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

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].

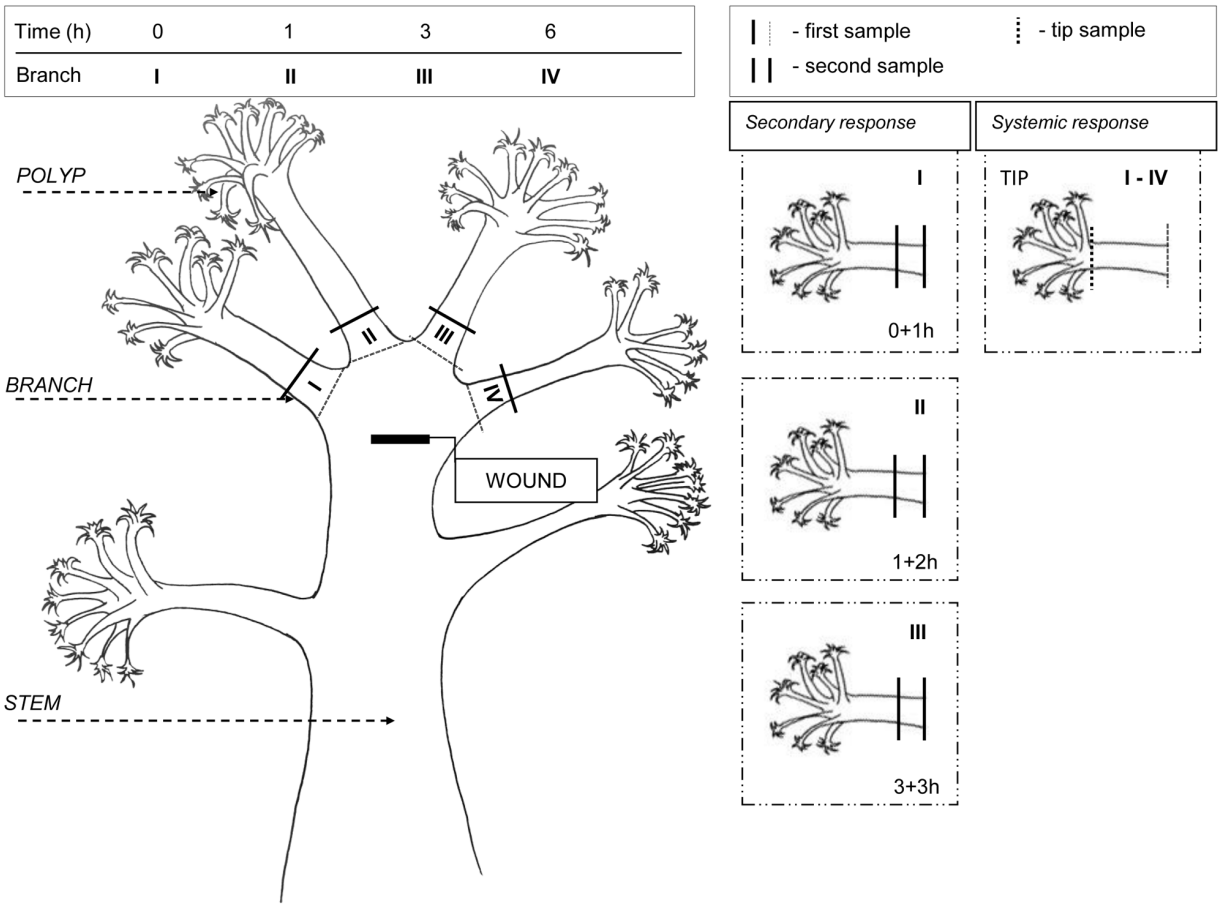

Figure 1. Experimental design of wounding stress. The location and time of sampling shown on a coral colony.
doi:10.1371/journal.pone.0089215.g001

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

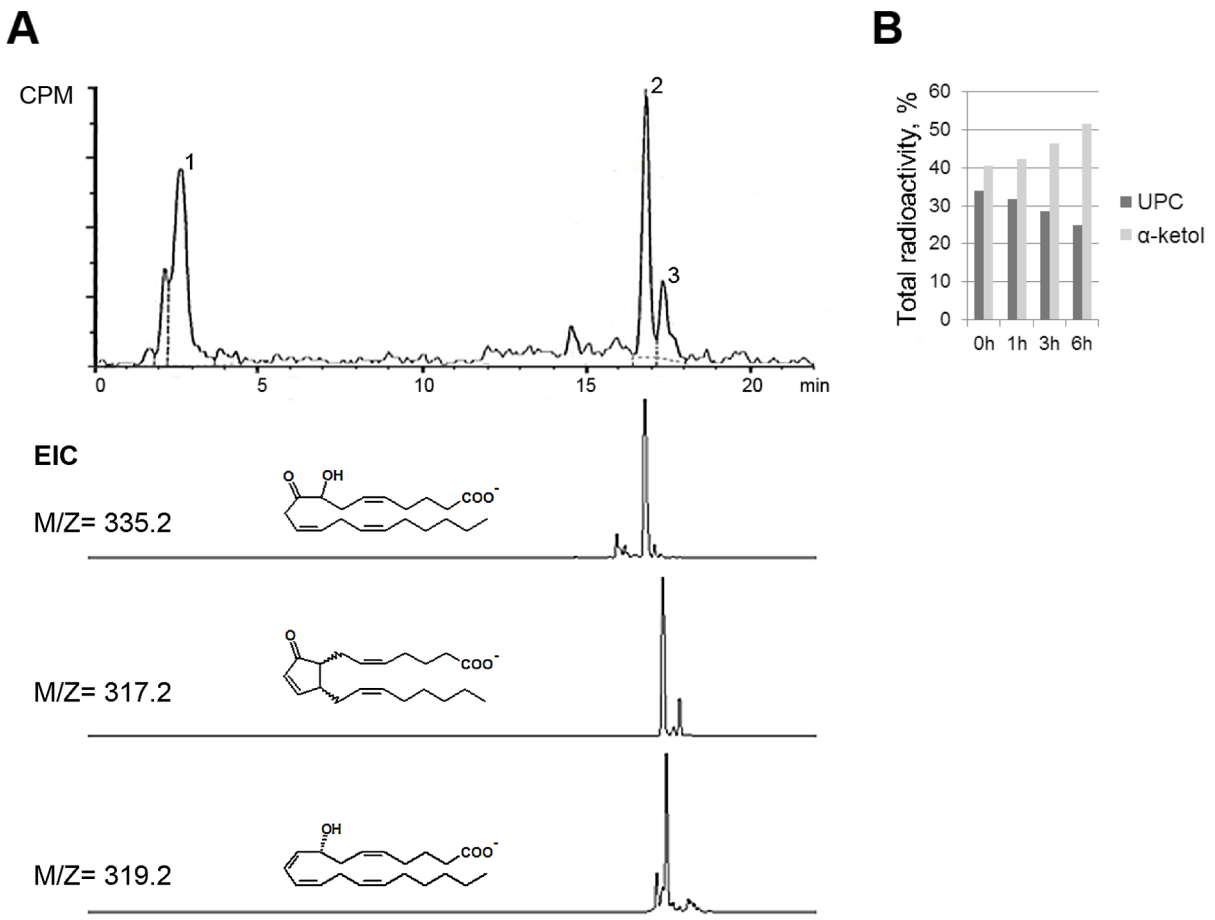

Figure 2. RP-HPLC analysis of incubation products of C. *imbricata* tissue homogenate. A) Radio chromatogram of the products formed from [1^{-14}C] AA by coral homogenate, extracted ion current (EIC) corresponding to α-ketol ([M^{-}] = 335.2, peak 2), cyclopentenone ([M^{-}] = 317.2, peak 3) and HETE ([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.

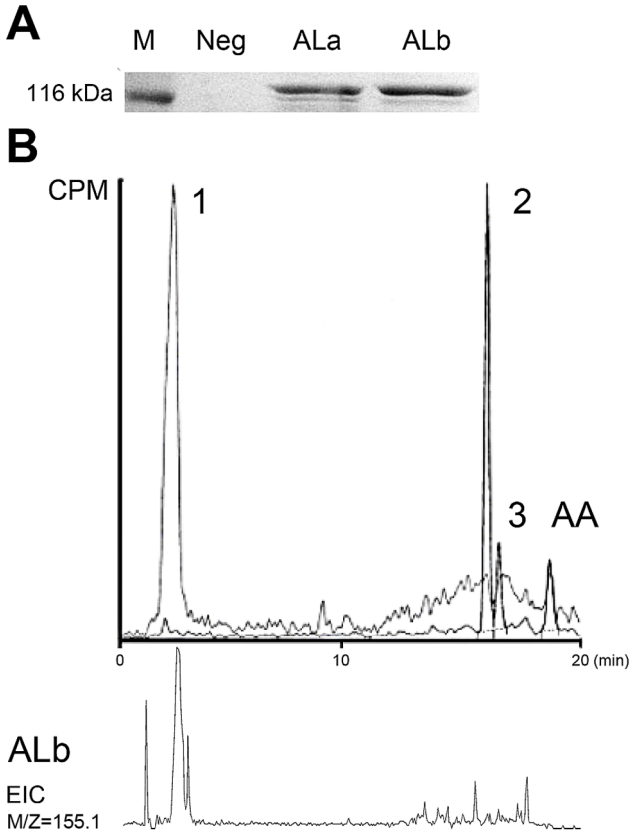

Figure 3. RP-HPLC analysis of incubation products of *C. imbricata* AOS-LOX fusion proteins expressed in *E. coli*. Bacterial extracts expressing the fusion proteins. M, protein molecular weight marker (Fermentas); Neg, negative control, pET11a vector without insert; ALa, AOS-LOXa; ALb, AOS-LOXb. The 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

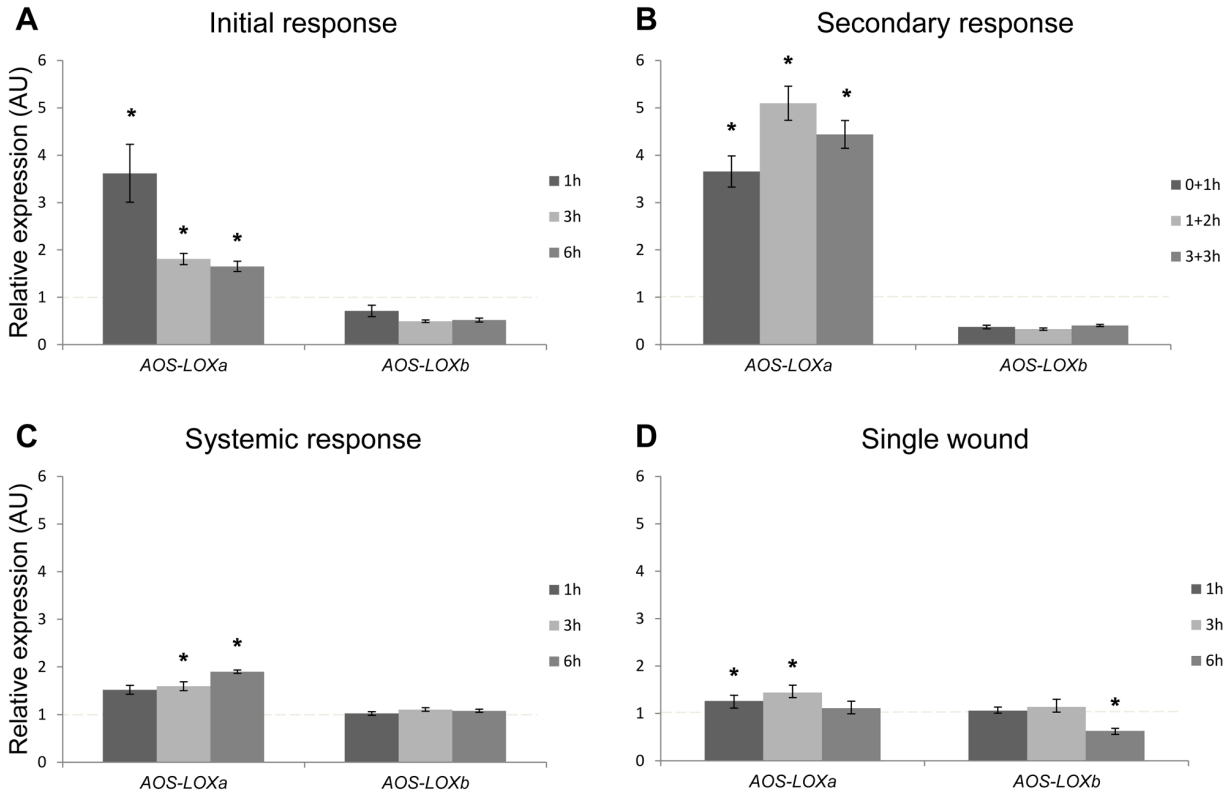

Figure 4. Quantitative real-time PCR analysis of transcript levels of *C. imbricata AOS-LOXa* and *AOS-LOXb*. Changes in gene expression in response to wounding within one colony: A) accumulative response; B) secondary response; C) systemic response. D) Response to a single wound. Data are means ± standard 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.