of Physiology 260:R839-R843
Gerwick WH, Moghaddam M, Hamberg M (1991) Oxylipin metabolism in the red alga Gracilariopsis lemaneiformis: Mechanism of formation of vicinal dihydroxy fatty acids. Archives of biochemistry and biophysics 290:436-444
Gfeller A, Baerenfaller K, Loscos J, Chetelat A, Baginsky S, Farmer EE (2011) Jasmonate Controls Polypeptide Patterning in Undamaged Tissue in Wounded Arabidopsis Leaves. Plant Physiology 156:1797-1807
Gilbert NC, Niebuhr M, Tsuruta H, Bordelon T, Ridderbusch O, Dassey A, Brash AR, Bartlett SG, Newcomer ME (2008) A Covalent Linker Allows for Membrane Targeting of an Oxylipin Biosynthetic Complex†‡. Biochemistry 47:10665-10676
Gillmor SA, Villasenor A, Fletterick R, Sigal E, Browner MF (1997) The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity. Nature structural biology 4:1003-1009
Glauser G, Dubugnon L, Mousavi SAR, Rudaz S, Wolfender JL, Farmer EE (2009) Velocity Estimates for Signal Propagation Leading to Systemic Jasmonic Acid Accumulation in Wounded Arabidopsis. Journal of Biological Chemistry 284:34506-34513
Goldstone JV (2008) Environmental sensing and response genes in cnidaria: the chemical defensome in the sea anemone Nematostella vectensis. Cell biology and toxicology 24:483-502
Grace PM, Hutchinson MR, Maier SF, Watkins LR (2014) Pathological pain and the neuroimmune interface. Nature Reviews Immunology 14:217-231
Grasso LC, Maindonald J, Rudd S, Hayward DC, Saint R, Miller DJ, Ball EE (2008) Microarray analysis identifies candidate genes for key roles in coral development. BMC genomics 9:540
Grechkin AN, Hamberg M (2004) The "heterolytic hydroperoxide lyase" is an isomerase producing a short-lived fatty acid hemiacetal. Bba-Mol Cell Biol L 1636:47-58

Gueldner RC, Wilson DM, Heidt AR (1985) Volatile Compounds Inhibiting Aspergillus-Flavus. Journal of agricultural and food chemistry 33:411-413

Guo AM, Arbab AS, Falck JR, Chen P, Edwards PA, Roman RJ, Scicli AG (2007) Activation of Vascular Endothelial Growth Factor through Reactive Oxygen Species Mediates 20-Hydroxyeicosatetraenoic Acid-Induced Endothelial Cell Proliferation. Journal of Pharmacology and Experimental Therapeutics 321:18-27

Haeggstrom JZ, Funk CD (2011) Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease. Chemical reviews 111:5866-5898

Haeggström JZ, Funk CD (2011) Lipoxygenase and Leukotriene Pathways: Biochemistry, Biology, and Roles in Disease. Chemical reviews 111:5866-5898

Halitschke R, Ziegler J, Keinänen M, Baldwin IT (2004) Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling crosstalk in Nicotiana attenuata. The Plant Journal 40:35-46

Hamberg M, Sanz A, Rodriguez MJ, Calvo AP, Castresana C (2003) Activation of the Fatty Acid - Dioxygenase Pathway during Bacterial Infection of Tobacco Leaves: FORMATION OF OXYLIPINS PROTECTING AGAINST CELL DEATH. Journal of Biological Chemistry 278:51796-51805

Hammarberg T, Provost P, Persson B, Radmark O (2000) The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity. The Journal of biological chemistry 275:38787-38793

Hansen J, Garreta A, Benincasa M, Fusté MC, Busquets M, Manresa A (2013) Bacterial lipoxygenases, a new subfamily of enzymes? A phylogenetic approach. Applied microbiology and biotechnology 97:4737-4747

Harden TK, Sondek J (2006) Regulation of phospholipase C isozymes by ras superfamily GTPases. Annual review of pharmacology and toxicology 46:355-379

Harms K, Ramirez II, Pena-Cortes H (1998) Inhibition of wound-induced accumulation of allene oxide synthase transcripts in flax leaves by aspirin and salicylic acid. Plant Physiol 118:1057-1065

Hashimoto N, Fujiwara S, Watanabe K, Iguchi K, Tsuzuki M (2003) Localization of clavulones, prostanoids with antitumor activity, within the Okinawan soft coral Clavularia viridis (Alcyonacea, Clavulariidae): Preparation of a high-purity Symbiodinium fraction using a protease and a detergent. Lipids 38:991-997

Hawkins DJ, Brash AR (1987) Eggs of the sea urchin, Strongylocentrotus purpuratus, contain a prominent (11R) and (12R) lipoxygenase activity. The Journal of biological chemistry 262:7629-7634

Higdon A, Diers AR, Oh JY, Landar A, Darley-Usmar VM (2012) Cell signalling by reactive lipid species: new concepts and molecular mechanisms. The Biochemical journal 442:453-464
Hoegh-Guldberg O (1999) Climate change, coral bleaching and the future of the world's coral reefs. Mar Freshwater Res 50:839-866
Hoegh-Guldberg O, Bruno JF (2010) The Impact of Climate Change on the World's Marine Ecosystems. Science 328:1523-1528
Hollien J (2013) Evolution of the unfolded protein response. Biochimica et biophysica acta 1833:2458-2463
Honda A, Mori Y, Iguchi K, Yamada Y (1987) Antiproliferative and cytotoxic effects of newly discovered halogenated coral prostanoids from the Japanese stolonifer Clavularia viridis on human myeloid leukemia cells in culture. Molecular pharmacology 32:530-535
Hoover CA, Slattery M, Marsh AG (2007) Gene expression profiling of two related soft corals, Sinularia polydactyla and S. maxima, and their putative hybrid at different life-history stages. Comp Biochem Physiol Part D Genomics Proteomics 2:135-143
Horn T, Reddy Kakularam K, Anton M, Richter C, Reddanna P, Kuhn H (2013) Functional characterization of genetic enzyme variations in human lipoxygenases. Redox Biology 1:566-577
Horvath I, Multhoff G, Sonnleitner A, Vigh L (2008) Membrane-associated stress proteins: more than simply chaperones. Biochimica et biophysica acta 1778:1653-1664
Howe GA, Schilmiller AL (2002) Oxylipin metabolism in response to stress. Curr Opin Plant Biol 5:230-236
Howe GA, Lee GI, Itoh A, Li L, DeRocher AE (2000) Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiol 123:711-724
Howie PW, Calder AA, Forbes CD, Prentice CR (1973) Effect of intravenous prostaglandin E 2 on platelet function, coagulation, and fibrinolysis. Journal of clinical pathology 26:354-358
Howlett BJ, Chehab EW, Kaspi R, Savchenko T, Rowe H, Negre-Zakharov F, Kliebenstein D, Dehesh K (2008) Distinct Roles of Jasmonates and Aldehydes in Plant-Defense Responses. PloS ONE 3:e1904
Hyman LH (1940) The invertebrates. McGraw-Hill, New York,
Iba K (2002) ACCLIMATIVE RESPONSE TO TEMPERATURE STRESS IN HIGHER PLANTS: Approaches of Gene Engineering for Temperature Tolerance. Annual Review of Plant Biology 53:225-245
Imbs AB, Yakovleva IM (2011) Dynamics of lipid and fatty acid composition of shallow-water corals under thermal stress: an experimental approach. Coral Reefs 31:41-53

Imbs AB, Demina OA, Demidkova DA (2006) Lipid class and fatty acid composition of the boreal soft coral Gersemia rubiformis. Lipids 41:721-725
Itoh A (2000) Molecular Cloning of a Divinyl Ether Synthase. IDENTIFICATION AS A CYP74 CYTOCHROME P-450. Journal of Biological Chemistry 276:3620-3627
Ivanov I, Heydeck D, Hofheinz K, Roffeis J, O'Donnell VB, Kuhn H, Walther M (2010) Molecular enzymology of lipoxygenases. Archives of biochemistry and biophysics 503:161-174
Ivanov I, Di Venere A, Horn T, Scheerer P, Nicolai E, Stehling S, Richter C, Skrzypczak-Jankun E, Mei G, Maccarrone M, Kuhn H (2011) Tight association of N-terminal and catalytic subunits of rabbit 12/15-lipoxygenase is important for protein stability and catalytic activity. Biochimica et biophysica acta 1811:1001-1010
Jacobs AT, Marnett LJ (2007) Heat Shock Factor 1 Attenuates 4-Hydroxynonenal-mediated Apoptosis: CRITICAL ROLE FOR HEAT SHOCK PROTEIN 70 INDUCTION AND STABILIZATION OF Bcl-XL. Journal of Biological Chemistry 282:33412-33420
Jarving R, Lookene A, Kurg R, Siimon L, Jarving I, Samel N (2012) Activation of 11R-lipoxygenase is fully Ca(2+)-dependent and controlled by the phospholipid composition of the target membrane. Biochemistry 51:3310-3320
Jisaka M, Kim RB, Boeglin WE, Brash AR (2000) Identification of amino acid determinants of the positional specificity of mouse 8S-lipoxygenase and human 15S-lipoxygenase-2. Journal of Biological Chemistry 275:1287-1293
Jokiel PL (2004) Temperature stress and coral bleaching. Coral Health and Disease:401-425
Jokiel PL, Coles SL (1990) Response of Hawaiian and other Indo-Pacific reef corals to elevated temperature. Coral Reefs 8:155-162
Jones HG, Flowers TJ, Jones MB (1989)
Jurivich DA, Pangas S, Qiu L, Welk JF (1996) Phospholipase A2 triggers the first phase of the thermal stress response and exhibits cell-type specificity. J Immunol 157:1669-1677
Kallenbach M, Gilardoni PA, Allmann S, Baldwin IT, Bonaventure G (2011) C12 derivatives of the hydroperoxide lyase pathway are produced by product recycling through lipoxygenase-2 in Nicotiana attenuata leaves. The New phytologist 191:1054-1068
Kelly AJ, Malik S, Smith L, Kavanagh J, Thomas J (2009) Vaginal prostaglandin (PGE2 and PGF2a) for induction of labour at term
Kenkel CD, Meyer E, Matz MV (2013) Gene expression under chronic heat stress in populations of the mustard hill coral (Porites astreoides) from different thermal environments. Molecular ecology 22:4322-4334

Kenkel CD, Sheridan C, Leal MC, Bhagooli R, Castillo KD, Kurata N, McGinty E, Goulet TL, Matz MV (2014) Diagnostic gene expression biomarkers of coral thermal stress. Molecular ecology resources 14:667-678

Kenkel CD, Aglyamova G, Alamaru A, Bhagooli R, Capper R, Cunning R, deVillers A, Haslun JA, Hedouin L, Keshavmurthy S, Kuehl KA, Mahmoud H, McGinty ES, Montoya-Maya PH, Palmer CV, Pantile R, Sanchez JA, Schils T, Silverstein RN, Squiers LB, Tang PC, Goulet TL, Matz MV (2011) Development of gene expression markers of acute heat-light stress in reef-building corals of the genus Porites. PloS ONE 6:e26914

Kobzar G, Mardla V, Järving I, Samel N, Lõhmus M (1997) Modulatory effect of 8-iso-PGE2 on platelets. General Pharmacology: The Vascular System 28:317-321

Koljak R, Boutaud O, Shieh BH, Samel N, Brash AR (1997) Identification of a naturally occurring peroxidase-lipoxygenase fusion protein. Science 277:1994-1996

Koljak R, Jarving I, Kurg R, Boeglin WE, Varvas K, Valmsen K, Ustav M, Brash AR, Samel N (2001) The basis of prostaglandin synthesis in coral: molecular cloning and expression of a cyclooxygenase from the Arctic soft coral Gersemia fruticosa. J Biol Chem 276:7033-7040

Kombrink E (2012) Chemical and genetic exploration of jasmonate biosynthesis and signaling paths. Planta 236:1351-1366

Kramell R, Miersch O, Atzorn R, Parthier B, Wasternack C (2000) Octadecanoid-derived alteration of gene expression and the "oxylipin signature" in stressed barley leaves. Implications for different signaling pathways. Plant Physiol 123:177-188

Krishnamoorthy S, Honn KV (2011) Eicosanoids and other lipid mediators and the tumor hypoxic microenvironment. Cancer and Metastasis Reviews 30:613-618

Kuhn H, Saam J, Eibach S, Holzhutter HG, Ivanov I, Walther M (2005) Structural biology of mammalian lipoxygenases: enzymatic consequences of targeted alterations of the protein structure. Biochemical and biophysical research communications 338:93-101

Kulkarni S, Das S, Funk CD, Murray D, Cho W (2002) Molecular basis of the specific subcellular localization of the C2-like domain of 5-lipoxygenase. The Journal of biological chemistry 277:13167-13174

Kultz D (2005) Molecular and evolutionary basis of the cellular stress response. Annual review of physiology 67:225-257

Larsen BT, Miura H, Hatoum OA, Campbell WB, Hammock BD, Zeldin DC, Falck JR, Gutterman DD (2006) Epoxyeicosatrienoic and dihydroxyeicosatrienoic acids dilate human coronary arterioles via BK(Ca) channels: implications for soluble epoxide hydrolase inhibition.

American journal of physiology Heart and circulatory physiology 290:H491-499
Latyshev NA, Naumenko NV, Svetashev VI, Latypov YY (1991) Fatty-Acids of Reef-Building Corals. Mar Ecol Prog Ser 76:295-301
Laudert D, Weiler EW (1998) Allene oxide synthase: a major control point in Arabidopsis thaliana octadecanoid signalling. The Plant journal : for cell and molecular biology 15:675-684
Laudert D, Pfannschmidt U, Lottspeich F, Hollander-Czytko H, Weiler EW (1996) Cloning, molecular and functional characterization of Arabidopsis thaliana allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant molecular biology 31:323-335
Lee AS (2005) The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods 35:373-381
Lee DS, Nioche P, Hamberg M, Raman CS (2008) Structural insights into the evolutionary paths of oxylipin biosynthetic enzymes. Nature 455:363-368
Leon J, Rojo E, Sanchez-Serrano JJ (2001) Wound signalling in plants. Journal of experimental botany 52:1-9
Lesser MP (2006) Oxidative stress in marine environments: biochemistry and physiological ecology. Annual review of physiology 68:253-278
Lesser MP, Farrell JH (2004) Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress. Coral Reefs 23:367-377
Lievremont JP, Rizzuto R, Hendershot L, Meldolesi J (1997) BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of Ca2+. The Journal of biological chemistry 272:30873-30879
Lindquist S (1986) The heat-shock response. Annual review of biochemistry 55:1151-1191
Liu Z, Shinzato C, Inoue M, Kusakabe M (2014) A Snapshot of a Coral "Holobiont": A Transcriptome Assembly of the Scleractinian Coral, Porites, Captures a Wide Variety of Genes from Both the Host and Symbiotic Zooxanthellae. PloS ONE 9:e85182
Luan W, Li F, Zhang J, Wang B, Xiang J (2009) Cloning and expression of glucose regulated protein 78 (GRP78) in Fenneropenaeus chinensis. Molecular biology reports 36:289-298
Maccarrone M, van Aarle PG, Veldink GA, Vliegenthart JF (1994) In vitro oxygenation of soybean biomembranes by lipoxygenase-2. Biochimica et biophysica acta 1190:164-169
Maderna P, Godson C (2009) Lipoxins: resolutionary road. British journal of pharmacology 158:947-959
Maffei ME, Mithöfer A, Boland W (2007) Before gene expression: early events in plant–insect interaction. Trends in plant science 12:310-316

Major RT, Marchini P, Sproston T (1960) Isolation from Ginkgo biloba L. of an inhibitor of fungus growth. The Journal of biological chemistry 235:3298-3299
Marcotte EM, Pellegrini M, Ng HL, Rice DW, Yeates TO, Eisenberg D (1999) Detecting protein function and protein-protein interactions from genome sequences. Science 285:751-753
Matz MV, Guest JR, Baird AH, Maynard JA, Muttaqin E, Edwards AJ, Campbell SJ, Yewdall K, Affendi YA, Chou LM (2012) Contrasting Patterns of Coral Bleaching Susceptibility in 2010 Suggest an Adaptive Response to Thermal Stress. PloS ONE 7:e33353
Maucher H, Hause B, Feussner I, Ziegler J, Wasternack C (2000) Allene oxide synthases of barley (Hordeum vulgare cv. Salome): tissue specific regulation in seedling development. The Plant journal : for cell and molecular biology 21:199-213
Meyer E, Aglyamova GV, Wang S, Buchanan-Carter J, Abrego D, Colbourne JK, Willis BL, Matz MV (2009) Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx. BMC genomics 10:219
Minor W, Steczko J, Stec B, Otwinowski Z, Bolin JT, Walter R, Axelrod B (1996) Crystal structure of soybean lipoxygenase L-1 at 1.4 A resolution. Biochemistry 35:10687-10701
Mittler R, Finka A, Goloubinoff P (2012) How do plants feel the heat? Trends in biochemical sciences 37:118-125
Moga T (2013) The 2-Series Eicosanoids in Cancer: Future Targets for Glioma Therapy? Journal of Cancer Therapy 04:338-352
Moreno AA, Orellana A (2011) The physiological role of the unfolded protein response in plants. Biological research 44:75-80
Moreno JJ (2009) New aspects of the role of hydroxyeicosatetraenoic acids in cell growth and cancer development. Biochemical pharmacology 77:1-10
Mortimer M, Jarving R, Brash AR, Samel N, Jarving I (2006) Identification and characterization of an arachidonate 11R-lipoxygenase. Archives of biochemistry and biophysics 445:147-155
Mosblech A, Feussner I, Heilmann I (2009) Oxylipins: Structurally diverse metabolites from fatty acid oxidation. Plant Physiology and Biochemistry 47:511-517
Moya A, Huisman L, Ball EE, Hayward DC, Grasso LC, Chua CM, Woo HN, Gattuso JP, Foret S, Miller DJ (2012) Whole transcriptome analysis of the coral Acropora millepora reveals complex responses to CO(2)-driven acidification during the initiation of calcification. Molecular ecology 21:2440-2454
Mueller MJ (2004) Archetype signals in plants: the phytoprostanes. Current Opinion in Plant Biology 7:441-448
Mueller MJ, Berger S (2009) Reactive electrophilic oxylipins: Pattern recognition and signalling. Phytochemistry 70:1511-1521

Murakami M (2011) Lipid mediators in life science. Experimental animals / Japanese Association for Laboratory Animal Science 60:7-20
Mydlarz LD, Jacobs RS (2006) An inducible release of reactive oxygen radicals in four species of gorgonian corals. Marine and Freshwater Behaviour and Physiology 39:143-152
Mydlarz LD, Holthouse SF, Peters EC, Harvell CD (2008) Cellular responses in sea fan corals: granular amoebocytes react to pathogen and climate stressors. PloS ONE 3:e1811
Nagy E, Balogi Z, Gombos I, Akerfelt M, Bjorkbom A, Balogh G, Torok Z, Maslyanko A, Fiszer-Kierzkowska A, Lisowska K, Slotte PJ, Sistonen L, Horvath I, Vigh L (2007) Hyperfluidization-coupled membrane microdomain reorganization is linked to activation of the heat shock response in a murine melanoma cell line. Proceedings of the National Academy of Sciences of the United States of America 104:7945-7950
Neau DB, Gilbert NC, Bartlett SG, Boeglin W, Brash AR, Newcomer ME (2009) The 1.85 A structure of an 8R-lipoxygenase suggests a general model for lipoxygenase product specificity. Biochemistry 48:7906-7915
Nelson DR, Goldstone JV, Stegeman JJ (2013) The cytochrome P450 genesis locus: the origin and evolution of animal cytochrome P450s. Philosophical transactions of the Royal Society of London Series B, Biological sciences 368:20120474
Nelson DR, Ming R, Alam M, Schuler MA (2008) Comparison of Cytochrome P450 Genes from Six Plant Genomes. Tropical Plant Biology 1:216-235
Nevalainen TJ, Peuravuori HJ, Quinn RJ, Llewellyn LE, Benzie JA, Fenner PJ, Winkel KD (2004) Phospholipase A2 in cnidaria. Comparative biochemistry and physiology Part B, Biochemistry & molecular biology 139:731-735
Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, Liao JK (1999) Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science 285:1276-1279
Noordermeer MA, Veldink GA, Vliegenthart JFG (2001a) Spectroscopic studies on the active site of hydroperoxide lyase; the influence of detergents on its conformation. FEBS letters 489:229-232
Noordermeer MA, Veldink GA, Vliegenthart JFG (2001b) Fatty acid hydroperoxide lyase: A plant cytochrome P450 enzyme involved in wound healing and pest resistance. Chembiochem 2:494-504
O'Neal W, Pawlik JR (2002) A reappraisal of the chemical and physical defenses of Caribbean gorgonian corals against predatory fishes. Mar Ecol Prog Ser 240:117-126
Oka T, Hayashida S, Kaneda Y, Takenaga M, Tamagawa Y, Tsuji S, Hatanaka A (2008) Green odor attenuates a cold pressor test-induced cardiovascular response in healthy adults. BioPsychoSocial Medicine 2:2

Olano CT, Bigger CH (2000) Phagocytic activities of the gorgonian coral Swiftia exserta. Journal of invertebrate pathology 76:176-184
Oldham ML, Brash AR, Newcomer ME (2005a) The structure of coral allene oxide synthase reveals a catalase adapted for metabolism of a fatty acid hydroperoxide. Proceedings of the National Academy of Sciences of the United States of America 102:297-302
Oldham ML, Brash AR, Newcomer ME (2005b) Insights from the X-ray crystal structure of coral 8R-lipoxygenase: calcium activation via a C2-like domain and a structural basis of product chirality. The Journal of biological chemistry 280:39545-39552
Oliw EH, Cristea M, Hamberg M (2004) Biosynthesis and isomerization of 11-hydroperoxylinoleates by manganese- and iron-dependent lipoxygenases. Lipids 39:319-323
Oren M, Amar KO, Douek J, Rosenzweig T, Paz G, Rinkevich B (2010) Assembled catalog of immune-related genes from allogeneic challenged corals that unveils the participation of vWF-like transcript. Developmental & Comparative Immunology 34:630-637
Palmer CV, Mydlarz LD, Willis BL (2008) Evidence of an inflammatory-like response in non-normally pigmented tissues of two scleractinian corals. Proceedings Biological sciences / The Royal Society 275:2687-2693
Palmer CV, Traylor-Knowles NG, Willis BL, Bythell JC (2011) Corals use similar immune cells and wound-healing processes as those of higher organisms. PloS ONE 6:e23992
Panigrahy D, Kalish BT, Huang S, Bielenberg DR, Le HD, Yang J, Edin ML, Lee CR, Benny O, Mudge DK, Butterfield CE, Mammoto A, Mammoto T, Inceoglu B, Jenkins RL, Simpson MA, Akino T, Lih FB, Tomer KB, Ingber DE, Hammock BD, Falck JR, Manthati VL, Kaipainen A, D'Amore PA, Puder M, Zeldin DC, Kieran MW (2013) Epoxyeicosanoids promote organ and tissue regeneration. Proceedings of the National Academy of Sciences of the United States of America 110:13528-13533
Park HG, Han SI, Oh SY, Kang HS (2005) Cellular responses to mild heat stress. Cellular and Molecular Life Sciences 62:10-23
Park JB, Lakes RS (2007) Characterization of Materials — I Biomaterials, an Introduction, pp41-81
Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. The Plant journal : for cell and molecular biology 31:1-12
Parker R, Phan T, Baumeister P, Roy B, Cheriyath V, Roy AL, Lee AS (2001) Identification of TFII-I as the endoplasmic reticulum stress response element binding factor ERSF: its autoregulation by stress and interaction with ATF6. Molecular and cellular biology 21:3220-3233

Pawlik JR, Burch MT, Fenical W (1987) Patterns of Chemical Defense among Caribbean Gorgonian Corals - a Preliminary Survey. Journal of Experimental Marine Biology and Ecology 108:55-66
Pidgeon GP, Lysaght J, Krishnamoorthy S, Reynolds JV, O'Byrne K, Nie D, Honn KV (2007) Lipoxygenase metabolism: roles in tumor progression and survival. Cancer and Metastasis Reviews 26:503-524
Polato NR, Vera JC, Baums IB (2011) Gene discovery in the threatened elkhorn coral: 454 sequencing of the Acropora palmata transcriptome. PloS ONE 6:e28634
Polato NR, Altman NS, Baums IB (2013) Variation in the transcriptional response of threatened coral larvae to elevated temperatures. Molecular ecology 22:1366-1382
Polato NR, Voolstra CR, Schnetzer J, DeSalvo MK, Randall CJ, Szmant AM, Medina M, Baums IB (2010) Location-specific responses to thermal stress in larvae of the reef-building coral Montastraea faveolata. PloS ONE 5:e11221
Pooyaei Mehr SF, DeSalle R, Kao HT, Narechania A, Han Z, Tchernov D, Pieribone V, Gruber DF (2013) Transcriptome deep-sequencing and clustering of expressed isoforms from Favia corals. BMC genomics 14:546
Portune KJ, Voolstra CR, Medina M, Szmant AM (2010) Development and heat stress-induced transcriptomic changes during embryogenesis of the scleractinian coral Acropora palmata. Marine genomics 3:51-62
Pozzi A, Macias-Perez I, Abair T, Wei S, Su Y, Zent R, Falck JR, Capdevila JH (2005) Characterization of 5,6- and 8,9-Epoxyeicosatrienoic Acids (5,6- and 8,9-EET) as Potent in Vivo Angiogenic Lipids. Journal of Biological Chemistry 280:27138-27146
Proksch E, Brandner JM, Jensen J-M (2008) The skin: an indispensable barrier. Experimental dermatology 17:1063-1072
Putnam NH, Srivastava M, Hellsten U, Dirks B, Chapman J, Salamov A, Terry A, Shapiro H, Lindquist E, Kapitonov VV, Jurka J, Genikhovich G, Grigoriev IV, Lucas SM, Steele RE, Finnerty JR, Technau U, Martindale MQ, Rokhsar DS (2007) Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. Science 317:86-94
Radmark O, Samuelsson B (2008) 5-Lipoxygenase: mechanisms of regulation. The Journal of Lipid Research 50:S40-S45
Radmark O, Samuelsson B (2009) 5-Lipoxygenase: mechanisms of regulation. Journal of lipid research 50 Suppl:S40-45
Reitzel AM, Sullivan JC, Traylor-Knowles N, Finnerty JR (2008) Genomic survey of candidate stress-response genes in the estuarine anemone Nematostella vectensis. The Biological bulletin 214:233-254
Resendez E, Jr., Wooden SK, Lee AS (1988) Identification of highly conserved regulatory domains and protein-binding sites in the promoters of the rat

and human genes encoding the stress-inducible 78-kilodalton glucose-regulated protein. Molecular and cellular biology 8:4579-4584
Reyes-Bermudez A, DeSalvo MK, Voolstra CR, Sunagawa S, Szmant AM, Iglesias-Prieto R, Medina M (2009) Gene expression microarray analysis encompassing metamorphosis and the onset of calcification in the scleractinian coral Montastraea faveolata. Marine genomics 2:149-159
Reymond P, Weber H, Damond M, Farmer EE (2000) Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. The Plant cell 12:707-720
Ricciotti E, FitzGerald GA (2011) Prostaglandins and Inflammation. Arteriosclerosis, Thrombosis, and Vascular Biology 31:986-1000
Rodriguez-Lanetty M, Harii S, Hoegh-Guldberg O (2009) Early molecular responses of coral larvae to hyperthermal stress. Molecular ecology 18:5101-5114
Rodriguez PG, Felix FN, Woodley DT, Shim EK (2008) The role of oxygen in wound healing: a review of the literature. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al] 34:1159-1169
Romero L, Marcussi S, Marchi-Salvador DP, Silva FP, Jr., Fuly AL, Stabeli RG, da Silva SL, Gonzalez J, Monte AD, Soares AM (2010) Enzymatic and structural characterization of a basic phospholipase A(2) from the sea anemone Condylactis gigantea. Biochimie 92:1063-1071
Rouzer CA, Marnett LJ (2003) Mechanism of free radical oxygenation of polyunsaturated fatty acids by cyclooxygenases. Chemical reviews 103:2239-2304
Rouzer CA, Marnett LJ (2005) Structural and functional differences between cyclooxygenases: Fatty acid oxygenases with a critical role in cell signaling. Biochemical and biophysical research communications 338:34-44
Rouzer CA, Marnett LJ (2011) Endocannabinoid Oxygenation by Cyclooxygenases, Lipoxygenases, and Cytochromes P450: Cross-Talk between the Eicosanoid and Endocannabinoid Signaling Pathways. Chemical reviews 111:5899-5921
Rowley AF, Vogan CL, Taylor GW, Clare AS (2005) Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems. The Journal of experimental biology 208:3-14
Schaloske RH, Dennis EA (2006) The phospholipase A2 superfamily and its group numbering system. Biochimica et biophysica acta 1761:1246-1259
Schilmiller AL, Howe GA (2005) Systemic signaling in the wound response. Current Opinion in Plant Biology 8:369-377

Schneider C, Pratt DA, Porter NA, Brash AR (2007) Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. Chemistry & biology 14:473-488
Schommer C, Palatnik JF, Aggarwal P, Chetelat A, Cubas P, Farmer EE, Nath U, Weigel D (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. Plos Biol 6:1991-2001
Schroder M, Kaufman RJ (2005) ER stress and the unfolded protein response. Mutation research 569:29-63
Schultz GS, Davidson JM, Kirsner RS, Bornstein P, Herman IM (2011) Dynamic reciprocity in the wound microenvironment. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 19:134-148
Seneca FO, Foret S, Ball EE, Smith-Keune C, Miller DJ, van Oppen MJ (2010) Patterns of gene expression in a scleractinian coral undergoing natural bleaching. Mar Biotechnol (NY) 12:594-604
Serhan CN, Chiang N (2002) Lipid-derived mediators in endogenous anti-inflammation and resolution: lipoxins and aspirin-triggered 15-epi-lipoxins. TheScientificWorldJournal 2:169-204
Serhan CN, Haeggstrom JZ, Leslie CC (1996) Lipid mediator networks in cell signaling: update and impact of cytokines. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 10:1147-1158
Serhan CN, Chiang N, Van Dyke TE (2008a) Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol 8:349-361
Serhan CN, Yacoubian S, Yang R (2008b) Anti-inflammatory and proresolving lipid mediators. Annu Rev Pathol 3:279-312
Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR, Mirick G, Moussignac RL (2002) Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. The Journal of experimental medicine 196:1025-1037
Serhan CN, Brain SD, Buckley CD, Gilroy DW, Haslett C, O'Neill LA, Perretti M, Rossi AG, Wallace JL (2007) Resolution of inflammation: state of the art, definitions and terms. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 21:325-332
Seubert JM, Zeldin DC, Nithipatikom K, Gross GJ (2007) Role of epoxyeicosatrienoic acids in protecting the myocardium following ischemia/reperfusion injury. Prostaglandins & other lipid mediators 82:50-59
Shinzato C, Shoguchi E, Kawashima T, Hamada M, Hisata K, Tanaka M, Fujie M, Fujiwara M, Koyanagi R, Ikuta T, Fujiyama A, Miller DJ, Satoh N

(2011) Using the Acropora digitifera genome to understand coral responses to environmental change. Nature 476:320-U382
Singer AJ, Clark RA (1999) Cutaneous wound healing. The New England journal of medicine 341:738-746
Sivasankar S, Sheldrick B, Rothstein SJ (2000) Expression of allene oxide synthase determines defense gene activation in tomato. Plant Physiol 122:1335-1342
Skrzypczak-Jankun E, Amzel LM, Kroa BA, Funk MO, Jr. (1997) Structure of soybean lipoxygenase L3 and a comparison with its L1 isoenzyme. Proteins 29:15-31
Song WC, Brash AR (1991a) Investigation of the Allene Oxide Pathway in the Coral Plexaura-Homomalla - Formation of Novel Ketols and Isomers of Prostaglandin-A2 from 15-Hydroxyeicosatetraenoic Acid. Archives of biochemistry and biophysics 290:427-435
Song WC, Brash AR (1991b) Purification of an Allene Oxide Synthase and Identification of the Enzyme as a Cytochrome-P-450. Science 253:781-784
Song WC, Funk CD, Brash AR (1993) Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. Proc Natl Acad Sci U S A 90:8519-8523
Søørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecology Letters 6:1025-1037
Souter P, Bay LK, Andreakis N, Csaszar N, Seneca FO, van Oppen MJ (2011) A multilocus, temperature stress-related gene expression profile assay in Acropora millepora, a dominant reef-building coral. Molecular ecology resources 11:328-334
Spector AA, Norris AW (2007) Action of epoxyeicosatrienoic acids on cellular function. American journal of physiology Cell physiology 292:C996-1012
Spriggs KA, Bushell M, Willis AE (2010) Translational Regulation of Gene Expression during Conditions of Cell Stress. Molecular cell 40:228-237
Stenzel I, Hause B, Maucher H, Pitzschke A, Miersch O, Ziegler J, Ryan CA, Wasternack C (2003) Allene oxide cyclase dependence of the wound response and vascular bundle-specific generation of jasmonates in tomato - amplification in wound signalling. The Plant journal : for cell and molecular biology 33:577-589
Steponkus PL (1984) Role of the Plasma-Membrane in Freezing-Injury and Cold-Acclimation. Annu Rev Plant Phys 35:543-584
Stintzi A, Weber H, Reymond P, Browse J, Farmer EE (2001) Plant defense in the absence of jasmonic acid: the role of cyclopentenones. Proceedings of the National Academy of Sciences of the United States of America 98:12837-12842

Sun J, Chen Q, Lun JCY, Xu J, Qiu J-W (2012) PcarnBase: Development of a Transcriptomic Database for the Brain Coral Platygyra carnosus. Marine Biotechnology 15:244-251
Sun J, Sui X, Bradbury JA, Zeldin DC, Conte MS, Liao JK (2002) Inhibition of vascular smooth muscle cell migration by cytochrome p450 epoxygenase-derived eicosanoids. Circulation research 90:1020-1027
Sung DY, Kaplan F, Lee KJ, Guy CL (2003) Acquired tolerance to temperature extremes. Trends in plant science 8:179-187
Söderhäll K, Libro S, Kaluziak ST, Vollmer SV (2013) RNA-seq Profiles of Immune Related Genes in the Staghorn Coral Acropora cervicornis Infected with White Band Disease. PloS ONE 8:e81821
Zacharias H, Anokhin B, Khalturin K, Bosch TCG (2004) Genome sizes and chromosomes in the basal metazoan Hydra. Zoology 107:219-227
Zeldin DC, Kobayashi J, Falck JR, Winder BS, Hammock BD, Snapper JR, Capdevila JH (1993) Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase. The Journal of biological chemistry 268:6402-6407
Zhang ZQ (2011) Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. Zootaxa:1-237
Zhong M, Orosz A, Wu C (1998) Direct sensing of heat and oxidation by Drosophila heat shock transcription factor. Molecular cell 2:101-108
Ziboh VA, Miller CC, Cho Y (2000) Metabolism of polyunsaturated fatty acids by skin epidermal enzymes: generation of antiinflammatory and antiproliferative metabolites. The American journal of clinical nutrition 71:361S-366S
Ziegler J, Hamberg M, Miersch O, Parthier B (1997) Purification and Characterization of Allene Oxide Cyclase from Dry Corn Seeds. Plant Physiol 114:565-573
Zimmerman DC, Coudron CA (1979) Identification of Traumatin, a Wound Hormone, as 12-Oxo-trans-10-dodecenoic Acid. Plant Physiology 63:536-541
Takahashi Y, Glasgow WC, Suzuki H, Taketani Y, Yamamoto S, Anton M, Kuhn H, Brash AR (1993) Investigation of the oxygenation of phospholipids by the porcine leukocyte and human platelet arachidonate 12-lipoxygenases. Eur J Biochem 218:165-171
Taki N, Sasaki-Sekimoto Y, Obayashi T, Kikuta A, Kobayashi K, Ainai T, Yagi K, Sakurai N, Suzuki H, Masuda T, Takamiya K, Shibata D, Kobayashi Y, Ohta H (2005) 12-oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in Arabidopsis. Plant Physiol 139:1268-1283
Tijet N, Brash AR (2002) Allene oxide synthases and allene oxides. Prostaglandins & other lipid mediators 68-69:423-431
Tijet N, Schneider C, Muller BL, Brash AR (2001) Biogenesis of Volatile Aldehydes from Fatty Acid Hydroperoxides: Molecular Cloning of a

Hydroperoxide Lyase (CYP74C) with Specificity for both the 9- and 13-Hydroperoxides of Linoleic and Linolenic Acids. Archives of biochemistry and biophysics 386:281-289

Toporkova YY, Gogolev YV, Mukhtarova LS, Grechkin AN (2008) Determinants governing the CYP74 catalysis: Conversion of allene oxide synthase into hydroperoxide lyase by site-directed mutagenesis. FEBS letters 582:3423-3428

Traylor-Knowles N, Granger BR, Lubinski TJ, Parikh JR, Garamszegi S, Xia Y, Marto JA, Kaufman L, Finnerty JR (2011) Production of a reference transcriptome and transcriptomic database (PocilloporaBase) for the cauliflower coral, Pocillopora damicornis. BMC genomics 12:585

Turlings TC, Loughrin JH, McCall PJ, Rose US, Lewis WJ, Tumlinson JH (1995) How caterpillar-damaged plants protect themselves by attracting parasitic wasps. Proceedings of the National Academy of Sciences of the United States of America 92:4169-4174

Upchurch RG (2008) Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. Biotechnology letters 30:967-977

Upston JM, Neuzil J, Witting PK, Alleva R, Stocker R (1997) Oxidation of free fatty acids in low density lipoprotein by 15-lipoxygenase stimulates nonenzymic, alpha-tocopherol-mediated peroxidation of cholesteryl esters. The Journal of biological chemistry 272:30067-30074

Valmsen K, Boeglin WE, Jarving I, Schneider C, Varvas K, Brash AR, Samel N (2004) Structural and functional comparison of 15S- and 15R-specific cyclooxygenases from the coral Plexaura homomalla. European journal of biochemistry / FEBS 271:3533-3538

Valmsen K, Jarving I, Boeglin WE, Varvas K, Koljak R, Pehk T, Brash AR, Samel N (2001) The origin of 15R-prostaglandins in the Caribbean coral Plexaura homomalla: molecular cloning and expression of a novel cyclooxygenase. Proc Natl Acad Sci U S A 98:7700-7705

Walther M, Wiesner R, Kuhn H (2004) Investigations into calcium-dependent membrane association of 15-lipoxygenase-1. Mechanistic roles of surface-exposed hydrophobic amino acids and calcium. The Journal of biological chemistry 279:3717-3725

Walther M, Hofheinz K, Vogel R, Roffeis J, Kuhn H (2011) The N-terminal beta-barrel domain of mammalian lipoxygenases including mouse 5-lipoxygenase is not essential for catalytic activity and membrane binding but exhibits regulatory functions. Archives of biochemistry and biophysics 516:1-9

van Oppen MJ, Gates RD (2006) Conservation genetics and the resilience of reef-building corals. Molecular ecology 15:3863-3883

Vane JR, Botting RM (1998) Anti-inflammatory drugs and their mechanism of action. Inflammation research : official journal of the European Histamine Research Society [et al] 47 Suppl 2:S78-87

Wang Y (2005) Arachidonic Acid Epoxygenase Metabolites Stimulate Endothelial Cell Growth and Angiogenesis via Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase/Akt Signaling Pathways. Journal of Pharmacology and Experimental Therapeutics 314:522-532

Vargas-Ángel B, Peters EC, Kramarsky-Winter E, Gilliam DS, Dodge RE (2007) Cellular reactions to sedimentation and temperature stress in the Caribbean coral Montastraea cavernosa. Journal of invertebrate pathology 95:140-145

Varvas K, Koljak R, Järving I, Pehk T, Samel N (1994a) Endoperoxide pathway in prostaglandin biosynthesis in the soft coral Gersemia fruticosa. Tetrahedron Letters 35:8267-8270

Varvas K, Koljak R, Jarving I, Pehk T, Samel N (1994b) Endoperoxide Pathway in Prostaglandin Biosynthesis in the Soft Coral Gersemia-Fruticosa. Tetrahedron Lett 35:8267-8270

Varvas K, Jarving I, Koljak R, Valmsen K, Brash AR, Samel N (1999) Evidence of a cyclooxygenase-related prostaglandin synthesis in coral. The allene oxide pathway is not involved in prostaglandin biosynthesis. J Biol Chem 274:9923-9929

Varvas K, Jarving I, Koljak R, Vahemets A, Pehk T, Muurisepp AM, Lille U, Samel N (1993) Invitro Biosynthesis of Prostaglandins in the White Sea Soft Coral Gersemia-Fruticosa - Formation of Optically-Active Pgd2, Pge2, Pgf2-Alpha and 15-Keto-Pgf2-Alpha from Arachidonic-Acid. Tetrahedron Letters 34:3643-3646

Wasternack C (2007) Jasmonates: An Update on Biosynthesis, Signal Transduction and Action in Plant Stress Response, Growth and Development. Annals of botany 100:681-697

Wasternack C, Kombrink E (2010) Jasmonates: structural requirements for lipid-derived signals active in plant stress responses and development. ACS chemical biology 5:63-77

Wasternack C, Hause B (2013a) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Annals of botany 111:1021-1058

Wasternack C, Hause B (2013b) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Annals of botany 111:1021-1058

Watanabe K, Sekine M, Takahashi H, Iguchi K (2001) New halogenated marine prostanoids with cytotoxic activity from the Okinawan soft coral Clavularia viridis. Journal of natural products 64:1421-1425

Webb MS, Uemura M, Steponkus PL (1994) A Comparison of Freezing-Injury in Oat and Rye - 2 Cereals at the Extremes of Freezing Tolerance. Plant Physiology 104:467-478

Weber H, Vick BA, Farmer EE (1997) Dinor-oxo-phytodienoic acid: a new hexadecanoid signal in the jasmonate family. Proceedings of the National Academy of Sciences of the United States of America 94:10473-10478

Weinheimer AJ, Spraggins RL (1969) The occurrence of two new prostaglandin derivatives (15-epi-PGA2 and its acetate, methyl ester) in the gorgonian Plexaura homomalla chemistry of coelenterates. XV. Tetrahedron letters:5185-5188

Wenemoser D, Lapan SW, Wilkinson AW, Bell GW, Reddien PW (2012) A molecular wound response program associated with regeneration initiation in planarians. Genes & development 26:988-1002

West JD, Wang Y, Morano KA (2012) Small molecule activators of the heat shock response: chemical properties, molecular targets, and therapeutic promise. Chemical research in toxicology 25:2036-2053

Whalen KE, Starczak VR, Nelson DR, Goldstone JV, Hahn ME (2010) Cytochrome P450 diversity and induction by gorgonian allelochemicals in the marine gastropod Cyphoma gibbosum. BMC ecology 10:24

Vick BA, Zimmerman DC (1987) Pathways of Fatty-Acid Hydroperoxide Metabolism in Spinach Leaf Chloroplasts. Plant Physiology 85:1073-1078

Vidal-Dupiol J, Zoccola D, Tambutte E, Grunau C, Cosseau C, Smith KM, Freitag M, Dheilly NM, Allemand D, Tambutte S (2013) Genes related to ion-transport and energy production are upregulated in response to CO2-driven pH decrease in corals: new insights from transcriptome analysis. PloS ONE 8:e58652

Voolstra CR, Dunn SR, Thomas MC, Nette GW, Dove SG (2012) A Lipidomic Approach to Understanding Free Fatty Acid Lipogenesis Derived from Dissolved Inorganic Carbon within Cnidarian-Dinoflagellate Symbiosis. PloS ONE 7:e46801

Voolstra CR, Schnetzer J, Peshkin L, Randall CJ, Szmant AM, Medina M (2009a) Effects of temperature on gene expression in embryos of the coral Montastraea faveolata. BMC genomics 10:627

Voolstra CR, Bay LK, Guérécheau A, Andreakis N, Ulstrup KE, Matz MV (2013) Gene Expression Signatures of Energetic Acclimatisation in the Reef Building Coral Acropora millepora. PloS ONE 8:e61736

Voolstra CR, Schwarz JA, Schnetzer J, Sunagawa S, Desalvo MK, Szmant AM, Coffroth MA, Medina MN (2009b) The host transcriptome remains unaltered during the establishment of coral - algal symbioses. Molecular ecology 18:1823-1833

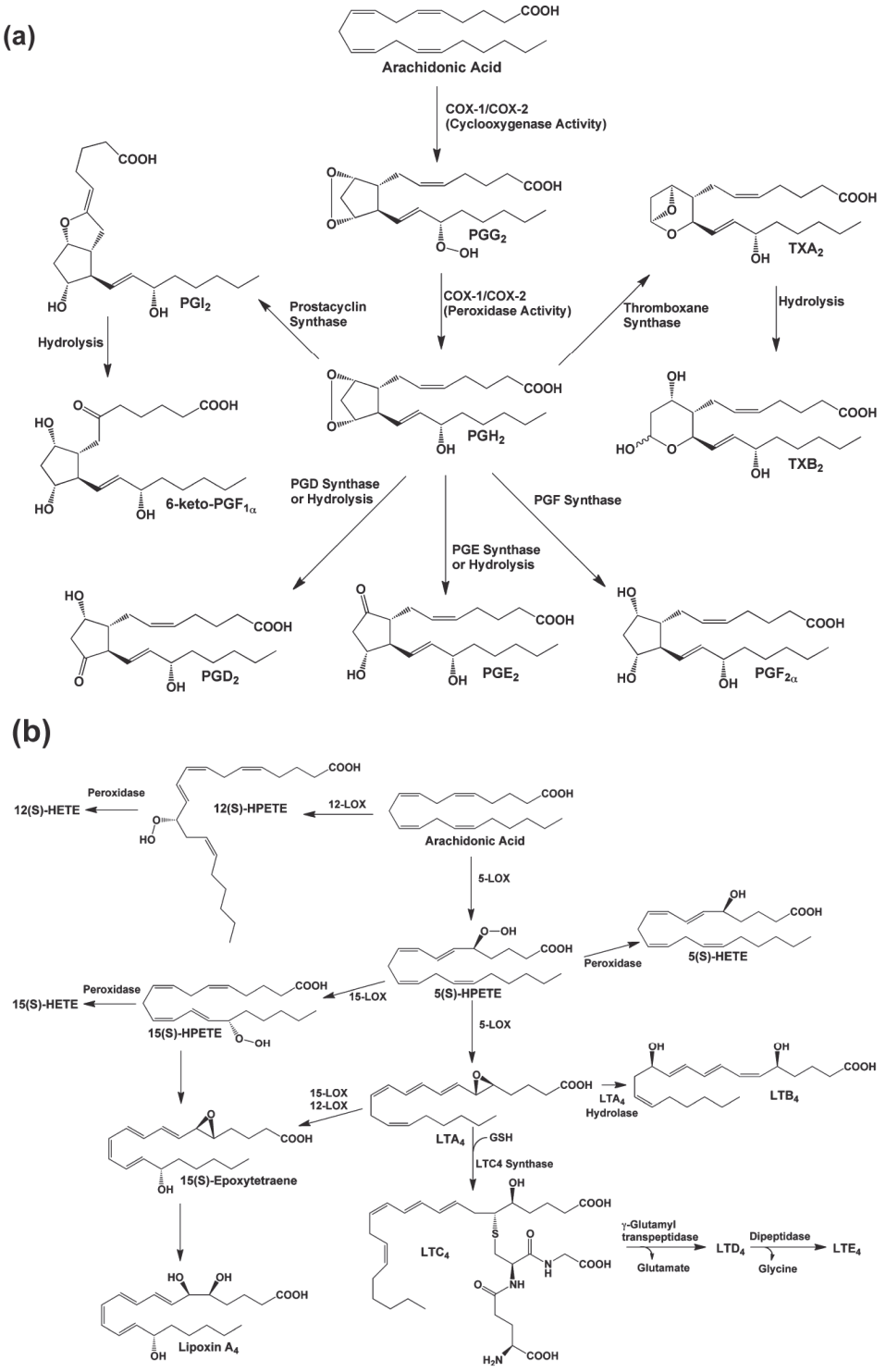
Voolstra CR, Karako-Lampert S, Zoccola D, Salmon-Divon M, Katzenellenbogen M, Tambutté S, Bertucci A, Hoegh-Guldberg O, Deleury E, Allemand D, Levy O (2014) Transcriptome Analysis of the Scleractinian Coral Stylophora pistillata. PloS ONE 9:e88615

Wu K, Heil M, Ibarra-Laclette E, Adame-Álvarez RM, Martínez O, Ramirez-Chávez E, Molina-Torres J, Herrera-Estrella L (2012) How Plants Sense Wounds: Damaged-Self Recognition Is Based on Plant-Derived Elicitors and Induces Octadecanoid Signaling. PloS ONE 7:e30537

Wymann MP, Schneiter R (2008) Lipid signalling in disease. Nature reviews Molecular cell biology 9:162-176

Xu S, Mueser Timothy C, Marnett Lawrence J, Funk Max O (2012) Crystal Structure of 12-Lipoxygenase Catalytic-Domain-Inhibitor Complex Identifies a Substrate-Binding Channel for Catalysis. Structure 20:1490-1497

APPENDIX 1


(a)
Arachidonic Acid
COX-1/COX-2
(Cyclooxygenase Activity)
PGG2
COX-1/COX-2
(Peroxidase Activity)
PGH2
Prostacyclin
Synthase
PGI2
Hydrolysis
6-keto-PGF1α
Thromboxane
Synthase
TXA2
Hydrolysis
TXB2
PGD Synthase
or Hydrolysis
PGD2
PGE Synthase
or Hydrolysis
PGE2
PGF Synthase
PGF2α
(b)
Arachidonic Acid
12-LOX
12(S)-HPETE
Peroxidase
12(S)-HETE
5-LOX
5(S)-HPETE
Peroxidase
5(S)-HETE
15-LOX
15(S)-HPETE
Peroxidase
15(S)-HETE
5-LOX
LTA4
LTA4
Hydrolase
LTB4
15-LOX
12-LOX
15(S)-Epoxytetraene
GSH
LTC4 Synthase
LTC4
γ-Glutamyl
transpeptidase
Glutamate
LTD4
Dipeptidase
Glycine
LTE4
Lipoxin A4

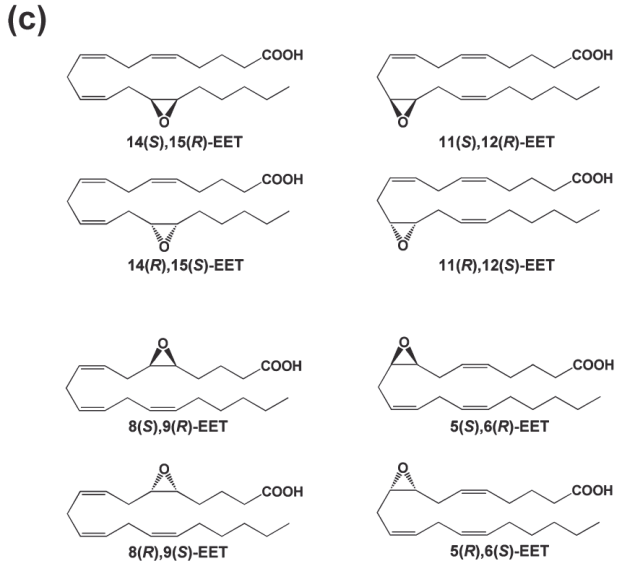


Figure 1. Animal eicosanoids. Cyclooxygenase (a), lipoxygenase (b) and cytochrome P450s pathways (c) of AA metabolism (Rouzer and Marnett 2011).

PUBLICATION I

Lõhelaid, H., Järving, R., Valmsen, K., Varvas, K., Kreen, M., Järving, I., Samel, N. (2008). "Identification of a functional allene oxide synthase-lipoxygenase fusion protein in the soft coral *Gersemia fruticosa* suggests the generality of this pathway in octocorals." Biochim. Biophys. Acta 1780(2): 315-321

Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1780 (2008) 315–321
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbagen

Short sequence paper

Identification of a functional allene oxide synthase-lipoxygenase fusion protein in the soft coral *Gersemia fruticosa* suggests the generality of this pathway in octocorals ☆

Helike Lõhelaid, Reet Järving, Karin Valmsen, Külliki Varvas, Malle Kreen, Ivar Järving, Nigulas Samel *

Department of Chemistry, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia

Received 16 August 2007; received in revised form 3 October 2007; accepted 15 October 2007
Available online 22 October 2007

Abstract

The conversion of fatty acid hydroperoxides to allene epoxides is catalysed by a cytochrome P450 in plants. In contrast, in the coral *Plexaura homomalla*, a catalase-related hemoprotein fused to the lipoxygenase (LOX) was found to function as an allene oxide synthase. This work reports the homology-based RT-PCR cloning and functional expression of a *Gersemia fruticosa* analogue of the allene oxide synthase-lipoxygenase (AOS-LOX) fusion protein. The *G. fruticosa* mRNA codes for a protein with 84% sequence identity to the *P. homomalla* AOS-LOX. Our data indicate that the AOS-LOX fusion protein pathway is used by another coral and *P. homomalla* represents no exception.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Allene oxide synthase; AOS-LOX; Lipoxygenase-peroxidase fusion protein; Coral; *Gersemia fruticosa*; HETE; Lipoxygenase; LC-MS

Allene epoxides (allene oxides) are short-lived products (half-lives 15–30 s) formed from certain lipoxygenase-derived fatty acid hydroperoxides by specific hydroperoxide dehydratases, the so-called allene oxide synthases (AOS). These enzymes are well characterised in plants where allene oxide plays a crucial role, being a precursor of the growth regulator jasmonic acid. The plant AOS belongs to the cytochrome P450 family of hemoproteins designated as CYP74 [1–3]. The allene oxide synthesis has also been observed in certain marine invertebrates like starfish [4] and coral [5,6]. However, all attempts to detect plant AOS-related sequences in coral have remained unsuccessful. Surprisingly, it was shown that the allene oxide synthesis in the Caribbean coral *Plexaura homomalla* is principally different from the pathway in plants [7]. Specifically, the conversion of fatty acid hydroperoxides to allene oxides in the coral is catalysed by a catalase-related hemoprotein fused to the lipoxygenase (LOX) that forms the 8*R*-hydroperoxyeicosatetraenoic acid (8*R*-HPETE) substrate [7–9]. The crystal structures of separate AOS and LOX domains of the AOS-LOX fusion protein from *P. homomalla* have by now been reported [10,11].

The formation of an α-ketol, the main degradation product of allene oxide, has also been observed in incubations of exogenous arachidonic acid with an acetone powder preparation of another coral, *Gersemia fruticosa* [12]. This cold-water soft coral is unique because of its very high prostaglandin (typical mammalian prostaglandins (PG) E_2, D_2, $F_{2\alpha}$ and corresponding 15-keto-PG) synthesizing activity in vitro [13]. The other products formed from arachidonic acid were 8*R*-hydroxyeicosatetraenoic acid (8*R*-HETE), 11*R*- hydroxyeicosatetraenoic acid (11*R*-HETE), 15*S*-hydroxyeicosatetraenoic acid (15*S*-HETE), and 8,15-dihydroxyeicosatetraenoic acid [13]. The metabolic routes to prostaglandins and 11*R*-HETE in *G. fruticosa* were unambiguously confirmed by the cloning and characterization of coral cyclooxygenases and corresponding genes [14,15], and a novel arachidonate 11*R*-lipoxygenase [16]. However, the metabolic

Abbreviations: AOS, allene oxide synthase; AOS-LOX, allene oxide synthase-lipoxygenase; HETE, hydroeicosatetraenoic acid; LC-MS, liquid chromatography- mass-spectroscopy; LOX, lipoxygenase; PCR, polymerase chain reaction; RACE-, rapid amplification of cDNA ends; RP, reversed phase; RT, reverse transcriptase

☆ *Gersemia fruticosa* AOS-LOX GenBank accession number, EU082210.

* Corresponding author. Tel.: +372 620 4376; fax: +372 620 2828.
E-mail address: samel@chemnet.ee (N. Samel).

0304-4165/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2007.10.010

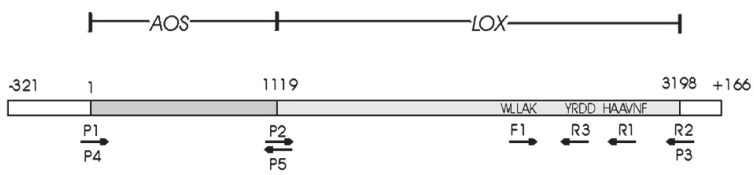


Fig. 1. The strategy of cloning of AOS-LOX from *G. fruticosa*. In the first round of PCR the degenerate primers were based on the conserved regions on plant and animal lipoxygenases WLLAK (WMLAK) (F1), and the downstream primers HAAVNF (R1) and near the C-terminus NS(V/I)SI (R2) [16]. For the half-nested second round of PCR HAAVNF (R1) or YRDD (R3) were used as downstream primers. The *full-length ORF* of the coral AOS-LOX cDNA was obtained by PCR. The *Nde*I sites were added at the 5′ end of the upstream (P1) 5′-GGAATCCCATATGGTTTGGAAAAATTATGGCTTTG-3′ and downstream (P3) 5′-GGCATTCCATATGTTAGATAGCAGTTCCATTGGG-3′ primers (DNA Tehnology A/S (Aarhus, Denmark)) to facilitate subcloning. PCR was run using 1 μl of the first strand cDNA and the Expand Long Template PCR System with buffer 3 (Roche Diagnostics GmbH). The PCR product was purified and cloned into the pET11a vector (Stratagene) *Nde*I site for expression in BL21(DE3) cells. *LOX domain*— The *Nde*I site was added at the 5′ end of an upstream primer (P2), 5′-TTCCATATGCATCACCATCACAACGCTCGCTACAATAT-3′, and a downstream primer (P3) to facilitate subcloning. PCR was run using AOS-LOX in a pET11a vector as a template, *Pfu* DNA polymerase and the buffer (Promega), 200 μM each dNTP, and 0.4 μM each primer. The PCR product was purified and cloned into the *Nde*I site of pET11a vector for expression in BL21(DE3) cells. *AOS-domain*—The *Bam*HI site was added at the 5′ end of the upstream primer (P4), 5′-AGTGGATCCACGATGGTTTGGAAAAATTATGGC-3′, and the downstream primer (P5), 5′-CTCGGATCCTTAATGGTGATGGTGTCGAGCGTTCTGTCCGGC-3′, to facilitate subcloning. PCR was run using AOS-LOX in a pET11a vector as a template, DNA was amplified with the *Pfu* DNA polymerase, 200 μM each dNTP, and 0.4 μM each primer. The PCR product was purified and cloned into the *Bam*HI site of pET11a vector for expression in BL21(DE3) cells. For sequencing the plasmid DNA (initial clones in pGEM-T Easy vector (Promega)) was isolated using the QIAGEN plasmid purification system and full-length-sequenced using an ABI Prism 310 Genetic Analyser.

origin of other products, including 8*R*-HETE, has remained unclear. Whether the arctic coral uses a separate 8*R*-LOX or the 8*R*-LOX activity is part of an AOS-LOX fusion protein, will require further study. Both types of 8*R*-LOX have been shown to co-exist in *P. homomalla* [6,7].

In this study, we report the cloning and characterisation of a fusion protein responsible for 8*R*-hydroperoxyeicosatetraenoic acid (HPETE) and allene oxide synthesis in the coral *G. fruticosa*. Our data clearly indicate that the AOS-LOX fusion protein pathway in allene oxide synthesis is used by a different coral and *P. homomalla* represents no exception.

Using a RT-PCR strategy based on the highly conserved LOX sequences we cloned and sequenced a cDNA encoding for an AOS-LOX fusion protein. The cDNA reactions were run as described previously using the total coral RNA [14] and an oligo (dT) sequence linked to an adaptor sequence at the 5′-end. The initial PCR clone was obtained by using the strategy described previously [16]. The full-length cDNA clones were obtained using a RACE-PCR methodology. The extension of the sequence in the 5′ direction gave a longer product than usual for lipoxygenases, similar in length to the *P. homomalla* AOS-LOX fusion protein [7] (Fig. 1).

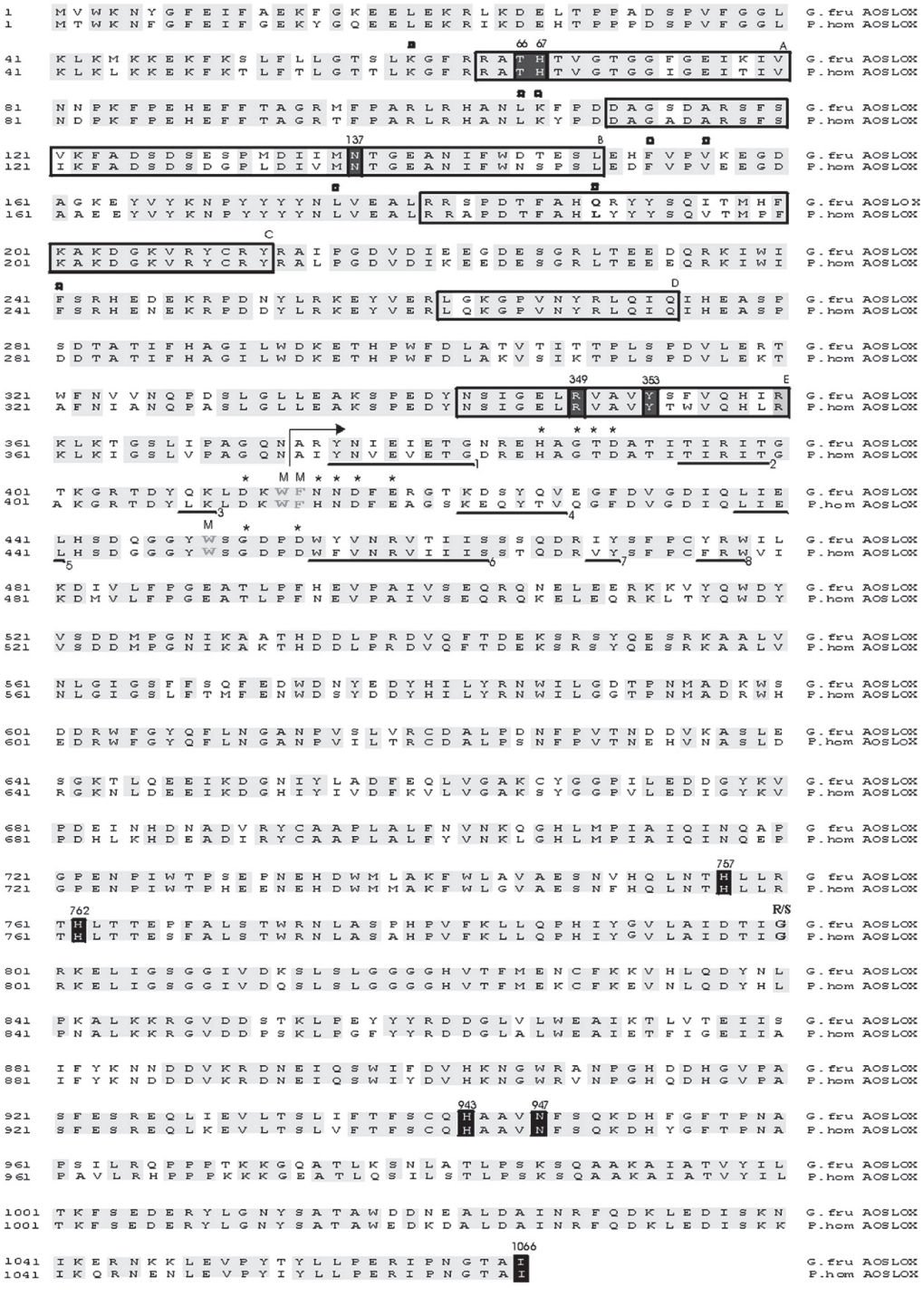
The full-length sequence of the *G. fruticosa* AOS-LOX mRNA is 3685 bp long, with 5′ UTR 321 bp and 3′ UTR 166 bp. The open reading frame region (3198 bp) consists of AOS- (1119 bp) and LOX- (2079 bp) related sequences. The border between the AOS and LOX domains (residue 374) was determined by an alignment of the *G. fruticosa* sequence with the *P. homomalla* AOS-LOX fusion protein sequence (Fig. 2). The predicted length of the fusion protein is 1066 amino acids with an estimated molecular mass of 122 kDa. The calculated molecular masses of LOX and AOS domains are 79 and 43 kDa, respectively.

The N-terminal AOS domain has five homologous regions with a catalase located in the correct sequential order along the polypeptide chain (Fig. 2). The threonine residue T66 shown to be responsible for the AOS activity in the *P. homomalla* AOS-LOX protein [17] is in the same position in the *G. fruticosa* AOS domain. The other AOS active site residues (H67, N137, R349, Y353) proposed by X-ray crystal study [10] are also present in the *G. fruticosa* AOS-LOX sequence (Fig. 2). The sequence identity compared to the *P. homomalla* AOS domain of the AOS-LOX protein is 84%. Except for the L190Q substitution between the AOS domains of the *P. homomalla* and *G. fruticosa* AOS-LOX, all the other residues (K60, L106, K107, F153, V156, L176, F241) proposed to be involved in the substrate binding are identical (Fig. 2) [10].

The C-terminal LOX domain shares the highest homology, 84%, with the LOX domain of the *P. homomalla* AOS-LOX protein. The sequence identity with the recently described *G. fruticosa* 11*R*-LOX [16] is about 70%. The deduced amino acid sequence alignment of lipoxygenases reveals a good conservation of amino acid residues shown to be important for the coordination of the non-heme iron (H757, H762, H943, N947, and I1066 via its C-terminus). Two tryptophanes, W413 and W449, along with a phenylalanine F414 associated to the membrane binding in the case of the *P. homomalla* AOS-LOX [11] are present in the LOX domain of the *G. fruticosa* AOS-LOX (Fig. 2).

Amino acids D391, D411, N416, D417 and E419, indicated to be involved in the calcium binding in the *P. homomalla* AOS-

Fig. 2. The alignment of *G. fruticosa* and *P. homomalla* AOS-LOX sequences using the Clustal program. The numbers represent the position of amino acid residues in the respective protein sequences. A light gray background designates identical residues; boxes marked with A, B, C, D, E—five sequence motifs of the catalase; boxed gray background — residues aligning with essential residues of the catalase active site; □—residues proposed to be involved in the substrate binding of *P. homomalla* AOS [10]; an arrow—a border between AOS and LOX domains; underlined regions—β strands of the LOX domain of the *P. homomalla* AOS-LOX; *—calcium binding residues in the LOX domain of *P. homomalla* AOS-LOX; M—membrane binding residues in the LOX domain of *P. homomalla* AOS-LOX [11]; a black background—residues implicated in the iron binding of lipoxygenases; R/S- a putative R/S determinant of lipoxygenases. The sequences used have the following GenBank accession numbers: *G. fruticosa* AOS-LOX, EU082210 and *P. homomalla* AOS-LOX, AF003692.


G.fru AOSLOX
P.hom AOSLOX
A
B
C
D
E
R/S

LOX [11] and human 5-LOX [18] are also conserved in the *G. fruticosa* 11*R*-LOX [16] and the LOX domain of the *G. fruticosa* AOS-LOX.

In the primary structure of the LOX domain of the *G. fruticosa* AOS-LOX, the residue to be found in the active site position equivalent to that reported as a determinant of *R* or *S* stereospecificity in lipoxygenases (glycine in *R*- and alanine in *S*-specific lipoxygenases) [19] is Gly800 (Fig. 2).

The amino acid sequence data were compared with entries in the GenBank™/EBI Data Bank using the BLAST search. The sequence homology was determined by the Clustal method using the Lasergene program (DNASTAR, Inc.). *P. homomalla* and *G. fruticosa* AOS-LOX sequences together with a related sequence from *Clavularia viridis* were used for the phylogenetic analysis. The *G. fruticosa* AOS-LOX sequence identity compared to its *P. homomalla* orthologue is 84%. It is even somewhat higher than the sequence identity between cyclooxygenase orthologues from the same corals that does not exceed 80%. The sequence homology compared to the putative AOS-LOX protein from *C. viridis* is 87% (GenBank accession number: AB188528). The higher sequence identity between the *G. fruticosa* and *C. viridis* AOS-LOX is in accordance with the coral origin tree in Comparative Toxicogenomics Database (http://ctd.mdibl.org) according to which *G. fruticosa* and *C. viridis* belong to the same Alcyonacea suborder while *P. homomalla* is a representative of Gorgonacea suborder.

To identify the products of the *G. fruticosa* AOS-LOX the enzyme was expressed in *E. coli* cells at 16 °C (Fig. 3, lane 2). The catalytic activity was recovered in the 13,000×*g* supernatant of sonicated cells. The supernatant was preincubated with $CaCl_2$ (the final concentration 10 mM) on ice for 5 min. The incubation with 50 μM arachidonic acid (AA) (Cayman Chemical Co.) was performed in 50 mM Tris–HCl buffer, pH 8.0, for 10 min at room temperature. To reduce the hydroperoxy products $SnCl_2$ was added. After acidification with HCl to pH 3, the products were extracted with ethyl acetate. The AOS-LOX fusion protein converted [1-^{14}C] AA into three products detected by RP-HPLC (Fig. 4, panel I, upper trace). In aqueous media allene oxide is non-enzymatically converted into an α-ketol (8-hydroxy, 9-keto-5Z,11Z,14Z-eicosatrienoic acid, as a major product (peak 1) and a minor cyclopentenone (peak 2). The formation of the α-ketol and cyclopentenone (retention times 10.4 and 11.3 min, respectively) is indicative of the allene oxide synthase activity of the *G. fruticosa* recombinant AOS-LOX. Peak 3 with the retention time of 17.2 s corresponds to 8-HETE, the known reduced product of the 8-lipoxygenase.

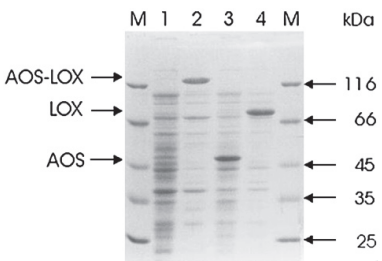


Fig. 3. The expression of the *G. fruticosa* fusion protein and the separate AOS and LOX domains in the *E. coli* strain BL21(DE3). SDS-PAGE (Coomassie Blue stained) of bacterial extracts expressing the fusion protein, the N-terminal AOS domain and the C-terminal LOX domain. The following IPTG induction cells were grown for 16–18h at 16 °C. M, molecular weight markers (Fermentas); 1, a negative control of expression (bacterial culture was transformed with a pET11a vector); 2, expression of AOS-LOX fusion protein; 3, expression of AOS domain separately; 4, expression of lipoxygenase domain separately.

The UV spectra for the same peaks 1–3 in the chromatogram obtained by a UV-detection (Fig. 4, panel I, lower trace) were recorded. Product 1 has no absorbance maxima above 210 nm. Product 2 shows λ_{max} at 218 nm, characteristic of a cyclic enone compound. Product 3 has λ_{max} at 236 nm characteristic of conjugated diene chromophores of HETEs (Fig. 4, panel II) [6].

LC-MS analysis was performed using an ion-trap mass spectrometer operating in a negative-ion mode. All AOS-LOX products gave strong signals corresponding to the deprotonated precursor ions $[M–H]^-$. The MS plot showed peaks at 10.4, 11.3, and 17.2 min (Fig. 4, panel III, trace A). The single ion monitoring at *m/z* values corresponding to the $[M–H]^-$ anions of the products predicted by UV spectra identified peak 1 as α-ketol (*m/z* 335.2), peak 2 as cyclopentenone (*m/z* 317.4), and peak 3 as 8-HETE (*m/z* 319.3) (Fig. 4, panel III, traces B–D).

To distinguish the allene oxide synthase and lipoxygenase activities of the fusion protein the LOX and AOS domains were expressed separately in *E. coli* BL21(DE3) cells (Fig. 3, lanes 3, 4). The expression of the C-terminal LOX domain at 16 °C gave a highly active enzyme with an activity recovered in the 200,000×*g* supernatant of sonicated cells. The supernatant was incubated with arachidonic acid under the same conditions as described above. The identity of the product as 8-HETE was first confirmed by an RP-HPLC/MS analysis (data not shown). To determine the stereochemistry of the lipoxygenase product, the sample was methylated and purified by SP-HPLC. The methyl ester derivative was subjected to the chiral-phase HPLC along with authentic methylated standards of ±8-HETE (±8-HETE-Me) and 8*R*-HETE (8*R*-HETE-Me). The product peak co-eluted with the peak of 8*R*-HETE-Me standard (Fig. 5C) indicating that the product was 8*R*-HETE.

The N-terminal AOS domain (amino acids 1–375) of the *G. fruticosa* fusion protein was expressed well at 16 °C (Fig. 3). In parallel, as a positive control the AOS domain of *P. homomalla* AOS-LOX fusion protein (amino acids 1–373) was expressed at identical conditions. The *G. fruticosa* AOS domain and AOS domain of *P. homomalla* AOS-LOX migrated in SDS-PAGE likewise. Both proteins migrated more slowly than predicted by the calculated molecular weight. The expressed *P. homomalla* AOS domain was fully active in incubations with 8*R*-HPETE as a substrate. In the incubations performed at identical conditions with the *G. fruticosa* AOS domain no AOS activity was recorded by neither radioactivity nor UV spectroscopy (data not shown). To be sure that the loss of activity is not due to the lack of heme, 1 μM of exogenous hemin was included in the incubations of *G. fruticosa* AOS. Yet, no AOS activity was detected (data not shown). Why the separately expressed AOS part of the *G. fruticosa* fusion protein was inactive remains to be elucidated in further studies.

In summary, the AOS-LOX fusion protein responsible for the allene oxide synthesis in *G. fruticosa* was cloned and

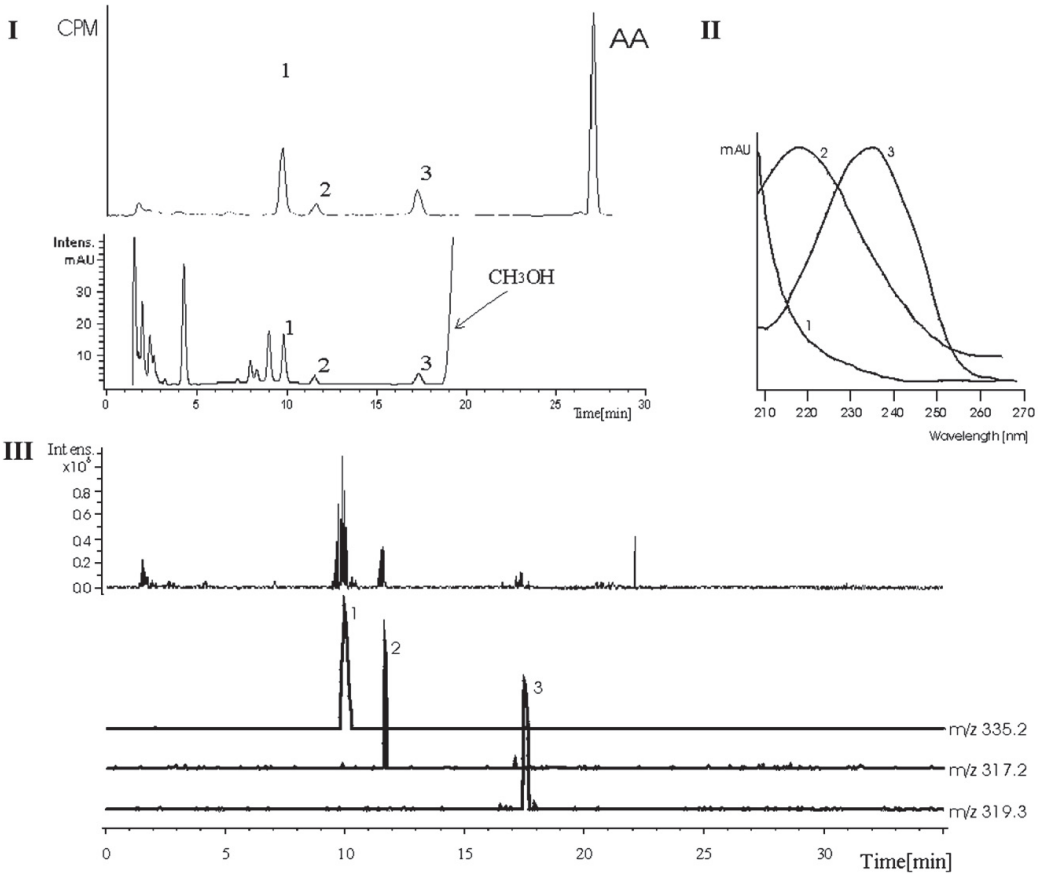


Fig. 4. Identification of the products formed from arachidonic acid (AA) in incubations with the *G. fruticosa* AOS-LOX fusion protein. Panel I: Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis of the products formed in incubation of [1-^{14}C]arachidonic acid with the *G. fruticosa* AOS-LOX. The frozen cell pellet (equal to 5 ml of the culture) was resuspended in 500 μl of 50 mM Tris–HCl, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and sonicated briefly (3×5 s) with a Cell Parmer TORBEO Ultrasonic cell disruptor. The cell suspension was centrifuged at 13,000×*g* for 20 min at 4 °C. The activity was recovered in the supernatant. In a standard assay the protein suspension was preincubated with $CaCl_2$ (the final concentration 10 mM) for 5 min on ice and suspended in 50 mM Tris–HCl (pH 8.0). The reaction was initiated by addition of 50 μM [1-^{14}C] AA and incubations were performed for 10 min at room temperature. The reaction was terminated with $SnCl_2$ (10 mM), acidified to pH 3 and the products were extracted with ethyl acetate. The extract was dried over Na_2SO_4, evaporated to dryness, dissolved in methanol:water (80:20) and analyzed by a RP-HPLC. There was no activity in bacteria expressing vector alone. The RP-HPLC analysis of the products was performed on a Zorbax Eclipse XDB-C18 column (3.5 μm silica, 2.1×150 mm, Agilent) with the solvent system of methanol:water:acetic acid (75:25:0.01 v/v/v) at a flow rate of 0.25 ml/min with an online detection of radioactivity using a Packard 500TR Series Flow Scintillation Analyzer (upper trace) and UV detection at 206 nm (lower trace). Panel II: the UV-spectra of major peaks. The numbers at the spectra correspond to the peak numbers on the chromatograms: 1, α-ketol, 2, cyclopentenone, and 3, 8-HETE. Panel III: the mass spectra of the products of the *G. fruticosa* AOS-LOX fusion protein with AA. The samples in MetOH: H_2O (8:2) were run as described above. The mass-spectrum was obtained by using a 6320 Ion Trap liquid chromatography- mass spectrometer (Agilent) operated in a negative mode. The data acquisition was performed by ±0.1 *m/z* units centered on each selected ion. Upper trace a reversed-phase LC-MS summation of all ion current transitions (total ion flow); trace 1, a single ion chromatogram for α-ketol $[M–H]^-$ at *m/z* ratio of 335.2; trace 2, a single ion chromatogram for cyclopentenone $[M–H]^-$ at *m/z* ratio of 317.4; trace 3, a single ion chromatogram for 8-HETE $[M–H]^-$ at *m/z* ratio of 319.3.

characterized. The expression of the separate LOX domain established the 8*R*-LOX activity of the C-terminal domain. The *G. fruticosa* AOS-LOX has a high sequence homology with the AOS-LOX fusion protein from *P. homomalla*. Our results illustrate that the AOS-LOX fusion protein pathway of the allene oxide synthesis is not limited to *P. homomalla* but is used by other corals.

Unfortunately, the detection of AOS-LOX related sequences and allene oxide synthesis in vitro in all three coral species did not answer the main issues of the biological role and metabolic fate of allene oxides in marine life. These coral species highly differ in the composition of endogenous eicosanoids as well as arachidonic acid metabolism in vitro. The Caribbean gorgonian *P. homomalla* is the richest known natural source of prostaglandins isolated mainly as 15-acetate methyl ester derivatives of prostaglandin A_2. At the same time, all attempts to detect prostaglandin synthesis from arachidonic acid by coral preparations in vitro have failed. The Hawaiian *C. viridis* does not contain prostaglandins but is

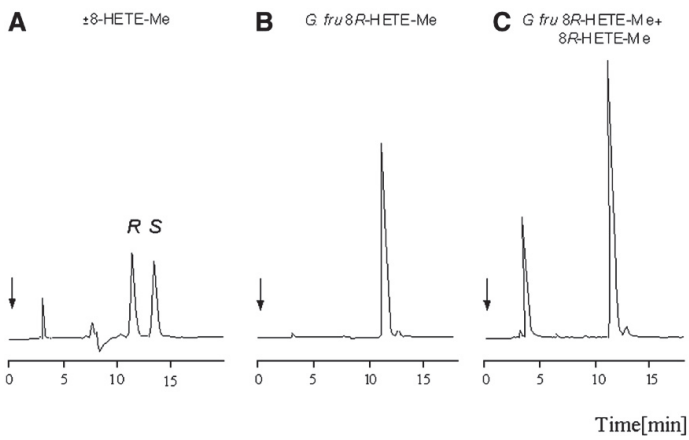


Fig. 5. Chiral-phase HPLC analyses of the product from the reaction of the arachidonic acid LOX domain of the *G. fruticosa* AOS-LOX. All samples were methylated with etheric diazomethane before analyses. (A) A ±8-HETE standard (Cayman Chemical Co.); (B) a product of the *G. fruticosa* LOX domain; C, a *G. fruticosa* LOX product coinjected with an 8*R*-HETE standard. The incubation of arachidonic acid with the *G. fruticosa* LOX domain was performed as described in the legend to Fig. 4 with one modification—0.5 mM $SnCl_2$ was present. The reaction was initiated by addition of 50 μM non-labeled arachidonic acid. The incubation was performed for 10 min at room temperature. The reaction mixture was acidified to pH 3 and the product was extracted with ethyl acetate. 1 ml of cell culture converted 150 nmol of arachidonic acid to 8*R*-HETE. The LOX product was purified by SP-HPLC on a Phenomenex Zorbax-Sil column (7 μm silica, 25 × 0.46 cm), with a solvent system of *n*-hexane:isopropyl alcohol (100:1.7, v/v) at a flow rate of 2 ml/min with the UV detection at 234 nm). The methylated reaction product was co-cromatographed with an authentic standard on a Chiralcel OD-H column (25 × 0.46 cm) using a solvent system of hexane:isopropanol (100:2, v/v) at a flow rate of 1 ml/min and with UV detection at 234 nm.

famous due to its high content of another group of cytotoxic cyclic eicosanoids, clavulones. However, the formation of prostaglandins or clavulones has never been observed in vitro incubations with arachidonic acid. The arctic soft coral *G. fruticosa* represents a third variant. The coral extracts contain typical mammalian prostaglandins that are all easily synthesized in vitro [20].

The initial presumption that allene oxides in coral may serve as intermediates in the prostaglandin synthesis has been rebutted by the cloning and expression of functional coral cyclooxygenases in *G. fruticosa* [14] and *P. homomalla* [2]. Another speculation that the synthesis of clavulones in *C. viridis* may occur through allene oxide intermediates, although not proved experimentally, remains quite likely. But irrespective of the route to clavulones, what is the metabolic fate of allene oxides in other corals? The presence of α-ketol, the main compound formed from allene oxide by non-enzymatic hydrolysis in vitro incubations, has never been detected in fresh *G. fruticosa* extracts. Moreover, there is no evidence of the presence of any endogenous allene oxide-originated products. There is a hypothesis that unstable allene oxides do not serve as intermediates of biologically active compounds but these short-lived compounds themselves may act as signalling molecules through the corresponding receptors [21]. However, the high content of active AOS-LOX fusion protein as well as the corresponding gene transcript contravenes this hypothesis. Thus, the biological role and mode of action of allene oxides in coral remain to be elucidated.

Acknowledgements

We thank Dr. A.R. Brash for the AOS domain construct of *P. homomalla* AOS-LOX. This work was financially supported by Estonian Science Foundation Grants 6911 (to Nigulas Samel); 6831 (to Külliki Varvas) and 6197 (to Ivar Järving).

References

[1] M. Hamberg, Fatty acid allene oxides, J. Am. Oil Chem. Soc. 66 (1989) 1445–1449.
[2] M. Hamberg, H.W. Gardner, Oxylipin pathway to jasmonates: biochemistry and biological significance, Biochim. Biophys. Acta 1165 (1992) 1–18.
[3] W.C. Song, C.D. Funk, A.R. Brash, Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 8519–8523.
[4] A.R. Brash, M.A. Hughes, D.J. Hawkins, W.E. Boeglin, W.-C. Song, L. Meijer, Allene oxide and aldehyde biosynthesis in starfish oocytes, J. Biol. Chem. 266 (1991) 22926–22931.
[5] E.J. Corey, M. d'Alarcao, S.P.T. Matsuda, P.T. Lansbury, Intermediacy of 8-R-HPETE in the conversion of arachidonic acid to pre-clavulone A by *Clavularia viridis*. Implication for the biosynthesis of marine prostanoids, J. Am. Chem. Soc. 109 (1987) 289–290.
[6] A.R. Brash, S.W. Baertschi, C.D. Ingram, T.M. Harris, On non-cyclooxygenase prostaglandin synthesis in the sea whip coral, *Plexaura homomalla*: an 8(R)-lipoxygenase pathway leads to formation of an alpha-ketol and racemic prostanoid, J. Biol. Chem. 262 (1987) 15829–15839.
[7] R. Koljak, O. Boutaud, B.-H. Shieh, N. Samel, A.R. Brash, Identification of a naturally occurring peroxidase-lipoxygenase fusion protein, Science 277 (1997) 1994–1996.
[8] O. Boutaud, A.R. Brash, Purification and catalytic activities of the two domains of the allene oxide synthase-lipoxygenase fusion protein of the coral *Plexaura homomalla*, J. Biol. Chem. 274 (1999) 33764–33770.
[9] B.D. Abraham, M. Sono, O. Boutaud, A. Shriner, J.H. Dawson, A.R. Brash, B.J. Gaffney, Characterization of the coral allene oxide synthase active site with UV- visible absorption, magnetic circular dichroism, and electron paramagneticresonance spectroscopy: evidence for tyrosinate ligation to the ferric enzyme heme iron, Biochemistry 40 (7) (2001) 2251–2259.

[10] M.L. Oldham, A.R. Brash, M.E. Newcomer, The structure of coral allene oxide synthase reveals a catalase adapted for metabolism of a fatty acid hydroperoxide, Proc. Natl. Acad. Sci. U. S. A. 102 (2) (2005) 297–302.
[11] M.L. Oldham, A.R. Brash, M.E. Newcomer, Insights from the X-ray crystal structure of coral 8R-lipoxygenase: calcium activation via a C2-like domain and a structural basis of product chirality, J. Biol. Chem. 280 (2005) 39545–39552.
[12] K. Varvas, I. Järving, R. Koljak, K. Valmsen, A.R. Brash, N. Samel, Evidence of a cyclooxygenase-related prostaglandin synthesis in coral. The allene oxide pathway is not involved in prostaglandin biosynthesis, J. Biol. Chem. 274 (1999) 9923–9929.
[13] K. Varvas, I. Järving, R. Koljak, A. Vahemets, T. Pehk, A.-M. Müürisepp, Ü. Lille, N. Samel, In vitro biosynthesis of prostaglandins in the white sea soft coral *Gersemia fruticosa*: Formation of optically active PGD_2, PGE_2, $PGF_{2\alpha}$ and 15-keto-PGF_{α} from arachidonic acid, Tetrahedron Lett. 34 (1993) 3643–3646.
[14] R. Koljak, I. Järving, R. Kurg, W.E. Boeglin, K. Varvas, K. Valmsen, M. Ustav, A.R. Brash, N. Samel, The basis of prostaglandin synthesis in coral: molecular cloning and expression of a cyclooxygenase from the Arctic soft coral *Gersemia fruticosa*, J. Biol. Chem. 276 (2001) 7033–7040.
[15] R. Järving, I. Järving, R. Kurg, A.R. Brash, N. Samel, On the evolutionary origin of cyclooxygenase (COX) isozymes: characterization of marine invertebrate COX genes points to independent duplication events in vertebrate and invertebrate lineages, J. Biol. Chem. 279 (2004) 13624–13633.
[16] M. Mortimer, R. Järving, A.R. Brash, N. Samel, I. Järving, Identification and characterization of an arachidonate 11R- lipoxygenase, Arch. Biochem. Biophys. 445 (2006) 147–155.
[17] T. Tosha, T. Uchida, A.R. Brash, T. Kitagawa, On the relationship of coral allene oxide synthase to catalase. A single active site mutation that induces catalase activity in coral allene oxide synthase, J. Biol. Chem. 281 (2006) 12610–12617.
[18] T. Hammarberg, P. Provost, B. Persson, O. Rådmark, The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity, J. Biol. Chem. 275 (2000) 38787–38793.
[19] G. Coffa, A.B. Brash, A single active site residue directs oxygenation stereospecificity in lipoxygenases: stereocontrol is linked to the position of oxygenation, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 15579–15584.
[20] K. Varvas, R. Koljak, I. Järving, T. Pehk, N. Samel, Endoperoxide pathway in prostaglandin biosynthesis in the soft coral *Gersemia fruticosa*, Tetrahedron Lett. 35 (1994) 8267–8270.
[21] N. Tijet, A.B Brash, Allene oxide synthases and allene oxides, Prostaglandins Other Lipid Mediat. 68–69 (2002) 423–431.

PUBLICATION II

Lõhelaid, H., Teder, T., Tõldsepp, K., Ekins, M., Samel, N. (2014). ”Up-regulated expression of AOS-LOXa and increased eicosanoid synthesis in response to coral wounding.” PLoS ONE 9(2): e89215

OPEN ACCESS Freely available online

PLOS ONE

Up-Regulated Expression of *AOS-LOXa* and Increased Eicosanoid Synthesis in Response to Coral Wounding

Helike Lõhelaid[1], Tarvi Teder[1], Kadri Tõldsepp[1¤], Merrick Ekins[2], Nigulas Samel[1]*

1 Department of Chemistry, Tallinn University of Technology, Tallinn, Estonia, **2** Sessile Marine Invertebrates, Queensland Museum, Brisbane, Queensland, Australia

Abstract

In octocorals, a catalase–like allene oxide synthase (AOS) and an 8*R*-lipoxygenase (LOX) gene are fused together encoding for a single AOS-LOX fusion protein. Although the AOS-LOX pathway is central to the arachidonate metabolism in corals, its biological function in coral homeostasis is unclear. Using an acute incision wound model in the soft coral *Capnella imbricata*, we here test whether LOX pathway, similar to its role in plants, can contribute to the coral damage response and regeneration. Analysis of metabolites formed from exogenous arachidonate before and after fixed time intervals following wounding indicated a significant increase in AOS-LOX activity in response to mechanical injury. Two AOS-LOX isoforms, *AOS-LOXa* and *AOS-LOXb,* were cloned and expressed in bacterial expression system as active fusion proteins. Transcription levels of corresponding genes were measured in normal and stressed coral by qPCR. After wounding, *AOS-LOXa* was markedly up-regulated in both, the tissue adjacent to the incision and distal parts of a coral colony (with the maximum reached at 1 h and 6 h post wounding, respectively), while *AOS-LOXb* was stable. According to mRNA expression analysis, combined with detection of eicosanoid product formation for the first time, the *AOS-LOX* was identified as an early stress response gene which is induced by mechanical injury in coral.

Citation: Lõhelaid H, Teder T, Tõldsepp K, Ekins M, Samel N (2014) Up-Regulated Expression of *AOS-LOXa* and Increased Eicosanoid Synthesis in Response to Coral Wounding. PLoS ONE 9(2): e89215. doi:10.1371/journal.pone.0089215

Editor: Douglas Andrew Campbell, Mount Allison University, Canada

Received September 19, 2013; **Accepted** January 17, 2014; **Published** February 14, 2014

Copyright: © 2014 Lõhelaid et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Estonian Science Foundation Grant 9410 and the Estonian Ministry of Education and Research Grant 0140010s08 (both to NS). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: nigulas.samel@ttu.ee

¤ Current address: Institute of Technology, University of Tartu, Tartu, Estonia

Introduction

Octocorals and hexacorals are affected by natural and human-induced stress factors [1]. Biological and chemical alterations, including changes in temperature, sedimentation, water conditions and biotic alterations all represent challenges to octocorals survival. Mechanical injury is also a major challenge to coral integrity and viability [2]. It is likely that each type of stress leads to an adaptation or repair response, aiming to re-establish homeostasis and survival [3].

For example, altered temperature (such as heat shock) leads to changes in coral heat stress responsive genes and elevated calcium levels [4]. It also results in elevated levels of heat shock proteins, reactive oxygen species, Ca^{2+} signaling and protein synthesis [5,6]. The identification of indicator pathways is relevant for the monitoring and prediction of environmental stress conditions in coral [7]. While coral response to stress has been well studied in reef-building corals (hexacorals) [4–7], the stress-response of soft corals (octocorals) remains largely elusive [8].

In both vertebrates and invertebrates, similar phases of wound healing (1- inflammation, 2- proliferation, and 3- matrix rebuilding and remodeling) have been described [9,10]. However, the coral wound response and wound-related stress have received little attention [11], most research has concentrated on the response at the tissue level [12–16].

In vertebrates and plants, oxylipins are important stress mediators. In mammals, eicosanoids (hydroperoxyeicosatetraenoic acids (HpETEs), leukotrienes, thromboxanes and prostaglandins) result from the conversion of arachidonic acid (AA) by lipoxygenase (LOX) and cyclooxygenase (COX) [17,18]. Due to their labile nature, these messengers act locally, in an auto- or paracrine manner, as part of inflammatory responses by immune cell activation during infection or anaphylaxis [19,20]. In plants, the conversion of α-linolenic acid by the LOX and allene oxide synthase (AOS) pathway results in 12-oxo-phytodienoic acid and jasmonic acid (JA), which regulate the expression of defense genes [21–24].

Soft corals expresses multiple eicosanoid biosynthesis pathways, including COX, LOX and allene oxide synthase- lipoxygenase (AOS-LOX) enzymes [25–32]. The unique AOS-LOX fusion protein catalyzes the formation of unstable allene oxide from AA via 8*R*-HpETE, a pathway common among octocorals [32]. Bioinformatics also indicate, that catalase like *AOS-LOX* is present in all cnidarian lineages (*Hydra*, *Acropora*, *Nematostella*) [33]. As related species often share similar metabolic routes, the data on biological role of eicosanoids in soft coral may be attributed to other *Cnidarian* lineages.

Whereas the spectrum of metabolites is largely known, the functional significance of AOS and LOX pathways in coral homeostasis and regeneration remains elusive. The current literature of coral eicosanoids contains data on the identification of naturally occurring compounds [27,34–36], the elucidation of metabolic pathways involved in their synthesis [26,28,29,37,38] and the effects of lipid extracts or isolated compounds on other

systems [39]. To date, only the role of prostaglandins in the chemical defense of the coral *Plexaura homomalla* has been revealed [40–43]. There is no data available about the function of eicosanoids produced via the AOS-LOX pathway in corals.

In the current study, using the model of *Capnella imbricata*, which is easily propagated and farmed in a laboratory marine aquarium, we address gene expression and eicosanoid synthesis through the AOS-LOX pathway in response to acute incision wounding of coral.

Materials and Methods

Coral Samples

Colonies of soft coral *C. imbricata* (*Cnidaria*, *Anthozoa*, *Octocorallia*, *Alcyonacea*, *Nephtheidae*) were purchased from a commercial source (Estonia), identified at Queensland Museum (specimen No: QM G317136), cultivated and propagated in a closed-circuit marine aquarium in the Department of Chemistry at Tallinn University of Technology at an ambient seawater temperature of 23±0.5°C, salinity 31 ppt, periodic day-night cycle (12 h–12 h) and 20% of biweekly water exchange.

Activity Assay

In a standard assay, the coral tissue (0.33 g mL^{-1}) was homogenized (Tissue Tearor, set 5) in 50 mM Tris-HCl pH 8.0 buffer, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) on ice. Immediately, an aliquot of homogenate (6.6 mg) was incubated with 50 μM [1-^{14}C] AA (GE Healthcare) in 1 mL (final volume) 50 mM Tris-HCl, 100 mM NaCl and 1 mM $CaCl_2$ pH 8.0 at room temperature, with constant stirring for 5 min. Incubation in the presence of a mild reducing agent (0.5 mM $SnCl_2$) was conducted in parallel. Reactions were terminated with $SnCl_2$ (10 mM) and, after acidification with HCl to pH 4.0 the products were extracted with ethyl acetate. The extract was dried over Na_2SO_4, evaporated to dryness and re-dissolved in methanol: water (4:1) for instant product analysis by reverse phase-high performance liquid chromatography (RP-HPLC).

RP-HPLC

Samples were analyzed by RP-HPLC, using a Zorbax Eclipse XDB-C_{18} column (5 μm, 4.6×150 mm), thermostat 35°C, run on an Agilent 1200 Series HPLC system, connected to a diode array detector (UV detection at 206 nm, 236 nm and 270 nm), followed by a 500TR Series Flow Scintillation Analyzer (Packard Bioscience) or Agilent 6540 UHD Accurate Quadrupole time of flight - MS/MS with Agilent Jet Stream ESI source. The HPLC was carried out with a solvent system of acetonitrile (ACN)/water/formic acid (98.9%/1.0%/0.1% v/v/v)(A) and water/formic acid (99.9/0.1% v/v)(B), 0–8 min isocratic (35% A:65% B), 9–17 min gradient to 100% A, 18–30 min 100% A at a flow rate of 1 mL min^{-1}. Mass spectra were acquired over a mass range of m/z 100–400 in a negative ion detection mode. Extracted ion current (EIC) was used for sensitive and specific detection of stable end products. The data acquisition was performed by ±0.1 m/z units centered on each selected ion.

Extraction of Total RNA and Synthesis of cDNA

For homology based RT-PCR. Total RNA was extracted from coral tissue using the phenol-chloroform extraction method [44]. First strand cDNA was synthesized from 20 μg of total RNA, using an oligo(dT)-adapter primer [ATGAATTCGG-TACCCGGGATCC$(T)_{17}$] for priming and M-MLV reverse transcriptase (Promega) according to the manufacturer's protocol.

For real-time quantitative PCR (qPCR). 100±2 mg of coral tissue was homogenized (IKA T18 basic ULTRA TURRAX) in a QIAzol Lysis Reagent. RNA was isolated using an RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. Isolated RNA was quantified with NanoDrop-3000 (Thermo Scientific), and the integrity was constantly confirmed by electrophoresis on 1% formaldehyde agarose gels. 1 μg of total RNA was treated with DNase I and used as a template in cDNA synthesis (QuantiTect Reverse Transcription Kit, Qiagen) with oligo(dT)primer in a total reaction volume of 20 μL. Negative controls without reverse transcriptase were included to test for genomic DNA contamination and the efficiency of cDNA synthesis.

Homology Based RT-PCR

The upstream degenerative primers were based on the conserved regions of coral AOS-LOX sequences HEFF and HPW (located on the AOS domain), and the downstream degenerative primers were based on QIQ and AGT (located on the AOS and LOX part of the AOS-LOX sequence, respectively). The first round PCR was run using 1 μL of first strand cDNA and the Expand Long Template PCR System with buffer 3 (Roche Diagnostics), 0.2 mM of each dNTP, and 0.3 μM primers (Table 1A). The PCR program was 1 cycle at 94°C for 2 min; 10 cycles at 93°C for 30 s, 52°C for 45 s, 68°C for 3 min; 20 cycles at 93°C for 30 s, 55°C for 45 s; 68°C for 3 min and 20 s for each cycle, and 68°C for 10 min. The half-nested second round PCR was run using 1 μL of 10 times diluted first round PCR reaction according to the same protocol. HEFF/QIQ; HEFF/AGT and HPW/AGT resulted in expected amplicon sizes (accordingly 586 bp, 981 bp and 300 bp). The PCR products were cloned (pGEM-T Easy Vector Systems, Promega) and sequenced (Agowa, Germany). Two different sequences homologous to coral *AOS-LOX*s were confirmed by the public BLAST platform at NCBI. Sequence alignments were created using the MegAlign program (DNAStar, Lasergene) with ClustalW. The 5′- and 3′-ends were extended using 5′-3′ RACE-PCR methodology (Promega), according to the manufacturer's instructions and sequence specific primers (Table 1B). The open reading frames coding full length *AOS-LOXs* were PCR amplified with specific primers (Table 1B) and Phusion High Fidelity DNA polymerase (Thermo Scientific). The PCR products were cloned, sequenced and submitted to a database (GenBank accession numbers: KF000373 and KF000374).

Bacterial Expression and Activity

The ORF of *AOS-LOXa* and *AOS-LOXb* fusion proteins were PCR amplified with specific primers (Table 1B), cloned into *Nhe*I or *Bam*HI restriction site (respectively) of pET11a expression vector (Stratagene) and expressed in *Escherichia coli* BL21(DE3)RP cells (Novagen) at 10°C as previously described [32]. Bacterial extracts expressing the fusion protein were separated on 10% SDS-PAGE (Coomassie Blue stained). The fusion proteins (corresponding to the 1 mL sonicated cell culture) were incubated with 50 μM [1-^{14}C] AA in 1 mL, and analyzed as described above.

Design of Wounding Experiments

Repeated wounding (one colony). A coral colony was injured at the stem by a cut (0.5×5 mm), and 7–8 cm branches of the same colony were cut away at different times: zero (I branch), 1 h (II branch), 3 h (III branch) and 6 h (IV branch) (Fig. 1). A tissue sample (adjacent to the cutting edge) was taken, weighted, homogenized, and RNA was extracted. The remaining branch

Table 1. List of primers: (a) degenerative primers used for isolation of the target genes, (b) primers used for 5′-3′ RACE and (c) qPCR primers used for gene expression analysis.

(a) Degenerative primers	
HEFF-up	CCTAAGTTYCCNGARCAYGARTTYTT
HPW-up	TGGGATAARGARACNCAYCCNTGG
QIQ-down	CTAGCYTCRTGDATYTGDATYTG
WDK-down	CCATGGRTGNGTYTCYTTRTCCCA
AGT-down	GTAATAGTNGCRTCNGTNCCNGC
(b) Specific primers	
5′ RACE	
FPV-down	TTGCATGACGTAATCTTACAGG
FWHT-down	AGTCTTCCAAGCTCGAAGTGTGC
FWNT-down	AGTCTTCAAAGCTTGAGGTGTTCC
GSD-down	AGAACGATCTAGCATCAGACCC
KYPD-down	CAGCACCTGCATCATCTGGATAC
LKLL-down	CCCTGCATCATCCAGTAGTTTAAG
3′ RACE	
DYHL-up	AAAGTTAACCTGCAAGACTATCATC
EESG-up	ATATCAAAGAGGAAGAAGAGAGTGG
ESLG-up	TCAACCAGCCGGAATCATTAGG
FAVS-up	ACAACTGAATCATTTGCTGTGTCG
FSRY-up	AAATATTTGGACATTCAGTCGTTATG
KTHG-up	AATATCAAGGCTAAAACACACGG
LGDT-up	CTGGATACTTGGTGATACGCC
QNAL-up	TTCCAACAGGACAGAACGCAC
RSRH-up	AACGTCTGGATTCGTAGTCGTCATC
YKWI-up	TGTGGCAGTGTACAAATGGATCC
AWED-up	AGCGCTACAGCTTGGGAGG
ERIP-up	TTCTTCCTGAGCGTATTCCC
***AOS-LOXa* ORF**	
ALA-up	ATCGGATCCATGACTTGGAAAAATTTTGGA
ALA-down	GTTCGGATCCCCGGGACATTAGATAGCAGTTCC
***AOS-LOXb* ORF**	
ALB-up	ATTGCTAGCATGGTTTGGAAAAATTTTGGTTACG
ALB-down	CAGCTAGCCTAGATTGCAGTTCCG

(c) Specific qPCR primers

Gene	*Primer*	*Sequence*	*Amplicon size*
AOS-LOXa	FSRY-up	AATATTTGGACATTCAGTCGTTATG	100 bp
	LKKG-down	CGATAGTTTACTGGGCCTTTCTTC	
AOS-LOXb	KLLD-up	CGTCATGCAAATCTTAAACTACTGG	180 bp
	VSSS-down	AGACTCTCCTGCACTTGATGATAC	
β-actin	HETC-up	TGTGGCATCCATGAGACCTG	95 bp
	TVLS-down	AGACAGCACTGTGTTGGCATAC	

Up - forward primer, down - reverse primer; all sequences are presented in the 5′ to 3′ direction.

doi:10.1371/journal.pone.0089215.t001

was additionally incubated until the next time point (1 h), resulting in a secondary incubation time of (0+1 h) (Fig. 1). The same scheme was repeated with branches II and III, and they were additionally incubated (for 2 and 3 h, respectively), marked as 1+ 2 h and 3+3 h (Fig. 1).

For the detection of a systemic response, transcript levels were estimated for the distal tips of the removed branches at given times (1 h, 3 h and 6 h), and they were compared to that of the time of wounding (control).

Single wounding (separate colonies). To obtain genetically identical coral, an adult coral colony was cut into six equal fragments and grown for 4 months to reduce the influence of fragmentation. A branch was removed from the stems of three coral colonies in parallel, serving the wounding event and used for the detection of the normal transcript level for each colony (control). The second sample adjacent to the wounding site was taken from the first to the third coral colony at 1 h, 3 h and 6 h post wounding.

qPCR and Gene Expression Analysis

β-actin was one of the three most stable genes identified in the cnidarian case study [45]. Corresponding sequence from *C. imbricata* was cloned, sequenced and used as the reference gene. Sequence specific quantitative real-time PCR primers (Table 1C) were designed on the *β-actin*, *AOS-LOXa* and *AOS-LOXb* genes by using PrimerSelect software (DNASTAR, Lasergene). Test-PCRs confirmed the specific amplification of the desired amplicons (95–180 bp). All sequenced qPCR products matched the expected product identities. First strand cDNA aliquots (1 µL) of each sample served as templates for a quantitative PCR reaction (total volume 10 µL) containing sequence-specific primers (500 nM) and a master mix (QuantiTect SYBR Green PCR Kit, Qiagen). Thermal cycling was performed on a *LightCycler* 480 Real-Time PCR system (Roche), under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 55°C for 20 s and 72°C for 20 s. To confirm the amplification of a single PCR product, a melting curve analysis (from 52°C to 95°C) was carried out after the end of the amplification cycle. All qPCR reactions were performed in triplicate, and negative controls consisting of un-transcribed RNA (no RT control) were performed for each RNA extraction. The normal expression levels were estimated in the "stem" and "branch" tissue of three different coral colonies (n = 3). The initial and single wound response data are the means of values obtained for the two independent biological replicates (n = 2) and the secondary and systemic response data are the means of three replicates (n = 3).

Statistical analysis. The expression ratio between the sample and control was determined using the comparative C_t method [46] by program REST - Relative Expression Software Tool-Multiple Condition Solver (REST-MCS) [47] (LightCycler Relative Quantification Software, Qiagen) with 2000 iterations. The results are presented as the mean ± standard error (SE).

Results

In *C. imbricata*, AA is an abundant polyunsaturated fatty acid, exceeding 20% of total fatty acids (unpublished data). The presence of endogenous eicosanoids of the AOS-LOX pathway, 8-HETE ($[M^-] = 319.2$), α-ketol ($[M^-] = 335.2$) and cyclopentenone ($[M^-] = 317.2$) in *C. imbricata* was confirmed by RP-HPLC/MSMS analysis (Fig. 2A, EIC). The identification of the compounds was based on identical retention times and mass-spectra with *G. fruticosa* AOS-LOX products as standards [32].

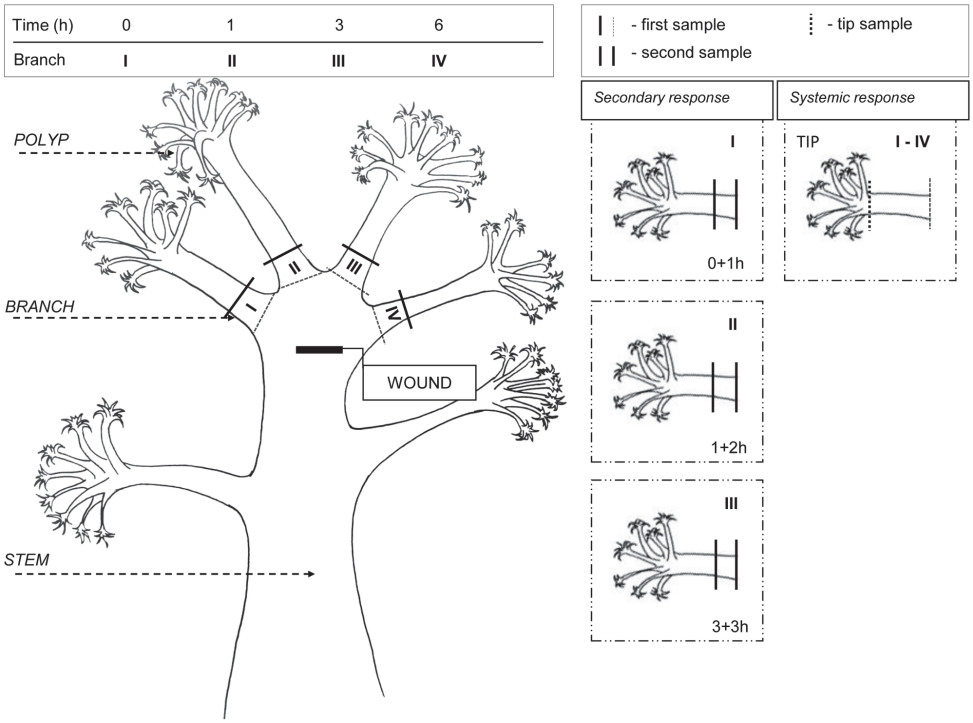


Figure 1. Experimental design of wounding stress. The location and time of sampling shown on a coral colony.
doi:10.1371/journal.pone.0089215.g001

Arachidonic Acid Metabolism in Response to Wounding

The metabolites of AA formed by *C. imbricata* were detected at the time of wounding and in response to incision (Fig. 1). The formation of 8-HETE, α-ketol, and cyclopentenone during the incubation of the coral tissue homogenate with radiolabeled AA confirmed the catalytic activity of AOS-LOX (Fig. 2A, peaks 2 and 3). In the presence of the reducing agent $SnCl_2$, the AOS reaction was avoided and the reduced intermediate 8-HETE was detected as the main product (data not shown). Besides the known products of the AOS-LOX pathway, the formation of additional compounds was detected (Fig. 2A, peak 1). As identification of these metabolites was out of the scope of the current research, two peaks visible on the radio chromatogram were not separated and the radioactivity was summarized. In the selected time frame, the level of α-ketol (Fig. 2A, peak 2) increased in response to wounding (in total 27%, Fig. 2B); at the same time the amount of unidentified polar compounds (UPC) (Fig. 2, peak 1) decreased equally (Fig. 2B).

Sequence Analysis of *AOS-LOX*

The cloning and sequencing of *C. imbricata AOS-LOX* cDNAs resulted in two complete *AOS-LOX* sequences of encoding ORFs, along with the 5′- and 3′-UTRs, designated as *AOS-LOXa* (NCBI ID: KF000373) and *AOS-LOXb* (NCBI ID: KF000374). BLASTp analysis revealed high sequence homology with other cnidarian *AOS-LOX*s. The identity to other coral *AOS-LOX*s *(G. fruticosa* GenBank *accession number* EU082210.1; *Clavularia viridis* AB188528.1; *Plexaura homomalla* AF003692.1) was between 81–87%. The sequence identity between *C. imbricata AOS-LOXa* and *b* (88%) was lower than between the corresponding *G. fruticosa* paralogs (98%, personal data).

Based on the sequence analysis of the coral AOS-LOX fusion proteins, all catalytically important amino acids of LOX domains are conserved. In the primary structure of the LOX domain of AOS-LOXa and AOS-LOXb, the active site residue equivalent to that reported to be determinant of *R* or *S* stereospecificity of LOXs (glycine in *R*- and alanine in *S*-specific LOXs) [48] is G800 and G801, respectively. Thus the LOX domains of AOS-LOX fusion proteins convert the substrate fatty acid (AA) into 8*R*-HpETE.

Except for a conservative L150F substitution in AOS-LOXb, all catalytically important amino acids (H67, N147, L150; R345, Y349) of the AOS domains of *C. imbricata* are conserved. On the other hand, in the substrate binding pocket of the AOS domain of AOS-LOXb, the substitution of phenyl alanine 150 (as in catalase) instead of leucine (as in other coral AOS-LOX fusion proteins) and additional amino acid substitutions (K60E; F90Y, V156S; L176/177S), including one amino acid insertion (S161), were detected.

To confirm their catalytic activity, the ORFs of the *AOS-LOX* fusion proteins were expressed in bacterial expression system, resulting in fusion proteins with expected size, 122.0 kDa for AOS-LOXa and 122.3 kDa for AOS-LOXb (Fig. 3, lanes ALa and ALb, respectively). The catalytic activity of AOS-LOXa was recovered in the 13000×g supernatant of sonicated cells with

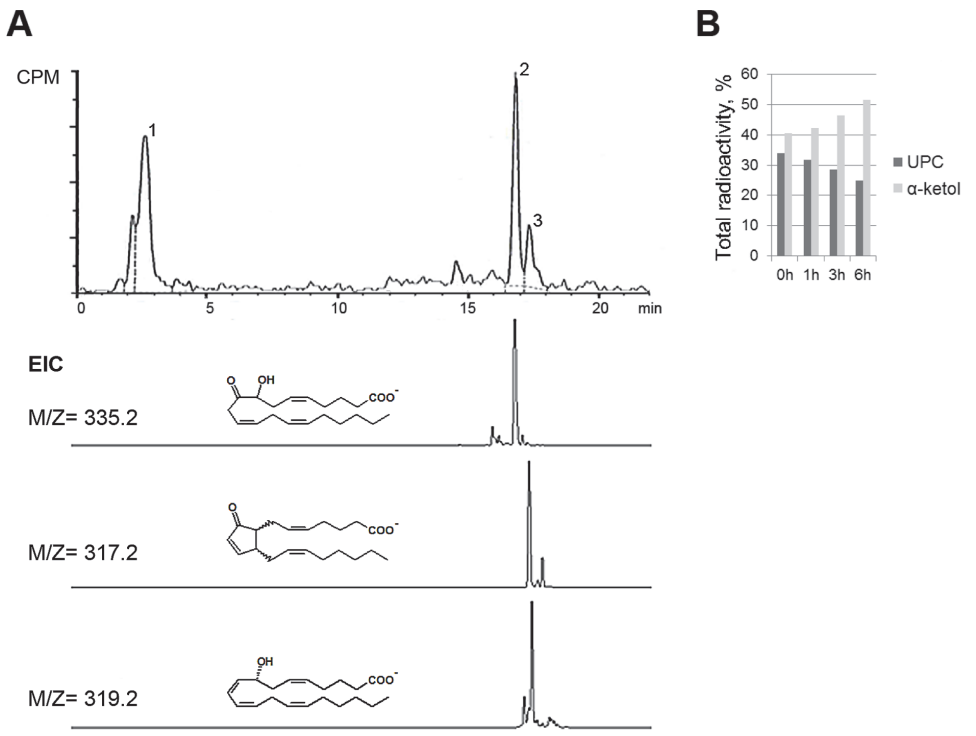


Figure 2. RP-HPLC analysis of incubation products of C. *imbricata* tissue homogenate. A) Radio chromatogram of the products formed from [1^{-14}C] AA by coral homogenate, extracted ion current (EIC) corresponding to α-ketol ([M$^-$] = 335.2, peak 2), cyclopentenone ([M$^-$] = 317.2, peak 3) and HETE ([M$^-$] = 319.2, peak 3). B) The conversion of [1^{-14}C] AA into unidentified polar compounds (UPC) (peak 1) and α-ketol (peak 2) in response to wounding. CPM - counts per minute.
doi:10.1371/journal.pone.0089215.g002

[1-^{14}C] AA as the substrate. The formation of α-ketol (70% of total radioactivity) and cyclopentenone (14%) by AOS-LOXa was confirmed by RP-HPLC (Fig. 3, peaks 2 and 3, respectively). Accordingly, the AOS-LOXa product profile is identical to that of previously characterized fusion proteins [28,32]. The main products formed from AA by AOS-LOXb (90% of total radioactivity) (Fig. 3, peak 1) co-eluted with the UPC (Fig. 2, peak 1). In the presence of mild reducing agent the LOX domains of both fusion proteins converted AA exclusively into 8-HETE. As incubation of AOS-LOXb with 8*R*- HpETE resulted in identical product profile compared to incubations with AA (data not shown), the novel activity can be attributed to the AOS domain of AOS-LOXb. Based on chromatographic behavior and mass-spectral data of AOS-LOXb products, we suggest the formation of an oxo-octenoic acid ([M$^-$] = 155.1) from 8-HpETE.

Accordingly, both coral *AOS-LOX* genes encode for functional fusion proteins. While AOS-LOXa is the main source of the known compounds of AOS-LOX pathway, AOS-LOXb exhibits a novel activity. The identification of the products of AOS-LOXb is a matter of future research.

Expression Analysis of AOS-LOX

Coral *C. imbricata* is also known by the common name Kenya tree coral because of its tree-like appearance. The normal expression levels of *AOS-LOXa* and *AOS-LOXb* were estimated in the "stem" and "branch" of the coral colony. Under normal conditions, higher expression levels of both transcripts were detected in stem tissue, while *AOS-LOXb* transcript was 2.8 times more abundant than *AOS-LOXa* ($P<0.05$). Consequently, all samples in one experimental set were taken from the same location (either stem or branch).

Repeated wounding (one colony). To exclude genetic variation between coral colonies, the expression levels of *AOS-LOXa* and *AOS-LOXb* in response to incision wounding were estimated within a single octocoral colony. Significant up-regulation of *AOS-LOXa* was confirmed adjacent to the wounding site. The maximum fold change 3.6 was recorded at one hour post wounding, decreasing at 3 h and 6 h to 1.8- and 1.6-fold, respectively ($P<0.05$) (Fig. 4A).

To assess the secondary response, the transcript levels were recorded in tissue adjacent to the wounding site at (0+1 h), (1 h+2 h) and (3 h+3 h). The fold change of *AOS-LOXa* at (0+1 h) was identical to the 1 h sample, indicating that a similar wound response was generated near the wounding site. An additional increase was detected in the (1 h+2 h) and (3 h+3 h) samples, with up-regulation of 5.0- and 4.4-fold ($P<0.05$), respectively (Fig. 4B). The data indicate that the severity of stress had a direct effect on *AOS-LOXa* transcription.

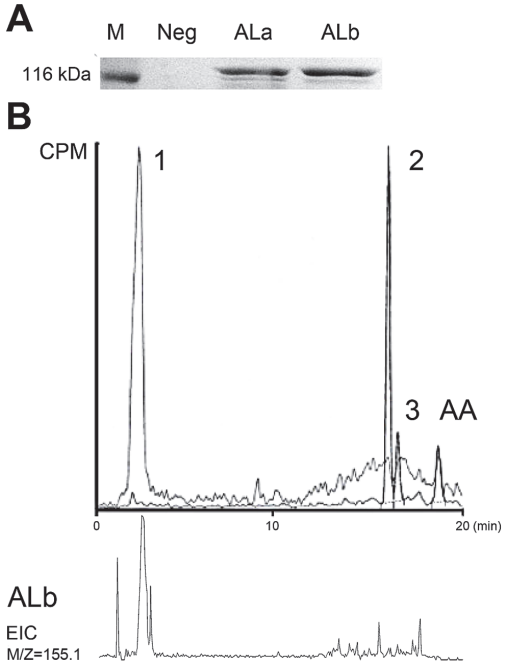


Figure 3. RP-HPLC analysis of incubation products of *C. imbricata* AOS-LOX fusion proteins expressed in *E. coli*. Bacterial extracts expressing the fusion proteins. M, protein molecular weight marker (Fermentas); Neg, negative control, pET11a vector without insert; ALa, AOS-LOXa; ALb, AOS-LOXb. The RP-HPLC analysis of the conversion of [1-^{14}C] AA by AOS-LOXa (peaks 2, 3) and AOS-LOXb (peak 1). The peak numbers indicate identical compounds formed by the coral homogenate (Fig. 2A, and corresponding EIC) and expressed AOS-LOX proteins. EIC [M$^-$] = 155.1 corresponding to the main AOS-LOXb product (peak 1). CPM - counts per minute.
doi:10.1371/journal.pone.0089215.g003

In a parallel experiment, the occurrence of a systemic response was investigated. The transcript level was recorded in distal parts of the coral colony at the tips of the branches. Although not reaching as high level as detected around the wounding site, *AOS-LOXa* was significantly up-regulated at 3 h and 6 h post wounding, by 1.5- and 1.9-fold ($P<0.05$), respectively. At the same time, the *AOS-LOXb* expression remained constant (Fig. 4C). The up-regulation of *AOS-LOXa* at distal parts of the colony indicated enhanced alertness of the whole colony.

It should be noted that after repeated wounding the coral was visibly stressed. After incision, all polyps of the colony instantly closed and remained closed while the area close to the wound contracted. Repeated wounding induced the color changes in the remaining colony. However, all treated colonies were able to recover and regenerate between four and ten days, depending on the severity of the injury.

Single wounding (separate colonies). In order to detect and compare the response between repeated and single incision wounding, the expression levels of *AOS-LOXa* and *AOS-LOXb* were estimated in separate coral colonies having identical genetic background. Adjacent to the wounding site, *AOS-LOXa* was significantly up-regulated at 1 h and 3 h post wounding by 1.2- and 1.4-fold ($P<0.05$), reaching the normal level at 6 h (Fig. 4D), while *AOS-LOXb* was significantly down-regulated only at 6 h with a relative expression ratio of 0.6 ($P<0.05$) (Fig. 4D). The moderate up-regulation of *AOS-LOXa* in response to a single wound also supports the contention that stress severity has an impact on *AOS-LOXa* expression.

Discussion

Using an acute incision wounding model, we showed that in response to wounding the soft coral *C. imbricata* undergoes rapid up-regulation of the AOS-LOX pathway *in vivo*. A major implication of our results is that the eicosanoid pathways used by coral during tissue injury and healing are similar to the oxylipin pathways in plant wound responses, highlighting a conserved pathway throughout the animal and plant kingdoms. The results of the current study establish coral AOS-LOX route as rapid-onset stress response pathway. Two *AOS-LOX* genes were found to be differentially responsive to mechanical injury, with *AOS-LOXa* transiently up-regulated (peaking at one hour post injury) while *AOS-LOXb* expression remained stable. The differential expression regulation and competition for the same upstream substrate (AA) implicate their distinct biological function in the wound response.

The initial wound response in animals, including corals, aims for rapid and efficient provisional plugging of the wound to minimize both the loss of vital fluids and environmental challenges (e.g. bacterial contamination) [10,49–51]. In vertebrate animals, the immediate release of cell-derived damage signals, including Ca^{2+}, ATP and reactive oxygen species (ROS), defines the wound area and severity of damage within the first minutes post injury [52]. Acute-onset signals initiate the secondary phases of wound healing through the transcription of wound response genes, including 1) immediate wave: genes expressed within 30 min to 1 h, the expression of which ceases within 6–12 h and in which protein synthesis is not required prior to induction, 2) fast phase: expression lasting from 30 min up to 12 h, and 3) sustained wave: lasting from 3 to 12 h [53]. Thus the time course of *AOS-LOXa* expression in *C. imbricata* is consistent with the immediate wave pattern.

Because of their sessile nature, corals share many plant-like physiological features. Likewise, the *AOS-LOXa* expression pattern in *C. imbricata* shows homology to lipoxygenase pathway expression in plant wound response. The wound response in plants includes the instant release of Ca^{2+}, ROS and leaf volatiles (e.g. short chain aldehydes) and traumatin [54,55], accompanied by the rapid synthesis and accumulation of the stress hormone JA via a lipoxygenase pathway, involving LOX, AOS, AOC steps [56,57]. To induce the synthesis of JA only one of four *Arabidopsis thaliana* 13(*S*)-LOXs (mainly found in plant plastids) is up-regulated an hour upon wounding [58–60]. Matching the same time window, the sequential step catalyzed in plastids by AOS is also up-regulated at the transcriptional [61,62] and translational level [24]. Thus, although the downstream mediators may diverge, the immediate wave expression of LOX and AOS after wounding is a shared, conserved pathway preparing the transit from provisional danger signaling to the initiation of structurally consolidating tissue repair. Whereas in plants the expression of *LOX* and *AOS* is regulated separately, in coral the AOS-LOX fusion protein enforces strict stoichiometric coupling of both catalytic steps.

The divergent product profile generated in the coral can be explained using plant oxylipin pathways as a guide. In plants the fatty acid hydroperoxide generated from α-linolenic acid by 13-LOX is converted in parallel by AOS [61,63,64] and hydroperoxide lyase (HPL) [65,66]. After wounding the AOS route leads to

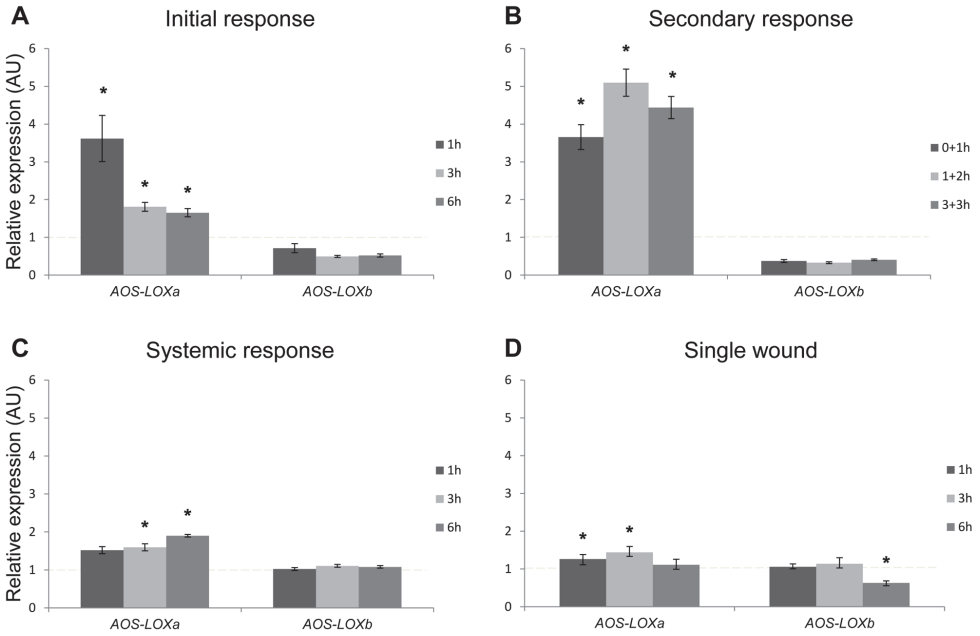


Figure 4. Quantitative real-time PCR analysis of transcript levels of *C. imbricata AOS-LOXa* and *AOS-LOXb*. Changes in gene expression in response to wounding within one colony: A) accumulative response; B) secondary response; C) systemic response. D) Response to a single wound. Data are means ± standard error, asterisk indicates significantly higher or lower expression relative to control ($P<0.05$).
doi:10.1371/journal.pone.0089215.g004

the formation of JA [61,63], while volatile C_6 aldehydes (e.g. Z-3-hexenal or E-2-hexenal) and non-volatile oxylipins (e.g. traumatin) are instantly generated by HPL [62,64,65,67]. Analogously to the reactions of plant LOX and AOS enzymes, the coral AOS-LOXa fusion protein catalyzes the formation of labile allene epoxide, although using AA instead of α-linolenic acid as a substrate.

Hypothetically, if the HPL-like reaction using 8-HpETE formed from AA by the 8-LOX domain of the AOS-LOX fusion protein takes place in the coral, two compounds with masses of 156 (C1–C8) and 180 (C9–C20) can be formed. The radiolabeled AA metabolite formed *in vitro* by *C. imbricata* AOS-LOXb has a mass of 156, which corresponds to (C1–C8) and also matches the location of [1-^{14}C] label, therefore suggesting that the HPL-like reaction has indeed taken place in coral by AOS-LOXb. The existence of parallel AOS-LOXa and AOX-LOXb pathways suggest that the coral initial wound response could also include the instant formation of aldehydes and oxo-acids.

As mentioned earlier, after wounding the concurrent conversion of the same substrate (AA) by *C. imbricata* (Fig. 2B) resulted in the gradual enhancement of α-ketol (Fig. 2A, peak 2) corresponding to AOS-LOXa and a decrease in AOS-LOXb metabolites (Fig. 2A, peak 1). The shift in metabolite spectrum after wounding is best explained by an altered balance between AOS-LOXa and AOS-LOXb, resulting in a dominance of AOS-LOXa products. A sustained increase in enzyme activity may be reached by up-regulation of gene expression and *de novo* protein synthesis or through the activation of AOS-LOX by increased cellular Ca^{2+}, shown to enhance the activity of *P. homomalla* and *G. fruticosa* AOS-LOXs *in vitro* [32,68]. Further studies are needed to identify all *in vivo* metabolites, as well as to specify the role and regulation of AOS-LOX isoforms in coral stress.

In conclusion, although plants and corals use different fatty acid precursors, the reactions and time course of oxylipin formation via LOX and AOS pathways in response to wounding are similar, suggesting conserved signaling pathways.

Acknowledgments

The authors gratefully acknowledge the valuable technical comments of Ivar Järving and Indrek Koppel. We thank Ken Veski and Aleksander-Mati Müürisepp for technical assistance.

Author Contributions

Conceived and designed the experiments: HL TT NS. Performed the experiments: HL TT KT ME. Analyzed the data: HL TT KT NS. Contributed reagents/materials/analysis tools: NS. Wrote the paper: HL TT NS. Identification of the coral species: ME.

References

1. Hughes TP, Baird AH, Bellwood DR, Card M, Connolly SR, et al. (2003) Climate change, human impacts, and the resilience of coral reefs. Science 301: 929–933.
2. Rotjan RD, Lewis SM (2008) Impact of coral predators on tropical reefs. Marine Ecology Progress Series 367: 73–91.
3. Kultz D (2005) Molecular and evolutionary basis of the cellular stress response. Annu Rev Physiol 67: 225–257.

4. Fang LS, Huang SP, Lin KL (1997) High temperature induces the synthesis of heat-shock proteins and the elevation of intracellular calcium in the coral Acropora grandis. Coral Reefs 16: 127–131.
5. DeSalvo MK, Voolstra CR, Sunagawa S, Schwarz JA, Stillman JH, et al. (2008) Differential gene expression during thermal stress and bleaching in the Caribbean coral Montastraea faveolata. Mol Ecol 17: 3952–3971.
6. Souter P, Bay LK, Andreakis N, Csaszar N, Seneca FO, et al. (2011) A multilocus, temperature stress-related gene expression profile assay in Acropora millepora, a dominant reef-building coral. Mol Ecol Resour 11: 328–334.
7. Seneca FO, Forêt S, Ball EE, Smith-Keune C, Miller DJ, et al. (2009) Patterns of Gene Expression in a Scleractinian Coral Undergoing Natural Bleaching. Marine Biotechnology 12: 594–604.
8. Woo S, Jeon H-y, Lee J, Song J-I, Park H-S, et al. (2010) Isolation of hyperthermal stress responsive genes in soft coral (Scleronephthya gracillimum). Molecular & Cellular Toxicology 6: 384–390.
9. Singer AJ, Clark RA (1999) Cutaneous wound healing. N Engl J Med 341: 738–746.
10. Palmer CV, Traylor-Knowles NG, Willis BL, Bythell JC (2011) Corals use similar immune cells and wound-healing processes as those of higher organisms. PLoS One 6: e23992.
11. Reitzel AM, Sullivan JC, Traylor-Knowles N, Finnerty JR (2008) Genomic survey of candidate stress-response genes in the estuarine anemone Nematostella vectensis. Biol Bull 214: 233–254.
12. Meszaros A, Bigger C (1999) Qualitative and quantitative study of wound healing processes in the coelenterate, Plexaurella fusifera: spatial, temporal, and environmental (light attenuation) influences. J Invertebr Pathol 73: 321–331.
13. Work TM, Aeby GS (2010) Wound repair in Montipora capitata. Journal of Invertebrate Pathology 105: 116–119.
14. Palmer CV, Mydlarz LD, Willis BL (2008) Evidence of an inflammatory-like response in non-normally pigmented tissues of two scleractinian corals. Proc Biol Sci 275: 2687–2693.
15. Mydlarz LD, Holthouse SF, Peters EC, Harvell CD (2008) Cellular responses in sea fan corals: granular amoebocytes react to pathogen and climate stressors. PLoS One 3: e1811.
16. Mydlarz LD, Harvell CD (2007) Peroxidase activity and inducibility in the sea fan coral exposed to a fungal pathogen. Comp Biochem Physiol A Mol Integr Physiol 146: 54–62.
17. Serhan CN, Chiang N, Van Dyke TE (2008) Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol 8: 349–361.
18. Schneider C, Pratt DA, Porter NA, Brash AR (2007) Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. Chem Biol 14: 473–488.
19. Kuhn H, O'Donnell VB (2006) Inflammation and immune regulation by 12/15-lipoxygenases. Prog Lipid Res 45: 334–356.
20. Kalish BT, Kieran MW, Puder M, Panigrahy D (2013) The growing role of eicosanoids in tissue regeneration, repair, and wound healing. Prostaglandins Other Lipid Mediat 104–105: 130–138.
21. Mauch F, Kmecl A, Schaffrath U, Volrath S, Gorlach J, et al. (1997) Mechanosensitive expression of a lipoxygenase gene in wheat. Plant Physiol 114: 1561–1566.
22. Sivasankar S, Sheldrick B, Rothstein SJ (2000) Expression of allene oxide synthase determines defense gene activation in tomato. Plant Physiol 122: 1335–1342.
23. Andreou A, Brodhun F, Feussner I (2009) Biosynthesis of oxylipins in non-mammals. Prog Lipid Res 48: 148–170.
24. Gfeller A, Baerenfaller K, Loscos J, Chetelat A, Baginsky S, et al. (2011) Jasmonate Controls Polypeptide Patterning in Undamaged Tissue in Wounded Arabidopsis Leaves. Plant Physiology 156: 1797–1807.
25. Bundy GL, Nidy EG, Epps DE, Mizsak SA, Wnuk RJ (1986) Discovery of an arachidonic acid C-8 lipoxygenase in the gorgonian coral Pseudoplexaura porosa. J Biol Chem 261: 747–751.
26. Brash AR, Baertschi SW, Ingram CD, Harris TM (1987) On non-cyclooxygenase prostaglandin synthesis in the sea whip coral, Plexaura homomalla: an 8(R)-lipoxygenase pathway leads to formation of an alpha-ketol and a Racemic prostanoid. J Biol Chem 262: 15829–15839.
27. Varvas K, Koljak R, Jarving I, Pehk T, Samel N (1994) Endoperoxide Pathway in Prostaglandin Biosynthesis in the Soft Coral Gersemia-Fruticosa. Tetrahedron Lett 35: 8267–8270.
28. Koljak R, Boutaud O, Shieh BH, Samel N, Brash AR (1997) Identification of a naturally occurring peroxidase-lipoxygenase fusion protein. Science 277: 1994–1996.
29. Koljak R, Jarving I, Kurg R, Boeglin WE, Varvas K, et al. (2001) The basis of prostaglandin synthesis in coral: molecular cloning and expression of a cyclooxygenase from the Arctic soft coral Gersemia fruticosa. J Biol Chem 276: 7033–7040.
30. Valmsen K, Boeglin WE, Jarving I, Schneider C, Varvas K, et al. (2004) Structural and functional comparison of 15S- and 15R-specific cyclooxygenases from the coral Plexaura homomalla. European Journal of Biochemistry 271: 3533–3538.
31. Mortimer M, Jarving R, Brash AR, Samel N, Jarving I (2006) Identification and characterization of an arachidonate 11R-lipoxygenase. Arch Biochem Biophys 445: 147–155.
32. Lohelaid H, Jarving R, Valmsen K, Varvas K, Kreen M, et al. (2008) Identification of a functional allene oxide synthase-lipoxygenase fusion protein in the soft coral Gersemia fruticosa suggests the generality of this pathway in octocorals. Biochim Biophys Acta 1780: 315–321.
33. Lee DS, Nioche P, Hamberg M, Raman CS (2008) Structural insights into the evolutionary paths of oxylipin biosynthetic enzymes. Nature 455: 363–368.
34. Corey EJ, Washburn WN, Chen JC (1973) Studies on the prostaglandin A 2 synthetase complex from Plexaura homomalla. J Am Chem Soc 95: 2054–2055.
35. Corey EJ, Lansbury PT, Yamada Y (1985) Identification of a New Eicosanoid from Invitro Biosynthetic Experiments with Clavularia-Viridis - Implications for the Biosynthesis of Clavulones. Tetrahedron Letters 26: 4171–4174.
36. Varvas K, Jarving I, Koljak R, Vahemets A, Pehk T, et al. (1993) Invitro Biosynthesis of Prostaglandins in the White Sea Soft Coral Gersemia-Fruticosa - Formation of Optically-Active Pgd2, Pge2, Pgf2-Alpha and 15-Keto-Pgf2-Alpha from Arachidonic-Acid. Tetrahedron Letters 34: 3643–3646.
37. Corey EJ, Dalarcao M, Matsuda SPT, Lansbury PT, Yamada Y (1987) Intermediacy of 8-(R)-Hpete in the Conversion of Arachidonic-Acid to Pre-Clavulone-a by Clavularia-Viridis - Implications for the Biosynthesis of Marine Prostanoids. J Am Chem Soc 109: 289–290.
38. Varvas K, Jarving I, Koljak R, Valmsen K, Brash AR, et al. (1999) Evidence of a cyclooxygenase-related prostaglandin synthesis in coral. The allene oxide pathway is not involved in prostaglandin biosynthesis. J Biol Chem 274: 9923–9929.
39. Hashimoto N, Fujiwara S, Watanabe K, Iguchi K, Tsuzuki M (2003) Localization of clavulones, prostanoids with antitumor activity, within the Okinawan soft coral Clavularia viridis (Alcyonacea, Clavulariidae): Preparation of a high-purity Symbiodinium fraction using a protease and a detergent. Lipids 38: 991–997.
40. Pawlik JR, Burch MT, Fenical W (1987) Patterns of Chemical Defense among Caribbean Gorgonian Corals - a Preliminary Survey. Journal of Experimental Marine Biology and Ecology 108: 55–66.
41. Gerhart DJ (1991) Emesis, Learned Aversion, and Chemical Defense in Octocorals - a Central Role for Prostaglandins. American Journal of Physiology 260: R839–R843.
42. O'Neal W, Pawlik JR (2002) A reappraisal of the chemical and physical defenses of Caribbean gorgonian corals against predatory fishes. Marine Ecology Progress Series 240: 117–126.
43. Whalen KE, Starczak VR, Nelson DR, Goldstone JV, Hahn ME (2010) Cytochrome P450 diversity and induction by gorgonian allelochemicals in the marine gastropod Cyphoma gibbosum. BMC Ecol 10: 24.
44. Su X, Gibor A (1988) A method for RNA isolation from marine macro-algae. Anal Biochem 174: 650–657.
45. Rodriguez-Lanetty M, Phillips WS, Dove S, Hoegh-Guldberg O, Weis VM (2008) Analytical approach for selecting normalizing genes from a cDNA microarray platform to be used in q-RT-PCR assays: a cnidarian case study. J Biochem Biophys Methods 70: 985–991.
46. Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2-\Delta\Delta CT$ Method. Methods 25: 402–408.
47. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: e36.
48. Coffa G, Schneider C, Brash AR (2005) A comprehensive model of positional and stereo control in lipoxygenases. Biochem Biophys Res Commun 338: 87–92.
49. Proksch E, Brandner JM, Jensen J-M (2008) The skin: an indispensable barrier. Experimental Dermatology 17: 1063–1072.
50. Rodriguez PG, Felix FN, Woodley DT, Shim EK (2008) The role of oxygen in wound healing: a review of the literature. Dermatol Surg 34: 1159–1169.
51. Ariel A, Timor O (2013) Hanging in the balance: endogenous anti-inflammatory mechanisms in tissue repair and fibrosis. The Journal of Pathology 229: 250–263.
52. Cordeiro JV, Jacinto A (2013) The role of transcription-independent damage signals in the initiation of epithelial wound healing. Nature Reviews Molecular Cell Biology 14: 249–262.
53. Wenemoser D, Lapan SW, Wilkinson AW, Bell GW, Reddien PW (2012) A molecular wound response program associated with regeneration initiation in planarians. Genes Dev 26: 988–1002.
54. Leon J, Rojo E, Sanchez-Serrano JJ (2001) Wound signalling in plants. J Exp Bot 52: 1–9.
55. Maffei ME, Mithöfer A, Boland W (2007) Before gene expression: early events in plant–insect interaction. Trends in Plant Science 12: 310–316.
56. Kombrink E (2012) Chemical and genetic exploration of jasmonate biosynthesis and signaling paths. Planta 236: 1351–1366.
57. Wasternack C, Hause B (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Annals of Botany 111: 1021–1058.
58. Bell E, Creelman RA, Mullet JE (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proc Natl Acad Sci U S A 92: 8675–8679.
59. Glauser G, Dubugnon L, Mousavi SAR, Rudaz S, Wolfender JL, et al. (2009) Velocity Estimates for Signal Propagation Leading to Systemic Jasmonic Acid Accumulation in Wounded Arabidopsis. Journal of Biological Chemistry 284: 34506–34513.

60. Schommer C, Palatnik JF, Aggarwal P, Chetelat A, Cubas P, et al. (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. Plos Biology 6: 1991–2001.
61. Laudert D, Pfannschmidt U, Lottspeich F, Hollander-Czytko H, Weiler EW (1996) Cloning, molecular and functional characterization of Arabidopsis thaliana allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant Mol Biol 31: 323–335.
62. Bate NJ, Sivasankar S, Moxon C, Riley JM, Thompson JE, et al. (1998) Molecular characterization of an Arabidopsis gene encoding hydroperoxide lyase, a cytochrome P-450 that is wound inducible. Plant Physiol 117: 1393–1400.
63. Song WC, Funk CD, Brash AR (1993) Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. Proc Natl Acad Sci U S A 90: 8519–8523.
64. Howe GA, Lee GI, Itoh A, Li L, DeRocher AE (2000) Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiol 123: 711–724.
65. Vick BA, Zimmerman DC (1987) Pathways of Fatty-Acid Hydroperoxide Metabolism in Spinach Leaf Chloroplasts. Plant Physiology 85: 1073–1078.
66. Grechkin AN, Hamberg M (2004) The "heterolytic hydroperoxide lyase" is an isomerase producing a short-lived fatty acid hemiacetal. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids 1636: 47–58.
67. Halitschke R, Ziegler J, Keinänen M, Baldwin IT (2004) Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling crosstalk in Nicotiana attenuata. The Plant Journal 40: 35–46.
68. Boutaud O, Brash AR (1999) Purification and catalytic activities of the two domains of the allene oxide synthase-lipoxygenase fusion protein of the coral Plexaura homomalla. J Biol Chem 274: 33764–33770.

PUBLICATION III

Lõhelaid, H., Teder, T., Samel, N. (2014). “Lipoxygenase-allene oxide synthase pathway in octocoral thermal stress response.” Coral Reefs DOI: 10.1007/s00338-014-1238-y

Coral Reefs
DOI 10.1007/s00338-014-1238-y

REPORT

Lipoxygenase-allene oxide synthase pathway in octocoral thermal stress response

H. Lõhelaid · T. Teder · N. Samel

Received: 16 April 2014 / Accepted: 28 October 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract Marine ecosystems are sensitive to elevated seawater temperature, with stony corals serving as model organisms for temperature-imposed declines in population viability and diversity. Several stress markers, including heat shock proteins, have been used for the detection and prediction of stress responses in stony corals. However, the stress indicators in soft corals remain elusive. In higher animals and plants, oxylipins synthesized by fatty acid di- and monooxygenases contribute to stress-induced signaling; however, the role of eicosanoid pathways in corals remains unclear. The eicosanoid gene specific to corals encodes for a natural fusion protein of allene oxide synthase and lipoxygenase (*AOS-LOX*). In this work, using the easily cultivated soft coral *Capnella imbricata* as the stress response model, we monitored the expression of the AOS-LOX and the formation of arachidonic acid metabolites in response to an acute rise in water temperature. Gene expression profiles of two 70 kDa heat shock proteins (*Hsps: Hsp70* and *Grp78*) were used as a positive control for the stress response. In comparison with normal seawater temperature (23 °C), *AOS-LOXa* and *Hsps* were all up-regulated after modest (28 °C) and severe (31 °C) temperature elevation. While the up-regulation of *AOS-LOXa* and *Grp78* was more sensitive to moderate temperature changes, *Hsp70*s were more responsive to severe heat shock. Concurrently, endogenous and exogenous AOS-LOXa-derived eicosanoids were up-regulated. Thus, together with the up-regulation of *AOS-LOX* by other abiotic and biotic stress stimuli, these data implicate AOS-LOX as part of the general stress response pathway in corals.

Keywords Coral thermal stress · Eicosanoids · AOS-LOX · Allene oxide synthase · Lipoxygenase · Hsp70

Communicated by Handling Editor Ruth Gates

H. Lõhelaid · T. Teder · N. Samel (✉)
Department of Chemistry, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia
e-mail: nigulas.samel@ttu.ee

Introduction

Global climate warming is a major threat affecting endangered coral ecosystems worldwide (Hoegh-Guldberg et al. 2007). Visible outcomes of the climate change and elevated sea water temperature includes coral bleaching, caused by the loss of symbiotic zooxanthellae (Jokiel and Coles 1990). Bleaching is a direct function of altered water temperature above the ambient level and occurs after short term (several degrees for 1–2 d) as well as long-term increments (1–2° for 1–2 weeks) (Jokiel and Coles 1990). In both cases, thermal stress affects coral physiology, with the altered composition of membrane lipids, energy reservoir and up-regulation of stress-responsive heat shock proteins (Søørensen et al. 2003), with the bleaching as a late cellular event (Ainsworth et al. 2008). In order to understand the cellular mechanisms underlying the stepwise alteration of coral physiology to environmental challenge, it is important to understand the early cellular stress response (Voolstra et al. 2009).

The molecular events involved in coral stress response are well studied in reef-building corals (DeSalvo et al. 2008; Rodriguez-Lanetty et al. 2009; Voolstra et al. 2009; Seneca et al. 2010; Kenkel et al. 2011; Polato et al. 2011; Souter et al. 2011), revealing a conserved response to elevated temperature via heat shock proteins and antioxidant enzyme systems (Lesser and Farrell 2004; DeSalvo et al. 2008; Fitt et al. 2009). 70 kDa heat shock proteins

Published online: 01 November 2014

(Hsp70) are molecular chaperones with important functions in protein biogenesis and stress tolerance (Lindquist 1986). Consequently, *Hsp70* up-regulation is widely used as a stress marker for estimating coral well-being (reviewed in van Oppen and Gates 2006). Although similarly affected by climate change and temperature, the heat stress response in soft corals remains poorly addressed.

Currently, coral stress studies focus on large-scale gene regulation, while the presence of active proteins or metabolites remains unknown. As an early consequence of cellular stress responses, membrane composition becomes restructured by lipid peroxidation, which results in protein rearrangement, permeability changes, and activation of phospholipases and downstream oxylipin pathways (Kultz 2005; Upchurch 2008). Oxylipins consist of oxygenated polyunsaturated fatty acids (PUFAs) formed on demand by mono- or dioxygenases (Gerwick et al. 1991), which function as lipid mediators in response to cell stress and inflammation (Wymann and Schneiter 2008). Oxylipins are synthesized by cyclooxygenases (COX) and lipoxygenases (LOX) in animals (Schneider et al. 2007) or LOX, allene oxide synthase (AOS) and allene oxide cyclase (AOC) in plants (Wasternack and Hause 2013), catalyzing the formation of prostaglandins, hydroperoxy fatty acids, allene oxides and 12-oxo-phytodienoic acids, respectively (Schneider et al. 2007; Andreou et al. 2009; Wasternack and Kombrink 2010). When oxylipins denote all oxygenated PUFAs, eicosanoids are a subset of oxylipins, formed from 20-carbon PUFAs only. In corals, eicosanoids are derived from arachidonic acid (AA) by COX (Varvas et al. 1994; Koljak et al. 2001; Valmsen et al. 2004), LOX (Mortimer et al. 2006) or AOS-LOX fusion proteins (Koljak et al. 1997; Lohelaid et al. 2008). Despite their widespread occurrence in marine invertebrates (Brash et al. 1987, 1991; Rowley et al. 2005), the physiological functions of eicosanoids, including hydroxy fatty acids, prostaglandins and their derivatives, in those organisms often remain unclear.

Recently, we showed that in the symbiotic Indo-Pacific soft coral *Capnella imbricata*, the increased generation of eicosanoids by an AOS-LOX pathway represents a stress response to mechanical wounding, mediated by two enzymatically active AOS-LOX isoforms (AOS-LOXa and AOS-LOXb), which convert exogenous AA via 8-hydroperoxyeicosatetraenoic acid (8-HpETE) into different end-products (Lõhelaid et al. 2014). Significantly, when AOS-LOXa represents a previously known AA metabolic pathway ("true AOS-LOX"), metabolites formed by AOS-LOXb have not been recorded in corals. Accordingly, from here on when the AOS-LOX pathway is mentioned, only the true AOS-LOX, AOS-LOXa and related activities are considered.

Representing a conserved coral pathway, the substrate AA as well as the sequential metabolizing enzymes is present in both, soft octocorals and reef-building hexacorals (Lee et al. 2008; Imbs and Yakovleva 2011; Figueiredo et al. 2012; Voolstra et al. 2012). In hexacorals, the up-regulation of *AOS-LOX* in response to white band disease (Söderhäll et al. 2013), elevated UV radiation (Aranda et al. 2011) and temperature (Polato et al. 2013) has been detected by transcriptome studies. Thus, although their species-specific downstream effectors remain unclear, coral eicosanoid pathways emerge as conserved regulators of cell stress responses, with potential as biomarkers to detect stress responses in both soft and stony corals.

The aim of this study was to address whether AOS-LOX expression and activity in the soft coral *C. imbricata* are both induced by temperature elevation. Using *Hsp70s* as a positive control for the stress response, we here demonstrate that the AOS-LOX pathway is part of the cellular stress response in corals.

Materials and methods

Coral samples

Colonies of the Indo-Pacific coral *C. imbricata* were purchased from a commercial source (Estonia), identified by M. Ekins at the Queensland Museum (specimen No: QM G317136), cultivated and propagated in a closed-circuit marine aquarium in the Department of Chemistry at Tallinn University of Technology at an ambient seawater temperature of 23 ± 0.2 °C, 31 ppt salinity, under a periodic day–night cycle (12–12 h) and 20 % biweekly water exchange.

Extraction of total RNA and synthesis of cDNA

Total RNA was extracted from 200 ± 2 mg coral branch tissue using an RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions and as described previously (Lõhelaid et al. 2014). Isolated RNA was quantified with a NanoDrop-3000 spectrophotometer (Thermo Scientific), and the integrity of isolated total RNA was constantly confirmed by electrophoresis on 1 % formaldehyde agarose gel. RNA extraction resulted in 0.4 μg of total RNA per mg of tissue. About 1 μg of total RNA was treated with DNase I, and the first-strand cDNA was synthesized with an oligo(dT)-adapter primer (Lõhelaid et al. 2014) in 20 μl reaction volume using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. To confirm the purity of the RNA sample and the efficiency of cDNA synthesis, control samples without reverse transcriptase were prepared in parallel.

Homology-based RT-PCR of partial Hsp70

Degenerative primers were generated based on conserved regions of *Hsp70*, the first round on SCVG and PDEA and the second round as half-nested PCR on YFND and PDEA (the PCR primers are listed in Table 1). Except for shorter elongation times (1 min 30 s in the first and 1 min in the second round), PCR conditions were identical to the protocol described earlier (Lõhelaid et al. 2014). PCR products with the expected amplicon sizes, 1,100 and 700 bp, respectively, were purified, cloned into pGEM-T Easy Vector (Promega) and sequenced (Agowa, Germany). Sequence analysis was performed using the BLAST (Basic Local Alignment Search Tool), and alignments were made by a MegAlign program (DNAStar, Lasergene) with ClustalW. Two partial sequences encoding for coral *Hsp70* were identified and submitted to a database (GenBank accession numbers: KJ452159 and KJ452160, respectively).

Experimental design

To exclude genetic variation, three adult coral colonies were fragmented, each into twelve identical sub-colonies. In order to reduce the influence of fragmentation, the coral nubbins were grown for an additional 4 months at 23 °C. Water temperatures above 30 °C induce natural bleaching, indicative of temperature stress (Glynn 1990; Brown 1997; Seneca et al. 2010). For the heat shock treatment, coral colonies were transferred to temperature-controlled tanks held either at 28 or 31 °C. It was established that the placement of corals from one tank to another was not stressful and the transcription and metabolite levels for genes studied remained stable (Lõhelaid et al. 2014). A sample of the coral branch tissue (200 ± 2 mg) was taken from untreated (at time zero) and treated (at time 1, 3, 6, 12 and 24 h after the beginning of the experiment) colonies (Fig. 1). The extraction of total RNA from coral tissue and the synthesis of cDNA were carried out as described above. The remaining colonies (2.5–3.0 g of tissue) were used to estimate AOS-LOX activity and endogenous AA metabolites at indicated time points.

Gene expression analysis

Sequence-specific qPCR primers for *AOS-LOXa* and *-b* and *beta-actin* of *C. imbricata* were used as described previously (Lõhelaid et al. 2014). qPCR primers for *Hsp70*s were designed using PrimerSelect software (DNASTAR, Lasergene) (Table 1). qPCR products were cloned and sequenced, and all matched the expected product identities. *Beta-actin* and *AOS-LOXb* were identified as stable genes during thermal stress by BestKeeper© Software (Pfaffl et al. 2004) and were used as a reference. Data were collected from three biologically independent replicates, and each value was calculated from the average of three technical replicates. The expression ratio between the sample and control using *beta-actin* and *AOS-LOXb* as the reference genes was determined using Relative Expression Software (REST-MCS) (www.qiagen.com; REST Software 2009) with 2000 iterations (Pfaffl et al. 2002). The threshold value indicative of the up-regulation of gene expression was set at 1.4. The results are presented as the mean ± standard error (SE), $n = 3$; fold changes were considered statistically significant at $p < 0.05$.

Table 1 List of primers

Primer	Sequence (5′–3′)	Gene	Product size (bp)	
(A) *Degenerative primers*				
SCVG-up	GGAACMACHTAYWSHTGYGTYGG	*Hsp70*	1,100	–
PDEA-down	CCRTAVGCHACRGCYTCRTCDGG	*Hsp70*		700
YFND-up	ACWGTDCCDGCWTAYTTCAAYG	*Hsp70*	–	
(B) *Specific primers for qPCR*				
Hsp70-up	TGAGGGGATCGACTTCTACACG	*Hsp70*	124	
Hsp70-down	TGTATTGATCCTTTATCAAGTCCAGC	*Hsp70*		
Grp78-up	GCGGCTGAAGAAATAAGTGC	*Grp78*	132	
Grp78-down	GTCTCTGCGCATCATTGAAA	*Grp78*		
ALa-up*	AATATTTGGACATTCAGTCGTTATG	*AOS-LOXa*	100	
ALa-down*	CGATAGTTTACTGGGCCTTTCTTC	*AOS-LOXa*		
ALb-up*	CGTCATGCAAATCTTAAACTACTGG	*AOS-LOXb*	180	
ALb-down*	AGACTCTCCTGCACTTGATGATAC	*AOS-LOXb*		
Act-up*	TGTGGCATCCATGAGACCTG	*Beta-actin*	95	
Act-down*	AGACAGCACTGTGTTGGCATAC	*Beta-actin*		

A, degenerative primers; B, qPCR primers used for gene expression analysis. Up, forward; down, reverse primer. * Primer sequences (Lõhelaid et al. 2014)

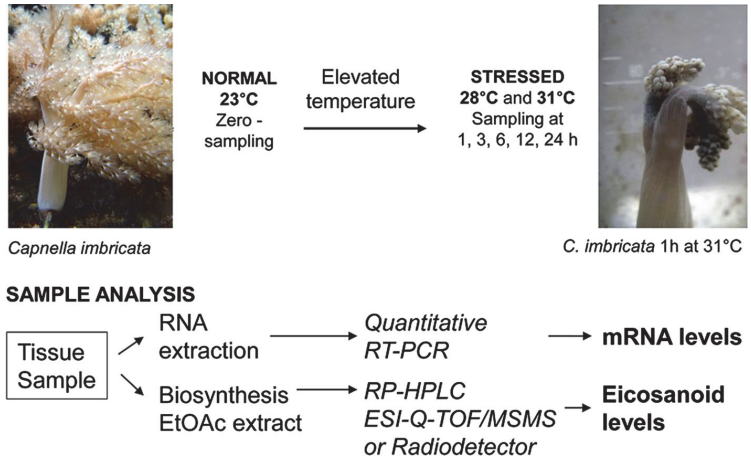


Fig. 1 Acute temperature-induced stress in *C. imbricata*: experimental approach. Three adult coral colonies were fragmented, each into twelve identical sub-colonies, and grown for 4 months at 23 °C. For heat shock treatment, coral colonies were placed in preheated (28 or 31 °C) tanks and sampled after 1, 3, 6, 12 and 24 h. The samples were used for gene expression and metabolite analysis

Analysis of AOS-LOX-derived metabolites

The AOS-LOX activity in the tissue homogenates of heat-stressed or nonstressed corals at indicated time points was estimated by in vitro incubations as described previously (Lõhelaid et al. 2014). In parallel, to analyze endogenous eicosanoids, the fresh coral homogenates were extracted with ethyl acetate (EtOAc) and dried over Na_2SO_4. The concentrated extracts (0.1 mg ml^{-1}) were stored at −20 °C. The samples were analyzed using a Zorbax Eclipse XDB-C_{18} column (5 µm, 4.6 × 150 mm), run on the Agilent 1200 Series HPLC system, connected to a diode array detector, followed by 500TR Series Flow Scintillation Analyzer (Packard Bioscience) or Agilent 6540 UHD Accurate Quadrupole time-of-flight MS/MS with an Agilent Jet Stream^{TM} ESI source in negative mode. Elution was performed with a solvent system of ACN/water/formic acid (98.9 %/1.0 %/0.1 % v/v)(A) and water/formic acid (99.9/0.1 % v/v)(B), 0–8 min isocratic (35 % A:65 % B), 9–17 min gradient to 100 % A, 18–30 min 100 % A at a flow rate of 1 ml min^{-1} (Lõhelaid et al. 2014). The data were analyzed by Agilent MassHunter Workstation Software Qualitative Analysis, version B.05.00 Build 5.0.519.0 and MassHunter Mass Profiler Software, version B03.00SP1 Build 35.0. The results are presented as the mean ± standard deviation (SD), $n = 3$. Values are statistically different as determined by Student's t test ($p < 0.05$).

Results

The soft coral *C. imbricata* was used to detect the coral stress response to thermal stress via altered transcription level and eicosanoid metabolite production of *AOS-LOXa,* using *Hsp70*s transcription as an established positive control for the stress response.

Hsp70 sequence analysis

Two partial sequences of *Hsp70* family members were identified in *C. imbricata*. Based on sequence analysis, they were designated as *heat shock protein* 70 kDa (*Hsp70*) (NCBI ID: KJ452160) and *glucose-regulated protein* 78 kDa (*Grp78*) (NCBI ID: KJ452159). The sequence identity between the identified *Hsp70* and *Grp78* segments was 65 %, and the deduced amino acid identity of *Hsp70* and *Grp78* with other *Hsp70* and *Grp78* family members was 83 and 87 %, respectively (Fig. 2). The first *Hsp70*s cloned from the soft coral confirm the high conservation between *Hsp70* protein families within different species.

Expression analysis

Beta-actin and *AOS-LOXb* were identified as the stable genes during thermal stress and were used as a reference. The change of expression levels of *AOS-LOXa*, *Hsp70* and *Grp78* was established for moderate thermal stress after 1, 3, 6, 12 and 24 h (Figs. 1, 3). The expression of both *AOS-LOXa* and *Grp78* mRNA was strongly up-regulated at 28 °C (Fig. 3). After rapid induction (3 h, by 8.0 ± 0.5-fold), the expression of *Grp78* remained at the same level. The initial increase of *AOS-LOXa* at 3 h was lower but the highest expression level was recorded at 12 h (by 11.3 ± 0.5 fold). In comparison, although the initial expression pattern of *Hsp70* was similar to *Grp78*, the detected fold changes were lower (Fig. 3). After up-regulation at 3 h (by 3.3 ± 0.3-fold), the expression of *Hsp70* remained stable, only moderately decreasing at 24 h.

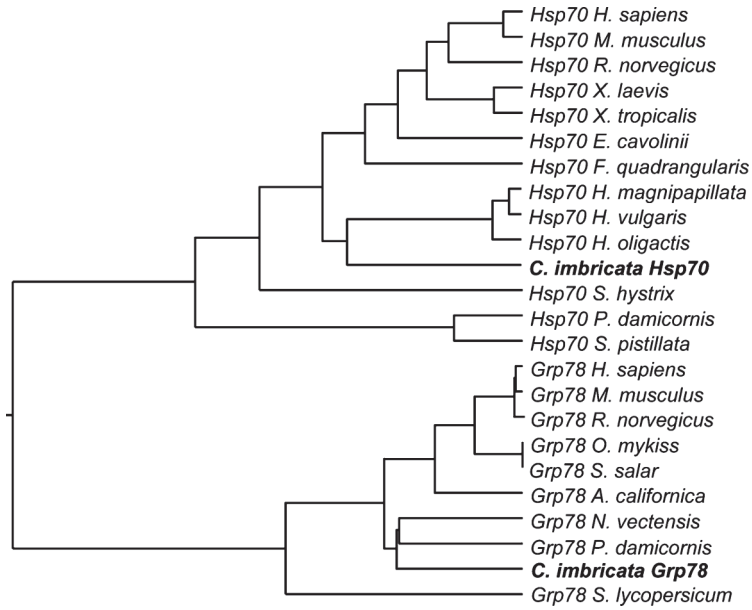


Fig. 2 Phylogenetic tree of partial amino acid sequences of *C. imbricata,* showing the relationship between other known *Grp78* and *Hsp70* sequences. The list of species and sequences is presented with gene accession numbers (NCBI Sequence ID): *Aplysia californica* (Z15041.1), *C. imbricata* (KJ452159; KJ452160), *Eunicella cavolinii* (AAC05363), *Funiculina quadrangularis* (AAC05361), *Homo sapiens* (NP_005336; M19645.1), *Hydra magnipapillata* (AAA29213), *H. oligactis* (CAA70893), *H. vulgaris* (ABC25030), *Mus musculus* (ABK96811; NM_022310.3), *Nematostella vectensis* (XP_001 625396), *Oncorhynchus mykiss* (AB196459.1), *Pocillopora damicornis* (BAD89541; BAD89540), *Rattus norvegicus* (CAA54424), *Salmo salar* (NM_001141642.1), *Seriatopora hystrix* (ADM13667), *Solanum lycopersicum*, *Stylophora pistillata* (AAF12746), *Xenopus laevis* (AAH78115), *X. tropicalis* (AAI35234)

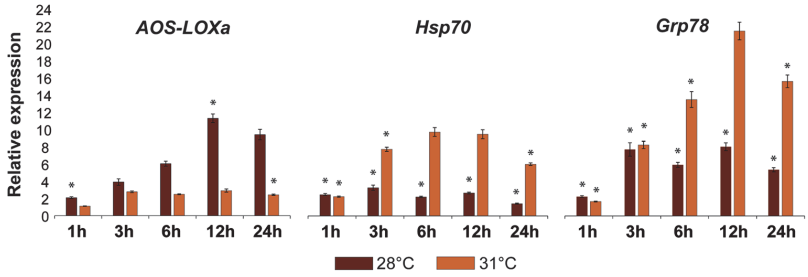


Fig. 3 Expression levels of *AOS-LOXa*, *Hsp70* and *Grp78* in response to acute thermal stress at 28 and 31 °C. Gene expression levels at indicated time points are presented relative to the normal temperature 23 °C (=1). All data are the mean ± SE, $n = 3$. *Asterisks* indicate significantly higher or lower expression relative to controls ($p < 0.05$)

To the contrary, severe heat stress at 31 °C caused a strong up-regulation of *Hsp70* (maximum at 6 h, by 9.7 ± 0.5-fold) and a somewhat delayed increase in *Grp78* transcript (maximum at 12 h, by 21.4 ± 1.0-fold), whereas *AOS-LOXa* regulation was less pronounced (Fig. 3). Thereafter, transcription levels of all three genes showed decline but remained above baseline until 24 h after heat shock induction (Fig. 3). Thus, *AOS-LOXa* regulation is most sensitive to moderate whereas *Hsp70* and *Grp78* respond preferentially to severe temperature elevation.

Estimation of AOS-LOX activity

To address the impact of *AOS-LOX* regulation on enzymatic activity and lipid mediator synthesis during thermal stress incubations with the coral tissue homogenate in the

presence of exogenous [1-^{14}C] AA were performed to estimate the metabolites formed from the single substrate in vitro. Next, coral EtOAc extracts were used to detect and estimate the formation of the same metabolites within the same time frame in vivo.

Analysis of the exogenous AA metabolites

Previous studies of [1-^{14}C] AA metabolism revealed the formation of known AOS-LOX products in *C. imbricata* (Lõhelaid et al. 2014). The enzymatic activity of the coral AOS-LOX pathway was estimated via the trapping of the intermediate 8-HpETE by $SnCl_2$ to 8-HETE (data not shown) or stable end-products α-ketol (8-hydroxy-9-keto-5Z, 9E, 11Z, 14Z-tetraenoic acid) and cyclopentenone (9-keto-prosta-5Z, 10, 14Z-trienoic acid) formed by AOS-LOXa (Fig. 4a) and novel, yet unidentified, short chain polar compounds (UPC) formed by AOS-LOXb.

The formation of AOS-LOXa products increased and AOS-LOXb products decreased in response to elevated temperatures, while cyclopentenone and AA levels remained stable (data not shown, $p > 0.05$). A negative correlation between α-ketol and UPC formation was observed at both temperatures tested (Fig. 4b). In comparison with the normal level at 23 °C, the highest enhanced activity of AOS-LOXa was detected at 31 °C after 24 h, when α-ketol increased by 139 % and UPC decreased by 55 %. At the same time, at 28 °C, the formation of α-ketol increased by 107 %, while UPC decreased by 54 % after 24 h (Fig. 4b). Compared to 28 °C, α-ketol formation at 31 °C was also significantly enhanced at 3 and 24 h ($p < 0.05$). According to the product profile generated from the exogenous AA (Fig. 4b), the substrate was mostly converted by the abundant AOS-LOXa. The detected increase in α-ketol level may have been due to the enhanced activity of existing enzyme or by de novo-synthesized AOS-LOXa fusion protein. Accordingly, the highest AOS-LOXa activity was detected after 24 h in response to severe temperature elevation.

Analysis of endogenous eicosanoids

Endogenous eicosanoids generated in vivo at different temperatures were determined by RP-HPLC/MSMS analysis of EtOAc extracts of freshly homogenized coral. The products of the AOS-LOXb fusion protein were detected in EtOAc extracts, but the level was too low for integration and data analysis. Similarly to the results of biosynthesis (Fig. 4b), the level of endogenous α-ketol increased in response to both moderate and severe heat stress (Fig. 4c). Initial changes were modest, moving in parallel up to 3 h and starting to diverge after 6 h, whereas the maximum α-ketol level was detected at 31 °C after 24 h. In contrast, the level of endogenous cyclopentenones remained stable throughout the experiment (Fig. 4c).

Thus, despite the reduction of *AOS-LOXa* transcription at 31 °C (Fig. 3), AOS-LOXa fusion protein metabolite levels with exogenous substrate were higher in response to severe thermal stress (Fig. 4b). Also, a higher level of endogenous α-ketol was detected at 31 °C (Fig. 4c). The upstream signals regulating AOS-LOX function in coral are not known. As the formation of AOS-LOX products may depend on the availability of substrate and enzyme, as well as enzyme activation, the regulation likely includes both transcriptional and enzyme activation mechanisms.

Discussion

The thermal stress response in *C. imbricata* described here includes elevated eicosanoid levels and preferential up-regulation of *AOS-LOXa* in response to moderately elevated temperature, which parallels with the up-regulation of the known stress proteins *Hsp70* and *Grp78*.

The particularly sensitive up-regulation of *AOS-LOXa* in response to moderately elevated water temperature is consistent with the concept of a graded response, which attenuates normal transcriptional processes during severe heat stress, and, instead, the transcription of heat shock protein genes is induced (Lindquist 1986). This could reflect an adaptation to varying levels of available energy and allow for a most adequate cell adaptation to overcome thermal stress (Feder and Hofmann 1999).

As a conserved mechanism shared between soft and stony coral thermal stress response, the up-regulation of heat shock protein transcripts in *C. imbricata* is in accordance with the studies conducted on the larvae of *Acropora millepora* (Rodriguez-Lanetty et al. 2009). The sudden up-regulation of *Hsp*s is a short-term process, and after temporary or permanent elevations in water temperature, the levels of *Hsp*s will stabilize or even decrease (Rodriguez-Lanetty et al. 2009; Meyer et al. 2011). A similar time course is also present in *C. imbricata Hsps* the levels of which, after transient up-regulation, decrease after 12 h of heat treatment. Consequently, taking into account the expression ratios, all genes under surveillance are involved in initial thermal stress response. The different up-regulation levels of two heat shock genes and *AOS-LOXa* illustrate the different aspects of cellular stress in an extensive way, discussed in detail below.

The detection of cyclopentenone as high or even higher level than α-ketol in coral EtOAc extracts (Fig. 4c) was unexpected, because in vitro preparation of the isolated *C. imbricata* AOS-LOXa produces α-ketol as the main and cyclopentenone as minor metabolite from [1-^{14}C] AA (Lõhelaid et al. 2014). This ratio of α-ketol and

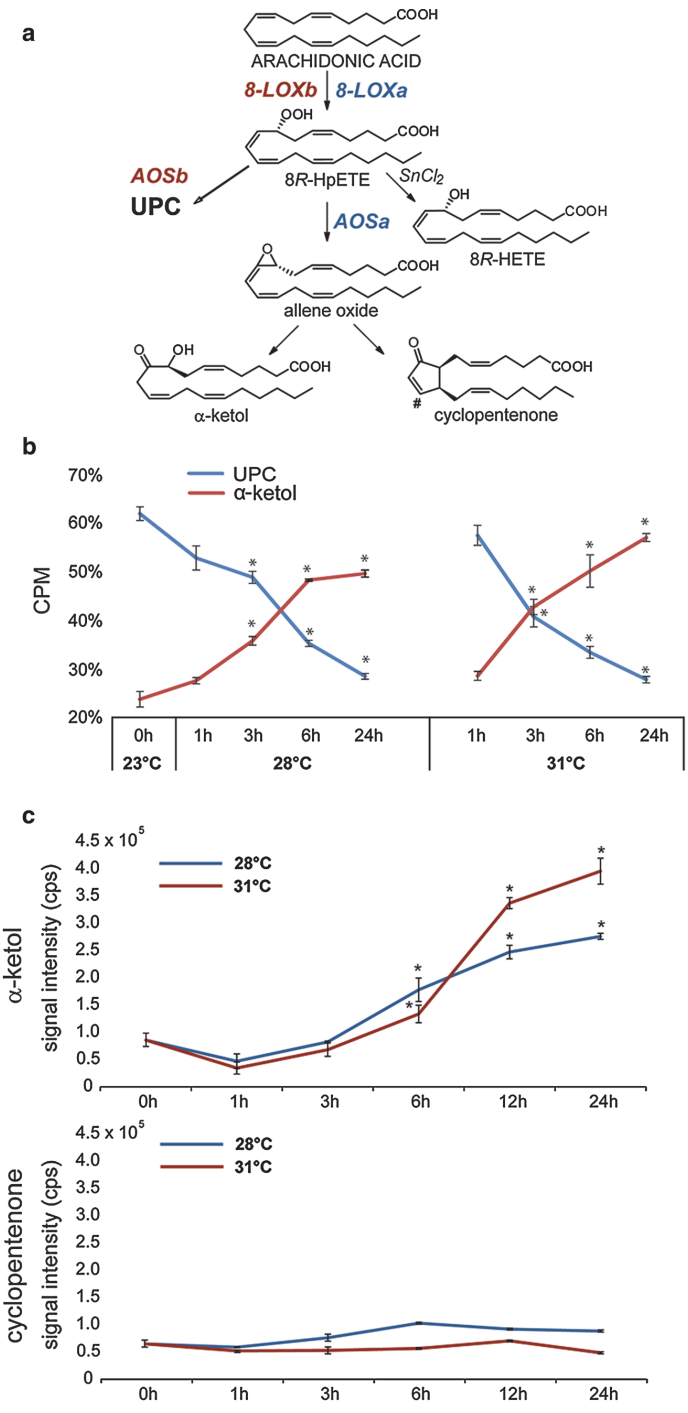


Fig. 4 Formation of eicosanoids by *C. imbricata*. **a** Arachidonic acid metabolites: 8-HpETE, 8-HETE, α-ketol (8-hydroxy-9-keto-5Z, 9E, 11Z, 14Z-tetraenoic acid), cyclopentenone (9-keto-prosta-5Z, 10, 14Z-trienoic acid) and UPC (unidentified polar compounds). The electrophilic center of the cyclopentenone is indicated by #; **b** the biosynthesis products formed from [1-^{14}C] AA by homogenate of *C. imbricata* under normal conditions and in response to elevated temperature; **c** endogenous eicosanoid levels (α-ketol $m/z = 336.2$, cyclopentenone $m/z = 318.2$) detected in EtOAc extracts, corresponding to **b**, (mean ± SD, $n = 3$). *Asterisks* values are statistically different as determined by Student's t test ($p < 0.05$). *CPM* counts per minute, *cps* counts per second

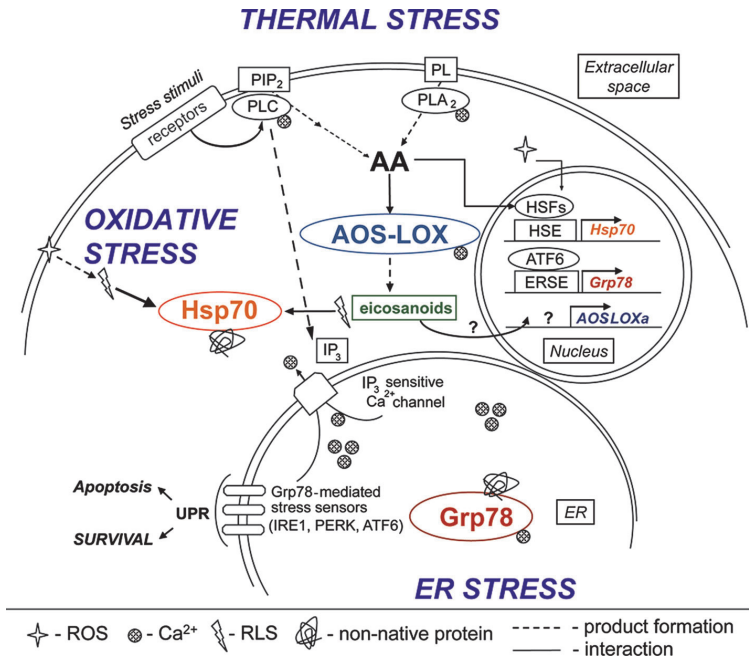


Fig. 5 Proposed model of coral stress response to elevated temperature. The intensity of stress response is coupled with the severity of stress. Stress triggers the influx of calcium to cytosol, which enhances the release of AA and the production of eicosanoids. ROS, RLS, AA and eicosanoids up-regulate the expression of Hsps. Coordinated by UPR, mildly elevated temperatures lead to transcriptional rearrangement acclimatization to elevated temperature and survival. In severe cases, the cellular overburden caused by misfolded proteins, ROS, RLS and eicosanoids leads to apoptosis. The model is based on our results, literature data and annotated databases of cnidarians. The figure is not intended to show the exact localization of all components. Abbreviations include: *AA* arachidonic acid, *AOS-LOX* allene oxide synthase-lipoxygenase fusion protein, *ATF6* activating transcription factor 6, *ER* endoplasmic reticulum, *ERSE* endoplasmic reticulum stress element, *Grp78* glucose-regulated protein 78 kDa, *HSE* heat shock element, *HSFs* heat shock factors, *Hsp70* heat shock protein 70 kDa, IP_3 inositol 1,4,5-triphosphate, *IRE1* inositol-requiring enzyme 1, *PERK* protein kinase RNA-like endoplasmic reticulum kinase, PIP_2 phosphatidylinositol 4,5-bisphosphate, *PL* phospholipid, PLA_2 phospholipase A_2, *PLC* phospholipase C, *RLS* reactive lipid species, *ROS* reactive oxygen species, *UPR* unfolded protein response

cyclopentenone formation is supported by previous in vitro studies on *Plexaura homomalla* and *Gersemia fruticosa* AOS-LOXs (Koljak et al. 1997; Lohelaid et al. 2008). Although the formation of eicosanoids was studied in octocorals *P. homomalla* (Corey et al. 1973) and *C. viridis* (Corey et al. 1987), the literature is lacking in comparable data of all metabolites formed, due to the focus on cyclic prostaglandin-like products only. Still, the divergence between in vitro and in vivo cyclopentenone formation may be explained by a preference for the formation of the cyclopentenone (Brash et al. 2013), or the cyclopentenone has been synthesized by another enzyme, such as AOC in plants (Wasternack and Hause 2013).

Integration of the AOS-LOX pathway in thermal stress response

As elevated temperatures affect the whole coral, its homeostasis is maintained by multiple molecular mechanisms, including the activation of Hsp70 and Hsp90 stress protein pathways and, as shown here, the rapid induction of eicosanoid pathways. As regards the AOS-LOX pathway, the emerging role of eicosanoids is to mediate the initial stress response, which eventually may initiate the heat shock response (HSR). As integrating concept for the thermal stress response in *C. imbricata,* the up-regulation of *Grp78* indicates ER stress, whereas *Hsp70* reflects heat and oxidative stress and *AOS-LOXa* denotes early stages of stress (Fig. 5).

To be able to respond quickly and adequately to the elevated temperature, and if needed to induce the HSR on time, the cell's sensory system must effectively integrate various stress signals (lipid mediators, ROS and unfolded proteins, and changes in RNA and DNA topology). One hallmark of the thermal stress is the unfolding of proteins and the resulting exposure of their hydrophobic interiors. Stress-induced accumulation of unfolded proteins is detected by

Hsps. Under normal conditions, Hsp70s are bound with heat shock factors (HSFs). When Hsps interact with unfolded proteins, HSF transcription factors are released, which in turn initiate the transcription of Hsp genes.

In corals, both Hsps and eicosanoid responses are preceded by additional signaling events, as cytosolic calcium increase ($[Ca^{2+}]_{cyt}$) triggered by heat (Fang et al. 1997; DeSalvo et al. 2008), which is further enhanced by expression of calcium channels and pumps (DeSalvo et al. 2008; Meyer et al. 2011).

Under normal conditions, the ER-specific glucose-regulated protein 78 (Grp78 or BiP), which is constitutively expressed and protects against protein denaturation, is up-regulated by elevated temperature and other stressors, indicative of ER stress (Resendez et al. 1988; Lee 2005; Luan et al. 2009). In coral, the up-regulation of *Grp78* has also been detected in response to UV radiation (Aranda et al. 2011). Although the majority of Ca^{2+} in ER lumen is free (Meldolesi and Pozzan 1998), approximately 25 % of ER Ca^{2+} is stored by Grp78 (Lievremont et al. 1997). Grp78 with other Ca^{2+}-buffering chaperones affects the Ca^{2+} level within the ER and the release into cytosol (Lievremont et al. 1997; Coe and Michalak 2009) (Fig. 5). Moreover, Grp78 is the key regulator of ER transmembrane proteins (Ire1, Atf6 and PERK and their homologs) (Fig. 5), which are the main initiators of the unfolded protein response (UPR) throughout *Metazoa* (Schroder and Kaufman 2005; Hollien 2013). Similar to other Hsp70s, the induction of Grp78 is regulated at the transcriptional level by transcription factor complexes (including ATF6) attached on the conserved stress response element (ERSE) (Fig. 5) (Parker et al. 2001). As the UPR directs survival and apoptotic signaling pathways, it is an important checkpoint measuring the severity of stress, which eventually determines the cell's fate.

Oxidative stress is closely connected with thermal stress (DeSalvo et al. 2008; Voolstra et al. 2009), both extensively studied in coral bleaching processes (Lesser 1997). ER stress signaling through chaperone networks and increase in $[Ca^{2+}]_{cyt}$ will activate several processes in mitochondria, including the production of reactive oxygen species (ROS) (Ermak and Davies 2002; Szabadkai and Rizzuto 2007). With the insufficient detoxification of ROS, oxidative stress occurs (Lesser 2006). Within a cell, ROS are produced mainly by the Ca^{2+} overload in mitochondria (Feissner et al. 2009), the activity of oxygenases (Radogna et al. 2009) and the formation of disulfide bonds by protein disulfide isomerase (Tu and Weissman 2004). In different coral species, elevated levels of ROS have been detected in response to heat stress (Mydlarz and Jacobs 2006). For instance, the direct sensing of hydrogen peroxide by HSFs leads to the induction of heat shock response (Zhong et al. 1998). The thermal-stress-induced LOX activity leads to lipid peroxidation and the production of reactive lipid species (RLS), oxidized PUFAs with electrophilic properties (Higdon et al. 2012). The cyclopentenone formed by AOS-LOXa possesses a high resemblance to stress mediators in plants (Stintzi et al. 2001) and other compounds with cyclopentenone moiety. Due to their electrophilic properties, cyclopentenones have the potential to disrupt the interaction between Hsps and HSF1, thus promoting the transcription of Hsp70s (Elia 1996; Jacobs and Marnett 2007; Higdon et al. 2012; West et al. 2012) (Fig. 5).

The production of eicosanoids is up-regulated in response to various stressors, and free AA is one of the heat shock response activators in heat stress (Balogh et al. 2013). Upstream regulation of AA release occurs predominantly via phospholipase A_2 (PLA_2) (Serhan et al. 1996; Schaloske and Dennis 2006) and to a lesser extent by phospholipase C (PLC) (Fig. 5) (Harden and Sondek 2006). Phospholipases are activated via different receptors (e.g. G-protein-coupled receptors) in response to a range of stimuli (Harden and Sondek 2006), including Ca^{2+} (Evans et al. 2001), lipid peroxides (Ermak and Davies 2002) and heat (Jurivich et al. 1996). Consistently, high activities of PLA_2 are detected across *Cnidaria* (Nevalainen et al. 2004; Romero et al. 2010), indicating a conserved pathway. Therefore, phospholipases and AA-metabolizing enzymes, including AOS-LOX, might be co-regulated to give the most adequate stress response.

Indicative of the cross talk between Ca^{2+} and eicosanoid formation in coral, the LOX domain of the AOS-LOX fusion protein binds membrane in a Ca^{2+}-dependent manner (Gilbert et al. 2008) and the enzymatic activity of AOS-LOX in vitro is enhanced by Ca^{2+} (Boutaud and Brash 1999). Elevated levels of α-ketol detected during thermal stress clearly illustrate the increased activity of AOS-LOX, orchestrated by cellular co-factors. In the early stages of cellular stress, the survival pathways are activated by stress mediators (Ainsworth et al. 2008). The mediating role can be attributed to the coral AOS-LOXa, as discussed above.

In conclusion, the coral AOS-LOXa is emerging as a sensitive early stress response gene induced by many biotic and abiotic stress factors, including elevated temperature.

Acknowledgments The authors gratefully acknowledge the valuable technical comments of Ivar Järving and Aleksander-Mati Müürisepp. This work was supported by Grant 0140010s08 and institutional research funding IUT 19-9 of the Estonian Ministry of Education and Research, and the Estonian Science Foundation Grant 9410 (all to NS).

References

Ainsworth TD, Hoegh-Guldberg O, Heron SF, Skirving WJ, Leggat W (2008) Early cellular changes are indicators of pre-bleaching thermal stress in the coral host. J Exp Mar Biol Ecol 364:63–71

Andreou A, Brodhun F, Feussner I (2009) Biosynthesis of oxylipins in non-mammals. Prog Lipid Res 48:148–170

Aranda M, Banaszak AT, Bayer T, Luyten JR, Medina M, Voolstra CR (2011) Differential sensitivity of coral larvae to natural levels of ultraviolet radiation during the onset of larval competence. Mol Ecol 20:2955–2972

Balogh G, Péter M, Glatz A, Gombos I, Török Z, Horváth I, Harwood JL, Vígh L (2013) Key role of lipids in heat stress management. FEBS Lett 587:1970–1980

Boutaud O, Brash AR (1999) Purification and catalytic activities of the two domains of the allene oxide synthase-lipoxygenase fusion protein of the coral *Plexaura homomalla*. J Biol Chem J Biol Chem 274:33764–33770

Brash AR, Baertschi SW, Ingram CD, Harris TM (1987) On non-cyclooxygenase prostaglandin synthesis in the sea whip coral, *Plexaura homomalla*: an 8(R)-lipoxygenase pathway leads to formation of an alpha-ketol and a Racemic prostanoid. J Biol Chem 262:15829–15839

Brash AR, Hughes MA, Hawkins DJ, Boeglin WE, Song WC, Meijer L (1991) Allene oxide and aldehyde biosynthesis in starfish oocytes. J Biol Chem 266:22926–22931

Brash AR, Boeglin WE, Stec DF, Voehler M, Schneider C, Cha JK (2013) Isolation and characterization of two geometric allene oxide isomers synthesized from 9S-hydroperoxylinoleic acid by cytochrome P450 CYP74C3: Stereochemical assignment of natural fatty acid allene oxides. J Biol Chem 288:20797–20806

Brown BE (1997) Coral bleaching: causes and consequences. Coral Reefs 16:S129–S138

Coe H, Michalak M (2009) Calcium binding chaperones of the endoplasmic reticulum. Gen Physiol Biophys 28 Spec No Focus:F96–F103

Corey EJ, Washburn WN, Chen JC (1973) Studies on the prostaglandin A 2 synthetase complex from *Plexaura homomalla*. J Am Chem Soc 95:2054–2055

Corey EJ, Dalarcao M, Matsuda SPT, Lansbury PT, Yamada Y (1987) Intermediacy of 8-(R)-HPETE in the conversion of arachidonic acid to pre-clavulone A by *Clavularia viridis*. Implications for the biosynthesis of marine prostanoids. J Am Chem Soc 109:289–290

DeSalvo MK, Voolstra CR, Sunagawa S, Schwarz JA, Stillman JH, Coffroth MA, Szmant AM, Medina M (2008) Differential gene expression during thermal stress and bleaching in the Caribbean coral *Montastraea faveolata*. Mol Ecol 17:3952–3971

Elia G (1996) 2-Cyclopenten-1-one, a new inducer of Heat Shock Protein 70 with antiviral activity. J Biol Chem 271:32192–32196

Ermak G, Davies KJ (2002) Calcium and oxidative stress: from cell signaling to cell death. Mol Immunol 38:713–721

Evans JH, Spencer DM, Zweifach A, Leslie CC (2001) Intracellular calcium signals regulating cytosolic phospholipase A2 translocation to internal membranes. J Biol Chem 276:30150–30160

Fang L-s, Huang S-p, Lin K-l (1997) High temperature induces the synthesis of heat-shock proteins and the elevation of intracellular calcium in the coral *Acropora grandis*. Coral Reefs 16:127–131

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282

Feissner RF, Skalska J, Gaum WE, Sheu SS (2009) Crosstalk signaling between mitochondrial Ca2+ and ROS. Front Biosci (Landmark Ed) 14:1197–1218

Figueiredo J, Baird AH, Cohen MF, Flot JF, Kamiki T, Meziane T, Tsuchiya M, Yamasaki H (2012) Ontogenetic change in the lipid and fatty acid composition of scleractinian coral larvae. Coral Reefs 31:613–619

Fitt WK, Gates RD, Hoegh-Guldberg O, Bythell JC, Jatkar A, Grottoli AG, Gomez M, Fisher P, Lajuenesse TC, Pantos O, Iglesias-Prieto R, Franklin DJ, Rodrigues LJ, Torregiani JM, van Woesik R, Lesser MP (2009) Response of two species of Indo-Pacific corals, *Porites cylindrica* and *Stylophora pistillata*, to short-term thermal stress: The host does matter in determining the tolerance of corals to bleaching. J Exp Mar Biol Ecol 373:102–110

Gerwick WH, Moghaddam M, Hamberg M (1991) Oxylipin metabolism in the red alga *Gracilariopsis lemaneiformis*: Mechanism of formation of vicinal dihydroxy fatty acids. Arch Biochem Biophys 290:436–444

Gilbert NC, Niebuhr M, Tsuruta H, Bordelon T, Ridderbusch O, Dassey A, Brash AR, Bartlett SG, Newcomer ME (2008) A covalent linker allows for membrane targeting of an oxylipin biosynthetic complex†‡. Biochemistry 47:10665–10676

Glynn PW (1990) Coral mortality and disturbances to coral reefs in the Tropical Eastern Pacific. In: Glynn PW (ed) Global ecological consequences of the 1980–83 El Nino-Southern Oscillation. Elsevier Oceanography Series 52, pp 44–126

Harden TK, Sondek J (2006) Regulation of phospholipase C isozymes by ras superfamily GTPases. Annu Rev Pharmacol Toxicol 46:355–379

Higdon A, Diers AR, Oh JY, Landar A, Darley-Usmar VM (2012) Cell signalling by reactive lipid species: new concepts and molecular mechanisms. Biochem J 442:453–464

Hoegh-Guldberg O, Mumby PJ, Hooten AJ, Steneck RS, Greenfield P, Gomez E, Harvell CD, Sale PF, Edwards AJ, Caldeira K, Knowlton N, Eakin CM, Iglesias-Prieto R, Muthiga N, Bradbury RH, Dubi A, Hatziolos ME (2007) Coral reefs under rapid climate change and ocean acidification. Science 318:1737–1742

Hollien J (2013) Evolution of the unfolded protein response. Biochim Biophys Acta 1833:2458–2463

Imbs AB, Yakovleva IM (2011) Dynamics of lipid and fatty acid composition of shallow-water corals under thermal stress: an experimental approach. Coral Reefs 31:41–53

Jacobs AT, Marnett LJ (2007) Heat Shock Factor 1 attenuates 4-hydroxynonenal-mediated apoptosis: Critical role for Heat Shock Protein 70 induction and stabililization of Bcl-XL. J Biol Chem 282:33412–33420

Jokiel PL, Coles SL (1990) Response of Hawaiian and other Indo-Pacific reef corals to elevated temperature. Coral Reefs 8:155–162

Jurivich DA, Pangas S, Qiu L, Welk JF (1996) Phospholipase A2 triggers the first phase of the thermal stress response and exhibits cell-type specificity. J Immunol 157:1669–1677

Kenkel CD, Aglyamova G, Alamaru A, Bhagooli R, Capper R, Cunning R, deVillers A, Haslun JA, Hedouin L, Keshavmurthy S, Kuehl KA, Mahmoud H, McGinty ES, Montoya-Maya PH, Palmer CV, Pantile R, Sanchez JA, Schils T, Silverstein RN, Squiers LB, Tang PC, Goulet TL, Matz MV (2011) Development of gene expression markers of acute heat-light stress in reef-building corals of the genus *Porites*. PLoS ONE 6:e26914

Koljak R, Boutaud O, Shieh BH, Samel N, Brash AR (1997) Identification of a naturally occurring peroxidase-lipoxygenase fusion protein. Science 277:1994–1996

Koljak R, Jarving I, Kurg R, Boeglin WE, Varvas K, Valmsen K, Ustav M, Brash AR, Samel N (2001) The basis of prostaglandin synthesis in coral: molecular cloning and expression of a cyclooxygenase from the Arctic soft coral *Gersemia fruticosa*. J Biol Chem 276:7033–7040

Kultz D (2005) Molecular and evolutionary basis of the cellular stress response. Annu Rev Physiol 67:225–257

Lee AS (2005) The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods 35:373–381

Lee DS, Nioche P, Hamberg M, Raman CS (2008) Structural insights into the evolutionary paths of oxylipin biosynthetic enzymes. Nature 455:363–368

Lesser MP (1997) Oxidative stress causes coral bleaching during exposure to elevated temperatures. Coral Reefs 16:187–192

Lesser MP (2006) Oxidative stress in marine environments: biochemistry and physiological ecology. Annu Rev Physiol 68:253–278

Lesser MP, Farrell JH (2004) Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress. Coral Reefs 23:367–377

Lievremont JP, Rizzuto R, Hendershot L, Meldolesi J (1997) BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of the rapidly exchanging pool of Ca2+. J Biol Chem 272:30873–30879

Lindquist S (1986) The heat-shock response. Annu Rev Biochem 55:1151–1191

Lõhelaid H, Teder T, Tõldsepp K, Ekins M, Samel N (2014) Up-regulated expression of AOS-LOXa and increased eicosanoid synthesis in response to coral wounding. PLoS ONE 9:e89215

Lohelaid H, Jarving R, Valmsen K, Varvas K, Kreen M, Jarving I, Samel N (2008) Identification of a functional allene oxide synthase-lipoxygenase fusion protein in the soft coral *Gersemia fruticosa* suggests the generality of this pathway in octocorals. Biochim Biophys Acta 1780:315–321

Luan W, Li F, Zhang J, Wang B, Xiang J (2009) Cloning and expression of glucose regulated protein 78 (GRP78) in *Fenneropenaeus chinensis*. Mol Biol Rep 36:289–298

Meldolesi J, Pozzan T (1998) The endoplasmic reticulum Ca2+ store: a view from the lumen. Trends Biochem Sci 23:10–14

Meyer E, Aglyamova GV, Matz MV (2011) Profiling gene expression responses of coral larvae (*Acropora millepora*) to elevated temperature and settlement inducers using a novel RNA-Seq procedure. Mol Ecol 20:3599–3616

Mortimer M, Jarving R, Brash AR, Samel N, Jarving I (2006) Identification and characterization of an arachidonate 11R-lipoxygenase. Arch Biochem Biophys 445:147–155

Mydlarz LD, Jacobs RS (2006) An inducible release of reactive oxygen radicals in four species of gorgonian corals. Mar Freshw Behav Physiol 39:143–152

Nevalainen TJ, Peuravuori HJ, Quinn RJ, Llewellyn LE, Benzie JA, Fenner PJ, Winkel KD (2004) Phospholipase A2 in cnidaria. Comp Biochem Physiol 139:731–735

Parker R, Phan T, Baumeister P, Roy B, Cheriyath V, Roy AL, Lee AS (2001) Identification of TFII-I as the endoplasmic reticulum stress response element binding factor ERSF: its autoregulation by stress and interaction with ATF6. Mol Cell Biol 21:3220–3233

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515

Polato NR, Vera JC, Baums IB (2011) Gene discovery in the threatened elkhorn coral: 454 sequencing of the *Acropora palmata* transcriptome. PLoS ONE 6:e28634

Polato NR, Altman NS, Baums IB (2013) Variation in the transcriptional response of threatened coral larvae to elevated temperatures. Mol Ecol 22:1366–1382

Radogna F, Sestili P, Martinelli C, Paolillo M, Paternoster L, Albertini MC, Accorsi A, Gualandi G, Ghibelli L (2009) Lipoxygenase-mediated pro-radical effect of melatonin via stimulation of arachidonic acid metabolism. Toxicol Appl Pharmacol 238:170–177

Resendez E Jr, Wooden SK, Lee AS (1988) Identification of highly conserved regulatory domains and protein-binding sites in the promoters of the rat and human genes encoding the stress-inducible 78-kilodalton glucose-regulated protein. Mol Cell Biol 8:4579–4584

Rodriguez-Lanetty M, Harii S, Hoegh-Guldberg O (2009) Early molecular responses of coral larvae to hyperthermal stress. Mol Ecol 18:5101–5114

Romero L, Marcussi S, Marchi-Salvador DP, Silva FP Jr, Fuly AL, Stabeli RG, da Silva SL, Gonzalez J, Monte AD, Soares AM (2010) Enzymatic and structural characterization of a basic phospholipase A(2) from the sea anemone *Condylactis gigantea*. Biochimie 92:1063–1071

Rowley AF, Vogan CL, Taylor GW, Clare AS (2005) Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems. J Exp Biol 208:3–14

Schaloske RH, Dennis EA (2006) The phospholipase A2 superfamily and its group numbering system. Biochim Biophys Acta 1761:1246–1259

Schneider C, Pratt DA, Porter NA, Brash AR (2007) Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. Chem Biol 14:473–488

Schroder M, Kaufman RJ (2005) ER stress and the unfolded protein response. Mutat Res 569:29–63

Seneca FO, Foret S, Ball EE, Smith-Keune C, Miller DJ, van Oppen MJ (2010) Patterns of gene expression in a scleractinian coral undergoing natural bleaching. Mar Biotechnol (NY) 12:594–604

Serhan CN, Haeggstrom JZ, Leslie CC (1996) Lipid mediator networks in cell signaling: update and impact of cytokines. FASEB J 10:1147–1158

Söderhäll K, Libro S, Kaluziak ST, Vollmer SV (2013) RNA-seq profiles of immune related genes in the staghorn coral *Acropora cervicornis* infected with White Band Disease. PLoS ONE 8:e81821

Søørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecol Lett 6:1025–1037

Souter P, Bay LK, Andreakis N, Csaszar N, Seneca FO, van Oppen MJ (2011) A multilocus, temperature stress-related gene expression profile assay in *Acropora millepora*, a dominant reef-building coral. Mol Ecol Resources 11:328–334

Stintzi A, Weber H, Reymond P, Browse J, Farmer EE (2001) Plant defense in the absence of jasmonic acid: the role of cyclopentenones. Proc Natl Acad Sci USA 98:12837–12842

Szabadkai G, Rizzuto R (2007) Chaperones as parts of organelle networks. Adv Exp Med Biol 594:64–77

Tu BP, Weissman JS (2004) Oxidative protein folding in eukaryotes: mechanisms and consequences. J Cell Biol 164:341–346

Upchurch RG (2008) Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. Biotechnol Lett 30:967–977

Valmsen K, Boeglin WE, Jarving I, Schneider C, Varvas K, Brash AR, Samel N (2004) Structural and functional comparison of 15S- and 15R-specific cyclooxygenases from the coral *Plexaura homomalla*. Eur J Biochem 271:3533–3538

van Oppen MJ, Gates RD (2006) Conservation genetics and the resilience of reef-building corals. Mol Ecol 15:3863–3883

Varvas K, Koljak R, Järving I, Pehk T, Samel N (1994) Endoperoxide pathway in prostaglandin biosynthesis in the soft coral *Gersemia fruticosa*. Tetrahedron Lett 35:8267–8270

Wasternack C, Kombrink E (2010) Jasmonates: structural requirements for lipid-derived signals active in plant stress responses and development. ACS Chem Biol 5:63–77

Wasternack C, Hause B (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann Bot 111:1021–1058

West JD, Wang Y, Morano KA (2012) Small molecule activators of the heat shock response: chemical properties, molecular targets, and therapeutic promise. Chem Res Toxicol 25:2036–2053

Voolstra CR, Dunn SR, Thomas MC, Nette GW, Dove SG (2012) A lipidomic approach to understanding free fatty acid lipogenesis derived from dissolved inorganic carbon within cnidarian-dinoflagellate symbiosis. PLoS ONE 7:e46801

Voolstra CR, Schnetzer J, Peshkin L, Randall CJ, Szmant AM, Medina M (2009) Effects of temperature on gene expression in embryos of the coral *Montastraea faveolata*. BMC Genomics 10:627

Wymann MP, Schneiter R (2008) Lipid signalling in disease. Nat Rev Mol Cell Biol 9:162–176

Zhong M, Orosz A, Wu C (1998) Direct sensing of heat and oxidation by *Drosophila* heat shock transcription factor. Mol Cell 2:101–108

ACKNOWLEDGEMENTS

"If you do not change direction, you may end up where you are heading."
– Lao Tzu

This study was carried out in the Chair of Bioorganic Chemistry of the Faculty of Science at Tallinn University of Technology (TUT).

I would like to thank:

- My supervisor, Professor Nigulas Samel for providing the space, facilities and support throughout the years.
- My partner, for his endless support, inspiration, guidance and constructive discussions.
- All colleagues, for their contribution to my work.
- My family and dear friends, for bringing out the light at many facets of my life.
- My children, Helika and Ülari for being a source of creativity and energy, keeping up a constant fire- burning brightly at both ends…, bringing me nothing but love, joy and pride.

ABSTRACT

In mammals and plants, oxylipins are recognized as important stress mediators. The allene oxide synthase-lipoxygenase (AOS-LOX) fusion protein, comprised of allene oxide synthase (AOS) and 8*R*-lipoxygenase (LOX) domains, is the central enzyme metabolizing arachidonic acid (AA) in certain corals. Recombinant AOS-LOX of *P. homomalla* converts AA via 8*R*-HpETE to a labile allene oxide, which spontaneously decomposes to the stable end-products α-ketol and cyclopentenone. However, the distribution and role of AOS-LOX and its metabolites in coral homeostasis *in vivo* remain unclear. In this thesis, the AOS-LOX pathway in the soft corals *G. fruticosa* and *C. imbricata* was resolved by biochemical and molecular strategies. As a central hypothesis, the up-regulation of gene expression and metabolite synthesis of coral AOS-LOX during the coral stress response to mechanical injury and thermal stress were established in *C. imbricata in vivo*.

An AOS-LOX was cloned and characterized as a functional enzyme from the Arctic coral *G. fruticosa***.** Recombinant AOS-LOX converted exogenous AA to α-ketol and cyclopentenone, identical with the products of *P. homomalla* AOS-LOX. However, when the Indo-Pacific coral *C. imbricata* was tested for AA conversion, additional novel products, designated UPC, were detected, consistent with C8 oxo-acid based on HPLC/MSMS analysis. Two AOS-LOX isoforms (*AOS-LOXa* and *AOS-LOXb*) which share 88% amino acid sequence were cloned from *C. imbricata,* and expressed as active fusion enzymes in *E. coli.* While the products of the recombinant AOS-LOXa were identified as α-ketol and cyclopentenone (70% and 14%, respectively), the AOS-LOXb products (90%) were identical to the previously detected UPC/C8 oxo-acid. The formation of C8 oxo-acid thus indicates the presence of a novel, plant hydroperoxide lyase-like activity in corals.

To assess the role of AOS-LOX in the coral stress response, mRNA of AOS-LOX isoforms and corresponding metabolite levels in *C. imbricata* under normal and incision wound conditions were determined by qPCR and HPLC, respectively. Wounding caused an increase in α-ketol and decrease in UPC level, with an increase in *AOS-LOXa* transcript levels in the tissue adjacent to the wound, as well as in distal tissues. Conversely, *AOS-LOXb* expression remained stable. Likewise, exclusive *AOS-LOXa* and downstream metabolite synthesis was increased in response to elevated water temperature, another environmentally relevant stressor. Two HSPs (*Hsp70* and *Grp78*), used as positive indicators of coral thermal stress were up-regulated concurrently with *AOS-LOXa*. In contrast to HSPs, *AOS-LOXa* was more responsive to moderate stress. Our results and data from literature suggest AOS-LOXa-produced eicosanoids are parts of a general stress response of corals, with Hsp70 as an indicator of heat and oxidative stress and Grp78 of ER stress.

In conclusion, the increase in α-ketol synthesis, both *in vitro* and *in vivo,* indicates that *AOS-LOXa* is a sensitive response element to moderate stress.

KOKKUVÕTE

Taimedes ja selgroogsetes organismides on oksülipiinid stressi vahendavad signaalmolekulid. Korallides leiduv alleenoksiidi süntaasist ja 8*R*-lipoksügenaasist koosnev liitvalk (AOS-LOX) on kesksel kohal nende organismide arahhidoonhappe (AA) metabolismis. Pehme koralli *Plexaura homomalla* AOS-LOX katalüüsib AAst läbi 8*R*-HpETE vaheühendi, labiilse alleenoksiidi (AO) sünteesi, mis vesikeskkonnas laguneb mitte-ensümaatiliselt stabiilseteks lõpp-produktideks, peamiselt α-ketooliks ja tsüklopentenooniks. Tänaseni on aga selgusetu funktsionaalse AOS-LOX raja olemasolu erinevates korallides ning sünteesitud produktide bioloogiline roll koralli homöostaasis. Käesolevas töös uuriti pehmetes korallides *Gersemia fruticosa* ja *Capnella imbricata* leiduvaid AOS-LOXi radu. Otsiti sobivat mudelorganismi *in vivo* stressikatsete jaoks ning testiti sobivaks osutunud korallis *C. imbricata* mehaanilise vigastuse ja temperatuuri mõju *AOS-LOX*-i geenide ekspressioonile ning vastavate metaboliitide sünteesile.

Esmalt tehti kindlaks funktsionaalse AOS-LOX-i olemasolu arktilises korallis *G. fruticosa*. Selleks kloneeriti ja ekspresseeriti *G. fruticosa* AOS-LOX ning kasutades kromatograafilisi meetodeid (HPLC) identifitseeriti liitvalgu poolt eksogeensest AA-st sünteesitud vahe- ning lõpp-produktid. Detekteeritud ühendid osutusid identseteks *P. homomalla* AOS-LOX liitvalgu produktidega. Sarnaselt määrati tekkivate produktide muster eksogeense AA inkubatsioonil *C. imbricata* koehomogenaadiga. Lisaks tuntud AOS-LOX-i raja produktidele sünteesiti AA-st ka tundmatud polaarsed ühendid (UPC - *unidentified polar compounds*), mis identifitseeriti ühendi väljumisaja ja massi-spektri järgi kui 8-oksoheksaeenhape. Seejärel kloneeriti korallist *C. imbricata* kaks *AOS-LOX*-i isovormi, *AOS-LOXa* ja *AOS-LOXb*. Vastavate liitvalkude tuletatud aminohappe järjestused on identsed 88% ulatuses. Mõlemad isovormid ekspresseeriti funktsionaalsena *E. coli* rakukultuuris. Rekombinantse AOS-LOXa peamiste produktidena identifitseeriti α-ketool ja tsüklopentenoon (vastavalt 70% ja 14%). Erinevalt oodatust detekteeriti AOS-LOXb produktidena ainult polaarseid ühendeid (90%), mis langesid kokku koralli homogenaadi inkubatsioonil detekteeritud UPC-ga. Ensüümi poolt spetsiifiliselt katalüüsitud 8-oksoheksaeenhappe tekkimine tõendab uudse, seni taimedele omase hüdroperoksiidlüaasse aktiivsuse olemasolu tuvastamist korallis.

Stressi mõju uurimiseks määrati *C. imbricata AOS-LOX*-i isovormide mRNA tasemed ning vastavate valkude poolt sünteesitud metaboliitide sisaldus nii koralli normaalolekus kui ka stressi tingimustes, kasutades vastavalt qPCR ja HPLC meetodeid. Vigastamise järgselt suurenes eksogeense AA konversioon α-ketooliks, samal ajal kui UPC sisaldus langes. Lõikehaava lähedal täheldati *AOS-LOXa* ekspressioonitaseme märkimisväärset tõusu juba üks tund peale vigastamist. Samas, *AOS-LOXb* ekspressiooni vigastamine ei mõjutanud. Vigastamisele sarnast stressivastust detekteeriti ka kõrgenenud veetemperatuuri puhul, kus samuti indutseeriti ainult *AOS-LOXa* ekspressioon ja vastavate

metaboliitide süntees. Seejuures, suurem tõus *AOS-LOXa* geenieksprессioonis detekteeriti vastuseks nõrgemale stressile. Samas, kui geenieksprессioon oli kõrgem nõrgema stressi korral, siis veidi suuremat α-ketooli taset detekteeriti kõrgema temperatuuri elik tugevama stressi korral.

AOS-LOXide kõrval kasutati positiivse temperatuuristressi indikaatorina koralli kuumašhoki valke, *Hsp70* ja *Grp78*. Tuginedes enda ja kirjanduse andmetele pakuti välja võimalik skeem AOS-LOX rollist koralli stressivastuses, kus Hsp70 vahendab oksüdatiivset ja termaalset stressi, Grp78 ER stressi ning AOS-LOXa produktided osalevad üldise stressivastuse vahendamise esmases faasis.

Kokkuvõtteks, AOS-LOX rada on laialt levinud nii pehmetes kui ka kõvades korallides. Töös kirjeldatud muutused, nii *AOS-LOXa* transkriptsiooni kui ka oksülipiinide tasemetes, viitavad AOS-LOX-i raja olulisusele korallide esmases stressi vastuses.

CURRICULUM VITAE

Name:	Helike Lõhelaid
Date of birth:	30.03.1979
Nationality:	Estonian
Citizenship:	Estonian
Contact information:	Department of Chemistry, Tallinn University of Technology, Akadeemia tee 15/1, 12618 Tallinn, Tel: +372 620 4386, e-mail: helike.lohelaid@ttu.ee

Education:
2003 - 2008 PhD Student; Dept. of Chemistry, Tallinn University of Technology
2001 - 2003 Dept. of Chemistry, Tallinn University of Technology (MSc)
1997 - 2001 Institute of Molecular and Cell Biology, University of Tartu (BSc), molecular biology and genetics

Employment:
01/09/2008 - ... Tallinn University of Technology, Faculty of Science, Department of Chemistry, Chair of Bioorganic Chemistry; Researcher
2008 - 31/08/2008 Tallinn University of Technology , Faculty of Science, Department of Chemistry, Chair of Bioorganic Chemistry; Extraordinary Researcher
2005 - 2007 Tallinn University of Technology, Faculty of Science, Department of Chemistry; Other staff

Dissertations supervised:
Tähti Kull, Bachelor's Degree, 2014, (sup) Helike Lõhelaid, Tarvi Teder, Molecular cloning of lipoxygenases from the soft coral *Capnella imbricata*, Tallinn University of Technology, Department of Chemistry

Ken Veski, Master's Degree, 2013, (sup) Helike Lõhelaid, Ivar Järving, Stress related eicosanoid synthesis in *Capnella sp.*, Tallinn University of Technology, Department of Chemistry

Tarvi Teder, Bachelor's Degree, 2012, (sup) Helike Lõhelaid, Identification of heat shock protein 70 family genes in coral *Capnella sp.* and detection of expression levels in response to wounding, Tallinn University of Technology, Department of Chemistry

Kadri Tõldsepp, Master's Degree, 2011, (sup) Helike Lõhelaid, Identification of AOS-LOX genes from coral *Capnella sp*. and their expression in response to wounding, Tallinn University of Technology, Department of Chemistry

Helin Veskiväli, Master's Degree, 2009, (sup) Helike Lõhelaid, Chimeric fusion proteins of allene oxide synthase-lipoxygenase, Tallinn University of Technology , Faculty of Science, Department of Chemistry

Kadri Tõldsepp, Bachelor's Degree, 2009, (sup) Helike Lõhelaid, Identification of fatty acids in corals, Tallinn University of Technology, Department of Chemistry

Helin Veskiväli, Bachelor's Degree, 2007, (sup) Helike Lõhelaid, Coral fusion proteins of allene oxide synthase-lipoxygenase, Tallinn University of Technology, Department of Chemistry

Publications:

Lõhelaid, H., Teder, T., Samel, N. (2014). Lipoxygenase-allene oxide synthase pathway in octocoral thermal stress response. Coral reefs DOI: 10.1007/s00338-014-1238-y

Lõhelaid, H., Teder, T., Tõldsepp, K., Ekins, M., Samel, N. (2014). Up-regulated expression of AOS-LOXa and increased eicosanoid synthesis in response to coral wounding. PLoS ONE 9(2): e89215

Varvas, K.; Kurg, R.; Hansen, K.; Järving, R.; Järving, I.; Valmsen, K.; **Lõhelaid, H.**; Samel, N. (2009). Direct evidence of the cyclooxygenase pathway of prostaglandin synthesis in arthropods: Genetic and biochemical characterization of two crustacean cyclooxygenases. Insect Biochemistry and Molecular Biology, 39(12), 851 - 860

Lõhelaid, H.; Järving, R.; Valmsen, K.; Varvas, K.; Kreen, M.; Järving, I.; Samel, N. (2008). Identification of a functional allene oxide synthase-lipoxygenase fusion protein in the soft coral Gersemia fruticosa suggests the generality of this pathway in octocorals. Biochimica et Biophysica Acta-General Subjects, 1780(2), 315 - 321

Published abstracts:

Lõhelaid, H.; Järving, R.; Järving, I.; Samel, N. (2010). Chimeric allene oxide synthase-lipoxygenase fusion proteins. FEBS Journal, 277(S1), 251

Lõhelaid, H.; Järving, R.; Samel, N. (2008). Generality of marine allene oxide synthesis by the allene oxide synthase-lipoxygenase fusion protein pathway. FEBS Journal, 275, 413

Conference presentations:

Lõhelaid, H.; Järving, R.; Järving, I.; Samel, N. (2010). Chimeric allene oxide synthase-lipoxygenase fusion proteins. FEBS Journal, 277(S1), 251

ELULOOKIRJELDUS

Nimi: Helike Lõhelaid
Sünniaeg: 30 märts 1979
Rahvus: Eestlane
Kodakondsus: Eesti
Kontaktandmed: Keemiainstituut, Tallinna Tehnikaülikool, Akadeemia tee 15/1, 12618 Tallinn, Tel: +372 620 4386, e-kiri: helike.lohelaid@ttu.ee

Haridustee:
2003 - 2008 Tallinna Tehnikaülikool, Keemiainstituut, doktorant
2001 - 2003 Tallinna Tehnikaülikool, Keemiainstituut, magistrikraad (MSc)
1997 - 2001 Tartu Ülikool, Bioloogia-geograafiateaduskond, (BSc) molekulaarbioloogia ja geneetika erialal

Töökoht ja amet:
01.09.2008 - ... Tallinna Tehnikaülikool, Keemiainstituut, Bioorgaanilise keemia õppetool; Teadur
2008 - 31.08.2008 Tallinna Tehnikaülikool, Keemiainstituut, Bioorgaanilise keemia õppetool; Erakorraline teadur
2005 - 2007 Tallinna Tehnikaülikool, Keemiainstituut, tehnik

Juhendatud lõputööd:
Tähti Kull, bakalaureusekraad, 2014, (juh) Helike Lõhelaid, Tarvi Teder, Osaliste lipoksügenaaside kloneerimine pehmest korallist *Capnella imbricata,* Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Keemiainstituut

Ken Veski, magistrikraad, 2013, (juh) Helike Lõhelaid, Ivar Järving, Stressist põhjustatud eikosanoidide taseme muutus korallis *Capnella sp*., Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Keemiainstituut

Tarvi Teder, bakalaureusekraad, 2012, (juh) Helike Lõhelaid, Vigastuse mõju Hsp70 kuumašoki valgu geenide ekspressioonile korallis *Capnella sp.*, Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Keemiainstituut

Kadri Tõldsepp, magistrikraad, 2011, (juh) Helike Lõhelaid, AOS-LOX geenide identifitseerimine korallis *Capnella sp*. ja vigastuse mõju nende ekspressioonitasemele, Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Keemiainstituut

Helin Veskiväli, magistrikraad, 2009, (juh) Helike Lõhelaid, Alleenoksiidi süntaas-lipoksügenaas kimäärsed rekombinantsed liitvalgud, Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Keemiainstituut

Kadri Tõldsepp, bakalaureusekraad, 2009, (juh) Helike Lõhelaid, Rasvhapete identifitseerimine korallides, Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Keemiainstituut

Helin Veskiväli, bakalaureusekraad, 2007, (juh) Helike Lõhelaid, Lipoksügenaas-alleenoksiidi süntaas liitvalgud korallides, Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Keemiainstituut

Publikatsioonid:

Lõhelaid, H., Teder, T., Samel, N. (2014). Lipoxygenase-allene oxide synthase pathway in octocoral thermal stress response. Coral reefs DOI: 10.1007/s00338-014-1238-y

Lõhelaid, H., Teder, T., Tõldsepp, K., Ekins, M., Samel, N. (2014). Up-regulated expression of AOS-LOXa and increased eicosanoid synthesis in response to coral wounding. PLoS ONE 9(2): e89215

Varvas, K.; Kurg, R.; Hansen, K.; Järving, R.; Järving, I.; Valmsen, K.; **Lõhelaid, H.**; Samel, N. (2009). Direct evidence of the cyclooxygenase pathway of prostaglandin synthesis in arthropods: Genetic and biochemical characterization of two crustacean cyclooxygenases. Insect Biochemistry and Molecular Biology, 39(12), 851 - 860

Lõhelaid, H.; Järving, R.; Valmsen, K.; Varvas, K.; Kreen, M.; Järving, I.; Samel, N. (2008). Identification of a functional allene oxide synthase-lipoxygenase fusion protein in the soft coral Gersemia fruticosa suggests the generality of this pathway in octocorals. Biochimica et Biophysica Acta-General Subjects, 1780(2), 315 - 321

Konverentsi teesid:

Lõhelaid, H.; Järving, R.; Järving, I.; Samel, N. (2010). Chimeric allene oxide synthase-lipoxygenase fusion proteins. FEBS Journal, 277(S1), 251

Lõhelaid, H.; Järving, R.; Samel, N. (2008). Generality of marine allene oxide synthesis by the allene oxide synthase-lipoxygenase fusion protein pathway. FEBS Journal, 275, 413

Suulised ettekanded:

Lõhelaid, H.; Järving, R.; Järving, I.; Samel, N. (2010). Chimeric allene oxide synthase-lipoxygenase fusion proteins. FEBS Journal, 277(S1), 251

DISSERTATIONS DEFENDED AT
TALLINN UNIVERSITY OF TECHNOLOGY ON
NATURAL AND EXACT SCIENCES

1. **Olav Kongas**. Nonlinear Dynamics in Modeling Cardiac Arrhytmias. 1998.

2. **Kalju Vanatalu**. Optimization of Processes of Microbial Biosynthesis of Isotopically Labeled Biomolecules and Their Complexes. 1999.

3. **Ahto Buldas**. An Algebraic Approach to the Structure of Graphs. 1999.

4. **Monika Drews**. A Metabolic Study of Insect Cells in Batch and Continuous Culture: Application of Chemostat and Turbidostat to the Production of Recombinant Proteins. 1999.

5. **Eola Valdre**. Endothelial-Specific Regulation of Vessel Formation: Role of Receptor Tyrosine Kinases. 2000.

6. **Kalju Lott**. Doping and Defect Thermodynamic Equilibrium in ZnS. 2000.

7. **Reet Koljak**. Novel Fatty Acid Dioxygenases from the Corals *Plexaura homomalla* and *Gersemia fruticosa*. 2001.

8. **Anne Paju**. Asymmetric oxidation of Prochiral and Racemic Ketones by Using Sharpless Catalyst. 2001.

9. **Marko Vendelin**. Cardiac Mechanoenergetics *in silico*. 2001.

10. **Pearu Peterson**. Multi-Soliton Interactions and the Inverse Problem of Wave Crest. 2001.

11. **Anne Menert**. Microcalorimetry of Anaerobic Digestion. 2001.

12. **Toomas Tiivel**. The Role of the Mitochondrial Outer Membrane in *in vivo* Regulation of Respiration in Normal Heart and Skeletal Muscle Cell. 2002.

13. **Olle Hints**. Ordovician Scolecodonts of Estonia and Neighbouring Areas: Taxonomy, Distribution, Palaeoecology, and Application. 2002.

14. **Jaak Nõlvak**. Chitinozoan Biostratigrapy in the Ordovician of Baltoscandia. 2002.

15. **Liivi Kluge**. On Algebraic Structure of Pre-Operad. 2002.

16. **Jaanus Lass**. Biosignal Interpretation: Study of Cardiac Arrhytmias and Electromagnetic Field Effects on Human Nervous System. 2002.

17. **Janek Peterson**. Synthesis, Structural Characterization and Modification of PAMAM Dendrimers. 2002.

18. **Merike Vaher**. Room Temperature Ionic Liquids as Background Electrolyte Additives in Capillary Electrophoresis. 2002.

19. **Valdek Mikli**. Electron Microscopy and Image Analysis Study of Powdered Hardmetal Materials and Optoelectronic Thin Films. 2003.

20. **Mart Viljus**. The Microstructure and Properties of Fine-Grained Cermets. 2003.

21. **Signe Kask**. Identification and Characterization of Dairy-Related *Lactobacillus.* 2003

22. **Tiiu-Mai Laht**. Influence of Microstructure of the Curd on Enzymatic and Microbiological Processes in Swiss-Type Cheese. 2003.

23. **Anne Kuusksalu**. 2–5A Synthetase in the Marine Sponge *Geodia cydonium*. 2003.

24. **Sergei Bereznev**. Solar Cells Based on Polycristalline Copper-Indium Chalcogenides and Conductive Polymers. 2003.

25. **Kadri Kriis**. Asymmetric Synthesis of C_2-Symmetric Bimorpholines and Their Application as Chiral Ligands in the Transfer Hydrogenation of Aromatic Ketones. 2004.

26. **Jekaterina Reut**. Polypyrrole Coatings on Conducting and Insulating Substracts. 2004.

27. **Sven Nõmm**. Realization and Identification of Discrete-Time Nonlinear Systems. 2004.

28. **Olga Kijatkina**. Deposition of Copper Indium Disulphide Films by Chemical Spray Pyrolysis. 2004.

29. **Gert Tamberg**. On Sampling Operators Defined by Rogosinski, Hann and Blackman Windows. 2004.

30. **Monika Übner**. Interaction of Humic Substances with Metal Cations. 2004.

31. **Kaarel Adamberg**. Growth Characteristics of Non-Starter Lactic Acid Bacteria from Cheese. 2004.

32. **Imre Vallikivi**. Lipase-Catalysed Reactions of Prostaglandins. 2004.

33. **Merike Peld**. Substituted Apatites as Sorbents for Heavy Metals. 2005.

34. **Vitali Syritski**. Study of Synthesis and Redox Switching of Polypyrrole and Poly(3,4-ethylenedioxythiophene) by Using *in-situ* Techniques. 2004.

35. **Lee Põllumaa**. Evaluation of Ecotoxicological Effects Related to Oil Shale Industry. 2004.

36. **Riina Aav**. Synthesis of 9,11-Secosterols Intermediates. 2005.

37. **Andres Braunbrück**. Wave Interaction in Weakly Inhomogeneous Materials. 2005.

38. **Robert Kitt**. Generalised Scale-Invariance in Financial Time Series. 2005.

39. **Juss Pavelson**. Mesoscale Physical Processes and the Related Impact on the Summer Nutrient Fields and Phytoplankton Blooms in the Western Gulf of Finland. 2005.

40. **Olari Ilison**. Solitons and Solitary Waves in Media with Higher Order Dispersive and Nonlinear Effects. 2005.

41. **Maksim Säkki**. Intermittency and Long-Range Structurization of Heart Rate. 2005.

42. **Enli Kiipli**. Modelling Seawater Chemistry of the East Baltic Basin in the Late Ordovician–Early Silurian. 2005.

43. **Igor Golovtsov**. Modification of Conductive Properties and Processability of Polyparaphenylene, Polypyrrole and polyaniline. 2005.

44. **Katrin Laos**. Interaction Between Furcellaran and the Globular Proteins (Bovine Serum Albumin β-Lactoglobulin). 2005.

45. **Arvo Mere**. Structural and Electrical Properties of Spray Deposited Copper Indium Disulphide Films for Solar Cells. 2006.

46. **Sille Ehala**. Development and Application of Various On- and Off-Line Analytical Methods for the Analysis of Bioactive Compounds. 2006.

47. **Maria Kulp**. Capillary Electrophoretic Monitoring of Biochemical Reaction Kinetics. 2006.

48. **Anu Aaspõllu.** Proteinases from *Vipera lebetina* Snake Venom Affecting Hemostasis. 2006.

49. **Lyudmila Chekulayeva**. Photosensitized Inactivation of Tumor Cells by Porphyrins and Chlorins. 2006.

50. **Merle Uudsemaa**. Quantum-Chemical Modeling of Solvated First Row Transition Metal Ions. 2006.

51. **Tagli Pitsi**. Nutrition Situation of Pre-School Children in Estonia from 1995 to 2004. 2006.

52. **Angela Ivask**. Luminescent Recombinant Sensor Bacteria for the Analysis of Bioavailable Heavy Metals. 2006.

53. **Tiina Lõugas**. Study on Physico-Chemical Properties and Some Bioactive Compounds of Sea Buckthorn (*Hippophae rhamnoides* L.). 2006.

54. **Kaja Kasemets**. Effect of Changing Environmental Conditions on the Fermentative Growth of *Saccharomyces cerevisae* S288C: Auxo-accelerostat Study. 2006.

55. **Ildar Nisamedtinov**. Application of ^{13}C and Fluorescence Labeling in Metabolic Studies of *Saccharomyces* spp. 2006.

56. **Alar Leibak**. On Additive Generalisation of Voronoï's Theory of Perfect Forms over Algebraic Number Fields. 2006.

57. **Andri Jagomägi**. Photoluminescence of Chalcopyrite Tellurides. 2006.

58. **Tõnu Martma**. Application of Carbon Isotopes to the Study of the Ordovician and Silurian of the Baltic. 2006.

59. **Marit Kauk**. Chemical Composition of $CuInSe_2$ Monograin Powders for Solar Cell Application. 2006.

60. **Julia Kois**. Electrochemical Deposition of $CuInSe_2$ Thin Films for Photovoltaic Applications. 2006.

61. **Ilona Oja Açik**. Sol-Gel Deposition of Titanium Dioxide Films. 2007.

62. **Tiia Anmann**. Integrated and Organized Cellular Bioenergetic Systems in Heart and Brain. 2007.

63. **Katrin Trummal**. Purification, Characterization and Specificity Studies of Metalloproteinases from *Vipera lebetina* Snake Venom. 2007.

64. **Gennadi Lessin**. Biochemical Definition of Coastal Zone Using Numerical Modeling and Measurement Data. 2007.

65. **Enno Pais**. Inverse problems to determine non-homogeneous degenerate memory kernels in heat flow. 2007.

66. **Maria Borissova**. Capillary Electrophoresis on Alkylimidazolium Salts. 2007.

67. **Karin Valmsen**. Prostaglandin Synthesis in the Coral *Plexaura homomalla*: Control of Prostaglandin Stereochemistry at Carbon 15 by Cyclooxygenases. 2007.

68. **Kristjan Piirimäe**. Long-Term Changes of Nutrient Fluxes in the Drainage Basin of the Gulf of Finland – Application of the PolFlow Model. 2007.

69. **Tatjana Dedova**. Chemical Spray Pyrolysis Deposition of Zinc Sulfide Thin Films and Zinc Oxide Nanostructured Layers. 2007.

70. **Katrin Tomson**. Production of Labelled Recombinant Proteins in Fed-Batch Systems in *Escherichia coli*. 2007.

71. **Cecilia Sarmiento**. Suppressors of RNA Silencing in Plants. 2008.

72. **Vilja Mardla**. Inhibition of Platelet Aggregation with Combination of Antiplatelet Agents. 2008.

73. **Maie Bachmann**. Effect of Modulated Microwave Radiation on Human Resting Electroencephalographic Signal. 2008.

74. **Dan Hüvonen**. Terahertz Spectroscopy of Low-Dimensional Spin Systems. 2008.

75. **Ly Villo**. Stereoselective Chemoenzymatic Synthesis of Deoxy Sugar Esters Involving *Candida antarctica* Lipase B. 2008.

76. **Johan Anton**. Technology of Integrated Photoelasticity for Residual Stress Measurement in Glass Articles of Axisymmetric Shape. 2008.

77. **Olga Volobujeva**. SEM Study of Selenization of Different Thin Metallic Films. 2008.

78. **Artur Jõgi**. Synthesis of 4'-Substituted 2,3'-dideoxynucleoside Analogues. 2008.

79. **Mario Kadastik**. Doubly Charged Higgs Boson Decays and Implications on Neutrino Physics. 2008.

80. **Fernando Pérez-Caballero**. Carbon Aerogels from 5-Methylresorcinol-Formaldehyde Gels. 2008.

81. **Sirje Vaask**. The Comparability, Reproducibility and Validity of Estonian Food Consumption Surveys. 2008.

82. **Anna Menaker**. Electrosynthesized Conducting Polymers, Polypyrrole and Poly(3,4-ethylenedioxythiophene), for Molecular Imprinting. 2009.

83. **Lauri Ilison**. Solitons and Solitary Waves in Hierarchical Korteweg-de Vries Type Systems. 2009.

84. **Kaia Ernits**. Study of In_2S_3 and ZnS Thin Films Deposited by Ultrasonic Spray Pyrolysis and Chemical Deposition. 2009.

85. **Veljo Sinivee**. Portable Spectrometer for Ionizing Radiation "Gammamapper". 2009.

86. **Jüri Virkepu**. On Lagrange Formalism for Lie Theory and Operadic Harmonic Oscillator in Low Dimensions. 2009.

87. **Marko Piirsoo**. Deciphering Molecular Basis of Schwann Cell Development. 2009.

88. **Kati Helmja**. Determination of Phenolic Compounds and Their Antioxidative Capability in Plant Extracts. 2010.

89. **Merike Sõmera**. Sobemoviruses: Genomic Organization, Potential for Recombination and Necessity of P1 in Systemic Infection. 2010.

90. **Kristjan Laes**. Preparation and Impedance Spectroscopy of Hybrid Structures Based on $CuIn_3Se_5$ Photoabsorber. 2010.

91. **Kristin Lippur**. Asymmetric Synthesis of 2,2'-Bimorpholine and its 5,5'-Substituted Derivatives. 2010.

92. **Merike Luman**. Dialysis Dose and Nutrition Assessment by an Optical Method. 2010.

93. **Mihhail Berezovski**. Numerical Simulation of Wave Propagation in Heterogeneous and Microstructured Materials. 2010.

94. **Tamara Aid-Pavlidis**. Structure and Regulation of BDNF Gene. 2010.

95. **Olga Bragina**. The Role of Sonic Hedgehog Pathway in Neuro- and Tumorigenesis. 2010.

96. **Merle Randrüüt**. Wave Propagation in Microstructured Solids: Solitary and Periodic Waves. 2010.

97. **Marju Laars**. Asymmetric Organocatalytic Michael and Aldol Reactions Mediated by Cyclic Amines. 2010.

98. **Maarja Grossberg**. Optical Properties of Multinary Semiconductor Compounds for Photovoltaic Applications. 2010.

99. **Alla Maloverjan**. Vertebrate Homologues of Drosophila Fused Kinase and Their Role in Sonic Hedgehog Signalling Pathway. 2010.

100. **Priit Pruunsild**. Neuronal Activity-Dependent Transcription Factors and Regulation of Human *BDNF* Gene. 2010.

101. **Tatjana Knjazeva**. New Approaches in Capillary Electrophoresis for Separation and Study of Proteins. 2011.

102. **Atanas Katerski**. Chemical Composition of Sprayed Copper Indium Disulfide Films for Nanostructured Solar Cells. 2011.

103. **Kristi Timmo.** Formation of Properties of $CuInSe_2$ and $Cu_2ZnSn(S,Se)_4$ Monograin Powders Synthesized in Molten KI. 2011.
104. **Kert Tamm**. Wave Propagation and Interaction in Mindlin-Type Microstructured Solids: Numerical Simulation. 2011.
105. **Adrian Popp**. Ordovician Proetid Trilobites in Baltoscandia and Germany. 2011.
106. **Ove Pärn**. Sea Ice Deformation Events in the Gulf of Finland and This Impact on Shipping. 2011.
107. **Germo Väli**. Numerical Experiments on Matter Transport in the Baltic Sea. 2011.
108. **Andrus Seiman**. Point-of-Care Analyser Based on Capillary Electrophoresis. 2011.
109. **Olga Katargina**. Tick-Borne Pathogens Circulating in Estonia (Tick-Borne Encephalitis Virus, *Anaplasma phagocytophilum*, *Babesia* Species): Their Prevalence and Genetic Characterization. 2011.
110. **Ingrid Sumeri**. The Study of Probiotic Bacteria in Human Gastrointestinal Tract Simulator. 2011.
111. **Kairit Zovo**. Functional Characterization of Cellular Copper Proteome. 2011.
112. **Natalja Makarytsheva**. Analysis of Organic Species in Sediments and Soil by High Performance Separation Methods. 2011.
113. **Monika Mortimer**. Evaluation of the Biological Effects of Engineered Nanoparticles on Unicellular Pro- and Eukaryotic Organisms. 2011.
114. **Kersti Tepp**. Molecular System Bioenergetics of Cardiac Cells: Quantitative Analysis of Structure-Function Relationship. 2011.
115. **Anna-Liisa Peikolainen**. Organic Aerogels Based on 5-Methylresorcinol. 2011.
116. **Leeli Amon**. Palaeoecological Reconstruction of Late-Glacial Vegetation Dynamics in Eastern Baltic Area: A View Based on Plant Macrofossil Analysis. 2011.
117. **Tanel Peets**. Dispersion Analysis of Wave Motion in Microstructured Solids. 2011.
118. **Liina Kaupmees**. Selenization of Molybdenum as Contact Material in Solar Cells. 2011.
119. **Allan Olspert**. Properties of VPg and Coat Protein of Sobemoviruses. 2011.
120. **Kadri Koppel**. Food Category Appraisal Using Sensory Methods. 2011.
121. **Jelena Gorbatšova**. Development of Methods for CE Analysis of Plant Phenolics and Vitamins. 2011.
122. **Karin Viipsi**. Impact of EDTA and Humic Substances on the Removal of Cd and Zn from Aqueous Solutions by Apatite. 2012.
123. **David Schryer**. Metabolic Flux Analysis of Compartmentalized Systems Using Dynamic Isotopologue Modeling. 2012.
124. **Ardo Illaste**. Analysis of Molecular Movements in Cardiac Myocytes. 2012.
125. **Indrek Reile**. 3-Alkylcyclopentane-1,2-Diones in Asymmetric Oxidation and Alkylation Reactions. 2012.
126. **Tatjana Tamberg**. Some Classes of Finite 2-Groups and Their Endomorphism Semigroups. 2012.

127. **Taavi Liblik**. Variability of Thermohaline Structure in the Gulf of Finland in Summer. 2012.
128. **Priidik Lagemaa**. Operational Forecasting in Estonian Marine Waters. 2012.
129. **Andrei Errapart**. Photoelastic Tomography in Linear and Non-linear Approximation. 2012.
130. **Külliki Krabbi**. Biochemical Diagnosis of Classical Galactosemia and Mucopolysaccharidoses in Estonia. 2012.
131. **Kristel Kaseleht**. Identification of Aroma Compounds in Food using SPME-GC/MS and GC-Olfactometry. 2012.
132. **Kristel Kodar**. Immunoglobulin G Glycosylation Profiling in Patients with Gastric Cancer. 2012.
133. **Kai Rosin**. Solar Radiation and Wind as Agents of the Formation of the Radiation Regime in Water Bodies. 2012.
134. **Ann Tiiman**. Interactions of Alzheimer's Amyloid-Beta Peptides with Zn(II) and Cu(II) Ions. 2012.
135. **Olga Gavrilova**. Application and Elaboration of Accounting Approaches for Sustainable Development. 2012.
136. **Olesja Bondarenko**. Development of Bacterial Biosensors and Human Stem Cell-Based *In Vitro* Assays for the Toxicological Profiling of Synthetic Nanoparticles. 2012.
137. **Katri Muska**. Study of Composition and Thermal Treatments of Quaternary Compounds for Monograin Layer Solar Cells. 2012.
138. **Ranno Nahku**. Validation of Critical Factors for the Quantitative Characterization of Bacterial Physiology in Accelerostat Cultures. 2012.
139. **Petri-Jaan Lahtvee**. Quantitative Omics-level Analysis of Growth Rate Dependent Energy Metabolism in *Lactococcus lactis*. 2012.
140. **Kerti Orumets**. Molecular Mechanisms Controlling Intracellular Glutathione Levels in Baker's Yeast *Saccharomyces cerevisiae* and its Random Mutagenized Glutathione Over-Accumulating Isolate. 2012.
141. **Loreida Timberg**. Spice-Cured Sprats Ripening, Sensory Parameters Development, and Quality Indicators. 2012.
142. **Anna Mihhalevski**. Rye Sourdough Fermentation and Bread Stability. 2012.
143. **Liisa Arike**. Quantitative Proteomics of *Escherichia coli*: From Relative to Absolute Scale. 2012.
144. **Kairi Otto**. Deposition of In_2S_3 Thin Films by Chemical Spray Pyrolysis. 2012.
145. **Mari Sepp**. Functions of the Basic Helix-Loop-Helix Transcription Factor TCF4 in Health and Disease. 2012.
146. **Anna Suhhova**. Detection of the Effect of Weak Stressors on Human Resting Electroencephalographic Signal. 2012.
147. **Aram Kazarjan**. Development and Production of Extruded Food and Feed Products Containing Probiotic Microorganisms. 2012.
148. **Rivo Uiboupin**. Application of Remote Sensing Methods for the Investigation of Spatio-Temporal Variability of Sea Surface Temperature and Chlorophyll Fields in the Gulf of Finland. 2013.
149. **Tiina Kriščiunaite**. A Study of Milk Coagulability. 2013.

150. **Tuuli Levandi**. Comparative Study of Cereal Varieties by Analytical Separation Methods and Chemometrics. 2013.
151. **Natalja Kabanova**. Development of a Microcalorimetric Method for the Study of Fermentation Processes. 2013.
152. **Himani Khanduri**. Magnetic Properties of Functional Oxides. 2013.
153. **Julia Smirnova**. Investigation of Properties and Reaction Mechanisms of Redox-Active Proteins by ESI MS. 2013.
154. **Mervi Sepp**. Estimation of Diffusion Restrictions in Cardiomyocytes Using Kinetic Measurements. 2013.
155. **Kersti Jääger**. Differentiation and Heterogeneity of Mesenchymal Stem Cells. 2013.
156. **Victor Alari**. Multi-Scale Wind Wave Modeling in the Baltic Sea. 2013.
157. **Taavi Päll**. Studies of CD44 Hyaluronan Binding Domain as Novel Angiogenesis Inhibitor. 2013.
158. **Allan Niidu**. Synthesis of Cyclopentane and Tetrahydrofuran Derivatives. 2013.
159. **Julia Geller**. Detection and Genetic Characterization of *Borrelia* Species Circulating in Tick Population in Estonia. 2013.
160. **Irina Stulova**. The Effects of Milk Composition and Treatment on the Growth of Lactic Acid Bacteria. 2013.
161. **Jana Holmar**. Optical Method for Uric Acid Removal Assessment During Dialysis. 2013.
162. **Kerti Ausmees**. Synthesis of Heterobicyclo[3.2.0]heptane Derivatives *via* Multicomponent Cascade Reaction. 2013.
163. **Minna Varikmaa**. Structural and Functional Studies of Mitochondrial Respiration Regulation in Muscle Cells. 2013.
164. **Indrek Koppel**. Transcriptional Mechanisms of BDNF Gene Regulation. 2014.
165. **Kristjan Pilt**. Optical Pulse Wave Signal Analysis for Determination of Early Arterial Ageing in Diabetic Patients. 2014.
166. **Andres Anier**. Estimation of the Complexity of the Electroencephalogram for Brain Monitoring in Intensive Care. 2014.
167. **Toivo Kallaste**. Pyroclastic Sanidine in the Lower Palaeozoic Bentonites – A Tool for Regional Geological Correlations. 2014.
168. **Erki Kärber**. Properties of ZnO-nanorod/In_2S_3/$CuInS_2$ Solar Cell and the Constituent Layers Deposited by Chemical Spray Method. 2014.
169. **Julia Lehner**. Formation of Cu_2ZnSnS_4 and $Cu_2ZnSnSe_4$ by Chalcogenisation of Electrochemically Deposited Precursor Layers. 2014.
170. **Peep Pitk**. Protein- and Lipid-rich Solid Slaughterhouse Waste Anaerobic Co-digestion: Resource Analysis and Process Optimization. 2014.
171. **Kaspar Valgepea**. Absolute Quantitative Multi-omics Characterization of Specific Growth Rate-dependent Metabolism of *Escherichia coli*. 2014.
172. **Artur Noole**. Asymmetric Organocatalytic Synthesis of 3,3'-Disubstituted Oxindoles. 2014.
173. **Robert Tsanev**. Identification and Structure-Functional Characterisation of the Gene Transcriptional Repressor Domain of Human Gli Proteins. 2014.
174. **Dmitri Kartofelev**. Nonlinear Sound Generation Mechanisms in Musical Acoustic. 2014.

175. **Sigrid Hade**. GIS Applications in the Studies of the Palaeozoic Graptolite Argillite and Landscape Change. 2014.
176. **Agne Velthut-Meikas**. Ovarian Follicle as the Environment of Oocyte Maturation: The Role of Granulosa Cells and Follicular Fluid at Pre-Ovulatory Development. 2014.
177. **Kristel Hälvin**. Determination of B-group Vitamins in Food Using an LC-MS Stable Isotope Dilution Assay. 2014.
178. **Mailis Päri**. Characterization of the Oligoadenylate Synthetase Subgroup from Phylum Porifera. 2014.
179. **Jekaterina Kazantseva**. Alternative Splicing of *TAF4*: A Dynamic Switch between Distinct Cell Functions. 2014.
180. **Jaanus Suurväli**. Regulator of G Protein Signalling 16 (RGS16): Functions in Immunity and Genomic Location in an Ancient MHC-Related Evolutionarily Conserved Synteny Group. 2014.
181. **Ene Viiard**. Diversity and Stability of Lactic Acid Bacteria During Rye Sourdough Propagation. 2014.