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Pathogen-Induced Defense and Innate Immunity in Macroalgae

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Abstract. Animals and vascular plants are known to defend themselves facultatively against pathogens, with innate receptors mediating their resistance. Macroalgal defense against microorganisms, in contrast, has until recently been regarded mainly as constitutive. Indeed, many macroalgae appear to be chemically defended at constantly high levels, and this is possibly one of the reasons why the first evidence of pathogen-aroused resistance in a macroalga was detected only a decade ago. Here, I summarize the results of studies that indicate the existence of pathogen-activated or pathogen-induced macroalgal defense. Most indications so far come from molecular investigations, which revealed major functional similarities among the defense systems of distant macroalgal clades and the innate immune systems of vascular plants and metazoans. Homologies exist in the primary and secondary defense-activating signals, as well as in the enzymes that are involved and the cellular responses that are activated. This strongly suggests that innate immunity also exists in relatively distinct macroalgal clades. However, a macroalgal receptor still needs to be isolated and characterized, and the molecular concept of macroalgal receptor-mediated immunity needs to be complemented with an ecological perspective on pathogen-induced defense, to develop a joint neuroecological perspective on seaweed-microbe interactions.

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Abbreviations: $CHBr_3$, bromoform; H_2O_2, hydrogen peroxide; HR, hypersensitive response; LPS, lipopolysaccharide; MAMP, microbe-associated molecular pattern; NO•, nitric oxide; O_2^-•, superoxide; PIMP, pathogen-induced molecular pattern; ROS, reactive oxygen species.

The (Missing?) Ecological Perspective: Pathogen-Induced Defense

As has been pointed out by Steinberg and deNys (2002), the defense ecology of macroalgae has until recently been dominated by investigations of seaweed/herbivore interactions. The first example of induced macroalgal defense against herbivores was detected two decades ago, and an increasing body of literature confirms that macroalgae often respond to grazers with an induced antiherbivore defense (for reviews, see Amsler and Fairhead, 2006; Toth and Pavia, 2007). Studies of seaweed-microbe interactions have lagged behind, mainly for methodological reasons. The characterization of alga-associated microorganisms is still in an early stage, since suitable tools for the analysis of epiphytic bacterial communities—including non-cultivatable components—were not available until rDNA sequencing techniques were introduced into this field of research (Ashen and Goff, 2000; Meusnier *et al.*, 2001). Seaweed-associated microorganisms typically live embedded in highly complex matrices; this, together with their relatively short life span, also complicates the design of ecological studies. Our understanding of the interactions between microorganisms and macroalgae and with each other is therefore rudimentary at best.

Microorganisms grow to higher densities in water than in air (Engel *et al.*, 2002). The aquatic environment generally favors the development of microbes and the formation of biofilms on surfaces, and macroalgae are associated and interact with these organisms. At the same time, mechanical defenses that are comparable to those of vascular plants are largely absent in macroalgae (Weinberger *et al.*, 1999). Although certain microorganisms have been shown to provide their host macroalgae with growth factors, nutrients, or

protection from settlement by other micro- or macrofoulers (Armstrong *et al.*, 2001; Dobretsov and Qian, 2002; Matsuo *et al.*, 2005; Zheng *et al.*, 2005), others have been identified as the causative agents of infectious diseases with characteristic symptoms such as rot, bleaching, lesions, or malformations (Correa, 1996). However, major outbreaks of diseases are relatively rarely reported, which may in part be due to the limitations of our perception, but which also indicates that macroalgae are in most situations capable of coping with pathogens.

The presence of potential pathogens on healthy macroalgae was demonstrated by Küpper *et al.* (2002). The authors reinfected axenic kelp (*Macrocystis pyrifera*) with microorganisms that originated from healthy kelp specimens and observed that the algae were rapidly decomposed when their defense was blocked with specific enzyme inhibitors. Control specimens that were either uninhibited or uninfected remained healthy, and the authors concluded that the decomposition was due to opportunistic pathogens that were unable to attack defended kelp.

Many of the microorganisms that are associated with apparently healthy macroalgae have the enzymatic capacity to disintegrate tissues of their host. For example, 0.4%–3.6% of the cultivatable bacteria associated with healthy *Gracilaria sp.* (as *Gracilaria conferta*) were capable of degrading its agar cell wall matrix (Weinberger *et al.*, 1994). Similar observations have been reported for other red macroalgae (Largo *et al.*, 1995; Jaffray *et al.*, 1997), as well as for kelps (Lin *et al.*, 2004). Degraders of phycocolloids are usually rare in nonalgal environmental samples (for this reason agar is traditionally used for the gellification of microbiological media), which indicates that their concentration on macroalgae has coevolutionary reasons. Massive increases in absolute and relative numbers of phycocolloid degraders are usually observed on stressed algal tissues with a reduced capacity for autodefense (Weinberger *et al.*, 1994; Lin *et al.*, 2004), suggesting that the microorganisms involved are opportunistic pathogens.

Optimal defense theory predicts that inducible defenses may evolve when pressure by pathogens is transient. Compared to constitutive resistance, facultative defenses imply a lower risk of pathogen adaptation (Boyd, 2006), as well as lower direct and indirect costs of defense under enemy-free conditions—for example, because energy is saved or exposure to autotoxic defensive compounds is reduced (Agrawal and Karban, 1999). On the other hand, facultative defenses also imply an increased risk of damage during the initial stage of pathogen attack (Järemo *et al.*, 1999). Given that relatively dense biofilms containing potential pathogens are more or less permanently present on macroalgal surfaces, ecological intuition would not necessarily expect mechanisms of inducible antimicrobial defense in algae.

The Molecular Perspective: Innate Immunity

Much of the evidence for induced antimicrobial defense in macroalgae comes from molecular and physiological studies. These demonstrate important similarities between the defense systems of macroalgae, vascular plants, and metazoans. Inducible defense requires that the defensive level can be upregulated fast enough to contain the attacker before irreversible damage is done. On the cellular level, such upregulation is usually based upon molecular recognition of pathogen infections by receptors.

Adaptive receptors exist only in jawed vertebrates, but all metazoans and vascular plants—and apparently macroalgae as well—express innate receptors. These perceive microbe-associated molecular patterns, or MAMPs (Nürnberger *et al.*, 2004), which are synonymously called general or exogenous elicitors. Usually MAMPs are highly conserved and not pathogen-specific, but specific of major groups of microorganisms that include pathogenic species. For example, lipopolysaccharides (LPS) and lipoteichoic acids—components of the outer cell envelopes of gram-negative and gram-positive bacteria, respectively—are examples of MAMPs that are perceived by many eukaryotes (Meyer *et al.*, 2001; Farnell *et al.*, 2003; He *et al.*, 2003; Remer *et al.*, 2003; Nürnberger *et al.*, 2004). In addition to MAMPs, vascular plants also perceive breakdown products of their own cell wall that are released by glucohydrolytic activities from attacking phytopathogenic microbes; these breakdown products are called pathogen-induced molecular patterns (PIMPs; Mackey and McFall, 2006), or endogenous elicitors. For example, pectin oligosaccharides are established as elicitors of defense responses in vascular plants (Mathieu *et al.*, 1991; Spiro *et al.*, 1998; Nürnberger *et al.*, 2004).

The responses and signaling pathways that are activated in vertebrates and vascular plants after MAMP or PIMP perception are complex and typical. As I will show in the next sections, the same pathways and responses are also activated in certain macroalgae after application of MAMPs or PIMPs. Such evidence today exists mainly for the brown macroalgal order of the Laminariales (kelps) and for red macroalgae of the genera *Gracilaria* and *Chondrus* (Table 1). These findings strongly indicate that macroalgal innate receptors exist, although no such receptor has yet been isolated and characterized.

Receptor-Mediated Immunity in Red Macroalgae

Vascular plants and metazoans typically respond after recognition of PIMPs or MAMPs with an oxidative burst, a transient production of reactive oxygen species (ROS) such as superoxide ions ($O_2^{-}\bullet$), hydrogen peroxide (H_2O_2), or hydroxyl radicals ($OH\bullet$) (Grant and Loake, 2000; Delledonne *et al.*, 2001; Nürnberger *et al.*, 2004; Torres and Dangl, 2005). An oxidative burst was also observed as a first physiological indication of PIMP perception when the

Table 1

Summary of main effects observed in receptor-mediated macroalga-pathogen interactions with key references

Macroalgal clade or species	Pathogen	MAMP / PIMP	Oxidative burst	NO• signaling activated	Oxylipin signaling activated	Protein expression modified	HR	Demonstrated activated defense	Demonstrated induced defense
Gracilaria sp.	Agar degrading bacteria	Agar oligosaccharide	+ Weinberger *et al.*, 1999 Weinberger *et al.*, 2005a	?	?	+ present article	+ ? Weinberger *et al.*, 1999	+ Weinberger and Friedlander, 2000b	?
Gracilaria chilensis	Agar degrading microorganisms	Agar oligosaccharide	− Weinberger *et al.*, 2005a	+ ? present article	− present article	+ present article	?	?	+ present article
Laminariales	Alginate degraders	Oligoguluronate	+ Küpper *et al.*, 2001 Huang *et al.*, 2002 Liu *et al.*, 2002b	?	+ Küpper *et al.*, 2006	+ Küpper *et al.*, 2002 Wang *et al.*, 2004c	+ Wang *et al.*, 2004a	+ Küpper *et al.*, 2002	+ Küpper *et al.*, 2002
Laminaria digitata	Gram-negative bacteria	LPS	+ Küpper *et al.*, 2006	− Küpper *et al.*, 2006	+ Küpper *et al.*, 2006	?	?	?	?
Chondrus crispus	Gram-negative bacteria	LPS	− present article	?	?	+ present article	?	?	?
C. crispus	*A. operculata*	?	+ Bouarab *et al.*, 1999	?	+ Bouarab *et al.*, 2004	+ Bou*is*arab *et al.*, 2004	?	+ Bouarab *et al.*, 2004	+ Bouarab *et al.*, 2004

Symbols: +, response detected; −, response not detected; ?, no observation available or observation unclear.

red macroalga *Gracilaria* sp. (as *Gracilaria conferta*) was exposed to products of the microbial degradation of its agar cell wall matrix (Weinberger *et al.*, 1999). After exposure of *Gracilaria* sp. to agar oligosaccharides, H_2O_2 accumulated in the algal medium within minutes.

As could be shown by transmission electron microscopy, the site of ROS production was the plasma membrane of epidermal and subepidermal cells (Weinberger *et al.*, 2005a). Further analysis of the oxidative burst response revealed that it was highly sensitive to diphenylene-iodonium, a specific inhibitor of NADPH-dependent enzymes, as well as to inhibitors of flavo-enzymes. The authors therefore concluded that NADPH oxidase (which is a flavo-enzyme) located in the plasma membrane was probably the source of ROS after *Gracilaria* sp. was exposed to an elicitor (Weinberger *et al.*, 2005a), as in vascular plants and metazoans (Torres and Dangl, 2005).

NADPH-oxidases of vascular plants and animals are stimulated by Ca^{2+}-ions (Keller *et al.*, 1998; Sagi and Fluhr, 2001; Banfi *et al.*, 2005), and Ca^{2+}-ions are also required for the activation of NADPH oxidase in *Gracilaria* sp. after agar oligosaccharide exposure. This is indicated by the observation that Ca^{2+}-channel inhibitors generally inhibit the response, while Ca^{2+}-ionophores increase its intensity (Weinberger *et al.*, 2005a).

The oxidative-burst response of *Gracilaria* sp. after agar oligosaccharide application was also prevented with protein kinase inhibitors and increased after application of phosphatase inhibitors, which indicated an involvement of phosphorylation events with NADPH oxidase activation (Weinberger *et al.*, 2005a). After a response was elicited, *Gracilaria* sp. remained incapable of responding for 6 h (Weinberger *et al.*, 2005a). Such a refractory state is typically observed after cellular recognition of chemical signals that activate phosphorylation events, because no unphosphorylated protein kinase substrate is available (Felix *et al.*, 1993). In conclusion, the early signaling events between stimulation of agar oligosacharide receptors and activation of NADPH oxidase in *Gracilaria* sp. include protein phosphorylation and Ca^{2+}-perception and appear similar in this way to those observed in vascular plants (Navazio *et al.*, 2002) and vertebrates (Meier, 1996).

A further similarity between *Gracilaria* sp. and vascular plants is in the specificity of their receptors for PIMPs that are functionally analogous. Agar is a functional analog of pectin in the cell wall matrices of red algae. Both pectin and agar are linear polysaccharides, consisting of monosaccharide and disaccharide repeating units, respectively. Both microbial agarases and pectinases are endo-hydrolases and generate the repeating units as final products. The perception of agar oligosaccharides by *Gracilaria* sp. is affected by the oligosaccharide size (Weinberger *et al.*, 2001). Saccharides consisting of six to eight disaccharide repeating units proved to be the most efficient in eliciting an oxidative burst and eliminating associated bacteria, while the disaccharide was not perceived. This is consistent with the observation that pectin oligosaccharides also appear to be best perceived when they are relatively long and consist of chains of 10 to 15 monosaccharides (Mathieu *et al.*, 1991).

An indication that MAMPs may also be perceived by red macroalgae comes from a study of *Chondrus crispus* and its endophytic algal pathogen *Acrochaete operculata* (Bouarab *et al.*, 1999). The authors reported that cell-free extract of *A. operculata* triggered an oxidative burst. The gametophytic generation of *C. crispus,* which is more resistant than the sporophytic generation to *A. operculata*, responded with a stronger release of ROS. The oxidative burst was sensitive to diphenylene iodonium and therefore probably catalyzed by an NADPH oxidase. Attempts to isolate the molecular signal were unsuccessful, because it was not consistently present in *A. operculata* extracts (F. Weinberger, unpubl. data). An origin from microorganisms that were associated with *A. operculata* rather than from *A. operculata* itself can therefore not be excluded.

Liposaccharide (LPS) and lipoteichoic acids isolated from the cell envelopes of gram-negative and gram-positive bacteria modified patterns of protein expression in *C. crispus* (Fig. 1), which strongly suggests that they are perceived by this alga. These compounds did not trigger an oxidative burst in *C. crispus*, which was also true in several confirmed cases of LPS perception in vascular plants (Erbs and Newman, 2003).

An oxidative burst *per se* is not necessarily a result of receptor activation, since sources other than NADPH oxidase may also generate ROS. In *Gracilaria chilensis,* a species closely related to *Gracilaria* sp., agar oligosaccharides did not activate NADPH oxidase, but were instead oxidized by an agar oligosaccharide oxidase located in the cell wall (Weinberger *et al.*, 2005a). Similarly, *A. operculata* often contains—and excretes—important amounts of asparagine, which may serve as a substrate of cell-wall-located amino acid oxidase in *C. crispus* (Weinberger *et al.*, 2002, 2005b). In both cases, important amounts of ROS may temporarily accumulate and even affect pathogens, but molecular perception is not required.

Receptor-Mediated Immunity in Kelps

Alginate is the functional analog of agar and pectin in kelps, and oligomeric degradation products of alginate rich in α-1,4-L-guluronic acid have been shown to elicit an oxidative burst in kelp sporophytes (Küpper *et al.*, 2001, 2002). Exposure of *Laminaria digitata* to 2.5 $\mu g\ l^{-1}$ or more of oligoguluronate triggered a release of superoxide radicals. Cortical and young tissues generated more ROS than medullary and old tissues, respectively. No response was measured for 3 h after exposure, and pharmacological investigation with specific enzyme inhibitors indicated that

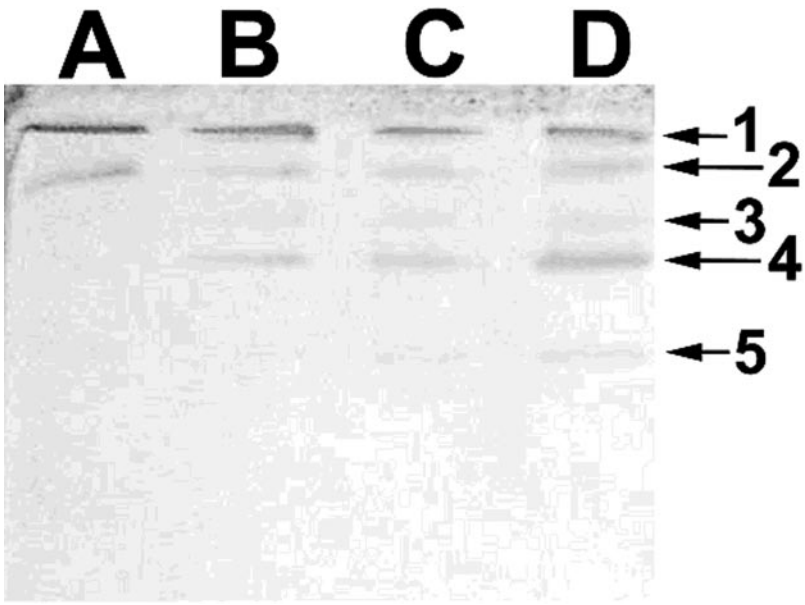

Figure 1. Expression of bromoperoxidases in *Chondrus crispus* as detected by gel electrophoresis under non-denaturating conditions. Lanes were loaded with 50 μg of crude protein of *C. crispus* incubated for 24 h in seawater alone (A) or seawater with addition of 1 μg ml^{-1} of lipoteichoic acid of *Staphylococcus aureus* (B), lipopolysaccharide of *Salmonella mortus-equi* (C), or lipoteichoic acid of *Bacillus subtilis* (D). Band 2 represents the red algal pigment phycoerythrin; bands 1 and 3 to 5 represent different bromoperoxidases. Visibly, bands 3 to 5 were absent in untreated *C. crispus*. For development, the gel was soaked with 25 mmol l^{-1} TRIS buffer, pH 7.4, in the presence of 10 μmol l^{-1} potassium bromide, 90 μmol l^{-1} *o*-dianisidine, and 0.45 mmol l^{-1} H_2O_2.

the signal transduction chain after treatment with oligoguluronate signals in *L. digitata* probably includes protein kinases, as well as Ca^{2+} channels and anion channels.

The authors also reported that oligoguluronate application resulted in a transient uptake of potassium for about 5 min, which was correlated with medium alkalinization and followed by a massive efflux of potassium ions. Interestingly, application of ionophores of K^+, H^+, and Ca^{2+}also activated NADPH oxidase, and the authors therefore concluded that plasma membrane depolarization must be directly involved in the activation of NADPH oxidase in *L. digitata*. This is similar to the situation in vertebrates (Amatore *et al.*, 2006), and different from that in vascular plants (Jabs *et al.*, 1997) and red macroalgae (Weinberger *et al.*, 2005b).

An NADPH-oxidase-mediated oxidative burst was also observed when *L. digitata* was challenged with LPS originating from the outer cell envelope of gram-negative bacteria (Küpper *et al.*, 2006). Such responses to LPS have also been observed in some vascular plants (Meyer *et al.*, 2001) and in metazoans (Farnell *et al.*, 2003; He *et al.*, 2003; Remer *et al.*, 2003).

Receptor-Mediated Protein Expression

After perception of MAMPs or PIMPs, animals and vascular plants typically respond not only with instantaneous activation of constitutive enzymes such as NADPH oxidase, but also with upregulated expression of defense-related proteins. There is evidence that macroalgae respond in an analogous way to MAMPs and PIMPs. For example, an upregulation of phenylalanine-ammonia lyase, a key enzyme of the biosynthesis of aromatic compounds, was reported for *Chondrus crispus* gametophytes after incubation with cell-free extracts of the endophytic pathogen *Acrochaete operculata*. Within 24 h, the enzyme was more strongly expressed, and this effect was correlated with increased resistance against *A. operculata* (Bouarab *et al.*, 2004).

A similar response was reported for *Laminaria digitata*, in which autofluorescent compounds—interpreted by the authors as defense-related aromatic compounds—accumulated within 7 d after treatment with oligoguluronate (Küpper *et al.*, 2002). This increase was positively correlated with resistance toward the endophyte *Laminariocolax tomentosoides*. In *Laminaria japonica*, the activity potential of polyphenol oxidase increased after infection with alginate-degrading microorganisms (Wang *et al.*, 2004c), which also indicates modifications in the metabolism of phenolic compounds.

Treatment of *C. crispus* with lipoteichoic acids or LPS originating from the outer cell envelopes of various bacteria triggered expression of new isoforms of bromoperoxidase 24 h later (Fig. 1), suggesting that these MAMPs increase the algal capacity for synthesis of brominated defense com-

pounds (see below). Exposure of different *Gracilaria* species to agar oligosaccharide resulted in an upregulation of agar oligosaccharide oxidoreductase 24 h later. The expression of this enzyme was positively correlated with resistance of *Gracilaria chilensis* toward attachment of the epiphytic filamentous alga *Acrochaetium* sp. (F. Weinberger, J. Beltrán, U. Lion, M.-L. Guillemin, unpubl. data).

Secondary Signal Transduction

Upregulation of specific proteins after MAMP or PIMP perception implies that intracellular signaling activates their transcription. ROS resulting from the oxidative burst, but also NO radicals, have been shown in animals and vascular plants to interact in a regulatory network that modifies protein expression (Jabs *et al.*, 1997; Grant and Loake, 2000; Delledonne *et al.*, 2001; Zeidler *et al.*, 2004; Pitzschke *et al.*, 2006). Using two different analytical methods, Küpper *et al.* (2006) failed to detected any NO• radicals in *Laminaria digitata* after exposure to LPS and concluded that they were probably not generated. In contrast, the upregulation of agar oligosaccharide oxidoreductase after treatment of *Gracilaria chilensis* with agar oligosaccharides could be prevented with various specific inhibitors of NO• synthase, suggesting that NO• radicals were possibly involved in the regulation (F. Weinberger, unpubl. data).

Signaling cascades involving oxidized polyunsaturated fatty acids—so-called oxylipins—are activated after perception of external molecular signals in vascular plants as well as in animals (Lee *et al.*, 2005), and this is also the case in macroalgae. Hydroperoxides derived from C20 polyunsaturated fatty acids (eicosanoids) regulate immune responses in metazoans (Soberman and Christmas, 2003; Stanley, 2006), whereas vascular plants use derivatives of C18 (octadecanoids) and C16 (hexadecanoids) fatty acids (Blee, 2002; Farmer *et al.*, 2003). In both phyla, lipoxygenases catalyze the oxygenation of polyunsaturated fatty acids into hydroperoxy derivatives and other secondary products (Feusner and Wasternack, 2002; Soberman and Christmas, 2003). Both red and brown macroalgae also produce oxylipins, which are derived from C18 as well as from C20 fatty acids, and several studies indicate regulatory roles for at least some of these compounds in algal defense (reviewed by Potin *et al.,* 2002; Pohnert, 2004).

Cellular perception of MAMPs has been shown to result in production of oxylipins and in biological effects. For example, concentrations of octadecanoid and eicosanoid oxylipins increased in *Chondrus crispus* after exposure to cell-free extracts of *Acrochaete operculata* (Bouarab *et al.*, 2004). Two lipoxygenase isoforms were upregulated within 1 d after elicitor application. Moreover, inhibition of lipoxygenase abolished the natural resistance of *C. crispus* gametophytes toward *A. operculata*, which demonstrated that this resistance requires oxylipin signaling. Further, treatments of *C. crispus* sporophytes—usually susceptible to *A. operculata* —with C20 or C18 oxylipins induced a transient resistance. The study by Bouarab *et al.* (2004) thus confirmed the involvement of both C18 and C20 oxylipins in defense signalling in *C. crispus*.

MAMP-activated production of oxylipins was also observed in *L.. digitata* (Küpper *et al.*, 2006). Within 30 min after treatment of *L. digitata* with two different LPS or with oligoguluronate, this brown alga released increased amounts of C18 and C20 free fatty acids. However, a concomitant accumulation of oxidized derivatives of linolenic and eicosapentaenoic acid was observed only after application of LPS, suggesting that oligoguluronate perception does not result in oxylipin production. Similarly, agar oligosaccharides did not activate oxylipin production in *G. chilensis* (U. Lion, Essen University, pers. comm.).

Further derivatization of C18 and C20 oxylipins leads to synthesis of jasmonic acid in vascular plants and of prostaglandins in metazoans. These cyclopentenones often act as defense hormones, in particular jasmonic acid (Farmer *et al.*, 2003; Soberman and Christmas, 2003; Schaller *et al.*, 2005; Stanley, 2006). Evidence exists that jasmonic acid, or structurally similar compounds (Pohnert, 2004), also play a role as secondary defense signals in macroalgae. For example, methyl jasmonate induced an increased biosynthesis of phlorotannins—often considered to be defense compounds—in the bladder wrack *Fucus vesiculosus* (Arnold *et al.*, 2001) and transient resistance to *A. operculata* in *C. crispus* (Bouarab *et al.*, 2004). Treatment of *C. crispus* with methyl jasmonate also resulted in a general increase in stress gene transcription, while the expression of genes involved in energy conversion and general metabolism was downregulated (Collén *et al.*, 2006), which confirmed the hormone-like function of jasmonic acid (or structurally similar compounds) in this macroalga.

Hypersensitive Response

At the site of attempted attack by an avirulent pathogen, vascular plants typically respond with hypersensitive cell death (Heath, 2000), and similar responses have been reported from macroalgae. During the hypersensitive response (HR), vascular plants sacrifice infected and adjacent cells to limit pathogen spreading. The HR is a result of programmed cell death (apoptosis) and different from the accidental cell death that may be caused by extrinsic factors such as accumulation of phytotoxic compounds after a traumatic stress (Van Breusegem and Dat, 2006). The regulation of the HR involves sensing of changes in intracellular homeostasis of ROS such as O_2^{-}• or NO• during the oxidative burst (Delledonne *et al.*, 2001), but due to the inherent toxic nature of these oxidants, their signaling function in the HR has long been masked.

Cell death is often observed during microbial infections

of macroalgae. For example, alginate-degrading bacteria have been shown to induce cell death in *Laminaria japonica* (Liu *et al.*, 2002a), but not in certain resistant clones that expressed a particularly high antioxidative capacity (Tang *et al.*, 2002), suggesting that ROS accumulation after oligoguluronate perception was crucial for this symptom. Cell death was also observed in gracilarioids after infection with bacteria (Weinberger *et al.*, 1994, 1997; Jaffray and Coyne, 1996; Schroeder *et al.*, 2003). Application of agar oligosaccharide into the medium of *Gracilaria* sp. caused this symptom in apical cells, and simultaneous application of catalase reduced the effect, which indicated an involvement of ROS into cell death (Weinberger, 1999).

Uncoupling of respiration and phosphorylation is a prerequisite for programmed cell death (Fleury *et al.*, 2002; Tiwari *et al.*, 2002; Van Breusegem and Dat, 2006), leading to amplified ROS production and changes in ROS homeostasis, and such uncoupling was observed after exposure of *Gracilaria* sp. to agar oligosaccharides (Weinberger, 1999). The resulting respiratory increase was sensitive toward respiration inhibitors, such as rotenone and antimycin A, and application of these agents also reduced agar oligosaccharide-induced cell death in *Gracilaria* sp. significantly. In addition to mitochondria, chloroplasts were also involved in the regulation of agar oligosaccharide-activated cell death in *Gracilaria* sp. The effect was dependent on small doses of light (Weinberger *et al.*, 1999), reminescent of findings with vascular plants which indicate an involvement of phytochrome light receptors in the regulation of the HR (Genoud *et al.*, 2002).

Key enzymes activated during apoptosis that catalyze programmed cell death in vascular plants and in metazoans are nucleases and caspases, a family of highly specific proteases (Heath, 2000; Lam and del Pozo, 2004). Investigations with *L. japonica* revealed that cell death after infection with alginate-degrading microorganisms was dependent upon caspases, and DNA cleavage was also observed (Wang *et al.*, 2004a). Alginate degrader-induced cell death in *L. japonica* therefore appears as a result of apoptosis, similar to the HR in vascular plants. Interestingly, overexpression of hypersensitive lesions in response to alginate-degrading bacteria may lead to massive losses of *L. japonica* sporelings in commercial kelp aquaculture and has been described as the so-called "rot disease" (Ding, 1992).

The Defensive Value of Receptor-Mediated Immunity

Irrespective of all similarities or differences with other host-pathogen systems, any defenses should, of course, result in a containment of pathogens. Indeed, exposure to MAMPs or PIMPs increases macroalgal resistance. In *Gracilaria* sp., treatment with agar oligosaccharides eliminated up to 55% of all associated bacteria within 1 h (Weinberger and Friedlander, 2000). Agarolytic bacterial strains that had been isolated from the surface of *Gracilaria* sp. proved to be particularly sensitive. Treatment with agar oligosaccharides also increased the resistance of *Gracilaria chilensis* against the epiphytic microalga *Acrochaetium* sp. (Weinberger, unpubl. data). After exposure to oligoguluronate, *Laminaria digitata* sporophytes became more resistant to infection with a brown algal endophyte, *Laminariocolax tomentosoides* (Küpper *et al.*, 2002). The resistance increased progressively, reached its maximum 7 d after exposure, and persisted for more than one week. Similarly, *Chondrus crispus* gained in resistance against the endophyte *Acrochaete operculata* when it was treated with cell-free extract from *A. operculata* (Bouarab *et al.,* 2004).

Innate immunity requires more than the general ability of an organism to perceive MAMPs or PIMPs and to activate a potentially defensive response after signal recognition: it also requires that the necessary concentrations of signals and defense compounds be reached under natural conditions. Host-pathogen interactions of vascular plants and animals typically take place in the phylloplane or lymph system, where concentrations of infochemicals and defense compounds can be more-or-less controlled by the host. In contrast, elicitors and defense compounds in macroalga-pathogen interactions may be subject to constant dilution in the aquatic environment. It is therefore an obvious question whether they can ever reach efficient concentrations.

Analytical tools that allow the real-time quantification of MAMPs or PIMPs directly at the site of their release do not yet exist, but several studies indicate that molecular signals are generated during pathogen infections in sufficient amounts to trigger responses. In *Laminaria japonica,* early stages of infections with alginate-degrading bacteria have been shown to be characterized by responses similar to those of oligoguluronane exposure; for example, by an oxidative burst (Huang *et al.*, 2002; Liu *et al.*, 2002b) and by Ca^{2+} translocation events (Wang *et al.*, 2004b), which indicates that oligoguluronates are apparently perceived. In pond aquaculture of *Gracilaria* sp., microbial decay of algal biomass resulted within 24 h in an accumulation of agar oligosaccharides that was potentially high enough to elicit an oxidative burst in all specimens of *Gracilaria* sp. that were present (Weinberger and Friedlander, 2000). A similar "neighbor effect" might also be possible in other closed water bodies, such as rock pools.

The quantification of unstable compounds such as ROS, in real time and at the site of their potential defensive action, poses a challenge similar to the quantification of MAMPs or PIMPs. Indications of direct defensive effects of algal responses after pathogen perception have nontheless been reported. Küpper *et al.* (2002) inhibited the capacity for an oxidative burst in *Laminaria digitata* and *Macrocystis pyrifera* by adding diphenylene iodonium to the medium, which rendered these kelps unable to defend themselves against decomposition by the same bacterial flora that under normal

conditions caused no damage to them. Seaweed-associated agarolytic bacteria (Weinberger and Friedlander, 2000) and alginate-hydrolyzing bacteria (Küpper *et al.*, 2001) were generally sensitive toward the concentrations of H_2O_2 that were observed in the algal medium after exposure to the elicitor, suggesting that direct cytotoxicity of ROS may lead to an elimination of these microorganisms.

In addition, many macroalgae have cell-wall-located haloperoxidases, which are limited in their activity by H_2O_2 and which generate hypohalous acids and various halocarbons, such as bromoform ($CHBr_3$) (Carpenter and Liss, 2000). It has long been disputed whether these compounds play a role in algal defense or are just byproducts of ROS detoxification (Manley, 2002). However, hypohalous acids generated by *L. digitata* have been shown to interfere with *N*-acyl-homoserine lactone-mediated quorum sensing, which regulates the formation of bacterial biofilms (Borchardt *et al.*, 2001). Moreover, a study by Paul *et al.* (2006) demonstrated that bacteria associated with the red alga *Asparagopsis armata* increased 14- to 20-fold when no $CHBr_3$ could be synthesized by their host due to bromide (Br^-) depletion. Bacteria that had been isolated from *A. armata* in Br^--depleted conditions proved to be more sensitive toward $CHBr_3$ than were bacteria that had been isolated when Br^- was present, which strongly suggests that the increase in bacterial numbers resulted from lack of $CHBr_3$ rather than from other effects of Br^--depletion.

Algal $CHBr_3$ production may therefore have a defensive value, provided that the rate of release is sufficiently high. The rate of $CHBr_3$ release by *A. armata* (Marshall *et al.*, 1999; Paul *et al.*, 2006) is in the same order of magnitude as in *L. digitata* that have not been exposed to an elicitor (Carpenter and Liss, 2000) or *Gracilaria* sp. (151 ng gDW^{-1} h^{-1} [where DW is dry weight of algal tissue], F. Weinberger, B. Coquimpot, S. Forner, P. Morin, B. Kloareg, P. Potin, unpubl. data). Exposure of *L. digitata* to oligoguluronate immediately accelerated $CHBr_3$ production by about 250% (Palmer *et al.*, 2005), and an increase by 780% was observed with *Gracilaria* sp. after treatment with agar oligosaccharide (Weinberger, Coquimpot *et al.*, unpubl. data). All together, these findings suggest that halogenated compounds and ROS can be generated by kelps and gracilarioids in sufficiently high amounts to kill or repel microorganisms and that their production is accelerated during the oxidative burst. Compared to the small number of studies that were conducted with macroalgae, relatively many indications of direct toxic effects of oxidative burst products upon associated microorganisms exist. This contrasts with the relatively few examples that have been described for vascular plants, suggesting that more ROS are excreted by algae, possibly to compensate for dilution effects.

The Evolution of Innate Immunity

Major principles of innate immunity appear strikingly similar among distant eukaryotic clades, which seemingly indicates that they are evolutionary ancient. However, comparisons of the molecular structures of innate receptors of animals and vascular plants reveal important differences (Nürnberger *et al.*, 2004; Ausubel, 2005), suggesting that similarities are the result of coevolutionary processes. The constitutive presence of innate receptors not only allows for particularly fast responses to pathogen attacks, but also is the necessary basis for a reliable cellular distinction between self and non-self (Nürnberger and Lipka, 2005), and this could be the reason that these receptors evolved several times independently.

Other components of innate immunity are apparently conserved. For example, the main subunits of NADPH oxidase in mammalian phagocytes, vascular plants, diatoms, and the red macroalgae *Porphyra yezoensis* and *Chondrus crispus* are structurally related (Keller *et al.*, 1998; Torres and Dangl, 2005; Hervé *et al.*, 2006), which confirms that this protein is evolutionary ancient. Eicosanoid and prostaglandin signaling may also be a common heritage of most or even all eukaryotes, which got lost only in spermatophytes (Bouarab *et al.*, 2004). Jasmonate signaling probably evolved after the primary chloroplast endosymbiosis but before any secondary endosymbiotic events (Fig. 2) and is therefore potentially a common trait of all photosynthetically active eukaryotes (Bouarab *et al.*, 2004). The same seems to be true for at least some components of apoptosis, which involves caspases in brown algae, vascular plants, and metazoans.

The fact that NADPH oxidase, oxylipin signaling pathways, and apoptosis appear to be evolutionary ancient is nonetheless no strong indication of a common origin of innate immunity. All three are not specific components of immunity regulation, but rather are generally employed in cellular stress management, and their similar functions in different eukaryotes could for this reason be a result of coevolution.

Interestingly, vascular plants and macroalgae share some immunological traits that are missing in metazoans—for example, the capacity to perceive PIMPs. Only the isolation and characterization of macroalgal MAMP and PIMP receptors in the future will allow for a final decision about whether innate immunity evolved only once or several times independently in photosynthetic eukaryotes.

Conclusion and Outlook: Challenges for Ecologists!

Considerable evidence of innate immunity in kelps, gracilarioids, and *Chondrus crispus* has accumulated during the last years (Table 1). Obvious similarities exist between the responses of kelps to alginate-degrading microorganisms and of vascular plants to pectin degraders. The re-

sponse of *Gracilaria* sp. to agar degraders also fits into this pattern, but differs in some aspects from that of the closely related *Gracilaria chilensis*. Nontheless, both responses affected pathogens in bioassays. This still needs to be demonstrated for macroalgal responses to LPS, which again appear similar to those of other eukaryotes. The response of *C. crispus* to cell-free extracts of *Acrochaete operculata* also shows important similarities with the innate immunity of other eukaryotes, while nature and origin of the active signal still remain enigmatic.

Yet relatively few examples of macroalgal innate immunity are known. This might mean that they are rare. However, the identification of molecular defense elicitors is time-consuming, and the lack of evidence of innate immunity in entire macroalgal clades such as chlorophytes and charophytes could simply reflect a lack of investigations.

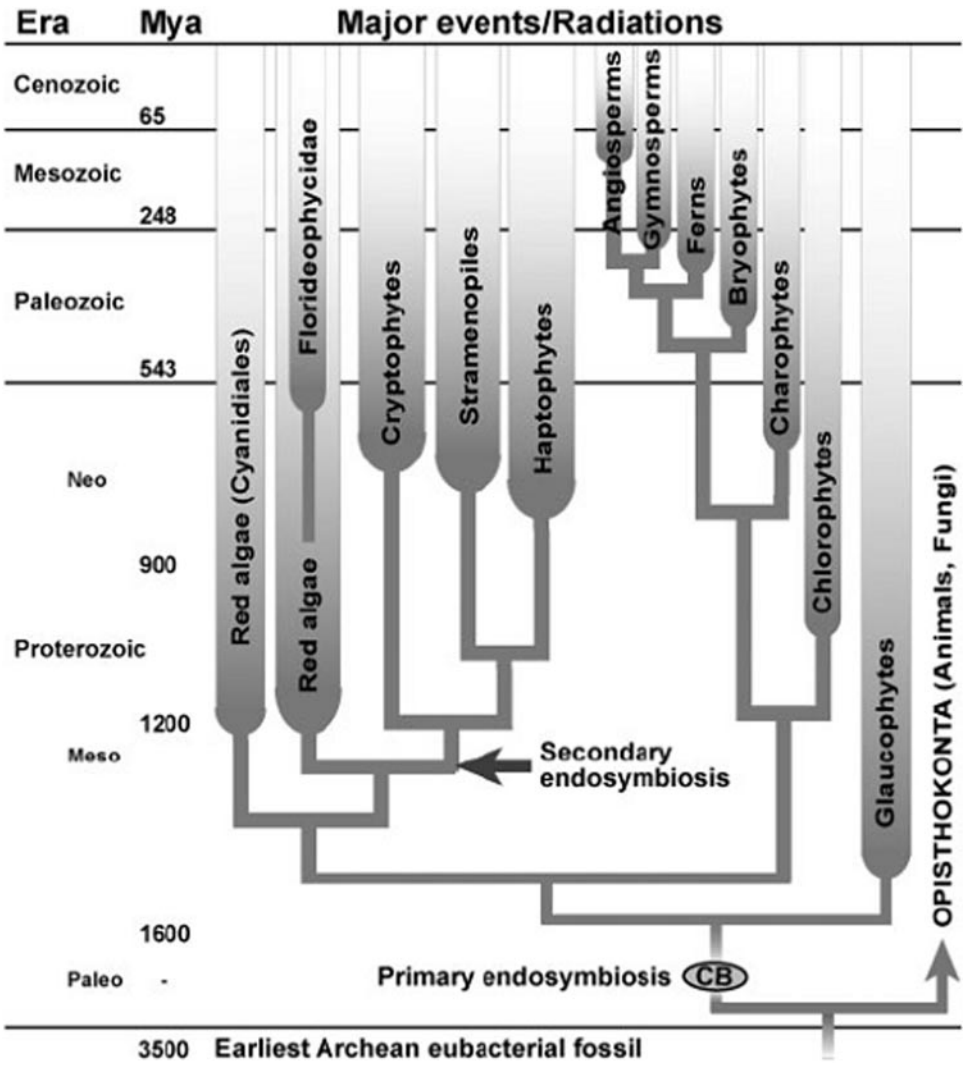

Figure 2. A schematic representation of the evolutionary relationships and divergence times for plastid-bearing eukaryotes (reproduced from Yoon, H. S., J. D. Hackett, C. Ciniglia, G. Pinto, and D. Bhattacharya. 2003. A molecular timeline for the origin of photosynthetic eukaryotes. *Mol. Biol. Evol.* **21**:809-818, by permission of the Society for Molecular Biology and Evolution and Oxford University Press). This tree was generated using maximum likelihood molecular clock methods, based upon a plastid-gene phylogeny with Bayesian inference. Nodes in the tree were constrained with six reliable fossil dates and a maximum age of 3500 millions of years based on the earliest known eubacterial fossil. The photosynthetic groups are outgroup-rooted with the Opisthokonta, which putatively ancestrally lacked a plastid. The branches on which the cyanobacterial (CB) primary and red algal secondary endosymbioses occurred are shown. Only red macroalgae (including Florideophycidae) and green macroalgae (including chlorophytes and charophytes) belong to the plant kingdom (Moreira *et al.,* 2000; Baldauf, 2003; Palmer, 2003). Brown macroalgae belong to the stramenopiles—and thus to the same phylum as diatoms—and have arisen from a secondary endosymbiosis between an ancestral unicellular red alga and a second colorless protist.

The first herbivore-associated molecular pattern that induces increased resistance to grazing in a macroalga has recently been identified (Coleman *et al.*, 2007): the protein α-amylase from mollusc saliva was apparently perceived by the brown alga *Ascophyllum nodosum*, which responded with increased synthesis of phlorotannin and reduced pallatability to the snail *Littorina obtusata*. This is reminescent of the recognition of specific components in the oral secretions of lepidopteran larvae by vascular plants (for a review, see Kessler and Baldwin, 2002).

So far, such examples of specific cellular recognition of grazers are an exception rather than a rule. Antigrazing defense in animals usually relies upon neuroreceptors, while vascular plants respond nonspecifically to wounding—irrespective of its cause. Wounding results in cell disintegration, in contact of phospholipases and lipids, subsequently in release of unsaturated fatty acids, and eventually in the formation of oxylipins (Kessler and Baldwin, 2002). Similar effects of wounding are also known from macroalgae (Schnitzler *et al.,* 2001; Lion *et al.,* 2006), and induction of macroalgal antiherbivore defenses by wounding has been reported (Toth and Pavia, 2007). However, more evidence of induced macroalgal antiherbivore defenses requiring chemical cues has been reported (Amsler, 2001; Toth and Pavia, 2007). These cues obviously must be perceived, and further examples of receptor-mediated defense against herbivores will possibly be detected in macroalgae. Additional examples will not only increase our understanding of seaweed-grazer interactions, but also allow us to widen the general concept of innate immunity. So far, innate immunity has been regarded only as the molecular basis of antimicrobial defenses (Nürnberger *et al.,* 2004; Ausubel, 2005), and the few known examples of cellular perception of grazers were seen as exceptional. However, information on nonmodel organisms such as macroalgae is still largely missing, and long-standing "exceptions" may turn out to be "rules" in the future. A striking example for such a devolopment is the recent finding of C20 oxylipins as relatively widely distributed signaling compounds that are missing only in spermatophytes.

Molecular biologists are now challenged to isolate and characterize the receptors that are the basis of macroalgal innate immunity. This will not only be the final proof that these receptors exist, but it will also allow for a clear-cut decision on their evolutionary origin. Another important task for the coming years—in addition to further elucidating the signaling pathways involved—will be to analyze patterns of gene expression after defenses were elicited. The few known examples in macroalgae of gene induction after perception of a defense signal were often detected on the basis of an informed guess. Systematic comparisons of gene transcripts in macroalgae that have and have not been exposed to an elicitor should give much more comprehensive indications of whether and which defense mechanisms are induced after signal perception.

The molecular concept of macroalgal innate immunity has provided important new insights into seaweed-microbe interactions, and it now needs to be complemented and further strengthened with a convincing ecological perspective on pathogen-induced defense. So far, the existence of macroalgal innate immunity against microorganisms has been demonstrated mainly in laboratory studies, using simplified host-pathogen systems and standardized environmental conditions. Ecologists are now challenged to evaluate the relevance and functionality of these mechanisms under natural conditions, where genetically more diverse macroalgae are subject to varying conditions and are associated with complex interacting populations of micro- and macroorganisms. Induced antimicrobial defense may potentially affect macrofoulers (since their settlement often depends upon bacterial biofilms; see also Steinberg and De-Nys, 2002) or the algal palatability to grazers.

Early stages of innate immune responses are typically characterized by transient signal and defense compound accumulation in and above the algal cell wall. The signals involved are usually complex and active at low concentrations in the nanomolar range, while the defense compounds involved are often short-lived (such as, for example, ROS). The demonstration that, under natural conditions, sufficiently high concentrations of these compounds are reached to cause an attributed effect is highly demanding, but necessary for the final proof of ecological relevance. In many cases this demonstration will probably require the development of new analytical tools that allow the quantification of signals and defense compounds *in vivo, in situ*, or both (La Barre *et al.*, 2004).

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