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Up-Regulation of Lipoxygenase, Phospholipase, and Oxylipin-Production in the Induced Chemical Defense of the Red Alga *Gracilaria chilensis* against Epiphytes

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Abstract The red alga *Gracilaria chilensis* is commercially farmed for the production of agar hydrocolloids, but some susceptible algae in farms suffer from intense epiphyte growth. We investigated the induced chemical defense response of *G. chilensis* against epiphytes and demonstrated that an extract of an epiphyte-challenged alga can trigger a defense response. The hormonally active metabolites were purified by RP-HPLC. Treatment with the extract or the purified fraction changed the chemical profile of the alga and increased resistance against epiphyte spores. Semi-quantitative RT-PCR and enzyme assays demonstrated that this metabolic response occurs after an increase in lipoxygenase and phospholipase A2 activity. Although this suggests the involvement of regulatory oxylipins, neither jasmonic acid nor the algal metabolite prostaglandin E2 triggers comparable defense responses.

Key Words Induced defence · Red algae · Hormones · Epiphytism · Gracilaria chilensis · Bioassays

Introduction

For at least two decades, from 1980–2000, cultivation of the marine red alga *Gracilaria chilensis* for the production of agar hydrocolloids was a major asset of Chilean aquaculture. More than 300 farming operations were established across the country, with annual yields of over 120,000 wet metric tons (Buschmann et al., 2001). However, the decade 2000–2010 was marked by a dramatic decline in farming operations due to a combination of negative economical and biological factors. The main nuisance to farmers is the widespread occurrence of

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infections by algal epiphytes, which negatively affect the crop growth rate, lead to biomass losses due to increased drag, and result in products with lower economic value. In this context, we initiated a study to understand the biological and biochemical basis of the interactions between *Gracilaria chilensis* and its main epiphytic pests.

Resistance against epibionts could be mediated by either structural or chemical means, with both mechanisms interfering with the settling and penetration of spores (Wikstrom and Pavia, 2004; Nylund and Pavia, 2005; Paul et al., 2006). Direct morphological comparison and ultrastructural investigations of resistant and susceptible *G. chilensis* isolates revealed that resistance against epiphytes is not linked to any structural properties (Leonardi et al., 2006). Either constitutive or induced chemical defense is the most likely basis for resistance. Algae may store toxins or other adverse compounds and rely on the action of these constitutive defense metabolites (Paul et al., 2006; Nylund et al., 2010). If predation risk or pathogen pressure is highly variable, dynamic defenses may evolve that rely on the recognition of a challenge and a subsequent metabolic response (Pohnert, 2004; Weinberger, 2007).

Wound-activated defense is based on the rapid transformation of stored metabolites after tissue disruption, and results in reactive products that act with a very short delay (Pohnert, 2004). Recently, we found that *G. chilensis* reacts to tissue damage like that which occurs upon attack by certain epiphytes (Leonardi et al., 2006) with the release of hydroxylated and dihydroxylated fatty acids (Lion et al., 2006). If administered in artificial substrates in naturally occurring concentrations, these metabolites significantly inhibit the settlement of epiphyte spores. The biosynthetic pathway for these defense metabolites involves both the release of arachidonic acid from phospholipids and its subsequent oxidation to 8-hydroxyeicosapentaenoic acid (8-HETE), and the direct release of 7,8-dihydroxyeicosapentaenoic acid (7,8-diHETE) from galactolipids (Lion et al., 2006).

Induced defenses rely on signals from an attacker with the subsequent production of defense metabolites, a strategy that is well documented in higher plants (Baldwin, 1998; Howe and Jander, 2008). The mechanistic details of induced defenses in algae are different from higher plants (Gaquerel et al., 2007). Most of the research on induced defenses of algae has focused on herbivore-induced chemical defense (Pavia and Toth, 2000; Pohnert, 2004; Molis et al., 2006), but dynamic defensive processes also can be induced under the influence of endo- or epiphytes (Potin et al., 2002; Weinberger et al., 2002; Bouarab et al., 2004; Weinberger, 2007). We report here that, in addition to a wound-activated defense, an induced process is stimulated in *G. chilensis* by epiphyte attack. We found that immunity can be triggered by exposing *G. chilensis* to hormonally active metabolites prior to inoculation with epiphyte spores, and that the response needs several days for the full defensive potential to build up.

Methods and Materials

Algal Culture Unialgal *G. chilensis* C.J. Bird, McLachlan & E.C. Oliveira strains CR14 and CS7 (maintained in the culture collection at U. Catholica, Santiago, Chile), originating from an intensive mariculture farm at Caldera (Chile), were cultivated at 15°C and under a photon flux density of 40–50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (D12:L12) in 500 ml glass flasks containing SFC medium (Correa and McLachlan, 1991). The medium was changed weekly. Fertile fragments of *Acrochaetium sp.* and *Sahlingia subintegra* were isolated from infected thalli of *G. chilensis* collected at Caldera, Chile. Unialgal cultures of the epiphytes were maintained actively growing and reproducing by using standard culture conditions in enriched modified seawater medium SFC with 2 mM $NaNO_3$ and 100 μM NaH_2PO_4 but without Tris, Na_2SiO_3, or Mo, and with only 0.02 pM Co.

Preparation and Fractionation of Active Extracts A total of 1.2 kg wet weight of frozen *G. chilensis* that was previously exposed to epiphytes and subsequently cleaned mechanically to remove epiphytes was brought to room temperature, homogenized in artificial seawater with an ultra Turrax, and extracted three times with 1 L of CH_2Cl_2. Tissue disruption in seawater leads to elevated oxylipin levels (Lion et al., 2006). The organic phases were combined, filtered, and concentrated to about 200 ml. Excess water was removed by freezing and subsequently by drying over Na_2SO_4, and the remaining CH_2Cl_2 was removed in vacuum.

Fractionation of the crude CH_2Cl_2 extract was done by HPLC using a preparative Gilson 321 pump connected to a Gilson 115 UV-detector. Separation was achieved with a Merck LiChroCart 250-10 Purosphere RP-18 cartridge. A gradient of water (A) and acetonitrile (B) (0 min 100% A, 25 min 100% B, 30 min 100% B at a flow rate of 6 ml min^{-1}) was used for elution. Fractions corresponding to 0–40%, 41–60%, 61–80%, and 81–100% acetonitrile were collected, and the acetonitrile was removed by rotary evaporation. The remaining water was removed by freeze drying, and the resulting residue was dissolved in ethanol for further bioassays.

Monitoring Metabolic Responses Gracilaria chilensis plantlets that were about 10 cm in length were cultured at a plant density of 50 $g L^{-1}$ in Petri dishes on a shaker using culture conditions as described above. Plantlets were treated with solutions of the crude dichloromethane extract, the

respective HPLC fractions, 7,8-di-HETE, prostaglandin E2 (Sigma-Aldrich, Seelze, Germany), methyl jasmonate (Givaudan, Vernier, Swizerland), linolenic acid (Sigma-Aldrich), coronalone (Schuler et al., 2004), or pure EtOH (control). Stock solutions (10 mg ml^{-1} ethanol) were prepared with all samples except the HPLC fractions, for which the final concentrations were adjusted to give a concentration equivalent to 10 mg ml^{-1} of the crude extract used for separation. The final concentrations in the medium were adjusted to 100 μg ml^{-1} or the amount of the HPLC fraction that was obtained from 100 μg ml^{-1} crude extract. After incubation (18 h), the algal biomass was transferred to fresh medium and cultured for an additional 48 h. Samples for chemical analysis were frozen in liquid nitrogen and stored at −80°C until further work-up as described below.

Analytical Procedures For metabolic profiling, *G. chilensis* samples were frozen in liquid nitrogen, spiked with 1 mg ml^{-1} phenyldodecanoic acid in ethanol as an internal standard (400 μl g^{-1} alga), ground with mortar and pestle, and immediately extracted with methanol (10 ml g^{-1} alga). The extracts were concentrated under vacuum, redissolved in methanol (400 μL g^{-1} alga), centrifuged, and submitted to analytical HPLC-MS analysis on an Agilent series 1100 HPLC equipped with a RP18 normal phase column GROM-SIL 120 ODS3 CP, 125×2 mm, and a DAD detector connected to a Finnigan LCQ with an ESI interface (Thermo Finnigan, Manchester, UK). A gradient with 0.5% aqueous acetic acid (A) and acetonitrile (B) (0 min 50% A, 30 min 100% B, 35 min 100% B at a flow rate of 0.2 ml min^{-1}) was used for separation. Spectra were recorded in the positive mode, needle voltage 4 kV, capillary temperature 120°C.

Bioassays Precultivated apical fragments (1 cm long) of *G. chilensis* from two different isolates were placed in beakers with 60 ml SFC medium, and were treated with 10 μg ml^{-1} of dichloromethane extract, HPLC fractions in ethanol at 1 mg ml^{-1}, or solvent controls. Incubation times were 18 h or 18 h followed by a second incubation of 54 h in 60 ml fresh SFC medium without extract. After treatment, the fragments were rinsed, transferred to 60 ml of fresh SFC medium, and 20 fertile *Acrochaetium sp* thalli and a 2× 2 cm glass slide were added in order to quantify spore settlement on *G. chilensis* in comparison with the inert support. Settlement of epiphytic spores was recorded under a light microscope 48 h after the start of the co-culturing. Spores were counted on 1 cm transects of the alga and on identical transects of the control glass slide. For each replicate, 3 transects were counted. For each isolate, 5 independent replicates consisting of treatments and control were run. In all of the experiments, spore settlement did not differ between the two isolates ($P>0.5$), and thus the results were pooled and the 10 replicates statistically evaluated.

Native Polyacrylamide Gel Electrophoresis For protein extraction, ground samples of *G. chilensis* were incubated at 4°C for 60 min in 1 ml g^{-1} of extraction buffer (50 mM Tris–HCl, pH 9.5, 500 mM potassium chloride and 10 mM β-mercaptoethanol). Protein concentrations were determined according to the method of Bradford (Bradford, 1976). Crude protein extracts (80 μg of total protein per lane) were separated by polyacrylamide gel electrophoresis (PAGE) on 12% native polyacrylamide gels. Lipoxygenase (LOX) activity was stained with o-dianisidine (Delumen and Kazeniac, 1976) using Na-pyrophosphate buffer (0.1 M, pH 7.95) containing 1 mM KCN instead of Na-acetate buffer. The intensities of bands appearing in the presence of 2 mM arachidonic acid or linoleic acid were quantified by image analysis, using the SigmaScan Pro 5.0 software package (SPSS Inc., Chicago, IL, USA).

Putative LOX Gene Expression Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to analyze gene expression. RNA was isolated from *G. chilensis* that was ground in liquid nitrogen using the plant RNeasy kit (Qiagen, Hilden, Germany). To design homologous primers for RT-PCR of LOX transcripts, the amplification and cloning of a putative lipoxygenase (LOX) cDNA fragment from *G. chilensis* was undertaken using degenerate primers designed from available sequences of *Porphyra purpurea* [GenBank accession number U08842 (Liu and Reith, 1994)] and *Porphyra yezoensis* [Kazusa accession number AU186969 (Asamizu et al., 2003)] and from conserved sections of a putative LOX coding region selected in an unpublished EST database from *Gracilaria tenuistipitata* (Jonas Collén, personal communication). The two primers used were 5'-GATAAGGTTGGCGTGCAAGCA-3' and 5'-GTTTGGGACGTAGGACAAGTA-3'. The 740 bp RT-PCR product was cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced (GenBank accession number JF896804). RNA from *G. chilensis* treated with dichloromethane extract for 0, 6, and 24 h was pooled and amplified in a RT-PCR reaction. Three independent replicates were examined. Subunit I of cytochrome c oxidase (COX, GenBank accession number EF434915, forward: 5'-AAGTCACTCTGGTGGTGC-3' and reverse: 5'-GTATC-TACATCAAGACCTAC-3') was used as control. Reactions were carried out in a final volume of 25 μl of the following reaction mix: 1 μL cDNA, 1× GoTaq Reaction Buffer (2.5 mM $MgCl_2$) (Promega) with 0.2 mM dNTP, 1.25 units of GoTaq DNA polymerase (Promega) and 0.2 μM gene-specific primers (Eurogentec, Seraing, Belgium). PCR was performed on a GenAmp PCR System 2700 (Applied Biosystems Foster City, LA, USA). The cycling program for PCR was: initial denaturation 5 min at 95°C, 25 to 30 cycles of 15 s at 95°C, 30 s at 50°C, and 45 s at 72°C, and

final elongation for 3 min at 72°C. For each gene, 5 µl of PCR product were loaded on a 1.8% agarose electrophoresis gel and run at 100 V for 30 min before incubation with ethidium bromide (0.07 µg ml^{-1}). Images were obtained using Biovision 1000/26 M (Vilber Lourmat, Marne-la-Vallée, France), and one representative image of each replicate was selected for quantification. The expression levels were determined by quantifying the gel bands using ImageQuant TL software (Molecular Dynamics, GE Healthcare, Piscataway, NJ, USA). The relative changes in expression among genes were calculated as x-fold changes to the appropriate control treatments.

Quantification of PLA_2-activity Aliquots of protein extract containing 100 µg crude protein were diluted with protein extraction buffer to a volume of 500 µl. The fluorescent probe PED6 (Molecular Probes, Leiden, The Netherlands) was added from a stock solution in ethanol to a final concentration of 4 µg ml^{-1} PED6 and 0.4% ethanol. The mixture was incubated on a shaker for exactly 10 min at room temperature and in darkness. Enzymatic decomposition of PED6 was stopped by addition of 500 µl chloroform/acetic acid (200:1 *v/v*, pH 2.6). For extraction of the released fluorescent label (BODIPY® FL C_5), the mixture was incubated for 10 min on a shaker. After separation of phases, the organic phase was evaporated under reduced pressure at room temperature, and the residue was taken up in 250 µl ethanol. The fluorescence of 100 µl of the resulting extract was observed with a Wallac Victor^2 (Perkin Elmer, Courtaboeuf, France) plate fluorimeter (excitation wave length: 485 nm, emission wave length: 535 nm). The background fluorescence was examined in parallel experiments without the addition of PED6, and proved to be close to zero, due to the two step extraction in aqueous buffer and in chloroform, which eliminates all potentially interfering pigments.

Statistical Analysis Data were analyzed with the Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA). Data distributions were tested for normality and heteroscedasticity using the Shapiro-Wilk test ($P<0.05$) and Levene's test ($P<0.05$), respectively. The Student *t*-test ($P<0.05$) was used for comparisons among pairs of treatments, while 2-way-ANOVA ($P<0.05$) and the Tukey-*posthoc* test ($P<0.05$) were employed where interactive effects of treatment and incubation time had to be analyzed.

Results and Discussion

Field and laboratory studies suggested that resistance of *G. chilensis* towards epiphytic algae is dynamically regulated (Lion et al., 2006). We initiated a mechanistic study to look for mechanisms of dynamic defense responses in this alga. While an immediate production of defensive metabolites in *G. chilensis* is based on rapid enzymatic reactions that are triggered by wounding (Lion et al., 2006), induced defenses require more time and a complex cascade of signaling events that includes hormonal regulation of metabolic responses (Pohnert, 2004; Devoto and Turner, 2005; Conrath et al., 2006). To search for underlying principles of regulation in the induced defense of *G. chilensis* we assayed extracts derived from field samples of epiphyte-stressed algae for hormonal activity. Treatment of *G. chilensis* with a crude lipophilic extract from epiphyte-challenged conspecific algae induced a significant metabolic response (Fig. 1). In contrast, the aqueous extract from the same individuals was inactive (data not shown). Concentrations as low as 1 µg ml^{-1} of the CH_2Cl_2-extract of *G. chilensis* triggered an increase of 8-hydroxyeicosatetraenoic acid (8-HETE) and 7,8-dihydroxyeicosatetraenoic acid (7,8-di-HETE), two metabolites previously identified as involved in the activated defense of this alga against epiphytes (Lion et al., 2006). In addition, unidentified metabolites that were not found in unchallenged algae or wounded tissue were induced (Fig. 1). This demonstrates that the dichloromethane fraction triggers the production of secondary metabolites, and thus might contain a hormone involved in the up-regulation of pathways required to produce defense metabolites. Because we cannot culture epiphyte-free algae, we cannot conclude whether or not the production or the respective hormone(s) is induced by epiphytes. Future studies are required to clarify how this activity is regulated upon challenge of the alga.

Separation of the active extract by preparative HPLC yielded four fractions, and the less polar ones were tested. Only the fraction of medium polarity induced the production of secondary metabolites (Fig. 1). Neither the more polar nor the more nonpolar fraction triggered any metabolic response. We concluded there was activity associated with one specific metabolite or a group of metabolites with a narrow distribution of polarities (Fig. 1).

To gain further insight into the inducing metabolite(s) we evaluated the activity of candidate molecules identified in the active HPLC fraction of the *G. chilensis* extract, and also of known plant hormones. Treatments with 7,8-di-HETE and prostaglandin E2 did not change the metabolic profile of the algae. While 7,8-di-HETE is a specific metabolite found only in *G. chilensis*, prostaglandin E2 is a mammalian hormone that also occurs in several *Gracilaria spp.* including *G. chilensis* (Fusetani and Hashimoto, 1984). In addition, we tested methyl jasmonate, a plant hormone that was not detected in *G. chilensis* but was active in the red alga *Chondrus crispus* (Farmer and Ryan, 1990; Bouarab et al., 2004); coronalone, a synthetic analogue of jasmonic acid that is also hormonally active in plants (Schüler et al. 2004); and linolenic acid, the biosynthetic precursor of jasmonic acid. None of these potential hormones or hormone

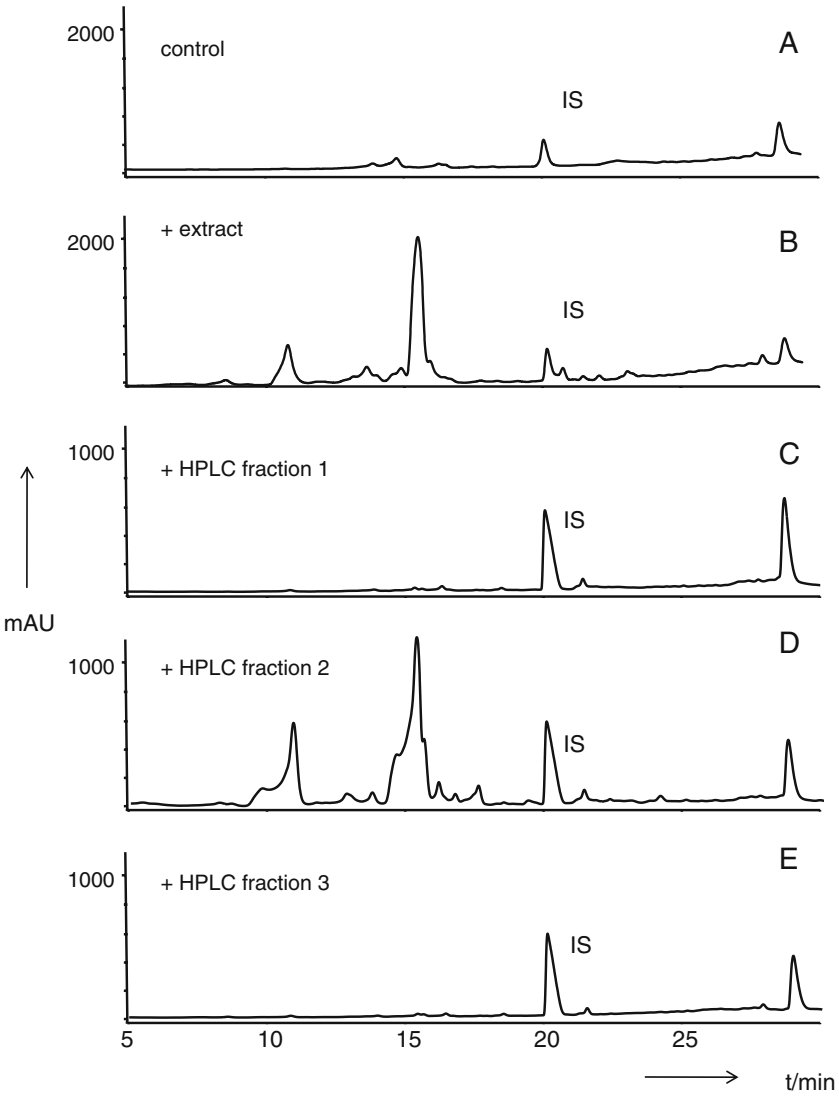

Fig. 1 Elicitation of the formation of oxylipins in *Gracilaria chilensis*. **a** Ethanol (control). **b** Crude dichloromethane extract (1 μg ml^{-1} in ethanol). The major peak at the retention time 11 min corresponds to 7,8-di-HETE and at 15.8 min to 8-HETE. **c**–**e** Fractions obtained by preparative RP-HPLC of the dichloromethane extract. Fractions eluted at 40–60% CH_3CN (**c**); 61–80% CH_3CN (**d**); 81–100% CH_3CN (**e**). The UV-trace at 254 nm is shown. Chromatograms were referenced in comparison to the internal standard (IS) phenyldocecenoic acid (retention time 20.5 min). Note the different scales of A, B and C-E

precursors triggered any detectable metabolic response in *G. chilensis* (data not shown). Concentrations of the plant hormones and coronalone that were tested exceeded those required to induce metabolic responses in higher plants and algae by about 5 to 50 fold, thus leading us to conclude that lack of activity was not a consequence of low concentration of the potential inducers (Bouarab et al., 2004; Schüler et al. 2004). The chemical nature of the active algal compound(s), which co-elutes with dihydroxylated C20 fatty acids in RP-HPLC, thus, remains unknown.

To determine whether the metabolic reaction observed after treatment with the crude, hormonally-active fraction is linked to an induced defense, we developed a bioassay in which settlement of epiphyte spores on induced and uninduced *G. chilensis* was monitored. In this assay, *G. chilensis* was incubated with fertile spores that were releasing *Acrochaetium sp.* thalli, and after defined time spans, the number of settled epiphyte spores on the algae were compared to the number of spores on a comparable

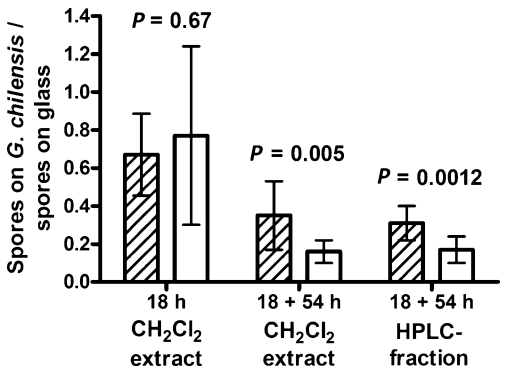

Fig. 2 Resistance of *Gracilaria chilensis* against spore settlement of the epiphyte *Acrochaetium* after different times of treatment with crude (10 μg ml^{-1}) or fractionated (corresponding to 10 μg ml^{-1} crude extract) CH_2Cl_2 extract in ethanol (*white bars*) or with ethanol only (*hatched bars*). Mean +/− standard deviation, N=10 (P values are indicated above bars)

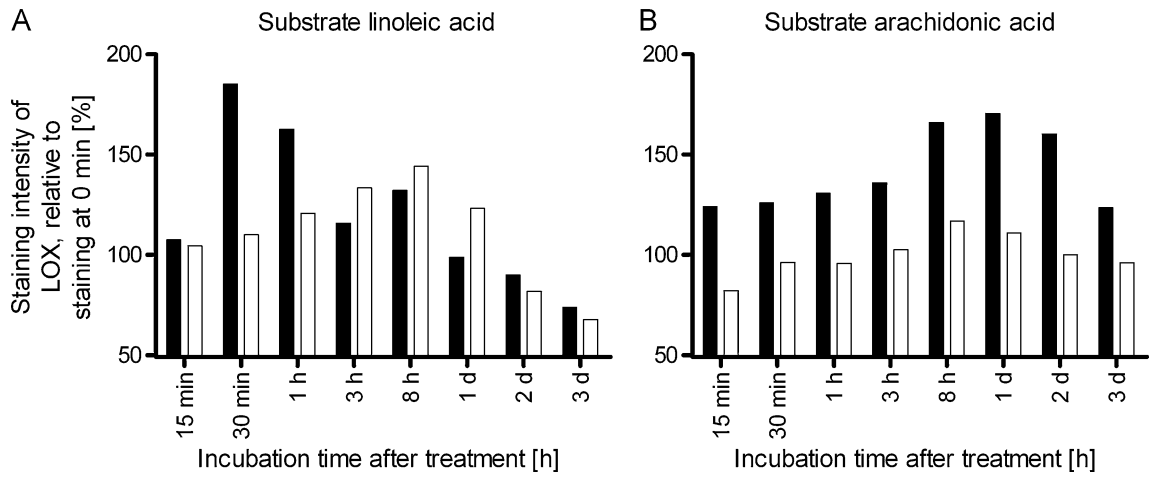

Fig. 3 Induction of lipoxygenase-type activity in *Gracilaria chilensis*. The activity was determined in native PAGE gels using 2 mM arachidonic acid or linoleic acid as a substrate. The protein extracts from algae that had been exposed to ethanol containing (*black bars*) or not containing (*white bars*) crude CH_2Cl_2-extract of *G. chilensis* for the times indicated

transect of a glass slide. This method allows normalization of the assays without the need to co-incubate treated and untreated *G. chilensis*, which could result in cross talk between the algae. Pre-treatment with the active extract for 18 h followed by a 54 h period in which the algae were allowed to develop metabolic reactions in pure culture medium resulted in a significantly reduced number of settled spores on the *G. chilensis* surface compared to algae that were treated with the solvent alone (P=0.005)(Fig. 2). The medium polarity HPLC fraction that induced a metabolic response also triggered a significant (P=0.001) reduction in the number of settled spores (Fig. 2). Resistance was observed only if an initial 18 h treatment with the active fraction was followed by a 54 h incubation period. Shorter incubations did not increase resistance. Results obtained with two different isolates of *G. chilensis* were statistically indistinguishable. Comparison of the uninduced isolates as controls also revealed no difference (P=0.63) in spore settlement. This significant response demonstrates clearly that a defense reaction against epiphyte spores can be triggered by algal compounds. Since shorter times of pre-incubation did not result in any significant biological response, it is likely that a slow up-regulation in defense genes is required for the establishment of the resistance. Comparable slow responses are known from the induced chemical defense of both plants and algae (Toth and Pavia, 2000; De Vos et al., 2005). It is important to note that the observed induced response is not a general defense against epiphytes, since parallel infection experiments with the red alga *Sahlingia subintegra* did not reveal any increased resistance (data not shown).

Recent work in algal chemical ecology suggests that induced defenses are widely distributed among seaweeds (Coleman et al., 2007; Weinberger, 2007; Molis et al., 2008; Rohde and Wahl, 2008). Nevertheless, besides studies on the activity of oxylipins from *Chondrus crispus,* little information about the nature of the involved signals is available (Bouarab et al., 2004; Gaquerel et al., 2007). Several hormones from a wide range of phyla from mammals to plants belong to the class of oxylipins. Since our HPLC fractionation of the crude extract also pointed

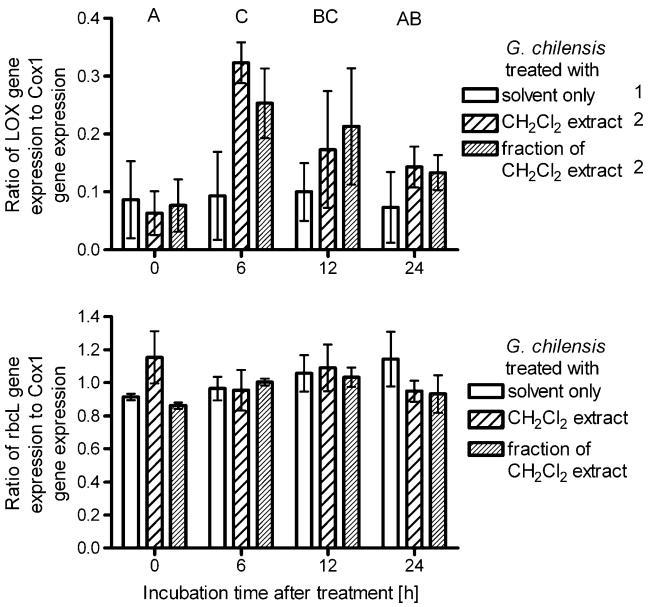

Fig. 4 Expression of a putative lipoxygenase gene in *Gracilaria chilensis* after treatment with dichloromethane extract. RNA from *G. chilensis* treated with solvent (control), with the dichloromethane extract or with fractions . Expression of LOX, the large subunit of ribulose-bisphosphate carboxylase (rbcL), or cytochrome c oxidase (COX) was quantified by semi-quantitative RT-PCRMean +/− standard deviation, N=3. Different capitals and different numbers indicate incubation times and treatments, respectively, that are significantly different (Tukey-test, P<0.05)

Table 1 Factorial ANOVA of (A) LOX to COX1 gene expression ratios and (B) rbcL to COX1 gene expression ratios in *Gracilaria chilensis* at four different times after treatment with CH_2Cl_2 extract, with a HPLC fraction of this extract or with solvent only. Incubation time and type of treatment were used as factors

Source	Sum of squares	Degrees of Freedom	Mean squares	*F*	*P*
A.					
Intercept	0.751111	1	0.751111	190.6911	0.000000
Time	0.108511	3	0.036170	9.1829	0.000316
Treatment	0.056939	2	0.028469	7.2278	0.003492
Time*Treatment	0.055706	6	0.009284	2.3571	0.062289
Error	0.094533	24	0.003939		
B.					
Intercept	33.78692	1	33.78692	3960.108	0.000000
Time	0.04101	3	0.01367	1.602	0.217416
Treatment	0.03413	2	0.01706	2.000	0.159185
Time*Treatment	0.15163	6	0.02527	2.962	0.028328
Error	0.18770	22	0.00853		

towards an active fraction that co-elutes with major C20-oxylipins from *G. chilensis*, such as 7,8-di-HETE, we decided to investigate the activity of key enzymes involved in the formation of these metabolites.

In-gel activity staining revealed that crude protein extracts of *G. chilensis* had lipoxygenase-activity towards linoleic and arachidonic acid (Fig. 3). Treatment of *G. chilensis* with dichloromethane-extract increased the algal capacity to oxidize linoleic acid within 30 min to 1 h after exposure (Fig. 3). The ability to oxidize arachidonic acid increased within 15 min of exposure, and was most substantially visible 8 h to 2 d after exposure (Fig. 3). The activity was insensitive to KCN, and was, therefore, not attributed to peroxidases or other heme proteins. but to lipoxygenases (Manchenko, 1994). Over the entire assay period, the up-regulation of linolenic acid LOX activity was not significant ($P>0.5$). When linoleic acid was used as substrate, only a weakly stained band was detected, although a relatively large amount of crude protein was loaded (80 μg per lane). The expression of a linolenic acid lipoxygenase was stronger in *C. crispus*, where more intense staining and two additional isoforms were detected on electrophoretic gels when only 30 μg protein were loaded (Bouarab et al., 2004). When arachidonic acid was used as the substrate during the gel staining procedure, a single stained band was detected accompanied by changes in arachidonic acid LOX activity upon challenge with the active extract ($P<0.001$). The stained band had a retention factor similar to the band observed after treatment with linoleic acid. However, the transformations were not catalyzed by the same enzyme because there were clear differences in the time courses of fatty acid hydroperoxide formation (Fig. 3). While induction with the dichloromethane extract resulted in a maximally increased arachidonic acid LOX activity after 24 h, linoleic acid was preferentially acted on after 30 min. The delayed metabolic response observed after the treatment with the extract is in accord with the delayed defense response in our bioassays. Such orchestrated expression of different lipoxygenases often is observed in higher plants, where oxylipins also play

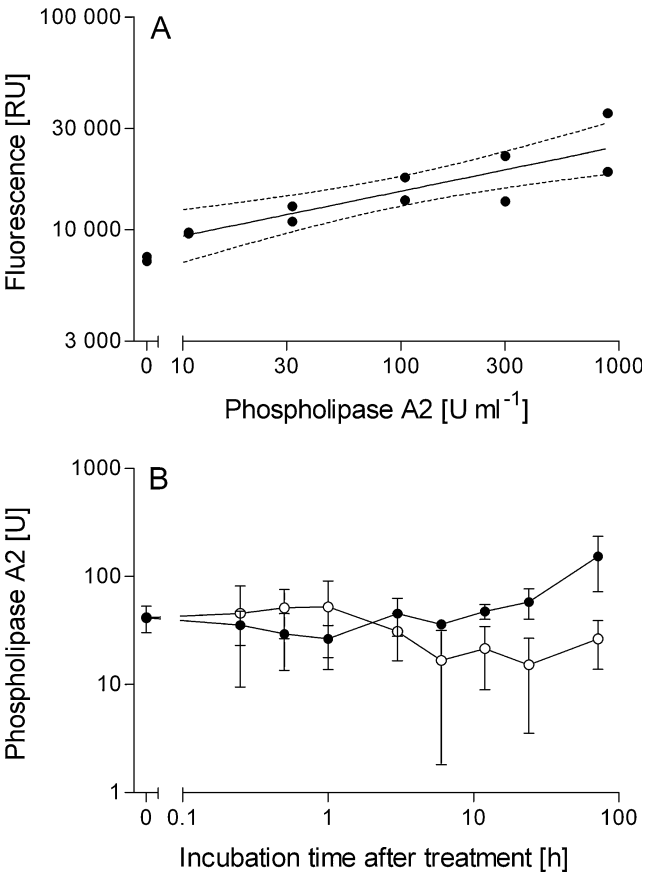

Fig. 5 Induction of phospholipase A2 in *Gracilaria chilensis*. **a** Calibration: Incubation of PED6 with increasing amounts of phospholipase A2 in 500 μl protein extraction buffer ($r^2=0.7366$, $P=0.002$ the dotted lines represent 95% confidence intervals). One U of enzyme hydrolyzes 1 μM phosphatidylcholine per min. **b** PLA2 activity in 100 μg crude protein originating from *G. chilensis* exposed (●) or unexposed (○) to dichlormethane extract. Averages of two replicates, bars represent minimum and maximum response

key roles in quick wound-activated and in slower induced defense reactions (Halitschke et al., 2000; Truman et al., 2007; Bonaventure and Baldwin, 2010).

The design of a set of primers based on an EST clone from a cDNA library of the red alga *Gracilaria tenuistipitata* (P. Nyvall, J. Collén et al., unpublished) allowed the cloning of a partial cDNA fragment from *G. chilensis*. This 764 bp fragment exhibited significant similarity to plant and animal LOX as well as to a putative red algal LOX (Supplemental Information). The corresponding amino acid sequence was 43% identical to the putative LOX from *Porphyra purpurea* (GenBank™ accession no. AAA61791). Sequence analysis shows that some of the residues that are involved in iron-ligand binding in all LOX (Prigge et al., 1997) are present in the partial sequence. Conserved sequences include the central histidine-rich region around His-510 and His-515 in the *P. purpurea* sequence, whereas the other residues including a distal histidine (His-638) and an asparagine (Asn-642) are missing in the partial fragment. The partial cDNA clone shared 87.4%, 52.2% and 50.6% similarity with the LOX cDNAs of *G. tenuistipitata* (J. Collén, pers. comm.), *Porphyra purpurea* (Liu and Reith, 1994), and *P. yezoensis* (Asamizu et al., 2003). The translated sequence also contained two determinants for substrate and regiospecificity (Sloane et al., 1991; Borngraber et al., 1996) as well as a generally conserved domain in lipoxygenases (Marchler-Bauer et al., 2005).

Expression of lipoxygenases was monitored by using semi-quantitative RT-PCR. A pronounced transcription of lipogygenase relative to cytochrome oxidase 1 was observed in *G. chilensis* after treatment with either the CH_2Cl_2 extract or the medium polar HPLC fraction (two-way-ANOVA and Tukey-test, $P<0.05$, Fig. 4, Table 1A). There were no changes in the expression of ribulose bisphosphate carboxylase relative to cytochrome oxidase 1 in the same samples, thus indicating a specific LOX upregulation (two-way-ANOVA and Tukey-test, $P>0.05$, Fig. 4). The effect was maximal after 6 h and still significant after 12 h, a delay that corresponds to the observed kinetics of the arachidonic acid LOX activity (Fig. 3). Only two-way ANOVA (Table 1B), but not the Tukey-test ($P<0.05$) detected a significant interaction of treatment and incubation time. The detected mRNA, therefore, probably encodes the enzyme that acts on arachidonic acid. To what extent the enzyme has a role in the direct synthesis of defensive oxylipins (Lion et al., 2006) vs. the production of regulatory active oxylipins can only be concluded indirectly. Since a further delay in the induced defense response is observed it might be more likely that LOX is involved in the triggering of further defensive metabolic reactions, also a well known phenomenon in higher plants.

Other enzymes that are crucial for the production of oxylipins are lipases. These enzymes release free unsaturated fatty acids, which are the substrates for LOX. They often are involved in the regulation of defense reactions in algae (Lion et al., 2006) and higher plants (Roy et al., 1995; Kallenbach et al., 2010). Treatment of *G. chilensis* with the dichloromethane extract also increased PLA_2 activity. In protein extraction buffer, enzyme activity could be quantified by the concentration-dependent release of fluorescent BODIPY FL C_5 from the phospholipase substrate PED6 (Fig. 5). Algae treated with dichlormethane extract for 6 h to 3 d had more activity than untreated plants (2-way-ANOVA, $P=0.03$). The strongest upregulation was observed after 3 d, when treated plants exhibited 5.8 times more PLA_2-activity than untreated plants. From the data on enzyme activity, we conclude that induction results after 6 h in an increased liberation of fatty acids from phospholipids, which probably fuel the lipoxygenase that is increasingly expressed after 8 h. To fully develop the defense potential the alga, however, requires additional time, since a response in the bioassay is delayed for another 48 h.

In summary, we provide evidence for an induced chemical defense mechanism of *G. chilensis* against epiphytes. This can be triggered by a hormonally active fraction of the *G. chilensis* extract, thereby supporting a general role of hormones in the induced defense of the alga. In addition, candidate genes and enzyme activities, commonly associated with an oxylipin based hormonal regulation of metabolic processes, are triggered by the addition of the active fraction. Further work will focus on the structural elucidation of the actual hormone and on an examination of how this hormone is regulated in response to biotic stress.

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