Oligoguluronates Elicit an Oxidative Burst in the Brown Algal Kelp *Laminaria digitata*[1]

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Oligomeric degradation products of alginate elicited a respiratory and oxidative burst in the sporophytes of the kelp *Laminaria digitata*. The generation of activated oxygen species (AOS), O_2^-, and H_2O_2 was detected at the single cell level, using nitroblue tetrazolium precipitation and a redox-sensitive fluorescent probe, respectively. The oxidative burst involved diphenyleneiodonium-sensitive AOS-generating machinery and its amplitude depended on the type of tissue. After a first elicitation plants were desensitized for about 3 h. The activity of alginate oligosaccharides was dose dependent, saturating around 40 μM. It was also structure-dependent, with homopolymeric blocks of α-1,4-L-guluronic acid, i.e. the functional analogs of oligogalacturonic blocks in pectins, being the most active signals. The perception of oligoguluronate signals resulted in a strong efflux of potassium. Pharmacological dissection of the early events preceding the emission of AOS indicated that the transduction chain of oligoguluronate signals in *L. digitata* is likely to feature protein kinases, phospholipase A_2, as well as K^+, Ca^{2+}, and anion channels.

The oxidative burst, i.e. a rapid, transient production of large amounts of activated oxygen species (AOS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl radicals (OH) was discovered several decades ago as a "respiratory burst" during phagocytosis in cells of the human immune system (Baldridge and Gerard, 1933). The same phenomenon was found later in plants (Doke, 1983a, 1983b) and it is recognized today as a ubiquitous characteristic of defense systems in these phyla (e.g. Baker and Orlandi, 1995; Bolwell et al., 1995; Lamb and Dixon, 1997; Wojtaszek, 1997). This defense response is most often triggered by cell-cell recognition, involving the perception at the plant cell membrane of signal molecules from the invading organism or from the host cell walls, referred to as elicitors. Common elicitors of non-host resistance are oligosaccharides, glycoproteins, and glycopeptides (e.g. Scheel and Parker, 1990; Boller, 1995). In higher plant pathogen recognition systems, xyloglucan (Fry et al., 1993) and pectin (e.g. Boudart et al., 1998) elicitors are of endogenous origin (i.e. from the plant), whereas glucans and chitins are exogenous (released from the pathogen during the interaction).

An increasing amount of scientific evidence (for review, see Wojtaszek, 1997) points out that the onset of the oxidative burst is controlled by a signal transduction cascade involving G proteins, adenylate cyclases, phopholipases, protein kinases, protein phosphatases, ion channels, changes in membrane potential, permeability and ion fluxes, and finally the activation of AOS-generating oxidases/dehydrogenases. The main enzymatic source of AOS in the oxidative burst of plant cells remains controversial, but most results point to three mechanisms: (a) an O_2^--generating plasmalemmic NAD(P) H oxidase (Desikan et al., 1996; Groom et al., 1996; Pugin et al., 1997; Jabs et al., 1997; Keller et al., 1998; Torres et al., 1998), analogous to that of mammalian cells (Morel et al., 1991); (b) other, apoplastic oxidases, such as oxalate oxidase (e.g. Dumas et al., 1993; Zhang et al., 1995; Thordal-Christensen et al., 1997) and amine oxidase (Allan and Fluhr, 1997); and (c) pH-dependent apoplastic peroxidases (Bolwell et al., 1995; 1998; Frahry and Schopfer, 1998; Martinez et al., 1998), which generate either O_2^- anions or hydrogen peroxide.

The high concentrations of AOS generated in the oxidative burst have direct, cytotoxic effects on invading pathogens (Peng and Kùc, 1992). In addition, the oxidative burst is known to orchestrate a variety of secondary defense responses, which include the production of low-M_r compounds with antimicrobial activity referred to as phytoalexins (Ebel et al., 1995; Apostol et al., 1987), synthesis of pathogenesis-related proteins (Hammond-Kossack and Jones, 1996; Fritig et al., 1998), and crosslinking of cell wall proteins (Brisson et al., 1994; Otte and Barz, 1996). Oxy-

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gen radicals are also thought to be involved in the hypersensitive response, i.e. limited necrosis at the site of pathogen attack (Levine et al., 1994; Tenhaken et al., 1995; Richberg et al., 1998), as well as in systemic acquired resistance (Chen et al., 1993; Dempsey and Klessig, 1994; Van Camp et al., 1998).

In contrast there is only limited knowledge about the cell-cell recognition mechanisms and chemical defenses involved in host-pathogen interactions in the marine environment (Potin et al., 1999) and we do not know whether the concepts derived from the study of terrestrial plant pathosystems also apply to the distantly related lineages found in the sea. A few observations, however, indicate that oligosaccharide recognition and the oxidative burst mediate host-microbe interactions in marine algae, too. Oxidative burst per se was first reported in the red alga *Eucheuma platycladum* following mechanical injury (Collén and Pedersén, 1994). More recently, oxidative burst induced by oligosaccharide elicitors was shown to induce protection against parasites in the red algae *Chondrus crispus* (Bouarab et al., 1999) and *Gracilaria conferta* (Weinberger et al., 1999), and AOS were demonstrated to play a key role in programmed cell death in blooms of the phytoplankton dinoflagellate *Peridinium gatunense* (Vardi et al., 1999).

This study was undertaken to investigate whether such phenomena also exist in brown algae, using as model system *Laminaria digitata*, a common kelp on the rocky shores of the North Atlantic. Oligosaccharides derived from alginate, the main brown algal cell wall polysaccharide (Fig. 1), are shown to elicit a marked oxidative burst in the cortical cells of *L. digitata* sporophytes, sufficient to control populations of epiphytic bacteria. Pharmacological evidence indicates that the transduction of alginate oligosaccharide signals involves some of the steps described for higher plants (Wojtaszek, 1997; Scheel, 1998), leading to the activation of a diphenylene iodonium chloride (DPI)-sensitive AOS-generating system.

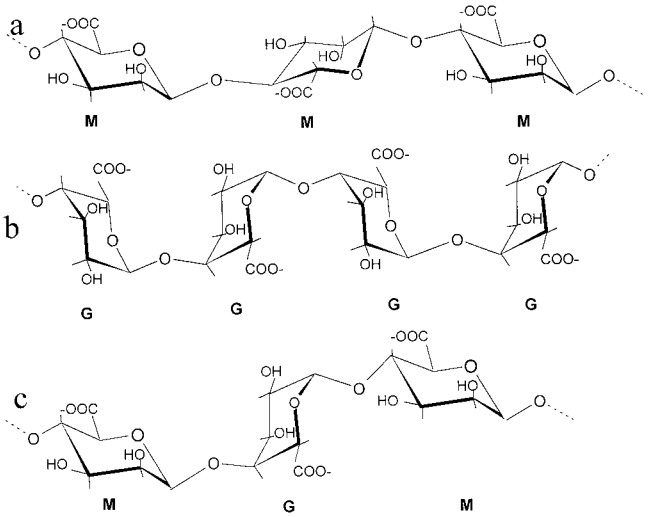

Figure 1. Chemical structure of alginates from brown algae. Alginates are linear anionic copolymers of β-1,4-D-mannuronic acid and of its C_5 epimer, α-1,4-L-guluronic acid. They consist of the alternation of homopolymeric blocks of poly-β-1,4-D-mannuronic acid, referred to here as MM blocks (a), of homopolymeric blocks of poly-α-1,4-L-guluronic acid (GG blocks; b), and of heteropolymeric blocks with random arrangements of both monomers (MG blocks; c).

RESULTS

Oligoguluronates Induce a Strong Respiratory and Oxidative Burst in *L. digitata* Sporophytes

Treatment of young sporophytic thalli of *L. digitata* with alginate oligosaccharides induced a massive increase in oxygen consumption within 2 to 3 min of their addition (Fig. 2A). As shown in Figure 2A, the increase in oxygen consumption was sometimes bimodal, first a sharp respiratory burst, with an initial rate up to 8 times that of basal respiration, followed a few minutes later by a second, less intense increase of apparent respiration. In the presence of oligoguluronates at the concentration of 50 μg mL^{-1}, the increase in oxygen consumption typically lasted 10 min, amounting to a total extra consumption of approximately 5 μmol O_2 g^{-1} fresh weight. Only the guluronate-containing alginate oligosaccharides, i.e. GG blocks and MG blocks, were able to elicit a respiratory response in *L. digitata* sporophytes. In contrast, MM blocks transiently decreased the apparent respiration (Fig. 2A).

At the same time a sudden and strong release of H_2O_2 into the incubation medium was observed (Fig. 2B). The peroxide concentration in the medium reached its maximum 5 to 10 min after the addition of alginate fragments, then decreased progressively back to the initial level after about 40 to 50 min. Addition of catalase (100 U mL^{-1}) prior to GG fragments completely inhibited the signal in the luminol assay (data not shown), indicating that this assay primarily detected H_2O_2. Superoxide dismutase (SOD; 200 U mL^{-1}) did not have a significant effect on the signal. The oxidative response was again dependent on the structure of the alginate oligosaccharides used as signal. MM did not elicit H_2O_2 release in *L. digitata* sporophytes (Fig. 2B). In contrast, when applied at the concentration of 50 μg mL^{-1} in experiments involving a biomass ratio of 20 to 50 mg fresh weight mL^{-1}, oligoguluronates typically initiated release of H_2O_2 up to concentrations in the 10 μM range. MG were also active in eliciting an oxidative response, yet to a much lesser extent than oligoguluronates (Fig. 2B).

The initial rate and amplitude of H_2O_2 release were also dependent on the concentration of alginate oligosaccharides. The threshold of elicitor concentration required to trigger an oxidative burst was between 1 (not yet active) and 2.5 μg mL^{-1} (already triggering a response), whereas the response saturated at about 150 μg mL^{-1} (Fig. 2C). A desensitizing effect was observed after the first elicitation: after a first elicita-

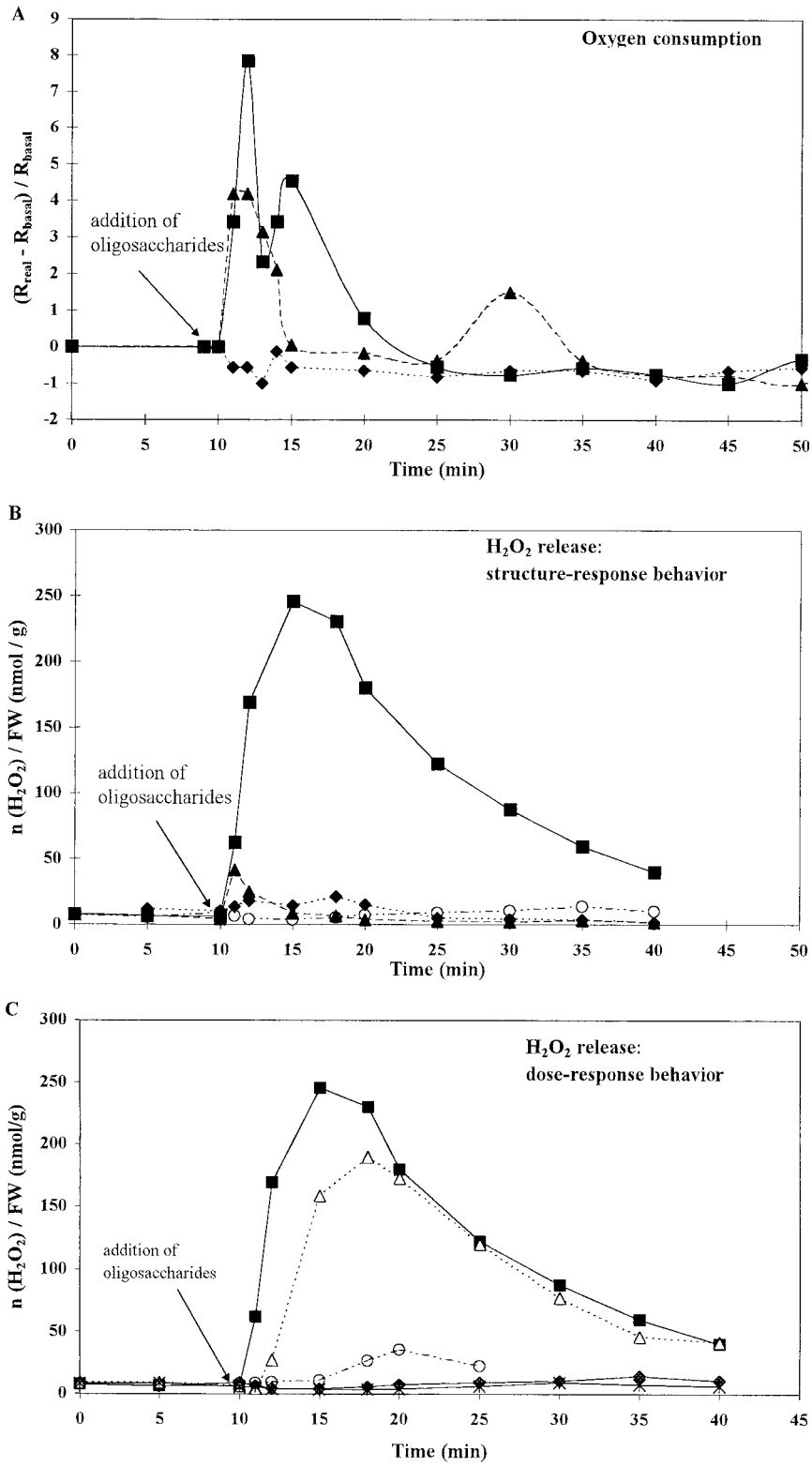

Figure 2. Oligoalginate-induced respiratory and oxidative bursts in *L. digitata*. A, Oligoguluronates stimulate oxygen consumption in young *L. digitata* plants. Young *L. digitata* plantlets were elicited with 50-μg mL^{-1} G blocks (■), MG (▲) blocks, or M blocks (♦), respectively. Their oxygen consumption was recorded with a Clark-type oxygen electrode and expressed relative to basal respiration (with R_{real} as the respiration rate at a given moment and R_{basal} as the basal respiration rate averaged over 15 min prior to elicitation). B, Oxidative burst in *L. digitata*: structure-response relationships. Kinetics of H_2O_2 release of *L. digitata* fronds challenged with G blocks (■), MG blocks (▲), M blocks (♦), and seawater (control, ○). C, Oxidative burst in *L. digitata*: dose-response behavior. Hydrogen peroxide production by *L. digitata* plantlets, after elicitation with 1 μg mL^{-1} (crosses), 10 μg mL^{-1} (△), and 100 μg mL^{-1} (■), respectively (control, ♦). The threshold for triggering a burst was at 2.5 μg mL^{-1} (○).

tion producing a burst, subsequent challenges with alginate oligosaccharides remained ineffective over a period of at least 3 h. When transferred to running seawater, elicited plantlets did not develop visible necroses in culture.

DPI, an irreversible inhibitor of the mammalian neutrophil NADPH oxidases (O'Donnell et al., 1993), inhibited the oxidative response in *L. digitata* sporophytes when used at a concentration of 10 μM (Fig. 3). Quinacrine, a general inhibitor of flavoprotein

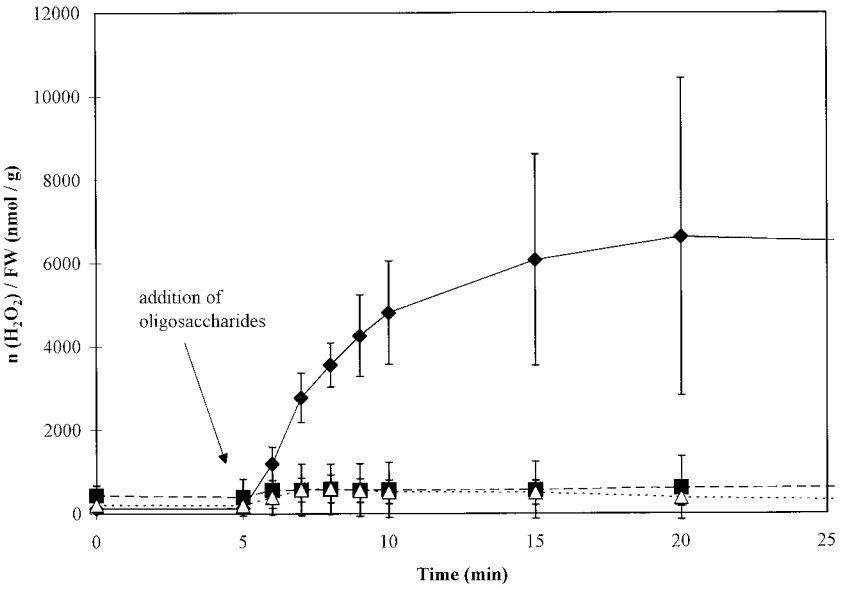

Figure 3. Effect of DPI on the oxidative burst in *L. digitata*. Kinetics of H_2O_2 emission by *L. digitata* plantlets challenged with 150 μg mL^{-1} GG (♦), 150 μg mL^{-1} GG after pre-incubation for 15 min with 10 μM DPI (△), and by unchallenged plantlets (■).

oxidases (Auh and Murphy, 1995), also blocked H_2O_2 release (data not shown).

Histology of the Oxidative Burst in *L. digitata* Sporophytes

Based on analyses by confocal microscopy with the fluorescent, redox-sensitive dye dichlorohydrofluorescin, AOS formation was triggered as soon as 1 min after elicitation of *L. digitata* plantlets with oligoguluronates and increased exponentially for at least 7 min. AOS mainly accumulated around epidermal and outer cortical cells, but not in the medulla (Fig. 4). Staining *L. digitata* with nitroblue tetrazolium (NBT) in the presence of oligoguluronates resulted in a blue precipitate, indicating that superoxide anions are formed during the oxidative burst (Fig. 5A). As indicated by a deeper blue stain, the accumulation of superoxide was enhanced by diethyldithiocarbamate (DDC), a strong inhibitor of SOD (Auh and Murphy, 1995; Jabs et al., 1997; Fig. 5C). However, attempts to prevent NBT accumulation by the addition of exogenous SOD were unsuccessful. In contrast and consistent with the effect of DPI on the release of hydrogen peroxide, pretreatment of young plantlets with this inhibitor (10 μM DPI, 15 min) did decrease precipitation of NBT upon elicitation with GG blocks (Fig. 5E). In agreement with the results of confocal microscopy, the staining with NBT of cross sections of *L. digitata* plantlets showed that O_2^- was accumulated mainly in the cortical cell layers, whereas it was hardly detectable in the medulla (Fig. 5, F and G).

Strong tissue-specific differences were observed when various tissues of adult *L. digitata* sporophytes were compared in their response with oligoguluronates. The young blade tissues were the most sensitive and the meristematic and older blade tissues were the least reactive parts. The sensitivity appeared to be confined to the cortex, the medulla (i.e. stipe tissue without cortex) showing no response upon elicitation (Fig. 6). Only *L. digitata* sporophytes and sporophyte suspended-cell cultures (data not shown) reacted with an oxidative burst upon elicitation with alginate oligosaccharides. *L. digitata* gametophytes, the filamentous, haploid life history phase, did notrespond to the presence of oligoalginate signals (data not shown).

Pharmacological Analysis of the Oxidative Burst in *L. digitata* Sporophytes

In an attempt to unravel the signal transduction pathways leading to AOS formation upon elicitation with alginate oligosaccharides in *L. digitata*, a number of compounds known to affect these processes in higher plants were tested. Pre-incubation of *L. digitata* plantlets in the presence of 10 μM staurosporine, a general inhibitor of protein kinases (Tavernier et al., 1995), inhibited the emission of H_2O_2 following elicitation with oligoguluronates. Inhibitors of calcium channels such as verapamil and methoxyverapamil effectively blocked the induction of the oxidative burst. The Ca^{2+} ionophore A23187 induced a strong oxidative burst, about 3 times higher than the control response to GG blocks. The inhibitor of higher plant anion channels, A9C (Cazalé et al., 1998), inhibited the burst, and so did two other anion channel inhibitors, 4,4′-diisothiocyanatestilbene-2, 2′-disulfonic acid (DIDS) and 5-nitro-2-(3-phenylpropylamino-) benzoic acid (NPPB; Lurin et al., 1996; Jabs et al., 1997). Chlorpromazine-HCl, an antagonist of phospholipase A_2 in higher plants (Chandra et al., 1996), completely blocked the induction of the response in *L. digitata*. Mastoparan, a specific activator of G proteins (Legendre et al., 1992, 1993), failed to

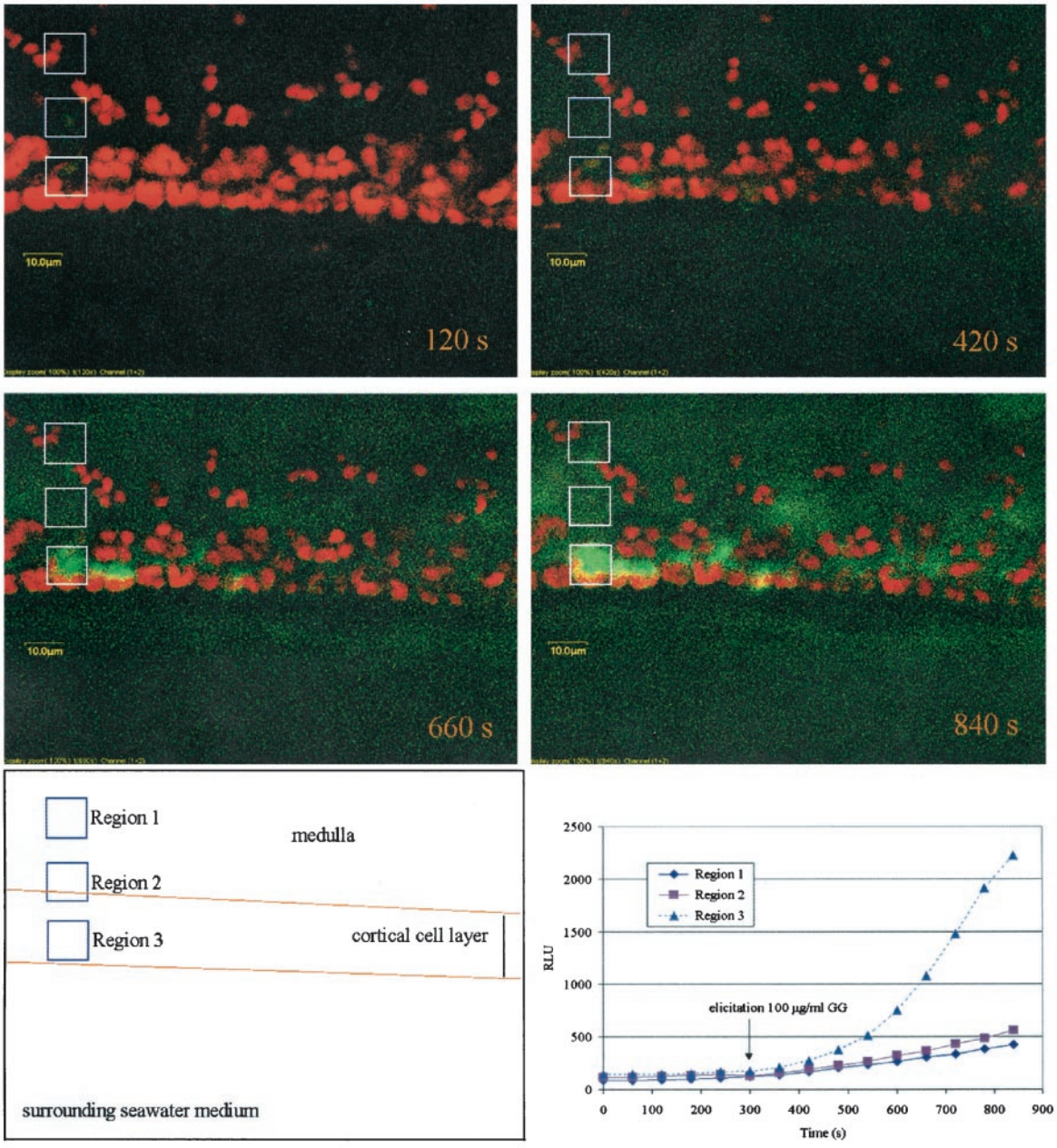

Figure 4. Laser scanning confocal imaging of elicited oxidative burst in *L. digitata* plantlets. *L. digitata* plantlets were loaded with the redox-sensitive fluorescent probe dichlorohydrofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) and examined by laser scanning confocal microscopy. Elicitors were added at $t = 300$ s during the time course of image acquisition. The green fluorescence of dichlorofluorescein was monitored on channel 1, concomitant with the red fluorescence of plastids on channel 2. Four images are presented, representative of the time course of fluorescence with flurorescences in channels 1 and 2 overlaid. Squares labeled 1, 2, and 3, respectively, were delineated from the outer cortex to the medulla, to record the three curves showing the respective integrated fluorescence. The data reported here correspond to one representative experiment out of three (bar corresponds to 10 μm and RLU refers to relative light units defined by the integrated luminosity of the pixels of the given area).

induce release of H_2O_2 from *L. digitata* sporophytes, even at concentrations as high as 10 μM.

All of the specific ionophores tested, namely valinomycin and nonactin (K^+), monensin (Na^+), A23187 ionophore (Ca^{2+}), and dinitrophenol (H^+) enhanced the oxidative response of *L. digitata* to oligoguluronate signals (Table I). When used alone at the concentration of 57 μM, valinomycin induced a strong oxidative burst, about one order of magnitude stronger than the usual response to GG blocks (Fig. 7). Both signals were capa-

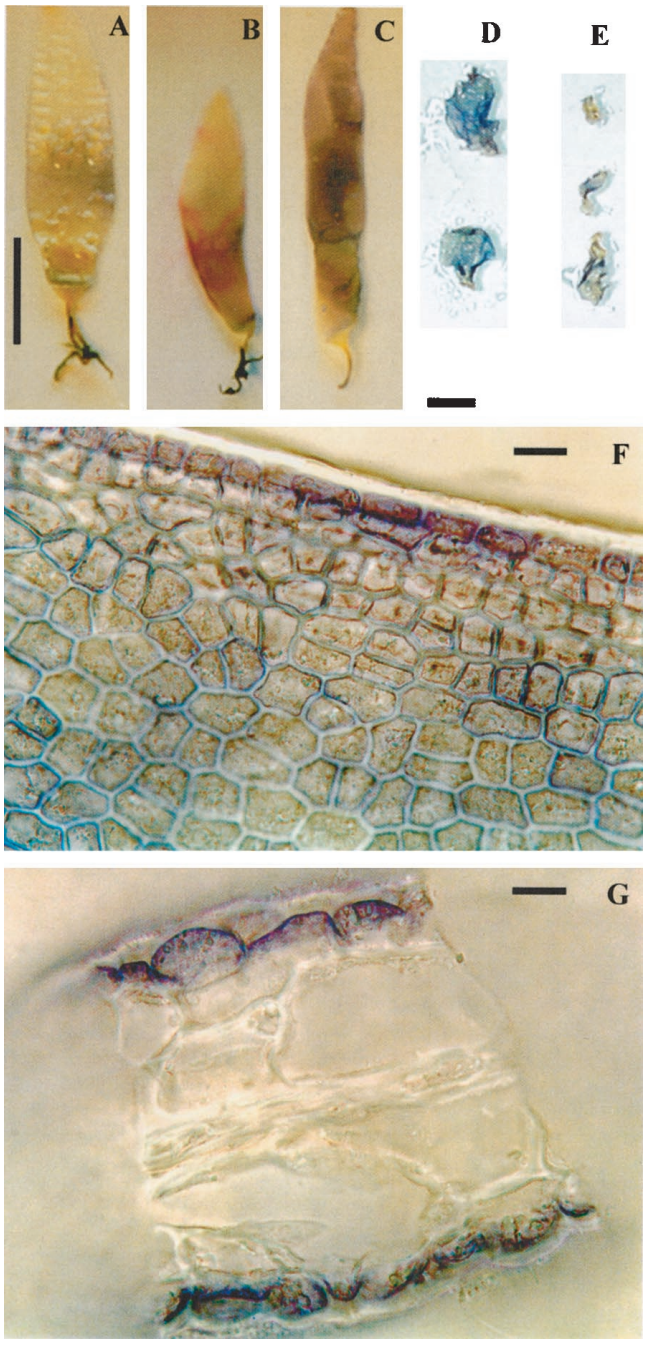

Figure 5. Superoxide generation in cortical tissues of *L. digitata* young sporophytes. After the addition of oligoguluronates, plantlets were stained with NBT from 15 to 30 min after elicitation to assay superoxide detected by a dark blue precipitate after bleaching of the plant pigments. The plants were treated as follows: A (control), pre-incubation with DDC, but without previous elicitation (almost no NBT staining due to low steady-state superoxide production); B, elicitation with 50 μg mL^{-1} GG (NBT staining 30 min later; some O_2^- detectable, but most of it is rapidly converted to H_2O_2); C, inhibition of SOD by pre-incubation with 1 mM DDC and elicitation with 50 μg mL^{-1} GG (NBT staining 15 min later; intensive stain due to massive accumulation of O_2^-); D, elicitation with 50 μg mL^{-1} GG (NBT staining 15 min later) on younger cultivated plantlets (control assay for the panel on the right); E, pre-incubation with 10 μM DPI and elicitation with 50 μg mL^{-1} GG (NBT staining 15 min later) on younger cultivated plantlets (almost no accumulation of O_2^- because the enzymatic source is inhibited). In A through E, the bar corresponds to 1 cm. F, Superficial view of the cortical cells near the margin of the blade of a young *L. digitata* plantlet pre-incubated with DDC and stained with NBT 15 min after elicitation (bar corresponds to 20 μm). A strong blue precipitate is visible in the surroundings of the epidermal cells. G, Cross section of the same blade (bar corresponds to 25 μm), showing the accumulation of blue precipitate around the cortical cells.

ble of acting synergistically, triggering bursts two orders of magnitude higher than the oxidative response induced by oligoguluronates (Fig. 7).

Oligoguluronates Trigger Potassium Efflux in *L. digitata* Sporophytes

A marked potassium efflux was observed from *L. digitata* sporophytes challenged with oligoguluronates in artificial seawater partially depleted in potassium (Fig. 8). After a slight initial decrease of the K^+ concentration in the surrounding medium, a steady net K^+ efflux was established, corresponding to a loss of about 10 μmol K^+ per gram fresh weight within 1 h. Concomitantly, a slight alkalinization (0.07 pH units) was detectable in unbuffered media within the first 3 min after elicitation, followed by an external acidification continuing for at least 1 h, from pH 8.25 to 7.85 (Fig. 8). Overnight, the leakage of potassium from a 50 mg mL^{-1} biomass of *L. digitata* plantlets raised the external medium concentration by an average of 4.16 mM in elicited samples, compared with 1.67 mM in the controls.

H_2O_2 Generated by the Oxidative Burst Controls Growth of Kelp Pathogenic and Epiphytic Bacteria

The question of whether the amount of AOS generated by the oxidative burst in *L. digitata* can be toxic for pathogens was then indirectly tested by cultivation of an alginate-degrading bacterial strain associated with *Laminaria japonica, Pseudoalteromonas elyakovii* (Sawabe et al., 2000), and the epiphytic strain referred to as Ldm2 in the presence of various concentrations of H_2O_2. Both strains were completely inhibited in their growth by H_2O_2 concentrations as low as 10 μM. An H_2O_2 concentration of 250 μM was sufficient to kill a culture of *P. laminariocolax* and to reduce the survival of strain Ldm2 by 60%.

DISCUSSION

The Brown Algal Kelp *L. digitata* Recognizes Guluronate-Rich Alginate Fragments as Signals to Trigger an Oxidative Burst

Alginate (Fig. 1) is the main cell wall component of Laminariales, amounting to approximately 60% of *L. digitata* isolated cell walls (Mabeau and Kloareg, 1987). It consists of three types of blocks (Haug et al.,

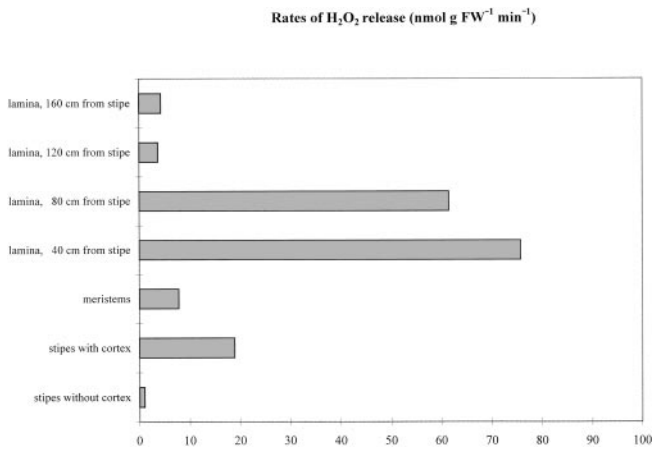

Figure 6. Comparison of the oxidative burst in various tissues of *L. digitata* after elicitation with oligoguluronates. Small tissue discs of an *L. digitata* plant were compared in their peak response with elicitation by GG blocks (50 μg mL^{-1}). The data reported here correspond to one representative experiment out of three.

1974), MM or GG, and blocks with mixtures of both monomers (MG). The above results demonstrate that reminiscent of the behavior of higher plants pectin oligosaccharides (e.g. Legendre et al., 1993), the sporophytes of the brown algal kelp *L. digitata* respond to incubation in the presence of alginate oligosaccharides by a sudden increase in oxygen consumption, concomitant with a marked release of hydrogen peroxide. The oxidative burst is rapid, starting within 2 to 3 min of the addition of oligosaccharides, and transient, lasting for no longer than 30 min.

External H_2O_2 concentrations reach their maximum 10 min after elicitation, i.e. at the same time or shortly after the peaks of oxygen consumption (Fig. 2, A and B). The average molar ratio between total oxygen consumption and the release of hydrogen peroxide upon the elicitation of *L. digitata* plantlets with alginate oligosaccharides, approximately five oxygen molecules consumed for the net release of one molecule of hydrogen peroxide, indicates that this brown alga rapidly detoxifies AOS during the oxidative burst. In higher plants detoxification of AOS involves protecting mechanisms such as the ascorbate-glutathione cycle, as well as scavenging enzymes such as catalases and peroxidases (Wojtaszek, 1997). No such mechanism has been described in brown algae yet. We are currently exploring the possibility that the mobilization of the intracellular iodine reservoir (Küpper et al., 1998) participates in the protection against oxidative stress in Laminariales (A. Baker, L. Carpenter, B. Kloareg, F. Küpper, P. Liss, and G. Malin, unpublished data).

L. digitata sporophytes react to the presence of alginate oligosaccharides in a dose-response dependent manner. Given that the oligoguluronate blocks used in these experiments had an average degree of polymerization of 20 to 25 (Heyraud et al., 1996), the threshold signal concentration required to elicit a significant burst is 0.5 μM, the oxidative response typically saturating around 40 μM (Fig. 2C). A clear structure-activity relationship was observed in the capacity of alginate oligosaccharides to elicit an oxidative burst in *L. digitata*. Alginate polymers and MM elicited no response. Only the homo-oligomeric fragments composed of GG and their co-oligomers with MG were recognized, with GG eliciting a stronger response than MG (Fig. 2, A and B). These observations indicate that stretches of guluronate residues are essential in the recognition of alginate oligosaccharides by *L. digitata*.

Oligoguluronate fractions obtained with the help of an alginate lyase, i.e. with unsaturated uronic acid residues at their non-reducing ends, were as active as those prepared by mild acid hydrolysis, indicating that the configuration of the non-reducing ends is not involved with signal recognition. However, the biodegradation products of sulfated fucans, a cell wall matricial polysaccharide that can be thought of as a structural and a functional analog of the fucoxyloglucans of higher plants (Kloareg and Quatrano, 1988), did not elicit any oxidative response in *L. digitata* sporophytes. Laminaran, the low molecular weight β-1,3-glucan storage polysaccharide of brown algae, which structurally mimics β-1,3–1,6-glucan fungal elicitors is known to elicit a variety of defense responses in higher plants (*Ebel, 1998*). Based on H_2O_2 assays, laminaran was not recognized as a defense signal by *L. digitata* sporophytes (data not shown). Altogether, alginate fragments made of guluronate residues appear as a relatively specific defense signal in *L. digitata*. It is worth noting here that the ordered conformation of poly-α-1,4-L-guluronic acid is very similar to that of the poly-α-1,4-L-GalUA blocks of pectins, both leading to intermolecular associations in the presence of calcium ions (Kohn, 1975). Oligogalacturonates indeed exhibited a slight elicitor activity on *L. digitata* sporophytes (data not shown).

AOS Production Involves an NADPH Oxidase-Like Enzyme in *L. digitata* Sporophytes

Histochemical evidence based on the superoxide-specific stain NBT and the SOD inhibitor DDC (Fig. 5) points out that the AOS first formed during the

Table I. *Effects of various ionophores on the oxidative burst in L. digitata*

Compound	Function	Concentration	% Increase/Inhibition
		μM	%
Nonactin	K^+ ionophore	1	+33.7[a]
			−54.7[b]
Monensin	Na^+ ionophore	25	+61.4
		250	−99.3
2,4-DNP	H^+ ionophore	1	−75.5
		100	+1,184.6

[a] Without pre-incubation. [b] After 1-h pre-incubation.

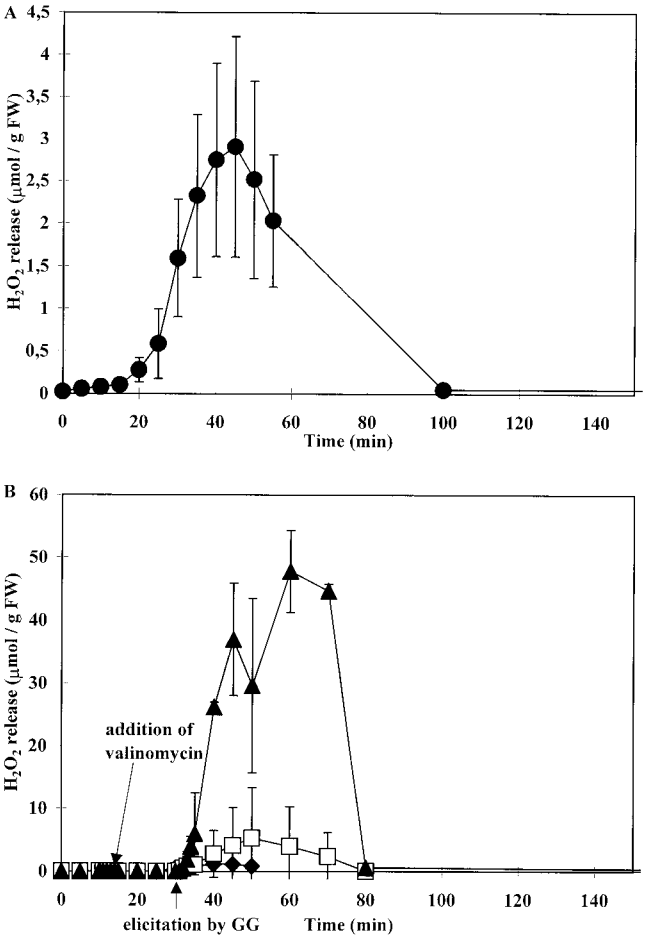

Figure 7. Effect of valinomycin on the oxidative burst triggered by oligoguluronates in *L. digitata*. A, Induction of an oxidative burst (without elicitation by GG) by 57 µM valinomycin (added at $t = 0$). B, Enhancement by valinomycin of the oxidative burst triggered by 150 µg mL^{-1} GG: dose-response behavior (control, ♦; 0.57 µM valinomycin, □; 5.7 µM valinomycin, ▲). Note that the addition of valinomycin enhances the oxidative burst, whereas concentrations around 57 µM are sufficient to trigger an oxidative burst without the physiological signal (GG).

oxidative burst is superoxide, most of which is subsequently converted to H_2O_2. This is further supported by the inhibition with DPI of the release of hydrogen peroxide (Fig. 3) and the staining with NBT (Fig. 5C) as diphenylene iodonium is known as an irreversible inhibitor of flavin-containing enzymes, including the superoxide-generating NAD(P) H oxidases of mammalian neutrophils (O'Donnell et al., 1993) and of higher plants (Auh and Murphy, 1995; Dwyer et al., 1996; Pugin et al., 1997). The fact that exogenous SOD did not affect the hydrogen peroxide release or NBT staining is likely due to a poor penetration of this enzyme through the thick brown algal cell wall.

The results obtained with quinacrine tend to further support that the oxidative-burst machinery of *L. digitata* contains a flavoprotein. Yet quinacrine is also a non-specific inhibitor of PLA_2 (Henderson et al., 1989) and consistent with the effect of chlorpromazine-HCl, this inhibitor may also indirectly affect AOS formation by interrupting a PLA_2-dependent transduction event. Altogether and keeping in mind the argument about the uses and misuses of inhibitors in studies of AOS sources in higher plants (Bestwick et al., 1999), we propose that a possible source of superoxide in *L. digitata* is an oxidase with a flavoprotein subunit (inhibited by DPI and likely by quinacrine).

Transduction of Oligoguluronate Signals Features Some Conserved Steps with Higher Plants

The general inhibitor of protein kinases, staurosporine, reported to block the protein phosphorylation events involved in induction of defense responses in tobacco cells upon elicitation by cryptogein (Viard et al., 1994) or oligogalacturonides (Mathieu et al., 1996), also inhibited the emission of H_2O_2 by *L. digitata* plantlets. Yet the protein phosphatase inhibitors calyculin A and cantharidin (Levine et al., 1994; Jabs et al., 1997) did not induce a release of H_2O_2 in *L. digitata*. Phospholipase A_2, another typical element of eukaryotic signal transduction pathways (Chapman, 1998), is also likely to participate in the oligoguluronate transduction chain, as suggested by the strong inhibition of H_2O_2 production by chlorpromazine-HCl, a selective inhibitor of this phospholipase following elicitation in higher plants (Chandra et al., 1996). In contrast no evidence was observed as to the involvement of G proteins in the transduction of oligoguluronate signals. Mastoparan, a specific activator of G proteins intrinsically capable of triggering an oxidative burst in soybean cells (Legendre et al., 1992), was ineffective to induce or enhance the production of AOS in *L. digitata* sporophytes, even at high concentrations.

Calcium entry, which is known to be involved with the activation of the oxidative machinery in higher plants (e.g. Jabs et al., 1997; Pugin et al., 1997) also appears to regulate the emission of AOS in *L. digitata*. This is demonstrated by the enhancing effect of the Ca^{2+} ionophore A23187 and the inhibitory effects of the antagonists of Ca^{2+} channels such as verapamil and methoxyverapamil (known to affect calcium channels in fucoid brown algae; Robinson, 1996), which completely blocked the oxidative burst.

Incubation of *L. digitata* sporophytes in the presence of oligoguluronates results in a marked potassium efflux (Fig. 8). Assuming that intracellular potassium concentrations are approximately 380 mM (see "Materials and Methods") and given the potassium concentration of the artificial seawater used in this study (500 µM instead of 10 mM in natural seawater), elicited *L. digitata* plantlets release about 2.6% of their internal potassium within the first h and about 13.1% overnight (14 h). Changes in plasma membrane permeability indeed appear to be directly involved in the regulation of oxidative burst in *L.*

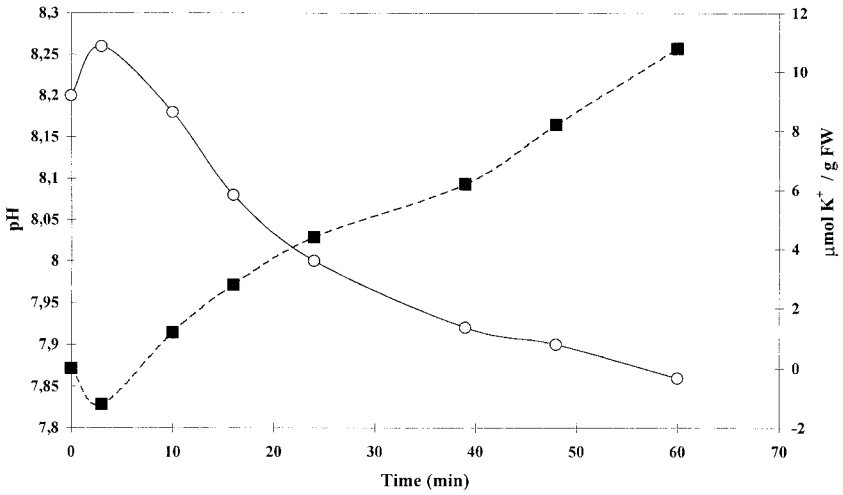

Figure 8. Time course of K^+ efflux (■) and pH variations (○) in the incubation medium of *L. digitata* plantlets (50 mg fresh weight mL^{-1}) treated at time 0 with 150 μg mL^{-1} oligoguluronates. The data reported here correspond to one representative experiment out of three.

digitata. The enormous enhancement of the effect of oligoguluronates by the K^+ ionophore valinomycin also points at the key role in the signal transduction chain of changes in membrane polarization by K^+ efflux. Yet the situation in *L. digitata* seems to be different from that in higher plants, where valinomycin fails to induce a burst when applied alone (Jabs et al., 1997). Pre-incubation with the uncoupler/H^+ carrier 2,4-dinitrophenol similarly enhanced the response, presumably also by changes in membrane polarization. Chloride channels may be involved too, as indicated by the partial inhibition of the burst by the anion channel antagonists, A9C, DIDS, and NPPB.

Establishing the hierarchical organization of the various transduction steps discussed above would now require an investigation of their links with other early events, such as ion fluxes, and the identification of defense responses that may function downstream or independently of the oxidative burst triggered by oligoguluronate recognition. The desensitizing effect of elicitation with alginate oligosaccharides suggests that such signaling pathway(s) cannot be repetitively activated over a certain period. A comparable refractory behavior during which cells remain insensitive to further elicitation for several hours is known in higher plants for various oligosaccharide elicitors, oligogalacturonides in tobacco (Rouet-Mayer et al., 1997) and soybean cells (Legendre et al., 1993), and chitin fragments in tomato cells (Felix et al., 1998).

Biological Significance of the Oxidative Burst in *L. digitata*

Enzymes that depolymerize alginate have been detected in a variety of marine molluscs, as well as in a number of marine bacteria associated with Laminariales, including *Pseudomonas alginovora*, a species epiphytic to *L. digitata* (Boyen et al., 1990), as well as strain H4 (Sawabe and Ezura, 1996) and *P. elyakovii* (Sawabe et al., 2000), two bacteria isolated from diseased cultures in *L. japonica* nurseries. Laminariales also are plagued by filamentous brown algal endophytes (Ellertsdottir and Peters, 1997), which are likely to express alginases when invading the host tissue. Therefore, recognition of alginate fragments by *L. digitata* sporophytes is likely to signal challenging with alginolytic, potentially detrimental organisms.

As reported for fungal pathogens of higher plants (Peng and Kùc, 1992), the oxidative burst may be viewed as an immediate, efficient defense in itself. The amounts of H_2O_2 released by *L. digitata* sporophytes upon recognition of oligoguluronates, up to 1 μmol g fresh $weight^{-1}$, together with the estimation that the apoplastic volume corresponds to approximately 10% of the tissue fresh weight in this brown alga (see Fig. 4), suggest that at the peak of production, the peroxide levels in the apoplasm can transiently reach concentrations as high as several millimolars, a figure comparable with those observed in soybean cells (Legendre et al., 1993). Such levels are at least one order of magnitude above the concentrations that were required to control the growth or even to kill the two kelp disease-associated bacteria investigated here, *P. elyakovii* and strain Ldm2. Exogenous applications of H_2O_2 in the mM range were indeed shown to control *Pseudoalteromonas teriolytica*, the causative agent of the red spot disease in *L. japonica* (Ezura et al., 1990; Sawabe et al., 1998).

Confocal microscopy (Fig. 4), histochemical staining (Fig. 5), as well as the comparison of algal fragments with and without the cortical cell layer (Fig. 6) indicate that reactivity to oligoguluronates is confined to cortical cells, i.e. cells that are the first to be exposed to pathogen attack. Young, fast-growing tissues of the blade are the most reactive upon elicitation. Yet meristematic areas showed almost no reaction. This suggests that the capability to produce AOS is linked to cell differentiation, as in cotton fibers (Potikha et al., 1999). The gametophyte generation, which in nature rapidly differentiates gametes

and thus has a short life span, consistently does not recognize oligoguluronates as defense signals.

In conclusion, guluronate-rich alginate oligosaccharides, which are likely to be released during interactions with alginolytic organisms, elicit an oxidative burst in *L. digitata*. Since they are readily recognized by whole plantlets, oligoguluronates may thus be used as a new tool to decipher transduction pathways and defense reactions in Laminariales. This order belongs to the phylum of Heterokonta, which emerged as an independent lineage during the so-called crown diversification of higher eukaryotes (Bhattacharya et al., 1991). As in the case of the rhodophytes *Gracilaria conferta* (Weinberger et al., 1999) and *Chondrus crispus* (Bouarab et al., 1999), it would be of particular interest to delineate in *L. digitata* conserved ancestral pathways and machineries from those that are unique to the marine environment. In this respect the results reported here suggest that brown algal kelps feature some of the transduction cascade components known for higher plants, as well as a superoxide-generating oxidase with similarities to the well-characterized mammalian NADPH oxidase. The hydrogen peroxide concentrations generated in the surroundings of the algae appear sufficient to exert an inhibitory effect toward potentially harmful micro-organisms. Other defenses may involve the oxidation of intracellular iodide, leading to the release of toxic, iodinated compounds (Potin et al., 1999).

MATERIALS AND METHODS

Plant Material

Young *Laminaria digitata* plantlets (2–15 cm in length) were seeded and grown in a well-aerated, running-seawater tank as follows. Adult fertile sporophytes were placed for 1 week in the tank and allowed to release spores. Gametophytes developed along the walls and fertilization yielded young *L. digitata* plantlets. Cultures were grown using a photoperiod of 16 h of light and 8 h of darkness at a photon flux density of 50 to 60 μmol photons m^{-2} s^{-1} and at temperatures corresponding to the surface seawater temperature at Roscoff, ranging from 9°C to 17°C throughout the seasonal cycle. Adult *L. digitata* sporophytes were collected by diving between Ile Verte and Ile de Batz, close to the Institute in Roscoff. In some experiments unialgal sporophytes were used, originating from the female gametophyte strain Lam dig Hel 1004 and the male gametophyte strain Lam dig Hel 1003 (kindly provided by D.G. Müller, Konstanz, Germany), and grown in the laboratory in Provasoli ES medium (Starr and Zeikus, 1987) prepared from artificial seawater (WIMEX, Krefeld, Germany, adjusted to 30‰ salinity). They were illuminated with daylight-type fluorescent lamps at an irradiance of 9 μE m^{-2} s^{-1} for 10 h per day and kept at 10°C ± 1°C. Cultures were transferred to fresh medium at 1- or 2-week intervals.

Inhibition of the Growth of Kelp-Associated Bacterial Strains

One of the alginolytic strains used, isolated from diseased *L. japonica* and identified as *Pseudoalteromonas elyakovii,* was provided by T. Sawabe (Hokkaido University, Japan). The other one lacks taxonomic classification so far and was termed strain "Ldm2." It is an isolate from epiphytic bacteria living on decaying diseased young *L. digitata* collected from the field. Bacteria were first grown for around 12 h in ZoBell medium (ZoBell and Upham, 1944). During their exponential growth phase, 200 μL of the cultures were transferred as inocula to a mixture of 15 mL ZoBell medium and 10 mL sterile, filtered seawater with various additions of H_2O_2. Growth of the cultures was followed for up to 16 h by measuring the A_{600}.

Elicitors

Alginate oligosaccharides with a polymerization degree ranging from 15 to 25 (Heyraud et al., 1996) were prepared in the laboratory according to Haug et al. (1974) using sodium alginate from *Laminaria hyperborea* stipes (provided by B. Larsen, Trondheim University, Norway) and yielding three categories of alginate oligosaccharides (GG, MM, and MG blocks). In an alternate manner, alginate oligosaccharides were obtained by enzyme degradation of sodium alginate with an alginate lyase from the abalone *Haliotis tuberculata* (Boyen et al., 1990). In elicitation experiments, GG, MM, and MG blocks were applied at final concentrations ranging from 1 to 150 μg mL^{-1}.

Inhibitors and Activators of AOS Generation and Signal Transduction Chains

Several compounds were screened for their potential activating or inhibiting activity on signal transduction and AOS generation after elicitation: KCN (final concentration in the seawater eliciting medium of 50 μM; target, heme-dependent enzymes) and NaN_3 (5 and 50 μM; target, a wide range of redox enzymes) from stock solutions dissolved in H_2O, respectively; quinacrine (500 μM; target, flavin-dependent redox enzymes, in particular oxidases), DDC (1 mM; target, SOD), nifedipin (100 μM; target, Ca^{2+} channels), and chlorpromazine-HCl (10 and 260 μM, 1 mM; target, phospholipase A) from stock solutions dissolved in ethanol, respectively; and DPI (10 and 100 μM, 1 mM; target, NADPH oxidases) and A9C (100 μM and 1 mM; target, anion channels) from a stock in dimethyl sulfoxide (DMSO) and purchased from Sigma (L'Isle d'Abeau Chesnes, France). Calyculin A (10 μM; target, protein phosphatases) and cantharidin (10 μM; target, protein phosphatases) were from stocks in DMSO; staurosporine (5 μM; target, protein kinases), valinomycin (57 and 570 nM, 5.7 μM; target, K^+ ionophore), nonactin (1 μM; target, K^+ ionophore), monensin (25 and 250 μM; target, Na^+ ionophore), A23187 (5 and 50 μM; target, Ca^{2+} ionophore), DIDS (1 mM; target, anion channels), NPPB (100 μM; target, anion channels), verapamil (D200; 100 μM; target, Ca^{2+} channels), and methoxyverapamil (D600; 100 μM; target,

Ca^{2+} channels) in ethanol, respectively, were purchased from Calbiochem (France Biochem, Meudon, France).

Hydrogen Peroxide/AOS Measurements

The concentration of hydrogen peroxide present in the medium around plants was determined using the luminol chemiluminescence method (Glazener et al., 1991) with a luminometer (LUMAT LB 9507, EG&G Berthold, Bad Wildbach, Germany). It was usual that 400-μL aliquots (out of initially 10 mL) were taken for one measurement. In the luminometer, 50 μL of 20 units mL^{-1} of 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) solution were added to the sample. Chemiluminescence was recorded immediately after the last injection with a signal integration time of 10 s. For calculating the concentration of H_2O_2 present in the samples, calibration with a standard curve was carried out at least once during any series of measurements. Control assays of this method revealed that *L. digitata* sporophytes and gametophytes maintain very low levels (<100 nM) of H_2O_2 in their surroundings under steady-state conditions. Catalase at 100 U mL^{-1} and SOD at 200 U mL^{-1} (Boehringer Mannheim) were used to test the types of AOS detected in the assay.

O_2 Measurements

Oxygen consumption was measured in darkness with an oxygen electrode (DW3 Clark-type, Hansatech Instruments, King's Lynn, UK). The 20-mL reaction vessel had a small opening allowing for the injection of microliter volumes of solutions (e.g. containing oligosaccharides) during the course of the experiments. Young *L. digitata* sporophytes approximately 3 cm in length and 50 mg in fresh weight were incubated in natural seawater to determine respiration rates.

Histochemical Staining for O_2^- Detection

Immediately after triggering an oxidative burst by elicitation (including in the presence of the SOD inhibitor DDC, 200 U mL^{-1} of SOD, or 10 μM DPI, respectively), plantlets were placed in 10 mM NaN_3 in 10 mM potassium phosphate buffer (pH 7.8) and immersed for 15 to 30 min in 3 mL of the same buffer containing 0.1% (w/v) NBT (Sigma) as described in Jabs et al. (1996). Plant pigments were subsequently cleared by boiling for 2 min in alcoholic lactophenol (95% [w/v] ethanol:lactophenol, 2:1). Macroscopic and microscopic photographs were taken of entire plantlets and of thin cross sections to localize superoxide formation.

Laser Scanning Confocal Microscopy

DCFH-DA was dissolved in DMSO or ethanol to produce 10 mM stock, which was frozen as aliquots. Young *L. digitata* thalli were cross-sectioned using razor blades, and cross sections were placed in the dark for 15 min into Petri dishes containing 5 mL of 22 μm-filtered seawater and 5 μL of DCFH-DA from the stock solution. Petri dishes 35 mm in diameter were prepared for microscopy by sealing a glass coverslip over a 18-mm hole drilled in the bottom of the dish. Cross sections were removed from the DCFH-DA-loading solution, rinsed with 22 μm filtered seawater, and affixed to the bottom of a Petri dish containing 2 mL of seawater for microscopy. Imaging was performed using a laser scanning confocal microscope (model IX 70/Fluoview, Olympus, Tokyo) with images recorded every 60 s. An argon/krypton OMNICHROM laser was used for excitation at 488 nm set on 20% power, with 525 nm emission (channel 1) and 580 nm emission (channel 2). Images were captured over a 15-min time course. Thalli were elicited 5 min after the beginning of the time course with 150 μg mL^{-1} oligoguluronates. Analysis of images was performed using the Fluoview software (version 2.0, Olympus).

K^+ and H^+ Fluxes

The protocol of Mathieu et al. (1991) for measurement of potassium efflux was modified as follows. The extracellular K^+ concentration was determined at intervals using a K^+-specific electrode (F2312K, Radiometer, Copenhagen). Artificial seawater (450 mM NaCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM $NaHCO_3$, and 40 mM $MgSO_4$) adjusted to 500 μM K^+ was used for all assays with living plants. A pH electrode was constantly inserted in the medium surrounding the plantlets. For comparability, readings were taken at every measurement of K^+. The intracellular K^+ concentration of *L. digitata* plantlets was determined by drying 10 samples of plantlets, which were subsequently analyzed by means of induction-coupled plasma-atom emission spectroscopy by the Service Central d'Analyse of Centre National de la Recherche Scientifique (Vernaison, France). The average potassium content of dried plantlets was 8.771% $\pm$ 1.814%. Given that, in *L. digitata*, 1 g of dry weight corresponds to approximately 6.56 g fresh weight (this study) and that the apoplastic volume represents around 10% of the tissue fresh weight (Mabeau and Kloareg, 1987), this corresponds to intracellular K^+ concentrations around 380 mM. This compares well with the 300 mM measured for other brown algae (Reed and Barron, 1983).

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