New Phytologist

Research

Kelps feature systemic defense responses: insights into the evolution of innate immunity in multicellular eukaryotes

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Received: *8 April 2014*
Accepted: *9 June 2014*

New Phytologist (2014)
doi: 10.1111/nph.12925

Key words: defense responses, elicitation, kelps, *Laminaria digitata*, oxidative burst, systemic immune response.

Summary

- Brown algae are one of the few eukaryotic lineages that have evolved complex multicellularity, together with Opisthokonts (animals, fungi) and Plantae (land plants, green and red algae). In these three lineages, biotic stresses induce similar local defense reactions. Animals and land plants also feature a systemic immune response, protecting the whole organism after an attack on one of its parts. However, the occurrence of systemic defenses has never been investigated in brown algae.
- We elicited selected parts of the kelp *Laminaria digitata* and monitored distant, nonchallenged areas of the same individual for subsequent defense reactions.
- A systemic reaction was detected following elicitation on a distant area, including an oxidative response, an increase in haloperoxidase activities and a stronger resistance against herbivory. Based on experiments with pharmacological inhibitors, the liberation of free fatty acids is proposed to play a key role in systemic signaling, reminiscent of what is known in land plants.
- This study is the first report, outside the phyla of Opisthokonts and Plantae, of an intraorganism communication leading to defense reactions. These findings indicate that systemic immunity emerged independently at least three times, as a consequence of convergent evolution in multicellular eukaryotic lineages.

Introduction

Brown algae are one of the few eukaryotic lineages that have evolved complex multicellularity, together with Opisthokonts (animals, fungi) and Plantae (land plants, green and red algae). Despite their phylogenetic distance, these lineages share common traits regarding local innate immunity (Hoffmann *et al.*, 1999; Cosse *et al.*, 2007; Weinberger, 2007). For instance, animal and plant immune systems use similar innate receptors for mediating their resistance against pathogens (Ausubel, 2005; Staal & Dixelius, 2007), and recently, candidate receptors potentially involved in algal defense were mined in the genome of the brown alga *Ectocarpus siliculosus* (Zambounis *et al.*, 2012). Another conserved feature is the oxidative burst that leads to a strong local concentration of reactive oxygen species (ROS). This early response was first discovered in human blood cells (Baldridge & Gerard, 1933) and later described in land plants (Wojtaszek, 1997) and marine algae (for a review, see Cosse *et al.*, 2007). Furthermore, pathogen infection induces the release of free fatty acids (C18 and/or C20 FA) from membrane lipids in the three lineages (Potin *et al.*, 2002; Yaqoob, 2003; Harizi *et al.*, 2008; Stanley *et al.*, 2009; Zoeller *et al.*, 2012; Yu *et al.*, 2013).

Besides these locally induced reactions, defense mechanisms can also be activated systemically in tissues that are not directly challenged. Systemic resistance aims to restrict the spread of the attacker within the challenged organism, as enhanced resistance is acquired in remote organs that are not yet affected. These phenomena are well known in vertebrates (Boehm, 2012; Dishaw & Litman, 2013), insects (Ferrandon *et al.*, 2007) and land plants (Dempsey & Klessig, 2012; Kachroo & Robin, 2013). In land plants, the activation of defense in systemic tissues is often termed systemic acquired resistance (SAR) or systemic acquired acclimation (SAA). The activation of SAR in uninfected or undamaged tissues requires transmission of signal(s) from the infected tissue via the vasculature, enhancing resistance to a second infection (Ross, 1961; Fu & Dong, 2013). During plant defense, volatile organic compounds (VOCs) are also emitted in the neighboring environment, functioning as an external signal for intraplant communication and systemic responses (Heil & Silva Bueno, 2007). These airborne cues act in synergy with systemic signals transported via the vasculature, optimizing the global plant resistance (Heil & Ton, 2008; Shah, 2009). In the terrestrial environment, some of these VOCs are also perceived in neighboring plants as warning signals and trigger a priming of

the defenses (Conrath, 2009). This state is an increased capacity to develop rapid and efficient responses to later biotic attacks, and has also evolved in animals (Netea *et al.*, 2011; Chambers & Schneider, 2012). In the context of innate immunity, priming and SAR are indeed complementary mechanisms using long-distance signaling.

Brown algal immunity has been most studied in the model organism *Laminaria digitata*. This brown algal kelp shares a number of common traits with immune responses in land plants and animals. Under attack, *L. digitata* specifically recognizes cell wall fragments, triggering a local oxidative burst (Küpper *et al.*, 2001, 2002), the expression of defense genes (Cosse *et al.*, 2009), and a rapid activation of specific pathways such as the release of free fatty acids (Goulitquer *et al.*, 2009) and halogen metabolism (Cosse *et al.*, 2009). These reactions are induced by bacterial attack or grazing by herbivores, which can be mimicked using cell wall fragments as defense elicitors (e.g. oligoguluronates, GG; Küpper *et al.*, 2002; Goulitquer *et al.*, 2009; Leblanc *et al.*, 2011) or free fatty acids and methyl jasmonate (Küpper *et al.*, 2009). Recently, the existence of priming mechanisms reminiscent of land plants was shown in *L. digitata*, where waterborne signals shape the elicitor-induced immunity of kelps (Thomas *et al.*, 2011). However, the occurrence of systemic signaling is still unknown in this brown alga. In order to explore this question, we hypothesized that, if a systemic propagation occurs in *L. digitata*, algal parts remote from the site of defense elicitation would display similar defense responses and/or efficient protection against later biotic attack. In this study, we investigated the effect of the incubation of a selected part of the lamina of *L. digitata* with GG defense elicitors on distant nonchallenged parts. Responses were analyzed at various levels, namely the emission of ROS, the transcription of defense marker genes, the induction of halogen-related enzymes, and the resistance against herbivory. Our data suggest the existence of an intraplant defense communication in *L. digitata*. This demonstrates the occurrence of systemic immunity in this brown alga, the first such evidence in an organism outside Metazoan and Plantae.

Materials and Methods

Biological material

The kelp life cycle consists of a microscopic haploid gametophyte phase, alternating with macroscopic diploid sporophytes. In this study, all experiments were done on the macroscopic diploid individuals. *L. digitata* (L) Lamouroux sporophytes (*c.* 30 cm in length) were collected from two sites (+48°43′3564, −3°58′697 and +48°42′2469, −4°3′5984) in the vicinity of Roscoff (Brittany, France) at low tide and kept for at least 4 d (recovering time) and up to 2 wk before experiments in well-aerated, filtered seawater (FSW) at 13°C under a photoperiod of 16 h of light (40 μmol photons m^{-2} s^{-1}) and 8 h of darkness. For each series of experiments, control and treated sporophytes were randomly selected from one batch of individuals, collected on the same day and kept in the same container. The blue-rayed limpets *Patella pellucida* Linnaeus (formerly named *Helcion pellucidum*) (*c.* 5 mm in length) were collected on *L. digitata* fronds from the same sites and starved in running seawater for 1 wk before use for herbivory bioassays.

Elicitors

Oligoguluronates were prepared using sodium alginate from *Laminaria hyperborea* stipes (Danisco, Landerneau, France) according to Heyraud *et al.* (1996). A 0.5% alginate solution was hydrolyzed 5 h at 100°C in 0.3 M HCl. After selective precipitation and centrifugation, GG blocks were dialyzed, freeze-dried and resuspended in distilled water.

Elicitation procedures

All elicitation experiments and associated controls were conducted at 13°C. Sporophytes were positioned over 3 Petri dishes (diameter 90 mm) filled with 60 ml of FSW, each corresponding to one of the three parts defined in Fig. 1. Challenged parts were elicited by application of GG stock solution at a final concentration of 150 μg ml^{-1} in FSW. At the end of the experiment, i.e. 3 h after the application of GG solution, algal parts were cut, frozen separately in liquid nitrogen and stored at −80°C until RNA extraction. In the cutting experiment, the algal parts 1 and 2 were separated using a scalpel just after elicitation on the meristematic part and left at the same position. For pretreatment with inhibitors, only the meristematic part of *L. digitata* sporophytes was incubated for 20 min with the chemicals. Algal tissues were then rinsed three times with FSW before elicitation. The stock solutions of inhibitors were prepared in dimethylsulfoxide (DMSO; Fisher Scientific, Loughborough, UK) and diluted in FSW at the following final concentrations: 20 μM chlorpromazine-HCl (CPZ, targeting phospholipase A1); 10 μM diphenylene iodonium (DPI, targeting NADPH oxidases); and 100 μM propyl gallate (PrG, targeting peroxidases and protectant of lipid oxidation). An equal volume of DMSO was used in each corresponding control treatment.

Hydrogen peroxide (H_2O_2) measurement

The concentration of H_2O_2 in FSW was measured over 3 h after elicitation using a luminometer (LUMAT LB9507; EG&G Berthold, Evry, France), as described previously (Küpper *et al.*, 2001). Briefly, 50 μl of 0.3 M luminol and 100 μl of

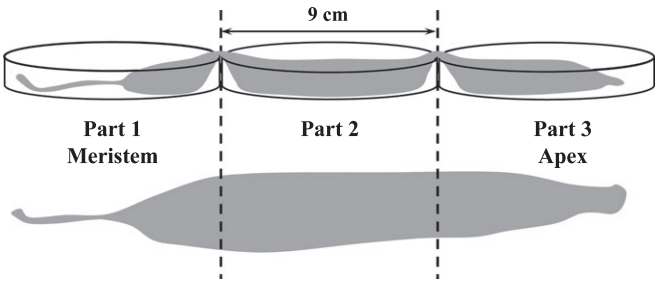

Fig. 1 Scheme showing the experimental setup used to test systemic responses in *Laminaria digitata* young sporophytes.

20 units ml^{-1} horseradish peroxidase in 10 mM phosphate buffer (pH 7.8) were added to 300 µl FSW aliquots. Chemiluminescence signal was integrated over 2 s and compared with a standard curve of H_2O_2 in FSW. H_2O_2 amounts were expressed as µmol g^{-1}, considering the volume of seawater surrounding each algal part and its FW.

Transmission electron microscopy

The cytochemical method used to localize H_2O_2 production was adapted from Weinberger *et al.* (2005) and based on the reaction of H_2O_2 with cerium chloride ($CeCl_3$) leading to insoluble, electron-dense precipitates of cerium perhydroxides $Ce[OH]_2OOH$ and $Ce[OH]_3OOH$. Using the experimental design shown in Fig. 1, sporophytes were challenged by GG elicitation on the apical part ($n = 3$). Experiments without GG elicitation were conducted at the same time (control treatment, $n = 3$). Tissue pieces (2–4 mm) were excised from apical, intermediate, and meristematic areas at 7 min, 1 h 40 min and 3 h after the beginning of the experiment, respectively. Samples were incubated for 1 h in FSW containing 5 mM $CeCl_3$ and 50 mM 3-(morpholino) propanesulfonic acid (pH 7.2; ACROS Organic, Geel, Belgium), fixed in 3% (v/v) glutaraldehyde for 1 h and transferred to another fixing solution containing 3% (v/v) glutaraldehyde and 1.5% (v/v) paraformaldehyde overnight. Samples were dehydrated in graded ethanol series (25, 50, 75, 100% (v/v), twice for 1 h each) and progressively embedded in Spurr's resin (25, 50, 75% (v/v), 24 h each). Tissues were transferred for 2 h into pure resin, followed by a change of fresh resin for 24 h, placed in blocks and polymerized at 60°C for 4 d. Sections were cut using a diamond knife on a Leica ultracut UCT ultramicrotome, stained with uranyl acetate and lead citrate and viewed with a JEOL JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan). Micrographs were taken using a Gatan Orius camera (Gatan France, Evry, France).

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using an adapted protocol from Apt *et al.* (1995) and digested with 2 U of Turbo DNase (Ambion) for 30 min at 37°C. Total RNA was quantified spectrophotometrically on a Nanodrop ND 1000 (Labtech, Palaiseau, France), checked for genomic DNA contamination by conventional PCR (35 cycles) of the actin gene and analyzed on 2% agarose gel to verify its integrity. cDNA was synthesized from 400 ng of total RNA using 2 µl of RT Improm II (Promega) according to the manufacturer's instructions. Quantitative PCR was performed as described previously (Cosse *et al.*, 2009). A fresh standard curve of genomic DNA (3–57 142 copies) was included in each plate to check the PCR efficiency and express the number of transcripts as gene copies. The specificity of amplification was checked by dissociation curve. Using sequence primers provided in Cosse *et al.* (2009), transcript abundances were quantified for four defense-related genes, namely glucose-6-phosphate dehydrogenase (g6pd), thioredoxin (trx), heat shock protein (hsp70) and vanadium-dependent bromoperoxidase 3 (vBPO3). The qRT-PCR data were normalized by transcript abundances of the tubulin gene in each sample.

Haloperoxidase activity measurement

Sporophytes were elicited on the apical area (part 3, Fig. 1), as described in the elicitation procedures. The control and elicited algae ($n = 3$) were regularly rehydrated with FSW to avoid desiccation as a result of the increased time of experiment. After 9 h, algal parts were cut and frozen separately in liquid nitrogen. Proteins were extracted from 1 g of ground tissues in 5 ml extraction buffer composed of 25 mM borate/NaOH buffer, pH 8.8, 15 mM EGTA, 15 mM $MgCl_2$, 2 mM dithiothreitol, 0.5% polyvinylpyrrolidone and a cocktail of antiproteases (Complete; Roche, Meylan, France). Samples were incubated for 1 h at room temperature under agitation and sonicated four times for 30 s on ice. After the addition of 0.3 vol. ethanol and incubation for 30 min at room temperature, samples were centrifuged for 15 min at 40 000 ***g***. Protein concentrations were determined in the supernatants following the method of Bradford with BSA as a standard. Vanadium-dependent iodoperoxidase (vIPO) and bromoperoxidase (vBPO) activities were measured using 5 µl of protein extracts by a colorimetric assay based on thymol blue halogenation as described in Verhaeghe *et al.* (2008). For each sample, technical duplicate activity tests were performed for vIPO and vBPO.

Herbivory bioassay

This bioassay was designed as a 'no choice' experiment to force *P. pellucida* to subsist on a single piece of alga, either an apical part which was previously elicited or not by GG or the distal meristematic part, which might systemically respond or not. *L. digitata* sporophytes (*c.* 20 cm in length) were placed over two Petri dishes on the same principle as shown in Fig. 1. Following incubation of the apical area in 15 ml FSW containing GG-oligosaccharides for 3 h, meristematic and apical parts were sectioned using a scalpel and placed separately in new Petri dishes with fresh FSW for 24 h. Non-elicited control sporophytes were handled in the same way without challenging with GG. After 24 h, each algal fragment was blotted dry, weighed to 10^{-4} g on an electronic scale (Scaltec SBA 33, Göttingen, Germany) to record the initial mass, and placed individually in FSW. Five randomly chosen blue-rayed limpets *P. pellucida* were then allowed to graze on each individual fragment. After 7 d, blue-rayed limpets were removed from the algal surface and the fragments were weighed to calculate the tissue consumption. Controls without herbivores were also prepared to check for nonfeeding-related (autogenic) weight variations during the experiment, and for all conditions, the FSW medium was changed every day. Consumption of each assayed algal piece was then calculated according to the following formula (Cronin & Hay, 1996): $\text{consumption} = T_{start} \times (C_{end}/C_{start}) - T_{end}$, where T_{start} and T_{end} represent the wet mass of an assayed algal piece before and after the feeding assay, respectively, and C_{start} and C_{end} represent pre- and postfeeding assay wet mass of the corresponding autogenic control alga, respectively.

Statistical procedures

Data were analyzed in R v2.15.0 (http://www.r-project.org). Statistical differences for H_2O_2 amounts and transcripts abundances were assessed by the Mann–Whitney test. Two-way ANOVA models were used to analyze the data for enzymatic activities and grazing impacts. In the first model, we tested the effect of the algal part (meristem, intermediate, apex) and treatment (control or elicited on apex), and their interactions, on the enzymatic activities of vBPO and vIPO. In the second model, we tested the effects of the algal part (meristem or apex) and treatment (control or elicited on apex), and their interactions, on the mass of tissue grazed by blue-rayed limpets. For this latter model, values were log-transformed to meet the normality and homogeneity of variance assumptions.

Results

Elicitation of *L. digitata* sporophytes with oligoguluronates triggers an oxidative response both locally and remotely

To investigate whether a local elicitation of a sporophytic blade of *L. digitata* with GG can induce responses in remote parts of the thallus, young sporophytes were placed in an experimental set up that allowed separate incubation of their meristematic, intermediate, and apical parts (Fig. 1). First, we followed the oxidative response induced with GG. The H_2O_2 concentration was monitored separately in seawater surrounding the different algal parts, following the application of GG on only one of them. As shown previously (Küpper *et al.*, 2001; Thomas *et al.*, 2011), GG elicitors induced a fast and transient release of H_2O_2 in the seawater surrounding the challenged part, reaching a maximum within 10 min and decreasing afterwards (see, e.g. Fig. 2a). The amount of H_2O_2 released by a challenged part depended on its position along the sporophyte, with maximum concentrations reaching, on average, 0.27 ± 0.15, 0.42 ± 0.13, and 1.10 ± 0.50 μmol g^{-1} FW for the meristematic, intermediate, and apical parts, respectively (Fig. 2b). Control sporophytes handled in the same experimental setup showed only a low, stable production of H_2O_2, on average a maximum release of 0.14 ± 0.02 μmol g^{-1} FW, irrespective of the site under investigation ($n = 36$).

Interestingly, increases of H_2O_2 concentration were also observed in the seawater surrounding algal tissues that were not in direct contact with elicitors. For example, elicitation of the kelp sporophytes in their intermediate part was followed by a marked increase in the production of H_2O_2 in both the distant, unchallenged apical and meristematic parts, reaching a maximum after 1 h 30 and 2 h, respectively (Fig. 2a). Overall, a distant oxidative response was observed whichever area was challenged with the elicitor, be it the meristematic, intermediate, or apical parts (Fig. 2b). In terms of kinetics, the remote responses always occurred from 30 min to 2 h after the local responses and were more spread out over time compared with the local and transient oxidative burst (see, e.g. Fig. 2a). However, within the 3 h timecourse of the experiments, no distant response was detected in the apical parts of those sporophytes that had been challenged in their meristematic areas (Fig. 2b).

Local and remote oxidative responses are comparable in terms of intensity and site of production

As shown in Fig. 2(b), the release of H_2O_2 from the unchallenged areas was equivalent to or even greater than from the directly GG-challenged tissues of *L. digitata* sporophytes. For example, elicited apical tissues produced, on average, 1.10 ± 0.50 μmol H_2O_2 g^{-1} FW, followed by a comparable release from the unchallenged intermediate (0.91 ± 0.43 μmol g^{-1} FW) and meristematic parts (0.83 ± 0.31 μmol g^{-1} FW). Interestingly, the meristematic part showed a more intense release of H_2O_2 when responding to an indirect challenge (0.75 ± 0.17 μmol g^{-1} FW, $n = 11$, irrespective of the site of elicitation) than locally (0.27 ± 0.15 μmol g^{-1} FW, $n = 8$).

To further compare the local and distant oxidative responses, we used a cytochemical method allowing the subcellular localization of the sites of H_2O_2 production within tissues. *L. digitata* sporophytes were either treated as controls or elicited with GG on their apical parts. The time points for the sampling of algal tissues were chosen according to the time of maximum H_2O_2 accumulation in the seawater as determined in the luminometry experiments. After 7 min, 1 h 40 min, and 3 h, fragments were taken from apical, intermediate, and meristematic areas, respectively, and then incubated in cerium chloride. Only very faint deposits of cerium perhydroxide were observed in tissues from control sporophytes (Fig. 2c). By contrast, precipitate accumulation was clearly observed in algal thin sections prepared from the three areas of algae elicited on their apical part. Deposits were mainly detected in the apoplasm, often featuring intense black spots close to the plasma membrane (Fig. 2c). In some cells, precipitates were also found along membranes of intracellular organelles, such as plastids or mitochondria (see Part 2, bottom picture in Fig. 2c for an example).

Distant responses are abolished by sectioning but not by the suppression of H_2O_2 accumulation at the initiation site

When sporophytes were sectioned just after elicitation of their meristematic area, the distant H_2O_2 increase in the intermediate part was fully abolished compared with intact algae (Fig. 3). In this case, a strong H_2O_2 production was detected only locally (1.19 ± 0.46 μmol H_2O_2 g^{-1} FW) and was significantly higher than in intact algae.

We next examined whether the localized oxidative burst was involved in the induction of the systemic cellular responses. Local application of various pharmacological inhibitors, known for blocking ROS accumulation (Küpper *et al.*, 2001, 2009), on the meristematic parts of *L. digitata* sporophytes before challenge with GG did not completely suppress the progression of the systemic signal. As shown earlier (Küpper *et al.*, 2001), preincubation with CPZ, possibly targeting phospholipases A, suppressed the local GG-induced release of H_2O_2 from the meristematic area (Fig. 3). The distant oxidative response of the remote

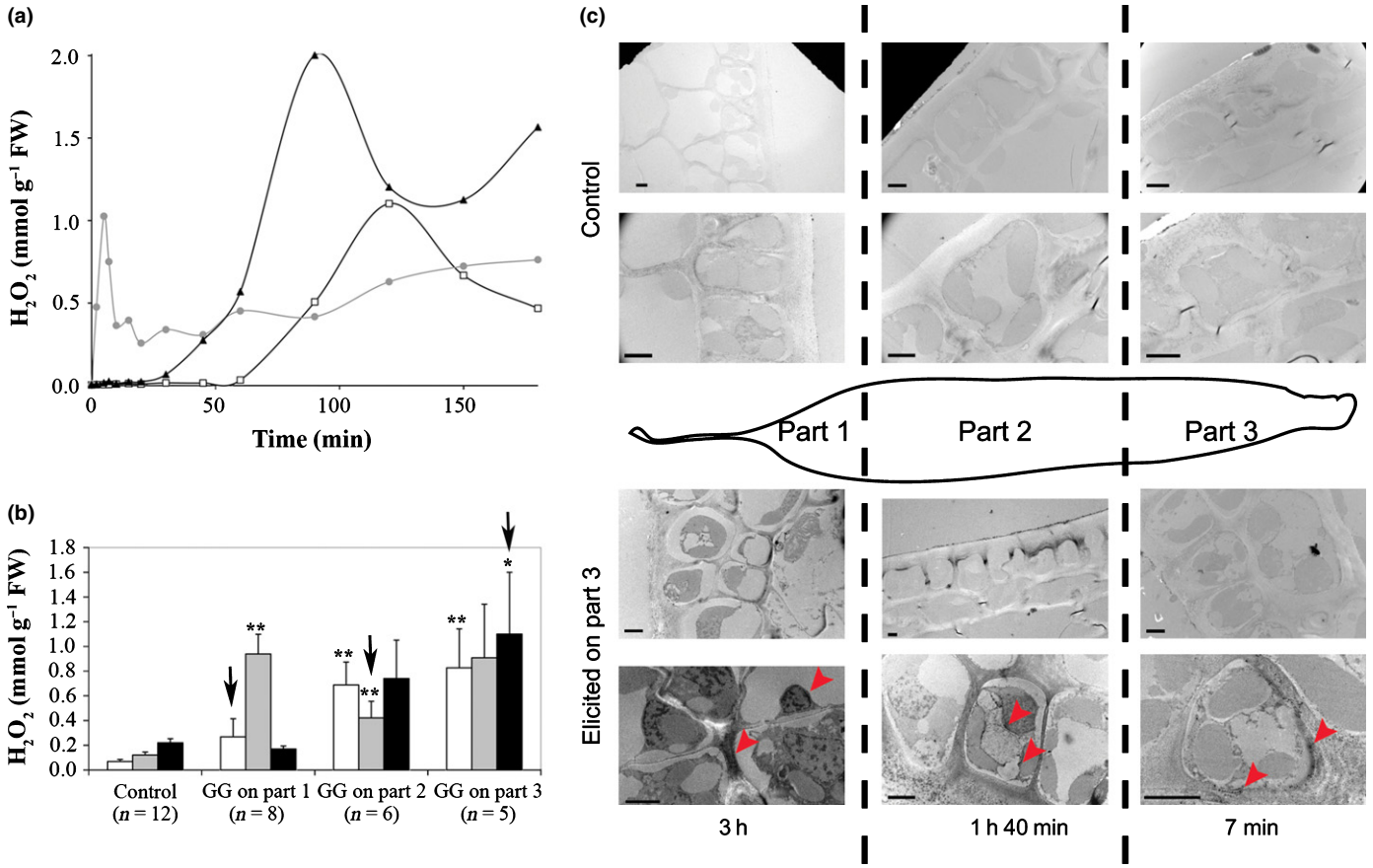

Fig. 2 Monitoring of the oligoguluronate (GG)-induced oxidative bursts locally and in remote parts of *Laminaria digitata* sporophytes. (a) Evolution of H_2O_2 concentration in seawater around meristematic part 1 (open squares), intermediate part 2 (grey circles) and apical part 3 (black triangles) after GG elicitation on part 2 over 3 h. All experiments were replicated three times and a typical result is shown. (b) Maximum H_2O_2 concentration measured in seawater over 3 h around part 1 (white), 2 (grey) or 3 (black) of *L. digitata* sporophytes after no treatment (control) or elicitation by GG of only one part (indicated by a black arrow). Data are means ± SE of *n* biological replicates (as indicated). The oxidative responses, locally (GG-induced part) and in surrounding parts, were compared with those of unelicited corresponding parts ('control' bars) using the Mann–Whitney test (*, $P \leq 0.065$; **, $P \leq 0.01$). (c) Detection and subcellular localization of H_2O_2 formation in *L. digitata* sporophytes elicited on the apical area (part 3), compared to unelicited controls. H_2O_2 formation was visualized by electron microscopy after precipitation of cerium hydroperoxide. Two typical results are shown for each case, and red arrows indicate precipitation along membranes of organelles. Bars, 2 μm.

intermediate part was also significantly reduced. DPI, a specific inhibitor of NADPH oxidases, abolished the emission of H_2O_2 from the elicited meristematic part. However, in this case, production of H_2O_2 still occurred in the remote parts, and was significantly higher in the apical part compared with algae untreated with DPI. Similarly, pretreatment with the inhibitor of peroxidases and protectant of lipid oxidation, PrG, also suppressed the local oxidative response to elicitation, but induced a significantly higher release of H_2O_2 from the unchallenged intermediate and apical parts.

Signal propagation does not lead to distant transcriptional activation of defense genes

In a previous study on young *L. digitata* plantlets (Cosse *et al.*, 2009), a variety of gene responses to the incubation in the presence of GG elicitors was identified. In the present work, we investigated the transcriptional activation of four of the most responsive genes that are induced within 3 h of elicitation with GG, namely the genes encoding g6pd, trx, hsp70 and vBPO3. *L. digitata* sporophytes were challenged with GG on their meristematic or apical parts for 3 h, then the expression of defense genes was quantified both locally (at the elicited site) and in remote parts of the sporophytes. The expression of the four genes under investigation was significantly induced in elicited meristematic parts (Table 1), as shown previously in Cosse *et al.* (2009). However, in the case of a challenge on the apical parts of *L. digitata* sporophytes, this GG-induced local effect was observed only for one of the marker genes (trx). The transcript abundances of the three other genes were not significantly higher than in controls. In addition, the expression of these four genes did not differ significantly from the controls in unchallenged, remote parts of the sporophytes, whatever the site of elicitation (Table 1).

Local GG elicitation and signal propagation increase haloperoxidase activities in *L. digitata* sporophytes

To correlate the occurrence of remote oxidative responses with other defense mechanisms, we measured the activity of vIPO and vBPO, a group of enzymes known to be involved in the

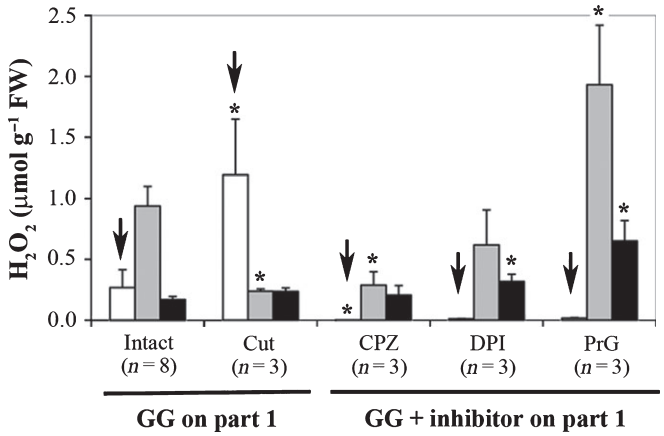

Fig. 3 Effect of cutting and pretreatment with inhibitors on the maximum H_2O_2 concentration measured in seawater over 3 h around part 1 (white), 2 (grey) or 3 (black) of *Laminaria digitata* sporophytes after elicitation of the meristematic area (part 1). Data are means ± SE of *n* biological replicates (as indicated) and were compared with the corresponding part from intact algae using the Mann–Whitney test (*, $P \leq 0.065$). CPZ, chlorpromazine-HCl, DPI, diphenylene iodonium, PrG, propyl gallate.

Table 1 Fold changes in transcript abundance of four defense-related genes in local and remote parts of *Laminaria digitata* after elicitation with oligoalginates (GG)

	GG on meristematic part		GG on apical part	
Gene	Local effect	Remote effect	Local effect	Remote effect
g6pd ($n = 7$)	**2.23 ± 0.4** ($P = 0.064$)	1.10 ± 0.20 ($P = 0.749$)	1.50 ± 0.21 ($P = 0.116$)	1.34 ± 0.31 ($P = 0.391$)
trx ($n = 7$)	**4.03 ± 0.71** ($P = 0.002$)	0.88 ± 0.28 ($P = 0.482$)	**2.49 ± 0.34** ($P = 0.013$)	1.04 ± 0.23 ($P = 0.286$)
hsp70 ($n = 3$)	**4.08 ± 1.30** ($P = 0.049$)	0.67 ± 0.37 ($P = 0.513$)	1.52 ± 0.24 ($P = 0.275$)	0.80 ± 0.37 ($P = 0.513$)
vBPO3 ($n = 7$)	**2.54 ± 0.66** ($P = 0.063$)	1.43 ± 0.55 ($P = 0.565$)	1.89 ± 0.39 ($P = 0.110$)	1.91 ± 0.75 ($P = 0.337$)

Transcript abundances were quantified by quantitative reverse transcription polymerase chain reaction (RT-PCR) and the fold changes were calculated relative to the appropriate control from unelicited algae. Values are means ± SE; *P*-values are the results of Mann–Whitney tests comparing elicited and control sporophytes; values in bold represent a significant increase in the transcript abundance compared with control ($\alpha = 6.5\%$). g6pd, glucose-6-phosphate dehydrogenase; trx, thioredoxin; hsp70, heat shock protein 70; vBPO3, vanadium-dependent bromoperoxidase 3.

halogen-related defense responses of brown algae (Cosse *et al.*, 2007). Protein extracts were prepared from the three different parts of *L. digitata*, 9 h after GG challenging on the apical part, and used for enzymatic tests (Fig. 4). For both haloperoxidases, there was no significant variation in the activities retrieved from the three different parts (two-way ANOVA, 'part effect'; $F = 0.67$, $P = 0.43$ for vBPO; $F = 0.11$, $P = 0.75$ for vIPO). Interestingly, the elicitation procedure induced a significant increase in these enzymatic activities in all parts compared with control algae, although only part 3 was challenged by GG (two-way ANOVA, 'treatment effect'; $F = 17.68$, $P = 8.8 \times 10^{-4}$ for vBPO; $F = 10.15$, $P = 6.6 \times 10^{-3}$ for vIPO). Indeed, stronger increases in the activity of both enzymes were found in algal parts distant from the challenged area. The highest inductions occurred in the remote, intermediate part (2-fold and 1.6-fold induction for vIPO and vBPO, respectively).

Signal propagation after local GG elicitation of *L. digitata* sporophytes stimulates resistance against grazing in remote tissues

To test whether elicitation on one part could favor systemic resistance against herbivory, we developed a grazing bioassay based on challenging with the blue-rayed limpet *P. pellucida*, which was recently confirmed as a specialist grazer of *L. digitata* using *in situ* observation and isotopic studies (Schaal *et al.*, 2010; Leblanc *et al.*, 2011). As shown in Table 2, in a 'no choice' experiment blue-rayed limpets grazed the meristematic part significantly more than the apex of *L. digitata* sporophytes (two-way ANOVA, 'part' effect, $F = 30.52$, $P = 4.6 \times 10^{-5}$), whatever the treatment. However, tissue consumption was significantly lower in algae elicited on the apical area than in the controls ('treatment' effect, $F = 6.05$, $P = 0.026$). Specifically, grazing of meristematic tissue, distant from the site of elicitation, was significantly decreased, by 55% compared with controls (*t*-test, $P = 0.038$; Table 2).

Discussion

L. digitata features both local and systemic defense responses

Elicitation with oligoguluronates, which mimic a biotic stress in *L. digitata* sporophytes and induce resistance against attackers (Küpper *et al.*, 2001, 2002), triggered defense responses locally. As shown previously (Küpper *et al.*, 2001; Cosse *et al.*, 2009; Thomas *et al.*, 2011), this includes an oxidative burst (Fig. 2) and the expression of specific defense genes (Table 1). In addition, we show here that this local response involves an increase in vanadium-dependent haloperoxidase activities in the challenged tissues (Fig. 4) and leads to resistance against grazing by the blue-rayed limpet *P. pellucida* (Table 2). Some differences were detected in the local responses, depending on the position of the challenged area along the *L. digitata* blade. Namely, the meristematic parts tended to accumulate lower amounts of H_2O_2 than the intermediate or apical areas when challenged directly, and the induction of defense genes was more intense in this area. It is also worth noting that in control algae, blue-rayed limpets grazed more on meristematic parts than on apex. This is consistent with field observations, which show that blue-rayed limpets are mainly found close to the meristem, stipe, or holdfast of *L. digitata* sporophytes (Graham & Fretter, 1947; Leblanc *et al.*, 2011). One explanation could be that older parts (apex) are less appetent or have a smaller nutritive value.

Interestingly, unchallenged parts of the sporophytes were affected by elicitation on a distant area. Notably, tissues distant from the initial spot of elicitation displayed production of H_2O_2

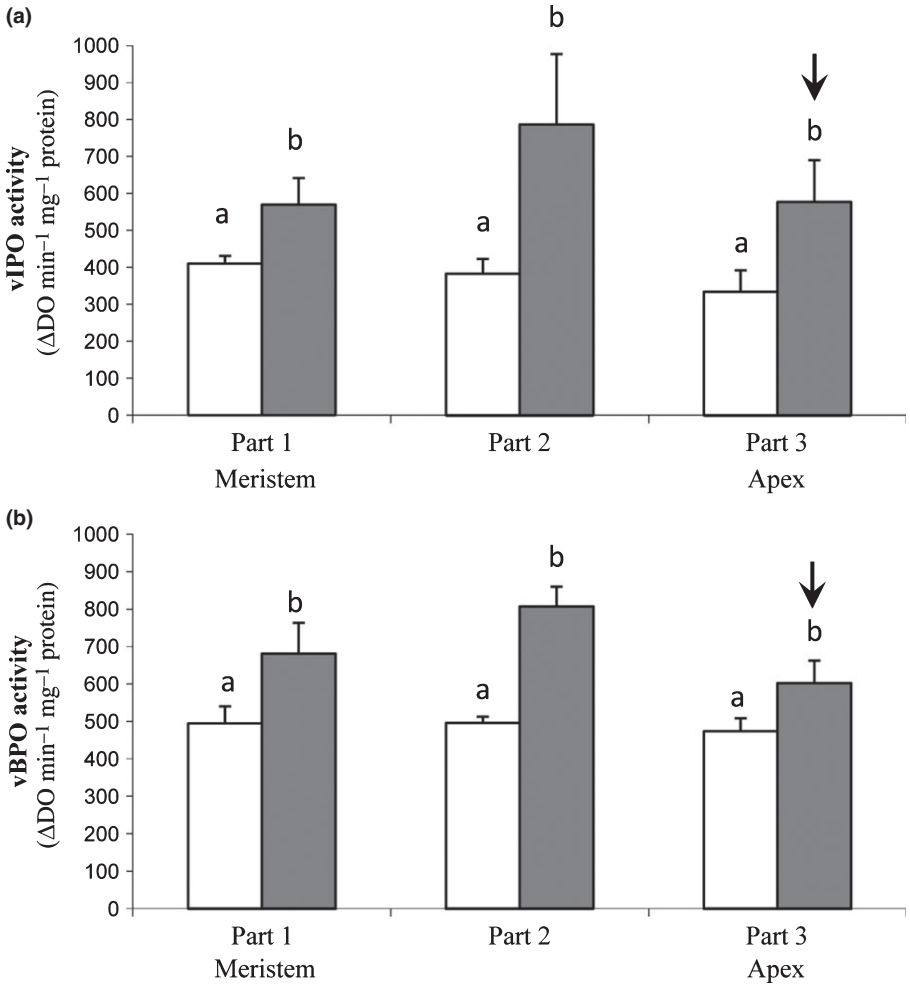

Fig. 4 Vanadium-dependent iodoperoxidase (vIPO, a) and bromoperoxidase (vBPO, b) activity measurements in crude protein extracts from the three different parts of *Laminaria digitata* sporophytes. Algae were elicited on the apical area (gray bars) or unelicited (control, white bars). Black arrows indicate the site of elicitation. The proteins were extracted 9 h after elicitation. Values are means ± SE of three biological replicates. Differences between treatments (control and elicited) and parts of the algae were tested using two-way ANOVA and letters above the error bars indicate groups that are significantly different ($P < 0.05$).

Table 2 Quantification of grazing impacts on control and elicited *Laminaria digitata*

	Mass of grazed tissue (mg)	
Treatment	Meristematic part	Apical part
Control	74.8 ± 19.3	16.7 ± 3.9
Oligoalginates (GG) on apical part	33.8 ± 6.7	11.1 ± 2.0

Patella pellucida herbivores were added to meristematic or apical parts of *L. digitata* sporophytes that had been submitted either to control treatment or elicitation on their apical parts. The mass of grazed tissue was recorded after 7 d. Values are means ± SE ($n = 5$).

(Fig. 2) and an increase in haloperoxidase activities (Fig. 4). Furthermore, the elicitation on the apex induced a stronger resistance against grazing in the meristem (Table 2). Altogether, these results reveal the existence of systemic defense responses in *L. digitata*. The intensity of the systemic responses was comparable to or even higher than that of the local responses. The cytochemical transmission electron microscopy method revealed that both the local and distant H_2O_2 production originate from similar cellular sites. In marine red and brown algae, local oxidative bursts are generally caused by the activation of NADPH oxidase-like enzymes, presumably membrane-bound (Küpper *et al.*, 2001; Weinberger *et al.*, 2005). Accordingly, in areas remote from the elicitation site, the appearance of cerium perhydroxide deposits suggests an abundant production of H_2O_2 along plasma membranes, diffusing into the apoplasm (Fig. 2c). Surprisingly, we did not detect any significant change in the transcription of the four marker (GG-responsive) genes in the algal parts remote from the site of elicitation (Table 1). One explanation could be that the sampling time (3 h after elicitation on one part) might have missed the peak of gene expression or did not allow enough delay for remote tissues to induce the gene expression after receiving the systemic signal. Another tempting hypothesis could be the occurrence of a priming mechanism in remote tissues, i.e. a potentiation to activate stronger expression of defense genes only when exposed again to any form of biotic stress. Recently, the existence of priming mechanisms has been shown for *L. digitata*, in the context of interindividual waterborne communication (Thomas *et al.*, 2011). A similar phenomenon might thus be involved in the systemic (i.e. intraindividual) defense responses of this kelp. However, the small number of genes measured at only one time point limits the interpretation of our results and clearly underlines the need to acquire global transcriptomic data over an extensive time-course to better dissect the systemic responses.

Physiology of the systemic signal

Separating the elicited part from the rest of the sporophyte abolished the remote oxidative response (Fig. 3), showing that tissue integrity is required for the systemic responses to occur. This result rules out the possibility that the remote effects involve volatile compounds emitted by the challenged area and perceived by distant parts. Rather, we propose that a signal is produced at the site of elicitation and then propagates within the alga towards distant parts.

Given that the production of H_2O_2 is delayed from the local to the remote part, we used the present data to estimate the propagation velocity of the putative systemic signal. For each algal part, the first time point showing an increase in H_2O_2 production was taken as an indication that the signal had reached this site. Delays of H_2O_2 production were then calculated and divided by the distance between the two parts to infer velocities. Although approximate, the transport velocity can thus be estimated at $21.5 \pm 7.7\ cm\ h^{-1}$ ($n = 24$, comprising all performed experiments). Although kelps are considered as nonvascular plants, members of the order Laminariales can transport molecules over a long distance, through sieve elements formed by series of cylindrical cells with perforated terminal end walls (Schmitz, 1981). These sieve tubes notably transport minerals, nutrients, and photoassimilates produced in mature blades towards the meristematic area (Floch & Penot, 1978). Transport velocities typically range from 5 to $10\ cm\ h^{-1}$ in *Laminaria* and can reach as high as $78\ cm\ h^{-1}$ in the giant kelp *Macrocystis pyrifera* (reviewed in Schmitz, 1981). Although approximate, the estimated transport velocity for the propagation of systemic defense signal in *L. digitata* is in agreement with these values. This systemic signal can travel in both directions, i.e. acropetal and basipetal, in contrast to the transport of photoassimilates. Based on the present data, no significant difference could be detected for the velocity in different directions (Mann–Whitney test, $P = 0.64$) and this suggests an active mechanism of signal translocation.

The pharmacological approach, even if based on only one time point, may give a first insight into the potential nature of the systemic signal. The three inhibitors abolished the local oxidative burst (Fig. 3). However, in the case of DPI or PrG, the local suppression of H_2O_2 production did not result in an inhibition of the systemic oxidative response. These results indicate that H_2O_2 or superoxide anions are not the systemic molecule and are not necessary for the systemic signal(s) to be produced, in contrast to what has been observed in land plants (Alvarez *et al.*, 1998; Miller *et al.*, 2009). The incubation of the elicited part with CPZ, a specific inhibitor of phospholipases A2 (PLA_2s), had a strong negative effect on the systemic response. In marine algae, PLA_2s are the first actors in the oxylipin pathway (Fig. 5), releasing polyunsaturated fatty acids (PUFAs) from membrane lipids (Gerwick *et al.*, 1999; Lion *et al.*, 2006). PUFAs are further metabolized by lipoperoxidases (LOX) and peroxidases into various octadecanoids (C18) and eicosanoids (C20). We observed that the inhibition of LOX and peroxidation by PrG in the elicited part lead to a drastic increase in the systemic oxidative response (Fig. 3). Our results therefore suggest that free PUFAs or some derivatives, which are not produced by PrG-sensitive enzymes, might play a role in the systemic signaling in *L. digitata*. While the transport and action of free PUFAs have still to be further validated in

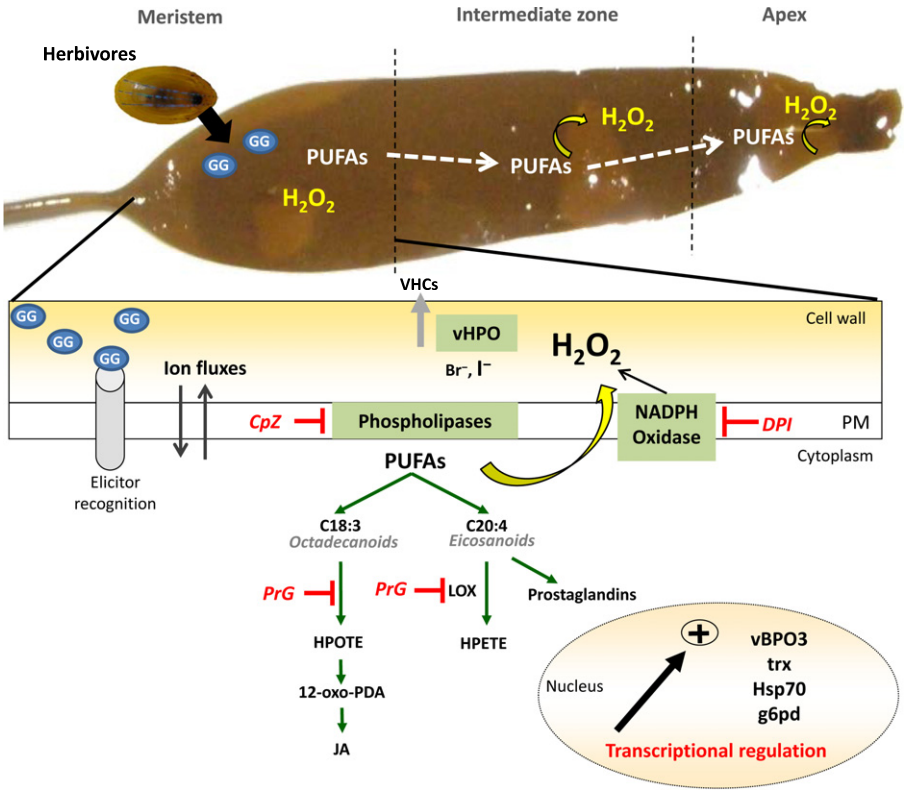

Fig. 5 Hypothetical model for the systemic propagation of defense signals in *Laminaria digitata*. Insert below: the recognition of oligoguluronate (GG) elicitors by algal cells triggers the production of signaling molecules, which activate either defense-specific biochemical pathways or general cell metabolism. PUFAs, polyunsaturated fatty acids; LOX, lipoxygenase; vHPO, vanadium-dependent haloperoxidase; VHCs, volatile halocarbons; HPOTE, hydroperoxy-octadecatetraenoic acid; HPETE, hydroperoxy-eicosatetraenoic acid; 12-oxo-PDA, 12-oxo-phytodienoic acid; JA, jasmonic acid; PM, plasma membrane; inhibitors – PrG, propyl gallate; CpZ, chlorpromazine-HCl; DPI, diphenylene iodonium; g6pd, glucose-6-phosphate dehydrogenase; trx, thioredoxin; hsp 70, heat shock protein; vBPO3, vanadium-dependent bromoperoxidase 3.

systemic response, this hypothesis is in agreement with the local induction of an oxidative burst by the free PUFAs C20:4 and C18:3 in *L. digitata* (Küpper *et al.*, 2009). The inhibition of peroxidation would favor the production of these putative signal molecule(s) by preventing the competition for substrate in the formation of octadecanoids and eicosanoids. Cutting the algal blade would prevent the translocation of these signals and lead to their accumulation in the challenged area. Consistent with this hypothesis, a fourfold production of H_2O_2 was found in elicited meristematic parts separated from the rest of the blade (Fig. 3). This effect cannot be a result of the physical stress of wounding induced by the cutting procedure, as this would also lead to an increase in the H_2O_2 production by the adjacent intermediate part.

Based on these preliminary observations, we propose a hypothetical model for the production and propagation of putative systemic signals (Fig. 5). After recognition of an attack, free PUFAs liberated by the activation of phospholipase at the plasma membrane or their derivatives could be translocated both acropetally and basipetally. In distal parts, they could activate the production of H_2O_2 and be further metabolized as oxylipins, which may prime the defense responses against a second aggression by a grazer (Table 2) or a pathogen (Küpper *et al.*, 2009).

Insights into the evolution of systemic immunity in Eukaryotes

Kelps such as *L. digitata* establish perennial populations in coastal ecosystems which, both physically and functionally, are described as the counterparts of terrestrial forests (Schaal *et al.*, 2010). In a previous study (Thomas *et al.*, 2011), we demonstrated that kelps are capable of emitting waterborne cues that signal pathogen attacks to neighboring individuals. We show here, for the first time, that upon challenging with defense elicitors they also are capable of systemic reactions, namely the intraindividual propagation of defense responses that lead to systemic protection against grazers. This suggests that kelps have developed defense strategies that are similar to other multicellular Eukaryotes: detect the attack; locally trigger immediate defense reactions; induce gene-regulated defense responses in the zone of attack; protect the individual as a whole (i.e. systemic defense through a vascular system); and possibly warn neighboring individuals or distal parts of the same individuals by volatile signaling (i.e. priming effect). As the ancestors of multicellular eukaryotes were all unicellular organisms, these elaborate strategies should have emerged independently at least three times in the evolution of Eukaryotes, in the Opisthokonta (animals), the Archaeplastida (land plants), and the Stramenopiles (brown algae). One question then arises: were the actors and mechanisms of innate immunity conserved throughout evolution or did they emerge through independent innovations? As a phylum that emerged as early as *c.* 1200 million yr ago and which now constitutes a lineage as distant from animals as from land plants, brown algae provide a new angle to address this question. Our pharmacological results further suggest that brown algae might rely for systemic signal propagation on derivatives of PUFAs, which are already known as signaling molecules in plants (Dempsey & Klessig, 2012; Zoeller *et al.*, 2012; Kachroo & Robin, 2013; Yu *et al.*, 2013) and animals (Yaqoob, 2003; Harizi *et al.*, 2008; Stanley *et al.*, 2009). If intercellular and long-distance signaling has evolved in brown algae using similar 'bricks', such as PUFAs, it is, however, much more probable that different oxylipin signals have emerged in the marine environment, as observed in diatom chemical interactions (for a review, see Leflaive & Ten-Hage, 2009) and viral interactions in haptophytes (for a review, see Bidle & Vardi, 2011).

In summary, this novel systemic signal network in the establishment of algal oxidative defense responses is shown to confer resistance against grazing in the brown algal kelp *L. digitata*. Investigations of infochemicals in *L. digitata* and other kelp species, including the giant kelp *M. pyrifera*, would provide exciting results to further understand the evolution of systemic signaling in brown algae with respect to their success in colonizing temperate and cold rocky ecosystems.

Acknowledgements

This work was conducted in the Laboratoire International Associé 'Dispersal and Adaptation of Marine Species' (LIA DIAMS) PUC, Chile and CNRS-UPMC, France. This work was partially funded by the ECOKELP program of the French National Research Agency (ANR) (ANR 06 BDIV 012). A PhD. fellowship to F.T. was awarded by the Ministry of Higher Education and Research. A.C. was supported by a PhD fellowship from the Brittany Regional Council and a postdoctoral fellowship from ECOKELP. This work benefited from the support of the French government through the National Research Agency with regard to an investment expenditure program, IDEALG (ANR-10-BTBR-04).

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