The Innate Immunity of a Marine Red Alga Involves Oxylipins from Both the Eicosanoid and Octadecanoid Pathways\(^1\)[w]

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The oxygenated derivatives of fatty acids, known as oxylipins, are pivotal signaling molecules in animals and terrestrial plants. In animal systems, eicosanoids regulate cell differentiation, immune responses, and homeostasis. In contrast, terrestrial plants use derivatives of C18 and C16 fatty acids as developmental or defense hormones. Marine algae have emerged early in the evolution of eukaryotes as several distinct phyla, independent from the animal and green-plant lineages. The occurrence of oxylipins of the eicosanoid family is well documented in marine red algae, but their biological roles remain an enigma. Here we address the hypothesis that they are involved with the defense mechanisms of the red alga *Chondrus crispus*. By investigating its association with a green algal endophyte *Acrochaete operculata*, which becomes invasive in the diploid generation of this red alga, we showed that (1) when challenged by pathogen extracts, the resistant haploid phase of *C. crispus* produced both C20 and C18 oxylipins, (2) elicitation with pathogen extracts or methyl jasmonate activated the metabolism of C20 and C18 polyunsaturated fatty acids to generate hydroperoxides and cyclopentenones such as prostaglandins and jasmonates, and (3) C20 and C18 hydroperoxides as well as methyl jasmonate did induce shikimate dehydrogenase and Phe ammonialyase activities in *C. crispus* and conferred an induced resistance to the diploid phase, while inhibitors of fatty acid oxidation reduced the natural resistance of the haploid generation. The dual nature of oxylipin metabolism in this alga suggests that early eukaryotes featured both animal- (eicosanoids) and plant-like (octadecanoids) oxylipins as essential components of innate immunity mechanisms.

Signaling cascades involving compounds derived from the oxidative metabolism of polyunsaturated fatty acids (PUFAs) are known to operate in response to external stimuli in both terrestrial plants and animals. In animal systems, eicosanoids, i.e. hydroperoxides derived from C20 PUFAs, regulate cell differentiation, immune responses, and homeostasis (Funk, 2001). In contrast, terrestrial plants use derivatives of C18 (octadecanoids) and C16 (hexadecanoids) fatty acids as developmental or defense hormones (Weber, 2002). The biosynthesis of these so-called oxylipins involves lipoxygenases (LOXs), which are multifunctional enzymes that catalyze the oxygenation of PUFAs into hydroperoxy derivatives, then on to other secondary products (Funk, 2001; Howe and Schilmiller, 2002). In terrestrial higher plants, this leads to the synthesis of the cyclopentenone jasmonic acid (JA), a key defense hormone described in a variety of crops or cell cultures (Heitz et al., 1997; Ishiguro et al., 2001; Seo et al., 2001; Turner et al., 2002). In animals, an alternative pathway involving cyclooxygenases also leads to the synthesis of important hormones with a cyclopentenone structure, known as prostaglandins (Funk, 2001). Another pathway derived from phospholipase-released arachidonic acid, involving 5-LOX, generates other potent eicosanoid lipid mediators referred to as leukotrienes (Funk, 2001).

Marine red algae, which emerged as an independent lineage early in the evolution of eukaryotes (Baldauf et al., 2000), contain oxylipins of the eicosanoid family, including prostaglandins and leukotrienes, as well as octadecanoids (Gerwick et al., 1999). On the basis of the structure of the oxylipins characterized so far, red algae are thought to feature 5\(R\), 8\(R\), 9\(S\), 12\(S\), and 15\(S\)-LOXs that act on eicosanoids, as well as 9\(S\), 11\(R\), and 13\(S\)-LOXs that act on octadecanoic acids (Gerwick et al., 1999). The occurrence of a functional 5R-lipoxygenase in *Rhodymenia pertusa* was recently inferred from the identification of four new oxylipins (Jiang et al., 2000). This finding strongly supports their generation through a leukotriene A-type intermediate.
Other red algae produce oxylipins by the pathways initiated by 8-, 9-, and 12-LOXs (Gerwick et al., 1999). An example is Gracilaria lemaneiformis, from which 12R, 13S-diHETE, and 12R, 13S-dihydroxy-eicosapentaenoic acid have been isolated (Jiang and Gerwick, 1994). At the molecular level, a cDNA encoding a putative 12-lipoxygenase was identified in the gametophyte of Porphyra purpurea (Liu and Reith, 1994) while a polyenoic fatty acid isomerase, which converts arachidonic acid into a conjugated triene, was purified and cloned from Ptilota filicina (Zhang et al., 2002).

Altogether, marine red algae are well documented to contain a variety of oxylipins of pharmacological interest, yet nothing is known of their biological functions in the algae (Gerwick et al., 1999). As sessile organisms, marine algae are challenged by a variety of potentially pathogenic organisms, including viruses, bacteria, fungi, other algae, and grazers. To survive in such a competitive environment, marine plants obviously had to evolve defenses such as the production of chemical deterrents (Kubanek et al., 2003). However, nothing is known of the signals that mediate the activation of cell-based induced defense responses (Potin et al., 2002). In this context, we have undertaken to study a model pathosystem in which the host is a parenchymatous red alga, Chondrus crispus, and the pathogen a filamentous green alga, Acrochaete operculata (Correa and McLachlan, 1991). C. crispus features an isomorphous life history, where the gametophytic and sporophytic generations differ by minor traits only. Interestingly, the gametophytic phase is naturally resistant to infection by the green algal endophyte, whereas the diploid sporophytic fronds are highly susceptible (Correa and McLachlan, 1991). In a previous study, we have shown that C. crispus gametophytes, and not the sporophytes, recognize cell-free extracts from A. operculata vegetative filaments as a defense elicitor, resulting in an oxidative burst, which appears as an essential component of the defense system of the red alga (Bouarab et al., 1999).

Given the functions of oxylipins in animals and terrestrial plants, we addressed the hypothesis that these compounds are also involved with the innate immunity of marine red algae. We show here that, when challenged by pathogen extracts, the red alga C. crispus produces both C20 and C18 oxylipins. These are shown to arise via the activation of enzymes involved in fatty acid oxidation and induce some other defensive responses, which mediate the resistance against A. operculata invasion.

RESULTS

Challenge with A. operculata Elicitors Induces Release of Free Fatty Acids and Activates Oxygenation Cascades in C. crispus Gametophytes

In this plant-pathogen system, we have previously reported that C. crispus gametophytes (the resistant generation) respond to recognition of A. operculata cell-free extracts by a burst of H2O2, whereas the sporophytes (invaded by the endophyte) release only limited amounts of H2O2 (Bouarab et al., 1999). As shown by LC/MS analyses of aqueous extracts at 24 h after elicitation and compared to control C. crispus gametophytes (Fig. 1A), challenging gametophytic thalli with A. operculata cell-free extracts dramatically changed their free fatty acid (FFA) composition. Only eicosapentaenoic acid (C20:5) and, in lesser amounts, linoleic acid (C18:2) were detected in control gametophytes, whereas significant levels of other free fatty acids and oxylipins were detected. The abundance of these compounds is shown in Figure 1B.

Figure 1. Fatty acid (FA) and oxylipin release in C. crispus gametophytes following challenge with A. operculata elicitors. Control gametophytes, gametophytes incubated with A. operculata elicitors, and gametophytes pretreated with n-PG before challenging with A. operculata elicitors were incubated for 24 h in culture conditions and were then extracted in a buffer as described in “Materials and Methods.” Fatty acids were extracted from cell-free extracts then resolved and characterized by RP-HPLC coupled to MS. A, FFA composition of the corresponding extracts, white bars, control, gray bars, A. operculata elicitor treatment, black bars, incubation with LOX inhibitors before elicitor treatment. Results are expressed in mg/g FW; mean and SD from three analyses of the same experiment are given. B, Chromatograms of control, elicited and inhibited gametophytes. In the chromatogram of the elicitor-treated gametophyte extract, eight eluting compounds were detected by ion monitoring and further identified by GC-MS analyses of collected fractions. Identified compounds correspond to (1) Ketols derivatives from C18:3, (2) Ketols derivatives from C18:2, (3) 13-HOTE, (4) 13-HODE, (5) 13-oxo-ODE, (6) 12-HETE, (7) 12,13-epoxy from C18:2, and (8) 9,10-epoxy from C18:2. Data are representative of three independent experiments.

Figure 1.
polyunsaturated fatty acids, including arachidonic acid (C20:4), linolenic acid (C18:3), and stearidonic acid (C18:4), were detected in the thalli challenged with the elicitor. Concomitantly with the detection of the release of FFA, hydroxy fatty acids, ketols, and epoxy, derived from C18:2 as well as from C18:3, and the hydroxylated derivative of C20:4, namely 12-HETE, were detected in the chromatograms of the extracts from the elicited thalli. These compounds (Fig. 1B) were identified by cochromatography with standards in reverse-phase HPLC as well as by gas chromatography-mass spectrometry (GC-MS), for which the compounds had fragmentation patterns identical to those of authentic, commercially available standards. In contrast, the FFA and hydroxylated FA composition of C. crispus gametophytes which, before elicitation, had been pretreated for 60 min with n-propylgallate (n-PG), a nonselective inhibitor of LOXs (Fournier et al., 1993), was the same as that of control thalli (Fig. 1). This indicates that the generation of a primary signal is prevented by treatment with n-PG, blocking the release of FFA. Therefore, the changes in the composition of the extracts could not be attributed to the uptake of FA added with the pathogen extract but to their release from the red algal membranes by the activation of lipase-like enzymes.

**Challenge with A. operculata Elicitors Activates Oxidative Metabolism in C. crispus Gametophytes**

To address the potential involvement of LOXs and other fatty acid oxidases in the generation of oxidized PUFAs in C. crispus, cell-free protein extracts were incubated with various PUFAs and the generated metabolites were analyzed by LC-MS (Fig. 2). Incubation of C18:3 with protein extracts from unchallenged gametophytes resulted in the production of 13-HOTE, which accounted for the conversion of only 2.30% of the added linolenic acid substrate. In contrast, this product was detected in larger amounts after incubation of C18:3 with the protein extracts from elicited gametophytes, accounting for a conversion of 8.40% of the substrate. Low amounts of 13-hydroperoxycatadecatrienoic acid (13-HPOTE) were also detected, representing a conversion of 0.80% of the initial substrate, likely because it might be rapidly converted into 13-HOTE and ketol derivatives (accounting for 4%). 13-HOTE was detected as only 1.40% of conversion of the substrate after incubation with extracts from elicited gametophytes which had been pretreated with n-PG. Similarly, incubation of linoleic or arachidonic acid with the protein extracts from elicited gametophytes yielded large amounts of oxylipins, mainly containing 13-hydroperoxy- and 13-hydroxyoctadecatrienoic acid [13-H(PO)DE] and 12-hydroperoxy- and 12-hydroxyeicosatetraenoic acid [12-H(PE)TE], respectively, while these compounds were not generated or were generated at very low levels only by the protein extracts from either unelicited gametophytes or elicited thalli pretreated with n-PG (data not shown).

Interestingly, in the presence of linolenic acid, both the protein fractions from control and elicited C. crispus generated a compound displaying a GC retention time (Rt; 9.5 min) and a mass spectrum (E.I. 70 eV) very similar to the fragmentation pattern of authentic methyl jasmonate (MeJA; see supplemental data, available at www.plantphysiol.org). Following incubation of protein extracts from elicited gametophytes with arachidonic acid, eluting compounds were detected with molecular masses [M-H]− at m/z 333 and m/z 335, corresponding to the expected masses for prostaglandins. Their reverse phase (RP)-HPLC Rt and their mass fragmentation spectra were indeed very similar to those from the authentic prostaglandins B2 (PGB2, [M-H]− m/z 333, Rt 7.3 min; Fig. 3) and B1 (PGB1, [M-H]− m/z 335, Rt 6.1 min). Two minor products, 12-hydroxy-5,8,10,14-eicosatetraenoic (12-HETE, Rt 25.8 min) and 11,12-epoxy-5,8,14-eicosatrienoic acids (11,12-EEt, Rt 37.8 min) were also characterized by their fragmentation patterns, displaying informative signals at m/z 319 (M-1), m/z 301 (M-18 loss of H2O), and by characteristic fragments due to the position of the epoxide or hydroxyl group at m/z 167 and m/z 149 for the 11,12-EEt and at m/z 208 and m/z 179 for 12-HETE, similar to the mass spectra of authentic standards. In addition, LC-MS analysis revealed noncharacteristic signals at m/z 295 (M-1) and m/z 277 (M-18, loss of water), corresponding to monoxygenated linoleic acid derivatives and characteristic fragments due to the position of the epoxide group at m/z 171 and 183 for the 9,10-epoxy-12-octadecenoic acid (Rt 36.9 min) and at m/z 223 and 195 for the 12,13-epoxy-9-octadecenoic acid (Rt 34.6 min). The RP-HPLC Rts and the fragmentation patterns were very similar to those obtained with authentic standards.

The induction of an oxidative cascade was then monitored by native PAGE and in-gel detection of C18:2 peroxidation (Fig. 4A). In elicited C. crispus gametophytes, three bands with lipoxigenase or/and...
peroxidase activity were up-regulated, from 24 h to at least 72 h after elicitation, whereas no such activation was observed in gametophytes pretreated with n-PG or salicylhydroxamic acid (SHAM), another nonspecific inhibitor of LOXs (Macri et al., 1994) nor in the unchallenged gametophytes (Fig. 4A). The corresponding gel bands were excised, crushed, and incubated with linoleic or arachidonic acid, and the oxygenation products were analyzed by LC-MS (Fig. 4, B and C).

Incubation of band I in the presence of linoleic acid yielded large amounts of a compound with a molecular mass [M-H] of 311 (Fig. 4B), which was further identified as the \( \alpha \) - and \( \gamma \)-ketols synthesized from 13-HPODE. Minor products, with a molecular mass [M-H] of 295 and showing Rts and mass spectra similar to authentic 9,10-epoxy-12-octadecenoic acid (coronaric acid) and 11,12-epoxy-9-octadecenoic acid (vernolic acid), were also generated. This band was not active in the presence of C20:4. Incubation of band II in the presence of C18:2 again yielded the \( \alpha \) - and \( \gamma \)-ketols of 13-HPODE, and it generated low amounts of oxidized compounds from C20:4 (data not shown). No product was generated at a detectable level from the incubation of band III with C18:2 and C20:4.

**Methyl Jasmonate Activates Oxidative Cascades in C. crispus Gametophytes**

We also investigated the effect of challenging C. crispus gametophytes with 100–\( \mu \)M MeJA for 6 h on their capacity to metabolize FFAs. Following the procedure described in “Materials and Methods,” metabolites from both C18:2 and C20:4 were characterized from their RP-HPLC Rts and their APCI mass spectra fragmentations. Incubation of protein extracts from unchallenged gametophytes in the presence of C18:2 (Fig. 5B) resulted in the production of 13-hydroxyoctadecadienoic acid (13-HODE, Rt 17.9 min), with a conversion of 4.9% of the substrate and of lower amounts of 11-hydroxyoctadecadienoic acid (11-HODE, Rt 13.3 min, 1.0%). Their fragmentation patterns displayed informative signals at \( m/z \) 295 (M-18 loss of water) and characteristic fragments due to the position of the hydroxyl group respectively at \( m/z \) 195 (\( \alpha \)-cleavage between C12 and C13 with gain of a hydrogen) and \( m/z \) 169 (\( \alpha \)-cleavage between C10 and C11 with gain of a hydrogen) in accordance with already published data (Bylund et al., 1998) and similar to the mass spectra of authentic standards (for 13-HODE). In contrast, 11-HODE was detected in large amounts after incubation with C18:2 of the protein extracts from gametophytes elicited with MeJA (Fig. 5A), accounting...
for a conversion of 12.5% of the substrate. In contrast to control gametophytes (Fig. 5D), incubation with C20:4 of the protein extracts from MeJA-elicited gametophytes (Fig. 5C) yielded a variety of metabolites with informative signals at m/z 319 (M-1), m/z 301 (M-18 loss of water). Those hydroxylated derivatives of C20:4 were identified as 15-HETE (Rt 19.5 min, 2.0% conversion), 13-HETE (Rt 16.3 min, 1.3% conversion), 12-HETE (Rt 25.8 min, 1.0%), 11-HETE (Rt 21.3 min, 1.0%), 9-HETE (Rt 23.8 min, 0.6%), and 8-HETE (not clearly separated from 12-HETE). Respectively, characteristic fragments due to the position of the hydroxyl group were detected at m/z 219 (α-cleavage between C14 and C15 with gain of a hydrogen), m/z 193 (α-cleavage between C12 and C13 with gain of a hydrogen), m/z 208 and 179 (α-cleavage between C11 and C12 with gain of a hydrogen), m/z 167 (α-cleavage between C10 and C11 with gain of a hydrogen), and m/z 155 (α-cleavage between C8 and C9 with loss of a hydrogen) in accordance with already published data (Bylund et al., 1998) and similar to the mass spectra of authentic standards (for 15-, 12-, 11-, and 8-HETEs). In contrast, only 13-HETE (2.5% conversion of the substrate) and 15-HETE (only 0.4% conversion of the substrate) were generated from C20:4 by the protein extracts from unelicited gametophytes (Fig. 5D). The fragmentation patterns obtained in GC-MS for the Me-TMS derivatives of 11-HODE and 13-HETE (data not shown), for which no standards were available, confirmed structural assignments and were in accordance with already published characterization in the red alga Lithothamnion corallioides (Hamberg et al., 1991; Gerwick et al., 1993).

Inhibitors of Fatty Acid Oxidation Reduced the Natural Resistance of C. crispus Gametophytes against A. operculata

The relevance of the activation of a fatty acid oxidation pathway in the defense system of C. crispus was then further investigated in pharmacological experiments in which we tested the effects of known inhibitors of fatty acid oxidation n-PG (60 μM) or SHAM (1 mM) on the resistance of C. crispus gametophytes to infection by A. operculata zoospores. Treatments with n-PG or with SHAM did not inhibit H2O2 release following challenge of C. crispus gametophytes with A. operculata elicitor (data not shown). However, the vulnerability of C. crispus to infection was markedly increased when the gametophytic thalli were treated with the inhibitors for 20 min before inoculation with the endophyte zoospores. In control C. crispus gametophytes, infection spots were scarce at the thallus surface (frequencies of infection at the tip of 6.2 ± 0.5 spots mm-2). In contrast, in the gametophytes treated with SHAM or n-PG, A. operculata zoospores settled at higher densities (72.5 ± 6.3 and 68.6 ± 5.8 spots mm-2, respectively), and developed into invasive filaments deeply embedded in the host medullary tissue (see supplemental data).

Oxylipins Elicit Shikimate Dehydrogenase and PAL Activities in C. crispus

The biological significance of oxylipin metabolism in C. crispus was further investigated by asking whether these compounds induce other defense responses in the
Figure 6. Oxylipins elicit shikimate dehydrogenase and PAL activities in C. crispus and confer induced resistance to the sporophytes. A and B, Shikimate dehydrogenase activity in protein extracts from C. crispus gametophytes (A) and sporophytes (B), following incubation with oxylipins and A. operculata elicitors. Shikimate dehydrogenase activity was revealed after native PAGE, using shikimic acid as substrate. Lanes correspond to protein extracts prepared at 24 h after challenge from 1, control C. crispus fronds; 2, 3, 4, 5, 6, 7, 8, and 9; fronds incubated with prostaglandins PGA1 (50 μg mL⁻¹), PGA2 (50 μg mL⁻¹), PGB1 (50 μg mL⁻¹), PGB2 (50 μg mL⁻¹), 12-HPETE (50 μg mL⁻¹), 13-HPODE (50 μg mL⁻¹), MeJA (20 μg mL⁻¹), and the pathogen elicitor, respectively. Arrowheads indicate the shikimate dehydrogenase isozyme under regulation. C, D, PAL activity in protein extracts from C. crispus gametophytes (C) and sporophytes (D) in control thalli and following incubation with oxylipins and A. operculata elicitors, as above. Reported values are the means of three independent experiments ± se. E to P, Effect of oxylipins on the resistance of C. crispus sporophytes to infection by A. operculata and on the accumulation of UV-fluorescent compounds around the infection sites. Surface views, under light microscopy (E–H) and UV excitation (I–L; Olympus BX 60 epifluorescence microscope), and cross sections (M–P) of C. crispus sporophytes fronds at 30 d after inoculation by A. operculata zoospores. E, I, and M, Control sporophytes. F, J, and N, Sporophytes pretreated with 12-HPETE (50 μg mL⁻¹), G, K, and O, Sporophytes pretreated with 13-HPODE (50 μg mL⁻¹). H, L, and P, Sporophytes pretreated with MeJA (20 μg mL⁻¹). Arrowheads point out the occurrence, or the absence, of UV-fluorescent compounds at the infection sites. Bars represent 40 μm. Note that the pathogen (arrows) invaded the medulla of the controls host thalli (M), whereas it was contained within the outer cortex of sporophytes treated with oxylipins (N–P). All of these data are representative of three independent experiments.

Table 1. Effect of oxylipins on the resistance of C. crispus sporophytes to A. operculata

<table>
<thead>
<tr>
<th>Host</th>
<th>Oxylipin Concentration</th>
<th>Frequency of Infection</th>
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<tbody>
<tr>
<td>Control sporophytes</td>
<td>0</td>
<td>70 ± 12</td>
</tr>
<tr>
<td>Sporophytes treated with 12-HPETE</td>
<td>10 μg mL⁻¹</td>
<td>52 ± 14</td>
</tr>
<tr>
<td>Sporophytes treated with 13-HPODE</td>
<td>50 μg mL⁻¹</td>
<td>24 ± 8</td>
</tr>
<tr>
<td>Sporophytes treated with MeJA</td>
<td>0.2 μg mL⁻¹</td>
<td>68 ± 11</td>
</tr>
<tr>
<td>Sporophytes treated with MeJA</td>
<td>2 μg mL⁻¹</td>
<td>45 ± 13</td>
</tr>
<tr>
<td>Sporophytes treated with MeJA</td>
<td>20 μg mL⁻¹</td>
<td>11 ± 5</td>
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*C. crispus sporophytes were treated for 1 h with various oxylipins, incubated in SFC medium for 24 h, and were then inoculated with A. operculata zoospores. *Ten independent replicates were performed for every inoculation condition. Frequencies of infection are given as the mean density of infection sites ± se from examinations under light microscopy of 1.0-mm² 64 areas at the host bases and apices.
completely invaded the host tissue (Fig. 6M). In contrast, in the sporophytes pretreated with 12-HPETE (50 μg.mL⁻¹), 13-HPODE (50 μg.mL⁻¹), or MeJA (20 μg.mL⁻¹), the infection rate was markedly reduced, with only a few infection spots (Fig. 6, F–H; Table I), and the filamentous germings were arrested between the outer and inner cortex of the host tissue (Fig. 3, N–P). Interestingly, in the sporophytes treated with oxylipins, accumulation of blue fluorescence was visible surrounding the sites of attempted penetration by A. operculata zoospores (Fig. 6, J–L), whereas no UV-fluorescent compounds were observed in the control, susceptible sporophytes (Fig. 6I). Similar features were found associated with the innate resistance of C. crispus gametophytes to the endophyte (see supplemental data). They were not observed, however, in the gametophytes pretreated with lipoxygenase inhibitors or in the sporophytes incubated with prostaglandins (data not shown). The above findings, reminiscent of the deposition of phenolic compounds in higher plant-pathogen interactions (McLusky et al., 1999), were consistent with the activation patterns of the marker enzymes of the shikimate and phenylpropanoid pathways (Fig. 6, A–D).

**DISCUSSION**

We here show that both C20 and C18 PUFAs and hydroxylated, epoxy, and ketol derivatives were generated (Fig. 1) and that several enzyme activities of the oxidative metabolism, including LOX, were upregulated (Figs. 2 and 4) in C. crispus gametophytes, 24 h following challenge with pathogen extracts. All of these responses were prevented by n-PG, a radical scavenger known to inhibit several enzyme activities of the fatty acid oxidation pathway (i.e. LOX, peroxidase), indicating that, as for mammalian (Parmentier et al., 2001) or plant (Sasaki et al., 2001) cells, this alga may be endowed by a positive feed-back loop by which oxygenated fatty acids mediate the release from membrane lipids of their own precursors by regulating the activities of lipase-like enzymes. We also show that when challenged by pathogen extracts, C. crispus gametophytes metabolize both C20 and C18 PUFAs into the corresponding hydroperoxides and derivatives (Figs. 2–4) and, likely, all the way down to the cyclopentenones such as prostaglandins (Fig. 3) and MeJA (see supplemental data), i.e. the ultimate products known for these oxygenation cascades in animals and higher plants, respectively. In addition, hydroperoxides derived from both C20 and C18 PUFAs induce defense enzymes in C. crispus and conferred an induced resistance to the sporophytic generation (Fig. 6; Table I), while inhibitors of fatty acid oxidation abolished the natural resistance of the gametophytic generation and the induced resistance of the sporophytic generation.

Thus, oxylipins originating from both C20 and C18 PUFAs identified in this study (Fig. 7), including 12-HPETE and 13-HPODE, respectively, appear as
essential intermediates in the innate immunity of this alga. To our knowledge, this is the first demonstration that the oxylipins of marine red algae, which so far have mainly been investigated for their potential uses as pharmaceutical agents in heterologous systems, naturally act as homologous defense mediators in the phylum Rhodophyta. Further experiments are now required to identify which products of 12-HPETE and of 13-HPODE are active in C. crispus defense responses. In this respect, it is worth noting here that, even though prostaglandins B1 and B2 were produced in the elicited alga, all of the prostaglandins investigated in this study (A1, A2, B1, and B2) and which are known as major defense cyclopentenones in mammalian cells (Funk, 2001), did not stimulate defense mechanisms in C. crispus (Fig. 6). Given the toxicity of red algal prostaglandins for human consumers (Noguchi et al., 1994), they may function as defense chemicals against grazers. In contrast, incubation with MeJA stimulated the synthesis of hydroxylated derivatives of both C18:2 and C20:4 (Fig. 5), up-regulated shikimate deshydrogenase and PAL activities in C. crispus gametophytes, and conferred induced resistance to the sporophytes (Fig. 6). Therefore, the key defense cyclopentenone of higher plants (Howe and Schilmiller, 2002), JA, or its methyl-ester form, also appears as an active metabolite in C. crispus.

Using cell-free homogenates of C. crispus gametophytes, we detected the presence of MeJA only after incubation with linolenic acid. Our previous attempts to identify JA in C. crispus cell homogenates using a protocol optimized for higher plants (Schittko et al., 2000) remained unsuccessful, and we were unable to show any up-regulation of JA synthesis after elicitation, as it is observed in elicited cells from higher plants (Howe and Schilmiller, 2002). In higher plants, it is well established that the allene oxide synthase (AOS) branch of the 13-LOX pathway gives rise to JA and MeJA, and their metabolic precursor 12-oxo-10,15-phytodienoic acid (12-OPDA; Howe and Schilmiller, 2002). AOS catalyzes the transformation of 13-hydroperoxy linolenic acid (13-HPOTE) to an allene oxide intermediate, 12,13-epoxyoctadecatrienoic acid (12,13-EOTE), which is converted to 12-OPDA by an allene oxide cyclase. Reduction of 12-OPDA by OPDA reductase and three cycles of β-oxidation then yields JA, while a carboxymethyl transferase catalyzes the formation of MeJA. During in vitro reactions carried out in the absence of allene oxide cyclase, 12,13-EOTE spontaneously hydrolyzes to α- and γ-ketols and can also undergo nonenzymatic cyclization to produce racemic 12-OPDA (Howe and Schilmiller, 2002). Our preliminary data indicate that MeJA is produced from linolenic acid in the cell-free system from C. crispus gametophytes, implying that the pathway, which lead to the synthesis of MeJA in higher plants, may exist in marine algae. In such a crude homogenate, it is not surprising that the enzymes of β-oxidation are still active. Further experiments are required to identify the different components of this pathway.

In conclusion, the red alga C. crispus is likely to use both animal-like (eicosanoid) and higher-plant-like (octadecanoid) oxylipins in the regulation of its metabolism toward protection against pathogens. This duality of the oxylipin metabolism parallels that of mammalian cells, where there is emerging evidence that, besides the C20:4 derivatives, the oxygenation products from C18:2 can act as defense compounds (Ishizaki et al., 1995a, 1995b). The most parsimonious scenario to account for the presence of both the C20 and C18 PUFA oxygenation cascades in these two phylogenetically distant lineages as well as in other eukaryotic branches (Fig. 8) is that these two categories of lipid derivatives preexisted before the radiation of extant eukaryotes.

Our findings also raise the question of which enzymes are involved with the generation of C20 and C18 cyclopentenones in red algae, either AOS as in plants and/or cyclooxygenase as in metazoans or else an enzyme machinery specific to this phylum. Presence of JA and MeJA has already been reported in several lineages of nonvascular plants (Hamberg and Gardner, 1992), including unicellular green algae.
(Ueda et al., 1991a; Fujii et al., 1997), Euglenophytes (Ueda et al., 1991b), and the Rhodophyte Gelidiella latifolia (Krupina and Dathe, 1991). The entire set of enzymes necessary for the biosynthesis of JA from linolenic acid have also been identified in the marine red algae Gracilaria sp. (Hamberg and Gerwick, 1993) and Lithothamnion corallioides (Hamberg, 1992). In addition, JA was reported from the cyanobacterium Spirulina sp. (Ueda et al., 1991a), an AOS gene was identified in the genome of another cyanobacterium, Anabaena (www.kazusa.or.jp/cyanobase/), and the envelope membrane from higher-plant chloroplasts is known to contain the whole set of enzymes for the oxygenation of octadecanoid acid hydroperoxides (BlÉe and Joyard, 1996). As photosynthetic eukaryotes have arisen from a single endosymbiosis event between a heterotrophic eukaryote and a photosynthetic cyanobacterium (Baldauf et al., 2000), it is thus tempting to speculate that the ability to synthesize jasmonate was acquired from the plastid genome. That transfer of this gene to the nucleus occurred before the radiation of the green lineage (Fig. 8) would account for the occurrence of JA in both red algae and higher plants. As powerful genomic approaches are now becoming applied to gene mining in red (Nikaido et al., 2000) and other algae, sequences for enzymes involved with oxylipin biosynthesis will soon be available in these phyla, providing additional data to assess the evolution of these pathways (Brash, 1999).

MATERIALS AND METHODS

Chemicals

Fatty acids (C14:0, C16:0, C18:1, C18:2, C18:3, C20:4, and C20:5) and prostaglandins A1, B1, A2, and B2 were purchased from Sigma (St. Louis); oxylipins 12-HETE, 13-HPODE, 13-HODE, (15, 12, 11-, 8-) HETES, 11,12-EET, and veronic acid and coronaric acids were from Cayman Chemicals (SPI Bio, Montigny le Bretonneux, France); and MeJa from Aldrich (Milwaukee, WI). Silylating reagent N, N-bistrimethylsilyltrifluoroacetamide containing 1% of trimethylchlorosilane (BSTFA + 1% TMCS) was from Pierce Europe (Ound-Beijerland, The Netherlands). All chemicals and solvents were from Merck (Darmstadt, Germany) and Sigma.

Plant Material and Inoculation Procedures

Unialgal cultures of Acrochaete operculata (isolates P 161085-2-1 and KH 040687-1-1) were established and grown in enriched seawater medium with full concentration of nutrients (SFC) as described previously (Correa and 040687-1-1) were established and grown in enriched seawater medium with C. crispus added in the culture medium to elicit defense responses in 1846 Plant Physiol. Vol. 135, 2004 (0.2–20 μM) and 100 μM of Tris-HCl pH 8.5 (+ 0.01% Tween 20). After a 15-min incubation at room temperature, metabolites and the residual substrates were extracted twice with 3 mL of diethyl ether, and the organic phase was dried under N2 dissolved in acetone and titrated by LC-MS.

LC-MS Analyses

The metabolites from linoleic, linolenic, and arachidonic acids were resolved and characterized by RP-HPLC coupled to a Navigator LC-MS mass spectrometer (Finnigan, Manchester, UK), equipped with an atmospheric pressure ionization source (APCI) running on a negative ion mode (cone voltage 30 V and 45 V for compounds fragmentation, as detailed in Adas et al., 1998). Metabolites were analyzed by RP-HPLC (SpectraSystem P400 with UV detector UV1000), using a 5-μm Ultrasphere C18 column 150 × 4.6 mm (Beckman, Paris). The mobile phase (0.2% acetic acid in water/acetone) program began isocratically with a 40:60 mixture (v/v) for 35 min followed by a 5-min linear gradient to 5/95 (v/v) mixture for 20 min at a flow rate of 1 mL/min, in order to elute lipophilic compounds, i.e. fatty acids and sterols, before returning to the initial conditions. Fatty acids and metabolites were identified by their Rts and their masses on the APCI(−) mode. Negative ions were monitored by full scan from m/z 60 to 600. The source heater was at 150°C and the APCI heater at 350°C with a cone voltage of either 30 V or 45 V to increase fragmentation. Detection of metabolites was achieved by monitoring selected ions corresponding to the expected carbohydrate anions [M-H]−. Compounds were then quantified with LC-MS from standard curves obtained by measuring the peak surfaces of authentic fatty acids, 13-HODE (11-HODE was quantified using the standard curve obtained for 13-HODE) and HETEs (13-HETE and 9-HETE was quantified using the standard curves obtained for 15-HETE and 11-HETE respectively).

GC-MS Analyses

GC-MS analyses were carried out on a HP 5890 Series II gas chromatograph equipped with a fused silica capillary column (HP-5MS 5% phenyl methyl siloxane; 30 m × 0.25 mm I.D.; film thickness 0.25 μm) and combined to a quadrupole mass-selective detector (HP 5971A, Agilent Technology, Massy, France). Mass spectra (F.I. mode) were recorded at 70 eV. Analyses were performed after methylation with ethereal diazomethane and silylation with a mixture of BSTFA (N,N-bistrimethylsilyltrifluoroacetamide)/TMCS (trimethylchlorosilane; 100:1, v/v) for 30 min at 60°C, in order to obtain trimethylsilyl derivatives for compounds containing hydroxyl group (Pinot et al., 1992). Compounds were dissolved in 1 mL of hexane and 2 μL were injected in the splitless mode at 60°C. After 5 min at 60°C, the oven temperature was increased to 200°C at 50°C/min and then linearly ramped to 280°C at 2°C/min that became stable for 10 min before returning to initial conditions.

Shikimate Dehydrogenase and Phenylalanine Ammonialyase Assays

C. crispus thalli were incubated for 1 h with oxylipins at the concentration of 50 μg/mL (20 μg mL−1 for MeJa). At 24 h after treatment, proteins were extracted and the 15,000g centrifugation supernatants were analyzed by Native-PAGE (Laemmli, 1970) on 12% acrylamide gels (30 μg of total proteins per lane). Shikimate dehydrogenase activity was evaluated by incubating the electrophoresis gels in the presence of shikimic acid and NADP+ (Sigma; Manchenko, 1994). PAL activity was assayed as described in Legrand et al. (1976).
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LITERATURE CITED


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