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New Perspectives for Addressing Patterns of Secondary Metabolites in Marine Macroalgae

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6.1 Interpreting Patterns of Chemical Defense in the Marine Environment

Natural products can structure relationships between organisms, affect resource allocation, influence competition, mediate species distributions, and select for traits leading to the potential diversification of species (e.g. see reviews by McClintock and Baker 2001; Potin et al. 2002; Pohnert 2004; Ianora et al. 2006). Although there have been numerous efforts to determine patterns of metabolite distribution in marine ecosystems, particularly in macroalgae, these efforts have focused primarily on a macroscale: global patterns, patterns within specific habitats (e.g. the intertidal zone), and patterns correlated with changes in biotic and abiotic factors. Work in terrestrial ecosystems has led to the development of ecological models that describe trade-offs, costs, and benefits, and have been applied to marine algal systems (e.g. Cronin 2001; Jormalainen et al. 2003; Honkanen and Jormalainen 2005; Dworjanyn et al. 2006b; Ianora et al. 2006). However, the response of algal secondary metabolites to stimuli is a complex process, and these models do not consistently predict patterns of macroalgal metabolite production.

Understanding this inherent complexity mandates the use of an approach with multiple perspectives that resolves the mechanisms behind regulation, expression, accumulation, localization, and transport of secondary metabolites. Macroscale patterns (e.g. global distribution patterns of secondary metabolites within or across algal taxa, or apparent predispositions within certain algal groups toward particular defense responses) are a direct function of what we are defining as microscale phenomena: molecular and biochemical processes occurring within an alga, the spatial distribution of compounds within the alga, and the varied temporal responses

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exhibited by an alga in the environment. It is the combination of factors at the microscale – within the cell (genetic history and biochemical machinery), within the thallus (differential distribution), and over time (short- and long-term responses) – that ultimately sculpts observed chemical profiles.

This review will primarily focus on "patterns" of chemical defense from the microscale perspective (cellular, subcellular, molecular, and temporal), exploring spatial and temporal patterns of response, as well as the innate molecular information and biochemical mechanisms involved in secondary metabolism that exist across different algal divisions (for additional reviews of various patterns of algal chemical defense, see Hay and Steinberg 1992; Van Alstyne et al. 2001a; Potin et al. 2002; Pohnert 2004; Amsler and Fairhead 2006; Ianora et al. 2006).

6.2 A Brief Review of Macroscale Patterns of Algal Chemical Defenses

Over 16,000 natural products have been characterized from marine organisms (Bhakuni and Rawat 2005), and include isoprenoids (e.g. terpenes, sterols, and quinones), eicosanoids (e.g. polyunsaturated aldehydes, algal hormones), phenolic compounds, polyketides, alkaloids, acetogenins, and compounds of mixed biogenesis (see Chap. 1). Marine natural products have a greater number of halogenated compounds and alkaloids relative to terrestrial systems. Specific metabolite classes may show prevalence within one of the three main macroalgal divisions: phaeophytes synthesize phlorotannins; rhodophytes produce halogenated compounds, primarily isoprenoids and acetogenins; chlorophytes produce an abundance of terpenoid compounds; however, there is also overlap of metabolites between algal divisions. Within each compound class small changes in similar structures (e.g. modified side groups) result in a broad diversity of marine natural products (e.g. Blunt et al. 2006; Moore 2006; Nicholas and Phillips 2006; see Chap. 1). Understanding the differences in ecological function resulting from the structural variations, and the factors that influence these changes in structure are important in considering patterns (both spatial and temporal) of metabolite production in algae.

Secondary metabolites in marine systems are commonly found from the tropics to the poles in varying concentrations (Van Alstyne and Paul 1990; Steinberg and Van Altena 1992; Targett et al. 1992; Amsler et al. 2005; Fairhead et al. 2005, see Chaps. 2–4), suggesting that local factors are as important as any latitude-associated factor in determining metabolite concentrations. This is contrary to terrestrial systems, where vigorous studies have shown that chemical and morphological traits vary with latitude (i.e., higher palatability of temperate plants, lower palatability of tropical plants) (Salgado and Pennings 2005; Pennings et al. 2007). Large-scale geographic surveys of marine secondary metabolites tend to obscure the contribution of local factors, yielding high levels of variability and no obvious patterns (Pavia and Åberg 1996). This variability is likely due to numerous microhabitats with different suites of selective forces leading to diverse and specific macroalgal chemical profiles. For example, the alga *Stypopodium zonale*, a member of the

brown algal family Dictyotaceae, produces diterpenes in both temperate and tropical populations (White and Jacobs 1983; Obrien et al. 1984; Gerwick et al. 1985). The dominant diterpene metabolite produced varies according to geographic location, with atomaric acid in temperate locales and stypoldione in tropical locales (e.g. Pereira et al. 2004). Both compounds showed activity against two different grazers; however, the bioactivity of atomaric acid, a dominant "temperate" diterpene, was more deterrent to the herbivores tested than was the "tropical" stypoldione. In this study, the bioactivity was correlated with the unique chemical structure, which in turn appears dependent on local selection factors. Similarly, minor modifications to side chain groups of furanones resulted in significantly different antifouling activities (Dworjanyn et al. 1999), while phlorotannins exhibit varied bioactivity based upon their chemical structure and molecular size (Steinberg 1988; Boettcher and Targett 1993; Dworjanyn et al. 1999). Thus, it is clear that the efficacy of secondary metabolites in mediating ecological interactions is dependent on their specific chemical structure, which in turn is affected by the forces at work in the local environment. Abiotic and biotic factors that structure microhabitats can select for subtle changes in metabolite structure and quantity, both of which relate to bioactivity.

The relationship between the specific structure and activity of natural products and their ecological function has been established in terrestrial systems. For example, phenylpropanoids may serve different ecological/physiological functions, and their relative abundances vary according to elicitors present and/or environmental conditions; there is no "catch-all" response. Plants will produce anthocyanins in response to freezing, but not to pathogen attack, during which coumarins might be produced in higher quantities (Dixon and Paiva 1995). These compounds, although relatively similar in structure, and derived from branch points of the same metabolic pathway, appear to have highly specific bioactivities. Both the marine and terrestrial studies cited earlier illustrate the need for understanding microscale aspects of secondary metabolites, including chemical structure of the compounds produced in response to specific environmental conditions and the biosynthetic mechanisms involved. Subtle differences in metabolite structure can result in significant changes in bioactivity at the organismal level and beyond.

Inducible and activated defenses complicate the search for universal patterns of secondary metabolites and continue to highlight the importance of localized phenomena. The ability of exogenous cues to induce the production of secondary metabolites was first reported in marine algae by Van Alstyne (1988) when phlorotannin production in *Fucus distichus* was induced by natural and artificial grazing. In brown algae, induced responses in the form of compound production or modified herbivore behavior have since been observed in response to herbivory (e.g. Van Alstyne 1988; Cronin and Hay 1996a; Pavia and Toth 2000; Sotka et al. 2002; Borell et al. 2004; Rohde et al. 2004; Weidner et al. 2004; Macaya et al. 2005; Toth et al. 2005; Molis et al. 2006), artificial grazing and wounding (Van Alstyne 1988; Peckol et al. 1996; Hammerstrom et al. 1998; Lüder and Clayton 2004), waterborne cues (Toth and Pavia 2000; Rohde et al. 2004; Macaya et al. 2005), volatile cues (Arnold et al. 2001; Pelletreau et al., unpublished data), and UV light (Pavia et al. 1997). However, induction of defense is not always detected, even in response to these same triggers (e.g. Pavia and Toth 2000; Toth and Pavia 2002; Dethier et al.

2005; Macaya et al. 2005; Fairhead et al. 2006; Molis et al. 2006). Induction of terpenoids in brown algae has also been reported (Cronin and Hay 1996a); however, this phenomenon has not received as much attention as the induction of phlorotannins.

In red algae induced response to herbivory was first observed in *Pterocladia capillacea* after exposure to grazing by amphipods (Weidner et al. 2004). Observed reduction of grazing on the same tissue in subsequent experiments implied initial grazing caused a chemical change, rendering the alga less palatable. Subsequent work (Ceh et al. 2005) showed that grazed tissue of *Hypnea pannosa* reduced amphipod consumption. For both *P. capillacea* and *H. pannosa*, amphipod herbivory on the induced tissue resumed after 2 weeks. Similarly, Diaz et al. (2006) found that previously grazed red alga *Galaxaura diessingiana* elicited significant reduction in herbivory relative to the nongrazed pieces, yet two additional red algal species, *Hypnea spicifera* and *Gracilaria capensis,* showed no response to grazers. Further work exploring the presence and magnitude of induced responses in rhodophytes is warranted. Induction of secondary metabolites in the chlorophytes has been documented only by Diaz et al. (2006); they found that terpenoid compounds in *Codium platylobium* were induced following the alga's exposure to waterborne cues of grazed conspecifics.

Two recent meta-analysis studies (Toth and Pavia 2007, and Chap. 3) have aided in clarifying patterns of induced defenses in macroalgae. These studies determined factors that do not play a role in inducing herbivore response (i.e., type of elicitor, artificial vs. natural food), highlighted factors that are important (i.e., size of the herbivore used and the timescale of the experiment), and illustrated distinct differences between algal groups in their ability to induce responses as observed through herbivore reactions (Toth and Pavia 2007, Chaps. 3 and 7).

In contrast to inducible defenses, activated defenses are common in chlorophytes (Paul and Van Alstyne 1992; Cetrulo and Hay 2000; Pohnert and Jung 2003), have been documented once in rhodophytes (Van Alstyne et al. 2001b), and are not yet verified in the phaeophytes (see Cetrulo and Hay 2000). Activated defenses occur when mechanical damage (such as grazing) alters the defense compound to produce a more toxic compound (Paul and Van Alstyne 1992). Examples of activated terpenoid compounds include the following: caulerpenyne in *Caulerpa* spp., which converts into oxytoxins; and halimedatetraacetate in *Halimeda* spp., which converts into the more toxic halimedatrial (Cimino et al. 1990; Paul and Van Alstyne 1992; Gavagnin et al. 1994; Jung and Pohnert 2001). The conversion of dimethylsulfoniopropionate (DMSP) to acrylic acid and dimethyl sulfide is another example common in ulvoids (see Chap. 9).

Specificity of molecular bioactivity and differentially induced defenses are only two examples of factors that can confound the interpretation of patterns at the macroscale. As our knowledge of marine systems continues to expand, the relative abundance of secondary metabolites in different geographic locations may be better understood. However, the literature supports the idea that local pressures and habitat, genetic composition, mode of response and metabolism of the algae play a significant role in shaping distribution patterns of secondary metabolites (e.g. Wright

et al. 2000, 2004; Edwards et al. 2006). Large-scale geographic patterns may be hard to deduce in light of the numerous potential microhabitats that likely generate varied chemical compositions within and between algal classes (cf. Pavia and Åberg 1996). Focusing research on metabolite production at a finer resolution and applying innovative molecular and biochemical techniques will better shape our understanding of the essential factors and selective forces involved in macroalgal secondary metabolism.

6.3 Patterns of Secondary Metabolites at the Microscale

6.3.1 Metabolite Distribution Within the Thallus

Studies that address the site of production, the transport, and the deposition of secondary metabolites provide another level of resolution that needs to be considered when evaluating the function of secondary metabolites and their patterns of distribution. Unlike approaches that quantify an alga's total metabolite concentration, studies that localize metabolites contained within algal tissues provide evidence of more subtle changes in metabolite distribution in response to stimuli and may clarify metabolite function.

The data from these studies support the hypothesis that secondary metabolites in marine algae have functions other than, or in addition to, herbivore deterrents. Localizing metabolites within certain regions of the thallus may have a minimal effect on large grazers that will potentially consume the entire algal thallus, and thus do not preferentially select tissue types or cell layers. Surface oriented distribution patterns imply defense against pathogen or bacterial colonization, epiphytism, mesograzers (small immobile herbivores), or protection against abiotic influences.

Although algae are under constant threat of bacterial infection, there are few observations of infections in vivo, which have made macroalgae the focus of intense research regarding antimicrobial or antifungal compounds (Smit 2004). Although it is assumed that macroalgae contain antimicrobial compounds, the nature of these metabolites and their location within an alga remain largely unknown in most algal species. In differentiated terrestrial plants, metabolites can be stored in vesicles, gland cells, cavities, canals, or other specialized structures (Fahn 1988; Duke et al. 1994; Wink 1997), and their distribution and localization within the plant have direct effects on their ecological function (Bernays and Chapman 1994). Although macroaglae may not have differentiated tissues, they do have particular cell types and have shown differential patterns of metabolite distribution within the thallus. To elucidate ecological function of some secondary metabolites, work has turned to determining the composition and concentration of algal surface metabolites, their site of production, and modes of delivery (reviewed by Steinberg et al. 2002 and the text that follows).

In brown algae, phlorotannins are localized in specialized bodies called physodes (Ragan 1976). Shifting the experimental approach, from chemical assays of total phlorotannin concentration to microscopic methods that describe physode transport and establish the timeframes at which phlorotannins accumulate in response to abiotic or biotic stimuli, has provided new insight into the understanding of phlorotannin production and function. It is known that physodes are derived from the endoplasmic reticulum (ER) and Golgi of the cell (Schoenwaelder and Clayton 2000). It appears that physodes are transferred across the cytosol and incorporated into the cell wall, where the phlorotannins are assumed to have a structural role and thus be involved in primary metabolism (Schoenwaelder and Clayton 2000; Arnold and Targett 2003).

Chemical measurements of tannin content in several brown algae have implied an induced response of phlorotannins in response to wounding (e.g. Van Alstyne 1988; Peckol et al. 1996; Hammerstrom et al. 1998). A recent combined microscopy approach was able to detect a rapid and dynamic production of phlorotannins within cells after wounding of proximal tissue (Lüder and Clayton 2004). Phlorotannin-containing physodes accumulated around the site of injury and extended outward. This accumulation was apparent as early as 24 h after wounding and was observed throughout the 9-day experiment (see Fulcher and McCully 1971). The active accumulation and mobilization of the phlorotannin pool were clearly demonstrated using light, fluorescence, and electron microscopy. Although this was considered to be a strong response on the part of the alga, the amount of phenolic accumulation could not have been detected via traditional spectrophotometric methods, even with their increased sensitivity (Stern et al. 1996). In this case, the application of combined microscopy clearly illustrated a sophisticated defense response in brown algae. It is possible that in many cases where phlorotannin induction has not been observed, it indeed occurred, but at levels too low to be detected with current methods.

Physodes accumulate in the outer region of mature brown algae, such as the epidermis and the cortical cell layers, as well as in meristematic or apical cells. Localization in these cell layers implies a defensive role against biotic (herbivores/pathogens) and abiotic (UV light) factors. *Hormosira banksii* develops a protective dark brown layer or "sunburn" which consists of oxidized phenolics leached from dead cells on the algal surface upon exposure to intense light treatment (Schoenwaelder 2002). Shibata et al. (2004) also found that the phlorotannins in the brown algae *Eisenia bicyclis* and *Ecklonia kurome* accumulated in the outer cortical cell layer, and were exuded only upon cell death. Neither phlorotannins, nor their monomeric building block 1,3,5-trihydroxybenzene, was secreted from healthy cells, but other phenolic compounds (2,4-dibromophenol and 2,4,6-tribromophenol) were secreted (Shibata et al. 2006) and exhibited some of the same functionalities as phlorotannins (e.g., UV absorbing capabilities). Studies have shown that phenolic compounds exuded into the surrounding seawater may serve a bioactive role in this capacity, perhaps as surface settlement inhibitors or sunscreens (Ragan and Glombitza 1986; Swanson and Druehl 2002).

The localization of several defensive compounds in red algae has clarified their ecological function, in a manner analogous to studies on terrestrial plants. The red alga *Delisea pulchra* is known for its production of halogenated furanones, which are implicated in a range of activities from herbivore deterrents to settlement inhibitors (Maximilien et al. 1998; Dworjanyn et al. 1999). Dworjanyn et al. (1999) combined microscopy techniques with chemical methods and culture studies to relate furanone function to algal biology. Fluorescence microscopy verified that furanones are stored in the large vacuole of specialized gland cells. The number of gland cells, the furanone concentration at the surface of the thallus, and the total furanone content was highest at the growing tip and lowest toward the base of the alga (Dworjanyn et al. 1999). The concentration of furanones in young actively growing tissue is consistent with the optimal defense theory (ODT; see Chap. 7), and suggests a protective role of furanones in the young tissue. The gradient of furanones from the surface toward the interior of the thallus was likely bactericidal or algicidal, since the concentration of furanones (~100 ng cm^{-2}) was well above a biologically active level (de Nys et al. 1995; Maximilien et al. 1998). And at biologically relevant concentrations, *D. pulchra* extracts, furanone fractions, and pure furanones significantly reduced settlement success by *Ulva* sp., *Ceramium* sp., *Polysiphonia* sp., and *Ectocarpus siliculosus* – common fouling macroalgae (Dworjanyn et al. 2006a). The red alga *Asparagopsis armata* contains more than 100 described halogenated compounds (McConnell and Fenical 1977) and specialized gland cells with tubular connections to a parent cell wall. This connection facilitates transport of halogenated compounds to the algal surface without cell damage (Paul et al. 2006a, b). The dominant halogenated compounds detected in the alga, bromoform and dibromoacetic acid, were detected in surrounding growth media, supporting the theory that these compounds are actively transported to the external region of the alga and secreted, likely as defense compounds.

Combining microscopy and traditional biochemical methods to examine the multiple factors involved in metabolite production and function, i.e., abundance of gland cells, the alga's ability to produce metabolites and form protective transport mechanisms, and the efficacy of specific metabolites against colonization, enabled the generation of a more thorough understanding of the ecological role of halogenated compounds in red algae (Dworjanyn et al. 1999, 2006a; Paul et al. 2006a, b). Lüder and Clayton (2004) observed de novo synthesis of tannin-containing physodes at wound sites, indicating that physodes are not transported from other parts of the alga. Adapting innovative methods to the study of secondary metabolites is a new area of exploration that will allow us to determine whether an alga has the ability to generate defense compounds in situ, or must transport compounds to specific sites in response to environmental stimuli. Understanding these cellular functions, i.e., the life of secondary metabolites within the cell and within the alga and the factors that affect the mobilization and production, will clarify patterns of distribution within the algal thallus, as well as variable patterns between individuals. Hence, subcellular phenomena involving secondary metabolites within algal cells have direct impacts on the observed patterns of distribution at broader perspectives.

6.3.2 Temporal Responses Within Algae: Seconds to Weeks

Timescales of defense responses, such as the variation in time to initiation and/or duration of response, affect patterns of distribution and abundance within an alga. Investigations conducted on a longer scale of days to weeks overlook short-term (<1 day) responses within the alga, such as the oxidative burst, (the rapid production and excretion of reactive oxygen species), signaling cascades, and gene transcription and translation. The synthesis, transport, bioaccumulation, and secretion of metabolites occur on longer timescales. With the exception of activated defenses, which have not been reported in brown algae, responses on various timescales ranging from seconds to weeks appear in all three algal divisions. The apparent pervasiveness of certain responses within algal groups, such as the induction of phlorotannins in the Fucales for several days to weeks, or the oxidative burst in the Laminariales, is most likely due to intensive study on certain phenomena in a few species and may not represent the overall prevalence of these mechanisms within certain algal groups.

Short-term, rapid responses of macroalgae in response to environmental stimuli are now well documented (Table 6.1). It is probable that many of the long-term responses studied to date, such as the accumulation of phlorotannins, are tightly linked to events on these shorter timescales. Oxidative burst appears to be a common physiological response within macroalgae (e.g. Collén and Pedersen 1994; Küpper et al. 2002, 2006; Ross and Van Alstyne 2007, see Chap. 12). For example, brown algae recognize self (alginate) and nonself (lipopolysaccharides) elicitors via a rapid induction of the oxidative burst response (Küpper et al. 2001, 2002, 2006). The efficacy of the oxidative burst against bacterial infection also has been shown in several studies (Küpper et al. 2002, 2006; Potin et al. 2002) and appears to be linked to other short-term responses such as fatty acid oxidation and the triggering of oxylipin cascades, similar to immune responses seen in higher plants (Bolwell et al. 1995; Wojtaszek 1997).

Investigations at these short timescales (within seconds) in marine systems are not numerous primarily because of methodological constraints. However, advancements in methodology may lead to discovery of this defense response in many algal species, where the capacity for rapid self-defense against infection would confer a selective advantage. The oxidative burst has been found in organisms ranging from bacteria to mammals, and its detailed mechanisms (signaling, gene information) have been well defined (e.g. Lamb and Dixon 1997; Pohnert and Boland 2002; Mahalingam and Fedoroff 2003; Halliwell and Gutteridge 2007). This established knowledge of the oxidative burst in other organisms has been directly applied to algae and has led to a rapid and thorough understanding of the mechanisms at work (e.g. Dring 2006; Lesser 2006 and references within). In contrast, some of the long-studied phenomena in algae (e.g. phlorotannin production in Fucales) remain uncharacterized, likely because of lack of the same mechanisms in better studied organisms.

It may be that temporal responses to environmental pressures occur in tandem at both the short and long terms, but further investigations are needed to confirm such

Table 6.1 Types of rapid defenses within the different macroalgal divisions, the relative response time of each mechanism, and the elicitor used in each study

Defensive mode of action	Phylum	Species	Time of response	Defense against	Source
Activated defense	Chlorophyceae	*Halimeda* spp.	Seconds	Wounding	1
		Caulerpa taxifola	Seconds	Wounding	2
		Ulva fenestrate	10 min	Wounding	3
	Rhodophyceae	*Polysiphonia hendryi*	10 min	Wounding	3
Oxidative burst	Phaeophyceae	*Laminaria* spp.	15 min	Oligogluronates	4
		Fucus vesiculosus	10 min	Oligogluronates	4
		Laminaria digitata	30 min	Pathogenic algae	4
		L. digitata	60–90 min	Bacterial LPS	5
	Chlorophyceae	*Dasycladus vermicularis*	35–45 min	Wounding	6
		Acrochaete aperculata	10 min	λ-Carrageenan	7
	Rhodophyceae	*Chondrus crispus*[a]	20 min	Pathogenic algae	7
		Gracilaria conferta	30 min	Pathogenic bacteria	8
		Eucheuma platycladum	10 min	Wounding	9
Fatty acid oxidation and oxylipin pathway[b]	Phaeophyceae	*L. digitata*	30–60 min	Bacterial LPS	5
	Rhodophyceae	*C. crispus*[a]	24 h	Pathogenic algae	10
		C. crispus[a]	24 h	Methyl jasmonate	10
		Gracilaria chilesis	30 min	Wounding	11

LPS lipopolysaccharides
1 Paul and Van Alstyne 1992; *2* Jung and Pohnert 2001; *3* Van Alstyne et al. 2001b; *4* Küpper et al. 2002; *5* Küpper et al. 2006; *6* Ross et al. 2005; *7* Bouarab et al. 1999; *8* Weinberger and Friedlander 2000; *9* Collén and Pederson 1994; *10* Bouarab et al. 2004; *11* Lion et al. 2006
[a]Gametophyte stage
[b]Oxylipin production directly involved in a defense response

interactions. The role of plant-plant signaling and induction of metabolite synthesis may, in fact, be linked by short-term reactions such as the oxidative burst and the oxylipin pathway, as seen in terrestrial plants (Liechti and Farmer 2002; Farmer et al. 2003). These multitemporal responses may aid in explaining how an induced defense can be an effective mechanism for fitness even against large grazers such as sea urchins, which can cause fatal damage to an alga in time periods much shorter than those reported for biosynthesis of metabolites. It warrants consideration that long-term defense responses may be (1) secondary to an initial (yet

undetected) defense response, (2) protection against secondary infection (pathogens) as a result of the grazing, (3) or a result of kin selection in response to a localized change in herbivore pressure. In the many systems where induced responses on timescales of days to weeks have been observed, additional investigation into immediate responses may identify additional protective mechanisms actively at work.

6.4 Advances in the Characterization of Patterns of Chemical Defenses

6.4.1 Phylogeny Meets Ecology

"What drives selection?" remains the underlying question behind the observed distribution patterns of secondary metabolites. In very broad terms there are two driving forces: phylogeny and ecology. Secondary metabolites are derived from a combination of phylogenetic factors, such as the genetic information and biochemical potential (i.e., what enzymes are present) and ecological factors, such as the biotic and abiotic components of an ecosystem. Terrestrial plant research has shown that these factors interact to shape the evolution of secondary metabolite regulation and production. Both phylogeny and ecology are critically important in the characterization of the distribution and function of natural products. Multiple models have emerged for predicting observed phenotypic, genotypic, and geographic differences in plant defenses, including trade-offs and costs of secondary metabolite production (see reviews by Loomis 1953; McKey 1974; Rhoades 1979; Bryant et al. 1983; Tuomi et al. 1988; Herms and Mattson 1992; Cronin and Hay 1996b; Cronin 2001; Strauss et al. 2002; Stamp 2003; see Chap. 7).

Several studies have addressed the popular concept of "trade-offs" in terrestrial plant chemical ecology. Leimu and Koricheva (2006) performed a meta-analysis study on 31 studies covering 17 plant species, to determine the relationship between tolerance traits (i.e., increased photosynthesis) and resistance traits (i.e., increased chemical defense). They concluded, "there is no overall trade-off in plants between tolerance and resistance to herbivores." Agrawal and Fishbein (2006) proposed reinstating the term "syndromes" when discussing plant defenses, thereby steering away from considerations of patterns as distinctly defined trade-offs and embracing a collective outcome of multiple selective pressures, including both phylogeny and ecology. Recent studies in marine systems have attempted to tease apart the roles of phylogeny versus ecology in production of secondary metabolites by investigating the genetic profile (genotype) of algae along with biotic or abiotic factors (Jormalainen et al. 2003; Jormalainen and Honkanen 2004; Wright et al. 2004; Honkanen and Jormalainen 2005; Dworjanyn et al. 2006a). Genetic variability of herbivores and their response to chemical defenses have also been investigated (Sotka 2003). Together, these studies have broadened our understanding of the role

of genetic variation in chemical defenses, better characterized costs associated with defense, and isolated the dominant selective pressures (i.e., phylogeny, herbivory, environment).

It is assumed that selection of chemical defense compounds in marine systems is driven primarily by the presence of herbivores and their selective grazing pressure, as is common in terrestrial systems. However, this assumption has not been rigorously tested in many marine habitats. While numerous studies identify factors that cause variation in the concentration or distribution of algal secondary metabolites, few address the role of genetic composition in the observed variation. Wright et al. (2004) were the first to investigate the relationship between genetic profile of the red alga *D. pulchra* and variability in chemical defense compounds. Specifically, they set out to determine whether the variability in chemical compounds was heritable (i.e., correlated to genotype), and whether this heritability responded to grazing selection pressure. In genetically identical individuals, there was a large variation of furanone concentration (3.8 ± 0.5 to 33.9 ± 2.5 mg/g dry mass). There was a significant relationship between genotype, total furanone concentration, and furanone structure, supporting a genetic basis for the observed variation in secondary metabolites (Wright et al. 2004). However, the calculated heritability values for chemical composition (both total and individual) implied that environment probably played a stronger role in the observed variation.

Jormalainen and Honkanen (2004) compared associations between genotypes and fouling organisms in *Fucus vesiculosus* to address the role of genotype in resistance to fouling, including the relationship of phlorotannin to epiphyte load. Past history of the alga, variable fitness costs, and genotype all played a role in phlorotannin production, but a large amount of variability remained within each of these measures (Jormalainen and Honkanen 2004). On closer examination, the researchers found that genotypes of *F. vesiculosus* taken from two distinct initial populations showed different tendencies toward resistance (i.e., chemical defense) or tolerance of fouling organisms (Honkanen and Jormalainen 2005). Patterns of genetic variation were not consistent across laboratory and field experiments. In the field, fouling varied across individuals, implying different levels of genetic resistance toward fouling throughout the community. However, in laboratory experiments, a different population of plants (different from those used in the field) showed no significant variation toward fouling (Honkanen and Jormalainen 2005). These methods illustrate that trends in fouling resistance are affected by both a genetic component and microhabitat and the observed variability does not appear to be uniform across different populations. Phlorotannin production also varied between different populations, but was not found to be correlated to epiphyte load.

Determining costs of secondary metabolites is inherently difficult as a result of the variable responses, multiple physiological roles of the metabolites, as well as the different types of costs involved in the production of secondary metabolites (production, transport, storage, maintenance) (Strauss et al. 2002). Emerging studies are now taking more of these variables into consideration. For example, work with clonal populations of *D. pulchra* has estimated costs of furanone production in relation to chemical defense and phenotype by investigations of reproductive

success, growth, and furanone content (the common trade-off approach), experimental and field correlations between genetic history, growth and furanone content, growth rate of algae with and without furanone production, and life history and furanone production (Dworjanyn et al. 2006a). In both laboratory and field experiments, growth rate was found to be positively linked with furanone production. The trait was family-dependent, implying a genetic correlation between furanones and growth. From this evidence, one would assume that there is little or no cost of furanone production, as there was no apparent trade-off in fitness correlates. However, when growth rate was compared across populations of furanone-producing and nonproducing populations, there was a significantly greater growth rate in the populations producing no furanones. Additionally, variation in growth rates was dependent on the clonal family that was used, supporting the idea that interindividual variability in chemical production exists.

Incorporating genetic history and the relationship of genetically driven variability of chemical defenses in these pioneering studies supports the importance of understanding genetic underpinnings and their involvement in the selection of traits involved in secondary metabolism. The merging of genotype (molecular information) and ecology (cues that trigger changes in that molecular information) will provide a stable scaffolding upon which to build a better framework for identifying patterns of metabolite production and their role in an alga's ecology. The investigations that incorporate genetic composition in their approach provide additional evidence for the importance of interindividual variability, for the idea that variability of chemical defenses can be genetically as well as environmentally based, and for the notion that populations of the same species may exhibit different patterns of genetic-based variability.

6.4.2 Metabolic Similarities Between Algal Groups

Although studies in marine chemical ecology often piggyback on terrestrial knowledge, in marine algae there is a lack of specific evidence for many of the secondary metabolite biochemical pathways, especially at the gene and enzyme levels. Radioisotopic labeling experiments have proven valuable in establishing the presence or absence of particular pathways in algal groups and the likely steps involved in compound production. Analysis of pathways can also be extremely valuable from an evolutionary perspective, since the same biosynthetic abilities are not observed universally across algal groups (Table 6.2); this is likely due to their unique evolutionary histories. Enzymatic inhibition of metabolic pathways and structural determination of metabolites are two other approaches most often used to determine the presence of secondary metabolic pathways. Molecular characterization of the specific genes encoding enzymes is also beginning to provide additional insight into the distribution patterns of metabolic pathways of secondary metabolism in algae. Such microscale techniques will enable us to define molecular switches (biochemical and environmental regulators of gene expression) and patterns

Table 6.2 Common biosynthetic pathways, their occurrence in cyanobacteria and algae, methods used to detect the pathways, enzymes verified as present, and indication of the presence of genetic information for the enzyme

Pathway	Algal group	Present	Absent	Enzymes verified[a]	Genetic information	Source
Eicosenoid	Cyanobacteria	ENZ/SC	–	LOX	No	1
	Phytoplankton	ENZ/SC	–	LOX	No	1
	Chlorophyta	ENZ/SC	–	LOX	No	1
	Rhodophyta	ENZ/SC	–	LOX	No	1
		MM	–	12-LOX	Yes	2
	Phaeophyta	ENZ/SC	–	LOX	No	3
Terpenoid MVA *(mevalonate-dependent)*	Cyanobacteria	–	X	–	–	–
	Phytoplankton	IL	–	–	No	4
	Chlorophyta	–	X	–	–	–
	Rhodophyta	IL	–	–	No	5
	Phaeophyta	IF	–	–	No	6
Terpenoid MEP *(mevalonate-independent)*	Cyanobacteria	IL	–	–	No	5
	Phytoplankton	IL	–	–	No	4
	Chlorophyta	IL	–	–	No	7
	Rhodophyta	IL	–	–	No	8
	Phaeophyta	–	UNK	–	–	–
Shikimate	Cyanobacteria	MM	–	EPSP synthase	Yes	9
	Phytoplankton	INH	–	EPSP synthase	No	10
		MM	–	Multiple	Yes	11
	Chlorophyta	MM	–	Multiple	Yes	11
	Rhodophyta	ENZ	–	SDH	No	12
		MM	–	Multiple	Yes	11
	Phaeophyta	INH	–	EPSP synthase	No	13
Phenylpropanoid	Cyanobacteria	–	UNK	–	No	–
	Phytoplankton	–	UNK	–	No	–
	Chlorophyta	–	UNK	–	No	–
	Rhodophyta	ENZ	–	PAL	No	12
	Phaeophyta	–	UNK	–	No	–
Polyketide	Cyanobacteria	MM	–	PKS/NRPS	Yes	14
	Phytoplankton	MM	–	PKS	Yes	15
	Chlorophyta	–	UNK	–	No	–
	Rhodophyta	–	UNK	–	No	–
	Phaeophyta	–	UNK	–	No	–

ENZ enzyme assays, *SC* structural composition, *MM* molecular methods, *IL* isotopic labeling, *IF* isotopic fractionation, *INH* inhibition studies, *UNK* unknown, *LOX* lipoxogenase, *EPSP synthase* 5-enolpyruvylshikimate-3-phosphate, *SDH* shikimate dehydrogenase, *PAL* phenylalanine ammonium lyase, *PKS* polyketide synthase, *NRPS* nonribosomal peptide synthase

1 Gerwick 1999; *2* Liu et al. 1994; *3* Boonprab et al. 2003; *4* Cvejic and Rohmer 1999; *5* Disch et al. 1998; *6* Chikaraishi et al. 2006; *7* Schwender et al. 2001; *8* Schwender et al. 1997; *9* Mayes et al. 1993; *10* Shick et al. 1999; *11* Richards et al. 2006; *12* Bouarab et al. 2004; *13* Pelletreau et al., unpublished data; *14* Dittman and Weigand 2006; *15* Rein and Barrone 1999

[a]Empty columns imply no *direct* evidence of these enzymes from these systems

of genetic regulation in marine algae. There are five biochemical pathways involved in the production of the most commonly observed secondary metabolites: the eicosanoid pathway, the isoprenoid pathway(s), the shikimate pathway, the phenylpropanoid pathway, and polyketide pathways.

The Eicosanoid Pathway: The most well characterized pathway for secondary metabolism is the eicosanoid pathway (Gerwick et al. 1993a, b; Gerwick 1999). Eicosanoids are produced by organisms ranging from bacteria to humans, and in terrestrial plants and animals the products of polyunsaturated fatty acid (PUFA) oxidation (oxylipins) are known to be involved in signaling and response to external stimuli. A well-characterized example is the jasmonic acid cascade involved in defense and signaling responses in higher plants (Farmer et al. 1992, 2003; Liechti and Farmer 2002). In algae this pathway is responsible for the formation of numerous oxylipins (potential signals), polyunsaturated aldehydes (potentially defensive), and gamete attractants (hormones) (Pohnert and Boland 2002; Potin et al. 2002; Boonprab et al. 2003; Paffenhofer et al. 2005; Pohnert 2005). The fatty acid precursors, as well as sites of oxidation, vary between algal groups, but eicosanoid metabolism as a whole appears to be well distributed among all algal groups, although certain algae exhibit unique properties. For example, the precursors in animals are C_{20} PUFAs, and in terrestrial plants, C_{18} or C_{16} PUFAs; however, the rhodophyte *Chondrus crispus* was found to metabolize *both* C_{20} and C_{18} PUFAs in response to pathogen attack (Bouarab et al. 2004). This work indicates that both the eicosanoid (C_{20} PUFA) and octadecanoid (C_{18} PUFA) pathways exist in red algae and both are involved in the defense of the *C. crispus* against the obligate algal endophyte *Acrochaete operculata*. This suggests the possibility that in early evolution of eukaryotic organisms, both pathways (eicosanoid and octadecanoid) may have been involved in generating signaling molecules and defense responses.

The Isoprenoid Pathway: Two pathways are known to exist for the synthesis of terpenes and steroids: a mevalonate-dependent pathway (MVA) and a mevalonate-independent pathway (2-*C*-methyl-D-erythritol-4-phosphate pathway; MEP). In animals the mevalonate-dependent pathway is prevalent, whereas both pathways exist in plants. The pathways are localized, with the MVA found in the cytosol and the MEP found in the plastids (Lichtenthaler et al. 1997; Lichtenthaler 1999). In algae, the distribution of these pathways is variable and may provide insight into an alga's evolutionary history. Similar to higher plants, a division between cytosolic and plastid-derived isoprenoids has also been observed in diatoms (Cvejic and Rohmer 2000) and red algae (Schwender et al. 1997; Wanke et al. 2001). This was not the case in cyanobacteria, where the MEP route was responsible for all isoprenoids produced, and in the euglenophytes, where the MVA pathway was solely responsible for production (Disch et al. 1998; Kim et al. 2004). Synthesis of terpenoids in Chlorophyta occurs exclusively via the MEP pathway (Schwender et al. 2001; Pohnert and Jung 2003). There is no division of terpene production between the two pathways, as seen in higher plants, possibly suggesting that the MVA pathway may have evolved multiple times or may have been lost in an early cholorphyte ancestor. Although terpenoid compounds are common in brown algae and have been extensively isolated and characterized from algal tissue, there is little

experimental evidence tracking the biosynthesis of these compounds in the phaeophytes. The similarity between brown and red algae of isotopic fractionation (ε) during sterol production suggests that the brown algal sterols are produced through a mechanism that is similar to that found in red algae (the MVA pathway) (Chikaraishi 2006), but no other experimental evidence exists to date to verify the mechanisms of isoprenoid production in brown algae.

The Shikimate Pathway: The shikimate pathway is responsible for the production of aromatic amino acids, and as such, its presence is necessary in phototrophic organisms. It is also responsible for making phenylalanine, the precursor of many plant secondary metabolites. Presence of the shikimate pathway in macroalgae was not experimentally verified until Richards et al. (2006) identified genes involved in all of the steps of the shikimate pathway in both green and red algae (*Chlamydomonas reinhardtii* and *Cyanidoschyzon merolae* respectively) as well as in the diatom *Thalassiosira pseudonana*. Work with *N*-phosphonomethlyglycine (glyphosate), which directly targets enzymes unique to the shikimate pathway, inhibited the production of mycosporine-like amino acids (MAAs), UV-protecting compounds (with potentially other ecological roles) commonly found among red algae and microalgae (Shick et al. 1999; Shick and Dunlap 2002). Glyphosate also caused a reduction in phlorotannin content and mortality in *F. vesiculosus*, providing anecdotal evidence that the shikimate pathway is present in brown algae (Pelletreau et al., unpublished data) Recently, presence and activity of shikimate dehydrogenase, a regulatory enzyme in the pathway, were measured in the red alga *D. pulchra* (Bouarab et al. 2004). The presence and upregulation of this enzyme in response to exposure to the signaling molecule methyl jasmonate (MeJA) provided the first enzymatic evidence that this pathway may be involved in defense response in *D. pulchra* (Bouarab et al. 2004). The detailed characterization of the shikimate pathway in higher plants and its role in primary and secondary metabolism via the generation of aromatic amino acids (which then leads to many secondary metabolites) render it an excellent candidate for comparing biochemical mechanisms across algal groups.

The Phenylpropanoid Pathway: The phenylpropanoid pathway, ubiquitous in higher plants, uses phenylalanine as a starting unit to generate an expansive range of metabolites. The only evidence to date of a phenylpropanoid pathway in marine algae is the presence and induced activity of the major regulatory enzyme phenylalanine ammonium lyase (PAL) in the red alga *C. crispus* (Bouarab et al. 2004). *C. crispus* exhibited an upregulation of this enzyme in response to oxylipins, resulting in lowered susceptibility to endophytic infection. Hence this pathway may be present in some macroalgae and involved in a similar defense response as is seen in higher plants. Although it would seem most likely that similar enzymes would be found in the chlorophytes because of their evolutionary relationship to higher plants, there is no experimental evidence of phenylpropanoid pathway enzymes in this algal group (Stafford 2000), or in phaeophytes.

The Polyketide Pathway: One metabolic process that has received much attention from natural product chemists is the production of secondary metabolites via polyketide synthase (PKS) enzymes. Polyketide synthases condense small starting

units, often acetate or malonate, into complex and diverse end products. Many of the toxins produced by harmful algal species and cyanobacteria are generated via polyketide synthases (e.g. Shimizu 2003; Dittmann and Wiegand 2006). Dinoflagellates are known to produce some of the largest and most complex polyketides yet identified (Rein and Borrone 1999; Berry et al. 2002; Snyder et al. 2003). These polyketides have been implicated in neurotoxic shellfish poisoning (brevetoxins), ciguatera fish poisoning (ciguatoxins), and diarrhetic shellfish poisoning (okadaic acid). In the macroalgae there has been little to no evidence of such an enzyme-mediated synthesis of secondary metabolites. This is curious for several reasons: (1) PKS are broadly distributed – found in bacteria, phytoplankton, diatoms, fungi, and higher plants; (2) the structure of many algal secondary metabolites implies the involvement of such a pathway; and (3) PKS enzymes are thought to have evolved from fatty acid synthases, which are necessary enzymes in all organisms because of their involvement in membrane production. Further investigation will provide evidence supporting the presence and function of this pathway in macroalgae.

Information regarding the presence of the metabolic pathways, their genetic underpinnings, and their regulation in marine algae has enhanced our understanding of the mechanisms by which certain secondary metabolites can be produced, and clarified our interpretations of distribution and production patterns of secondary metabolites both within and between algal groups. This detailed information strengthens our experimental capabilities, allowing researchers to directly examine responses to cues at the molecular level and enabling studies that address specific cellular responses, on-off switches, and potentially even the discovery of novel metabolites and pathways. A finely resolved understanding of what pathways are involved in metabolite production, what regulates these pathways, and what elicits pathway production enables a holistic understanding of all factors involved in shaping patterns of metabolite distribution.

6.4.3 The Use of Molecular Tools to Characterize Patterns of Gene Response Involved in Macroalgal Defenses

Plant chemical ecologists have embraced the use of molecular tools to characterize plant natural products and interactions between organisms at the cellular and molecular level (Dixon et al. 2002; Dixon 2005a, b; Xie and Dixon 2005; Ouborg and Vriezen 2007). The application of molecular tools to macroalgal chemical ecology has been slow to develop, mostly because of logistical difficulties such as effective nucleic acid extraction from algal tissue that contain high levels of inhibitors and coprecipitates (Phillips et al. 2001; Pearson et al. 2006; Varela-Alvarez et al. 2006), and the effects of these inhibitors on downstream applications. In terrestrial systems, advances in molecular understanding have been predicated on model organisms that are collectively studied by the scientific community (i.e., *Arabadopsis*). In marine algal systems no model organism has yet emerged. Despite

these hindrances, there have been several studies that have incorporated the use of molecular tools to better characterize metabolic processes of chemical defense at the cellular level in response to various cues (discussed below). In response to specific agents (e.g. methyl jasmonate), researchers can observe which of the genes and enzymes involved in secondary metabolism are up- or downregulated. The available information for molecular study in phycology is limited, especially when compared to the knowledge base for plant and fungal systems; however, this is changing rapidly. The presence of this information (expressed sequence tag (EST) data in the NCBI database, genome sequencing) serves as a positive feedback mechanism for future studies by increasing the collective knowledge of algal molecular composition.

To date, the only full size genome sequenced from a nonparasitic protist is of the diatom, *Thalassiosira pseudonana,* (Armbrust et al. 2004). The two other complete algal genomes are highly reduced: the unicellular red alga *Cyanidioschyzon merolae* (Matsuzaki et al. 2004), and the cryptophyte *Guillardia theta* (Douglas et al. 2001). At present, no macroalgal genomes have been fully sequenced, although several have been proposed, including the phaeophyte *Ectocarpus siliculosus* (Peters et al. 2004), and the rhodophyte *Porphyra yezoensis* (Waaland et al. 2004). These algal genomes will be particularly valuable in light of the unique evolutionary history that exists between the major algal divisions, and for their potential to yield insight into the evolution and modification of metabolic pathways and mechanisms in biosynthesis. It is also worthwhile noting that such novel metabolic mechanisms may also lead to new drug discoveries and applications.

Phycological molecular work has focused on phylogeny, using molecular techniques to trace endosymbiont lineages and determine the correct characterization of algal groups and evolutionary processes (e.g. Keeling 2004; Keeling et al. 2005; Patron et al. 2006; Waller et al. 2006; Rogers et al. 2007). One of the first molecular approaches to macroalgal ecological study was to characterize, at the genetic level, the processes occurring during desiccation in the brown alga *F. vesiculosus* (Pearson et al. 2001). Subtractive suppressive hybridization, followed by reverse Northern blot analysis, showed that 60–70% of the randomly selected clones (genes) were differentially expressed in hydrated versus desiccated algae. When these genes were compared to other genes in the NCBI database, the majority showed little or no homology to known genes. This dissimilarity is likely biased by the paucity of information on this algal group in the NCBI database, but may also reflect the fact that Phaeophyta contain distinct genetic patterns and information as a result of its unique evolutionary path. Pearson et al. (2001) were able to identify the upregulation of several photosynthetic genes in response to desiccation. Their work has served as a starting point for investigating genetic response to environmental stress.

More recently, EST libraries from protoplasts and mature specimens of the rhodopyhte *C. crispus* were generated to investigate the genes involved in stress response (which may involve production of secondary metabolites) and cell wall regeneration (Collén et al. 2006a). Eighteen percent of the genes in the stressed library were attributed to cell rescue, defense, and cell death, whereas only 3.5% of

the genes in the unstressed library were allocated to these processes, indicating significant effort into defense and/or protection. A large number of heat shock proteins (HSP), vanadium bromoperoxidase, antioxidant enzymes, and detoxification genes (potentially involved in the breakdown of halogenated compounds) were also identified. Red algae show unique traits in their metabolic pathways and in their genetic composition relative to other algae. Only genetic information can afford us a level of understanding that allows us to question whether we can assume that the defense systems between algal groups will function identically if they have fundamentally different building blocks underlying these responses.

Using the information generated from the EST library, Collén et al. (2006b) investigated the role of volatile MeJA on gene expression of *C. crispus* through microarrays and verification with quantitative real-time PCR, the first application of these methods in red macroalgae. Methyl jasmonate is a widely known signaling molecule in defense response in terrestrial plants, and there is evidence that it (or structurally similar molecules) functions as an inducer of defense response in marine algae (Arnold et al. 2001; Bouarab et al. 2004). *C. crispus* was exposed to MeJA for a 24-h period. DNA samples were taken at regular intervals and the expression patterns were compared. Six percent of the genes examined showed a response to MeJA some time during the course of the experiment (Collén et al. 2006b). Potential stress- and defense-related genes that were upregulated overlapped with some of those described by Collén et al. (2006a). Other genes involved in metabolism and in unknown functions were also upregulated following exposure to MeJA, while several others were downregulated. From this study, the authors concluded that algae undergo a dynamic response upon exposure to MeJA; that MeJA functions as a stress hormone by increasing the expression of stress-related genes; and that based on the genes expressed, the reactive oxygen mechanism may be involved. Using molecular methods, Collén et al. provided strong evidence supporting the role of MeJA in defense response of red algae, implying the presence of a valid signaling system in red algae.

6.5 Conclusion

Research in marine chemical ecology traditionally has focused primarily on the effects of herbivory and abiotic factors on the production of secondary metabolites in macroalgae. Standard techniques measured the total amount of secondary metabolites produced in response to manipulation and attempted to characterize tradeoffs in fitness versus cost. A microscale approach (i.e., subcellular, microscopic and molecular) combined with knowledge of genetic variability and composition can provide more comprehensive insight into the triggers for observed patterns of chemical defense and the up- and downregulation of metabolites. Similarly, understanding the regulatory factors involved in gene expression, the signaling molecules and cascades involved in cellular response, and the total timescale involved in the defense response from perception of cue to production of metabolite are all important

considerations. By incorporating these considerations, we will enhance our present understanding of chemical responses and better interpret observed patterns of metabolites at the cellular, organism, and ecosystem levels.

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