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Oxidative Burst and Related Responses in Biotic Interactions of Algae

P. Potin

12.1 Introduction

Herbivores and pathogens can have strong effects on algal fitness, regulate population dynamics, and cause considerable damage in marine ecosystems. This was exemplified in kelp forests by dramatic changes associated with the reduction or extinction of local populations of some key predators controlling macroalgal grazers (Estes and Duggins 1995), or in coral reef ecosystems, where coralline lethal orange disease (CLOD), a disease affecting various coralline algae, has led to the destruction of thousands of kilometers of reefs (Littler and Littler 1995). Such diseases or predation may also be highly destructive in managed ecosystems, as reported for *Laminaria japonica* (Ishikawa and Saga 1989), *Porphyra yezoensis* (Fujita et al. 1972), and *Eucheuma/Kappaphycus* (Ask and Azanza 2002; Hurtado et al. 2006) aquaculture fields. In the context of global change, including human impacts and introduction of alien species, the frequency of pathogens and epidemics has increased in recent decades and sessile invertebrate and algal populations will have to adapt their defense strategies to cope with new challenges (Harvell et al. 1999, 2002; Mydlarz et al. 2006). Marine algae have evolved a variety of defensive mechanisms against grazers and pathogens (Pohnert 2004). They strongly depend on their chemical repertoire to influence interactions with other organisms and with the environment. A portion of these chemicals may provide constitutive barriers against grazers or parasites. Constitutive production of secondary metabolites provides antimicrobial compounds (see Chap. 11; de Nys and Steinberg 2002; Kubanek et al. 2003) and grazer deterrents (see Chaps. 2–6, 9; Paul and Puglisi 2004; Paul et al. 2006c). Considering the fundamental question of the investment of physiological resources in defense structures or metabolites (see Chap. 7; Amsler and Fairhead 2006; Ianora et al. 2006), however, it is obvious that marine algae have also developed activated and induced defense mechanisms. Nonetheless, in striking contrast with the knowledge

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on host-pest interactions in terrestrial crop or wild plants (e.g., Nürnberger et al. 2004), very little is known about signaling or defense induction and regulation in marine algae (Bouarab et al. 2001a; Potin et al. 2002).

In terrestrial plant-pathogen interactions, it is believed that most microorganisms fail to successfully colonize most plant species because plants possess an elaborate surveillance system that readily uncovers the presence of potentially pathogenic agents. Plant cells are able to sense this invasion, for example, by associated mechanical wounding or the detection of released plant wall fragments (John et al. 1997; De Lorenzo et al. 2001; Vorwerk et al. 2004) and conserved pathogen-derived molecules which are referred to as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs). A subset of the latter has been found to act as general elicitors of basal plant immune responses (Nürnberger et al. 2004; Nürnberger and Lipka 2005), part of which take place at the cell wall (Hauck et al. 2003; Schulze-Lefert 2004). Local generation of reactive oxygen species (ROS) at the cell periphery, for example, is a frequent plant response to attack by many microbial pathogens. ROS is a collective term that includes oxygen-derived small molecules, including oxygen radicals [superoxide (O_2•–), hydroxyl (•OH), peroxyl (RO_2•), and alkoxyl (RO•)], and certain nonradicals that are either oxidizing agents and/or are easily converted into radicals, such as hypohalous acid (HOX), ozone (O_3), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge 1999). This transient and rapid production of large amounts of ROS associated with increased O_2 consumption is reminiscent of the oxidative burst, 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 described more recently in terrestrial plants (Doke 1983a, b), and it is recognized today as a ubiquitous characteristic of defense systems in these phyla (e.g., Baker and Orlandi 1995; Lamb and Dixon 1997; Wojtaszek 1997; Bolwell et al. 1998).

Although the role of ROS in mediating compatibility and/or resistance in plant-pathogen interactions is still controversial (for review, see Hückelhoven and Kogel 2003), these molecules are generally thought to function in oxidative cell wall cross-linking and plant defense signaling. The key role of reactive oxygen metabolism in the defensive responses of marine algae and invertebrates to environmental stresses and to infection by potential pathogens has been recently reviewed (Dring 2006; Lesser 2006). Thus, this review will concentrate on aspects that have received little attention in marine algae, such as methods to detect ROS in seaweeds, the oxidative-burst machinery, other sources of ROS, and also the ecological significance of the oxidative burst and related responses. Particular attention will be given to current views on algal defense elicitors and to recent developments in determining the identity of associated defenses, which are mounted concomitantly with the perception of such elicitors.

12.2 Reactive Oxygen Species and Detection Methods

ROS generation is generally a cascade of reactions that starts with the production of superoxide. Superoxide rapidly dismutates to hydrogen peroxide either spontaneously, particularly at low pH, or catalyzed by superoxide dismutase (SOD).

Other elements in the cascade of ROS generation include the reaction of superoxide with nitric oxide (NO) to form peroxynitrite (RNOO), peroxidase-catalyzed formation of hypochlorous acid from hydrogen peroxide, and the iron-catalyzed Fenton reaction leading to hydroxyl radical generation (Klebanoff 1980; Thannickal and Fanburg 2000). Nitrogen-containing oxidants, such as NO, are called reactive nitrogen species (RNS). ROS and RNS react with a large number of molecules, including other small inorganic molecules as well as proteins, lipids, carbohydrates, and nucleic acids. Through such interactions, ROS may irreversibly destroy or alter the function of the target molecule. Consequently, ROS are major contributors to cell damage in biological organisms but can be beneficial as a host defense. This latter point became particularly clear when the link was made between deficiency in ROS generation and reduced killing ability in leukocytes (Baehner and Nathan 1967) and more recently in plant cells with inactivated *rboh* genes (Torres and Dangl 2005). However, in the past decades ROS involvement has proven important in many reversible regulatory processes in virtually all cells and tissues (Thannickal and Fanburg 2000; for terrestrial plants, see Torres et al. 2006).

Facile detection of ROS and RNS in biologic systems is often problematic (for detailed methodological reviews, see Halliwell and Whiteman 2004; Tarpey et al. 2004). This is a result of numerous cellular mechanisms, both enzymatic and nonenzymatic, involved in their catabolism/decomposition, the complex and overlapping nature of their reactivities, as well as the often limited intracellular access of detector systems. In marine algae, the first monitoring of extracellular ROS emission upon mechanical damage was conducted using luminol-dependent chemiluminescence (Glazener et al. 1991) in the red alga *Eucheuma platycladum* (Collén et al. 1994). This technique is routinely used to monitor production in culture media of animal or plant cells and allows one to detect H_2O_2 and other forms of ROS such as O_2•– and •OH. Dring (2006) recently described reservations concerning the reliability of this method as an indicator of biological ROS. Luminol must first be oxidized in a one-electron step by H_2O_2 and a peroxidase or chemical catalyzer such as ferricyanide. Hence luminol may be an unreliable probe and should be used with appropriate controls to detect any contaminating oxidizing agent that could cause light emission. Nevertheless, this method was used in a number of studies depicting ROS emission in biotic interactions of marine algae (Bouarab et al. 1999, 2001b; Küpper et al. 2001, 2002, 2006; Weinberger et al. 2002, 2005a, b) and these results were validated using the following methods.

Since the pioneering work of Collén and Davison (1997) in *Fucus evanescens*, the use of fluorescent probes such as 2′,7′-dichlorohydrofluorescein diacetate (DCFDA) has also become popular to detect "cellular peroxides" in marine algae (Vardi et al. 1999; Weinberger et al. 1999; Collén and Davison 1999a–c; Coelho et al. 2002; Ross et al. 2005b). However, it is unlikely to be effective because it reacts slowly with H_2O_2 or lipid peroxides and because DCFDA can detect cellular peroxides only if they are decomposed to radicals. It appears that DCF fluorescence is an assay of global oxidative stress rather than of any particular ROS (Halliwell and Whiteman 2004). During the past decade, it was used in many studies of oxidative stress in marine algae (reviewed by Dring 2006) and is particularly suitable for cell imaging of an oxidative burst by using fluorescence and confocal microscopy (Vardi

et al. 1999; Küpper et al. 2001; Coelho et al. 2002; Ross et al. 2005b). Recently, diaminofluorescein diacetate (DAF2-DA), a specific reporting probe of NO production, was successfully used to detect NO in a wounded siphonous green alga (Ross et al. 2006) and in diatoms (Vardi et al. 2006). However, this probe did not detect RNS in lipopolysaccharide (LPS)-treated *Laminaria digitata* (Küpper et al. 2006).

Other indirect methods have also been used to detect or localize ROS production in marine algae. Küpper et al. (2001) used nitroblue tetrazolium salts to demonstrate O_2•– production in *L. digitata* challenged with alginate oligosaccharides. More recently, a method utilizing precipitation of cerium salts to detect subcellular sites of H_2O_2 production was successfully used in marine red algae (Weinberger et al. 2005a).

12.3 Inducers and Sources of ROS Emission in Biotic Interactions of Marine Algae

An increasing number of observations indicate that emission of ROS following inducer recognition mediates host-pathogen interactions in algae (Table 12.1). The physiological generation of ROS can occur as a by-product of other cellular reactions in mitochondria, chloroplasts and peroxisomes, cytochrome P450, and other cellular elements (Dring 2006). However, in biotic interactions, emission of ROS or RNS is most often triggered by cell-cell recognition, involving perception at the cell membrane of a signal from an invading organism or liberated from the host surfaces (referred to as elicitors in terrestrial plant pathology) (Fig. 12.1).

12.3.1 Elicitors and Sources of ROS in Terrestrial Plant-Pathogen Interactions

PAMPs include cell wall components of microorganisms such as the LPS of Gram-negative bacteria and peptidoglycans or lipoteichoic acid (LTA) of Gram-positive bacteria. In terrestrial plants they also include bacterial proteins or peptide motifs such as flagellin and elongation factor Tu (EF-Tu) (reviewed by Nürnberger and Lipka 2005; Abramovitch et al. 2006). LPS are now recognized as elicitors of innate immunity in terrestrial plants (Silipo et al. 2005). Challenges with various sources of LPS trigger the induction of an oxidative burst (Meyer et al. 2001; Gerber et al. 2004), activation of phosphorylation cascades (Gerber and Dubery 2004), as well as the production of NO (Zeidler et al. 2004). The relevance of elicitors such as oligosaccharins as in vivo participants in defense systems is supported by their possible natural occurrence during plant-microbe interactions (Fritig et al. 1998). 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 (Klarzynski et al. 2000) and chitins are exogenous (released from the pathogen).

In most terrestrial plant-pathogen interactions a diphenylene-iodonium (DPI)-sensitive (O'Donnell et al. 1993), membrane-located, and receptor-activated NADPH oxidase generates superoxide radicals (Levine et al. 1994; Doke and Miura 1995; Lamb and Dixon 1997; Bolwell et al. 1998), which eventually dismutate into H_2O_2 and O_2 (Sutherland 1991). Apoplastic peroxidases (Bolwell et al. 1998; Martinez et al. 1998), as well as various oxidases such as oxalate oxidase (Zhang et al. 1995; Thordal-Christensen et al. 1997) or amine oxidase (Laurenzi et al. 2001; Rea et al. 2002), have also been identified as sources of ROS in higher plants.

12.3.2 A Growing Repertoire of ROS Inducers

Until recently, a few inducers of ROS emission were recognized as true elicitors of defense responses in marine plant-microbe interactions, and these have already been discussed in previous reviews (Potin et al. 1999, 2002; Pohnert 2004; Dring 2006). This list has slightly increased recently (Table 12.1).

An oxidative burst has been reported both after the perception of algal cell-wall-derived elicitors (Bouarab et al. 1999; Weinberger et al. 2001, 2005a; Küpper et al. 2001, 2002) and in response to PAMPs and MAMPs from various mammalian and marine pathogenic bacteria (Küpper et al. 2006). The most efficient elicitor of an oxidative burst in *L. digitata* is the LPS from *Salmonella arbotus equi,* which induced severe inflammatory responses, including oxidative burst in mammalian hosts. Although the oxidative burst induced by this LPS occurs considerably later, it has a range similar to that observed after elicitation by oligoguluronates in *L. digitata* and is also sensitive to DPI (Küpper et al. 2006). This study was the first report on an oxidative burst response to LPS perception in any algal group, although it is likely that recognition of LPS and other MAMPs will prove to be ubiquitous in several groups of algae. Precise structure-function studies will reveal the minimum structure required for perception, and it will be useful to isolate and test the LPS from algal pathogenic bacteria such as *Pseudoalteromonas bacteriolytica*, the causative agent of red spot disease in maricultured *L. japonica* (Sawabe et al. 1998).

12.3.3 New Insights into ROS Sources in Algae

In contrast with the ubiquitous response of higher plant cells to either oligogalacturonans or oligoglucans, the capacity to recognize a specific oligosaccharide structure seems to be confined to particular algal taxa. In a survey of 45 species belonging to 11 orders, the capacity to recognize alginate oligosaccharides as defense signals is limited to a few members of brown algae belonging to the orders Laminariales, Desmaretiales, Ectocarpales, and Fucales (Küpper et al. 2002). In red

Table 12.1 Host-released elicitors, pathogen-associated molecular patterns, and substrates of ROS-generating enzymes involved in algal defenses

Molecule	Source	Minimal structural motif required for defence activation	Biological response	Reference
Agar	Cell walls of agarophyte red algae	Agar hexasaccharide: neogarohexaose	Oxidative burst, and all associated responses in *G. conferta* in response to bacterial infection	Weinberger et al. 1999, 2001, 2005a
	Gracilaria conferta	Optimum degree of polymerization > 10	Tip bleaching in *G. gracilis*	Schroeder et al. 2003
Carrageenans	Cell walls of carrageophyte red algae	Carrageenan oligosaccharides	Modulation of the virulence of the endophytic green alga *Acrochaete operculata*. Oxidative burst + carrageenolytic enzymes for lambda + secretion of L-Asn for kappa	Bouarab et al. 1999, 2001
	Chondrus crispus	Lambda (3 sulfate per disaccharide repeating units) and kappa (1S) families		Weinberger et al. 2002, 2005b
Alginate	Cell walls of brown algae	Homo-oligomeric fragments of poly-α-1,4-L-guluronic acid (degree of polymerization > 15)	Oxidative burst, and all associated responses in *Laminaria digitata* and *L. japonica* + VHOCs	Küpper et al. 2001, 2002
			Induced resistance against bacteria and endophytes in kelps	Palmer et al. 2005; Teissier et al., unpublished results
			Gene-regulated responses	Cosse et al., unpublished results
Lipopolysaccharides	Human pathogenic and marine bacteria	Lipid A + various attached oligosaccharide decorations	Oxidative burst, and oxylipin production in *L. digitata*	Küpper et al. 2006

Unknown	Unidentified epiphytic bacterium of *G. conferta*	Small peptide (700–1,500 Da)?	ROS emission in *G. conferta*	Weinberger and Friedlander 2000
Unknown	Marine fungus *Lindra thalassiae*	Nonelucidated	ROS emission in the green alga *Dictyosphaeria cavernosa*	Ross 2005; www.sms.si.edu/2005final_screen.pdf
Unknown	Cell-free extracts of endophytic green alga *A. operculata*	Nonelucidated	Oxidative burst, and all associated responses in *Chondrus crispus*, including oxylipin pathway and VHOCs	Bouarab et al. 1999, 2004 Bouarab et al., unpublished results
Amino acid	Secreted by endophytic green alga *A. operculata* challenged with kappa-carrageenan oligosaccharides	L-Asparagine	ROS production by an extracellular amino acid oxidase	Weinberger et al. 2002, 2005b
Agar	Cell walls of agarophyte red algae *Gracilaria chilensis*	Agarose decasaccharide	ROS production by an extracellular agar oligosaccharide oxidase	Weinberger et al. 2005a
Agar	Cell walls of agarophyte red algae	Sulfated agars and porphyran-agaroid from *Porphyra* spp.	Appressoria formation in the specific pathogenic oomycete *Pythium porpyrae*	Uppalapaati and Fujita 2000

ROS reactive oxygen species, *VHOCs* volatile halogenated organic compounds

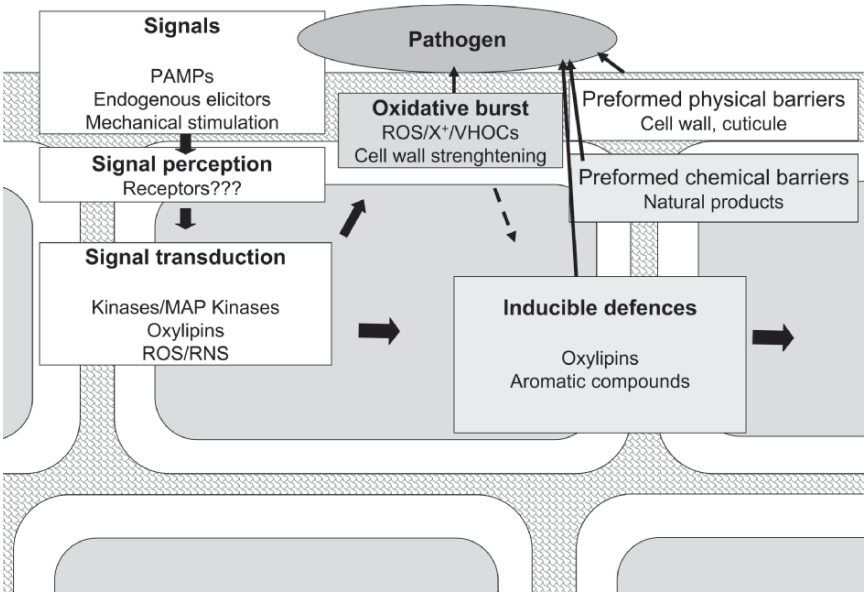

Fig. 12.1 Hypothetical scheme of the complex sensory, signaling and executive mechanisms following the oxidative burst induced by recognition of exogenous pathogen-associated molecular patterns (PAMPs) or endogenous elicitors (Table 12.1) based on the current knowledge about marine algae. The occurrence of receptors for PAMPs or oligosaccharides is not yet proven in algae. Pharmacological evidence and some direct measurements of ion fluxes indicate that initiation of signaling cascades controls ROS emission and activated responses and the subsequent gene-regulated inducible defenses. (Redrawn and adapted from a drawing of analogous mechanisms that constitute the basis of nonhost resistance in terrestrial plants (Nürnberger and Lipka 2005))

algae, respiratory bursts in response to agar oligosaccharides were observed in six species of the family Gracilariaceae but were not observed when other agarophyte or carrageenophyte red algae were tested (Weinberger et al. 1999). Sensitivity to agar oligosaccharides therefore seems to be a ubiquitous, but specific feature in Gracilariaceae. However, the respective sources of ROS production were not clearly determined in these previous reports. Recently, the two agar-producing red algae *Gracilaria chilensis* and *G. conferta* were compared for their respective capacity to generate H_2O_2 in the presence of agar oligosaccharides in their culture medium (Weinberger et al. 2005a). In *G. conferta*, a transient release of H_2O_2 was observed, followed by a refractory state of 6 h. This response was sensitive to DPI and other chemical inhibitors of NADPH oxidase, protein kinases, protein phosphatases, and calcium translocation in the cell, while it was insensitive to inhibitors of metalloenzymes. Transmission electron microscopic observation of the H_2O_2-dependent formation of ceriumperoxide from cerium chloride indicated that large amounts of oxygen were activated at the plasma membrane of *G. conferta* within few minutes after challenge with agar oligosaccharides. These results indicate that

a putative system consisting of a receptor specific to agar oligosaccharides and a plasma-membrane-located NADPH oxidase is responsible for the release of H_2O_2 in *G. conferta*. In contrast, subcellular examination of *G. chilensis* showed that the H_2O_2 release after exposure to agar oligosaccharides was located in the cell wall. It was sensitive to inhibitors of metalloenzymes and flavoenzymes, but not to DPI, and no refractory state was observed, indicating that the activation of an oxidase is not linked to a receptor. The release of H_2O_2 was correlated with accumulation of an aldehyde in the algal medium, suggesting that an oxidase is present in the apoplast of *G. chilensis*. The enzyme acted exclusively on agar oligosaccharides larger than disaccharides and was inhibited by reduced agar oligosaccharides. As revealed by native polyacrylamide gel electrophoresis, the expression of several isoforms of agar oligosaccharide oxidase was constitutive in *G. chilensis*. In contrast, elicitation of *G. conferta* with agar oligosaccharides resulted within 24 h in a strong expression of two isoforms of an agar oligosaccharide oxidizing enzyme, which were absent in nonelicited plants. Both species thus appear to be responding to agar oligosaccharides by releasing H_2O_2 but using different biochemical mechanisms. However, both responses display a defensive value. *G. chilensis* plants with a high potential for oxidation of agar oligosaccharides proved to be less susceptible to settlement by the epiphytic red alga *Acrochaetium* sp. than did plants with a low potential, and a single addition of agar oligosaccharides to the medium of *G. chilensis* also resulted in reduced settlement of *Acrochaetium* spores (Weinberger et al. 2005a).

The interaction between the red alga *Chondrus crispus* and its green algal endophyte *Acrochaete operculata* provides one of the most studied host-pathogen interaction in marine algae. *C. crispus* cell wall matrix polysaccharides include either κ- or λ-type carrageenans, which are found in gametophytes and tetrasporophytes respectively. These were shown to control endophyte penetration, with λ-carrageenans increasing and κ-carrageenans reducing the endophyte virulence (Bouarab et al. 1999). Contact of *A. operculata* with κ-carrageenans enhances secretion of the nitrogen storage compound L-asparagine (L-Asn), which in turn induces a release of H_2O_2 by *C. crispus* (Weinberger et al. 2002). This is an interesting example in chemical ecology where the induced signal of an attacker serves directly as a substrate for the production of a chemical defense metabolite. Only the presence of an amino acid oxidase that can produce elevated levels of H_2O_2 is required to control the early stages of the infection by *A. operculata*. The H_2O_2 concentrations generated by *C. crispus* in the presence of physiologically relevant amounts of L-Asn were shown to be sufficient to significantly prevent settlement of *A. operculata* zoospores (Weinberger et al. 2005b). However, this is not the only way in which C. *crispus* uses ROS in the control of *A. operculata*.

Gametophytes of *C. crispus*, when challenged with cell-free extracts of *A. operculata*, generate an oxidative burst. This is inhibited by DPI, an inhibitor of flavoenzymes such as NADPH oxidases. Moreover, when incubated with DPI the gametophytes lost their resistance to *A. operculata* infection (Bouarab et al. 1999). These results suggest the involvement of a NADPH oxidase homologue in the generation of the oxidative burst and demonstrate that the oxidative burst is an essential element in *Chondrus* immunity. The identity of this oxidase was recently elucidated.

A single copy gene encoding a homologue of respiratory burst oxidase gp91^{phox}, named *Ccrboh* has been identified from the red alga *C. crispus* (Hervé et al. 2006). A search performed using the *Ccrboh* gene in available algal genome and expressed sequence tag (EST) databases identified sequences showing common features of NADPH oxidases in other algae, such as the two diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseunonana*, the red, unicellular *Cyanidioschyzon merolae*, and macroalga *Porphyra yezoensis*, whereas no sequence showing similarity to *rboh* genes has yet been found in the brown alga *L. digitata* (Hervé et al. 2006). Indeed, phylogenetic analysis revealed that homologues of NADPH oxidases in red algae and diatoms constitute an independent cluster, which emerged early in evolution from a common ancestor of the ferric reductase and NADPH oxidases (Hervé et al. 2006). These results invalidate the hypothesis of Lalucque and Silar (2003), which postulated that NADPH oxidase constitutes the redox signaling system that contributed to the emergence of multicellular organisms in the course of evolution.

Elicitor- or stress-activated, DPI-sensitive oxidases, presumably membrane-bound NADPH oxidases, have been reported in *C. crispus* (Bouarab et al. 1999) and other red (Weinberger et al. 1999), as well as in brown (Küpper et al. 2001; Coelho et al. 2002) and green siphonous algae (Ross et al. 2005b, 2006). The reactions catalyzed by these enzymes were shown to control infections by pathogens, including bacteria (Weinberger and Friedlander 2000; Küpper et al. 2001, 2002) and endophytic filamentous algae (Bouarab et al. 1999; Küpper et al. 2002).

12.4 Oxidative-Burst-Associated Responses

12.4.1 Emission of Volatile Halogenated Organic Compounds

A rapid response, likely to constitute a chemical defense against foulers, pathogens, and herbivores in marine algae, is the emission of volatile halogenated organic compounds (VHOCs). Reminiscent of the oxidative burst of mammalian phagocytes, rapid and intense production of ROS following pathogen recognition in marine algae is concomitant with production of hypohalous acids, which can halogenate various organic substrates (Weinberger et al. 1999). In mammals, neutrophil activation results in the production of cytotoxic, bactericidal compounds that contribute to protection from invading organisms such as bacteria and fungi (Hampton et al. 1998). The combination of H_2O_2 production by an NADPH oxidase and the release of myeloperoxidase by the activated neutrophils results in the production of hypohalous acids, including hypobromous acid and hypochlorous acid (Gaut et al. 2001). In marine algae, an increased production of iodinated, brominated, or chlorinated organic compounds is associated with oxidative stress induced by excess light (Mtolera et al. 1996), ultra-violet (UV) exposure (Laturnus et al. 2004), or temperature changes (Abrahamsson et al. 2003). An increased production of

VHOCs was also observed in response to grazing pressure in *Ascophyllum nodosum* beds (Nightingale et al. 1995). Their biogenesis involves vanadium haloperoxidases (vHPO), which catalyse the oxidation of halides (X^-) and generate X^+ to yield hypohalous acid (XIO) and other forms of oxidized halides (X_2, X_3^-). Marine organisms and especially seaweeds have been known for a long time to concentrate halides from their environment (for a recent review, see Leblanc et al. 2006).

In the brown alga *L. digitata*, the oligoguluronate-induced oxidative burst is followed by a rapid increase in the emission of iodine-containing halocarbons and molecular iodine (I_2) (Palmer et al. 2005). Similarly, agar oligosaccharides caused H_2O_2 release, which resulted in an immediate increase in the brominating activity within the red alga *G. conferta* (Weinberger et al. 1999). This led to the bleaching of thallus tips described earlier (Friedlander and Gunkel 1992) and was similar to the symptoms of "ice-ice" white powdery disease in *Eucheuma* and *Kappaphycus* species (Lavilla-Pitogo 1992; Largo et al. 1995). Recent investigations in red algae have also shown that elicitation of an oxidative burst by agar oligosaccharides in *G. conferta* (Weinberger et al., unpublished results) and by pathogen extracts in *C. crispus* (Bouarab et al., unpublished results; Bouarab 2000) triggers an upregulation of VHOC production. Interestingly, when interactions involved molecules that provide substrates for agar oxidases in *G. chilensis* (Weinberger et al. 2005a) or amino acid oxidases in *C. crispus* (Weinberger et al. 2002, 2005b), production of VHOCs remained unchanged between control and challenged thalli of these species (Bouarab et al.; Weinberger et al., unpublished results). This suggests that membrane-associated ROS production and signaling events are required to provide additional substrates for vanadium haloperoxidases.

More recently, a novel type of halogenating enzyme, named hydroperoxide halolyase, which generates halogenated aldehydes, has been described in the marine diatom *Stephanopyxis turris* (Wichard and Pohnert 2006). In other microalgae, halogenation of organic compounds was shown to mainly involve methyl halide transferases (Moore et al. 1996; Manley 2002), and no vHPO has yet been identified on genomic data obtained from diatoms (Scala et al. 2002; Armbrust et al. 2004). Clearly, these emissions are not directly associated with an oxidative burst.

12.4.2 Lipid Peroxidation and Generation of Oxylipins

Lipids can be oxidized by some ROS such as hydroxyl (•OH), but not by H_2O_2, NO, or O_2•– (Halliwell and Gutteridge 1999). However, both enzymatic and nonenzymatic cellular mechanisms can oxidize fatty acids. Challenging *L. digitata* sporophytes with lipopolysaccharides from various sources resulted in a rapid release of free fatty acids (FFAs) with a concomitant accumulation of oxidized derivatives of linolenic (C18:2) and eicosapentaenoic acid (C20:5) (Küpper et al. 2006). Other strong inducers of the oxidative burst, such as oligoguluronates, in *Laminaria* could induce neither the release of FFAs nor the oxylipin production. These results

suggest that different signaling pathways are implicated in the control of oxylipin production depending on the nature of ROS inducers (Küpper et al. 2006). In *C. crispus* gametophytes, the oxidative burst induced by cell-free extracts of *A. operculata* cultures is followed by activation of lipases, which liberate free fatty acids to fuel lipoxygenase pathways (Bouarab et al. 2004). Fatty acid hydroperoxides and hydroxides are produced, which were identified by a combination of liquid chromatography and mass spectrometry (LC-MS) in the first study which identified a function for oxylipin in mediating algal innate immunity mechanisms (Bouarab et al. 2004). Two lipoxygenase isoforms, which were specific for the metabolism of linoleic acid, were upregulated following the oxidative burst in *C. crispus*, and lipoxygenase inhibitors abolished the natural resistance of *C. crispus* gametophytes, showing that induced resistance, which is dependent on the oxidative burst, involved downstream activation of the oxylipin pathways (Bouarab et al. 2004). Although the wound response of *G. chilensis* was not shown to be linked to an oxidative burst (see Sect. 12.4.3), it involved release of free fatty acids as well as the hydroxylated eicosanoids, 8*R*-hydroxy eicosatetraenoic acid (8-HETE) and 7*S*,8*R*-dihydroxy eicosatetraenoic acid (7,8-di-HETE). While the release of free arachidonic acid and subsequent formation of 8-HETE is likely controlled by phospholipase A, 7,8-di-HETE production is independent of this lipase. This dihydroxylated fatty acid might be directly released from galactolipids that contained 7-HETE or 7,8-di-HETE (Lion et al. 2006). Further investigations of oxidative-burst-related FFA release and oxylipin production in algae are required to fully understand the involvement of these pathways in the regulation of defense responses either as signaling or as antimicrobial compounds.

12.4.3 Phenolics, Cell-Wall Cross-Linking, and Responses to Wounding

Reminiscent of the deposition of phenolics in higher plants in response to pathogen attack, *L. digitata* plantlets inoculated with the epi-endophytic pathogen *Laminariocolax tomentosoides* show a strong, localized accumulation of UV-blue autofluorescent compounds in the cortical cells surrounding the penetrating spore germ tubes. In contrast, oligoguluronate-treated plantlets resistant to endophyte penetration exhibit a less intense, but ubiquitous, cortical accumulation, suggesting that synthesis of aromatic secondary metabolites such as phlorotannins is induced following elicitation of an oxidative burst in *L. digitata* (Küpper et al. 2002). Remarkably, *C. crispus* gametophytes synthesize UV-absorbing compounds around the sites of *A. operculata* zoospore penetration, whereas this response is absent in the sensitive tetrasporophyte generation (Bouarab et al. 2004 and unpublished data). Although the exact structure of these UV-absorbing compounds remains to be identified in *C. crispus*, analysis by high-pressure liquid chromatography (HPLC) confirms that they are aromatic in nature, suggesting the involvement of phenylpropanoid

metabolism. Indeed, *A. operculata* extracts and several oxylipins activate two key enzymes in the induction of this pathway, shikimate dehydrogenase and phenylalanine ammonia lyase (Bouarab et al. 2004 and unpublished results). A pharmacological approach using two inhibitors of these pathways, glyphosate and L-α-aminooxy-β-phenylpropionic acid (AOPP), consistently inhibited activation of these enzymes, prevented the accumulation of UV-fluorescent compounds, and abolished the resistance of *C. crispus* gametophytes to *A. operculata* (Bouarab et al., unpublished data). In the filamentous brown alga *Pylaiella littoralis,* cells infected by the biotrophic parasite *Chytridium polysiphoniae* accumulate fluorescent compounds, indicating that an increase in nonphotochemical quenching is concomitant with the onset of active defense mechanisms in the infected cells (Gachon et al. 2006).

Vreeland and Laetsch (1990) and Vreeland et al. (1998) proposed a brown algal cell wall synthesis model in which oxidative cross-linking of extracellular alginate and phlorotannin polymers leads to adhesion, cell wall strengthening, or both. Assuming this model is correct, targeted cross-linking of cell wall material by haloperoxidase catalysis requires H_2O_2, which as described earlier, is abundantly produced during oxidative bursts. In vitro cross-linked polyphenols have been obtained using purified vHPO in the presence of H_2O_2, bromide, or iodide (Berglin et al. 2004; Bitton et al. 2006), leading to cell wall strengthening. Although it is known that oxidative burst responses in terrestrial plants provide mechanical protection and activate the metabolism against pathogens (Brisson et al. 1994) or herbivores (Orozco-Cardenas and Ryan 1999; Orozco-Cardenas et al. 2001), there are no published reports of an oxidative burst in response to direct herbivore attacks in algae.

ROS production in the red alga *E. platycladum* (Collén et al. 1994) and in the green alga *D. vermicularis* (Ross et al. 2005a) has been monitored following mechanical injuries. The latter study emphasized the pharmacological inhibition of a DPI-sensitive, putative NADPH oxidase that contributes to ROS accumulation. *D. vermicularis* is a coenocytic alga that upon injury is able to rapidly form wound plugs to prevent cytoplasmic loss (Ross et al. 2005b). DPI inhibition of ROS production suggests that the second phase of wound repair is based on activation of a putative NADPH oxidase enzyme, 35 min after injury, leading to micromolar-level production of H_2O_2. This latent oxidative burst is proposed to be involved, through catalysis by peroxidases, in oxidative cross-linking of coumarins during wound plug hardening and browning (Ross et al. 2005a). More recently, *D. vermicularis* have been shown to also produce RNS, including NO, and pharmacological approaches have suggested that the signaling pathways leading to the production of ROS and RNS share similarities with defense and wounding signal transduction in higher plant systems (Ross et al. 2006). Although other mechanisms of wound-activated responses such as transformation of halimedatetraacetate or esterase-mediated transformation of caulerpenynine exist (reviewed in Pohnert 2004), it is possible that a wound-induced oxidative burst may represent a common theme in the defense strategies of siphonous green and other marine algae.

12.4.4 Gene-Regulated Responses

The capability to monitor gene-regulated responses is essential to identify the various steps that lead from the perception of elicitors to their activation of the oxidative burst machinery for defense induction. Powerful genomic approaches have now become available for the main algal models mentioned in this chapter and have allowed investigators to mine putative defense genes.

Exploitation of libraries of Expressed Sequence Tags (EST) to compare transcripts in macroscopic sporophytes of *L. digitat*a with those in microscopic gametophytes (Crépineau et al. 2000) revealed that the sporophyte library contained higher levels of a vHPO transcript when compared with those of the gametophyte library (Crépineau et al. 2000). Using these ESTs and complementary proteomic approaches for cDNA cloning, several genes of vanadium-dependent bromoperoxidase (vBPO) and iodoperoxidase (vIPO) have been characterized, representing multigenic families in *L. digitata* (Colin et al. 2003). One constitutive vIPO (Colin et al. 2003) is likely responsible for iodide oxidation and transport in *L. digitata* sporophytes (Küpper et al. 1998; Leblanc et al. 2006). A cDNA library, utilized for transcript analysis of *L. digitata* protoplasts that were exposed to severe oxidative stress, revealed numerous copies of a vHPO that is markedly different from the constitutive isoform (Roeder et al. 2005). These may have special defense-related functions such as the halogenation of organic compounds. Surprisingly, ESTs coding for other key ROS-scavenging enzymes such as catalase, superoxide dismutase, gluthation peroxidase, or ascorbate peroxidase were lacking from the libraries (Roeder et al. 2005) even though these enzymes are expressed under oxidative stress conditions in terrestrial plants (Mittler 2002).

As with *L. digitata*, EST analysis of protoplasts from *C. crispus* gametophytes yielded a high proportion of detoxification and heat-shock proteins (Collén et al. 2006a), validating this approach for discovery of stress-related proteins in algae. A total of 10,154 ESTs are currently available from the leafy gametophyte and 10,265 for the filamentous sporophyte of the red alga *Porphyra yezoensis* (Asamizu et al. 2003). As mentioned earlier, a *rboh* gene was identified in the gametophyte library and was used for cloning the *Ccrboh* homolog in *C. crispus* (Hervé et al. 2006). Interestingly, in comparison with that in control thalli, expression of *Ccrboh* is induced and maintained at higher levels for at least 24 h following the inoculation of *C. crispus* gametophytes with zoospores of *A. operculata*. Induction of *Ccrboh* mRNA accumulation occurred when germinating zoospores attempt to penetrate through the host cell wall, confirming the involvement of a DPI-sensitive NADPH oxidase in the defensive oxidative burst response (Bouarab et al. 1999).

The many genes generated by EST libraries from *C. crispus* have been used to construct the first cDNA microarray for a macroalga and it was first used for expression profiling of *C. crispus* after exposure to the plant stress hormone methyl jasmonate (MeJA) (Collén et al. 2006b). The study showed that 6% of the genes responded to the addition of MeJA and the most dynamic response was seen after 6 h. A comparison between different functional groups showed an upregulation of stress-related genes and a downregulation of genes involved in energy conversion

and general metabolism. Interestingly, the DHAP synthase gene, which is the first enzyme in shikimate pathway, is upregulated by MeJA. This confirms the regulation of secondary metabolite synthesis by the oxylipin pathway identified using biochemical approaches (Bouarab et al. 2004).

A DNA filter array has been constructed in order to provide the first characterization of the transcriptional reorganization occurring after oligoguluronate perception in *L. digitata* sporophytes. The array contains about 100 genes related to stress and defense as deduced in EST libraries from either the *L. digitata* thallus (Crépineau et al. 2000) or the protoplasts (Roeder et al. 2005) or obtained in a subtractive library from plants challenged with or without oligoguluronates (Cosse et al., unpublished results). This filter has been used to study the kinetics of gene expression in response to elicitors of the oxidative burst response, and several genes under regulation were validated by real-time PCR experiments. This includes several transcripts involved in the pentose phosphate pathway and coding for vHPO, indicating that they are clearly important for establishing defense responses (Cosse et al., unpublished results). This first DNA filter array is currently being used to develop strategies to monitor defense-gene-regulated responses in field studies.

12.5 Functions of the Oxidative Burst in an Ecological Context, the Hallmark of Parasite or Disease Resistance

12.5.1 Ecological Functions of the Oxidative Burst

From the previously discussed studies, the oxidative burst response appears to integrate and/or orchestrate several defense responses. These provide an adaptive mechanism to counteract attacks on or injury to algal cells and thalli. As shown in Fig. 12.2, the occurrence of an oxidative burst associated with the perception of cell-free extracts of *A. operculata* was shown to play a crucial role in the natural resistance of *C. crispus* gametophytes (Bouarab et al. 1999).

In the kelp *L. digitata* (Fig. 12.3), the integration of diverse downstream responses to the oxidative burst was shown to control the growth of epiphytic, potentially pathogenic bacteria (Küpper et al. 2001, 2002). Elicitation with oligoguluronates also increased resistance of the alga to infection by its brown algal endophyte *Laminariocolax tomentosoides* (Küpper et al. 2002). Interestingly, programmed cell death (PCD), which is associated with hypersensitive response (HR) in higher plants, is not observed in response to a challenge of *L. digitata* with oligoguluronates, whereas it was described in *L. japonica* infected with alginic decomposing bacteria (Wang et al. 2004). This suggests that PCD in inoculated *L. japonica* may be induced by bacterial effectors or upon recognition of PAMPs (Abramovitch et al. 2006).

In the red alga *G. conferta*, agar oligosaccharide-induced oxidative bursts also result in the elimination of the bacterial epiflora (Weinberger and Friedlander 2000). Since a positive correlation was observed between the presence of agarolytic

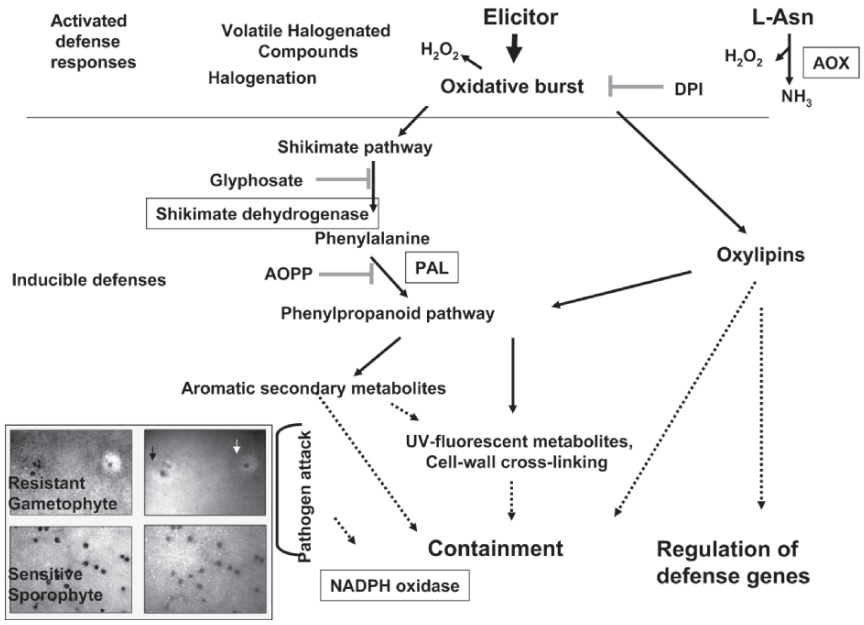

Fig. 12.2 Simplified scheme of defense reactions deciphered from studies of the interaction between the red alga *Chondrus crispus* and the green algal pathogenic endophyte *Acrochaete operculata*. Recognition of pathogen-derived elicitors triggers a diphenylene-iodonium (DPI)-sensitive oxidative burst and the production of signaling molecules, which either induce defense-specific biochemical pathways or activate halogenation reactions which directly affect pathogenic cells. In contrast, L-Asn provides a substrate for the AOX enzyme to yield H_2O_2 and NH_3 as by-products of amino acid oxidation. As illustrated in the *inset*, UV-fluorescent compounds accumulate in *C. crispus* gametophytes at the sites of attempted penetration by *A. operculata* zoospores, whereas sensitive sporophytes do not display any fluorescence. *PAL* phenylalanineammonia lyase, *AOPP* L-α-aminooxy-β-phenylpropionic acid, *AOX* L-amino acid oxidase

epiphytes and bacterial pathogenicity against *Gracilaria* spp. (Schroeder et al. 2003), it is clear that this mechanism provides a way to prevent the effect of virulence factors, such as bacterial exoenzyme agarase in these algae. Red algal halogenated furanones were recently reported to inhibit expression of exoenzyme virulence factors both in the phytopathogenic bacterium *Erwinia carotovora* (Manefield et al. 2001) and in the human pathogen *Pseudomonas aeruginosa* (Hentzer et al. 2003). Similar mechanisms are likely to occur with algal bacterial pathogens. In mammals, neutrophil activation results in the production of cytotoxic, bactericidal compounds that contribute to protection from invading foreign organisms such as bacteria and fungi (Hampton et al. 1998). The combination of H_2O_2 production by an NADPH oxidase and the release of myeloperoxidase by activated neutrophils results in production of hypohalous acids, including hypobromous acid and hypochlorous acid (Gaut et al. 2001).

Similarly, DPI-sensitive activation of H_2O_2 in marine algae and the concomitant formation of volatile and nonvolatile halogenated compounds are likely to play a

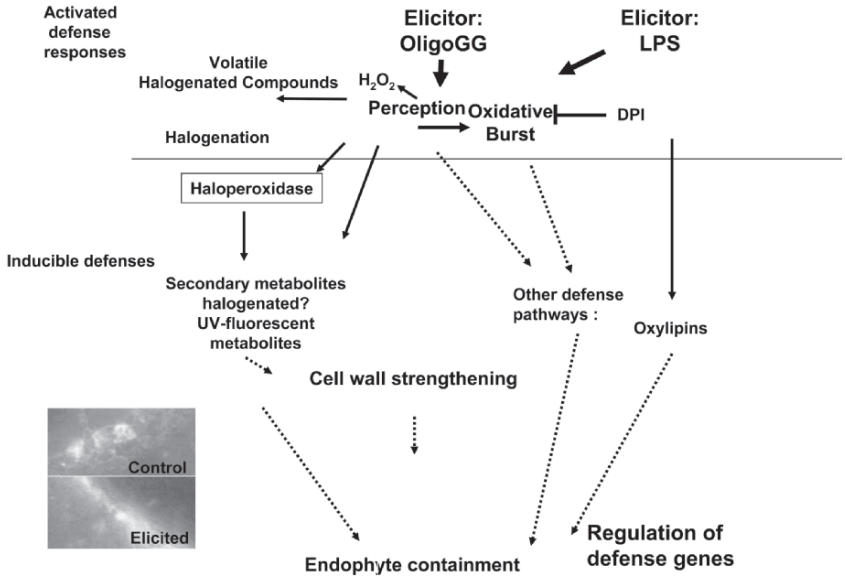

Fig. 12.3 Simplified scheme of defense reactions deciphered from studies of the oligoguluronate-induced oxidative burst in the brown alga *Laminaria digitata* in its association with the brown algal pathogenic epi-endophyte *Laminariocolax tomentosoides*. Recognition of cell-wall released oligoguluronate triggers a diphenylene-iodonium (DPI)-sensitive oxidative burst and production of signaling molecules, which either induce defense-specific biochemical pathways or activate halogenation reactions which directly affect pathogenic cells. In addition, lipopolysaccharide (LPS) triggers a DPI-sensitive oxidative burst and the production of oxylipins. In the *inset*, *L. digitata* plantlets inoculated with the epi-endophytic pathogen *L. tomentosoides* show a strong, localized accumulation of UV-blue autofluorescent compounds in cortical cells surrounding the penetrating spore germ tubes, whereas the oligoguluronate-treated plantlets resistant to endophyte penetration exhibit a less intense, but ubiquitous cortical accumulation

critical role in the defense systems of marine organisms. The correlation between stress-activated oxidative metabolism and increases in VHOC emission has led some authors to propose that halogenated compounds are only secondary waste products of ROS detoxification processes (Pedersén et al. 1996). Their specific selection as defensive compounds in algae has been controversial for a long time even though a number of these compounds are known to have potent antibiotic activities (Wever et al. 1991; Manley 2002). Algal halogenated compounds were shown to interfere with bacterial signaling systems such as the quorum sensing (QS) of Gram-negative bacteria and to induce the dispersal of bacterial biofilms (see Chap. 11). A natural haloperoxidase system from *L. digitata*, producing hypohalous acid (XOH) and X_2 plus X_3^-, was shown to inactivate acylated homoserine lactones (AHLs), which mediate QS communication between Gram-negative bacteria (Borchardt et al. 2001). Recent investigations of iodovolatilization, (i.e., molecular iodine emission and VHOCs emission) have shown that it follows oxidative stresses (Palmer et al. 2005). It is tempting to speculate that in iodine-concentrating brown

algae such as *L. digitata*, the oxidative bursts and concomitant iodine efflux in the presence of extracellular vHPOs are part of a very efficient early defense response that eliminates or controls microbes using molecular iodine. I_2 is formed chemically by oxidation of I^- in the presence of H_2O_2, but its rapid formation can be catalyzed by vHPOs. Strong antiseptic activities of iodine species have been known for a long time, and aqueous or alcoholic povidone-iodine solutions are commercially available as disinfectants. As I_2 is the most reactive chemical form of iodine, it provides the highest biocide potential (Gottardi 1999). Ohsawa et al. (2001) demonstrated that bromoform ($CHBr_3$) produced by the red calcareous algae *Corallina pilulifera* and *Lithophyllum yessoense* could eliminate epiphytic organisms, especially diatoms, from the macroalgal surface and this antiproliferating effect was dependent on vBPO enzyme activity. In the red alga *Asparagopsis armata*, $CHBr_3$ and dibromoacetic acid are the dominant brominated compounds among an impressive number of metabolites (Kladi et al. 2004) stored in specialized gland cells (Paul et al. 2006a). When released at the surface of the thallus, they displayed antibiotic activity against epiphytic bacteria (Paul et al. 2006b). They also function as mesograzer feeding deterrents as inferred from the increased consumption of bromide-starved algal cultures (Paul et al. 2006c). In *C. crispus* gametophytes, three VHOCs were upregulated after defense elicitation and were toxic to spores and germlings of *A. operculata* (Bouarab et al., unpublished results). Indeed, these compounds fulfil all criteria necessary to assign a natural antifouling role: they provide resistance to epi-/endophytism, they are produced on the surface, and they affect spore settlement at the thallus surface. In red algae, VHOCs seem to play an important physiological role in activated defense responses, acting as biocidal or repelling substances against microorganisms and herbivores, and also in controlling the development of epi-/endophytic, parasitic, or pathogenic algae.

Thus, together these studies suggest that the oxidative burst machinery has evolved before the crown diversification of eukaryotes (Baldauf 2003) to provide marine algal lineages with natural and induced innate immunity mechanisms. These play a role similar to the HR in terrestrial plants infected by incompatible pathogens and they share important common traits with the innate immunity response of mammalian phagocytes.

12.5.2 Toward New Approaches to Test the Ecological Relevance of Oxidative-Burst-Associated Responses

Monitoring the occurrence and dynamics of an oxidative burst in natural populations and testing its functions in field experiments is not a simple task. Some indirect evidence of the occurrence of oxidative bursts may be inferred from chlorophyll fluorescence parameters (Collén and Davison 1999a, 1999c; Gachon et al. 2006) and/or lipoperoxide accumulation, but it will be difficult to distinguish these responses from other environmental stresses. Indirect approaches need to be developed to investigate the impact of an oxidative burst on the fitness of algal species.

An epidemiological survey of complementary symptoms, reminiscent of the HR of terrestrial resistant plants, such as tip bleaching or accumulation of fluorescent aromatic compounds around the site of penetration of parasite propagules (Figs. 12.2 and 12.3), may facilitate the evaluation of the impact of host-microbe interactions in natural populations (Correa and Sanchez 1996; Ellertsdottir and Peters 1997; Bouarab et al. 2001a). This could allow one to test the importance of oxidative-burst-associated defense reactions in various biotic interactions.

A sophisticated, but likely more precise approach would be to develop comparative monitoring of defense-responsive gene expression patterns in controlled mesocosm experiments or in natural populations experiencing varying grazing or fouling intensities or infectious diseases. Using quantitative screening methods for the induction of transcripts identified as being upregulated, stable, or downregulated, one might identify gene networks and the interplay of defense pathways in response to specific pests. As mentioned earlier, development and optimization of screening tools, such as dedicated DNA chips or filter arrays, has been conducted in *C. crispus* and *L. digitata* (Collén et al. 2006b). These new molecular tools are available to start assessing patterns of differential gene expression and to identify major defense-responsive genes whose expressions vary within a population or are dependent on environment cues. These new approaches will also benefit the development of quantitative high- or medium-throughput methods for metabolite profiling of several individuals or within a population (La Barre et al. 2004). Knowledge of the complete genome of the brown alga *Ectocarpus siliculosus* by the end of 2007 (Peters et al. 2004) should also be of enormous help in identifying new candidate defense genes and in identifying gene function in mutants impaired in the resistance against pathogens or grazers. Furthermore, the availability of pangenomic DNA microarrays will be an important tool for investigating the signaling function and pathways of defense regulators, including waterborne signals (Toth and Pavia 2000). Although this favors *Ectocarpus* as a model alga for research in defence signaling, there is no doubt that some of the available molecular and genetic tools, such as invalidation of master or specific genes in other ecologically relevant species, will allow an integration of molecular ecology with more classical approaches in marine chemical ecology.

12.6 Conclusions

This chapter was an attempt to summarize how much important work has been done in the context of the oxidative-burst-associated responses in algal cells. The past decade has witnessed a substantial progress in our understanding of ROS metabolism (Dring 2006), the generation of halogen oxidants (Leblanc et al. 2006), and the oxidative metabolism of fatty acids in algae (Bouarab et al. 2004; Küpper et al. 2006). These active defense pathways against pathogens and possibly foulers and herbivores are likely to be important in terms of control of ecological interactions and in contributing a major role in the structure and function of ecosystems

based on macroalgal communities. Until recently, a common view of algal defense mechanisms was based on a lack of cell-based immunity mechanisms in algae. As it is true that algae, like terrestrial plants, do not display acquired immunity specialized cells, they have evolved several innate immunity traits that are conserved with other eukaryotes and that provide them with an efficient system to cope with pathogen aggression and to fight diseases. The dynamics of these activated and induced defense responses, most often orchestrated by an oxidative burst and/or by RNS production, is a key element to take into account in the study of the ecological roles of defense strategies based on the production of secondary metabolites (Chap. 11).

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