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Prostaglandin A_2 triggers a strong oxidative burst in *Laminaria*: a novel defense inducer in brown algae?

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We report an oxidative burst triggered by prostaglandin A_2 (PGA_2) in the brown algal kelp *Laminaria digitata*, constituting the first such discovery in an alga and the second finding of an oxidative burst triggered by a prostaglandin in a living organism. The response is more powerful than the oxidative burst triggered by most other chemical elicitors in *Laminaria*. Also, it is dose-dependent and cannot be inhibited by diphenylene iodonium, suggesting that another source than NAD(P)H oxidase is operational in the production of reactive oxygen species. Despite the very strong oxidative response, rather few effects at other levels of signal transduction pathways could be identified. PGA_2 does not increase lipolysis (free fatty acids) in *Laminaria*, and only one oxylipin (15-hydroxyeicosatetraenoic acid; 15-HETE) was found to be upregulated in *Laminaria*. In a subsequent set of experiments in the genome model *Ectocarpus siliculosus*, none of 5 selected candidate genes, all established participants in various stress responses, showed any significant differences in their expression profiles.

Key Words: Cyclopentenone; diphenylene iodonium; Phaeophyta; polyunsaturated fatty acids; prostaglandin A_2

INTRODUCTION

Brown algae (Phaeophyta) make up most of the benthic biomass on temperate and polar rocky shores. Kelp forests are key ecosystems of such coastal areas and they constitute habitat and breeding grounds for a very large diversity of marine life forms - besides offering a physical protection by providing a wave energy-absorbing buffer for coasts. Brown algae belong to a lineage that has been evolving independently of other major photosynthetic

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[†]We would like to dedicate this publication to the memory of our friend Jean-Pierre Salaün, who untimely passed away on June 22, 2011, shortly before the submission of this article. His enthusiasm and inspiration for science - in particular, oxylipin biochemistry - will be unforgotten.

lineages, in particular the green plants (Chlorophyta) and red algae (Rhodophyta). Instead, they are classified within the Stramenopiles and Chromalveolates together with diatoms, golden-brown algae and oomycetes (Baldauf 2003). As a consequence of this singular evolutionary history, brown algae exhibit many unusual, and often unique, features. These features are adaptations to the marine coastal environments in which brown algae are usually the dominant organisms in terms of biomass, in particular by forming extensive kelp forests structuring these ecosystems. The key role of kelp forests, effectively constituting an interface between the ocean, the atmosphere and land masses, in the biogeochemical cycle of halogens is well established (Carpenter et al. 2000, Küpper et al. 2008).

Ectocarpus siliculosus is a filamentous brown alga with a worldwide distribution along temperate coastlines, and is a nuisance as a "fouling" organism on many man-made surfaces in the sea. It has some significant advantages as an experimental model and constitutes one of the best-studied seaweeds (Peters et al. 2004, Charrier et al. 2008); it can easily be cultivated in small volumes of seawater media both axenically and with associated bacteria (Müller et al. 2008); it belongs to a sister group of the ecologically and economically very important kelps; its entire, well-known life cycle can be completed within a few months in culture; many molecular tools are available, including mutant collections (Peters et al. 2004), microarrays (Dittami et al. 2009) and proteomics (Ritter et al. 2010). It has also recently become the first seaweed of which the entire genome has been sequenced and thus offers unprecedented opportunities for study (Cock et al. 2010).

Like all other marine organisms, marine algae are subject to a virtually permanent onslaught of a broad spectrum of pathogens - including viruses (Müller and Knippers 2001), chytrid fungi (Gleason et al. 2011), oomycetes (Strittmatter et al. 2009) and endophytic / parasitic algae (Ellertsdóttir and Peters 1997). While some algal pathogens have been known since the 19th century (e.g., Wright 1877), mechanistic aspects have been studied only for little more than a decade (e.g., Potin et al. 2002). A significant finding of recent years was that brown and red algae share key defense mechanisms with animals and higher plants (Küpper et al. 2001, Potin et al. 2002).

The mammalian response to microbial attack involves the activation of immune response genes, including those involved in the arachidonic acid cascades, leading to the synthesis of leukotrienes and prostaglandins (Funk 2001, Gachon et al. 2010). In comparison, plants synthesize compounds of direct antimicrobial toxicity such as reactive oxygen species (ROS), phytoalexins and pathogenesis-related proteins. Higher plants are also known to activate genes involved in the oxylipin cascade such as lipoxygenases, in a process that appears similar to the arachidonic acid cascade in mammals (Andreou et al. 2009). Many inducible defense genes are regulated by signalling pathways involving one or more low molecular weight signal molecules, such as jasmonic acid, salicylic acid, ethylene, and possibly H_2O_2 (Reymond and Farmer 1998, Orozco-Cárdenas et al. 2001).

The oxidative burst was initially discovered in human macrophages (Baldridge and Gerard 1933). It is now recognized also as a key element of plant (Wojtaszek 1997, Mittler et al. 2004) and algal (Potin et al. 2002, Dring 2005, Cosse et al. 2007) defense, where it is considered both as a rapid defense response and an internal emergency signal. It consists of the rapid and massive production of ROS such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$)which are likely produced via the interaction of plasma membrane-associated NAD(P)H oxidases (Wojtaszek 1997). Hence, pathogen attack triggers both the production of ROS and the oxidation cascade of fatty acids leading to the production of bioactive oxylipins in plants. In algal systems, the oxidative burst was initially discovered in response to injury in a red alga (Collén et al. 1994). Over the last decade, oxidative bursts in response to oligosaccharide elicitors were observed in a number of red and brown algal models, in most cases to control the growth of bacterial biofilms (Weinberger et al. 1999, Weinberger and Friedlander 2000*a*, 2000*b*, Küpper et al. 2001, 2002, 2006, 2009) or the attack of eukaryotic endophytes (Bouarab et al. 1999, Küpper et al. 2002). More recently, the involvement of an oxidative burst in the wound healing response of a siphonalean green alga was highlighted (Ross et al. 2005), including its cross-communication with nitric oxide signalling (Ross et al. 2006).

Most polyunsaturated fatty acids (PUFAs) in higher plants belong to the C_{18} family, whilst polyunsaturated C_{20} fatty acids seem to occur only in very few species. In contrast, algal lineages such as the Phaeophyceae (Hofmann and Eichenberger 1997) produce both C_{18} and C_{20} PUFAs in significant amounts. A number of algae have become industrial sources of biotechnologically important PUFAs such as arachidonic acid (C20:4) or eicosapentaenoic acid (C20:5) (Colombo et al. 2006, Spolaore et al. 2006).

Oxylipins and prostaglandins in particular are important intra- and intercellular messengers in all living organisms. Among the cellular signalling responses which

ROS generation can trigger is the activation of fatty acids by the production of oxygenated PUFAs called oxylipins which play a key role against abiotic and biotic stress in plants and metazoans. In mammals, these compounds (which include leukotrienes and prostaglandins) are produced from the oxidation of C_{20} PUFAs. Prostaglandins mediate pivotal functions in immunity processes and cell development, allergic reactions and, in a broader sense, defensive stress responses (Funk 2001). Furthermore, in plants, their recruitment during wounding stress, pathogen and herbivore invasions has been fully confirmed in recent years (Farmer et al. 2003, Farmer and Schulze-Lefert 2005). In our previous work, we had shown that bacterial surface macromolecules (especially lipopolysaccharides) can trigger both an oxidative burst and induce the release of free fatty acids and synthesis of oxylipins in *Laminaria* (Küpper et al. 2006). More recently, we observed similar effects following treatment of *Laminaria* with linolenic and arachidonic acid as well as methyl jasmonate (Küpper et al. 2009): besides triggering an oxidative burst, these compounds led to a release of free fatty acids and oxylipin synthesis and, significantly, to an induced resistance against the kelp endophyte *Laminariocolax tomentosoides*. However, in neither of these studies the formation of cyclopentenones such as prostaglandins or jasmonate was observed. However, cyclopentenones have recently been found to be synthesized in *Laminaria* under copper stress (Ritter et al. 2008) constituting the only report of cyclopentenones in brown algae to date. Other than that, all reports of cyclopentenones reported from seaweeds originate from red, not brown algae - e.g., methyl jasmonate in *Chondrus crispus* (Bouarab et al. 2004), PGA_2, PGE_2, PGF_2 and 15-keto-prostaglandins in *Gracilaria verrucosa* (Sajiki 1997, Imbs et al. 2001, Dang et al. 2010) and PGE_2 in *Gracilaria tenuistipitata* (Hsu et al. 2008) and *G. rhodocaudata* (Sajiki 1997). In none of these cases, a function could be associated with these compounds yet, even though in several cases prostaglandin production was found to be upregulated by some form of stress (e.g., Sajiki 1997, Imbs et al. 2001, Bouarab et al. 2004, Gaquerel et al. 2007, Hsu et al. 2008). Interestingly, prostaglandin content (or production, respectively) has been associated with occasional cases of toxicity of seaweeds to humans (Higa and Kuniyoshi 2000). Contrasting with these, the observation of phlorotannin induction by airborne methyl jasmonate in *Fucus* by Arnold et al. (2001) and our finding of the activity of exogenous methyl jasmonate in *Laminaria* (Küpper et al. 2009) so far remain the only cases of a cyclopentenone showing a biological effect in a brown alga. Contrasting with these studies, Wiesemeier et al. (2008) did not observe defense-related effects of jasmonate in other species of brown algae.

Considering the paramount importance of prostaglandins in the defense of mammalian cells, the scope of this study was to screen 5 prostaglandins as potential defense elicitors in brown algae. In analogy to our previous studies, we monitored *Laminaria digitata* for an oxidative burst response and changes in its fatty acid and oxylipin profile. Then, following the identification of PGA_2 as a potent trigger of an oxidative burst in *Laminaria* and taking advantage from the availability of the *Ectocarpus* genome (Cock et al. 2010), we investigated prostaglandin effects on the transcriptional induction of 5 candidate genes potentially related with oxidative stress, defense and cell rescue.

MATERIALS AND METHODS

Chemicals

Prostaglandins (PGE_1, PGE_2, $PF_2\alpha$, PGB_1, PGA_2) were obtained from FLUKA and Cayman Europe (Tallinn, Estonia), dissolved in dimethyl sulfoxide and stored at -20℃ until further use. These prostaglandins had been preselected among a multitude of compounds due to their established, stress-related roles in other organisms.

Laminaria digitata

For experiments involving *Laminaria*, essentially the same procedures as described previously for studying the effects of lipopolysaccharides (Küpper et al. 2006) and free fatty acids and methyl jasmonate (Küpper et al. 2009) were used. Briefly, young *L. digitata* sporophytes [produced from gametophyte strains CCAP 1321/1 (♀) and CCAP 1321/2 (♂) from Helgoland] were grown in half-strength Provasoli ES (PES) medium (Starr and Zeikus 1987) prepared from natural Atlantic open-ocean water. They were illuminated with daylight-type fluorescent lamps at an irradiance of 9 $\mu E\ m^{-2}\ s^{-1}$ for 10 h per day and kept at 10 ± 1°C. Sporophyte thalli were transferred to fresh medium at one-or two-week intervals. They were used for experiments when they had reached a length of 5 mm to 3 cm.

For assessing the effects of prostaglandins on oxidative metabolism in *Laminaria*, thalli were incubated in 10 mL filtered seawater in petri dishes at 12°C, to which dilutions of PGE_1, PGE_2, $PF_2\alpha$, PGB_1 or PGA_2 were added

at final concentrations of 100 µM. The concentration of hydrogen peroxide in the medium around algal thalli was determined using the luminol chemiluminescence method (Glazener et al. 1991) with a LUMAT LB 9507 luminometer (EG&G Berthold, Bad Wildbach, Germany), as described before (Küpper et al. 2001).

For all further studies following the finding of PGA_2 as a potent inducer of the oxidative burst, a dose-response curve for the oxidative burst was measured using the aforementioned chemiluminescence assay, with final PGA_2 concentrations of 0.1, 1, 3, 6 and 10 µM, respectively. For analyses of free fatty acids and oxylipins, *Laminaria* thalli were incubated with PGA_2 at a final concentration of 10 µM. Tissue samples were subsequently frozen and ground in liquid nitrogen. The frozen powder was transferred into borosilicate tubes and homogenized with 2 mL of ethyl acetate. Lipids were extracted by mixing on a rotary shaker for 1 h at 4°C and then 1 mL of ice cold water was added. The mixture was kept 5 min on ice and then centrifuged at 4°C and 3,500 g. After recovery of the organic phase, ethyl acetate was evaporated under a stream of argon. Lipid analyses were performed as described previously (Küpper et al. 2006). Oxylipins were identified by reverse phase-high performance liquid chromatography coupled to a mass spectrometer (HPLC-MS) (Finnigan, Manchester, UK) in atmospheric pressure ionisation (APCI) and negative ion mode. Structural assignments were confirmed by gas chromatography (GC) (HP 5890 Series II; Hewlett Packard, Palo Alto, CA, USA) coupled to mass spectrometry (HP 5971A; Agilent Technologies, Santa Clara, CA, USA) after appropriate derivatizations. Fatty acids were quantified by GC-MS as methyl esters from standard curves obtained by measuring the peak surfaces of authentic standards. Statistic significance for increases of PUFAs and oxylipins amounts after treatments compared to controls was tested by a Student t-test.

Ectocarpus siliculosus

All experiments with *Ectocarpus* used the genome-sequenced strain *E. siliculosus* CCAP 1310/4 (Cock et al. 2010). Monoeukaryotic, clonal cultures of this strain were grown in 650 mL filter cap suspension flasks (175 cm²; Greiner Bio-One, Courtaboeuf, France) at 15°C using half-strength modified Provasoli-enriched sea water (West and McBride 1999), at 10 µE m^{-2} s^{-1} (daylight-type fluorescent lamps, 12 h photoperiod).

Treatments with PGA_2 (obtained from FLUKA and Cayman Europe, as above) were performed at a final concentration of 10 µM in an experimental procedure developed by Zambounis, Strittmatter and Gachon (unpublished / personal communication). Briefly, algal cell cultures were treated as follows: PGA_2 or ethyl ester (controls; 10 µL per 15 mL of culture) was added for 2 h each day during seven consecutive days. Following incubation the material was rinsed and placed in fresh half-strength modified

Table 1. Overview of the primers used for monitoring the response of stress gene candidates in *Ectocarpus siliculosus*

Primer name	Primer sequence Forward (5´-3´) Reverse (5´-3´)	ID *Ectocarpus siliculosus* genome[a]	Reference
EF1a	GCAAGGGCCTCAGCTCTG ACAAGCCGTCTGGGTATATGTTAGC	Esi0387_0021	Le Bail et al. (2008)
vBPO_3	GGAACTGCCCAACCTTTCGG GCTCGTCACAGAAGTCGTCTCC	Esi0009_0080	Strittmatter et al. (unpublished)
MnSOD	CGGTGGTGTCTGAGAACTAC GTGGTGGAAGATAATCGCAATG	Esi0091_0024	Strittmatter et al. (unpublished)
HSP70_1	AGGCGTTGGATTGGCTGGAC ACAACTCGTCGTCTCCGAAGTC	Esi0002_0284	Strittmatter et al. (unpublished)
GRX	CGGCTTCTAAACAGGGCAGGAG GGTCTACGCATCAGGGCTAACAC	Esi0010_0089	Zambounis, Strittmatter and Gachon (unpublished / personal communication)
GST	ATGCCCGTGTTGGAGGTAGATG GCACTGTCGGCGAAAGCG-	Esi0191_0054	De Franco et al. (2008)
mN	TCATTTTTCATGTGGAGGTCTCTG GCCAAACAAACAACAACCCTC	Esi0092_0006	Le Bail et al. (2008)

EF1a, elongation factor 1α; *vBPO*, vanadium-dependent bromoperoxidase; *MnSOD*, manganese superoxide dismutase; *HSP70*, heat shock protein 70; *GRX*, glutaredoxin; *GST*, glutathione S-transferase.
[a]Cock et al. (2010).

Provasoli-enriched seawater in 650 mL filter cap suspension flasks (175 cm²; Greiner Bio-One). The experimental procedure included two biological and three technical replicates each, respectively. Cultures were harvested for RNA extraction by concentrating the culture on 70 µm nylon mesh filters (Cell Strainers™; BD Biosciences, Oxford, UK), quickly dried with an autoclaved paper towel, and immediately immersed in RNALater™ solution (Ambion, Austin, TX, USA), left for perfusion for 1 day at 4°C, followed by storage at -80°C.

Total RNA was extracted from approximately 70 mg (fresh weight) of tissue using the Qiagen RNeasy® Plant Mini-kit (Qiagen, Crawley, UK) and DNase-treated (RNAse-free DNAse I; Invitrogen, Paisley, UK), according to the manufacturer's instructions with the following modifications: Two volumes (900 µL) of buffer RLC was added to the disrupted tissue (Step 2; see RNeasy® Plant Mini-kit protocol; Qiagen) which resulted in cleaner, higher yield RNA extraction. Quality and integrity assessments as well as quantification of the RNA were performed using a Bioanalyzer 2100™ (Agilent Technologies) with the corresponding Agilent RNA 6000 Nano Kit. From each RNA sample, 0.5 µg was reverse transcribed to cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Subsequently, first strand cDNAs were diluted with nuclease-free water to a final concentration of 1 ng μL^{-1}.

Potential molecular responses of *E. silicilosus* to PGA_2 treatment were monitored with five stress marker genes (Table 1), following the protocol of Zambounis, Strittmatter and Gachon (unpublished / personal communication). The specificity, efficiency and dynamic range of the primers were tested on 1/5 serial dilutions of cDNA (range, 0.24-150 ng for elongation factor 1α [*EF1a*], vanadium-dependent bromoperoxidase [*vBPO*], manganese superoxide dismutase [*MnSOD*] and heat shock protein 70 [*HSP70*]; 0.12-75 ng for glutaredoxin [*GRX*]; 0.048-30 ng for glutathione S-transferase [*GST*]). The absence of genomic DNA contamination was examined in each sample with the intron-specific primer mN (Table 1) (Le Bail et al. 2008).

Quantitative expression analysis was performed using a QUANTICA™ real-time PCR system (Techne-Barloworld, Stone, Staffordshire, UK). Quantitative polymerase chain reactions (qPCRs) were run in a final volume of 20 µL with 2 ng cDNA and 300 µM of each primer with MesaGreen™ reaction mix (Eurogentec, Southampton, UK). Reactions were run in triplicates. The cycle parameters were 10 min at 95°C, followed by 45 runs of 15 s at 95°C and 1 min at 60°C. Dissociation curves were obtained by melting curve analysis from 65 to 95°C with readings every 0.5°C and 10 s hold between them.

Relative variation in gene expression ratios was calculated as fold changes relative to the control experiments using REST 2009 software (Pfaffl et al. 2002). *EF1a* was used as internal reference gene. Expression ratios of tested genes were determined between the control samples and the corresponding, treated samples using the geometric means of each group. Statistical significance of the results was tested [p(H1)] with pairwise fixed reallocation randomization tests using the REST 2009 software (M. Pfaffl, Munich; Qiagen, Hilden, Germany).

RESULTS

Oxidative burst

Among the five prostaglandins (PGE_1, PGE_2, $PF_2\alpha$, PGB_1, PGA_2) tested in *Laminaria*, only PGA_2 was found to be active (Fig. 1). The threshold concentration was observed to be between 0.1 and 1 µM, and the response occurred within seconds after adding the compound (Fig. 1). The strongest response was in the range of 5 µmol hydrogen peroxide per gram fresh weight. Also, diphenylene iodonium (DPI) at 10 µM did not inhibit the response of *L. digitata* to PGA_2 (data not shown).

Lipid metabolism

Fig. 2 provides an overview of free saturated and unsaturated fatty acid contents in *Laminaria* upon incubation with PGA_2. No significant differences in the amounts of individual and total free fatty acids, compared to controls, were observed after this treatment.

Three oxylipins, previously identified as relevant markers of fatty acid oxidation in *Laminaria* (Küpper et al. 2006), were quantified (Fig. 3). When compared to control conditions, PGA_2 treatment resulted, after 10 min, in significantly ($p < 0.001$) increased levels of free 15-hydroxyeicosatetraenoic acid (15-HETE). In contrast, no significant differences were observed for 13-hydroxyoctadecatreienoic (13-HOTE) and 15-hydroxyeicosapentaenoic acids (15-HEPE).

Gene expression analysis

In order to monitor the gene expression profiles obtained after PGA_2 treatment in *Ectocarpus*, five candidate genes (*vBPO*, *MnSOD*, *HSP70*, *GRX*, *GST*) were selected

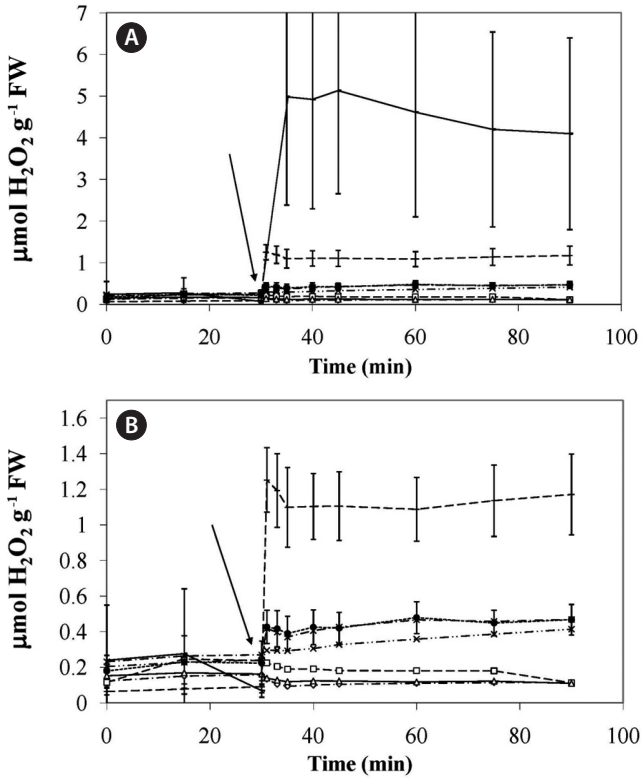

Fig. 1. Prostaglandin A_2 (PGA_2) triggers an oxidative burst in *Laminaria digitata*. The graphs show the time course of hydrogen peroxide production, following the addition of PGA_2 to the medium at t = 30 min (indicated by an arrow). (B) is a magnification of the data for the control series as well as 0.1, 1, 3, 6, 10 and 50 µM PGA_2, respectively, while (A) includes the response curve at 100 µM PGA_2. (A, B) Dashes, 100 µM; crosses, 50 µM; circles, 10 µM; asterisks, 6 µM; × crosses, 3 µM; triangles, 1 µM; squares, 0.1 µM; diamonds, control.

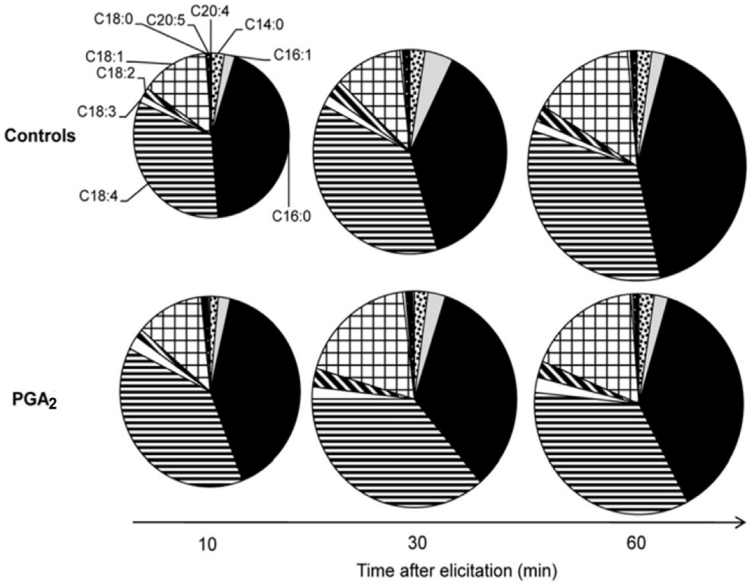

Fig. 2. Effects of prostaglandin A_2 (PGA_2) on the liberation of free fatty acids in *Laminaria*. *Laminaria* seedlings were kept in seawater (control) for 10, 30 and 60 min and treated with: PGA_2 (10 µM). Free saturated and unsaturated fatty acids from C14:0 to C20:5 (C14:0, C16:0, C18:0, C16:1, C18:1, C18:2, C18:3, C18:4, C20:4, C20:5) were quantified by gas chromatography-mass spectrometry. Results are the mean of triplicate experiments and pie charts display the relative free fatty acid composition at each sampling time. Sizes of the pie charts are proportional to total free fatty acid pools. No changes were observed compared to controls in relative composition and overall total pool of free fatty acids. Statistical significance of the level of each free fatty acid after treatment against control conditions was tested using a Student t-test.

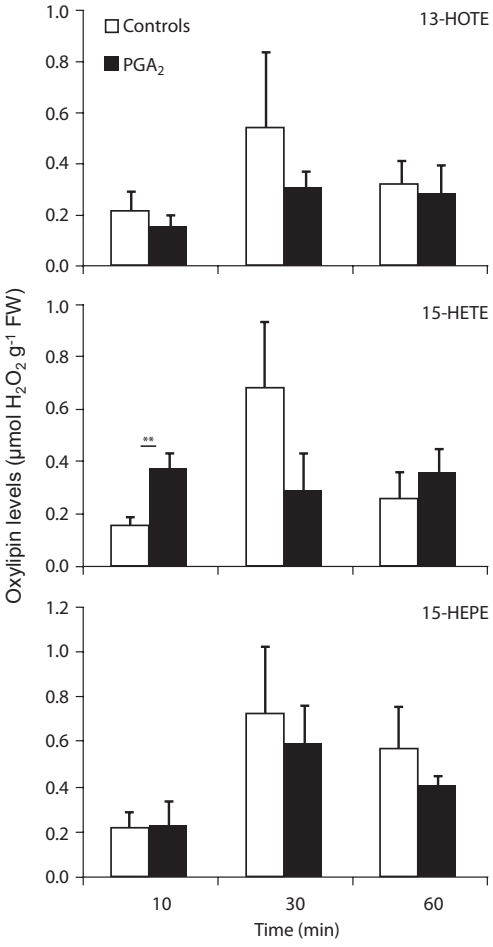

Fig. 3. Effects of prostaglandin A_2 (PGA_2) on the synthesis of oxidized fatty acids in *Laminaria*. *Laminaria* seedlings were kept in seawater (control) for 10, 30 and 60 min and treated with PGA_2 (10 µM, the same periods of time as the control). 13-hydroxyoctadecatrienoic acid (13-HOTE) (A), 15-hydroxyeicosatetraenoic acid (15-HETE) (B), and 15-hydroxyeicosapentaenoic acid (15-HEPE) (C) were quantified by HPLC-$APCI^{(-)}$-MS. Results are the mean of triplicate experiments and expressed (+/- SE) in µg g^{-1} of wet mass. Statistical significance of treatments against control conditions were tested using a Student t-test (**$p < 0.001$). HPLC-$APCI^{(-)}$-MS, reverse phase-high performance liquid chromatography coupled to a mass spectrometer in atmospheric pressure ionisation and negative ion mode.

based on our results in a parallel proteomics study of *Ectocarpus* subject to biotic stress (infection by *Eurychasma dicksonii*; Strittmatter, Gachon, Grenville-Briggs, van West, Potin and Küpper unpublished) in order to represent different functional categories related to oxidative stress, defense and cell rescue. Their respective transcript accumulations were quantified by a qPCR approach; in both biological repetitions after PGA_2 treatment the relative expression ratios of all target genes did not exhibit any significant differences compared to control samples [$p(H1) > 0.05$] (Table 2).

DISCUSSION

Significantly, this study reports the first case of an oxidative burst triggered by a prostaglandin in an algal or plant-like organism. The finding is very remarkable even with regard to animal models, where prostaglandins are known as modulators rather than actual inducers of oxidative bursts (Vaidya et al. 1999). To our knowledge, only one case of an oxidative burst triggered by a prostaglandin has been reported in eukaryotes, that of 15-deoxy-delta(12,14)-prostaglandin J_2, an agonist of peroxisome proliferator activated receptor gamma, which induces superoxide anion production in human cells, with an efficacy similar to its action on ERK phosphorylation (Huang et al. 2002). In contrast, some prostaglandins have been reported to inhibit the oxidative burst (e.g., Ottonello et al. 1995). Also, the oxidative burst observed here is among the strongest in amplitude ever observed in a brown algal system: The PGA_2-triggered oxidative burst is considerably stronger with up to 5 µmol H_2O_2 g^{-1} FW observed, in contrast to around 1 mol H_2O_2 g^{-1} FW for oligoguluronates (Küpper et al. 2001, 2002), lipopolysaccharides (Küpper et al. 2006), polyunsaturated free fatty acids and methyl jasmonate (Küpper et al. 2009). In contrast to arachidonic acid and methyljasmonate (MeJA) (Küpper et al. 2009), the oxidative burst response to PGA_2 in *Laminaria* increased in a dose-response-like manner towards higher concentrations, with the strongest response observed at 100 µM. Also, contrary to the response triggered by lipopolysaccharides and methyl jasmonate, the oxidative burst in response to PGA_2 occurs within seconds after treatment.

Whilst the oxidative burst triggered by arachidonic acid, MeJA (Küpper et al. 2009), oligoguluronates (Küpper et al. 2001, 2002) and lipopolysaccharides (Küpper et al. 2006) can be inhibited by DPI, this does not apply to the response to PGA_2. This finding indicates that at least two different sources of ROS co-operate in oxidative burst responses in brown algae. This is consistent with certain results in higher plants (Bolwell et al. 1998, Bestwick et al. 1999) which suggest that both an NAD(P)H oxidase, a neutrophil-like superoxide-generating enzyme, susceptible to inhibition by DPI, a suicide substrate of flavin-containing oxidases (O'Donnell et al. 1993), and peroxidases should be involved in the oxidative burst.

Moreover, it is noteworthy that besides activating an oxidative burst, lipopolysaccharides (Küpper et al. 2006), polyunsaturated free fatty acids, MeJA (Küpper et al. 2009) and PGA_2 (this study) also induce fatty acid oxidation in *Laminaria* leading to the synthesis of hydroxyl-PUFAs

- even though in the case of PGA_2 only one product (15-HETE) was present in significantly increased amounts. The major compounds observed in these studies, 13-HOTE, 15-HETE and 15-HEPE, have been described in *Laminaria* (Gerwick 1994) as putative ω-6 lipoxygenase metabolites. These three compounds (derivatives of C18:3, C20:4 and C20:5, respectively) have been reported to be anti-inflammatory mediators in mammals (Miller et al. 1990) but their physiological relevance in brown algae remains enigmatic. In our recent studies, we demonstrated that levels of these fatty acid hydroxides were highly increased after treatment of *Laminaria* plantlets with either bacterial lipopolysaccharides (Küpper et al. 2006), PUFAs or arachidonic acid (Küpper et al. 2009) and excess of copper (Ritter et al. 2008). The activation of such an intensity of PUFA oxidation was not reproduced here after treatment with PGA_2 - in fact, the low levels of free fatty acid release and fatty acid oxidation after PGA_2 treatment compared to the effects of lipopolysaccharide, MeJA and PUFAs are quite remarkable. This would support the notion that fatty acid oxidation is either not essential for the establishment of an oxidative burst when thalli are treated with PGA_2, i.e., that this prostaglandin activates signalling events considerably down-stream of PUFA oxidation - or that PGA_2 itself is a major agonist in defense reactions not recognized in previous studies. Further studies will be needed to address this question.

Furthermore and like for the peroxisome-proliferator activated receptors α and γ (PPAR α and γ) in mammals (Kliewer et al. 1995), arachidonic acid could be recognized by the same target receptors in *L. digitata* as its prostaglandin derivative: The absence of effects of inhibitors of cyclooxygenase and prostaglandin biosynthesis, indomethacin and aspirin (Jaworek et al. 2001), allene oxide synthase, aspirin (Pan et al. 1998) and of lipoxygenase (salicylhydroxamic acid) on the oxidative burst triggered by arachidonic acid in *Laminaria* which we have recently reported (Küpper et al. 2009) may be considered as first indications in this aspect. But neither in terrestrial plants nor in algae, any evidence exists so far for PPAR-like receptors. On the other hand, the possibility that arachidonate and PGA_2 selectively activate the production of two independent sources of ROS could explain the lack of inhibitor effects.

Evidence is growing that if terrestrial plants recognize their own lipid-based signals, they may also be able to recognize those of herbivores. For instance, caterpillar regurgitant contains aminated eicosanoids (peptidolipids) that are perceived by higher plants and induce elevated defenses (Arimura et al. 2000, Turlings et al. 2000). In this respect, it is noteworthy that prostaglandins have been described as elicitors from the oral secretions of herbivores (Schultz and Appel 2004) recognized by plants. A similar interpretation might be considered for the role of prostaglandins in the defense of *Laminaria* - even though PGA_2 has been shown to be synthesized in brown algae (Ritter et al. 2008), it cannot be ruled out to be produced by grazers and pathogens attacking brown algae as well.

The substantial knowledge about the important model *Ectocarpus* (Peters et al. 2004, Charrier et al. 2008), including the recently-accomplished genome sequencing project (Cock et al. 2010), prompted us to investigate this alga for potential molecular responses to PGA_2 treatment. However, our results with *Ectocarpus* showed no expres-

Table 2. Effects of prostaglandin A_2 (PGA_2) on relative gene expression in *Ectocarpus siliculosus*

Gene	Reaction efficiency	Expression ratio		Standard error		p(H1)	
		1st biological repetition	2nd biological repetition	1st biological repetition	2nd biological repetition	1st biological repetition	2nd biological repetition
EF1a	1.300	1.000	1.000				
vBPO	1.078	0.748	1.140	0.355-1,231	0.604-1.764	0.623	0.767
MnSOD	1.292	0.802	0.631	0.622-1,000	0.498-0.819	0.297	0.087
HSP70	1.273	0.716	0.580	0.492-0.971	0.383-0.899	0.153	0.107
GRX	1.144	0.611	0.821	0.450-0.768	0.355-2.015	0.202	0.702
GST	1.369	0.901	1.161	0.556-1.327	0.821-1.671	0.749	0.429

Relative expression ratios of the five *E. siliculosus* genes (after seven days of PGA_2 treatment). The results represent two independent experiments (exp.) (n = 3). p(H1) values were calculated based on a pairwise fixed reallocation randomization test statistical model inferring 2,000 iterations and indicate the probability of alternate hypothesis that difference between sample and control groups is due only to chance ($p < 0.05$).
EF1a, elongation factor 1α; *vBPO*, vanadium-dependent bromoperoxidase; *MnSOD*, manganese superoxide dismutase; *HSP70*, heat shock protein 70; *GRX*, glutaredoxin; *GST*, glutathione S-transferase.

sion response to PGA_2 treatment for all five genes tested, highlighting that at most levels including gene expression, the overall effects of PGA_2 remain unclear at the current stage. Nevertheless, the unprecedented finding of a strong oxidative burst triggered by PGA_2 in *Laminaria*, suggesting that this compound is a potent defense inducer, warrants more in-depth mechanistic investigations going beyond the scope of this manuscript.

In conclusion, the discovery of an oxidative burst in an alga triggered by a prostaglandin is intriguing. Prostaglandins - in particular, PGA_2 and its derivatives - are well established as feeding deterrents in corals (Gerhart 1984, Whalen et al. 2010): the findings presented in this study highlight new perspectives of PGA_2 being a more widespread defense molecule and inducer in the marine environment, in very distantly related lineages.

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