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Aldehyde perception induces specific molecular responses in *Laminaria digitata* and affects algal consumption by a specialist grazer

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SUMMARY

In the marine environment, distance signaling based on water-borne cues occurs during interactions between macroalgae and herbivores. In the brown alga *Laminaria digitata* from North-Atlantic Brittany, oligoalginates elicitation or grazing was shown to induce chemical and transcriptomic regulations, as well as emission of a wide range of volatile aldehydes, but their biological roles as potential defense or warning signals in response to herbivores remain unknown. In this context, bioassays using the limpet *Patella pellucida* and *L. digitata* were carried out for determining the effects of algal transient incubation with 4-hydroxyhexenal (4-HHE), 4-hydroxynonenal (4-HNE) and dodecadienal on algal consumption by grazers. Simultaneously, we have developed metabolomic and transcriptomic approaches to study algal molecular responses after treatments of *L. digitata* with these chemical compounds. The results indicated that, unlike the treatment of the plantlets with 4-HNE or dodecadienal, treatment with 4-HHE decreases algal consumption by herbivores at 100 ng.ml^{-1}. Moreover, we showed that algal metabolome was significantly modified according to the type of aldehydes, and more specifically the metabolite pathways linked to fatty acid degradation. RNAseq analysis further showed that 4-HHE at 100 ng.ml^{-1} can activate the regulation of genes related to oxylipin signaling pathways and specific responses, compared to oligoalginates elicitation. As kelp beds constitute complex ecosystems consisting of habitat and food source for marine herbivores, the algal perception of specific aldehydes leading to targeted molecular regulations could have an important biological role on kelps/grazers interactions.

Keywords: Brown algae, biotic interactions, defense and signaling responses, transcriptomics, metabolomics.

INTRODUCTION

As the major primary producers of temperate rocky coasts, large brown macroalgae or kelps are known as food resources for a variety of marine animals. Although kelps have great biomass around the world, over-grazing can still cause destructive consequences to the kelp forests. For instance, mass occurrences of sea urchins grazed kelp forest to lead to barren grounds, resulting in serious ecological and environmental issues (Steneck et al., 2002; Steneck et al., 2004). However, similarly, to land plants, kelps may have developed complex defense responses following biotic stress to protect undamaged tissues or whole individual from some grazers. Indeed, systemic responses were detected in apex part of *L. digitata* after treating meristem part with defense elicitors, such as oligoguluronates (GG) (Thomas et al., 2014). It has also been shown that grazing by specialized herbivores can induce both transcriptomic regulation and the production of free fatty acids and oxylipins in *L. digitata* (Ritter et al., 2017). During low tides, volatile organic compound amounts into the

seawater and in the air drastically increased above kelp beds, highlighting specific emissions of C6- and C9-aldehydes (Goulitquer et al., 2009), and water-borne cues present in seawater surrounding kelps at low tides have shown to later modify and amplify early responses upon elicitation in young *L. digitata* (Thomas et al., 2011). These results suggest that oxylipin signaling pathways might play an important role in the systemic and distant signaling of kelps during the interaction with herbivores.

Oxylipin pathways share common biochemical traits among Eukaryotes and play important roles in defense responses. In land plants, aldehydes are considered as an important group of signal or defense molecules. In Arabidopsis, the application of C6 aldehydes such as (Z)-3-hexenal induces up-regulation of defense-related genes in the phenylpropanoid and jasmonate biosynthetic pathways (Bate & Rothstein, 1998; Kishimoto et al., 2005) and increase of lignin content in leaves (Kishimoto et al., 2006), enhancing the resistance of Arabidopsis to fungal pathogens. Similarly, the C9 aldehyde, nonanal, also primes the defense in *Phaseolus lunatus* to a bacterial pathogen, *Pseudomonas syringae* pv syringae (Yi et al., 2009). In addition, some C6 aldehydes might also have strong antimicrobial effects against bacteria (Croft et al., 1993) or fungi (Matsui et al., 2006). In a recent study, the cinnamic aldehyde was reported to have phytotoxic effect against some weed species such as *Amaranthus retroflexus*, *Avena fatua*, *Portulaca oleracea*, and *Erigeron bonariensis* (Muñoz et al., 2020). Animals, such as lizards, also use aldehydes for communication and the content of aldehydes changes due to different behavior or climate (Campos et al., 2020). As reactive compounds, aldehydes might have toxic effects on animal cells by causing DNA damage or protein modification (Duryee et al., 2004; Langevin et al., 2011). In animals, the aldehyde 4-HHE comes from the peroxidation of n-3 PUFAs like linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid, while the aldehyde 4-HNE is provided by the peroxidation of n-6 PUFAs, such as arachidonic and linoleic acids and their 15-lipoxygenase metabolites (Riahi et al., 2010). In a wide range of Eukaryotic lineages, the generation of oxidized fatty acid derivatives proceeds from oxygenation of free polyunsaturated fatty acids (PUFAs) mostly through intervention of lipoxygenases (LOXs), which generate hydroperoxides, until production of aldehydes by hydroperoxide lyases (HPLs) (Feussner & Wasternack, 2002; Fontana et al., 2007). LOXs and HPLs are well characterized in the animal and plant lineages, in the red algal lineage like in *Pyropia haitanensis* (Chen et al., 2015) and in the diatom lineage (Leflaive & Ten-Hage, 2009). The access to genomic data in few brown algal species, first in *Ectocarpus siliculosus* (Cock et al., 2010) and later in *Saccharina japonica* (Ye et al., 2015), has allowed one to analyze the phylogeny of two key gene families, encoding lipoxygenases (LOXs) (Teng et al., 2017) and cytochromes P450 (CYPs), the later family containing most of the HPLs (Teng et al., 2019), showing a large diversity of homologous enzymes, with unknown biochemical functions in Stramenopiles. Recently, two CYP genes belonging to CYP5164 clan in *E. siliculosus* were expressed in *E. coli* and the recombinant proteins showed epoxyalcohol synthase (Toporkova et al., 2017) or hydroperoxide bicyclase (Toporkova et al., 2022) activities when incubated with oxylipins. However, the oxylipin pathways and functions in algae are still elusive in many cases despite some oxylipin-derived compounds have been characterized (Chen et al., 2019). The derivatives of C18 (octadecanoids) and C16 (hexadecanoids) fatty acids are known as defense hormones involved in intra- or interplant distance signaling (Fu & Dong, 2013; Meng & Zhang, 2013). Among the biogenic volatile organic compounds, 1-octen-3-ol has been shown to activate defensive and protective responses in *Arabidopsis thaliana* (Kishimoto et al., 2007) and has therefore been used as an external inducer of defense-related responses in some red or brown algae species (Chen et al., 2019; Zhang et al., 2021). This compound was indeed found to be present in several brown algae, including kelps, even if the conditions triggering its production are still unidentified (Rubiño et al., 2022).

Marine macroalgae were found to generate C20 and C18 PUFAs and oxidized derivatives during innate immunity responses (Bouarab et al., 2004; Potin et al., 2002). In response to bacterial-derived compounds, the kelp *L. digitata* induced a rapid release of Free Fatty Acids (FFAs) with a simultaneous accumulation of oxidized derivatives of linoleic (C18:2) and eicosapentaenoic acids (C20:5), as well as oxylipins like 13-hydroxyoctadecatrienoic acid (13-HOTrE) and 15-hydroxyeicosapentaenoic acid (15-HEPE) (Küpper et al., 2006). In this species, the incubation with linolenic acid (C18:3) and arachidonic acid (C20:4) and methyl jasmonate (MeJA) was also shown to induce an oxidative burst and the production of some FFAs, leading to a protection against algal endophytes (Küpper et al., 2009). In *L. angustata*, C9- and C6-aldehydes and hydroperoxides were found constitutively present into algal tissues (Boonprab et al., 2006; Boonprab, Matsui, Akakabe, et al., 2003; Boonprab, Matsui, Yoshida, et al., 2003), whereas diverse C6-, C7-, C8-, and C9-aldehydes, such as 4-HHE and dodecadienal, were highly released in the surrounding seawater following elicitation by oligoguluronates or in response to copper stress (Goulitquer et al., 2009; Thomas et al., 2011).

Kelp beds are therefore the natural sources of a wide range of aldehydes, for which the ecological and biological roles have hardly been explored. In this context, we questioned their potential roles as bioactive signals during interactions between kelps and herbivores. Thus, we investigated the effects on algal protection of three specific aldehydes, namely 4-hydroxy-hexenal (4-HHE),

4-hydroxy-nonenal (4-HNE) and dodecadienal that were previously shown to be significantly released into the surrounding seawater by *L. digitata* after oligoalginates elicitation or during low tides in the field (Goulitquer et al., 2009). In this purpose, we monitored the effects of algal pre-incubation with these aldehydes on subsequent consumption of *L. digitata* by its specialist herbivore *Patella pellucida* using a bioassay developed in the laboratory (Leblanc et al., 2011). We also studied the impacts of aldehyde treatments on algal physiology, metabolism, and gene regulations, by following oxidative responses and by analyzing the modifications of the algal endometabolome and transcriptome.

RESULTS

Aldehyde incubation showed different effects on later algal consumption by specialist herbivores according to chemical nature and concentration

The effects of three aldehydes on algal physiological responses were first assessed by measuring consumption of *L. digitata* tissues by *P. pellucida* during 3 days using a bioassay developed in laboratory-controlled conditions (Figure 1), following a 4-h-incubation with different concentrations of aldehydes and 24 h in FSW. Oligoguluronates (GG) elicitation of algae was tested in similar conditions. Consumption of elicited algae was higher than consumption of control algae, but not significantly (Wilcoxon test: W = 248, *P*-value = 0.22). Dodecadienal increased significantly the consumption by herbivores when applied at 100 ng.ml^{-1} compared to control (W = 24, *P*-value = 0.02). 4-HNE did not induce significant changes in consumption by herbivores at 1 μg.ml^{-1} (W = 14, *P*-value = 0.84) and 100 ng.ml^{-1} (W = 17, *P*-value = 0.42). However, effects of 4-HHE on algal consumption depended on the concentration of this aldehyde applied to the alga. Over 500 ng.ml^{-1}, 4-HHE had no significant effect on algal consumption by *P. pellucida* (W = 22, *P*-value = 0.06 and W = 17, *P*-value = 0.42 for 1 μg.ml^{-1} and 500 ng.ml^{-1}, respectively). Interestingly, at the concentration of 100 ng.ml^{-1}, 4-HHE decreased significantly grazing by herbivores (Wilcoxon test: W = 52, *P*-value = 0.01). At lower concentrations, 4-HHE had no effect, neither at 50 ng.ml^{-1} (W = 13, *P*-value = 1), nor at 10 ng.ml^{-1} (W = 46, *P*-value = 0.79).

Aldehydes do not trigger an oxidative burst in L. digitata plantlets

As already established for *L. digitata* (Küpper et al., 2001), the amount of hydrogen peroxide (H_2O_2) was significantly increased over 60 min, in seawater surrounding *L. digitata* culture plantlets, after elicitation by oligoguluronates (GG), compared to control, showing the occurrence of a rapid oxidative burst. On the contrary, no significant difference regarding H_2O_2 accumulation was recorded in surrounding seawater of algae after addition of one of the three aldehydes, compared to control (Figure 2; ANOVA F-value = 19.98, *P*-value <0.001, SNK test: C=H=N=D<E). Elicitation by GG triggered H_2O_2 production of 3 to 4 times more than that measured in control *L. digitata* or treated with 4-HHE, 4-HNE, or dodecadienal.

Aldehyde treatments induced different metabolic modifications in algal tissues compared to controls

Partial least squares discriminant analyses (PLS-DA) were carried out from the relative abundance of the 177 ions detected by LC–MS analyses (Figure 3a; Table S1). The statistical post-tests were not conclusive at each sampling time (PLS-DA: 4 h: NMC = 0.69, *P*-value = 0.18; 8 h: NMC = 0.82, *P*-value = 0.76; 28 h: NMC = 0.78, *P*-value = 0.51). This could reflect a high biological variability of the metabolic responses, avoiding to statistically discriminate the different treatments from each other. However, some general patterns could be highlighted. At 4 h, the dispersion of the four replicates was higher for the algae treated with aldehydes, than those elicited with GG or controls, suggesting more variability in metabolic profiles after aldehyde treatment. At 4, 8, and 28 h, the aldehyde-treated samples were clearly separated from the control points, even if some were close to each other or with the elicited samples, suggesting distinct metabolic profiles in elicited and aldehyde-treated algae compared to controls at each sampling time (Figure 3a).

Aldehydes triggered the production of FFAs and potentially related compounds

Among the 10 most discriminant ions between different aldehydes or oligoalginates treatments compared to controls (without any chemical pre-incubation) at each sampling time, five ions were selected, because annotated as putative FFAs or oxidized derivatives. One of these *in silico* annotations was confirmed by further LC–MS/MS analysis (Figure S2), that is, the stearidonic acid (m/z 275.20, rt 603 s). Another ion (m/z 301.22, rt 679 s) was very close to eicosapentaenoic acid, but not identical, and identified at level 3 as long-chain fatty acid with an aliphatic tail between 13 and 20 carbon atoms, with a raw formula determined as $C_{20}H_{30}O_2$. Another ion (m/z 309.2, rt 403 s), firstly annotated as (13)-HOTrE, was identified as a derivative from lineolic acid with a proposed formula of $C_{18}H_{30}O_4$ (Figure S2). The m/z 209.12, rt 364 s ion has a confirmed formula of $C_{12}H_{18}O_3$ and may be related to an oxidative derivative of jasmonic acid (Figure S2). The last ion (m/z 351.22, rt 346 s) was not confirmed to be 20-hydroxy-leukotriene B4 (data not shown).

The relative amounts of these 5 ions in *L. digitata* are presented in Figure 3b, along the time course, according to the different treatments (see Table S1 for complete dataset). Globally, incubation in 4-HNE and 4-HHE led to a

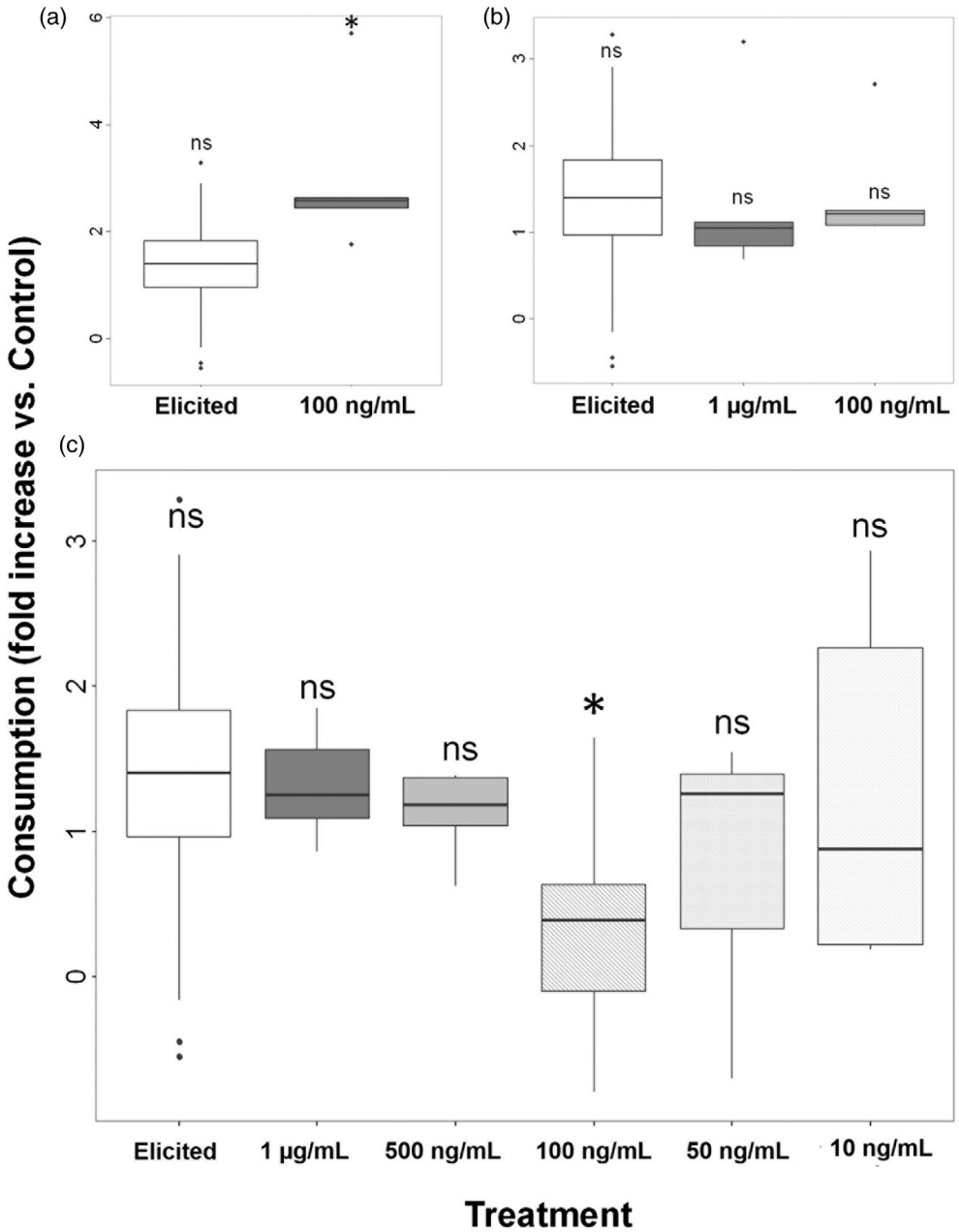

Figure 1. Effects of different treatments on consumption of *L. digitata* by *P. pellucida* grazers after 3 days. 24 h before putting in contact with herbivores, algae were incubated either with GG for 1 h (Elicited, E treatment) or with different aldehydes for 4 h, that is, dodecadienal (a, D treatment), 4-HNE (b, N treatment) or 4-HHE (c, H treatment), before rinsing. Results are expressed in algal consumption relative to control (without chemical treatments of algae) and represented as boxplots with whiskers drawn to the min/max values (see M&M). Elicited: 150 $\mu g.ml^{-1}$ (n = 25), identical values were reported on each graph for comparison. Aldehydes were applied on algae at the following concentrations: dodecadienal (D) 100 $ng.ml^{-1}$ (n = 5); 4-HNE (N) 1 $\mu g.ml^{-1}$ (n = 5) and 100 $ng.ml^{-1}$ (n = 5); 4-HHE (H) 1 $\mu g.ml^{-1}$ (n = 5), 500 $ng.ml^{-1}$ (n = 5), 100 $ng.ml^{-1}$ (n = 15), 50 $ng.ml^{-1}$ (n = 5) and 10 $ng.ml^{-1}$ (n = 10). Wilcoxon tests were performed for each treatment and concentration, to compare the consumption of treated algae with the consumption of the control algae (without treatment). * significantly different from the control; ns: non-significantly different from the control.

strong increase of their production 4 h after incubation. This rapid induction was less pronounced for the dodecadienal and elicitation treatments, lasted up to 8 h for the 4-HHE treatment, and was followed by a relative decrease for the 4-HNE and dodecadienal treatments. After 28 h, the plantlets submitted to the dodecadienal treatment showed a stronger production peak for these five compounds. More specifically, all the samples treated with the three aldehydes contained two to over four times more of the unknown compound m/z 351.22 compared to a control kelp at 4 h, whereas its relative amount tended to be downregulated after 8 or 28 h. The stearidonic acid was rapidly more

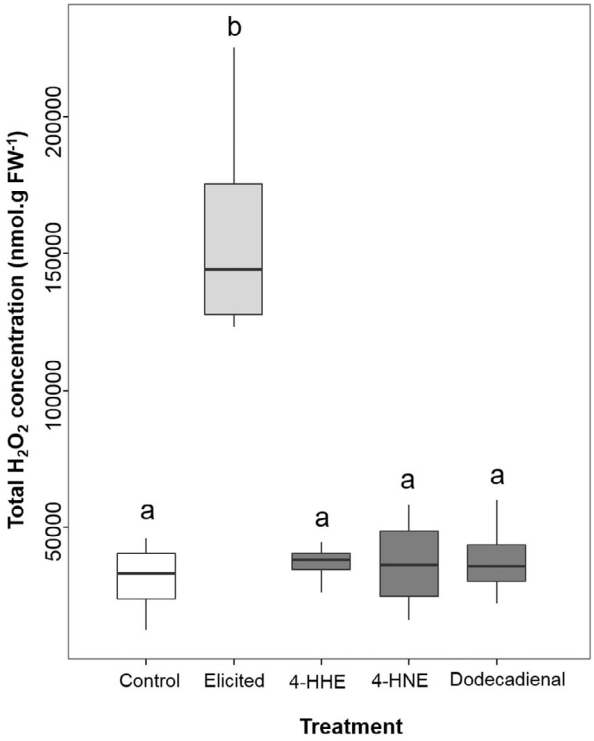

Figure 2. H_2O_2 production over 60 min in algal surrounding seawater after application of oligoguluronates at 150 $\mu g.ml^{-1}$ (Elicited, E treatment) or of 4-HHE (H), 4-HNE (N), or dodecadienal (D) at 100 $ng.ml^{-1}$ or nothing (Control, C). The data are represented as boxplots with whiskers drawn to the min/max values (see M&M). After checking normality, ANOVA analysis was performed: F-value = 19.976, *P*-value = 7.165e-06, SNK test: C=H=N=D < E (n = 4). Letters above the error bars indicate groups that are significantly different ($P < 0.05$).

produced after 4-h incubation in 4-HNE and 4-HHE than without treatment and after 8 h following the dodecadienal treatment. After 28 h, the relative amount of this FFA was similar to that of the control algae, unlike in elicited algae where it reached twice more than the control level. For the 4-HHE treatment, a higher production of the $C_{12}H_{18}O_3$ compound was observed after 8 h compared to other treatments. The relative amount of the unknown ion m/z 309.20 was similar or slightly increased in aldehyde-treated algae compared to control algae after 4 h. However, after 28 h, its relative amount and that of the putative eicosapentaenoic acid were, respectively, 3 and 3.5 times more important after incubation in dodecadienal than after no incubation or GG-elicitation.

4-HHE treatment induced specific transcriptomic regulation in L. digitata

The *de novo* assembled transcriptome of *L. digitata* contains 270 492 transcripts (Table S2). After mapping all the reads to the transcriptome, 90% of total expression was present in 34 032 transcripts with an average contig length of 1226 bp, which were used to perform downstream analysis. The average GC content of this *de novo* transcriptome from *L. digitata* was 54.8%. The result of the BUSCO analysis revealed a near-complete gene sequence information for the *de novo* transcriptome with 82.6% complete BUSCO matches with eukaryotic dataset.

After 12 h of 4-HHE treatment, a small fraction of genes showed significant expression differences compared to the control group (1.05%, including 163 upregulated and 197 downregulated differentially expressed genes (DEGs), Table S3). Following GG treatment, a more important fraction of the transcriptome was differentially expressed (4.16%) with 787 upregulated genes and 629 downregulated genes after 12 h, when compared to control samples (Table S3). The comparison of DEGs showed that 115 upregulated genes (Figure 4a) and 24 downregulated genes (Figure 4b) were shared between 4-HHE and GG treatment.

To further analyze the transcriptional regulation of *L. digitata*, *in silico* annotation and classification were performed on DEGs. For 4-HHE and GG treatments, the annotation rates of DEGs were, respectively, 41.11% and 37.99% (Table S3). However, the annotation rate of DEGs, specifically associated with 4HHE treatment (18.34%), was much lower than the overall rate. Nevertheless, the result of the annotation showed that 4-HHE and GG treatments induced the regulation of genes related to fatty acid metabolism. Two genes, putatively encoding acyl-CoA dehydrogenase and aldehyde dehydrogenase, were upregulated in both conditions. After 4-HHE treatment, among six other DEGs detected, two downregulated genes were annotated as putative cytochrome P450 (CYP). In GG treatment, eight upregulated genes include genes encoding putative lipoxygenases and phospholipases, also involved in oxylipin biosynthesis (Table 1). Concerning the signaling process, one upregulated myosin light chain kinase (MLCK) gene and one downregulated putative serine/threonine-protein kinase SRPK1 gene were potentially detected after 4-HHE treatment. While in GG treatment, the same MLCK gene was also found upregulated after 12 h, and several putative calcium-dependent protein kinase (CDPK) genes and serine/threonine protein kinase genes were upregulated (Table 1). Five genes encoding putative transcription regulators were upregulated in response to GG treatment. Two of them were annotated as MYB transcription factors and two other genes were annotated as heat shock transcription factors. One gene encoding putative RNA polymerase sigma factor SigD was upregulated in both 4-HHE and GG treatment (Table 1).

4-HHE treatment also induced the up-regulation of some genes potentially involved in stress responses such as genes encoding catalase, ascorbate peroxidase, DnaJ domain-containing protein and glutathione S-transferase (Table 2), similar to the GG treatment. At the same time, GG treatment also triggered the regulation of genes

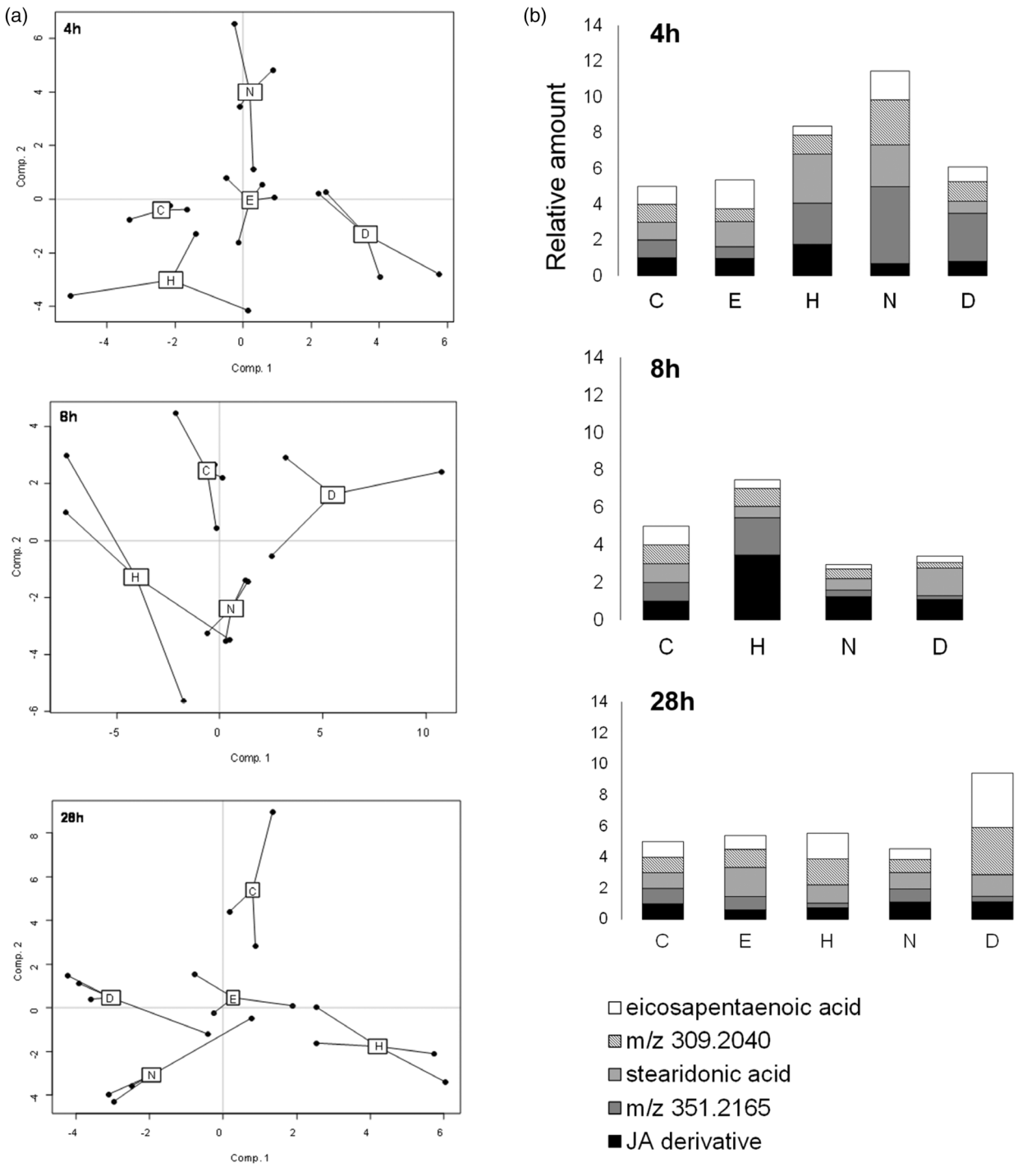

Figure 3. Endo-metabolome analysis of *L. digitata* sampled at 4, 8, and 28 h after incubation in aldehydes 4-HHE (H), 4-HNE (N), or dodecadienal (D), and compared with a control without treatment (C) and an elicited alga (E).

(a) PLS-DA analysis of the metabolic profiling based on 177 ions. MVA test, 999 permutations 4 h: NMC = 0.69, *P*-value = 0.18; 8 h: NMC = 0.82, *P*-value = 0.76; 28 h: NMC = 0.78, *P*-value = 0.51.

(b) Relative amount of five compounds in *L. digitata* sampled after 4, 8 and 28 h of incubation. For each compound, the control level was set to a relative unit of 1 to express the fold variation in the other conditions (absolute concentration values are provided in Table S1). These compounds were further identified by LC–MS/MS analyses (Figure S2) as stearidonic acid (m/z 275.20, rt 603 s), close to eicosapentaenoic acid (m/z 301.22, rt 679 s, raw formula $C_{20}H_{30}O_2$), or derivate from lineolic acid (m/z 309.20, rt 403 s, raw formula $C_{18}H_{30}O_4$). The m/z 209.12, rt 364 s ion has a raw formula of $C_{12}H_{18}O_3$, and the ion m/z 351.22, rt 346 s is still unidentified.

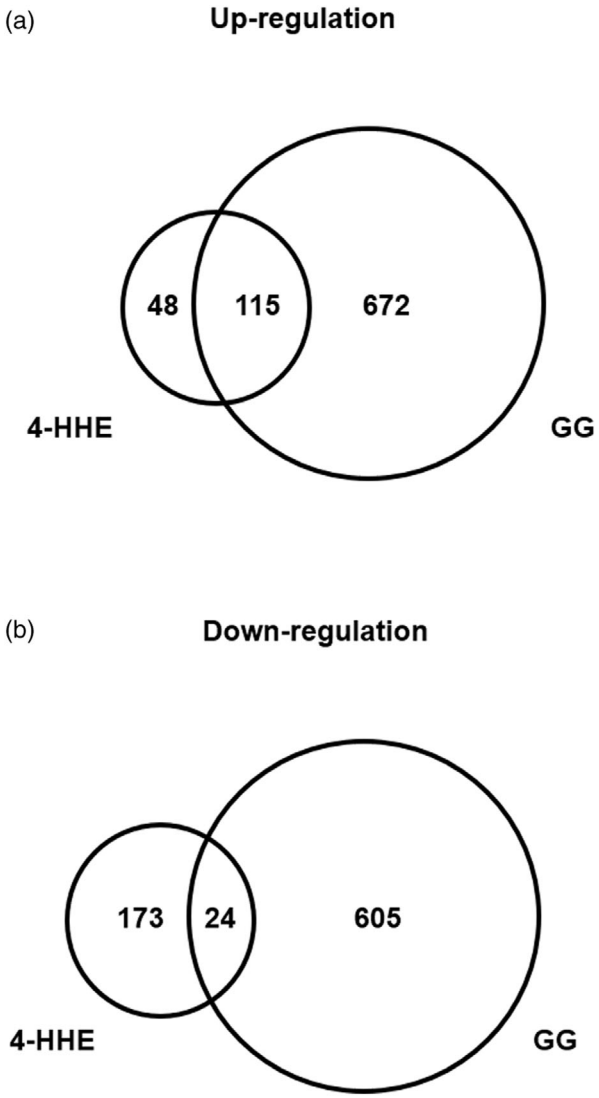

Figure 4. Venn diagram of upregulated (a) and downregulated genes (b) after 12 h of 4-HHE and GG treatments compared to control samples.

encoding putative chaperones (Heat shock protein 40 and 70) and halide oxidation enzymes (vanadium-dependent bromoperoxidases). Two putative defense-related genes, metacaspase and LanC lantibiotic synthetase component C-like 2, were upregulated after the 4-HHE treatment. Similarly, two genes, annotated as LanC lantibiotic synthetase component C-like 2, were upregulated in GG treatment, including one also detected after the 4-HHE treatment. Two other defense-related genes, annotated as pathogenesis-related protein and putative respiratory burst oxidase protein, were also upregulated after the GG treatment (Table 2).

Additionally, the GG elicitation induced up- or down-regulation of several genes potentially involved in photosynthesis, carbon fixation, and polysaccharide metabolism, such as genes encoding light-harvesting complex (LHC) proteins, Ribulose-1,5-bisphosphate carboxylase, or endo-1,3-beta-glucanase (Table S3). Similar gene regulation was not observed after 4-HHE treatment and some identified genes even showed opposite expression patterns. For example, two genes potentially involved in cell wall modification, cellulose synthase gene and mannuronan C-5-epimerase gene, were significantly repressed by 4-HHE treatment, but not regulated upon GG treatment.

DISCUSSION

Several studies have attempted to characterize molecular changes in transcriptomic and/or metabolomic profiles in macroalgae submitted to potential chemical signals (Chen et al., 2019; Zhang et al., 2021), but this study was performed using treatments with molecules that are released in the seawater surrounding the alga upon defense elicitation. Indeed, the elicitation by oligoalginates (GG) induced the production of several aldehydes in *L. digitata* (Goulitquer et al., 2009) and we showed here that only one of the tested compounds, 4-HHE, seemed to have not only the ability of activating the oxylipin signaling pathway but to induce the protection of the algae against herbivores based on laboratory bioassays. Our study investigated both physiological and metabolomic responses of *L. digitata* to the incubation with different aldehydes, and further analyzed the transcriptomic regulation in response to the 4-HHE treatment. Up to now, the biological roles of these oxylipin-derived compounds were mainly unknown in kelps. Based on these molecular data, we discuss the potential biological functions of aldehyde-based signaling in kelps during interactions with specialist herbivores (Figure 5).

4-HHE perception induces specific transcriptomic regulations, independently of the oxidative burst, compared to GG elicitation

We first demonstrated that 4-HNE, 4-HHE, and dodecadienal, applied at 100 ng.ml^{-1}, did not induce an oxidative burst in *L. digitata*, in contrary with GG elicitors (Küpper et al., 2001, 2002) or as observed in response to some free fatty acids and methyl jasmonate (Küpper et al., 2009). This could suggest that these C6-, C9-, and C12-aldehydes did not trigger oxidative responses as the level of H_2O_2 in surrounding seawater was similar between aldehyde-treated and control algae. We also hypothesize that they do not act as exogenous elicitors like oligoguluronates (Küpper et al., 2001) and that they may not activate the NADPH oxidase. The result of transcriptome analysis also showed that, unlike the GG treatment, the gene regulation of oxidative burst was not observed in 4-HHE-incubated plantlets. Reactive oxygen species (ROS) have already been shown to play roles in algal defense against pathogens. In red algae, ROS emissions were shown to have an antibacterial

Table 1 Selected significant differentially expressed genes (DEGs), with putative functions in signaling, oxylipin biosynthesis, and transcription regulation, after 12 h treatment with 4-HHE or GG compared to the control group. The significantly differential expression fold-changes ($P \leq 0.01$, $|\log2\ FC| \geq 1$) were labeled in bold

		Log2Foldchange	
Gene ID	Putative gene product	4-HHE	GG
TRINITY_DN336_c2_g1_i1	Calcium-dependent protein kinase	0.74	**1.44**
TRINITY_DN8143_c0_g1_i5	Calcium-dependent protein kinase	0.84	**1.48**
TRINITY_DN9292_c0_g1_i1	Calcium-dependent protein kinase	0.93	**1.25**
TRINITY_DN351_c0_g2_i1	Calcium-dependent protein kinase	−0.51	**1.80**
TRINITY_DN2829_c1_g1_i1	Myosin light chain kinase (MLCK)	**1.72**	**1.87**
TRINITY_DN4660_c0_g1_i3	Serine/threonine-protein kinase SRPK1	−**1.14**	0.27
TRINITY_DN2610_c0_g1_i1	Serine/threonine-specific protein kinase-like protein	0.66	**1.56**
TRINITY_DN8748_c0_g1_i1	Cytochrome P450	−**3.53**	−0.38
TRINITY_DN9178_c0_g1_i2	Cytochrome P450	−**3.44**	−0.48
TRINITY_DN5135_c0_g1_i4	Lipoxygenase	0.34	**1.10**
TRINITY_DN9021_c0_g1_i1	Putative phospholipase	0.58	**1.29**
TRINITY_DN19539_c0_g1_i1	Calcium-independent phospholipase a2-gamma	0.85	**1.87**
TRINITY_DN485_c7_g1_i1	MYB-like DNA-binding protein	0.83	**1.50**
TRINITY_DN659_c4_g2_i1	Heat Shock transcription factor	0.92	**1.65**
TRINITY_DN10087_c0_g1_i2	Transcriptional activator MYB	0.52	**1.15**
TRINITY_DN4325_c4_g1_i1	Putative Heat Shock transcription factor	0.46	**1.35**
TRINITY_DN657_c0_g1_i2	RNA polymerase sigma factor SigD	**1.15**	**1.37**

Table 2 Selected significant differentially expressed genes (DEGs) with putative functions in defense and oxidative responses, after 12 h treatment with 4-HHE or GG compared to the control group. The significantly differential expression fold-changes ($P \leq 0.01$, $|\log2\ FC| \geq 1$) were labeled in bold

		Log2Foldchange	
Gene ID	Putative gene product	4-HHE	GG
TRINITY_DN1232_c1_g1_i3	Metacaspase	**1.03**	0.95
TRINITY_DN1741_c0_g1_i1	Pathogenesis-related protein, class 1	0.33	**3.21**
TRINITY_DN4799_c9_g1_i1	LanC lantibiotic synthetase component C-like 2	**1.09**	**1.38**
TRINITY_DN855_c3_g1_i1	LanC lantibiotic synthetase component C-like 2	0.35	**1.46**
TRINITY_DN964_c0_g2_i4	Putative respiratory burst oxidase protein	0.52	**1.22**
TRINITY_DN1585_c3_g1_i2	Catalase	**1.66**	**2.01**
TRINITY_DN13_c0_g2_i2	L-ascorbate peroxidase	**1.30**	0.65
TRINITY_DN13_c2_g1_i1	Ascorbate peroxidase	−0.05	**1.09**
TRINITY_DN46310_c0_g2_i2	Glutathione S-transferase	**2.40**	**4.34**
TRINITY_DN679_c1_g3_i2	Glutathione S-transferase	**14.54**	**25.40**
TRINITY_DN2952_c2_g1_i2	Heat shock protein 40 like protein/ DnaJ domain-containing protein	0.22	**1.21**
TRINITY_DN3892_c0_g1_i1	Heat shock protein 40 like protein/ DnaJ domain-containing protein	−0.65	−**9.18**
TRINITY_DN2265_c0_g2_i1	DnaJ domain-containing protein	**1.58**	**1.53**
TRINITY_DN4387_c0_g1_i1	Heat shock protein 70	−0.05	**1.04**
TRINITY_DN535_c0_g1_i3	Heat shock protein 70	−0.20	−**1.54**
TRINITY_DN535_c0_g1_i5	Heat shock protein 70	0.12	**1.76**
TRINITY_DN5130_c1_g1_i1	Vanadium-dependent bromoperoxidase 1	0.46	**1.33**
TRINITY_DN957_c0_g2_i1	Vanadium-dependent bromoperoxidase 1	0.45	**1.02**

effect on *Gracilaria conferta* epiflora after oligoagar elicitation (Weinberger & Friedlander, 2000). In *L. digitata*, the oxidative response is also an essential element of natural resistance against bacterial degraders and the GG-triggered oxidative burst was shown to significantly increase resistance against infection by endophytic pathogens (Küpper et al., 2002). Similar to all tested aldehyde compounds, 4-HHE does not trigger ROS emissions, but it is the only one that led to significant protection levels in plantlets during subsequent grazing bioassays, suggesting the activation of defense responses through ROS-independent signaling pathways.

Further transcriptomic analysis was then conducted to explore 4-HHE signaling effects in *L. digitata*. The global analysis showed that 4-HHE treatment only induced the regulation of a small portion of the transcriptome when

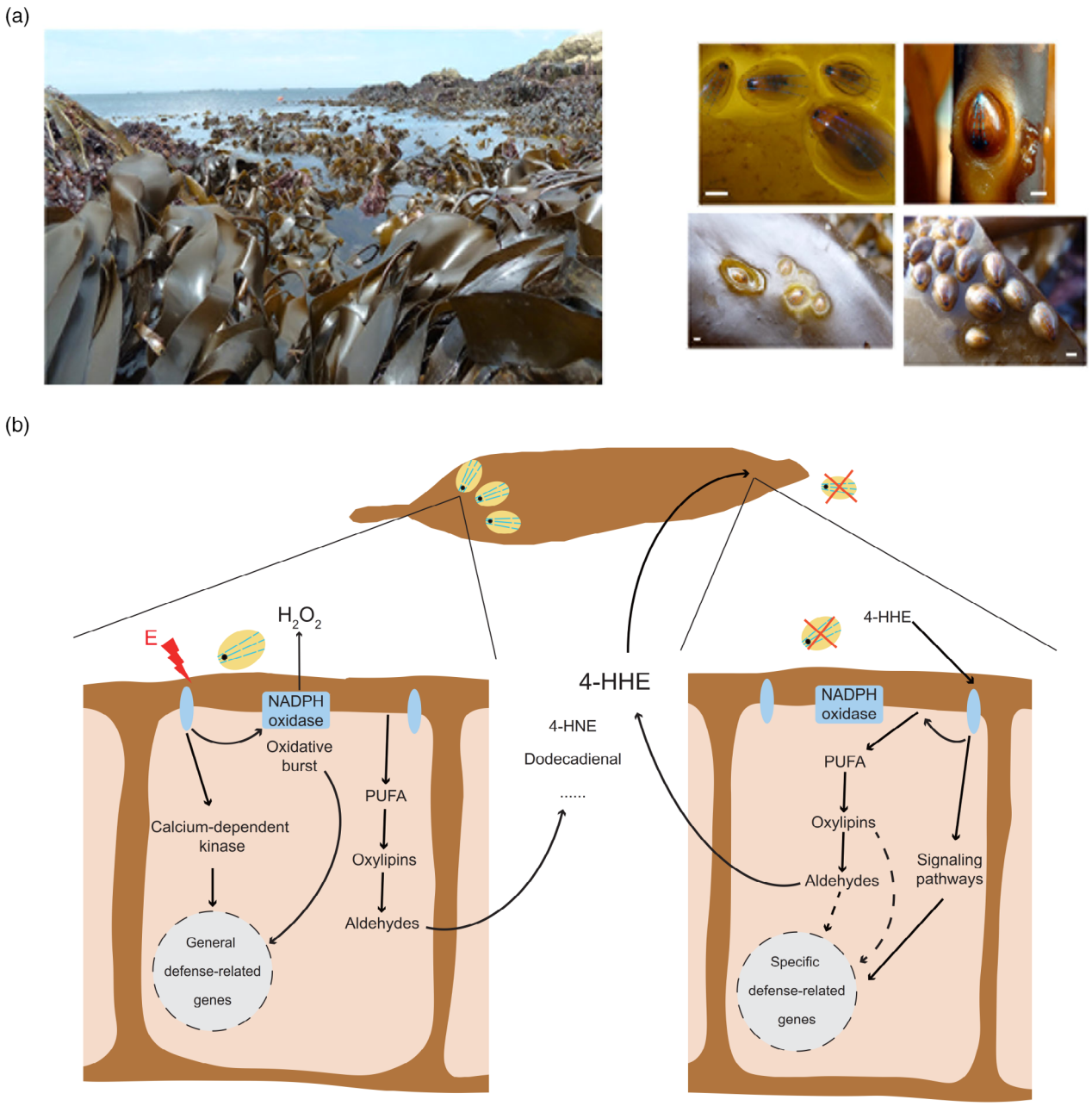

Figure 5. (a) Field pictures showing a typical kelp bed of *Laminaria digitata* and specialist herbivores, *Patella pellucida*, grazing on algal blades and stipes (white scale bars: 1 mm). Photos by LC and CL (b) Hypothetical model of local and general molecular responses and aldehyde release (left) induced by GG elicitation (E) or by its specialized grazer, *P. pellucida*, and systemic/distant, specific defense responses (right) induced by perception of 4-HHE in *L. digitata*.

compared to GG elicitation. As said above, GG treatment activated massive ROS production in *L. digitata*, leading to a strong oxidative stress in cells. Therefore, many genes putatively involved in anti-oxidative responses were differentially expressed after GG treatment, as previously shown (Cosse et al., 2009). For instance, catalase and ascorbate peroxidase can convert H_2O_2 into H_2O and O_2 through different processes, protecting algal cells from oxidative damage (Rezayian et al., 2019). Genes encoding both putative enzymes were upregulated after GG or 4-HHE treatment, suggesting that 4-HHE treatment might also activate anti-oxidative responses in *L. digitata*. However, the upregulation of two genes encoding vanadium-dependent bromoperoxidases (vBPO) confirmed that GG elicitation may induce a stronger oxidative stress than 4-HHE, as these enzymes were likely to be key actors of anti-oxidative

responses in brown algae through the oxidation of halides (Bischof & Rautenberger, 2012; Cosse et al., 2009; Xu et al., 2019). The activation of signal transduction pathways is another important step to initiate adaptive reactions when facing stresses. Calcium-dependent protein kinases, as members of the big family of serine/threonine protein kinases, play important roles in plant adaptions upon abiotic stresses such as salt and osmotic stress (Asano et al., 2012; Singh et al., 2017). Upon GG elicitation (E treatment), two genes encoding putative calcium-dependent protein kinases and five genes encoding putative serine/threonine protein kinases were upregulated. However, only the down-regulation of one serine/threonine protein kinase was observed in 4-HHE treatment, indicating that specific signaling processes might be activated in that case. Regarding signal transduction, MYB transcription factors are downstream effectors in various processes induced by jasmonic acid in land plants, such as trichome and anther development, or glucosinolate biosynthesis (Wasternack & Feussner, 2018). A MYB gene is also induced upon MeJa treatment in one red alga (Garcia-Jimenez et al., 2017). Given the high number of lineage-specific amplification in this gene family (Zeng et al., 2022), the orthology relationships with some genes activated by 4-HHE and GG here remain undetermined. Another difference of transcriptomic regulations observed after 4-HHE treatment compared to GG elicitation concerns photosynthesis-related processes and carbon metabolism. For example, several genes encoding light harvesting complex (LHC) protein were significantly repressed after GG treatment (Table S3). It has been demonstrated in the green alga *Chlamydomonas reinhardtii* that LHCs were damaged following ROS production at high salinity condition, whereas a down-regulation of LHC genes was also observed (Wang et al., 2018). In contrast, after 4-HHE treatment, no significant down- or up-regulation of photosynthesis-related genes was observed, in relationships with no strong oxidative burst. Although further work is required to verify annotated gene functions, altogether our results suggest that, whereas GG elicited general molecular responses, including antioxidative responses as those observed upon abiotic stresses, 4-HHE may act through distinct signaling pathways and regulate more specific responses, which could lead to increasing protection against herbivores (Figure 5).

4-HHE activates the oxylipin pathways in L. digitata

Following incubation with 4-HNE, 4-HHE and dodecadienal, we have investigated the changes of the algal endometabolome during a time-course up to 1 day. The comparison of metabolic profiles based on 177 detected ions did not allow to strongly discriminate the different treatments (incubation in aldehydes, control or GG elicitation) whatever the sampling time. However, in the PLS-DA, the metabolic profiles of the control algae were not grouped with those of aldehyde-treated plantlets, neither with those of GG-elicited *L. digitata*, at 4, 8 and 28 h. It has already been shown that elicitation by GG specifically induced metabolic regulation, with a massive release of halogenated volatile compounds and aldehydes in the medium surrounding *L. digitata* 1 h after GG addition (Thomas et al., 2011). Similarly, the differences observed in metabolic profiles of the aldehyde-treated algae are likely to correspond to different metabolic regulations. All the three aldehydes tested are therefore perceived as signals by *L. digitata* and seem to induce production of metabolites at different timeframes depending on their chemical nature.

We could follow the profiling of some compounds that were already known as induced in response to abiotic and biotic stresses (Goulitquer et al., 2009; Ritter et al., 2008; Ritter et al., 2017). An increase of the production and release of free fatty acids and oxylipins was previously described in *L. digitata* 8 h after copper stress (Ritter et al., 2008). Interestingly, both aldehydes, 4-HNE and 4-HHE, strongly induced an early production of the 5 compounds in *L. digitata* compared with control and GG-elicited algae after 4-h or 8-h incubations, whereas the dodecadienal's treatment revealed their increases 1 day later. Four out of the five targeted ions were related to FFA and oxylipin pathways, such as stearidonic acid, compound closely-related to EPA and potential derivatives from lineolic acid and jasmonic acid, as determined by LC–MS/MS. On the other hand, the transcriptomic analysis showed that, like the GG treatment, the 4-HHE treatment increased the expression of genes involved in FFA metabolism. We identified two upregulated genes putatively encoding enoyl-CoA hydratase and propionyl-CoA carboxylase which may be involved in the β-oxidation of free fatty acids. The up-regulation of β-oxidation-related genes was also observed in the red alga *Laurencia dendroidea* in response to bacterial infection and was considered to provide alternative sources of respiratory substrates (de Oliveira et al., 2017). Besides the energy metabolism, β-oxidation was also participating in the biosynthesis of oxylipins such as jasmonic acid in land plants (Wasternack & Feussner, 2018). Concerning oxylipin pathway, only two CYP genes were shown to be downregulated after 4-HHE treatment, and no related genes was identified as upregulated. Altogether, these results suggested the activation of FFA and oxylipin pathways and are consistent with the study of Bouarab et al. (2004) which demonstrated that oligosaccharide elicitation or methyl jasmonate activated the metabolism of C20- and C18- PUFAs in the red alga *Chondrus crispus*, generating prostaglandins and jasmonates. Similarly, LPS and GG were shown to induce an increased release of C18- and C20- FFAs in *L. digitata* within 30 min (Küpper et al., 2006). However, unlike LPS, GG did not induce a significant accumulation of some oxidized

derivatives of linolenic and eicosapentaenoic acid after 1 h, suggesting that oligoguluronates perception would activate specific oxylipin biosynthetic pathways. Interestingly, 4-HHE applied on *L. digitata* at the concentration of 1 $\mu g.ml^{-1}$ was previously shown to significantly increase the production of the oxylipin 13-(HOTrE) after 24 h (Goulitquer et al., 2009). Moreover, the aldehyde, dodecadienal, induced a stronger release of stearidonic acid compared to the other tested aldehydes. This fatty acid derivative was already shown to be strongly produced in the red alga *C. crispus* in response to infection by the endophyte *A. operculata*, concomitantly with arachidonic acid and linolenic acid (Bouarab et al., 2004). Altogether, our results suggest that 4-HHE, 4-HNE, and dodecadienal could trigger the production of their own precursors by the activation of different metabolic pathways, including oxylipin pathways, and that 4-HHE perception by the alga could activate its own protection against grazers by intensifying downstream defense responses (Figure 5b).

Aldehydes showed antagonistic effects on algal consumption according to their chemical nature

According to the nature of the aldehyde, *L. digitata* responded differently to a subsequent grazing by *P. pellucida*. Dodecadienal increased significantly the consumption by herbivores, while 4-HHE decreased it when applied at 100 $ng.ml^{-1}$, and 4-HNE had no effect. The metabolic regulation observed in response to the aldehyde incubation suggested that dodecadienal, 4-HNE, and 4-HHE were perceived by the kelp, but inducing different metabolic pathways in the kelp plantlet. If 4-HNE incubation did not trigger later protective responses towards herbivores, this could suggest that this aldehyde perception would not be sufficient to activate defense responses against grazers. The different effect of 4-HNE and 4-HHE could also be due to the chemical properties of the compounds, such as solubility. Indeed, challenging of *L. digitata* with oligoguluronates or copper stress showed a strong release of 4-HHE mostly in the water phase, while 4-HNE was rapidly transferred in the air phase (Goulitquer et al., 2009), related to a lower amount in the water phase. Dodecadienal was released in an equivalent way between the two phases (Goulitquer et al., 2009), but showed an antagonistic effect than 4-HHE on algal consumption by grazers. Even if experimental conditions are far from *in situ* conditions (see below), the contrasting biological effect of these two aldehydes might reflect the complexity and integration of regulatory mechanisms and later algal physiological responses during grazing. Indeed, age- and tissue-specific grazing pressures by *P. pellucida* were already observed for *L. digitata* thalli *in situ* (Leblanc et al., 2011). The feeding preference for different tissue within plant was also observed in other kelps. For instance, feeding by pod grazers (*Parhyalella penai*) differed between reproductive and vegetative blades of *Lessonia nigrescens* and *Macrocystis integrifolia*, and this was only observed in living algal samples (Pansch et al., 2008). These results suggested that the feeding preference observed may be due to tissue-specific structure or specific inducible responses along the thallus, which still have to be deciphered.

We showed that the effective concentration of aldehydes for observation of algal protection against grazing was 100 $ng.ml^{-1}$ of 4-HHE in laboratory conditions. These aldehydes were previously described in animals to induce cytotoxic effects at high concentrations (Tsuchiya et al., 2005; Uchida et al., 2003), but to have a role in cellular signals at lower concentrations (Riahi et al., 2010). They were found as highly reactive and involved in pathophysiological interactions, which could lead to cell death (Coll et al., 2011). We could hypothesize that in these laboratory conditions, 4-HHE perception can induce specific defense responses against grazers when applied at 100 $ng.ml^{-1}$ but not below. However, at higher concentrations, a cytotoxic effect still needs to be explored, because *L. digitata* plantlets treated with 500 $ng.ml^{-1}$ or 1 $\mu g.ml^{-1}$ of 4-HHE for 4 h did not present any visible damage when maintained in culture for 1 week, during grazing bioassay. At the transcriptomic level, the treatment with 100 $ng.ml^{-1}$ 4-HHE induced not only the regulation of some genes involved in stress responses but also the up-regulation of putative defense-related genes, such as metacaspase-encoding genes. Metacaspases belong to a family of cysteine proteases and the up-regulation of two metacaspase genes was considered as part of the defense responses in the red alga *Pyropia yezoensis* to the infection of a pathogen oomycete, *Pythium porphyrae* (Tang et al., 2019). Additionally, a significant part of differentially expressed genes, were only identified after 100 $ng.ml^{-1}$ 4-HHE treatment, were not annotated, suggesting that these specific responses might lead to grazing protection. Due to the lack of researches on the defense responses in macroalgae, it is difficult to directly correlate genes and metabolites to specific defense responses. We have therefore proposed a hypothetical model of local and general defense molecular responses (Figure 5) and information on specific metabolites and differentially expressed genes provided in our study will be useful to decipher the mechanisms of macroalgae/herbivore interactions. On the other hand, if the 4 h incubation with 100 $ng.ml^{-1}$ 4-HHE is sufficient to activate specific defense-related responses against grazers after 24 h in *L. digitata*, this concentration is substantially higher than aldehyde amounts measured in the environment or released by *L. digitata* following stress in laboratory experiments. Indeed, measured concentrations after 1 h elicitation in the laboratory ranged between 0.2 and 2.0 $ng.ml^{-1}$, for 4-HHE and 4-HNE. In the field, the highest concentrations of aldehydes measured over kelp beds were around 0.015 $ng.ml^{-1}$ 4-HHE in seawater 1 h after low tide and 0.03 $ng.ml^{-1}$ 4-HNE 30 min after low tide

(Goulitquer et al., 2009). This strong difference between the effective concentration in the laboratory-conducted experiments and expected natural concentrations of 4-HHE might be due to additional unknown mechanisms occurring *in situ*, such as the specific amplification of aldehyde signals. In land plants, the abscisic acid signal can be amplified more than 40-fold under salt and drought stress conditions (Verslues et al., 2006). A previous study has shown that waterborne signals present in the kelp bed and/or released from elicited neighboring plants enhanced *L. digitata* defense-related responses, through a yet unknown mechanism reminiscent of priming in land plants (Thomas et al., 2011). The amplification of chemical signal has been well studied in land plants (Li et al., 2019), but rarely reported in kelps. In a context of kelps-herbivores interactions, future work still needs to be done to validate and decipher the hypothetical mechanism of *in situ* signal perception and amplification in kelps.

In conclusion, *in L. digitata*, the pre-incubation with three different aldehydes in laboratory conditions modified algal metabolic profiles after few hours and later algal consumption by specialist herbivores, suggesting a potential role as bioactive signals. Interestingly, according to their chemical nature, aldehyde signals have different effects on metabolic regulation and antagonist effects on algal consumption by grazers. For instance, 4-HHE pre-incubation seems to increase the resistance of the kelp within 1 day against the specialist herbivore *P. pellucida*, compared to untreated algae. On the contrary, dodecadienal appeared to induce some metabolisms favoring algal consumption by herbivores. Metabolomic and transcriptomic analyses suggest that both aldehydes might activate the oxylipin pathways. Furthermore, the comparative analysis of 4-HHE and oligoalginates treatments showed that 4-HHE perception might activate some specific defense-related genes in *L. digitata*,but not an oxidative burst. These results support the hypothesis that 4-HHE plays a role as signal molecule and highlight the potential biological importance of aldehyde-based signals during kelps-herbivores interactions.

MATERIALS AND METHODS

Algal material and herbivore sampling

All experiments were performed using young macroscopic diploid sporophytes of *Laminaria digitata*. Unialgal cultures of *L. digitata* sporophytes were obtained from random crosses of gametophyte batches. The latter were yielded in the laboratory from mature wild sporophytes collected in the same population. Two weeks after crossing, the sporophytes were transferred to 10 L flasks and grown until they reached a size of about 3–4 cm, in Provasoli Enriched Seawater (PES) culture media prepared with natural filtered seawater (FSW), collected off shore of Roscoff, as described in (Thomas et al., 2011). Algal cultures were illuminated with daylight-type fluorescent lamps at an irradiance of 25 $\mu E.m^{-2}.sec^{-1}$ for 10 h per day and kept at 13 ± 1°C.

The blue-rayed limpets *Patella pellucida* of 3–4 mm long were collected either at low tide or by diving, on *L. digitata* fronds. In laboratory, grazers were kept in an aquarium, and fed on *L. digitata* fronds until bioassay. Running seawater was constantly supplied and chilled at 15 ± 1°C, with constant air bubbling in half-light conditions.

Elicitation and aldehydes treatments

Alginate oligoguluronates (poly-alpha-1,4-L-guluronic acid blocks, GG blocks) were prepared by acid hydrolysis according to Haug et al. (1974), using sodium alginate from *Laminaria digitata* stipes (Danisco, Landerneau, France). Elicitation treatment was performed by placing one *L. digitata* plantlet individually in 30 ml FSW with GG at a final concentration of 150 $\mu g.ml^{-1}$ for 1 h (E treatment) at 13°C under agitation. Its control was treated equally, with seawater instead of GG solution.

Stock solutions of (±)-4-hydroxy-2E-hexenal (4-HHE; 10 $mg.ml^{-1}$) and (±)-4-hydroxy-2E-nonenal (4-HNE; 10 $mg.ml^{-1}$) from Interchim were prepared in EtOH 100% and the solution of 2,4-Dodecadienal (20 $mg.ml^{-1}$) from Sigma was dissolved in distilled water. For each treatment, *L. digitata* plantlets were incubated together in 50 ml (4 individuals), in 70 ml (8 or 12 individuals) of FSW for 4 h at 13°C, under continuous agitation. 4-HHE (H treatment), 4-HNE (N treatment), and Dodecadienal (D treatment) were added to the plantlets after dilution in 10 µl of EtOH 70%, in order to obtain a final concentration between 10 $ng.ml^{-1}$ and 1 $\mu g.ml^{-1}$ according to the aldehyde and the experiment. After 4-h treatment, plantlets were then rinsed twice in FSW and put individually into clean glass beakers with 30 ml fresh FSW, under agitation, up to independent sampling for metabolomic or transcriptomic analyses and for grazing bioassay up to the addition of herbivores. The control algae were treated in the same way, by adding only EtOH 70% to the plantlets. The Figure S1 summarizes the timing of the independent sampling and treatments conducted during this study according to each different analysis, detailed below.

For metabolomic analyses, each aldehyde treatment (H, N, D) was performed at a concentration of 100 $ng.ml^{-1}$ with 12 plantlets incubated together for 4 h, rinsed, and put individually in 30 ml FSW under agitation. Four plantlets (corresponding to four pseudo-replicates) were frozen in liquid nitrogen after 4 h incubation in aldehydes, as well as 4 and 24 h after rinsing and individual incubation in FSW, corresponding to 4, 8, and 28 h sampling times, respectively. For elicitation treatment (E), the individual elicited and control plantlets were rinsed after 1 h, put in 30 ml FSW under agitation and freeze-dried after 3 or 24 h, corresponding to 4 and 28 h sampling times respectively.

For transcriptomic analysis, four plantlets of *L. digitata* were submitted to GG elicitation as described above (E treatment) and control plantlets were maintained in the same conditions. In parallel, four plantlets were incubated with 100 $ng.ml^{-1}$ 4-HHE (H treatment) for 4 h, then rinsed twice with FSW and transferred individually in 30 ml FSW. The three groups of plantlets (E, H, and control) were collected after 12 h, frozen in liquid nitrogen, and stored at −80°C.

Before bioassays with *P. pellucida*, algal treatments were performed using eight plantlets incubated together for 4 h in FSW for control ones or with the addition of an aldehyde at different concentrations, or with GG solution at 150 $\mu g.ml^{-1}$ for 1 h. Treated and control algae were then rinsed twice in FSW and placed individually in 30 ml fresh FSW for 24 h under agitation, before addition of grazers.

Bioassays of algal consumption by grazers

For the 'no-choice' feeding-based bioassay and each chemical (H, N, D, and E) or control (C) treatment, 5 *L. digitata* plantlets were submitted to grazing by *P. pellucida*, while the three other plantlets treated in the same way we were used to control autogenic changes of mass during experimental setup, without the addition of grazers. Six *P. pellucida* individuals were placed on one plantlet in 50 ml filtered seawater at 13°C for up to 6 days. Algal mass was measured at the beginning of bioassay and 3 days after exposition to grazing, as already done previously (Leblanc et al., 2011; Thomas et al., 2014). The algal consumption for each treated or control plantlet corresponded to the algal mass difference between day 0 and day 3, corrected by the averaged mass difference of the three ungrazed plantlets maintained in the same conditions. Each grazing bioassay was conducted at least on five plantlets for chemical treatment, repeated two to three times for 4-HHE (H treatment) and up to five times for GG-treated algae (E treatment). The data were represented as boxplots, showing the distribution of data around the median between Q1 and Q3 percentiles, with whiskers drawn to the minimum/maximum values calculated as Q1/Q3 −/+ 1.5 * IQR (interquartile range), using R. The data were analyzed using Wilcoxon tests for comparison of mean algal consumption between the different treated (H, N, D, and E) and control (C) algae, using the free software R.

Hydrogen peroxide measurements

The concentration of hydrogen peroxide in the medium around algae was determined using the luminol chemiluminescence method (Glazener et al., 1991) with a Tristar luminometer (EG&G Berthold, Bad Wild-bach, Germany). During Elicitation (E) and Aldehyde treatments (H, N, D) performed at 100 $ng.ml^{-1}$, 150 µl of seawater was taken for one measurement, just before incubation in aldehyde or GG, and then every 5 min during 1 h after addition of aldehyde or GG solutions. Aliquots were put in 96-well microplates (Lumitrac 200, White, Greiner Bio-One). In the luminometer, 50 µl of 20 $U.ml^{-1}$ horseradish peroxidase (Boehringer Mannheim, Meylan, France), dissolved in pH 7.8 phosphate buffer, and 100 µl of 0.3 M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich, St. Louis, MO, USA) solution were added to the sample. Chemiluminescence was recorded immediately after the last injection with a signal integration time of 0.55 sec. The concentration of H_2O_2 in $nmol.g.FW^{-1}$ was calculated for each sample based on a standard calibration curve. The H_2O_2 production was estimated by integrating the total amount of H_2O_2 monitored over 1 h. The experiment was repeated f times for each treatment and the data plot as boxplot using R. One-way ANOVA was performed, followed by Student–Newman–Keuls (SNK) post hoc tests for comparisons of the mean production, using the free software R.

Metabolomic analyses and metabolic profiling of FFAs and putative derivatives

For each algal treatment (H, N, D, and E) and sampling time (4, 8, and 28 h), metabolic extracts were obtained from 100 mg of frozen algal powder with 1 ml MeOH:H_2O (8:2), as described in (Ritter et al., 2017). In each sample, 1.25 $\mu g.ml^{-1}$ 12-OH-lauric acid was added as an internal standard for mass spectrometry analysis in the negative mode. To separate FFAs and their derivatives from polar compounds, samples were first dried under nitrogen flow at 40°C, and metabolic extraction was made with 200 µl of ethyl acetate and 200 µl H_2O. The ethyl acetate phase was recovered and dried under nitrogen flow at 40°C. For metabolomic analyses carried out on the mass spectrometry facility of Corsaire-MetaboMer (Roscoff), 90 µl EtOH were added to each sample, and aliquots of 50 µl of each extract were used for analyses. Chemical compounds were first separated by ultrahigh-pressure liquid chromatography (UPLC) and analyzed by mass spectrometry (MS) on a Thermo Scientific LTQ-Orbitrap Discovery™ mass spectrometer (Thermo Scientific) equipped with an Electro Spray Ionization (ESI) source running on the negative ionization mode, as described in Ritter et al. (2014). Metabolic samples were analyzed using a 1.9 µm Thermo Hypersil Gold C18 column (100 × 2.1 mm) and the mobile phase A was composed of 0.1% acetic acid in H_2O, and mobile phase B was 0.1% acetic acid in acetonitrile. The gradient, starting at 0% B, consisted of an initial hold at 60% mobile phase B from 1 to 3 min, followed by a gradient to 95% B in 12 min and a hold for 4 min, followed by re-equilibration for 4 min at 0% B, for a total run time of 23 min. Xcalibur 2.1 software was used for instrument control and data acquisition.

Mass spectra data were processed by XCMS on the online version (https://workflow4metabolomics.usegalaxy.fr/; Giacomoni et al., 2015), after conversion of raw spectra to mzXML format. Data processing was performed using the centWave method for the peak picking, with a maximum deviation of 4 ppm. The signal/noise threshold was fixed at 10, the prefilter at 3100 and the noise filter at 5000. For the first group step, density method was used, with the band width set at 30 and the minimum fraction of samples necessary at 0.7. For correction of retention time, the obiwarp method was used and a step size of 0.1 m/z. The second group step was performed using density method and a band width of 20, and 10 for the third group step. Fillpeaks step was used with the chrom filling method. Finally, annotation by CAMERA was set using a max ion charge of 2, a general ppm error of 5 and a precision of 4 decimals of m/z values. The final table contained ion relative abundances in each sample, which corresponded to peak areas normalized by the standard peak area in the same sample. Multivariable statistical analyses were performed using Partial Least Squares - Discriminant Analysis (PLS-DA), and Number of MisClassifications (NMC) and *P*-value of PLS-DA were obtained, using R. Then, extraction of the 10 most discriminant ions using Variable Importance in Projection (VIP) and putative annotation of some of those VIP were made based on previous studies (Ritter et al., 2008).

For the chemical identification of five selected compounds, complementary analyses were carried out on the mass spectrometry facility of Corsaire-Laberca (Nantes), using a high-performance liquid chromatography (HPLC) system (Ultimate® 3000 Dionex, Thermo Fisher Scientific) coupled to high-resolution mass spectrometer described below. For this purpose, the following chemical standards from Interchim were used: jasmonic acid, 9(S)-hydroperoxyoctadecatrienoic acid (9(S)-HpOTrE), 13(S)-hydroperoxyoctadecatrienoic acid (13(S)-HpOTrE), 20-hydroxy-leukotriene B4 and stearidonic acid. The chromatographic separation was performed on Hypersil Gold C18 column (2.1 mm × 100 mm, 1.9 µm particle size, Thermo Fisher Scientific). Mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% acetic acid (A). The used elution gradient (A:B, v/v) was as follows: 100:0 from 0 to 1.0 min; 40:60 at 3 min; 5:95 at 15 min for 4 min then 100:0 for 4 min. The injected volume was 5 µl, the flow rate was 350 $\mu l.min^{-1}$ and the temperature of the column was maintained at 35 °C. The LC system was coupled to a Hybrid Quadrupole-Orbitrap Mass Spectrometer (Q Exactive™, Thermo Fisher Scientific) with a heated electrospray ionization source (HESI). Nitrogen was used as sheath gas and auxiliary gas at flow rates of 55 and 10 a.u. (arbitrary units), respectively. The ion transfer tube temperature was set at 350°C, the vaporizer

temperature at 300°C, and the electrospray voltage was set at 3.0 kV in positive mode and − 3.0 kV in negative with a s-lens RF level of 50%. For fullMS acquisition a polarity switching ion mode (positive/negative) with a mass range of m/z 65–975 at a mass resolving power of 70 000 Full Width Half Maximum (FWHM at 200 m/z) is used. The automatic gain control target and maximum injection time were 1e6 counts and 100 ms, respectively. The normalized collision energy (NCE) used for targeted MS/MS and data-dependent MS/MS modes was 35. Each target m/z was monitored with a 40 sec window (retention time ± 20 sec), 2-amu isolation window (target m/z ± 1 amu). For injection, the extract was diluted by 2 in ACN:H_2O (50:50), and 5 μl were injected the same day on the LC-HRMS system.

RNA-Seq and data processing

For each control, elicited and 4-HHE plantlets, sampled after 12 h, RNA was extracted with the method described by Heinrich et al. (2012) with a combination of a classical CTAB-based method and the RNeasy Mini kit (QIAGEN, Hilden, Germany) including an on-column DNA digestion. After the extraction, the quantity and quality of extracted RNA were tested on a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, US) and on a 2% agarose gel electrophoresis. Then the RNA samples passing the quality assessment were used to perform sequencing library preparation and Illumina Novaseq S4 (PE 150 bp) at the Plateforme Génomique du Genopole Toulouse Midi-Pyrénées GeT (France).

The quality control of raw data produced by sequencing was performed using FastQC (Andrews, 2010). Low quality reads (Phred score < 33 or length < 50 nucleotides) and sequencing adapters were removed using Trimmomatic (Bolger et al., 2014) and residual rRNA was removed with SortmeRNA (Kopylova et al., 2012). The data cleaning step was followed by another quality check with FastQC to ensure the quality of data reach the requirements of downstream analysis.

A *de novo* transcriptome was assembled based on the pooled reads of all conditions using Trinity (Grabherr et al., 2011) with the default options. Then reads were mapped on the transcriptome to calculate the transcript abundance with Bowtie2 aligner and RSEM (Langmead & Salzberg, 2012) and transcripts that represent 90% of reads were kept for downstream analysis. After obtaining a transcriptome with low redundancy, reads were mapped to this new transcriptome again with Bowtie 2 aligner and transcript abundance was estimated with RSEM. Gene annotation was performed with a Blastx search against the NCBI-nr and the Uniprot database with an E-value cut-off of 10^{-5}.

Differentially expressed gene analysis between control and treatment (4-HHE and GG) groups was performed separately using R package DESeq2 (Love et al., 2014). Transcripts with Log2Foldchange value ≥1 or ≤ −1 and adjusted *P*-value <0.05 were considered as differentially expressed genes (DEGs), and fasta sequence data of DEGs are listed in the supplementary Data S1 and S2.

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AUTHOR CONTRIBUTIONS

QX, LC, SF, and CL conceived the experiments. LD and ER produced the algal material. QX, LC, AD, and SR performed the experiments and generated the data. QX, LC, GLC, and YG performed the bioinformatic and metabolomic analyses. QX, LC, PP, GVM, SF, and CL interpreted the data. QX, LC, GVM, and CL wrote the first draft of the manuscript. All authors contributed to the revisions, and read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The RNA-seq datasets analyzed during the current study are available in the EMBL databases under the accession ID: PRJEB58330. All other data generated during this study are either included in this published article and its supplementary information files, or available from the corresponding author on request.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Scheme summarizing the kinetics of the independent sampling and treatments conducted according to each analysis (i.e., metabolomic, transcriptomic, and grazing bioassay).

Figure S2. Comparison of (a) MS chromatograms and (b) MS/MS fragmentation mass spectra of targeted compounds (QC) and commercial standards (STD).

Table S1. Relative Ion abundances from *L. digitata* samples from control, GG elicitation, or pre-incubated with 4-HHE, 4-HNE, and Dodecadienal. The five targeted ions shown in Figure 3a are highlighted in yellow.

Table S2. Summary of the Trinity assembly and annotation of *L. digitata* RNAseq dataset.

Table S3. Log2Foldchange values and annotations of Differentially-Expressed Genes (DEGs) in *L.digitata* after 12 h of treatment with 4-HHE and GG.

Data S1. Sequences of DEGs induced by 4-HHE treatment.

Data S2. Sequences of DEGs induced by GG treatment.

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