Sulfated Oligosaccharides Mediate the Interaction between a Marine Red Alga and Its Green Algal Pathogenic Endophyte

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The endophytic green alga Acrochaete operculata completely colonizes the sporophytes of the red alga Chondrus crispus; however, it does not penetrate beyond the outer cell layers of the gametophytes. Given that the life cycle phases of C. crispus differ in the sulfation pattern of their extracellular matrix carrageenans, we investigated whether carrageenan fragments could modulate parasite virulence. \(\alpha\)-Carrageenan oligosaccharides induced release of \(H_2O_2\), stimulated protein synthesis, increased carrageenolytic activity, and induced specific polypeptides in the pathogen, resulting in a marked increase in pathogenicity. In contrast, \(\kappa\)-carrageenan oligosaccharides did not induce a marked release of \(H_2O_2\) from A. operculata but hindered amino acid uptake and enhanced their recognition by the host, resulting in a reduced virulence. Moreover, C. crispus life cycle phases were shown to behave differently in their response to challenge with cell-free extracts of A. operculata. Gametophytes exhibited a large burst of \(H_2O_2\), whereas only low levels were released from the sporophytes.

INTRODUCTION

In terrestrial plants, it is now well established that host-pathogen interactions involve extracellular signals that are known as elicitors. These signals are recognized by and initiate defense reactions in the host (Clark et al., 1992; Boller, 1995; Baker et al., 1997; Yang et al., 1997; Ebel and Mithöfer, 1998). Many elicitors of nonhost resistance in plants consist of oligosaccharins, that is, polysaccharide fragments with signaling activities (Ryan and Farmer, 1991; Darvill et al., 1992; Côté and Hahn, 1994; John et al., 1997; Côté et al., 1998). Endogenous elicitors, such as oligopectins, are released from the host cell walls. Critical structural features that modulate the biological activity of oligopectins as defense signals are chain length (Mathieu et al., 1991, 1998; Simpson et al., 1998), the configuration of reducing ends (Spiro et al., 1998), and substitution by methoxy groups (Boudart et al., 1998). Exogenous elicitors, such as \(\beta\)-1,3-glucans or oligochitins, are released from the cell walls of pathogenic fungi. Other interactions between plants and microorganisms, such as root nodule formation in the symbiosis between legumes and rhizobia, involve lipochitooligosaccharide signals in which sulfate substituents are determinants of host specificity (Lerouge et al., 1990; Roche et al., 1991; Truchet et al., 1991).

Oligosaccharins can trigger a variety of defense responses in plants. One typical rapid reaction is the release of a burst of activated oxygen species (AOS), which are toxic to the pathogen and act as defense secondary messengers for the host (Levine et al., 1994; Mehdy, 1994; Baker and Orlandi, 1995; Dixon and Lamb, 1997). Other short-term (i.e., within minutes) defense reactions consist of signal transduction events that include calcium efflux, plasma membrane depolarization, apoplast alkalinization, protein kinase cascade activation, and phosphorylation and/or dephosphorylation of numerous proteins (reviewed in Yang et al., 1997; Scheel, 1998). Oligosaccharins also induce longer term gene-regulated responses in the host, such as synthesis of pathogenesis-related proteins, production of protease or pectinase inhibitors, stimulation of secondary metabolite pathways, cell wall cross-linking and lignification, and the hypersensitive response, that is, limited necrosis at the site of infection (reviewed in Mehdy, 1994; Hammond-Kosack and J ones, 1996; Low and Merida, 1996; Lamb and Dixon, 1997; Scheel, 1998; Sommsich and Halbrock, 1998). Class II (glucanases) and classes III, IV, VIII, and XI (chitinases) pathogenesis-related proteins attack fungal cell walls and amplify the production of oligosaccharidic elicitors (Fritig et al., 1998).

In marine plants, infectious diseases can be highly destructive. Examples include the kelp Undaria pinnatifida (Ishikawa and Saga, 1989) and the red alga Porphyra yezoensis (Fujita et al., 1972), two major mariculture crops in Japan, commonly named wakame and nori, respectively.

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However, little is known about the biochemical basis of pathogen recognition or the related host defense responses (Correa, 1997).

Chondrus crispus (Gigartinales, Rhodophyceae) is a marine red alga with an isomorphic life history comprising morphologically similar haploid gametophytes and diploid sporophytes. The alga is exploited for industrial production of its cell wall polysaccharides, known as carrageenans, sporophytes. The alga is exploited for industrial production

The carrageenans differ by the number and position of sulfate substitutions on the α-(1→3) and β-(1→4) galactan chains.

| A | \(\kappa\)-Carrageenan, β-D-galactopyranosyl-4-sulfate (1→4)-O-3,6-anhydro-α-D-galactopyranosyl. |
| B | \(\iota\)-Carrageenan, β-D-galactopyranosyl-4-sulfate (1→4)-O-3,6-anhydro-α-D-galactopyranosyl-2-sulfate. |
| C | \(\lambda\)-Carrageenan, β-D-galactopyranosyl-2-sulfate (1→4)-O-\(\alpha\)-D-galactopyranosyl-2,6-disulfate. |

The carrageenans differ by the number and position of sulfate substitutions on the α-(1→3) and β-(1→4) galactan chains.

DISCUSSION

- The carrageenans differ by the number and position of sulfate substitutions on the α-(1→3) and β-(1→4) galactan chains.
- When carrageenan oligosaccharide signals were recognized by the pathogen, we monitored A. operculata cultures for the emission of O₂ and H₂O₂. Carrageenan oligomers were added to the seawater in concentrations up to 3 hr after the addition of the oligosaccharide signal. H₂O₂ was released in seawater in concentrations up to the micromolar range (Figure 2) and was not completely scavenged until 3 hr after the addition of \(\lambda\)-carrageenan oligosaccharides. Similar observations were made when \(\lambda\)-carrageenan oligosaccharides were added to the incubation medium; however, the H₂O₂ levels in the seawater did not rise so high.

**RESULTS**

**A. operculata Possesses Carrageenolytic Activity**

To address whether A. operculata produces enzymes that can degrade C. crispus cell wall polysaccharides, we assayed cell-free extracts from A. operculata cultures for carrageenolytic activity. Unelicited A. operculata cultures displayed virtually no carrageenolytic activity toward the carrageenans extracted from C. crispus gametophytes, whereas they were able to degrade those extracted from the sporophytes (Table 1). Similar observations were made when A. operculata cultures had been incubated in the presence of \(\kappa\)-carrageenan oligosaccharides for 4 days. In contrast, A. operculata cultures elicited with either \(\lambda\)- or \(\iota\)-carrageenan oligomers exhibited marked carrageenolytic activities toward cell wall polysaccharides from both the gametophytic and the sporophytic phases.

**A. operculata Recognizes Oligocarrageenans**

To determine whether C. crispus extracellular matrix components are recognized by the pathogen, we monitored A. operculata cultures for the emission of H₂O₂ when carrageenan oligomers were added (Figure 2). A. operculata cultures constitutively released very low levels of H₂O₂. Samples incubated with \(\kappa\)-carrageenan oligosaccharides also released low levels of H₂O₂. In contrast, samples challenged with \(\lambda\)-carrageenan oligomers showed a significant emission of H₂O₂ within minutes of the addition of the oligosaccharide signal. H₂O₂ was released in seawater in concentrations up to the micromolar range (Figure 2) and was not completely scavenged until 3 hr after the addition of \(\lambda\)-carrageenan oligosaccharides. Similar observations were made when \(\lambda\)-carrageenan oligosaccharides were added to the incubation medium; however, the H₂O₂ levels in the seawater did not rise so high.
Unialgal cultures of *A. operculata* were incubated with κ-, λ-, or υ-carrageenans or with their respective oligomeric constituents, and the uptake of 35S-labeled amino acids and new protein synthesis were monitored at various intervals after the addition of the polysaccharides or oligosaccharides. These experiments showed that high molecular weight carrageenans had no effect on amino acid uptake or new protein synthesis in *A. operculata*. When compared with seawater controls, the cultures treated with either υ- or λ-carrageenan oligomers displayed an enhanced uptake of 35S-cysteine and methionine for at least 2 days after the addition of the elicitor. In contrast, incubation in the presence of κ-carrageenan oligosaccharides resulted in a marked decrease of label uptake for at least 4 days after the addition of the oligosaccharides (Figure 3).

Treatment with υ- or λ-carrageenan oligomers also specifically elicited the production of various polypeptides in *A. operculata* (Figures 4C and 4D). When compared with cultures grown in seawater, no changes were detected in the two-dimensional electrophoresis protein patterns during the first 4 days of elicitation (data not shown). However, a novel 70-kD acidic protein, referred to as P38 and P40, were markedly upregulated by both λ- and υ-carrageenan oligomers (Figures 4C and 4D), whereas only traces of these polypeptides could be detected under the other incubation conditions (Figures 4A and 4B).

### Oligocarrageenans Control *A. operculata* Pathogenicity

The potential effects of *C. crispus* extracellular matrix (ECM) components on pathogen virulence were investigated further by incubating *A. operculata* filaments with oligocarrageenans for 1 week before zoospore release. In a first series of experiments, zoospores were used to challenge *C. crispus* gametophytes during the life cycle phase known to resist invasion by the pathogen (Correa and McLachlan, 1991, 1994). As expected for zoospores from untreated *A. operculata* cultures, few infection spots could be observed on the thallus surface (Figure 5A and Table 2), and filamentous germings were unable to penetrate beyond the cortex of the host gametophytes (Figure 5B). Respective to this control, *A. operculata* cultures incubated with κ-carrageenan oligosaccharides released zoospores with a markedly reduced virulence (Table 2). In contrast, zoospores from *A. operculata* cultures elicited with λ-carrageenan oligosaccharides settled at high densities (Figure 5C and Table 2) and developed into invasive filaments deeply embedded in the medullary tissue of the host gametophytes (Figure 5D). This type of infection pattern had previously been reported only for the *C. crispus* sporophytic susceptible phase (Correa and McLachlan, 1991, 1994; Table 2).

### Table 1. Carrageenolytic Activity of *A. operculata* Protein Extracts toward Carrageenans from the Gametophytes or the Sporophytes of *C. crispus*

<table>
<thead>
<tr>
<th>Elicitation Conditions</th>
<th>Substrate Degradation (%)</th>
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<tbody>
<tr>
<td></td>
<td>G Carrageenans&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0.7</td>
</tr>
<tr>
<td>κ-Carrageenan oligosaccharides</td>
<td>2.0</td>
</tr>
<tr>
<td>υ-Carrageenan oligosaccharides</td>
<td>11.1</td>
</tr>
<tr>
<td>λ-Carrageenan oligosaccharides</td>
<td>13.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Extracts prepared from pathogen cultures elicited for 4 days with carrageenan oligomers were assayed by scintillation counting for the release of oligocarrageenans from the 35S-labeled substrates. Carrageenolytic activity is expressed as the proportion of released label relative to the initial substrate radioactivity.

<sup>b</sup> G, gametophytic.

<sup>c</sup> S, sporophytic.
addressed by monitoring the production of H$_2$O$_2$ after chal-
tophytes respond differently to pathogen signals then was 
The question of whether

A. operculata

C. crispus

between the epidermis and the cortex (Figure 6D). 
face (Figure 6C), and filamentous germlings were arrested
Only a few infection spots were observed on the thallus sur-
charides released zoospores with a markedly reduced

carrageenans. Sporophytes released low amounts of H$_2$O$_2$,
neither sporophytic nor gametophytic fronds of

C. crispus

operculata

entirely invaded the host tissues (Figure 6B). Zoospores
control cultures of the pathogen settled
throughout the sporophytic fronds (Figure 6A), and filaments
A. operculata cultures incubated with
k-carrageenan oligosaccharides released zoospores with a markedly reduced
pathogenicity toward the host sporophytic fronds (Table 2). Only a few infection spots were observed on the thallus sur-
C. crispus

Gametophytes in Response to
A. operculata Extracts

To further understand the influence of host ECM fragments on A. operculata pathogenicity, we then challenged C. cris-
pus gametophytes with A. operculata extracts prepared from unialgal cultures that had been grown under various
conditions of elicitation with oligocarrageenans. The intensity
and the duration of the oxidative burst by the host ga-
metophytes depended on the oligocarrageenan that had
been used to elicit the A. operculata cultures (Figure 7B). A
higher, more transient oxidative burst was observed with ex-	racts from cultures elicited with $\kappa$-carrageenan oligosac-
charides. Extracts prepared from unelicited A. operculata
filaments or cultures elicited with $\iota$-carrageenan oligosac-
charides resulted in lower production of H$_2$O$_2$ by gameto-
phytes. Even more remarkably, gametophytes challenged
with extracts of A. operculata previously elicited with $\lambda$-car-
rageenan oligosaccharides did not release significant
amounts of H$_2$O$_2$ (Figure 7B).

Preincubation of C. crispus gametophytic thalli with ex-
tracts of A. operculata previously elicited with $\lambda$-carr-
rageenan oligosaccharides quenched in a dose-dependent
manner the oxidative burst triggered by the extracts from A.
operculata elicited with $\iota$-carrageenan oligosaccharides
(Figure 8A). Induction of various AOS scavenging enzymes
known to be involved with the control of host defenses by
pathogens, namely, catalase (Garre et al., 1998), superoxide
dismutase (Lamboy et al., 1995), and peroxidases (Brisson
et al., 1994), then was investigated by using native PAGE
followed by in-gel activity assays. Among those, a peroxi-
dase isozyme was specifically induced in A. operculata
cultures elicited with $\lambda$-carrageenan oligosaccharides (Figure
8B). The peroxidase isozyme was not produced under any
of the other elicitation conditions.

C. crispus Releases AOS in Response to
A. operculata Extracts

The question of whether C. crispus sporophytes and game-
tophytes respond differently to pathogen signals then was
addressed by monitoring the production of H$_2$O$_2$ after chal-
lenge with A. operculata cell-free extracts. Neither the
sporophytes nor the gametophytes constitutively produced
extracellular H$_2$O$_2$ (Figures 7A and 7B). It is worth noting that
neither sporophytic nor gametophytic fronds of C. crispus
released H$_2$O$_2$ in response to direct challenge with oligo-
carrageenans. Sporophytes released low amounts of H$_2$O$_2$,
regardless of which extract from the pathogen was used for
challenging (Figure 7A). In contrast, cell-free extracts of A.
operculata triggered a significant oxidative burst in C. cris-
pus gametophytes. After a lag period of $\sim$20 min, H$_2$O$_2$ was
released in seawater at concentrations up to the 10-$\mu$M
range, that is, an order of magnitude higher than the corre-
20 min, H$_2$O$_2$ was

The Oxidative Burst in C. crispus Is a Key Element in
Establishing Resistance to A. operculata

Treatment with 5 $\mu$M diphenyleneiodonium (DPI), a suicide
inhibitor of NADPH oxidase, inhibited the production of
H$_2$O$_2$ by C. crispus gametophytes that had been challenged
with A. operculata cell-free extracts (Figure 9A). As shown
in Table 3, this inhibition resulted in the loss of resistance of C.

![Figure 3. Oligocarrageenans Modulate Amino Acid Uptake in Unialgal A. operculata Cultures.](image)

The pathogen was incubated in SFC medium without oligocarra-
geenans (open bars) or in the presence of $\lambda$- (filled bars), $\iota$- (cross-hatched bars), or $\kappa$- (hatched bars) carrageenan oligomers. Data are averages from three independent experiments. Bars indicate ±SD.
crispus gametophytes to invasion by the pathogen. In contrast to control plants (Figures 9B and 9D), gametophytes pretreated with DPI immediately before inoculation with A. operculata zoospores became covered with high densities of zoospores (Figure 9C and Table 3), and they were deeply penetrated by green algal filaments (Figure 9E).

**DISCUSSION**

**Oligosaccharide Signals from the Host ECM Control Pathogen Physiology and Virulence**

Plant cell walls are physical barriers that hinder the penetration of alien organisms. Pathogenic microorganisms therefore are equipped with a variety of cell wall-degrading enzymes (Côté et al., 1998). A. operculata also appears to constitutively synthesize carrageenolytic enzymes, and it thus can be inferred that oligocarrageenans are produced by C. crispus during the infection process. However, compared with the carrageenans from the resistant generation, protein extracts from the unelicited pathogen exhibited a higher activity when the cell wall components from the susceptible C. crispus phase were used as a substrate. Interestingly, incubation of the pathogen in the presence of oligocarrageenans also resulted in a buildup of carrageenolytic activity. θ-Carrageenan oligosaccharides, however, failed to elicit carrageenolytic activity toward the cell wall components from the resistant host generation.

As indicated by the differences in the emission of H₂O₂ when A. operculata cultures were incubated in the presence...
of various oligocarrageenans, the oligosaccharide structural motives are perceived by the pathogen as different signals (Figure 2). It appears that λ-carrageenan fragments elicit a strong release of H$_2$O$_2$ (Figure 2), stimulate amino acid uptake (Figure 3), and upregulate several specific polypeptides (Figure 4) in the green algal pathogen, enhancing its virulence and making colonization possible during the host’s resistant phase (Figure 5). A. operculata filaments elicited with λ-carrageenan oligosaccharides consistently exhibited a marked hydrolytic activity toward gametophytic carrageenan extracts (Table 1). In contrast, oligo-κ-carrageenan oligosaccharides are poor inducers of AOS emission (Figure 2), hinder
Oligosaccharide Signaling in Algal Pathogens

Table 2. Effect of Oligocarrageenans on the Virulence of A. operculata toward C. crispus Gametophytes and Sporophytes

<table>
<thead>
<tr>
<th>Host</th>
<th>Pathogen Inoculum</th>
<th>Frequency of Infection (Number of Spots mm(^{-2}))</th>
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<tbody>
<tr>
<td></td>
<td>Tips</td>
<td>Bases</td>
</tr>
<tr>
<td>Gametophytes(^b)</td>
<td>Control zoospores</td>
<td>5.4 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Zoospores elicited with (\kappa)-carrageenan oligosaccharides</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Zoospores elicited with (\lambda)-carrageenan oligosaccharides</td>
<td>68.3 ± 11.5</td>
</tr>
<tr>
<td>Sporophytes(^b)</td>
<td>Control zoospores</td>
<td>68.9 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>Zoospores elicited with (\lambda)-carrageenan oligosaccharides</td>
<td>82.0 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>Zoospores elicited with (\kappa)-carrageenan oligosaccharides</td>
<td>20.9 ± 2.8</td>
</tr>
</tbody>
</table>

\(^a\) C. crispus gametophytes and sporophytes were inoculated with zoospores released from A. operculata cultures previously elicited for 1 week with oligocarrageenans. Three weeks after inoculation, endophytic cells were scored at the surface of C. crispus fronds.

\(^b\) Ten independent replicate experiments 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-mm\(^2\) areas at the host bases and apices.

Amino acid uptake (Figure 3), enhance pathogen recognition by C. crispus gametophytes (Figure 7), and reduce A. operculata virulence (Table 2 and Figure 6).

Therefore, the green algal endophyte A. operculata can recognize the number and position of sulfate substituents on the oligogalactan glycosidic backbone. These observations are reminiscent of the recognition by alfalfa of 6-O-sulfate substituents on the reducing-end glucosamine residue of nodulation factors (Lerouge et al., 1990; Roche et al., 1991; Truchet et al., 1991). Given that carrageenans display a variety of sulfation patterns (Figure 1), the aquatic facultative endophyte A. operculata thus provides an interesting, additional model in which to investigate the perception and transduction of sulfated oligosaccharide signals by chlorophytic plants.

In this study, we demonstrate that in plant–pathogen interactions, oligosaccharide signals also can be recognized by the pathogenic partner. In response to the composition of the host ECM, the pathogen can modulate its physiology and its behavior in the interaction. Notably and in contrast to challenging with extracts prepared from A. operculata filaments that had been preincubated with \(\kappa\)-carrageenan oligosaccharides, incubating pathogen cultures with \(\lambda\)-carrageenan oligosaccharides resulted in a complete quenching of the oxidative burst in C. crispus gametophytes. \(\lambda\)-Carrageenan oligosaccharides, therefore, may signal the pathogen to avoid or quench the host defense reactions, for example, by enhancing the synthesis in A. operculata of components that interfere with its recognition by C. crispus gametophytes and/or with the increase of AOS levels in the host apoplasm.

Consistent with this latter hypothesis, a peroxidase isozyme was induced specifically by \(\lambda\)-carrageenan oligosaccharides in A. operculata (Figure 8B). Because the enzyme is not induced until several days after elicitation with \(\lambda\)-carrageenan oligosaccharides, it cannot protect against

The AOS produced by the pathogen itself upon recognition of the signal (Figure 2). We suggest that this peroxidase is involved in scavenging the AOS that may be produced in the host apoplasm during the infection. It is also tempting to speculate that this isoform corresponds to one of the proteins that is upregulated in response to incubation in the presence of \(\lambda\)-carrageenan oligosaccharides (Figure 4D). A precise assignment, however, will require the molecular characterization of these polypeptides or the raising of antibodies against the purified peroxidase isoform.

The Marine Red Alga C. crispus Features an Oxidative Burst When the Pathogen is Recognized

In this plant-pathogen system, C. crispus sporophytes (the host life cycle phase that is sensitive to the pathogen) release low amounts of \( \text{H}_2\text{O}_2 \) when challenged by A. operculata extracts (Figure 7B). In contrast, C. crispus gametophytes (the resistant generation) respond to recognition of A. operculata extracts by a burst of AOS, with levels of \( \text{H}_2\text{O}_2 \) 10 to 15 times higher than those observed with the sporophytes (Figure 7A). Because the A. operculata extracts used for challenging the host were AOS free, the oxidative burst recorded in these experiments is a genuine host response. This indicates that as in higher plant-pathogen interactions, cell–cell recognition in this marine pathosystem is followed by a release of AOS. Assuming that C. crispus cell walls represent ~20% of the tissue fresh weight, the peak peroxide levels correspond to local concentrations of ~2 mM in the apoplasm, which is similar to those reported in the oxidative burst of higher plants (Legendre et al., 1993). Inhibition of the oxidative burst by DPI suggests that C. crispus gametophytes might possess a flavoprotein-containing NADPH oxidase complex that is analogous to those involved in the production of AOS in animals (Segal and Abo, 1993) and
higher plants (Groom et al., 1996; Jabs et al., 1997; Pugin et al., 1997; Keller et al., 1998; Torres et al., 1998). However, because DPI also was shown to inhibit animal nitric oxide synthase (Stuehr et al., 1991) as well as the peroxidase-mediated generation of H$_2$O$_2$ in higher plants (Boilwell et al., 1998; Frahry and Schopfer, 1998), no conclusion can be drawn regarding the machinery of AOS production in this marine red alga.

The differences in the oxidative responses to pathogen extracts of the susceptible and resistant host generations indicate that, as in higher plants and animals, accumulation of AOS is an essential component of the defense system of the
red alga C. crispus. This is substantiated further by the dramatic decrease in the resistance of C. crispus gametophytes when the oxidative burst was inhibited by DPI (Table 3 and Figure 9). These observations are in agreement with recent reports indicating a major role of H\textsubscript{2}O\textsubscript{2} in the induction of systemic acquired resistance in higher plants (Alvarez et al., 1998; Van Camp et al., 1998). By analogy, it is likely that AOS are involved in the protection of the red algal host as secondary messengers or in mediating cell wall modifications. No cell death, however, is associated with the oxidative burst in the C. crispus—A. operculata pathosystem. Finally, that the oxidative burst is a typical defense reaction

\[ \text{Figure 7. Production of Hydrogen Peroxide by C. crispus Fronds Challenged with A. operculata Cell-Free Extracts.} \]

(A) Kinetics of H\textsubscript{2}O\textsubscript{2} release by sporophytic fronds. (B) Kinetics of H\textsubscript{2}O\textsubscript{2} release by gametophytic fronds. Controls (open triangles) were C. crispus fronds challenged with seawater only; C. crispus fronds were challenged with extracts from unstimulated A. operculata (open diamonds); extracts from A. operculata grown in the presence of \kappa-carrageenan oligosaccharides (filled diamonds); extracts from A. operculata cultures stimulated with \kappa-carrageenan oligosaccharides (filled triangles); and extracts from A. operculata cultures elicited with \lambda-carrageenan oligosaccharides (filled circles). Average data are from three independent experiments. Bars indicate ±SD.

\[ \text{Figure 8. Effects of \lambda-Carrageenan Oligosaccharides on the Ability of A. operculata to Control the Oxidative Burst in Its Host.} \]

(A) Treatments with extracts of A. operculata elicited with \lambda-carrageenan oligosaccharides quenched the release of H\textsubscript{2}O\textsubscript{2} by C. crispus gametophytes. C. crispus gametophytes were preincubated for 15 min with seawater (filled diamonds), with 150 μL of the quenching extract (open circles), or with 450 μL of the same extract (filled circles) and then challenged with 150 μL of extracts from A. operculata grown in the presence of \kappa-carrageenan oligosaccharides. Average data are from three independent experiments. Bars indicate ±SD. (B) \lambda-Carrageenan oligosaccharides induce a de novo peroxidase in protein extracts from A. operculata cultures. Lane 1 contains an extract from A. operculata cultures elicited with \lambda-carrageenan oligosaccharides; lane 2, an extract from A. operculata cultures elicited with \kappa-carrageenan oligosaccharides; and lane 3, an extract from unstimulated A. operculata filaments. Arrowhead points to the induced peroxidase isoform.
Figure 9. DPI Inhibits the Oxidative Burst in C. crispus Gametophytes and Abolishes Their Resistance to A. operculata.
in this red alga is supported by the fact that, as discussed above, A. operculata filaments incubated in the presence of \(\lambda\)-carrageenan oligosaccharides both were capable of quenching the oxidative response of C. crispus gametophytes (Figure 8A) and exhibited a markedly higher virulence (Figure 5). This finding is a first step toward establishing a causal relationship between carrageenan signaling and the modifications of pathogen virulence.

Because oligocarrageenans do not trigger an oxidative burst in C. crispus thalli, endogenous cell wall fragments are not recognized as defense signals in this alga. In this respect, C. crispus departs from higher plants in which endogenous oligogalacturonin elicitors directly trigger defense responses in the host (e.g., Mathieu et al., 1991; Boudart et al., 1998; Simpson et al., 1998). The chemical nature of the A. operculata signal(s) recognized by the C. crispus-resistant generation remains an open question. Based on the oxidative response of C. crispus gametophytes, no signals were released in A. operculata culture medium (data not shown). Yet, recognition signals are readily solubilized from A. operculata filaments, that is, within 30 min of extraction at room temperature, and it is likely that they are perceived by the host during the infection. Partial purification by using the production of AOS by C. crispus gametophytes as a bioassay indicates that the A. operculata signal(s) consists of small molecules, saccharidic in nature and comprising uronic residues (K. Bouarab, B. Kloareg, and P. Potin, unpublished results). Based on their capability to trigger an oxidative burst, these molecules appear to be poorly recognized by the susceptible sporophytic host generation (Figure 7A), suggesting that C. crispus sporophytes lack the very receptors involved in the perception of Acrochaete filaments by the gametophytes.

Sulfated Oligosaccharides Mediate the Interaction between C. crispus and A. operculata

In conclusion and consistent with the relationships between carrageenan distribution in C. crispus life cycle phases (McCandless et al., 1973) and the differences in their resistance to the pathogen (Correa and MacLachlan, 1991), our data suggest that oligocarrageenans govern the biological interaction between the red alga and its green endophyte (Figure 10). In this interaction, the host-susceptible generation does not properly recognize A. operculata filaments. Moreover, fragments of \(\lambda\)-carrageenan, which is the cell wall polymer prevalent throughout the host-susceptible phase, induce in the pathogen the production of specific carrageenolytic and AOS-scavenging enzymes, enhancing pathogenicity (Figure 10A). In contrast, in the presence of oligomers of \(\kappa\)-carrageenan, the main ECM polysaccharide of the host-resistant generation, the above-mentioned virulence proteins, is not produced; rather, the pathogen appears to be better recognized (Figure 10B). This results in a marked inhibition of the virulence of A. operculata, which remains contained in the outer cortex of C. crispus gametophytes.

**Table 3.** Effect of DPI on the Susceptibility of C. crispus Gametophytes toward Infection by A. operculata

<table>
<thead>
<tr>
<th>Host</th>
<th>Frequency of Infection (Number of Spots mm(^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control gametophytes(^a)</td>
<td>6.2 ± 0.5</td>
</tr>
<tr>
<td>DPI-treated gametophytes(^b)</td>
<td>68.3 ± 8.5</td>
</tr>
</tbody>
</table>

\(^a\) C. crispus gametophytes treated with 5 \(\mu\)M DPI or left untreated were inoculated with zoospores released from A. operculata cultures. Three weeks after inoculation, endophytic cells were scored at the surface of C. crispus fronds.

\(^b\) Ten independent replicate experiments 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-mm\(^2\) areas at the host bases and apices.

**METHODS**

**Preparation of Oligocarrageenans**

Oligocarrageenans were prepared by enzymatic digestion of commercial \(\kappa\)-carrageenan (batch HMR XZ 4804; Sanofi BioIndustries, Baup, France), \(\lambda\)-carrageenan (batch HMR XSI 4104; Sanofi BioIndustries), or...
Figure 10. Sulfated Oligosaccharide Signaling in the C. crispus–A. operculata Host-Pathogen Association.

(A) In the case of the C. crispus sporophytic phase, $\lambda$-carrageenan oligosaccharides released from the extracellular matrix during infection signal the pathogen to control the host defense reactions. In particular, $\lambda$-carrageenan fragments enhance the synthesis of specific polypeptides in A. operculata including a peroxidase isoform that is likely to be involved in quenching the AOS released by the host. This results in stimulation of the virulence of the pathogen.

(B) In the case of gametophytic plants, $\kappa$-carrageenan oligosaccharides released during infection from the host inner cell layers do not induce the production of AOS in the pathogen and hinder the expression of genes involved with virulence. Furthermore, pathogen recognition is enhanced, triggering an oxidative burst in the host, which appears essential in preventing further invasion. AOS may be detrimental to the pathogen, and they are likely to participate in oxidative cross-linking of the host cell walls and to induce gene-regulated defense responses. Altogether, pathogen virulence is inhibited.

Filled hexagons represent recognition signals from A. operculata; half-square-ended bars, putative receptors for $\lambda$-carrageenan oligosaccharides; half-circle-ended bars, putative receptors for $\kappa$-carrageenan oligosaccharides; R and R, putative receptors for signals from A. operculata; scissors, carrageenolytic activity; T-bars, inhibiting effects. Broken lines and italicized captions refer to inhibited processes or pathways.
λ-carrageenan (batch PSN MS 92; Sanofi Bioindustries). For the preparation of κ-carrageenan oligosaccharides, the commercial polymeric substrate first was digested with purified λ-carrageenase (Potin et al., 1991) to remove contaminating λ-carrageenan molecules. The undigested fraction then was precipitated with isopropanol and incubated in the presence of purified κ-carrageenase (Potin et al., 1991), and the oligosaccharic products were fractionated by preparative steric exclusion chromatography (Rochas and Heyraud, 1981). A parallel procedure was applied to the preparation of ω-carrageenan oligosaccharides. For the preparation of λ-carrageenan oligomers, λ-carrageenan was repurified by precipitation with 3 M KCl, dialyzed, treated with ω-carrageenase, and finally digested with λ-carrageenase from *Pseudoalteromonas carrageenovora* (Johnston and McCandless, 1973) by using 0.2 enzyme units per mg of polymer. After removing the undigested fraction by isopropanol precipitation, the λ-carrageenan oligosaccharides were desalted and freeze-dried.

Oligosaccharide purity was checked by 13C-nuclear magnetic resonance spectroscopy (Potin et al., 1991) and PAGE (Zablackis et al., 1991). Oligocarrageenan fractions consisted of repeated disaccharidic motifs of κ-, ω-, or λ-carrageenan, respectively (Figures 1A to 1C). They ranged in size from the hexasaccharide (n = 3) to the dodecasaccharide (n = 6).

### Plant Material and Inoculation Procedures

Unialgal cultures of *Acrochaete operculata* (isolate P 161085-2-1) were established and grown in enriched seawater medium SFC as described previously (Correa et al., 1988). Large-scale cultures were grown aseptically at 15°C by using a photoperiod of 16 hr of light and 8 hr of darkness at a photon flux density of 40 μmol m⁻² sec⁻¹. The culture medium was changed weekly. Bacterial contamination was controlled by monthly addition (0.1% [v/v]) of a penicillin/streptomycin solution from Sigma.

Infection trials were performed with gametophytic (strain J C 002 PC-G) or sporophytic (strain J C 001 PC-S) *Chondrus crispus* unialgal isolates, by culturing 10 freshly cut fragments in 30 mL of SFC medium, as described in Correa and McMclachlan (1991). Inocula consisted of 5 × 10⁴ zoospores mL⁻¹ from unialgal *A. operculata* cultures (isolate KH 040677-1-1), maintained for 8 days in SFC medium supplemented with or without oligocarrageenans at the concentration of 150 μg mL⁻¹. The culture medium was changed every 3 days. After 3 weeks, thallus fragments were brushed gently to remove gerrms that had not penetrated through the host cell wall, and infection was quantified by counting the endophytic cells at the surface of *C. crispus* fronds with a light microscope. In each thallus fragment, three randomly selected 1.0-mm² areas at both the host bases and apices were scored for the presence of settled spores, and cross-sections were examined for the development of *A. operculata* green filaments. The effect of diphenyleleniodonium (DPI; Sigma) on the outcome of the infections was tested by incubating *C. crispus* gametophytes for 20 min in the presence of 5 μM DPI. This treatment proved not to be detrimental to *C. crispus* thalli. Thallus fragments then were extensively rinsed in fresh SFC medium, and the inoculations were performed as described above.

Host oxidative responses were investigated with wild *C. crispus* sporophytes and gametophytes collected from the shore at Roscoff, France, and maintained in running seawater tanks. Asexually propagated gametophytes from the large-scale culture ponds of *Systèmes* *Bioindustries* (Bouin, France) also were used.

### Incubation of *A. operculata* with Oligocarrageenans

Three milliliters of aliquoted cultures of *A. operculata* in SFC medium, containing ~300 mg of tissue, was incubated for up to 8 days in the presence of various carrageenans or oligocarrageenans at the concentration of 150 μg mL⁻¹. After various times of elicitation (i.e., 16 hr or 2, 4, 6, or 8 days), cells were pelleted, rinsed three times with SFC medium, and then assayed for carrageenolytic activity, label uptake, and new protein synthesis or used to prepare cell-free extracts. Controls consisted of cultures grown in the absence of oligocarrageenans or of polymeric carrageenans.

### Detection of Carrageenolytic Activity in *A. operculata*

35SO₄⁻ labeled carrageenans were prepared according to the procedure described by McLean and Williamson (1979). Briefly, *C. crispus* gametophytic or sporophytic fronds (blotted fresh weight of 5 g) were incubated in artificial, sulfate-free seawater (450 mM NaCl, 10 mM KCl, 46 mM MgCl₂, and 2 mM NaHCO₃) and supplemented with 2 μM Na₃SO₄, 2.16 μL L⁻¹; DuPont New England Nuclear, Le Blanc Mesnil, France) and the nutrients of SFC medium (Correa et al., 1988). Thalli were grown for 4 days, rinsed extensively, ground in liquid nitrogen, and extracted with 50 mM NaCl (15 mL g⁻¹) for 2 hr in a water bath at 90°C. Extracts then were centrifuged at 1600g for 15 min, and carrageenans were precipitated with 3 volumes of ethanol, rinsed in 75% ethanol, and dried. Specific radioactivities were 90,000 and 175,000 dpm per mg of carrageenans from the gametophytes and the sporophytes, respectively.

To assay for carrageenolytic activity, we ground *A. operculata* fragments in liquid nitrogen, and we resuspended the resulting algal powder in 50 mM Tris·HCl, pH 7.5, buffer containing 5 mM MgCl₂ and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). *A. operculata* protein extracts (30 μL) then were incubated for 3 days with 30 μL of radiolabeled carrageenans resuspended at the concentration of 5 mg mL⁻¹ in 10 mM sodium phosphate buffer containing 50 mM NaCl. At the end of the incubation, 3 volumes of ethanol were added, the reaction mixture was centrifuged at 12,000g for 10 min, and radioactivity of the supernatant was assayed by liquid scintillation, as a measurement of the release of oligocarrageenans. Controls consisted of incubating carrageenanol solutions in the presence of SFC medium only.

### Monitoring H₂O₂ Emission

The kinetics of H₂O₂ release by *A. operculata* cultures (500 mg blotted fresh weight in 10 mL of 0.22 μm–filtered seawater) in response to challenge with various oligocarrageenans at the concentration of 150 μg mL⁻¹ were monitored by luminescent-dependent luminescence, by using a Lumat LB 9507 Berthold luminometer (EG&G Berthold, Evry, France). At intervals, 400-μL aliquots of the reaction medium were assayed for H₂O₂ by recording luminescence counts for 10 sec after adding 100 μL of horseradish peroxidase (Boehringer Mannheim; 20 units mL⁻¹ in 10 mM potassium phosphate buffer, pH 7.8) and 50 μL of 0.3 mM luminol (Aldrich, Steinheim, Germany).

Extracts were prepared from *A. operculata* cultures incubated for 8 days with various oligocarrageenans by grinding 300 mg of blotted fresh weight in liquid nitrogen, followed by homogenization for 30 min in 200 μL of 50 mM Tris·HCl buffer, pH 6.0, 50 mM NaCl, 10 mM MgCl₂, and 1 mM PMSF. The kinetics of H₂O₂ release by *C. crispus* gametophytic or sporophytic fronds (500 mg of blotted fresh weight in 10 mL of 0.22 μm–filtered seawater) in response to challenging
with A. operculata cell-free extracts (300 µL) were monitored by luminol-dependent luminescence, as described above. No H₂O₂ was detected in the A. operculata extracts.

**Monitoring Protein Synthesis in A. operculata**

Labeling with a sulfur-35 mixture (Pro-Mix; Amersham; 70% methionine and 30% cysteine) was added 16 hr before each measurement point so that the initial radioactivity of the batch was 0.135 mCi mL⁻¹, and cells were allowed to take up the label for 16 hr. For measurement of amino acid uptake, cells were rinsed extensively, ground in liquid nitrogen, and homogenized in Laemmli (1970) buffer. Homogenates then were centrifuged at 12,000g for 10 min, and the radioactivity was measured in the supernatant with a Packard Tri-Carb 1500 counter (Packard, Rungis, France). Aliquots (3 mL) of A. operculata cultures, containing ~300 mg of tissue, were incubated for up to 8 days in the presence of oligocarrageenans at the concentration of 150 µg mL⁻¹.

In these experiments, incubation with cycloheximide strongly reduced the label uptake by A. operculata, indicating that new protein synthesis from bacterial contamination or in organelles was negligible.

**Two-Dimensional Gel Electrophoresis and Native PAGE**

For the analysis of newly synthesized soluble proteins, aliquots of ground algal powder (300 mg blotted fresh weight) were incubated at 0°C for at least 10 min in 800 µL of extraction buffer (500 mM Tris-HCl, pH 8.0, 1 M KCl), 50 mM EDTA, 2 mM PMSE, and 2% β-mercaptoethanol). Phenol (800 µL) then was added, and the samples were mixed, left for 1 hr at room temperature, and centrifuged for 5 min at 12,000g (Schuster and Davies, 1983). The phenol layer was washed with 3 × 600 µL of extraction buffer, and proteins were precipitated overnight at −20°C by the addition of 4 volumes of 0.1 M sodium acetate in methanol. Precipitates were washed twice with 400 µL of methanol and once with 200 µL of acetone, and then solubilized in O’Farrell’s two-dimensional electrophoresis buffer (Hilbert et al., 1991). Isoelectric focusing was conducted at 400 V for 8 hr by using a Mini Protean apparatus from Bio-Rad, with 4% acrylamide gels in 9.2 M urea and 2% Triton X-100 and with a 2% ampholyte solution (1.6% at pH 5.0 to 7.0 and 0.4% at pH 3.0 to 10.0; Bio-Rad). Gels were loaded with the same amount of radioactivity, irrespective of the protein content. SDS-PAGE was run at 100 V in a 12% acrylamide gel, with a buffer consisting of 25 mM Tris, 0.192 M glycine, pH 8.3, and 0.1% SDS. Two-dimensional gels were vacuum-dried and exposed for 2 days to X-Ovit-AER 5 Kodak films (Eastman Kodak, Rochester, NY).

For the analysis of peroxidase induction in A. operculata cultures, proteins were extracted 8 days after the addition of λ-carrageenan oligosaccharides. After centrifugation at 15,000g, the cleared supernatant was separated by native PAGE by using the procedure of Laemmli (1970), but without denaturing agents (40 µg of total protein per lane). Peroxidase activity was determined by using 3-amino-9-ethylcarbazole as a substrate (Manchenko, 1994).

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**REFERENCES**


Oligosaccharide Signaling in Algal Pathogens 1649
and nodPQ genes encode the sulfation of lipo-oligosaccharide signals. Cell 67, 1131–1143.


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