UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

Transcriptomic profile and defense strategies in the red seaweed *Laurencia dendroidea* J. Agardh

Louisi Souza de Oliveira

2015

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Tese submetida ao Programa de Pós-graduação em Biodiversidade e Biologia Evolutiva como parte dos requisitos necessários à obtenção do título de doutor.

Orientadores: Dr. Renato C. Pereira

Dr. Fabiano L. Thompson

2015

De Oliveira, Louisi Souza

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/ Louisi Souza de Oliveira – Rio de Janeiro: Instituto de Biologia, 2015.

x, 157 f. : il. ; 31 cm. Orientador: Renato Crespo Pereira. Co-orientador: Fabiano Lopes Thompson

Tese (doutorado) – UFRJ, IB, Programa de Pós-Graduação em Biodiversidade e Biologia Evolutiva, 2015.

1. Holobionte. 2. Alga. 3. Bactéria**.** 4. Metabólito secundário. 5. Terpeno. 6. Patógeno. 7. Defesa. 8. Expressão Diferencial. - Tese. I. Pereira, Renato Crespo. II. Universidade Federal do Rio de Janeiro, IB, Programa de Pós-Graduação em Biodiversidade e Biologia Evolutiva. III. Título.

FOLHA DE APROVAÇÃO

LOUISI SOUZA DE OLIVEIRA

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Tese de doutorado submetida ao Programa de Pós-graduação em Biodiversidade e Biologia Evolutiva, Instituto de Biologia, Universidade Federal do Rio de Janeiro, como requisito para a obtenção do título de Doutor em Ciências.

Aprovada em: ____ / ____ / ____

Banca examinadora:

_____
Dra. Yocie Yoneshigue Valentin
Universidade Federal do Rio de Janeiro

_____
Dra. Mariana Cabral de Oliveira
Universidade de São Paulo

_____
Dra. Angelica Ribeiro Soares
Universidade Federal do Rio de Janeiro

_____
Dr. Ricardo Henrique Kruger
Universidade de Brasília

_____
Dr. Paulo Sergio Salomon
Universidade Federal do Rio de Janeiro

Agradecimentos

Ao longo dos anos de dedicação que resultaram nessa tese, tive a felicidade de contar com o apoio de pessoas muito especiais, sem as quais a caminhada seria muito mais árdua. Aos meus orientadores, prof. Renato Crespo Pereira e prof. Fabiano Thompson, agradeço pelos valiosos ensinamentos, pela dedicação e incentivo em todas as fases do trabalho, e por oferecerem as condições necessárias para o desenvolvimento dessa tese. Agradeço, ainda, aos meus caros colegas e amigos do Laboratório de Microbiologia pelo auxílio na execução dos experimentos e pela convivência enriquecedora. À Patrícia Malheiros e Rosa Otsuki, obrigada pelo companheirismo e pelo auxílio nas tarefas burocráticas. À Angélica do Carmo agradeço pelo carinho e por tornar a rotina mais leve.

Em especial, agradeço ao Gustavo Gregoracci, Diogo Tschoeke, Genivaldo Gueiros, Ana Carolina Rubens, Marcelle Gerolimich, Monique Anjos, Gabriela Calegario, Pedro Meirelles e Luciana Leomil sem os quais esse trabalho não seria possível. Aos colaboradores do Jardim Botânico do Rio de Janeiro, prof. Gilberto Amado-Filho, prof. Leonardo Salgado, Lilian Hill e Vanessa Reis, meu sincero agradecimento pela importante contribuição científica e pelo auxílio nas coletas. Às colaboradoras e amigas Daniela Sudatti e Aline Oliveira agradeço por cederem os clones de *Laurencia* utilizados no trabalho e pela contribuição nas mais diversas etapas. Agradeço à Lina Felix e ao prof. Marcio Alves por me ensinarem a dar os primeiros passos no mundo da biologia molecular.

Ao Programa de Pós-graduação em Biodiversidade e Biologia Evolutiva e aos professores a ele vinculados, agradeço por receber meu projeto e oferecer as condições acadêmicas necessárias à sua execução. À Universidade Federal do Rio de Janeiro, agradeço pela infraestrutura para o desenvolvimento desta tese e por me receber de forma acolhedora em seu quadro de funcionários. Agradeço às agências de fomento CAPES, FAPERJ e CNPq pelo auxílio financeiro para o desenvolvimento deste estudo.

Aos meus pais agradeço pelo amor e pelos valores essenciais a mim transmitidos, vocês foram e sempre serão meus maiores incentivadores e meu referencial. Ao meu irmão, minha querida cunhada e nossa pequena Helena, obrigada pelo companheirismo e doçura nos momentos mais difíceis. Ao meu namorado, Caio Rangel, agradeço pelo

amor e compreensão diante da constante correria que representou a conclusão deste trabalho. Aos primos João e Marisa Tulli, agradeço pela acolhida na Cidade Maravilhosa. Agradeço à minha grande família pelo apoio incondicional e pelos momentos de alegria incomparáveis.

Aos meus amigos espalhados por esse país, mas muito próximos do coração, agradeço imensamente pela torcida, vocês tornam meus dias muito mais alegres e ensolarados. De forma especial agradeço a Daniela Magalhães, Marina Sales, Ticiellen Macedo e Mayara Tulli, amigas de longa data e sempre presentes, apesar da distância. Às queridas Giselle Cavalcanti, Cecília do Valle, Gilda Amaral, Gizele Garcia, Cintia Rua, Ana Carolina Soares, Adriana Fróes, Juline Walter e Cynthia Silveira pelos momentos de descontração e risadas inesquecíveis. Ao amigo Rafael Pinho pela companhia e pelo bom humor inabalável. À "galera do bem" pela alegria de sempre e pelo apoio nessa trajetória. A todos os que torceram e intercederam por mim, aos queridos amigos e familiares que não me deixaram desanimar diante das adversidades, muito obrigada.

“There is no chance, no destiny, and no fate that can circumvent, or hinder, or control the firm resolve of a determined soul”.

Ella Wheeler Wilcox

Articles published during the PhD program

De Oliveira LS, Tschoeke DA, de Oliveira AS, Hill LJ, Paradas WC, Salgado LT, Thompson CC, Pereira RC, Thompson FL: **New Insights on the terpenome of the red seaweed *Laurencia dendroidea* (Florideophyceae, Rhodophyta).** *Mar Drugs* 2015, **13**:879–902.

Moreira APB, Duytschaever G, Chimetto Tonon LA, Fróes AM, de Oliveira LS, Amado-Filho GM, Francini-Filho RB, De Vos P, Swings J, Thompson CC, Thompson FL: ***Photobacterium sanctipauli* sp. nov. isolated from bleached *Madracis decactis* (Scleractinia) in the St Peter & St Paul Archipelago, Mid-Atlantic Ridge, Brazil.** *PeerJ* 2014, **2**:e427.

Reis VM, Oliveira LS, Passos RMF, Viana NB, Mermelstein C, Sant'anna C, Pereira RC, Paradas WC, Thompson FL, Amado-Filho GM, Salgado LT: **Traffic of secondary metabolites to cell surface in the red alga *Laurencia dendroidea* depends on a two-step transport by the cytoskeleton.** *PLoS One* 2013, **8**:e63929.

De Oliveira LS, Gregoracci GB, Silva GGZ, Salgado LT, Filho GA, Alves-Ferreira M, Pereira RC, Thompson FL: **Transcriptomic analysis of the red seaweed *Laurencia dendroidea* (Florideophyceae, Rhodophyta) and its microbiome.** *BMC Genomics* 2012, **13**:487.

Articles submitted during the PhD program

Moura RL, Rezende CE, Almeida MG, Silva Jr. JM, Araujo BF, Brito F., Rangel TP, Oliveira BCV, Salomon PS, Moraes FC, Amado Filho G., Bahia R., Paranhos R, Dias RJ., Mahiques MM, Siegle E, Bastos AC, Figueiredo A, Silva C, Hajdu E, Asp N, Gregoracci G, Leitão S., Yager P, Froes A, Campeão M, Silva BS, Moreira A., de Oliveira LS, Soares AC, et al.: **Discovery of a new reef biome at the mouth of the Amazon River**. *Science Advances* 2015, *submitted*.

Walter JM, Tschoeke DA, de Oliveira L, Leomil L, Tenório M, Moura RL, Meirelles, PM, Amado-Filho G., Valle R, Coutinho R, Salomon P., Thompson C., Thompson F.: **Metagenomic plasticity explains widespread distribution of turf algae holobionts in reef systems**. *ISME J* 2015, *submitted*.

Appolinario LR, Tschoeke D, Venas T, Campeão ME, Amaral G, Leomil L, de Oliveira LS, Otsuki RK, Swings J, Thompson FL, Thompson CC: **Description of *Endozoicomonas arenosclerae* sp. nov. using a genomic taxonomy approach**. *Antonie van Leeuwenhoek J Microbiol* 2015, *submitted*.

Calegario G, de Oliveira LS, Amado Filho G, Pereira RC, Thompson CC, Thompson FL: **Genetic diversity of the endemic red algae *Plocamium brasiliense* in a South Atlantic upwelling region**. *J Phycol* 2015, *submitted*.

Articles in preparation

De Oliveira LS, Tschoeke DA, Lopes ACRM, Sudatti DB, Meirelles PM, Pereira RC, Thompson F: **Transcriptomic reprogramming of the red seaweed *Laurencia dendroidea* in response to the potentially pathogenic bacteria *Vibrio madracius***. *In preparation* 2015.

Tschoeke DA, de Oliveira LS, Meirelles PM, Thompson CC, Coutinho R, Sergey D, Thompson FL: **Metagenomic analysis and quorum sensing inhibitory activity of microbial mats from a hypersaline coastal lagoon**. *In preparation* 2015.

Silva BSO, Leomil L, de Oliveira LS, Fróes A, Gregoracci GB, Yager PL, Thompson FL: **Virioplankton diversity in the river-ocean continuum of the Amazon river**. *In preparation* 2015.

Nardeli SM, Artico S, de Moura SM, Aoyagi G, Silva TF, de Oliveira LS, Tschoeke DA, Romane E, Tompson FL, Alves-Ferreira M: **Genome-wide identification and phylogenetic analysis of MADS-box gene family in *Gossypium hirsutum* and in diploid parental cotton species**. *In preparation* 2015.

Silveira CB, Gregoracci GB, Coutinho FH, Soares AC, Silva GG, Haggerty JM, de Oliveira LS, Cabral A, Dinsdale EA, Edwards RA, Paranhos R, Francini-Filho RB, Rohwer F, Thompson F: **Shifts towards anaerobic microbial community in the coral holobiont *Mussismilia braziliensis* during the night**. *In preparation* 2015.

Silveira CB, Coutinho FH, Silva GG, Cavalcanti G, de Oliveira LS, Cabral A, Paranhos R, Francini-Filho RB, Moura RL, Thompson CC, Gregoracci GB, Wegley-Kelly L, Dinsdale EA, Edwards RA, Rohwer F, Thompson F: **Seasonal patterns of taxonomic and functional diversity in the Abrolhos Bank viral assemblages**. *In preparation* 2015.

Cavalcanti G, Silveira CB, de Oliveira LS, Carvalho R, Willemes MJ, Salgado LT, Amado-Filho G, Thompson F: **Assessing rhodoliths holobionts in a high CO2 ocean: Transcriptomic reprogramming of free-living crustose calcifying algae and their associated microbiome in response to acidification**. *In preparation* 2015.

Meirelles PM, Soares ACS, Almeida RTB, de Oliveira LS, Leomil L, Moura RL, Francini-Filho RB, Amado-Filho GM, Paranhos R, Rezende CE, Thompson C, Sawabe T, Thompson FL: **Coral reefs resilience affected by turf algae and possible links with microbialization**. *In preparation* 2015.

Participation in scientific expeditions

Abrolhos Bank; October, 2013 - Collection of turfs for metagenomic analysis; Diving-PAM measurements to estimate the photosynthetic rates of different types of turfs.

Abrolhos Bank; February, 2014 – Diel collection of seawater to evaluate changes in bacterial composition (through metagenomic analysis), and also in dissolved and particulate organic carbon and chlorophyll concentration.

Amazon river mouth; September 2014 - Seawater sampling to analyze the bacterial and viral taxonomic composition and metabolic activities (through metagenome and proteome), to quantify the dissolved and particulate organic carbon and to analyze the chlorophyll concentration. Collection of benthic organisms for metagenomic analysis.

Resumo

A macroalga vermelha *Laurencia dendroidea* sintetiza diversos metabólitos secundários que exibem importantes atividades ecológicas e aplicabilidades biotecnológicas. A maioria dos estudos acerca do gênero *Laurencia* tem sido dedicada ao isolamento e caracterização dessas substâncias, embora o conhecimento molecular desse gênero seja ainda limitado. Nessa tese foi descrito o primeiro perfil transcriptômico de *L. dendroidea*, fornecendo informações relevantes sobre as interações a nível molecular entre essa alga e a microbiota associada. Através de análises de transcriptoma, verificou-se uma importante contribuição de *L. dendroidea* para a produção primária do holobionte, bem como o papel das bactérias como consumidoras de matéria orgânica e possíveis fontes de nitrogênio. Apesar da ocorrência de uma microbiota predominantemente benéfica associada a *L. dendroidea*, algumas bactérias marinhas têm o potencial de causar doenças em macroalgas. Nesse contexto a habilidade das algas de se defender contra patógenos deve ter sido evolutivamente selecionada. No presente estudo, verificou-se uma reprogramação do transcriptoma de *L. dendroidea* em resposta à bactéria potencialmente patogênica *Vibrio madracius*. Alguns processos metabólicos induzidos em *L. dendroidea* na presença de *V. madracius* incluem a produção de espécies reativas de oxigênio, a sinalização intracelular, o tráfego de vesículas e a conversão de energia. Além disso, a maioria dos genes relacionados à síntese de terpenos em *L. dendroidea* foram descritos nessa tese, oferecendo novas possibilidades para a biossíntese heteróloga dessas substâncias com aplicabilidade biotecnológica. Essa tese contribui notavelmente para o conhecimento atual referente aos mecanismos moleculares envolvidos no processo de defesa altamente dinâmico das macroalgas.

Palavras-chave: holobionte, alga, bactéria, metabólito secundário, terpeno, patógeno, defesa, expressão diferencial.

Abstract

The red seaweed *Laurencia dendroidea* synthesizes diverse secondary metabolites that exhibit relevant ecological roles and biotechnological applications. Most of the studies regarding the genus *Laurencia* were dedicated to isolate and characterize these compounds although the molecular knowledge was still limited. This thesis describes the first transcriptomic profile of *L. dendroidea* and provides relevant information comprising the interactions between this seaweed and the associated microbiota in the molecular level. Using transcriptomic analysis, we verified the important contribution of *L. dendroidea* for the primary production of the holobiont and the role of Bacteria as consumers of organic matter and possibly as a nitrogen source. Despite the occurrence of a predominantly beneficial microbiome associated to *L. dendroidea*, some marine bacteria have the potential to cause diseases in seaweeds. In this context, the ability of seaweeds to defend against pathogens might have been evolutionarily selected. Here we describe the transcriptomic reprogramming of *L. dendroidea* in response to the potentially pathogenic bacteria *Vibrio madracius*. Some relevant metabolic processes over-represented in *L. dendroidea* in the presence of *V. madracius* were related to the production of reactive oxygen species, intracellular signaling, vesicle trafficking and energy conversion. Furthermore, most of the genes related to the biosynthesis of terpenes in *L. dendroidea* were described, which offers new possibilities for a heterologous biosynthesis of these compounds with biotechnological application. This thesis notably contributes to the current knowledge of the molecular mechanisms involved in the highly dynamic defense process in seaweeds.

Keywords: holobiont, algae, bacteria, secondary metabolite, terpene, pathogen, defense, differential expression.

Sumário

CHAPTER 1 - General Introduction

The red seaweed genus *Laurencia*: a study model for bioactive chemicals in the applied and ecological context

The seaweeds that belong to the genus *Laurencia* are widely distributed from the temperate to the tropical oceans worldwide and occur in the intertidal and subtidal zones [1]. The high morphological plasticity and poor taxonomic definition of many species led to incorrect identifications and to the assignment of a large number of infraespecific taxa to the *Laurencia* complex. The extensive review of morphological characters associated to the use of molecular markers, such as the plastidial rbcL gene, allowed a significant change in the taxonomy of this group. One important modification was the recognition of specimens from Brazil previously mentioned as *L. filiformis*, *L. majuscula* and *L. obtusa* as the same taxonomic entity, currently defined as *L. dendroidea* [2].

L. dendroidea is characterized by forming brown-purple or violet-greenish thalli which are 4–20 cm high, and its type locality is in Brazil [2] (Figure 1). This species has a broad geographic distribution along the Brazilian coast, occupying diverse environments from sheltered bays to wave exposed rocky shores, and is considered a relevant component of the benthic communities in the intertidal and subtidal zones [3].

Taxonomic classification

Kingdom Plantae

Phylum Rhodophyta

Subphylum Eurhodophytina

Class Florideophyceae

Subclass Rhodymeniophycidae

Order Ceramiales

Family Rhodomelaceae

Genus *Laurencia* J.V. Lamouroux

Species *Laurencia dendroidea* J. Agardh

Figure 1. *Laurencia dendroidea* specimen [4].

Since the first studies on marine natural products, *Laurencia* is recognized as a prolific source of secondary metabolites, specially terpenoids and acetogenins [5]. The secondary metabolites, or natural products, are defined as organic compounds that are not involved in the basal metabolism which promotes the development, growth or reproduction, but are essential to the survival of the organisms, participating in their ecological interactions [6]. Secondary metabolites are in general biosynthesized through chemical modifications of intermediary compounds derived from the primary metabolism [6].

One of the major secondary metabolites from *Laurencia dendroidea* is the halogenated sesquiterpene (-)-elatol, which plays relevant biological and ecological roles (Figure 2). This metabolite inhibits the growth of pathogenic bacteria [7] and prevents the consumption of *L. dendroidea* by herbivores, such as the crab *Pachygrapsus transversus* and the sea urchin *Lytechinus variegatus* [8]. Also, natural products from *L. dendroidea* exhibit antifouling activities, avoiding the deleterious effects of biofouling that include decreases on light and nutrient availability and mechanical damage of seaweeds surfaces [9].

The intra-tallus concentration of (-)-elatol is highly variable among specimens of *L. dendroidea* in the same population, ranging from 0.1 to 2.2% d.w.[10]. Moreover, an interpopulational variation in the content of (-)-elatol from 0.001% to 1.24% d.w. was

reported when comparing four different populations of *L. dendroidea* along the Brazilian coast [11].

In addition, a quantitative variation in the content of (-)-elatol was observed as a result of changes in abiotic conditions, such as temperature and salinity [11, 12]. Finally, a biotic influence on the biosynthesis of (-)-elatol is inferred, since a simulated herbivory experiment induced the increase in the concentration of this metabolite in *L. dendroidea* [13]. Thus, the elatol content in *L. dendroidea* seems to be influenced by genetic and environmental factors [11].

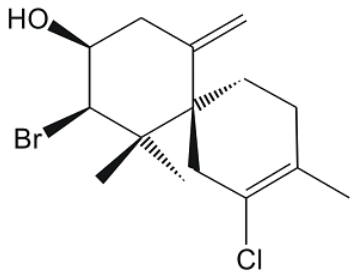

Figure 2. Structural formula of the halogenated sesquiterpene (-)-elatol [4].

Nevertheless, it is important to take into account that the concentration of (-)-elatol in *L. dendroidea* thallus at a given moment is the reflection of a dynamic process. The halogenated secondary metabolites from *L. dendroidea* are stored in specialized cellular structures, so-called corps en cerise (CC), to avoid autotoxicity [14]. In order to play its ecological roles, especially as antifoulant and antibiotic compounds, these metabolites need to be transported to the thallus surface. The intracellular traffic of these halogenated metabolites occur inside vesicles that are transported through membranous tubular connections to the cell periphery [15, 16].

The traffic of vesicles in *L. dendroidea* occurs in a two step process dependent on cytoskeleton. Actin filaments are involved in the transport of vesicles from the CC to the cell periphery [17]. Microtubules are essential to the positioning of these vesicles to specific regions where exocytosis takes place and are also involved in anchoring the CC [17]. Indeed, the vesicle traffic is influenced by temperature, irradiance, desiccation and bacterial fouling [18]. Under stressful conditions the membranous tubular connections are lost and the transport of vesicles is interrupted [18].

Therefore, the chemical defense mechanisms in *L. dendroidea* are based on a dynamic process influenced by several abiotic and biotic factors that affect the synthesis, storage and release of halogenated compounds to the cell surface. Considering the ecological roles of the natural products, especially the (-)-elatol, synthesized by *L. dendroidea*, the variation in the content of these compounds might influence the interactions between this seaweed and the associated microbiota or potential herbivores.

Beyond the aforementioned ecological relevance of the terpenes produced by *L. dendroidea*, some of these natural products present a pharmacologically relevant potential due to their strong antiviral [19], antibiotic [20], antimalarial [21], antileishmanial [22], antitrypanosomal [23], anti-inflammatory [24] and anti-carcinoma [25] activities. Therewithal, the strong anti-epibiosis activity of (-)-elatol led to the filing of the patent in Brazil to use this compound as a component of antifouling paints.

Despite the high number of studies regarding the isolation and characterization of natural products from *Laurencia* species, the genes related to the biosynthesis of these compounds are unknown. In fact, uncovering the molecular mechanisms involved in the biosynthesis of secondary metabolites in *L. dendroidea* may provide ecologically and economically valuable information. This knowledge is an important step to the heterologous synthesis of biotechnologically relevant compounds from *L. dendroidea*, possibly using genetically modified organisms in fermentation processes, following the strategy used to synthesize several plant terpenes [26].

The seaweed holobiont: tightly regulated host-microbe interactions

Seaweeds are essential components of benthic communities contributing to marine primary productivity, acting as food sources for herbivores and providing a sheltered habitat for diverse invertebrates [27, 28]. Besides, macroalgae release carbon compounds that are consumed by bacteria and may also act as chemical cues for bacterial colonization [29] (Figure 3). Indeed, the composition of the microbiota associated to seaweeds tends to be species-specific and different from the surrounding seawater [30], suggesting that selective mechanisms are involved in the establishment of biofilms on seaweed thalli.

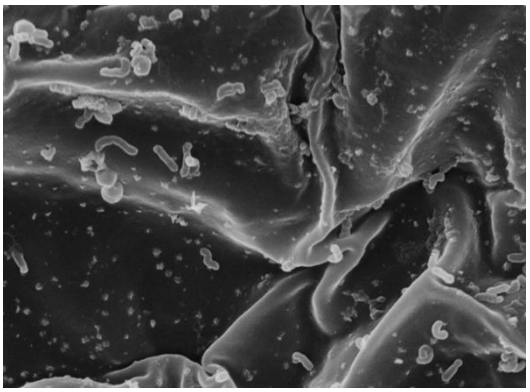

Figure 3. Scanning electron microscope image showing bacteria associated to the thallus of the red seaweed *Laurencia dendroidea* [18].

Seaweeds synthesize diverse antibiotic compounds that selectively inhibit the growth of specific bacteria, affecting the density and composition of the associated microbiome [31]. Beyond that, bacteria associated to macroalgae synthesize antibacterial compounds that inhibit the growth of other marine bacteria, which is relevant for space competition on host surfaces and also affect the biofilm species composition [32]. The antibiotic produced by bacteria associated to seaweeds defend their hosts against pathogens [33]. Epiphytic bacteria also produce antifouling compounds that protect seaweeds from micro and macrofouling organisms, such as diatom and mussels, respectively [34].

The interactions between seaweeds and microbes occur at different levels (reviewed in [35, 36]). Bacteria associated to macroalgae benefit from organic compounds released by their hosts [29], while providing essential nutrients for seaweeds morphological development [37]. In addition, some bacteria associated to macroalgae are able to fix nitrogen and enhance seaweed growth [38], specially on nitrogen limited tropical marine ecosystems.

Moreover, bacteria-derived substances affect seaweed reproduction traits. The N-acyl-homoserine lactones (AHLs) are a class of molecules produced by Gram-negative bacteria and involved in quorum-sensing, which is a necessary process for biofilm establishment [39]. In fact, AHLs can also act as cues for the settlement of zoospores of the green macroalga *Ulva* sp. [40]. The presence of AHLs also induces spore release in the red macroalga *Acrochaetium* sp. [41]. Further, extracellular polymeric substances (EPSs) secreted by an epiphytic marine bacteria induces the settlement of zoospores of *Ulva fasciata* [42].

The evidences for an ancient and close relationship between macroalgae and microbes were recently reinforced. The complete genome sequencing of *Ectocarpus siliculosus* and *Chondrus cripus* enabled the discovery of seaweed genes acquired from marine bacteria through horizontal gene transfer, including some relevant pathways for the biosynthesis of cell wall components and storage metabolites [43, 44].

Host-microbe interaction is a relevant force for co-evolution in the marine environment, leading to the establishment of beneficial microbiomes. The tightly regulated interactions between seaweeds and the associated microbiota led to the recent trend of studding the macroalgae in a holobiont perspective [36]. The term holobiont refers to a long-term association between different organisms forming an entity that, according to the hologenome theory, is the unit under natural selection in evolution [45].

However, the seaweed holobiont homeostasis can be disrupted under stress and by the activity of pathogens. Diverse taxonomic groups are recognized as causative agents of algal diseases, such as viruses, bacteria, protists and phycomycetes [46]. Among bacteria, the Gram-negative taxa are frequently related to disease symptoms is seaweeds, and can promote disease outbreaks on farming of economically relevant macroalgae [47, 48].

Besides promoting tissue damage, the diseases can increase the vulnerability of seaweeds to herbivores, impacting host population dynamics [49]. In this context, the ability to defend against pathogens is essential for the seaweeds to survive in the marine environment and may have been evolutionarily selected. Nevertheless, the molecular mechanisms involved in seaweed-microbe positive and negative interactions have rarely been evaluated.

Seaweed defense strategy against pathogens

Seaweeds rely on a multi-level strategy to defend against pathogens. The innate immunity, a mechanism highly conserved in plants and animals is also the first line of defense in seaweeds [50]. The ability to recognize the enemy depends on membrane receptors that are sensitive to microbe-associated molecular patterns (MAMPs) or pathogen-induced molecular patterns (PIMPs) [51]. MAMPs refer to molecules that are characteristic of microbes but are absent in hosts, such as bacterial cell wall components or flagellin, and PIMPs are the products of the microbial degradation of seaweed cell wall matrix, including oligoagars and oligoguluronates [51]. The recognition of MAMPs and PIMPs was previously detected in red

[52] and brown macroalgae [53, 54], although the compatible membrane receptors are not yet characterized in these organisms (summarized in Table 1).

After the recognition of pathogen-related cues, a transient production of reactive oxygen species (ROS) by a membrane located NADPH oxidase is induced. This oxidative burst limits pathogen spread and also act as a signal to activate other levels of defense responses [52]. Secondary signal messengers were suggested to participate in seaweed defense mechanisms. For example, Ca^{2+} ions, which are considered master messengers for defense reactions in plants are also necessary for the activation of NADPH oxidase after *Gracilaria* sp. is exposed to oligoagars [55].

Moreover, free fatty acids and methyl jasmonate induce an oxidative burst in *Laminaria digitata* [56]. Jasmonic acid (JA) is a plant hormone that regulates diverse physiological processes, including plant response to biotic and abiotic stresses [57]. Methyl jasmonate, a jasmonic acid derivative, also induces the expression of stress-related genes in *Chondrus crispus* [58], and the biosynthesis of phlorotannins in *Fucus vesiculosus* [59], indicating a role of this class of compounds in seaweed defense signaling (Table 1).

Pathogen recognition and intracellular signaling ultimately results in the activation of the expression of genes related to the biosynthesis of seaweed secondary metabolites [60, 61]. However, the genes involved in the biosynthesis of these compounds in seaweeds are largely unknown. Besides, the molecular mechanisms involved in the multi-level defense strategies of seaweeds are still poorly understood. Indeed the genes involved in pathogen perception and intracellular signaling were rarely described and the studies available are mainly based on indirect stimulus through the application of MAMPs and PIMPs (Table 1).

Table 1. Most relevant studies comprising the innate immunity process in seaweeds.

Elicitor	Elicitor type	Seaweed	Response evaluated	Reference
Oligoagars	PIMP	*Gracilaria conferta*	Inhibition of bacteria growth	[32]
Oligoalginate	PIMP	Laminariales	Oxidative burst	[33]
Oligoagar	PIMP	*Gracilaria chilensis* and *Gracilaria conferta*	Oxidative burst	[35]
Oligoguluronates	PIMP	*Laminaria digitata*	Change in expression level	[60]
Oligoguluronates	PIMP	*Laminaria digitata*	Oxidative burst	[62]
Lipopolysaccharides	MAMP	*Laminaria digitata*	Oxidative burst and fatty acid oxidation	[34]
Free fatty acids and methyl jasmonate	Signaling molecules and hormone	*Laminaria digitata*	Oxidative burst	[36]
Methyl jasmonate	Hormone	*Chondrus crispus*	Change in expression level	[63]
Methyl jasmonate	Hormone	*Fucus vesiculosus*	Phlorotannin production	[59]
Eurychasma dicksonii (oomycete)	Pathogen	*Ectocarpus siliculosus*	Oxidative stress and halogen metabolism	[61]

Large-scale transcriptome sequencing: a revolutionary tool in the molecular study of non-model species

Recent advances on next-generation sequencing platforms allowed the massively parallel sequencing of DNA and cDNA with a reduced cost [64]. Currently, the sequencing technology developed by Illumina is one of the most used for RNA-seq studies. The method is based on a sequencing-by-synthesis approach and uses fluorescent reversible-terminator nucleotides to sequence clonally amplified DNA fragments immobilized on a flowcell [64].

Through large-scale RNA-seq approaches, it is now possible to analyze the transcriptomic profile of complex non-model organisms [65]. Transcriptome is the complete set of transcripts in an organism at a specific life stage or environmental condition. The results of RNA-seq studies show high levels of reproducibility and offer an accurate quantitation of gene expression levels, as validated by real-time PCR (qPCR) analysis [65]. In contrast to the microarray and suppression subtractive hybridization (SSH) techniques, which frequently monitor changes in the expression level of a limited number of genes, the RNA-seq allow the analysis of the complete transcriptomic profile of any organism in response to a particular condition [65].

In the past few years, several RNA-seq studies arose as a tentative to describe the transcriptome of seaweeds without a reference genome, clarify phylogenetic relationships and also to evaluate transcriptomic changes in response to abiotic stresses [66–68]. Nonetheless, the knowledge about the genes involved in seaweed secondary metabolism is still scarce and researches regarding the molecular mechanisms involved in macroalgal defense are rare. The large-scale transcriptome sequencing techniques also open up new avenues in the study of seaweed-microbe interactions.

Main goal

This thesis aims to study the holobiont *L. dendroidea*, characterize the transcriptomic profile under natural and cultured conditions, and unveil the molecular mechanisms involved in the defense of this seaweed.

Specific goals

- Characterize the transcriptomic profile of the red seaweed *Laurencia dendroidea* (Chapter 2).
- Explore the taxonomic and functional composition of the *L. dendroidea* microbiome and the interactions among these holobiont components in the molecular level (Chapter 2).
- Identify the genes involved in the biosynthesis of terpenes in *L. dendroidea* (Chapters 2 and 3).
- Evaluate the influence of the potentially pathogenic bacteria *Vibrio madracius* on the gene expression patterns of *L. dendroidea* (Chapter 4).

References

1. **AlgaeBase. World-wide electronic publication, National University of Ireland, Galway.** [http://www.algaebase.org]
2. Cassano V, Metti Y, Millar AJK, Gil-Rodríguez MC, Sentíes A, Díaz-Larrea J, Oliveira MC, Fujii MT: **Redefining the taxonomic status of *Laurencia dendroidea* (Ceramiales, Rhodophyta) from Brazil and the Canary Islands**. *Eur J Phycol* 2012, **47**:67–81.

3. Yoneshigue Y: **Taxonomie et écologie des algues marines dans la région de Cabo frio (Rio de Janeiro, Brésil) = Taxonomy and ecology of marine algae in the area of Cabo Frio (Rio de Janeiro, Brazil)**. Université d'AixMarseille III, Aix-en-Provence; 1985.

4. Oliveira AS de: **Variabilidade intraespecífica na produção do sesquiterpeno elatol em *Laurencia dendroidea* (Ceramiales, Rhodophyta): influência de aspectos ambientais e genéticos.** 2011.

5. Irie T, Suzuki M, Kurosawa E, Masamune T: **Laurinterol, debromolaurinterol and isolaurinterol, constituents of *Laurencia* intermedia Yamada**. *Tetrahedron* 1970, **26**:3271–3277.

6. Williams DH, Stone MJ, Hauck PR, Rahman SK: **Why are secondary metabolites (Natural Products) biosynthesized?** *J Nat Prod* 1989, **52**:1189–1208.

7. Vairappan CS, Anangdan SP, Tan KL, Matsunaga S: **Role of secondary metabolites as defense chemicals against ice-ice disease bacteria in biofouler at carrageenophyte farms**. *J Appl Phycol* 2010, **22**:305–311.

8. Pereira RC, Da Gama B a P, Teixeira VL, Yoneshigue-Valentin Y: **Ecological roles of natural products of the Brazilian red seaweed *Laurencia obtusa* .** *Braz J Biol* 2003, **63**:665–72.

9. Da Gama B a, Pereira RC, Soares AR, Teixeira VL, Yoneshigue-Valentin Y: **Is the mussel test a good indicator of antifouling activity? A comparison between laboratory and field assays.** *Biofouling* 2003, **19 Suppl**:161–9.

10. Sudatti DB, Rodrigues SV, Pereira RC: **Quantitative GC-ECD analysis of halogenated metabolites: determination of surface and within-thallus elatol of *Laurencia obtusa* .** *J Chem Ecol* 2006, **32**:835–43.

11. Oliveira AS, Sudatti DB, Fujii MT, Rodrigues SV, Pereira RC: **Inter- and intrapopulation variation in the defensive chemistry of the red seaweed *Laurencia dendroidea* (Ceramiales, Rhodophyta)**. *Phycologia* 2013, **52**:130–136.

12. Sudatti DB, Fujii MT, Rodrigues SV, Turra A, Pereira RC: **Effects of abiotic factors on growth and chemical defenses in cultivated clones of *Laurencia dendroidea* J. Agardh (Ceramiales, Rhodophyta)**. *Mar Biol* 2011, **158**:1439–1446.

13. Sudatti DB: **Influência de fatores abióticos e bióticos na química defensiva da macroalga marinha *Laurencia dendroidea* J. Agardh (Ceramiales: Rhodophyta)**. Universidade Federal Fluminense; 2010.

14. Young DN, Howard BM, Fenical W: **Subcellular localization of brominated secondarymetabolites in the red alga *Laurencia snyderae* .** *J Phycol* 1980, **16**:182–185.

15. Salgado LT, Viana NB, Andrade LR, Leal RN, da Gama BAP, Attias M, Pereira RC, Amado Filho GM: **Intra-cellular storage, transport and exocytosis of halogenated compounds in marine red alga *Laurencia obtusa* .** *J Struct Biol* 2008, **162**:345–55.

16. Sudatti DB, Rodrigues S V., Coutinho R, da Gama B a. P, Salgado LT, Amado Filho GM, Pereira RC: **Transport and defensive role of elatol at the surface of the red seaweed *Laurencia obtusa* (Ceramiales, Rhodophyta)**. *J Phycol* 2008, **44**:584–591.

17. Reis VM, Oliveira LS, Passos RMF, Viana NB, Mermelstein C, Sant'anna C, Pereira RC, Paradas WC, Thompson FL, Amado-Filho GM, Salgado LT: **Traffic of secondary metabolites to cell surface in**

the red alga *Laurencia dendroidea* depends on a two-step transport by the cytoskeleton. *PLoS One* 2013, **8**:e63929.

18. Paradas WC, Salgado LT, Sudatti DB, Crapez M a, Fujii MT, Coutinho R, Pereira RC, Amado Filho GM: **Induction of halogenated vesicle transport in cells of the red seaweed *Laurencia obtusa* .** *Biofouling* 2010, **26**:277–86.
19. Sakemi S, Higa T, Jefford CW, Bernardinelli G: **Venustatriol. A new, anti-viral, triterpene tetracyclic ether from *Laurencia venusta* .** *Tetrahedron Lett* 1986, **27**:4287–4290.
20. Vairappan CS, Kawamoto T, Miwa H, Suzuki M: **Potent antibacterial activity of halogenated compounds against antibiotic-resistant bacteria.** *Planta Med* 2004, **70**:1087–90.
21. Topcu G, Aydogmus Z, Imre S, Gören AC, Pezzuto JM, Clement JA, Kingston DGI: **Brominated sesquiterpenes from the red alga *Laurencia obtusa* .** *J Nat Prod* 2003, **66**:1505–8.
22. Dos Santos AO, Veiga-Santos P, Ueda-Nakamura T, Filho BPD, Sudatti DB, Bianco EM, Pereira RC, Nakamura CV: **Effect of elatol, isolated from red seaweed *Laurencia dendroidea* , on *Leishmania amazonensis* .** *Mar Drugs* 2010, **8**:2733–43.
23. Veiga-Santos P, Pelizzaro-Rocha KJ, Santos a O, Ueda-Nakamura T, Dias Filho BP, Silva SO, Sudatti DB, Bianco EM, Pereira RC, Nakamura C V.: **In vitro anti-trypanosomal activity of elatol isolated from red seaweed *Laurencia dendroidea* .** *Parasitology* 2010, **137**:1661–70.
24. Chatter R, Ben Othman R, Rabhi S, Kladi M, Tarhouni S, Vagias C, Roussis V, Guizani-Tabbane L, Kharrat R: **In vivo and in vitro anti-inflammatory activity of neorogioltriol, a new diterpene extracted from the red algae *Laurencia glandulifera* .** *Mar Drugs* 2011, **9**:1293–306.
25. Lhullier C, Falkenberg M, Ioannou E, Quesada A, Papazafiri P, Horta PA, Schenkel EP, Vagias C, Roussis V: **Cytotoxic halogenated metabolites from the Brazilian red alga *Laurencia catarinensis* .** *J Nat Prod* 2010, **73**:27–32.
26. Ignea C, Cvetkovic I, Loupassaki S, Kefalas P, Johnson CB, Kampranis SC, Makris AM: **Improving yeast strains using recyclable integration cassettes, for the production of plant terpenoids.** *Microb Cell Fact* 2011, **10**:4.
27. Best RJ, Chaudoin AL, Bracken MES, Graham MH, Stachowicz JJ: **Plant–animal diversity relationships in a rocky intertidal system depend on invertebrate body size and algal cover**. *Ecology* 2014, **95**:1308–1322.
28. Wernberg T, Thomsen MS, Kotta J: **Complex plant–herbivore–predator interactions in a brackish water seaweed habitat**. *J Exp Mar Bio Ecol* 2013, **449**:51–56.
29. Haas AF, Nelson CE, Wegley Kelly L, Carlson CA, Rohwer F, Leichter JJ, Wyatt A, Smith JE: **Effects of coral reef benthic primary producers on dissolved organic carbon and microbial activity.** *PLoS One* 2011, **6**:e27973.
30. Barott KL, Rodriguez-Brito B, Janouškovec J, Marhaver KL, Smith JE, Keeling P, Rohwer FL: **Microbial diversity associated with four functional groups of benthic reef algae and the reef-building coral Montastraea annularis.** *Environ Microbiol* 2011, **13**:1192–204.
31. Persson F, Svensson R, Nylund GM, Fredriksson NJ, Pavia H, Hermansson M: **Ecological role of a seaweed secondary metabolite for a colonizing bacterial community.** *Biofouling* 2011, **27**:579–88.

32. Rao D, Webb JS, Kjelleberg S: **Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicata*.** *Appl Environ Microbiol* 2005, **71**:1729–36.

33. Goecke F, Labes A, Wiese J, Imhoff JF: **Phylogenetic analysis and antibiotic activity of bacteria isolated from the surface of two co-occurring macroalgae from the Baltic Sea**. *Eur J Phycol* 2013, **48**:47–60.

34. Hong Y-K, Cho JY: **Effect of seaweed epibiotic bacterium *Streptomyces violaceoruber* SCH-09 on marine fouling organisms**. *Fish Sci* 2013, **79**:469–475.

35. Singh RP, Reddy CRK: **Seaweed-microbial interactions: key functions of seaweed-associated bacteria.** *FEMS Microbiol Ecol* 2014, **88**:213–30.

36. Egan S, Harder T, Burke C, Steinberg P, Kjelleberg S, Thomas T: **The seaweed holobiont: understanding seaweed-bacteria interactions.** *FEMS Microbiol Rev* 2013, **37**:462–76.

37. Singh R, Mantri V, Reddy C, Jha B: **Isolation of seaweed-associated bacteria and their morphogenesis-inducing capability in axenic cultures of the green alga *Ulva fasciata***. *Aquat Biol* 2011, **12**:13–21.

38. Singh RP, Bijo AJ, Baghel RS, Reddy CRK, Jha B: **Role of bacterial isolates in enhancing the bud induction in the industrially important red alga *Gracilaria dura*.** *FEMS Microbiol Ecol* 2011, **76**:381–92.

39. Dobretsov S, Teplitski M, Paul V: **Mini-review: quorum sensing in the marine environment and its relationship to biofouling.** *Biofouling* 2009, **25**:413–27.

40. Joint I, Tait K, Wheeler G: **Cross-kingdom signalling: exploitation of bacterial quorum sensing molecules by the green seaweed *Ulva*.** *Philos Trans R Soc Lond B Biol Sci* 2007, **362**:1223–33.

41. Weinberger F, Beltran J, Correa JA, Lion U, Pohnert G, Kumar N, Steinberg P, Kloareg B, Potin P: **Spore release in *Acrochaetium* sp. (Rhodophyta) is bacterially controlled**. *J Phycol* 2007, **43**:235–241.

42. Singh RP, Shukla MK, Mishra A, Reddy CRK, Jha B: **Bacterial extracellular polymeric substances and their effect on settlement of zoospore of *Ulva fasciata*.** *Colloids Surf B Biointerfaces* 2013, **103**:223–30.

43. Collén J, Porcel B, Carré W, Ball SG, Chaparro C, Tonon T, Barbeyron T, Michel G, Noel B, Valentin K, Elias M, Artiguenave F, Arun A, Aury J-M, Barbosa-Neto JF, Bothwell JH, Bouget F-Y, Brillet L, Cabello-Hurtado F, Capella-Gutiérrez S, Charrier B, Cladière L, Cock JM, Coelho SM, Colleoni C, Czjzek M, Da Silva C, Delage L, Denoeud F, Deschamps P, et al.: **Genome structure and metabolic features in the red seaweed *Chondrus crispus* shed light on evolution of the Archaeplastida.** *Proc Natl Acad Sci U S A* 2013, **110**:5247–52.

44. Cock JM, Sterck L, Rouzé P, Scornet D, Allen AE, Amoutzias G, Anthouard V, Artiguenave F, Aury J-M, Badger JH, Beszteri B, Billiau K, Bonnet E, Bothwell JH, Bowler C, Boyen C, Brownlee C, Carrano CJ, Charrier B, Cho GY, Coelho SM, Collén J, Corre E, Da Silva C, Delage L, Delaroque N, Dittami SM, Doulbeau S, Elias M, Farnham G, et al.: **The *Ectocarpus* genome and the independent evolution of multicellularity in brown algae.** *Nature* 2010, **465**:617–21.

45. Zilber-Rosenberg I, Rosenberg E: **Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution.** *FEMS Microbiol Rev* 2008, **32**:723–35.

46. Gachon CMM, Sime-Ngando T, Strittmatter M, Chambouvet A, Kim GH: **Algal diseases: spotlight on a black box.** *Trends Plant Sci* 2010, **15**:633–40.

47. Largo DB, Fukami K, Nishijima T: **Occasional pathogenic bacteria promoting ice-ice disease in the carrageenan-producing red algae *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Solieriaceae, Gigartinales, Rhodophyta)**. *J Appl Phycol* 1995, **7**:545–554.

48. Wang G, Shuai L, Li Y, Lin W, Zhao X, Duan D: **Phylogenetic analysis of epiphytic marine bacteria on Hole-Rotten diseased sporophytes of *Laminaria japonica***. *J Appl Phycol* 2008, **20**:403–409.

49. Campbell AH, Vergés A, Steinberg PD: **Demographic consequences of disease in a habitat-forming seaweed and impacts on interactions between natural enemies**. *Ecology* 2014, **95**:142–152.

50. Nurnberger T, Brunner F, Kemmerling B, Piater L: **Innate immunity in plants and animals: striking similarities and obvious differences**. *Immunol Rev* 2004, **198**:249–266.

51. Weinberger F: **Pathogen-induced defense and innate immunity in macroalgae.** *Biol Bull* 2007, **213**:290–302.

52. Weinberger F, Friedlander M: **Response of *Gracilaria conferta* (Rhodophyta) to oligoagars results in defense against agar-degrading epiphytes**. *J Phycol* 2000, **36**:1079–1086.

53. Küpper FC, Müller DG, Peters AF, Kloareg B, Potin P: **Oligoalginate recognition and oxidative burst play a key role in natural and induced resistance of sporophytes of Laminariales**. *J Chem Ecol* 2002, **28**:2057–2081.

54. Küpper FC, Gaquerel E, Boneberg E-M, Morath S, Salaün J-P, Potin P: **Early events in the perception of lipopolysaccharides in the brown alga *Laminaria digitata* include an oxidative burst and activation of fatty acid oxidation cascades.** *J Exp Bot* 2006, **57**:1991–9.

55. Weinberger F, Leonardi P, Miravalles A, Correa JA, Lion U, Kloareg B, Potin P: **Dissection of two distinct defense-related responses to agar oligosaccharides in *Gracilaria chilensis* (Rhodophyta) and *Gracilaria conferta* (Rhodophyta)**. *J Phycol* 2005, **41**:863–873.

56. Küpper FC, Gaquerel E, Cosse A, Adas F, Peters AF, Müller DG, Kloareg B, Salaün J-P, Potin P: **Free fatty acids and methyl jasmonate trigger defense reactions in *Laminaria digitata*.** *Plant Cell Physiol* 2009, **50**:789–800.

57. Bosch M, Wright LP, Gershenzon J, Wasternack C, Hause B, Schaller A, Stintzi A: **Jasmonic acid and its precursor 12-oxophytodienoic acid control different aspects of constitutive and induced herbivore defenses in tomato.** *Plant Physiol* 2014, **166**:396–410.

58. Collén J, Hervé C, Guisle-Marsollier I, Léger JJ, Boyen C: **Expression profiling of *Chondrus crispus* (Rhodophyta) after exposure to methyl jasmonate.** *J Exp Bot* 2006, **57**:3869–81.

59. Arnold TM, Targett NM, Tanner CE, Hatch WI, Ferrari KE: **Evidence for methyl jasmonate-induced phlorotannin production in *Fucus vesiculosus* (Phaeophyceae)**. *J Phycol* 2001, **37**:1026–1029.

60. Cosse A, Potin P, Leblanc C: **Patterns of gene expression induced by oligoguluronates reveal conserved and environment-specific molecular defense responses in the brown alga *Laminaria digitata*.** *New Phytol* 2009, **182**:239–50.

61. Strittmatter M, Grenville-Briggs LJ, Breithut L, VAN West P, Gachon CMM, Küpper FC: **Infection of the brown alga *Ectocarpus siliculosus* by the oomycete *Eurychasma dicksonii* induces oxidative stress and halogen metabolism.** *Plant Cell Environ* 2015.

62. Küpper FC: **Oligoguluronates elicit an oxidative burst in the brown algal kelp *Laminaria digitata***. *PLANT Physiol* 2001, **125**:278–291.

63. Collén J, Hervé C, Guisle-Marsollier I, Léger JJ, Boyen C: **Expression profiling of *Chondrus crispus* (Rhodophyta) after exposure to methyl jasmonate.** *J Exp Bot* 2006, **57**:3869–81.

64. Van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C: **Ten years of next-generation sequencing technology**. *Trends Genet* 2014, **30**:418–426.

65. Wang Z, Gerstein M, Snyder M: **RNA-Seq: a revolutionary tool for transcriptomics.** *Nat Rev Genet* 2009, **10**:57–63.

66. Sun P, Mao Y, Li G, Cao M, Kong F, Wang L, Bi G: **Comparative transcriptome profiling of *Pyropia yezoensis* (Ueda) M.S. Hwang & H.G. Choi in response to temperature stresses.** *BMC Genomics* 2015, **16**:463.

67. Liu F, Sun X, Wang W, Liang Z, Wang F: **De novo transcriptome analysis-gained insights into physiological and metabolic characteristics of *Sargassum thunbergii* (Fucales, Phaeophyceae)**. *J Appl Phycol* 2013, **26**:1519–1526.

68. Wu S, Sun J, Chi S, Wang L, Wang X, Liu C, Li X, Yin J, Liu T, Yu J: **Transcriptome sequencing of essential marine brown and red algal species in China and its significance in algal biology and phylogeny**. *Acta Oceanol Sin* 2014, **33**:1–12.

de Oliveira *et al. BMC Genomics* 2012, **13**:487
http://www.biomedcentral.com/1471-2164/13/487

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CHAPTER 2 - Transcriptomic analysis of the red seaweed *Laurencia dendroidea* (Florideophyceae, Rhodophyta) and its microbiome

Louisi Souza de Oliveira[1], Gustavo Bueno Gregoracci[1], Genivaldo Gueiros Zacarias Silva[2], Leonardo Tavares Salgado[3], Gilberto Amado Filho[3], Marcio Alves-Ferreira[4], Renato Crespo Pereira[5] and Fabiano L Thompson[1]*.

Abstract

Background: Seaweeds of the *Laurencia* genus have a broad geographic distribution and are largely recognized as important sources of secondary metabolites, mainly halogenated compounds exhibiting diverse potential pharmacological activities and relevant ecological role as anti-epibiosis. Host-microbe interaction is a driving force for co-evolution in the marine environment, but molecular studies of seaweed-associated microbial communities are still rare. Despite the large amount of research describing the chemical compositions of *Laurencia* species, the genetic knowledge regarding this genus is currently restricted to taxonomic markers and general genome features. In this work we analyze the transcriptomic profile of *L. dendroidea* J. Agardh, unveil the genes involved on the biosynthesis of terpenoid compounds in this seaweed and explore the interactions between this host and its associated microbiome.

Results: A total of 6 transcriptomes were obtained from specimens of *L. dendroidea* sampled in three different coastal locations of the Rio de Janeiro state. Functional annotations revealed predominantly basic cellular metabolic pathways. Bacteria was the dominant active group in the microbiome of *L. dendroidea*, standing out nitrogen fixing Cyanobacteria and aerobic heterotrophic Proteobacteria. The analysis of the relative contribution of each domain highlighted bacterial features related to glycolysis, lipid and polysaccharide breakdown, and also recognition of seaweed surface and establishment of biofilm. Eukaryotic transcripts, on the other hand, were associated with photosynthesis, synthesis of carbohydrate reserves, and defense mechanisms, including the biosynthesis of terpenoids through the mevalonate-independent pathway.

Conclusions: This work describes the first transcriptomic profile of the red seaweed *L. dendroidea*, increasing the knowledge about ESTs from the Florideophyceae algal class. Our data suggest an important role for *L. dendroidea* in the primary production of the holobiont and the role of Bacteria as consumers of organic matter and possibly also as nitrogen source. Furthermore, this seaweed expressed sequences related to terpene biosynthesis, including the complete mevalonate-independent pathway, which offers new possibilities for biotechnological applications using secondary metabolites from *L. dendroidea*.

Keywords: Red seaweed, Terpene, Bacteria, Holobiont, Metabolic pathway, EST

Introdution

Laurencia dendroidea is a red seaweed species widespread in the Atlantic Ocean, whose type locality is in Brazil. It is found from the intertidal to the subtidal zone at 3m depth. The thalli are erect, forming dense tufts 4–20 cm high, brown-purple or violet-greenish in color [1]. The genus *Laurencia* [2] was recognized, since the first studies on natural products in the 1960s, as an important source of secondary metabolites, mainly halogenated compounds [3,4]. The secondary metabolites of *Laurencia* play a relevant ecological role as chemical defenses against bacterial colonization and infection [5-7].

Seaweeds are especially susceptible to microbial colonization due to the biosynthesis and release of large amounts of organic compounds, which may serve as chemo-attractants and nutrient source for microbes [8]. In this context, secondary metabolites and exudates may act together selecting the microbial community associated with the surfaces and tissues of seaweeds [9,10]. Host-microbe interaction is widely recognized as one of the main driving forces for co-evolution in the marine environment, leading to the establishment of beneficial microbiomes. For instance, microbes associated with seaweed tissues may possess the ability to fix nitrogen, mineralize the organic substrates and also supply the seaweeds with carbon dioxide and growth factors [11-14]. The microbiome on seaweeds tends to be species-specific and different from the surrounding seawater [15]. However, the characterization of the microbial community living at the surface of macroalgae is still limited and the molecular studies of these communities are rare [15-17].

The untapped diversity of the secondary metabolites of *Laurencia*, particularly terpenes, has attracted considerable attention of different research groups worldwide. The pharmacological potential of these compounds comprises the strong antibiotic [18,19], antiviral [20], antimalarial [21], antitrypanosomal [22], antileishmanial [23], anti-inflammatory [24] and anti-carcinoma [25-27] activities. A major secondary metabolite of *L. dendroidea* is the sesquiterpene (C15) (-)-elatol, a substance that has a high biocidal and anti-epibiosis activity and could be used for the preparation of antifouling paints, or for the development of antimicrobials [28-30]. A first attempt for the commercial application of (-)-elatol resulted in the filing of the patent in Brazil to use this compound as an antifouling agent. However, technological developments are still needed to ensure its commercial viability [31]. This obstacle stems from the low yield of the extraction process, the complexity of the organic total synthesis of (-)-elatol in laboratory [32], and the failure of the large scale cultivation of this species. A possible alternative to circumvent this problem is the synthesis of (-)-elatol in the laboratory using genetically modified organisms [31].

The cellular location and the environmental factors that induce the production of this compound by *L. dendroidea* are known [33,34], but the genes involved in the biosynthesis of this compound were not yet determined, representing a new research frontier in the technological use of (-)-elatol. Recent studies have determined some of the genes responsible for the biosynthesis of terpenes (i.e. cyclases or synthases) in bacteria [35], fungi [36], and plants [37]. The sequence homology observed among at least some classes of terpene synthases from these organisms [38] may facilitate the search for homolog genes in *L. dendroidea*.

Despite the large number of studies based on the chemical composition of *Laurencia* species, the genetic knowledge regarding this genus is currently restricted to taxonomic markers [39,40]. The genome size of *L. dendroidea* is estimated to be about 833 Mbp, based on a study of another species of the same genus [41], but gene sequences from this species have not previously been described. In this work we analyze the transcriptomic profile of *L. dendroidea* at different geographic locations, unveil the genes involved on the biosynthesis of terpenoid compounds in this seaweed and also explore the interactions between the alga and the associated microbiome.

Methods

Specimens collection

Specimens of *L. dendroidea* were randomly collected in the intertidal zone during high tide at Azedinha (22°44'28.76"S, 41°52'55.70"W) and Forno beaches (22°45'42.72"S, 41°52'29.81"W), both in Búzios, and at Ibicuí beach (22°57'45.02"S, 41°01'29.05"W) located in Mangaratiba, all these places on the coast of the Rio de Janeiro state, Brazil (Figure 1). Seaweeds were collected from nearly the same depth in two subsequent days, at approximately the same hour, with the same climatic characteristics to minimize the variation in abiotic factors. The collected thalli were rapidly cleaned of macroscopic epiphytes using tweezers, without damage to the host seaweed, and the samples were immediately frozen in liquid nitrogen, to better preserve the holobiont.

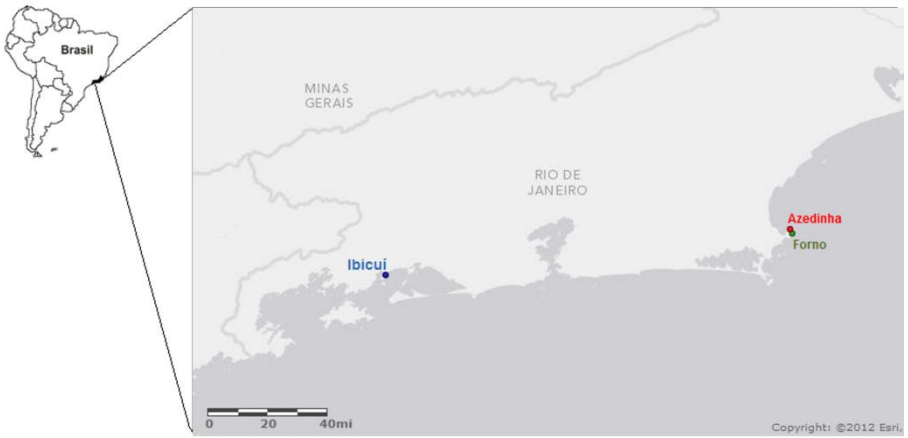

Figure 1. Collection sites of specimens of *L. dendroidea* in Búzios and Mangaratiba, on the coast of the Rio de Janeiro state, Brazil. Scale bar presented in miles (mi).

RNA extraction, reverse-transcription and pyrosequencing

Two specimens of. *L. dendroidea* from each location were separately ground in liquid nitrogen using a mortar and pestle to obtain a fine powder. Then, 100 mg of powder from each sample was suspended in 1 mL of extraction buffer (6.5 M guanidinium hydrochloride, 100 mM Tris-HCl pH 8.0, 0.1 M sodium acetate pH 5.5, 0.1 M β-mercaptoethanol, 0.2 M KOAc). Total RNA was extracted following the method previously proposed for another red seaweed [42], but we performed an extra centrifugation step and transferred the supernatant phase before adding the chloroform, which improved the RNA quality. In order to eliminate

DNA residues, all the samples were treated with DNAse (RNAse free, PROMEGA, Madison, USA). The double-stranded cDNAs (ds cDNAs) were synthesized and amplified using the SMARTer cDNA synthesis kit and the Advantage2 polymerase (Clontech, Califórnia, USA) starting from 1 μg of total RNA. The optimal number of amplification cycles was determined to be 23. This amplification did not exclude the prokaryotic portion of the holobiont, allowing the study of the microbiome along with the host. The PCR amplification products were purified using the NucleoSpinW Extract II kit (Macherey-Nagel, Düren, Alemanha). Finally the ds cDNAs were eluted in TE buffer (10 mM Tris-HCl pH 7.6; 1 mM EDTA) and sequenced using 454 pyrosequencing technology [43].

Transcriptome analysis

The sequences from each sample were preprocessed using the software Prinseq [44] to trim poly-A/T tails at least 20 bp long and to remove reads shorter than 75 bp, and then assembled into contigs using the Roche's algorithm Newbler (minimum overlap length = 40 bp, minimum overlap identity = 95%). In our analysis we annotated both contigs and singlets after assembly (hereafter referred as transcripts), since they contained different sequences and relevant information. We downloaded all the EST sequences deposited for the class Florideophyceae in the NCBI (comprising 11 species) and assembled the reads using the TGICL software from TIGR [45]. Afterwards, the assembly of all sequences derived from *L. dendroidea* was aligned against the Florideophyceae EST NCBI database using the Promer alignment tool (MUMmer 3.0) using the 'maxmatch' parameter [46]. The results were parsed using the show-coords script with – k and - r parameters and only reciprocal matches were considered for calculations. Sequences annotated as Bacteria were treated separately in this analysis, but eventual micro-eukaryotic sequences could not be removed, since the database is not complete regarding eukaryotic marine life and no *Laurencia* sequences aside from taxonomic markers are available.

Taxonomic and functional analysis were performed on assembled sequences from all samples, using the Newbler software, and automatically annotated, using the MG-RAST server, through BLAST, against the GenBank, COG, KEGG and Subsystems databases with maximum e-value cutoff of 10^{-5} [47]. The sequences obtained in this project are publicly available in the MG-RAST database and were organized in a file for each sample, named according to the site of origin, and a file containing the assembler of all reads

(http://metagenomics.anl.gov/linkin.cgi?project=1274). To characterize the major phenotypic features of the microbial community associated with *L. dendroidea*, features of bacterial genera identified against Genbank (through MG-Rast) were manually annotated using the Bergey's manuals of Systematic Bacteriology (2nd ed.). Additionally, we explored the relative contributions of Bacteria and Eukarya to the functional profile. Sequences annotated against the Genbank corresponding to these domains were extracted using the Workbench tool from MG-RAST server, and re-annotated against functional hierarchies (COG, Subsystems). The functional profiles of the domains were compared using the Statistical Analysis of Metagenomic Profiles (STAMP) bioinformatics software v2.0 [48]. Statistical significance ($p < 0.05$) was calculated pairwise using two sided G-test (with Yates' correction) and Fisher's exact test, and the confidence intervals for each proportion were calculated using the asymptotic method with a continuity correction considering the threshold of 95%. Furthermore, a specific search for two profiles using hidden markov models was performed, through the HMMER 3.0 software [49]. The first HMM profile was based on the alignment of all vanadium bromoperoxidases deposited in the protein database of NCBI, and the second one, based on the universal metal-binding domain of terpene synthases (PF03936), was obtained from PFAM as previously described [35].

Results

A total of 6 transcriptomes (235,572 reads, 52 Mbp) were obtained for specimens of the seaweed *L. dendroidea* originated from three different locations in the Rio de Janeiro coast. The assembly of the sequences from each replicate resulted on 500–1,000 contigs and 10,000–16,000 singlets (see Table 1 for detailed information). The COG functional annotation and the GenBank taxonomic annotation indicated that the transcriptomic profile of *L. dendroidea* was highly similar among the samples (Additional files 1 and 2). Since no significant differences were observed, all the reads of the 6 transcriptomes were assembled in order to represent a transcriptomic profile for this species, resulting on 3,887 contigs and 38,010 singlets. A total of 30,585 tentative unigenes (73% of the transcripts) were identified as genes coding for proteins with unknown function, indicating the need for further molecular studies in order to unravel the function of a large portion of the transcriptome of this seaweed.

Table 1. Characteristics of the sequencing and assembly of the cDNA libraries from the *Laurencia dendroidea* holobiont.

Location	Azedinha1	Azedinha2	Forno1	Fono2	Ibicuí1	Ibicuí2
Total Nucleotides (basepairs)	11,635,249	9,384,269	11,049,671	7,101,334	5,550,607	8,011,563
N. of Sequences	51,592	42,577	49,001	31,434	24,423	36,545
N. of Contigs	1,079	926	985	556	586	683
Avg. Size of Contigs	492.24 ± 190.19	489.62 ± 195.59	481.88 ±195.80	466.06 ± 182.92	465.71 ± 164.32	487.58 ± 193.41
N. of Singlets	15,755	14,480	14,830	10,935	10,522	11,719
Avg. Size of Singlets	202.17± 78.19	198.52 ± 74.80	198.01± 77.76	197.09 ± 76.42	212.72 ± 80.09	195.50 ± 75.30

The closest red algal genus with sequences deposited in the database is *Griffithsia*, classified in the order Ceramiales, for which we found only 1,277 ESTs, most of them (99.76%) derived from *Griffithsia okiensis* [50]. Searching at a higher taxonomic level, the total number of ESTs from the class Florideophyceae deposited in NCBI was 37,198, comprising 21,475 unigenes, from which only 5.95% matched with 3.34% unigenes from this study (Figure 2). These numbers include the sequences of Bacteria associated with the *Laurencia* holobiont (1.94%), from which 0.3% matched with 1.39% of the sequences in the Florideophyceae database, indicating that the reference database itself contains bacterial sequences. Excluding those bacterial sequences from our analysis, 3.04% of the remaining sequences are left matching 4.56% of sequences from the Florideophyceae database (Figure 2). Therefore, 95.02% of the sequences provided by this work could potentially enrich our current knowledge regarding Florideophyceae as they represent unknown genes.

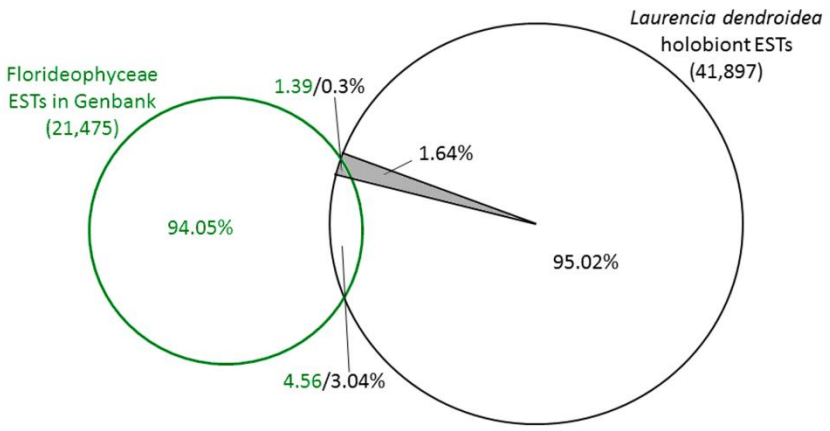

Figure 2. MUMMER-based identification of shared sequences between this study and the dbEST for the class Florideophyceae. The shaded area corresponds to sequences annotated as bacteria in this study.

Major groups of transcripts of *L. dendroidea*.

The functional classification of the ESTs revealed that most of the transcripts were related to the basal metabolism of the *Laurencia* holobiont (Figures 3 and 4). The most represented COG categories were associated to Translation, Ribosomal Structure and Biogenesis (18.65%), Posttranslational Modification, Protein Turnover and Chaperones (14.90%), and Amino acid Transport and Metabolism (7.57%). Additionally, functions associated with Energy Production and Conversion were relatively common (7.37%). Moreover, the sequences related to Replication, Recombination and Repair (7.37%), and the ESTs involved in Carbohydrate Transport and Metabolism (5.42%) were among the most represented categories in the transcriptome of *L. dendroidea* (Figure 3).

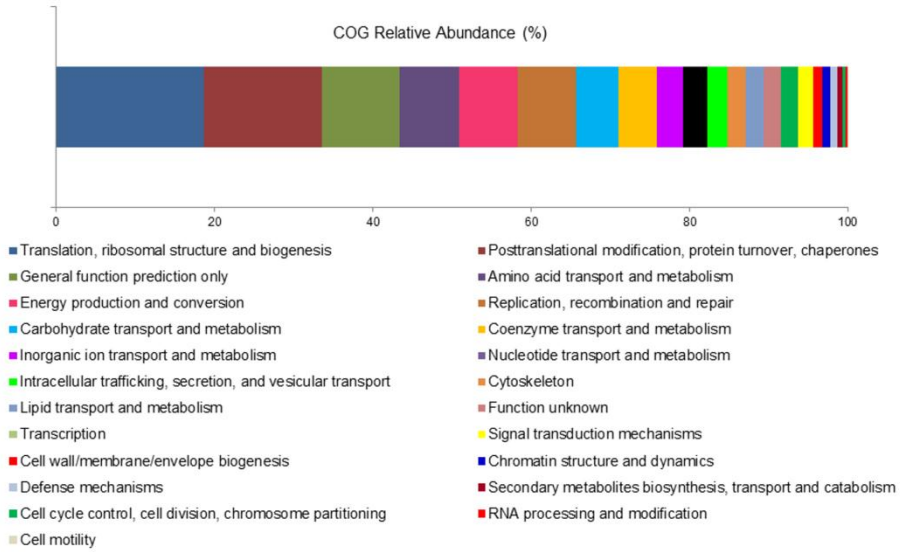

Figure 3. COG functional profile overview of the transcriptome of *L. dendroidea*.

The Subsystems annotation corroborated further the general expression profile of *Laurencia*. The main recognized features are Protein Metabolism (19.20%) and Carbohydrates (13.11%). Transcripts related to Cofactors, Vitamins, Prosthetic Groups, Pigments (8.88%), Amino Acids and Derivatives (8.77%) and RNA Metabolism (8.71%) were also numerous (Figure 4).

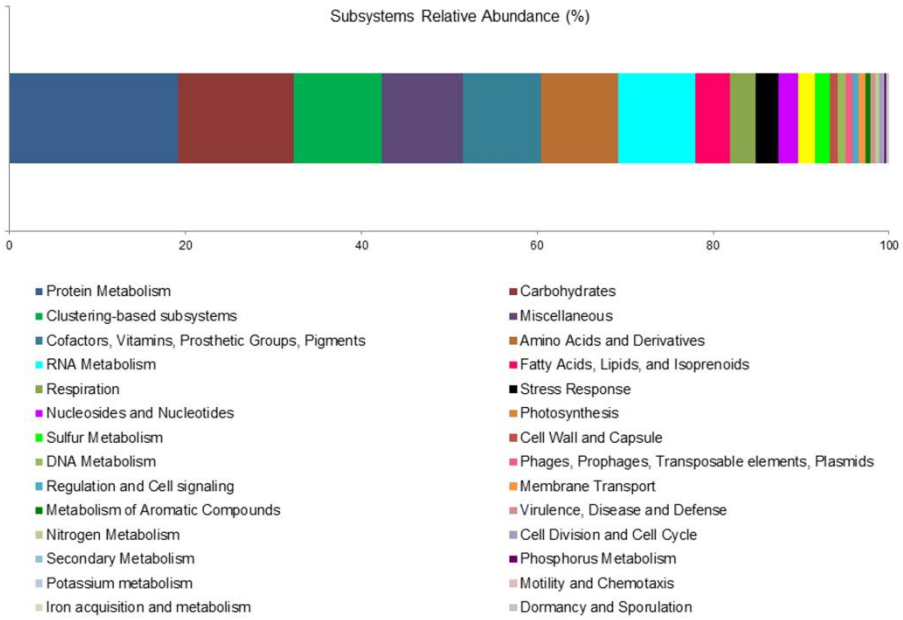

Figure 4. Subsystems functional profile overview of the transcriptome of *L. dendroidea*.

Transcriptome of *L. dendroidea*-associated microbiome

The functional analysis of the transcriptome revealed bacterial genes that are important for surface colonization, such as the transcripts related to flagellum (0.11% of the total), CheY-like receiver domain (0.04% of the total), and S-adenosylmethionine synthetase (0.03% of the total). Indeed, we detected fewer sequences involved in Motility and Chemotaxis (0.11% of the total) in comparison with the ones related to Capsular and extracellular polysaccharides (0.53% of the total).

A total of 6,154 reads (14.69% of the total) were assigned to taxonomic categories using the GenBank database. Among them, 17.26% were classified in the domain Bacteria (Figure 5a). The most abundant bacterial transcripts were assigned to the phylum Cyanobacteria (35.97%), mainly to the orders Chroococcales, Oscillatoriales and Nostocales. The second most represented phylum is Proteobacteria (32.86%) with Gammaproteobacteria and Alphaproteobacteria as the dominant classes (Figure 5b).

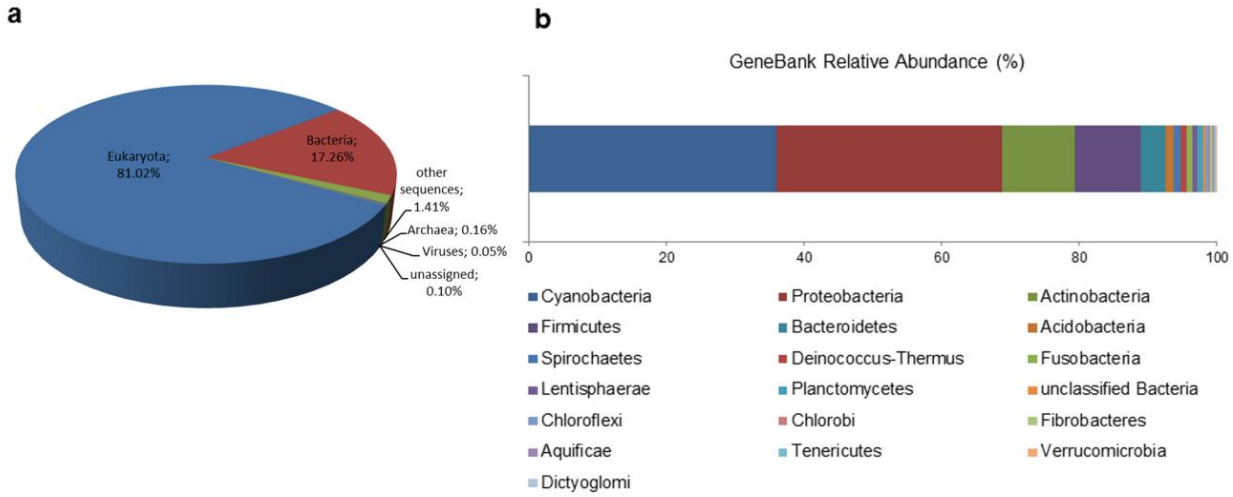

Figure 5. Taxonomic classification for the transcriptome of *L. dendroidea*. (a) Taxonomy overview ; (b) Relative abundance of bacterial phyla.

Manual annotation revealed the majority of the bacterial transcripts (to which a description of respiratory metabolism could be found in Bergey's manuals) as ascribed to aerobic (62.30%) or aerotolerant groups (14.00%). We also verified a higher abundance of transcripts related to respiration (2.96%) in comparison with the ones involved in the fermentative metabolism (0.64%). Furthermore, Bacteria expressed genes, such as Superoxide dismutase (0.51%), Glutaredoxins (0.42%), Alkyl hydroperoxide reductase (0.21%), and the chaperones GroEL (3.17%), DnaJ (1.37%) and DnaK (0.84%), related to protection from reactive oxygen species produced during aerobic metabolism (Additional file 3).

Genes involved in Photosynthesis (3.18%) and in the biosynthesis of starch (0.66%) were more abundant in eukaryotes, while ESTs related to Carbohydrate (5.63%) and Lipid Transport and Metabolism (3.58%), and to Energy Production and Conversion (11.38%) were more represented in Bacteria. Transcripts associated to Amino acid metabolism (11.50%) were also more represented in Bacteria, except for the glutamate biosynthesis that was preferentially expressed by Eukarya (0.58%, Additional file 3).

Additionally, several transcripts were attributed to bacterial genera known to be heterotrophs (> 51.9%) or motile (> 28.4%). Furthermore, 25.4% of the heterotrophy associated transcripts belong to genera recognized as pathogens or closely associated to eukaryotes. Along this context, the Hmmer search for vanadium-dependent bromoperoxidases, which could be involved in response to infection, resulted on 10 hits, and their functional classification was confirmed by Blastx.

Terpenoid biosynthesis in the holobiont

Within the functional annotations, 34 transcripts associated to the terpenoid backbone biosynthesis in *L. dendroidea* were found, representing all the required enzymes involved in the mevalonate-independent pathway (Table 2, Figure 6). The identified genes participate in important steps for the synthesis of dimethylallyl diphosphate (EC 2.2.1.7; EC: 1.1.1.267; EC: 2.7.7.60; EC: 2.7.1.148; EC 4.6.1.12; EC: 1.17.7.1; EC: 1.17.1.2), its isomerization to isopentenyl diphosphate (EC: 5.3.3.2), and the condensation of these two C5-units, through the action of prenyltransferases, generating geranyl diphosphate (GDP, EC: 2.5.1.1), farnesyl diphosphate (FDP, EC: 2.5.1.10), and geranylgeranyl diphosphate (GGDP, EC: 2.5.1.29). We also found genes involved in the subsequent steps to the synthesis of chlorophylls (EC: 1.3.1.83), plastoquinone, phylloquinone, ubiquinone (EC: 2.5.1.84, EC: 2.5.1.85, EC: 2.5.1.91) and N-glycans, (EC: 2.5.1.87). The Hmmer search for the metal binding conserved domain (PF03936) in the transcriptome of *L. dendroidea* resulted on 3 hits, and the subsequent manual annotation confirmed their classification as terpene synthases.

Table 2. Description of the enzymes involved on terpenoid backbone biosynthesis

Enzyme codes	Enzyme names	Databases
EC 2.2.1.7	1-deoxy-D-xylulose-5-phosphate synthase	SEED
EC: 1.1.1.267	1-deoxy-D-xylulose-5-phosphate reductoisomerase	KEGG/SEED
EC: 2.7.7.60	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	KEGG
EC: 2.7.1.148	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase	KEGG/SEED
EC 4.6.1.12	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	SEED
EC: 1.17.7.1	(E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase	KEGG/SEED
EC: 1.17.1.2	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	KEGG/SEED
EC: 5.3.3.2	Isopentenyl-diphosphate Delta-isomerase	KEGG/SEED
EC: 2.5.1.1	Dimethylallyltranstransferase	KEGG/SEED
EC: 2.5.1.10	(2E,6E)-farnesyl diphosphate synthase	KEGG/SEED
EC: 2.5.1.29	Geranylgeranyl diphosphate synthase	KEGG/SEED
EC: 2.5.1.87	Ditrans,polycis-polyprenyl diphosphate synthase ((2E,6E)-farnesyl diphosphate specific)	KEGG
EC: 1.3.1.83	Geranylgeranyl diphosphate reductase.	KEGG/SEED
EC: 2.5.1.85	All-trans-nonaprenyl-diphosphate synthase (geranylgeranyl-diphosphate specific)	KEGG
EC: 2.5.1.84	All-trans-nonaprenyl-diphosphate synthase (geranyl-diphosphate specific)	KEGG
EC: 2.5.1.91	All-trans-decaprenyl-diphosphate synthase	KEGG

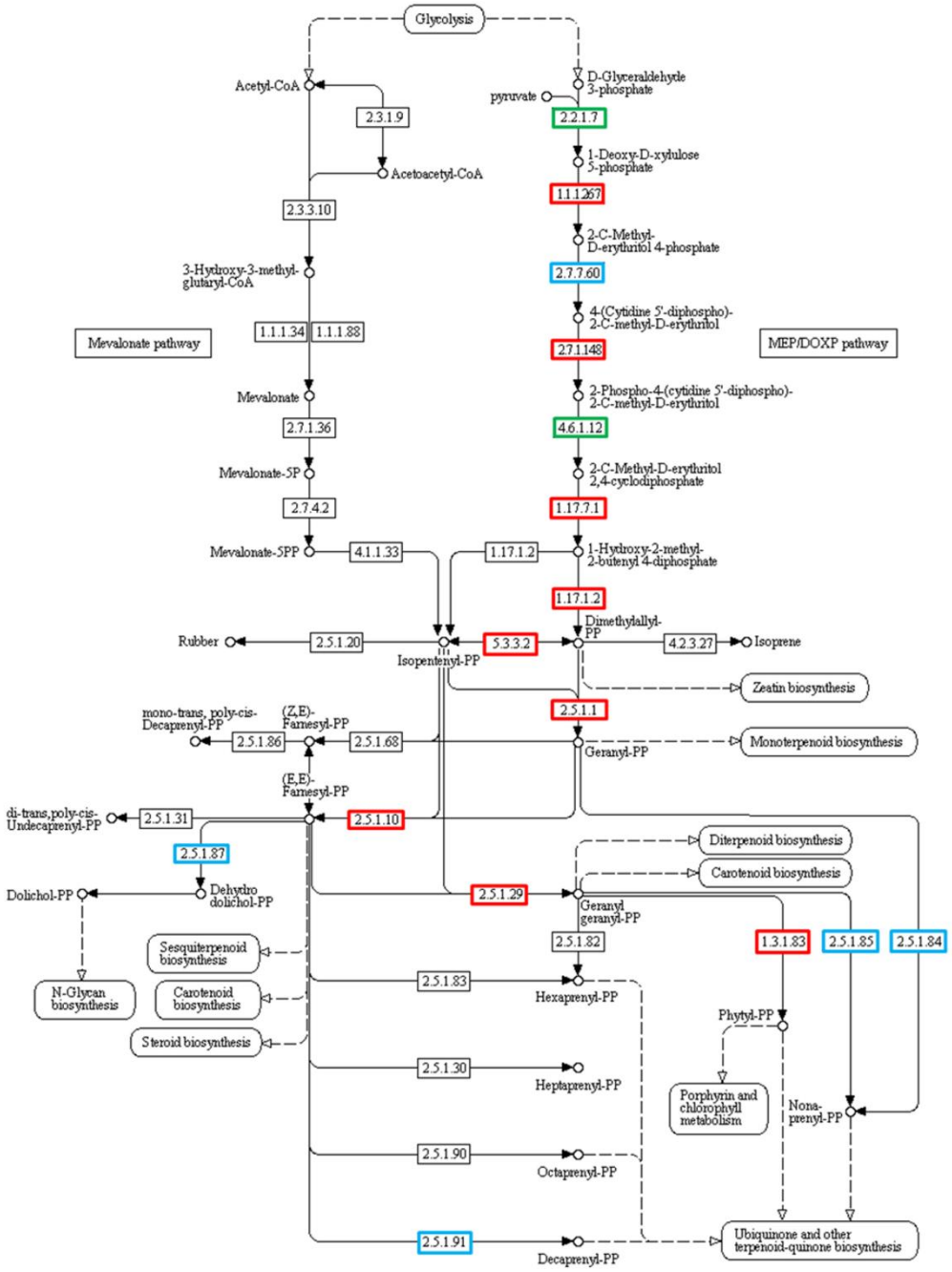

Figure 6. Terpenoid backbone biosynthetic pathway. Blue squares represent the genes identified through the KEGG database, green squares points the genes identified using the SEED database and red squares highlight the genes identified using both databases.

Discussion

The present study provides the largest transcriptome dataset for the class Florideophyceae and represents the first transcriptomic characterization of the seaweed

Laurencia dendroidea. The presented numbers could be an overestimate of the contribution of *L. dendroidea* to the Florideophyceae database, since we worked with complex samples. Nevertheless, at least some of the sequencing projects in the Florideophyceae dbEST are also based on non-axenic field samples [51,52], hampering the achievement of a more accurate estimate. Indeed, it is notable the presence of sequences deposited in this database that matched our bacterial sequences.

Recent advances in the field of algal genomics included only the complete sequencing of the nuclear genome of the microalgae *Cyanidioschyzon merolae* [53], *Ostreococcus tauri* [54], *Chlamydomonas reinhardtii* [55], and *Cyanophora paradoxa* [56] and the brown macroalga *Ectocarpus siliculosus* [57]. Moreover, EST projects have provided valuable information in the transcriptomic profile of some species of Rhodophyta [50,51,58-62] in the phylogenetic relationships among photosynthetic eukaryotes [63,64] and have also unveiled genes involved in stress response [52,65,66] and in life phase differentiation [67-70].

The transcriptomic profile of *L. dendroidea* and its corresponding associated microbiome was closely similar among all the samples, regardless of their place of origin. Likewise, a previous study verified a higher similarity between bacterial populations from seaweeds of the same species sampled at different sites than between those from different species growing at the same habitat, emphasizing the specificity of this association [71]. Our data reinforces these findings as we observed a high similarity in the taxonomic composition of the active microbiome associated with *L. dendroidea* in different sample sites.

Major groups of transcripts of *L. dendroidea*

The functional annotation of the transcripts revealed predominantly basic cellular metabolic pathways. In general, functions related to translation and protein synthesis, from amino acid precursors to post-translational modifications are the most abundantly expressed in the transcriptome of *L. dendroidea*. Besides, complete pathways for energy production were well represented, mainly related to the pyruvate dehydrogenase complex, electron transfer, thioredoxins, citric acid cycle and NADH dehydrogenase. The ESTs involved in carbohydrate transport and metabolism (mainly glycolysis, starch and sucrose metabolism, and pentose phosphate pathway), Cofactors, Vitamins, Prosthetic Groups, Pigments (including Folate and Pterines, Tetrapyrroles and Pyridoxine), RNA Metabolism (mainly

RNA Processing and Modification) were among the most represented categories in the transcriptome of *L. dendroidea*.

Other relevant features in this transcriptome are related to DNA replication, recombination and repair, which are important to the survival and growth of the seaweed, especially in the rocky-shore coastal environment where the organisms are subject to high UVB levels that causes serious damages to DNA [72]. The ability to resist to UV-exposure influences the vertical distribution of seaweeds [73], and *L. dendroidea* typically grows in the lower midlittoral zone where UV-damage repair may be necessary. The same set of expressed sequences relevant in the transcriptome of *L. dendroidea* are among the most represented in the EST databases of *Gracilaria gracilis* [62], *G. changii* [51], *G. tenuistipitata* [60], *Porphyra yezoensis* [61,67], *P. haitanensis* [59], *Eucheuma denticulatum* [74], *Furcellaria lumbricalis* [52], and *Kappaphycus alvarezii* [66], possibly indicating a general pattern of expression in red seaweeds.

Transcriptome of *L. dendroidea*-associated microbiome

Seaweeds are especially susceptible to epibiosis because they inhabit environments with strong competition for space [75], and release large amounts of organic compounds that induce the microbial colonization [76], but the interaction between seaweeds and their microbiomes is little known to the molecular level.

The functional analysis of the holobiont transcriptome revealed the expression of bacterial genes involved on cell motility and chemotaxis, for example the ESTs related to flagellum and CheY-like receiver domain which are important, respectively, for the recognition of the surface of the seaweed and the establishment of the biofilm [77,78]. However, the relatively low abundance of these transcripts in comparison with the ones involved in extracellular polysaccharide synthesis suggests a mature biofilm with some level of detachment, possibly of dispersal cells [79]. Transcripts coding for the enzyme S-adenosylmethionine synthetase, which participates in the synthesis of quorum sensing autoinducers, were also detected [80]. Quorum sensing (QS) is a bacterial cell to cell communication mechanism based on the release and perception of signaling molecules such as oligopeptides, N-acyl homoserine lactones (AHL) and autoinducers that allow bacteria to monitor their own population density and to coordinate swarming, biofilm formation, stress resistance, and biosynthesis of toxins and secondary metabolites [81], and it exhibits an

important role in the interactions between bacteria and their eukaryotic hosts. Several red seaweeds are able to inhibit bacterial QS signaling, such as *Delisea pulchra* [82] and *Ahnfeltiopsis flabelliformis* [83], and a small inhibitory activity against QS signaling was previously detected in the ethyl acetate extract from a *Laurencia* sp. [84].

The taxonomic analysis of the transcriptome showed Bacteria as the dominant active group in the microbiome of *L. dendroidea*, with Cyanobacteria and Proteobacteria as the most represented bacterial phyla. These groups were also verified as predominant in the evaluation of the microbial diversity associated with four functional groups of seaweeds through metagenomics [17].

Among the cyanobacterial transcripts associated with the thalli of *L. dendroidea*, the Chroococcales, Oscillatoriales and Nostocales were the dominant orders, all of them comprising nitrogen fixing species. In a previous study, Phlips and Zeman [85] reported the occurrence and the nitrogen fixing activity of epiphytic forms of *Oscillatoria* associated to *Sargassum* thalli. Nitrogen can be the limiting nutrient in coastal ecosystems [86] and under this situation, nitrogen fixing cyanobacteria may be favored and gain in growth and reproductive success. In fact, Hoffman [87] pointed that despite their important contribution to benthic primary production, the main role of Cyanobacteria in the tropical marine ecosystems appears to be as nitrogen fixers.

However, no sequences related to nitrogen fixation were observed in our data. This is expected since our data clearly indicates an oxygenic environment, and the nitrogenase expression is inhibited by oxygen [88]. Our samples, collected near the peak of photosynthetic activity (right before midday) should have a very low expression of this nitrogenase [89]. In fact, the most abundant cyanobacteria genus were *Synechococcus* and *Cyanothece*, which together with *Lyngbya* and *Synechocystis* were previously reported to rely on temporal separation between photosynthesis and nitrogen fixation, the last occurring mainly at night [90,91]. Further studies on the diel variation of the transcriptome profile could verify this hypothesis.

Analyzing the functional relative contribution of specific domains, we noticed a higher involvement of Bacteria in the Amino acid metabolism, except for the biosynthesis of glutamate, more represented in eukaryotes. Such situation was reported for *Rhizobium* nodules, where plants provide glutamate and a carbon source and in turn the nitrogen fixing Bacteria provide ammonium and amino acids such as alanine and aspartate for asparagine

biosynthesis in the plant cytosol [92]. Although specialized mechanisms like nodules are not known in red algae, our data suggests a similar interaction between the seaweed and the associated microbiome, involving the exchange of nitrogen compounds.

Proteobacteria was the second largest active group with assigned sequences mostly to the classes Gammaproteobacteria and Alphaproteobacteria. The higher abundance of these classes was previously reported for the surface microbiome of the macroalgae *Ulva australis* [93] and *Laminaria hyperborean* [94], through denaturing gradient gel electrophoresis (DGGE) analysis. Predominantly heterotrophs, these groups would be opportunists, exploring an oxic productive environment [95]. The high prevalence of aerobic and aerotolerant groups reflects a photosynthesizing environment, also noted by Barott et al. [17]. The predominance of respiration over fermentative metabolism in the holobiont transcriptomic profile reinforces these findings. The aerobic metabolism generates reactive oxygen species (ROS) [96] that can damage DNA, lipids, and proteins [97]. In order to cope with oxygen toxicity and grow in aerobic conditions, Bacteria expressed genes correlated to oxidative stress, such as Superoxide dismutase, Glutaredoxins and Alkyl hydroperoxide reductase [98], and also stress related chaperones such as GroEL, DnaJ and DnaK [99,100].

Transcripts associated to photosynthesis and to the biosynthesis of carbohydrate reserves, such as starch, were more represented in eukaryotes, which indicate an important role of *L. dendroidea* in the primary production of the holobiont, generating carbon in excess to its immediate demand. The typical starch from Rhodophyta is called floridean starch and it shows structural similarities with starch granules from higher plants except for the lack of amylose in most of the species [101]. On the other hand the Bacteria contributed more to Carbohydrate and Lipid Transport and Metabolism, and to Energy Production and Conversion, standing out genes related to glycolysis and also to lipid and polysaccharide breakdown, reinforcing the role of Bacteria as consumers of organic matter in this holobiont [102].

Despite the beneficial or neutral interaction processes depicted here between *L. dendroidea* and its microbiome, some bacteria may also offer threats to the health and survival of seaweeds in their natural environment [103]. As such, defense mechanisms, such as the aforementioned secondary compounds of *L. dendroidea* [18], may have been evolutionarily selected. The expression of vanadium-dependent bromoperoxidases, involved on the halogenation and cyclization of terpenes in Rhodophyta [104], was detected in the

transcriptomic profile of *L. dendroidea*. Additionally the previously reported increase on the bromination activity of red algae in response to infection signals, such as agar oligosaccharide [105], indicates an important role of this enzyme in the chemical defense of Rhodophyta.

Terpenoid biosynthesis in the holobiont

The biosynthesis of terpenoid backbones provides precursors for the biosynthesis of diverse compounds that display relevant roles in plant and algal physiology [106]. The identified genes are involved in important steps for the biosynthesis of the building blocks dimethylallyl diphosphate, isopentenyl diphosphate and the higher-order building blocks geranyl diphosphate, farnesyl diphosphate and geranylgeranyl diphosphate, which are the precursors of monoterpenoids (C10), sesquiterpenoids (C15), and diterpenoids (C20), respectively [107].

The subsequent addition of isoprene units leads to the biosynthesis of sterols (isoprenoids with a C30 backbone) which are components of cell membranes; carotenoids (C40) and chlorophylls (with a C20 isoprenoid side-chain) that act as photosynthetic pigments; and plastoquinone, phylloquinone and ubiquinone (with long isoprenoid sidechains) that participate in electron transport systems for respiration or photosynthesis [106]. Terpenoid backbones are also required for the biosynthesis of N-glycans, important components for the proper folding of proteins in eukaryotic cells [108].

The biosynthesis of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the central intermediates in the biosynthesis of isoprenoids, occur through two different pathways in plants, one dependent (MVA) and other independent of mevalonate (DOXP/MEP). The mevalonate (MVA) pathway, located in the cytosol, is responsible for the production of sterols, triterpenes and some sesquiterpenes [109]. The MVA-independent pathway operates in plastids and provides the precursors to monoterpenes, diterpenes, certain sesquiterpenes, carotenoids and the side chains of chlorophyll and plastoquinone [110]. This division between isoprenoids derived from plastids and cytoplasm was also observed in red algae [111,112]. Despite the occurrence of both biosynthetic routes in Rhodophyta, this study found only transcripts associated with the mevalonate-independent pathway. Furthermore, three transcripts were identified containing the terpene synthase family metal-binding domain [35], representing new possible targets for further functional clarification. Phylogenetic reconstruction based on genes of terpene synthases was attempted, using the fragments (50–

310 amino acids) we obtained from our whole transcriptome strategy (data not shown). However, it is difficult to infer a phylogenetic relationship among taxonomic groups using the gene fragments of this pathway because, in nearly all cases, the bootstrap support for the branches is low when homologous sequences were available for analysis. Nevertheless, it is notable that in most cases, the sequences from *L. dendroidea* holobiont and other red algae cluster together with a relatively high bootstrap support.

These findings associated to the reconstruction of a complete pathway for the biosynthesis of terpenoid backbones in *L. dendroidea* are important steps to enable the heterologous biosynthesis of terpenes of interest, such as (-)-elatol, in genetically modified organisms. The molecular engineering of *Escherichia coli* and *Saccharomyces cerevisiae* has recently allowed the use of these microorganisms as cell factories to synthesize plant terpenes such as the antimalarial drug artemisinin [113,114], opening up new avenues for the scalable biosynthesis of terpenoid compounds. Our research provides a comparative basis for prospecting more specific terpene synthases genes for (-)-elatol and other commercially relevant terpenes, which could be explored in cell factories. This could be accomplished through the use of high producing strains of *L. dendroidea* under favorable conditions.

Conclusions

Our work describes the first transcriptomic profile of the red seaweed *L. dendroidea*, increasing the knowledge of ESTs from the Florideophyceae class. Basic cellular metabolic functions were the most represented in this profile, as observed in other seaweeds. The associated microbial transcriptome was independent of the location of collect, and the holobiont transcriptome indicated interesting interactions such as biofilm formation, the possible exchange of nitrogen compounds between bacteria and eukaryotes, the role of *L. dendroidea* in photosynthesis and of bacteria as consumers of excess carbon, and the bacterial molecular strategies to cope with the oxidative stress generated during aerobic metabolism. In addition, seaweeds defense mechanisms were also suggested with the disclosure of a complete mevalonate-independent pathway. The present study is a first contribution to the transcriptomic analysis of *L. dendroidea*, and opens up new avenues for biotechnological applications using this seaweed.

Additional files

Additional file 1: COG functional profile of the transcriptome of *L. dendroidea* (separate samples).

Additional file 2: Bacterial phyla recognized on the transcriptome of *L. dendroidea* (separate samples).

Additional file 3: Relevant functions for the interaction between Bacteria and Eukarya in the transcriptomic profile of the holobiont.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LSO carried out the samples collection, and RNA extraction, participated in the bioinformatic analysis and drafted the manuscript. GBG participated in the bioinformatic analysis and in the discussions and draft of the manuscript. GGZS carried out the bioinformatic analysis and participated in the discussion of the results. LTS participated in the sample collection, the discussion of the results, and the acquisition of funding. GAF participated in the acquisition of funding, the work planning and the discussion of the results, MAF participated in RNA extraction, EST library construction and discussion of the results. RCP participated in the acquisition of funding, work planning, discussion of the results, and draft of the manuscript. FLT participated in the acquisition of funding, work planning, data interpretation and draft of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We thank the financial support of CAPES, CNPq, and FAPERJ.

Author details

[1]Departamento de Biologia Marinha, Instituto de Biologia, Universidade Federal do Rio de Janeiro (UFRJ) Av. Carlos Chagas Filho, 373-CCS - IB -BLOCO A (ANEXO) A3- 202, Rio de Janeiro 21941-599, Brazil.
[2]Laboratório de Bioinformática e Biologia Evolutiva, Universidade Federal de Pernambuco. Av. Prof. Moraes Rego 1235, Cidade Universitária, Recife 50670-901PE, Brazil.
[3]Instituto de Pesquisa Jardim Botânico do Rio de Janeiro, Rua Pacheco Leão, 915. Jardim Botânico, Rio de Janeiro 22460-030RJ, Brazil.
[4]Departamento de Genética. Instituto de Biologia. Av. Prof. Rodolpho Paulo Rocco, s/n, CCS, Sala A2-93, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro 21941-599RJ, Brazil.

[5]Departamento de Biologia Marinha, Universidade Federal Fluminense (UFF). Morro do Valonguinho, s/n. Centro, Niteroi 24001-970RJ, Brazil.

Received: 26 March 2012 Accepted: 31 August 2012

Published: 17 September 2012

References

1. Cassano V, Metti Y, Millar AJK, Gil-Rodríguez MC, Sentíes A, Díaz-Larrea J, Oliveira MC, Fujii MT: Redefining the taxonomic status of *Laurencia dendroidea* (Ceramiales, Rhodophyta) from Brazil and the Canary Islands. Eur J Phycol 2012, 47(1):67–81.
2. Guiry MD, Guiry GM: AlgaeBase. In Edited by. Galway: National University of Ireland; 2010. http://wwwalgaebaseorg.
3. Irie T, Suzuki M, Masamune T: Laurencin, a constituent from *Laurencia* species. Tetrahedron Lett 1965, 6:1091–1099.
4. Irie T, Suzuki M, Kurosawa E, Masamune T: Laurinterol, debromolaurinterol and isolaurinterol, constituents of *Laurencia intermedia* Yamada. Tetrahedron 1970, 26(13):3271–3277.
5. Vairappan CS, Daitoh M, Suzuki M, Abe T, Masuda M: Antibacterial halogenated metabolites from the Malaysian *Laurencia* species. Phytochemistry 2001, 58(2):291–297.
6. Vairappan CS, Suzuki M, Ishii T, Okino T, Abe T, Masuda M: Antibacteria activity of halogenated sesquiterpenes from Malaysian *Laurencia* spp. Phytochemistry 2008, 69(13):2490–2494.
7. Vairappan CS, Anangdan SP, Tan KL, Matsunaga S: Role of secondary metabolites as defense chemicals against ice-ice disease bacteria in biofouler at carrageenophyte farms. J Appl Phycol 2010, 22(3):305–311.
8. Haas AF, Nelson CE, Kelly LW, Carlson CA, Rohwer F, Leichter JJ, Wyatt A, Smith JE: Effects of coral reef benthic primary producers on dissolved organic carbon and microbial activity. PLoS One 2011, 6(11):e27973.
9. Goecke F, Labes A, Wiese J, Imhoff JF: Chemical interactions between marine macroalgae and bacteria. Mar Ecol Prog Ser 2010, 409:267–300.
10. Persson F, Svensson R, Nylund GM, Fredriksson NJ, Pavia H, Hermansson M: Ecological role of a seaweed secondary metabolite for a colonizing bacterial community. Biofouling 2011, 27(6):579–588.
11. Cole JJ: Interactions between bacteria and algae in aquatic ecosystems. Annu Rev Ecol Syst 1982, 13:291–314.

12. Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG: Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 2005, 438(7064):90–93.
13. Tanaka Y, Ogawa H, Miyajima T: Bacterial decomposition of coral mucus as evaluated by long-term and quantitative observation. Coral Reefs 2011, 30(2):443–449.
14. Rosenberg G, Paerl H: Nitrogen fixation by blue-green algae associated with the siphonous green seaweed *Codium decorticatum*: effects on ammonium uptake. Mar Biol 1981, 61(2):151–158.
15. Burke C, Steinberg P, Rusch D, Kjelleberg S, Thomas T: Bacterial community assembly based on functional genes rather than species. Proc Natl Acad Sci 2011, 108(34):14288–14293.
16. Staufenberger T, Thiel V, Wiese J, Imhoff JF: Phylogenetic analysis of bacteria associated with *Laminaria saccharina*. FEMS Microbiol Ecol 2008, 64(1):65–77.
17. Barott KL, Rodriguez Brito B, Janouškovec J, Marhaver K, Smith JE, Keeling P, Rohwer FL: Microbial diversity associated with four functional groups of benthic reef algae and the reef building coral *Montastraea annularis*. Environ Microbiol 2011, 13(5):1192–1204.
18. Vairappan CS: Potent antibacterial activity of halogenated metabolites from Malaysian red algae, *Laurencia majuscula* (Rhodomelaceae, Ceramiales). Biomol Eng 2003, 20(4–6):255–259.
19. Vairappan CS, Kawamoto T, Miwa H, Suzuki M: Potent antibacterial activity of halogenated compounds against antibiotic-resistant bacteria. Planta Med 2004, 70(11):1087–1090.
20. Sakemi S, Higa T, Jefford CW: Venustatriol. A new, anti-viral, triterpene tetracyclic ether from *Laurencia venusta*. Tetrahedron Lett 1986, 27(36):4287–4290.
21. Topcu G, Aydogmus Z, Imre S, Gören AC, Pezzuto JM, Clement JA, Kingston DGI: Brominated sesquiterpenes from the red alga *Laurencia obtusa*. J Nat Prod 2003, 66(11):1505–1508.
22. Veiga-Santos P, Pelizzaro-Rocha KJ, Santos AO, Ueda-Nakamura T, Dias Filho BP, Silva SO, Sudatti DB, Bianco EM, Pereira RC, Nakamura CV: In vitro antitrypanosomal activity of elatol isolated from red seaweed *Laurencia dendroidea*. Parasitology 2010, 137(11):1661–1670.
23. Santos AO, Veiga-Santos P, Ueda-Nakamura T, Dias-Filho BP, Sudatti DB, Bianco EM, Pereira RC, Nakamura CV: Effect of elatol, isolated from red seaweed *Laurencia dendroidea*, on *Leishmania amazonensis*. Mar Drugs 2010, 8(11):2733–2743.
24. Chatter R, Othman RB, Rabhi S, Kladi M, Tarhouni S, Vagias C, Roussis V, Guizani-Tabbane L, Kharrat R: In vivo and in vitro anti-inflammatory activity of neorogioltriol, a new diterpene extracted from the red algae *Laurencia glandulifera*. Mar Drugs 2011, 9(7):1293–1306.

25. Kladi M, Xenaki H, Vagias C, Papazafiri P, Roussis V: New cytotoxic sesquiterpenes from the red algae *Laurencia obtusa* and *Laurencia microcladia*. Tetrahedron 2006, 62(1):182–189.
26. Kim MM, Mendis E, Kim SK: *Laurencia okamurai* extract containing laurinterol induces apoptosis in melanoma cells. J Med Food 2008, 11(2):260–266.
27. Lhullier C, Falkenberg M, Ioannou E, Quesada A, Papazafiri P, Antunes Horta P, Paulo Schenkel E, Vagias C, Roussis V: Cytotoxic halogenated metabolites from the Brazilian red alga *Laurencia catarinensis*. J Nat Prod 2010, 73(1):27–32.
28. Pereira RC, Da Gama BA, Teixeira VL, Yoneshigue-Valentin Y: Ecological roles of natural products of the Brazilian red seaweed *Laurencia obtusa*. Braz J Biol 2003, 63(4):665–672.
29. Da Gama BAP, Pereira RC, Carvalho AGV, Coutinho R: The effects of seaweed secondary metabolites on biofouling. Biofouling 2002, 18(1):13–20.
30. Da Gama BAP, Pereira RC, Soares AR, Teixeira VL, Yoneshigue-Valentin Y: Is the mussel test a good indicator of antifouling activity? A comparison between laboratory and field assays. Biofouling 2003, 19(SUPPL):161–169.
31. Pereira RC, Costa-Lotufo LV: Bioprospecting of bioactives from seaweeds: potential, obstacles and alternatives. Braz J Pharmacog 2012, 22(4):894–905.
32. White DE, Stewart IC, Grubbs RH, Stoltz BM: The catalytic asymmetric total synthesis of elatol. J Am Chem Soc 2008, 130(3):810–811.
33. Salgado LT, Viana NB, Andrade LR, Leal RN, Da Gama BAP, Attias M, Pereira RC, Amado Filho GM: Intra-cellular storage, transport and exocytosis of halogenated compounds in marine red alga *Laurencia obtusa*. J Struct Biol 2008, 162(2):345–355.
34. Sudatti DB, Fujii MT, Rodrigues SV, Turra A, Pereira RC: Effects of abiotic factors on growth and chemical defenses in cultivated clones of *Laurencia dendroidea* J. Agardh (Ceramiales, Rhodophyta). Mar Biol 2011, 158(7):1439–1446.
35. Cane DE, Ikeda H: Exploration and mining of the bacterial terpenome. Accounts Chem Res 2011, 45(3):463–472.
36. Agger S, Lopez-Gallego F, Schmidt-Dannert C: Diversity of sesquiterpene synthases in the basidiomycete *Coprinus cinereus*. Mol Microbiol 2009, 72(5):1181–1195.
37. Degenhardt J, Kollner TG, Gershenzon J: Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. Phytochemistry 2009, 70:1621–1637.
38. Morrone D, Chambers J, Lowry L, Kim G, Anterola A, Bender K, Peters R: Gibberellin biosynthesis in bacteria: separate ent-copalyl diphosphate and ent-kaurene synthases in *Bradyrhizobium japonicum*. FEBS Lett 2009, 583(2):475–480.
39. Gil-Rodríguez M, Sentíes A, Díaz-Larrea J, Cassano V, Fujii MT: *Laurencia marilzae* sp. nov. (Ceramiales, Rhodophyta) from the Canary Islands, Spain, based on morphological and molecular evidence. J Phycol 2009, 45(1):264–271.
40. Martin-Lescanne J, Rousseau F, De Reviers B, Payri C, Couloux A, Cruaud C, Le Gall L: Phylogenetic analyses of the *Laurencia* complex (Rhodomelaceae, Ceramiales) support

recognition of five genera: *Chondrophycus*, *Laurencia*, *Osmundea*, *Palisada* and *Yuzurua* stat. nov. Eur J Phycol 2010, 45(1):51–61.

41. Kapraun DF: Nuclear DNA content estimates in multicellular green, red and brown algae: phylogenetic considerations. Ann Bot 2005, 95(1):7–44.
42. Falcão VR, Tonon AP, Oliveira MC, Colepicolo P: RNA Isolation method for polysaccharide rich algae: agar producing *Gracilaria tenuistipitata* (Rhodophyta). J Appl Phycol 2008, 20(1):9–12.
43. MarguliesM, EgholmM, AltmanWE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, et al: Genome sequencing in microfabricated high-density picolitre reactors. Nature 2005, 437(7057):376–380.
44. Schmieder R, Edwards R: Quality control and preprocessing of metagenomic datasets. Bioinformatics 2011, 27(6):863–864.
45. Pertea G, Huang X, Liang F, Antonescu V, Sultana R, Karamycheva S, Lee Y, White J, Cheung F, Parvizi B: TIGR Gene indices clustering tools (TGICL): a software system for fast clustering of large EST datasets. Bioinformatics 2003, 19(5):651–652.
46. Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, Salzberg SL: Versatile and open software for comparing large genomes. Genome Biol 2004, 5(2):R12.
47. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, et al: The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 2008, 9:386.
48. Parks DH, Beiko RG: Identifying biologically relevant differences between metagenomic communities. Bioinformatics 2010, 26(6):715–721.
49. HMMER Biosequence analysis using profile hidden Markov models. http://hmmer.org/.
50. Lee H, Lee HK, An G, Lee YK: Analysis of expressed sequence tags from the red alga *Griffithsia okiensis*. J Microbiol (Seoul) 2007, 45(6):541.
51. Teo SS, Ho CH, Teoh S, Lee WW, Tee JM, Rahim RA, Phang SM: Analyses of expressed sequence tags from an agarophyte, *Gracilaria changii* (Gracilariales, Rhodophyta). Eur J Phycol 2007, 42(1):41–46.
52. Kostamo K, Olsson S, Korpelainen H: Search for stress-responsive genes in the red alga *Furcellaria lumbricalis* (Rhodophyta) by expressed sequence tag analysis. J Exp Mar Biol Ecol 2011, 404(1–2):21–25.
53. Matsuzaki M, Misumi O, Shin-i T, Maruyama S, Takahara M, Miyagishima S, Mori T, Nishida K, Yagisawa F, Nishida K: Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D. Nature 2004, 428(6983):653–657.
54. Derelle E, Ferraz C, Rombauts S, Rouzé P, Worden AZ, Robbens S, Partensky F, Degroeve S, Echeynié S, Cooke R: Genome analysis of the smallest freeliving eukaryote *Ostreococcus tauri* unveils many unique features. Proc Natl Acad Sci 2006, 103(31):11647–11652.

55. Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, Terry A, Salamov A, Fritz-Laylin LK, Maréchal-Drouard L: The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science 2007, 318(5848):245–251.
56. Price DC, Chan CX, Yoon HS, Yang EC, Qiu H, Weber AP, Schwacke R, Gross J, Blouin NA, Lane C, et al: *Cyanophora paradoxa* genome elucidates origin of photosynthesis in algae and plants. Science 2012, 335(6070):843–847.
57. Cock JM, Sterck L, Rouzé P, Scornet D, Allen AE, Amoutzias G, Anthouard V, Artiguenave F, Aury JM, Badger JH: The *Ectocarpus* genome and the independent evolution of multicellularity in brown algae. Nature 2010, 465(7298):617–621.
58. Nikaido I, Asamizu E, Nakajima M, Nakamura Y, Saga N, Tabata S: Generation of 10,154 expressed sequence tags from a leafy gametophyte of a marine red alga, *Porphyra yezoensis*. DNA Res 2000, 7(3):223–227.
59. Xiaolei F, Yongjun F, Songnian H, Guangce W: Generation and analysis of 5318 expressed sequence tags from the filamentous sporophyte of *Porphyra haitanensis* (Rhodophyta). J Phycol 2007, 43(6):1287–1294.
60. Collén PN, Collén J, da Silva Reis M, Pedersén M, Setubal JC, Varani AM, Colepicolo P, Oliveira MC: Analysis of expressed sequence tags from the agarophyte *Gracilaria tenuistipitata* (Rhodophyta). J Appl Phycol 2012, 24(4):641–647.
61. Yang H, Mao YX, Kong FN, Yang GP, Ma F, Wang L: Profiling of the transcriptome of *Porphyra yezoensis* with Solexa sequencing technology. Chinese Sci Bull 2011, 56(20):2119–2130.
62. Lluisma AO, Ragan MA: Expressed sequence tags (ESTs) from the marine red alga *Gracilaria gracilis*. J Appl Phycol 1997, 9(3):287–293.
63. Chan CX, Yang EC, Banerjee T, Yoon HS, Martone PT, Estevez JM, Bhattacharya D: Red and green algal monophyly and extensive gene sharing found in a rich repertoire of red algal genes. Curr Biol 2011, 21(4):328–333.
64. Chan CX, Zauner S, Wheeler G, Grossman AR, Prochnik SE, Blouin NA, Zhuang Y, Benning C, Berg GM, Yarish C, et al: Analysis of *Porphyra* membrane transporters demonstrates gene transfer among photosynthetic eukaryotes and numerous sodium-coupled transport systems. Plant Physiol 2012, 158(4):2001–2012.
65. Collén J, Roeder V, Rousvoal S, Collin O, Kloareg B, Boyen C: An expressed sequence tag analysis of thallus and regenerating protoplasts of *Chondrus crispus* (Gigartinales, Rhodophyceae). J Phycol 2006, 42(1):104–112.
66. Liu C, Wang X, Huang X, Liu J: Identification of hypo-osmotically induced genes in *Kappaphycus alvarezii* (Solieriaceae, Rhodophyta) through expressed sequence tag analysis. Bot Mar 2011, 54(6):557–562.
67. Asamizu E, Nakajima M, Kitade Y, Saga N, Nakamura Y, Tabata S: Comparison of RNA expression profiles between the two generations of *Porphyra yezoensis* (Rhodophyta), based on expressed sequence tag frequency analysis. J Phycol 2003, 39(5):923–930.

68. Ren X, Zhang X, Sui Z: Identification of phase relative genes in tetrasporophytes and female gametophytes of *Gracilaria*/*Gracilariopsis lemaneiformis* (Gracilariales, Rhodophyta). Electron J Biotechn 2006, 9(2):127–132.
69. Kamiya M, Kawai H, Moon D, Goff LJ: Isolation and characterization of phase-specific cDNAs from carposporophytes of *Gracilariopsis andersonii* (Gracilariales, Rhodophyta). Eur J Phycol 2011, 46(1):27–35.
70. Shen S, Zhang G, Li Y, Wang L, Xu P, Yi L: Comparison of RNA expression profiles on generations of *Porphyra yezoensis* (Rhodophyta), based on suppression subtractive hybridization (SSH). BMC Res Not 2011, 4(1):428.
71. Lachnit T, Blumel M, Imhoff JF, Wahl M: Specific epibacterial communities on macroalgae: phylogeny matters more than habitat. Aquat Biol 2009, 5(2):181–186.
72. Huovinen P, Gómez I, Lovengreen C: A five year study of solar ultraviolet radiation in Southern Chile (39°S): Potential impact on physiology of coastal marine algae? Photochem Photobiol 2006, 82(2):515–522.
73. Bischof K, Gomez I, Molis M, Hanelt D, Karsten U, Lüder U, Roleda MY, Zacher K, Wiencke C: Ultraviolet radiation shapes seaweed communities. Life in Extreme Environments 2007, 5:187–212.
74. Aspilla PS, Antonio A, Zuccarello GC, Rojas NRL: A partial expressed sequence tag (EST) library of the economically important red alga *Eucheuma denticulatum* (NL Burham) FC Collins and Hervey. Phil Sci Lett 2010, 3:109–120.
75. Sandin SA, McNamara DE: Spatial dynamics of benthic competition on coral reefs. Oecologia 2011, 168(4):1079–1090.
76. Steinberg PD, de Nys R: Chemical mediation of colonization of seaweed surfaces. J Phycol 2002, 38(4):621–629.
77. O'Toole GA, Kolter R: Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 1998, 30(2):295–304.
78. Miller TR, Hnilicka K, Dziedzic A, Desplats P, Belas R: Chemotaxis of *Silicibacter* sp. strain TM1040 toward dinoflagellate products. Appl Environ Microbiol 2004, 70(8):4692.
79. McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S: Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat Rev Microbiol 2011, 10(1):39–50.
80. Hanzelka BL, Greenberg E: Quorum sensing in *Vibrio fischeri*: evidence that S-adenosylmethionine is the amino acid substrate for autoinducer synthesis. J Bacteriol 1996, 178(17):5291.
81. Dobretsov S, Teplitski M, Paul V: Mini-review: quorum sensing in the marine environment and its relationship to biofouling. Biofouling 2009, 25(5):413–427.
82. Manefield M, de Nys R, Naresh K, Roger R, Givskov M, Peter S, Kjelleberg S: Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone

(AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. Microbiology 1999, 145(2):283–291.

83. Kim J, Kim Y, Seo Y, Park S: Quorum sensing inhibitors from the red alga, *Ahnfeltiopsis flabelliformis*. Biotechnol Bioproc E 2007, 12(3):308–311.
84. Skindersoe ME, Ettinger-Epstein P, Rasmussen TB, Bjarnsholt T, De Nys R, Givskov M: Quorum sensing antagonism from marine organisms. Marine Biotechnol 2008, 10(1):56–63.
85. Phlips E, Zeman C: Photosynthesis, growth and nitrogen fixation by epiphytic forms of filamentous cyanobacteria from pelagic *Sargassum*. B Mar Sci 1990, 47(3):613–621.
86. Howarth RW, Anderson DM, Church TM, Greening H, Hopkinson CS, Huber WC, Marcus N, Naiman RJ, Segerson K, Sharpley AN, et al (Eds): Clean coastal waters: understanding and reducing the effects of nutrient pollution. Washington, DC: National Academy Press; 2000.
87. Hoffman L: Marine cyanobacteria in tropical regions: diversity and ecology. Eur J Phycol 1999, 34(4):371–379.
88. Berman-Frank I, Chen Y-B, Gerchman Y, Dismukes G, Falkowski P: Inhibition of nitrogenase by oxygen in marine cyanobacteria controls the global nitrogen and oxygen cycles. Biogeoscs Discuss 2005, 2:261–273.
89. Toepel J, Welsh E, Summerfield TC, Pakrasi HB, Sherman LA: Differential transcriptional analysis of the cyanobacterium *Cyanothece* sp. strain ATCC 51142 during light-dark and continuous-light growth. J Bacteriol 2008, 190(11):3904–3913.
90. Berman-Frank I, Lundgren P, Falkowski P: Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. Res Microbiol 2003, 154(3):157–164.
91. Huang TC, Lin RF, Chu MK, Chen HM: Organization and expression of nitrogen-fixation genes in the aerobic nitrogen-fixing unicellular cyanobacterium *Synechococcus* sp. strain RF-1. Microbiology 1999, 145(Pt 3):743–753.
92. Lodwig EM, Hosie AH, Bourdes A, Findlay K, Allaway D, Karunakaran R, Downie JA, Poole PS: Amino-acid cycling drives nitrogen fixation in the legume-*Rhizobium* symbiosis. Nature 2003, 422(6933):722–726.
93. Tujula NA, Crocetti GR, Burke C, Thomas T, Holmström C, Kjelleberg S: Variability and abundance of the epiphytic bacterial community associated with a green marine Ulvacean alga. ISME J 2009, 4(2):301–311.
94. Bengtsson MM, Sjøtun K, Øvreås L: Seasonal dynamics of bacterial biofilms on the kelp *Laminaria hyperborea*. Aq Microb Ecol 2010, 60(1):71–83.
95. Stevens H, Stubner M, Simon M, Brinkhoff T: Phylogeny of Proteobacteria and Bacteroidetes from oxic habitats of a tidal flat ecosystem. FEMS Microbiol Ecol 2005, 54(3):351–365.
96. Messner KR, Imlay JA: The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. J Biol Chem 1999, 274 (15):10119–10128.

97. Kirkland JB: Lipid peroxidation, protein thiol oxidation and DNA damage in hydrogen peroxide-induced injury to endothelial cells: role of activation of poly (ADP-ribose) polymerase. Biochim Biophys Acta 1991, 1092(3):319–325.
98. Zheng M, Storz G: Redox sensing by prokaryotic transcription factors. Biochem Pharmacol 2000, 59(1):1–6.
99. Farr SB, Kogoma T: Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol Rev 1991, 55(4):561–585.
100. Fredriksson A, Ballesteros M, Dukan S, Nystrom T: Defense against protein carbonylation by DnaK/DnaJ and proteases of the heat shock regulon. J Bacteriol 2005, 187(12):4207–4213.
101. McCracken D, Cain J: Amylose in floridean starch. New Phytol 1981, 88(1):67–71.
102. Cottrell MT, Kirchman DL: Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. Appl Environ Microbiol 2000, 66(4):1692–1697.
103. Largo DB, Fukami K, Nishijima T: Occasional pathogenic bacteria promoting ice-ice disease in the carrageenan-producing red algae *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Solieriaceae, Gigartinales, Rhodophyta). J Appl Phycol 1995, 7(6):545–554.
104. Carter-Franklin JN, Parrish JD, Tschirret-Guth RA, Little RD, Butler A: Vanadium haloperoxidase-catalyzed bromination and cyclization of terpenes. J Am Chem Soc 2003, 125(13):3688–3689.
105. Weinberger F, Coquempot B, Forner S, Morin P, Kloareg B, Potin P: Different regulation of haloperoxidation during agar oligosaccharide-activated defence mechanisms in two related red algae, *Gracilaria* sp. and *Gracilaria chilensis*. J Exp Bot 2007, 58(15–16):4365–4372.
106. McGarvey DJ, Croteau R: Terpenoid metabolism. Plant Cell 1995, 7(7):1015.
107. Kuzuyama T: Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units. Biosci Biotechnol Biochem 2002, 66(8):1619–1627.
108. Schwarz F, Aebi M: Mechanisms and principles of N-linked protein glycosylation. Curr Opin Struct Biol 2011, 21(5):576–582.
109. Laule O, Fürholz A, Chang HS, Zhu T, Wang X, Heifetz PB, Gruissem W, Lange M: Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 2003, 100(11):6866.
110. Rohmer M: The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. 1999, 16:565–574.
111. Schwender J, Zeidler J, Groner R, Muller C, Focke M, Braun S, Lichtenthaler FW, Lichtenthaler HK: Incorporation of 1-deoxy-D-xylulose into isoprene and phytol by higher plants and algae. FEBS Lett 1997, 414:129–134.

112. Wanke M, Skorupinska-Tudek K, Swiezewska E: Isoprenoid biosynthesis via 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway. Acta Biochim Polon 2001, 48:663–672.
113. Ignea C, Cvetkovic I, Loupassaki S, Kefalas P, Johnson CB, Kampranis SC, Makris AM: Improving yeast strains using recyclable integration cassettes, for the production of plant terpenoids. Microb Cell Fact 2011, 10:4.
114. Martin VJ, Pitera DJ, Withers ST, Newman JD, Keasling JD: Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 2003, 21(7):796–802.

doi:10.1186/1471-2164-13-487
Cite this article as: de Oliveira *et al.*: Transcriptomic analysis of the red seaweed *Laurencia dendroidea* (Florideophyceae, Rhodophyta) and its microbiome. *BMC Genomics* 2012 **13**:487.

Mar. Drugs **2015**, *13*, 879-902; doi:10.3390/md13020879

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marine drugs

ISSN 1660-3397

www.mdpi.com/journal/marinedrugs

CHAPTER 3 - New Insights on the Terpenome of the Red Seaweed *Laurencia dendroidea* (Florideophyceae, Rhodophyta)

Louisi Souza de Oliveira [1], Diogo Antonio Tschoeke [1], Aline Santos de Oliveira [2], Lilian Jorge Hill [3], Wladimir Costa Paradas [3], Leonardo Tavares Salgado [3], Cristiane Carneiro Thompson [1], Renato Crespo Pereira [4] and Fabiano L. Thompson [1,*]

Abstract

The red seaweeds belonging to the genus *Laurencia* are well known as halogenated secondary metabolites producers, mainly terpenoids and acetogennins. Several of these chemicals exhibit important ecological roles and biotechnological applications. However, knowledge regarding the genes involved in the biosynthesis of these compounds is still very limited. We detected 20 different genes involved in the biosynthesis of terpenoid precursors, and 21 different genes coding for terpene synthases that are responsible for the chemical modifications of the terpenoid precursors, resulting in a high diversity of carbon chemical skeletons. In addition, we demonstrate through molecular and cytochemical approaches the occurrence of the mevalonate pathway involved in the biosynthesis of terpenes in *L. dendroidea*. This is the first report on terpene synthase genes in seaweeds, enabling further studies on possible heterologous biosynthesis of terpenes from *L. dendroidea* exhibiting ecological or biotechnological interest.

Keywords: seaweed; terpene synthase; prenyl transferase; gene; secondary metabolite; metabolic pathway; mevalonate pathway; transcriptome; *Laurencia*

Introduction

Several secondary metabolites produced by marine organisms may interfere in biological interactions at the population, community, and ecosystem levels [1]. Seaweeds commonly produce secondary metabolites to defend themselves against herbivory and biofouling [2] and also to mediate the competitive interactions for space in benthic habitat, acting as allelochemicals [3]. Seaweed species of the genus *Laurencia* are recognized for the biosynthesis of a high diversity of secondary compounds, especially terpenes and acetogennins. This macroalgal genus has been chemically investigated since 1960 [4], but it remains the subject of considerable interest, as evidenced by the recent discovery of new secondary metabolites [5]. The halogenated sesquiterpene (−)-elatol is one interesting example of the major secondary metabolites produced by *Laurencia* species worldwide and it may interact with other compounds to defend this seaweed against herbivory and biofouling [6,7]. Recent studies revealed the variability of (−)-elatol concentration in *L. dendroidea* at the intra- and interpopulation levels [8,9], suggesting that this variability is most likely influenced by environmental factors such as temperature and salinity [10].

Some terpenes biosynthesized by *Laurencia* species exhibit a pharmacologically relevant potential due to their strong antiviral [11], antibiotic [12,13], antimalarial [14], antileishmanial [15], antitrypanosomal [16], anti-inflammatory [17] and anti-carcinoma [18–20] activities. In addition, the terpenoids from *Laurencia* species have a pronounced anti-epibiosis activity and they could be used for the preparation of antifouling paints [6,7,21]. For example, a first attempt at the commercial application of the sesquiterpene (−)-elatol resulted in a filing of the patent in Brazil to use this compound as an antifouling agent. However, the failure of the large-scale cultivation of *Laurencia* species, the low yield of the extraction process, and the complexity of the organic total synthesis of (−)-elatol in the laboratory [22] are current obstacles to the commercial exploitation of this compound in a biotechnological context. A possible alternative for overcoming this obstacle is the synthesis of terpenes of interest in the laboratory using genetically modified organisms [23]. The molecular engineering of *Escherichia coli* and *Saccharomyces cerevisiae* is a promising alternative, since it allows for the biosynthesis of plant terpenes such as the antimalarial drug artemisinin [24,25], opening up new avenues for the sustainable obtention of terpenoid compounds of biotechnological interest.

In plants and several algae, the biosynthesis of terpenoid precursors can occur through the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway. In a recent work, we demonstrated the expression of the genes involved in the MEP pathway in *L. dendroidea* [26], but there are still no studies reporting the occurrence of the MVA pathway in this species. In fact, the evidence for the simultaneous occurrence of the MEP and MVA pathways in Rhodophyta is currently restricted to biochemical characterizations and gene cloning in *Cyanidium caldarium* and *Galdieria sulphuraria* [27].

The halogenation of organic compounds is a relatively common process in red seaweeds and apparently provides these substances with important features [28,29]. *Laurencia* species biosynthesize a large array of halogenated compounds, which are stored in specialized cellular structures—so-called *corps en cerise* (CC)—in order to avoid autotoxicity [30]. The release of these compounds to the surface of the seaweed is a necessary process to allow their action as defense, and this process occurs in a regulated manner [31]. It depends on the activity of microfilaments that are relevant for the traffic of vesicles containing halogenated metabolites from the CC to the cell periphery [31], and microtubules that participate in the positioning of the vesicles along the cell periphery to specific regions where exocytosis occurs [32]. The frequency of vesicle transport from the CC to the cell surface is influenced by irradiance, desiccation, temperature, and bacterial fouling [33].

Despite the large number of studies analyzing the secondary metabolites, especially terpenes, of *Laurencia* and its chemical, cytological, and ecological aspects, the current knowledge regarding the genes, the biochemical processes, and the cell structures involved in the biosynthesis of these compounds is still limited. In a previous study, we obtained the almost complete set of genes involved in the methylerythritol phosphate (MEP) pathway from *L. dendroidea* living under different environmental conditions [26]. In the present study, we expanded our transcriptomic analysis of *L. dendroidea* using clonal axenic cultures under light and dark controlled laboratory conditions and used a cytochemical labeling approach to detect and localize the activity of enzymes involved in the mevalonate (MVA) pathway in this seaweed. Our aim was to analyze the existence of the MVA pathway in *L. dendroidea* and unveil the genes related to the biosynthesis of monoterpene, diterpene, triterpene, and sesquiterpene skeletons.

Methods

Laurencia dendroidea was sampled in the intertidal zone at Castelhanos beach in Anchieta municipality, Espírito Santo State, Brazil (20°51'40"S, 40°37'00"W) in 2008 and has been maintained in the laboratory since then. In order to establish a unialgal culture of this seaweed, the apices were successively excised and grown in sterile seawater enriched with 25% Provasoli solution [114]. These algal clones were treated with an antibiotic mix to reduce the bacteria in the culture (100 µg/mL ampicillin, 120 µg/mL streptomycin, and 60 µg/mL gentamicin) and were grown in sterile seawater with germanium dioxide (1 mg/L) and 50% Provasoli solution for 2 days before the experiment. The culture conditions were salinity 32 ± 1, temperature 22 ± 1 °C, irradiance 80 ± 5 µmol photons.m^{-2}·s^{-1}, and 14 h light/10 h dark. To increase the scope of this work and maximize the detection of genes related to the biosynthesis of terpenes, we analyzed the trascriptome of *L. dendroidea* after exposure to dark and light conditions. Thus, one clone of this seaweed was sampled at the end of the dark period and the other was sampled after 10 h in the light.

The algal clones were separately ground in liquid nitrogen using a mortar and pestle to obtain a fine powder. The total RNA was extracted using the TRIzol reagent (Life Technologies-Invitrogen, Carlsbad, CA, USA) protocol. The DNA residues were eliminated with DNAse (RNAse free, PROMEGA, Madison, WI, USA), and the double-stranded cDNAs (ds cDNAs) were synthesized and amplified using the SMARTer cDNA synthesis kit and the Advantage2 polymerase (Clontech, Foster City, CA, USA) starting from 1 µg of total RNA. The optimal number of amplification cycles was determined to be 23. The PCR amplification products were purified using the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Alemanha) and the ds cDNAs were eluted in nuclease-free water.

The ds cDNA libraries were prepared using the Nextera XT Sample Preparation Kit (Illumina, San Diego, CA, USA) and the size distribution was accessed using the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and the High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA). The accurate quantification of the libraries was accomplished using the 7500 Real Time PCR (Applied Biosystems, Foster City, CA, USA) and the KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA, USA). Paired-end sequencing (2 × 150 bp and 2 × 250 bp) was performed on a MiSeq (Illumina, San Diego, CA, USA).

The sequences from each sample were preprocessed using the software Prinseq [115] to trim poly-A/T tails at least 20 bp long, to remove reads shorter than 35 bp, and to trim

sequences with a quality score lower than 30. Then the sequences were assembled using the software Trinity, which is based on de Brujin graphs [116], and both contigs and singlets were used in the downstream analysis. To identify the transcripts associated to the synthesis of terpenoid compounds, we prospected the transcriptome of *L. dendroidea* using hidden markov models generated from the alignment of sequences available in the KEGG database through the HMMER 3.0 software [117]. Other searches using specific HMM profiles were based on the alignment of all vanadium-dependent bromoperoxidase sequences deposited in the protein database of NCBI and on the universal metal-binding domain (PF03936) and *N*-terminal domain (PF01397) of terpene synthases obtained from PFAM as previously described [118]. The sequences matching all these profiles were annotated through BLAST search against the NCBI-nr, PlantCyc, and Uniprot databases. The functional identifications were manually confirmed.

To perform the cytochemical labeling of the activity of HMGS and/or HMGR, the release of spores was induced in the laboratory by subjecting *L. dendroidea* sporophytes to light deprivation. The spores were maintained for 12 h in sterile seawater enriched with 50% Von Stosch solution [119] at 20 °C, and 60 μmol phtons.$m^2 \cdot s^{-1}$. The viable spores that adhered to the bottom of the Petri dishes were selected for the experiment. Spores are proliferative and developing cells, which can better reveal the primary regions involved in mevalonic acid production.

The cytochemical labeling method, previously described by Curry (1987) [120], is based on the reaction catalyzed by the enzyme HMGS that converts acetyl CoA and acetoacetyl CoA in 3-hydroxy-3-methylglutaryl-CoA, which is subsequently reduced by HMGR. Both reactions produce free Coenzyme A-SH (CoA-SH) [121], which reacts with potassium ferricyanide, reducing it to ferrocyanide. The ferrocyanide then reacts with added uranyl acetate to form uranyl ferrocyanide, which precipitates and appears as a highly electron-dense material in transmission electron microscopy (TEM). In this way, the localization of HGMR is indistinguishable from that of HGMS because both reactions produce CoA-SH, which precipitates as uranyl ferrocyanide.

The spores of *L. dendroidea* were fixed for 30 min in 4% formaldehyde and 1% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.6) diluted in sterile seawater, followed by a buffer rinse (0.05 M cacodylate, pH 7.6). Subsequently, the algal spores were pre-incubated at room temperature for 20 min in 3 mM potassium ferrocyanide in 0.05 M cacodylate buffer (pH 7.6), followed by a buffer rinse [120]. Then, a group of spores was

maintained for 45 min at room temperature with the complete incubation solution composed by acetyl-CoA sodium salt (0.8 $mg \cdot mL^{-1}$), acetoacetyl-CoA sodium salt (1.6 $mg \cdot mL^{-1}$), potassium ferricyanide (2.0 $mg \cdot mL^{-1}$), uranyl acetate (1.0 $mg \cdot mL^{-1}$), and sodium cacodylate buffer (0.05 M, pH 7.0); a second group received the same compounds except for the acetyl-CoA sodium salt (substrate control), and a third group received the same compounds that were in the complete incubation solution except for acetoacetyl-CoA sodium salt (reaction product control). The last two groups were used as a control to test for unspecific precipitations of uranyl ferrocyanide caused by the action of any enzyme other than HMGS and HMGR. After that, the algal spores were post-fixed for one hour at room temperature in 2% (w/v) osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.0), followed by a buffer rinse. The algal spores were dehydrated in a crescent acetone series (up to 100%) and embedded in Spurr resin. The polymerization process was performed at 70 °C and the ultrathin sections (70 nm) were obtained in a Reichert ultramicrotome and collected on copper grids (300 mesh). The grids were contrasted with uranyl acetate and lead citrate and 50 spore cells from each treatment were observed in a JEOL 1200 EX transmission electron microscope (JEOL, Peabody, MA, USA).

Results

A total of 5,896,520 sequences were obtained for the transcriptome of *L. dendroidea* (Table 1). The relatively large amount of high-quality sequences (with a quality score above 30) obtained from unialgal clones of *L. dendroidea*, previously treated with an antibiotic mix and exposed to alternate light and dark conditions, provided an unprecedentedly high coverage of the transcriptome of this seaweed and the possibility to detect new genes associated with the biosynthesis of terpenes. The assembly of all sequences resulted in 54,255 contigs and singlets (Table 1). The genome size of Rhodophyta appears to be highly variable, although the exact values are largely unknown for most species since the complete genome sequence of red macroalgae is currently available only for *Pyropia yezoensis* (43 Mbp) [34] and *Chondrus crispus* (105 Mbp) [35]. The genome size estimate for the species *Laurencia papillosa* is 833 Mbp, based on microspectrophotometry [36]. Taking this value as a reference, and considering that in *Chondrus crispus* around 8% of the genetic material codes

for proteins [35], our study would contribute with a 11.5-fold coverage of the transcriptome of *L. dendroidea*.

Table 1. Characteristics of the sequencing and assembly of the cDNA libraries from the unialgal clones of *Laurencia dendroidea* (SD = Standard deviation).

Sample	Number of Sequences	Total Nucleotides (bp)	Average Size (bp) ± SD
Light	5,825,960	749,118,981	128.0 ± 30
Dark	70,560	15,816,057	224.0 ± 45
Assembled sequences	54,255	19,504,276	359.5 ± 589

The functional annotation of the transcripts revealed for the first time the expression of the genes encoding acetyl-CoA *C*-acetyltransferase (EC 2.3.1.9, Blast e-value: 0.0; similarity: 75%) and mevalonate kinase (EC 2.7.1.36, Blast e-value: 2e-24; similarity: 87%) in *L. dendroidea*, comprising two essential steps for the biosynthesis of isoprenoid precursors through the mevalonate (MVA) pathway (Figure 1).

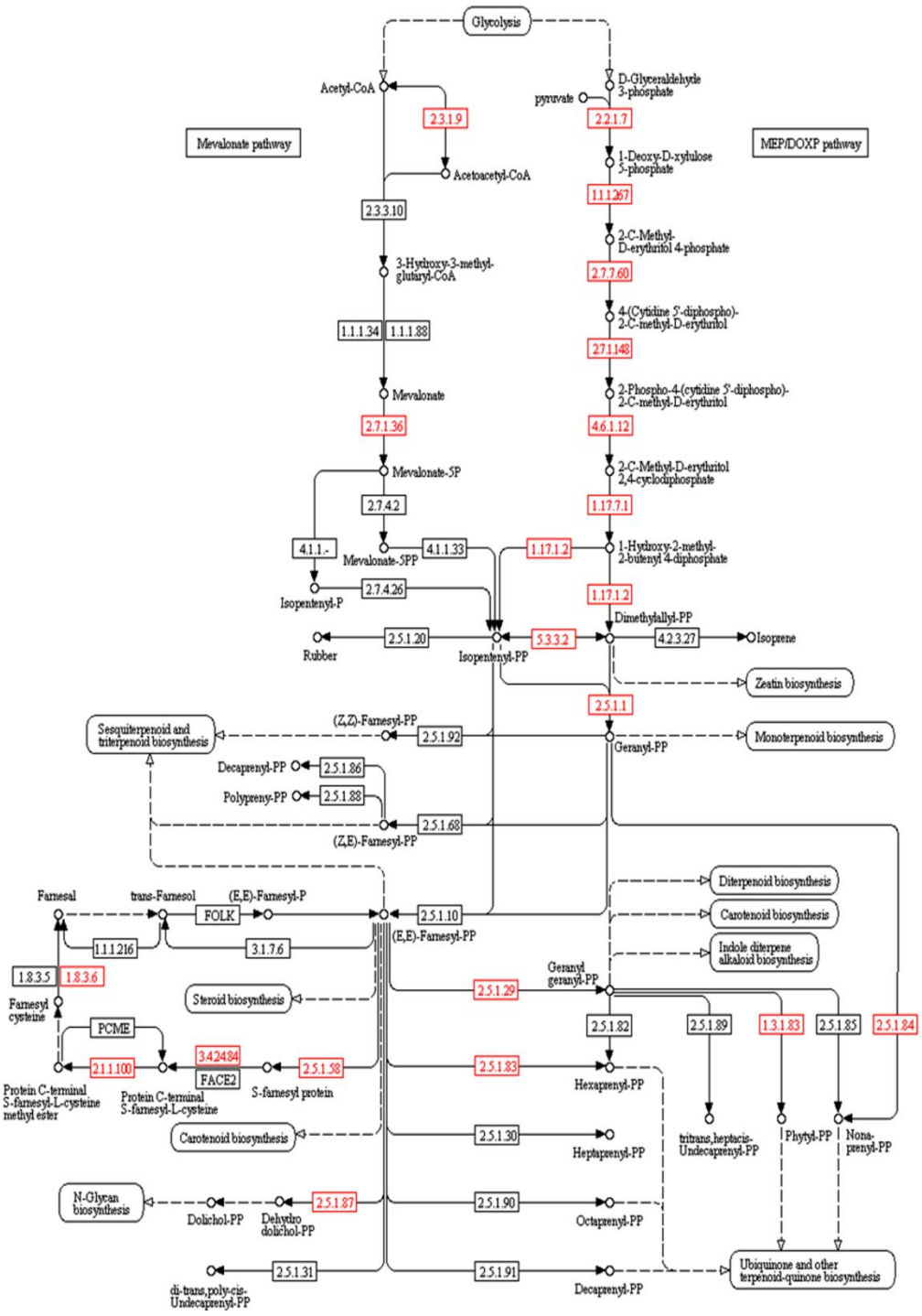


Figure 1. Biosynthetic routes to the terpenoid precursors through the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway. The red squares represent the genes detected in the transcriptomic analysis of *L. dendroidea*. Modified from the Kegg Pathway Database website—Terpenoid backbone biosynthesis reference pathway [122].

Additionally, the activity of 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS, EC 2.3.3.10) and/or 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR, EC 1.1.1.34) was detected through a cytochemical assay. The electron-dense structures indicating the activity of

these enzymes were found in spore cells treated with the complete incubation solution containing acetyl-CoA and acetoacetyl-CoA, specifically in lamellar structures surrounding the chloroplasts (Figure 2A,C), in vesicle-like structures near the chloroplasts (Figure 2B), and also in large lamellar structures between chloroplasts (Figure 2D). Electron-dense small spherical structures within the chloroplasts corresponded to the plastoglubules and were present in all samples. The control samples showed no electron-dense deposits (Figure 2E,F). Moreover, we detected the expression of all the genes involved in the methylerythritol phosphate (MEP) pathway (Figure 1). These results indicate the simultaneous occurrence of the MVA and the MEP pathway in *L. dendroidea*.

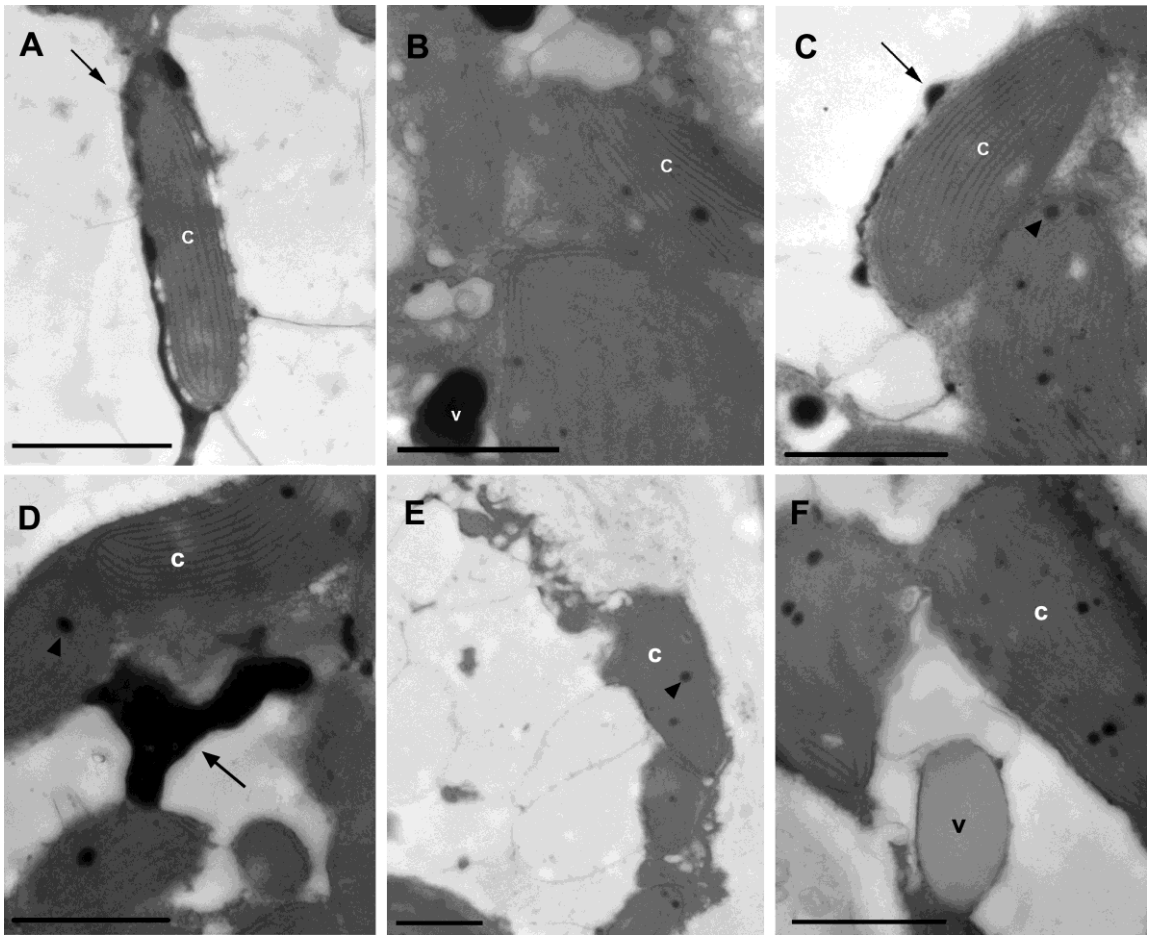


Figure 2. Transmission electron microscopy (TEM) images of *Laurencia dendroidea* spore cells submitted to cytochemical mevalonic acid enzyme assay. (A–D) indicate the spore cells submitted to the complete acetoacetyl-CoA medium, (E) indicates the cells submitted to the substrate control for acetoacetyl-CoA medium, and (F) indicates cells submitted to the reaction product control medium. In images (A–D), arrows are pointing to electron-dense granular material, indicating the HGMS/HGMR activity inside lamellar structures surrounding the chloroplasts (c) (A,C), in vesicle-like structures (v) near the chloroplasts (B), and in large lamellar structures between chloroplasts (D). In the control samples (E,F), no precipitation was verified. The arrowheads indicate plastoglobules. Bars = 1 µm.

Despite limited knowledge about terpenoid synthase (TPS) in seaweeds, we were able to identify through Blast searches 21 genes from *L. dendroidea* that were similar to well-characterized plant TPS (Table 2 and Supplementary Information). The search for the metal-binding domain (PF03936) and the *N*-terminal domain (PF01397) of terpene synthases did not return new sequences. Still, the search for vanadium-dependent bromoperoxidases returned 11 sequences.

The enzymes involved in the biosynthesis of monoterpenes identified in this study were (3*R*)-linalool synthase (EC 4.2.3.26), (+)-trans-carveol dehydrogenase (EC 1.1.1.275), (+)-pulegone reductase (EC 1.3.1.81), (−)-isopiperitenone reductase (EC 1.3.1.82), and secologaninsynthase (EC 1.3.3.9). The genes coding for farnesyl-diphosphate farnesyltransferase (EC 2.5.1.21) and squalene monooxygenase (EC 1.14.13.132), detected in the transcriptome of *L.dendroidea*, are involved in the biosynthesis of triterpenoid precursors (Figure 3). In this work,we also detected genes involved in the biosynthesis of two different types of triterpenes, represented by the genes coding for squalene-hopene/tetraprenyl-beta-curcumene cyclase (EC 4.2.1.129) and lupeol synthase 1 (EC 5.4.99.41).

Furthermore, the transcriptome of *L. dendroidea* revealed genes associated with the biosynthesis of different classes of sesquiterpenes. The nerolidol synthase gene (EC 4.2.3.48) detected in this transcriptome profile is involved in the biosynthesis of the acyclic sesquiterpene nerolidol; the gene coding for alpha-bisabolene synthase (EC 4.2.3.38) is responsible for the biosynthesis of sesquiterpenes of the bisabolene-type; and the genes coding for germacrene-A synthase (EC 4.2.3.23), germacrene A oxidase (EC 1.1.1.314), aristolochene synthase (EC 4.2.3.9), and 5-epiaristolochene 1,3-dihydroxylase (EC 1.14.13.119) are involved in the biosynthesis of germacrene-type sesquiterpenes. Moreover, we detected the expression of the genes encoding pentalenene synthase (EC 4.2.3.7) and (+)-delta-cadinene synthase (EC 4.2.3.13), which are involved in the biosynthesis of humulene-type and canydil-type sesquiterpenes, respectively. Additionally, the sequence annotation revealed a gene coding for zerumbone synthase (EC 1.1.1.326), which is responsible for the biosynthesis of the sesquiterpene zerumbone.

Finally, three genes involved in the biosynthesis of diterpenes were also detected in the transcriptome of *L. dendroidea*: gibberellin 20-oxidase (EC 1.14.11.12), gibberellin 2-oxidase (EC 1.14.11.13), and abietadienol/abietadienal oxidase (EC 1.14.13.109).

Table 2. List of gene names for mono-(C_{10}), di-(C_{20}), tri-(C_{30}), and sesquiterpenes (C_{15}) found in the transcriptome of *L. dendroidea*, with their products, EC number, Blast e-value and similarity, ecological roles (ER), and biotechnological potential (BP).

Gene Name	EC Number	Blast e-Value	Similarity	Gene Product	Terpene Class	Role	Reference
(3R)-linalool synthase	4.2.3.26	3e-07	68%	Linalool	Monoterpene	Defense (ER); antibiotic, antifungal anticonvulsant, antitumor (BP)	[33–35,46,47]
(+)-trans-carveol dehydrogenase	1.1.1.275	2e-08	54%	(+)-(*S*)-carvone	Monoterpene	Anti-herbivore, antifungal (ER); anticonvulsant, antibiotic, cytotoxic, anti-sprouting agent in potatoes (BP).	[38–41]
(+)-pulegone reductase	1.3.1.81	3e-39	52%	(+)-pulegone	Monoterpene	Defense (ER); analgesic, antibacterial, antifungal, insecticide, acaricidal (BP)	[42–47]
(−)-isopiperitenone reductase	1.3.1.82	1e-04	56%	(−)-isopiperitenone (intermediate compound to (−)-menthone)	Monoterpene	Defense (ER); acaricidal, antibiotic (BP)	[45–48]
secologanin synthase	1.3.3.9	2e-16	52%	Secologanin	Monoterpene	Precursor to indole alkaloids; antimicrobial (BP)	[48,78]
farnesyl-diphosphate farnesyltransferase	2.5.1.21	3e-12	64%	Squalene	Triterpene	Precursor to triterpene	[89]
squalene monooxygenase	1.14.13.132	3e-96	69%	(*S*)-squalene-2,3-epoxide	Triterpene	Precursor to triterpene	[89]
squalene-hopene/tetraprenyl-beta-curcumene cyclase	4.2.1.129	7e-04	62%	hopan-22-ol	Triterpene	Precursor to triterpene with chair-chair-chair-chair conformation	[90]
lupeol synthase 1	5.4.99.41	2e-04	41%	lupeol,β-amyrin	Triterpene	Antibacterial, anti-fungal, anti-inflammatory, antineoplastic, antihypertensive, antiurolithiatic (BP)	[61–63]
nerolidol synthase	4.2.3.48	9e-04	62%	Nerolidol	Sesquiterpene	Precursor to α- and β-snyderols	[20,43]

Table 2. *Cont*.

alpha-bisabolene synthase	4.2.3.38	6e-04	43%	(*E*)-alpha-bisabolene	Sesquiterpene	Precursor to (−)-elatol and caespitol; Defense (ER); antileishmanial, anti-trypanosomal, antibiotic, anti-tumor (BP)	[6,7,21,22,26,55–57]
germacrene-A synthase	4.2.3.23	6e-20	43%	(+)-(*R*)-gemacrene A	Sesquiterpene	Precursor to germacrene-type sesquiterpenes	[95,96]
germacrene A oxidase	1.1.1.314	3e-9	52%	germacra-1(10),4,11(13)-trien-12-oate	Sesquiterpene	Precursor to germacrene-type sesquiterpenes	[95,96]
aristolochene synthase	4.2.3.9	5e-04	44%	Aristolochene	Sesquiterpene	Precursor to germacrene-type sesquiterpenes	[95,96]
5-epiaristolochene 1,3-dihydroxylase	1.14.13.119	3e-20	53%	Capsidiol	Sesquiterpene	Plant defense (ER); Antibiotic, prostaglandin inhibitor (BP)	[58–61]
pentalenene synthase	4.2.3.7	1e-07	42%	Pentalenene	Sesquiterpene	Precursor to humulene-type sesquiterpene	[64]
(+)-delta-cadinene synthase	4.2.3.13	3e-05	48%	Precursor to (−)-δ-cadinene and (+)-α-cadinol	Sesquiterpene	Plant defense (ER), antibiotic (BP)	[58,59]
zerumbone synthase	1.1.1.326	5e-08	43%	Zerumbone	Sesquiterpene	Antitumor, anti Alzheimer's disease (BP)	[60,61]
gibberellin 20-oxidase	1.14.11.12	2e-12	43%	gibberellin 44	Diterpene	Endogenous growth regulators (ER)	[62,63]
gibberellin 2-oxidase	1.14.11.13	7e-11	45%	2beta-hydroxygibberellin 1	Diterpene	Endogenous growth regulators (ER)	[62,63]
abietadienol/abietadienal oxidase	1.14.13.109	1e-13	46%	diterpene acids	Diterpene	Intermediate to diverse diterpene skeletons	[106]

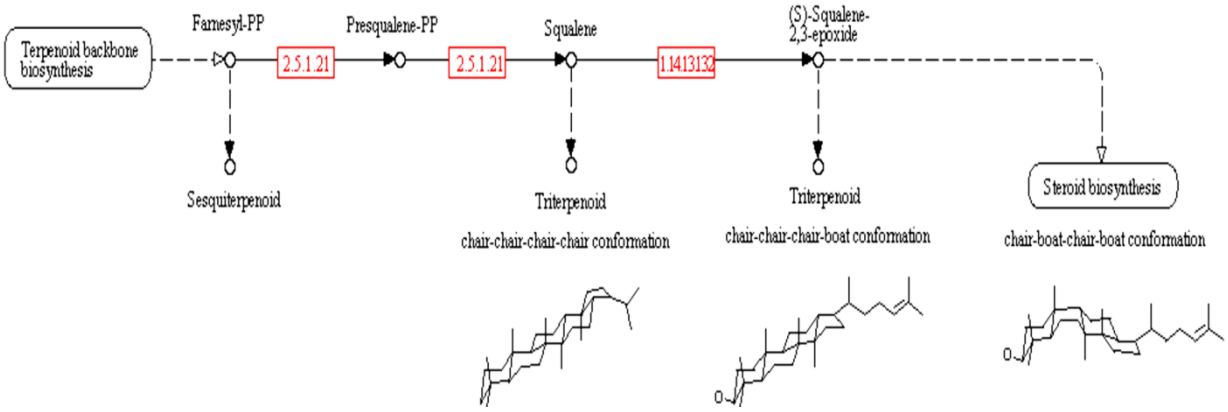


Figure 3. Biosynthetic pathway to the triterpenoid precursors. Modified from the Kegg Pathway Database website—Sesquiterpenoid and triterpenoid biosynthesis reference pathway [123].

Discussion

The class of compounds known as terpenes includes some primary metabolites, such as sterols and carotenes; they are part of the molecules of chlorophylls (with a C_{20} isoprenoid side-chain) and of plastoquinone, phylloquinone, and ubiquinone (with long isoprenoid side-chains), which are essential for the survival of the producing organisms [78]. Nevertheless, most of the terpenes found in plants are classified as secondary metabolites, acting mainly as defensive compounds. More than 30,000 terpenoid compounds have been identified to date, all of them derived from diverse combinations of dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) by the activity of prenyl transferases, resulting in the biosynthesis of monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}), and triterpenoids (C_{30}). In this work we detected 20 different genes involved in the biosynthesis of these terpenoid precursors.

The transcriptomic analysis of *L. dendroidea* revealed for the first time the expression of the genes encoding acetyl-CoA C-acetyltransferase (EC 2.3.1.9) and mevalonate kinase (EC 2.7.1.36), which are involved in the biosynthesis of isoprenoid precursors through the mevalonate (MVA) pathway. Furthermore, in the cytochemical assay, some spherical (vesicle-like) and lamellar intracellular structures of *L. dendroidea* spores were positively labeled, indicating the activity of 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS, EC 2.3.3.10) and/or 3-hydroxy-3-methylglutaryl-

CoA reductase (HMGR, EC 1.1.1.34), and thus supporting the occurrence of the MVA pathway in *L. dendroidea*.

Two different and nonhomologous pathways are known to produce isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the central intermediates in the biosynthesis of isoprenoids: the mevalonate (MVA) pathway in some eukaryotes and archaea, and the methylerythritol phosphate (MEP) pathway in bacteria and several photosynthetic eukaryotes. In the MVA pathway, the acetyl-coenzyme A is converted to IPP through mevalonate. In *L. dendroidea*, the main sites presenting the activity of the enzymes from the mevalonate pathway HMGS and/or HMGR were vesicle-like structures and well-defined lamellar structures. Similarly, HMGR-enriched vesicles were observed in *Arabipdosi*s cells [79], which might have characteristic enzymatic composition specialized in the biosynthesis of isoprenoids. Furthermore, a major HMGR activity was detected in microsomal fractions in *Pisum sativum* seedlings, possibly corresponding to Golgi apparatus and membrane profiles [80]. In addition, we observed HMGS/HMGR activity surrounding the chloroplasts in lamellar inclusions not delimited by membrane, indicating an initial step of the synthesis occurring in the cytoplasm (Figure 2). Likewise, the HMG-CoA reductase was also localized outside the chloroplasts in *Nepeta cataria* leaves [81]. The labeling of regions near the chloroplasts can be related to a role of this organelle in the mevalonate pathway, since the chloroplasts are known to produce acetyl-CoA [82], the first substrate used in the mevalonate dependent pathway. Thus, considering a possible initial step in the cytoplasm, these findings suggest a posterior multi-organelle compartmentalization of the mevalonate pathway, which can be an important intracellular channeling mechanism for the biosynthesis of the different classes of terpenes. Several hypotheses regarding the evolutionary origin of these metabolic pathways have been put forward in the past years, but a recent study suggests that the MVA pathway was probably present in the last common ancestor of all organisms [83], and secondarily lost in some evolutionary lineages, such as the green algae (Chlorophyta) [84].

In addition, we detected all the genes involved in the methylerythritol phosphate (MEP) pathway in *L. dendroidea*. The MEP pathway, present in cyanobacteria, was probably transferred to photosynthetic eukaryotes through endosymbiotic events [84]. Plants and red algae retained the ability to biosynthesize terpenoid precursors through both the MVA and the MEP pathways [27]. In general, the MVA pathway is involved in the biosynthesis of sterols, triterpenes, and some sesquiterpenes, while the MEP pathway

provides the precursors to monoterpenes, diterpenes, certain sesquiterpenes, carotenoids, and the side chains of chlorophyll and plastoquinone [85]. However, several terpenes have a mixed origin, suggesting some level of interaction between these pathways [86]. In this context, a cross-talk mechanism is relevant to regulate the IPP/ DMAPP supply according to the cell demands.

The huge diversity of terpenoid compounds stems from the skeleton modifications catalyzed by terpene synthases (TPS), a mid-size family, with variable gene numbers in sequenced plant genomes [87] and basically uncharacterized in seaweeds. The skeletal diversity arises from the number of terpene synthases, but also from the ability of a single TPS enzyme to generate multiple products using a single substrate, because of the stochastic nature of bond rearrangements that follow the creation of the carbocation intermediates, which undergo a series of cyclizations, isomerizations, hydride shifts, methyl shifts, or other rearrangements [88].

Species of the genus *Laurencia* are recognized as the most prolific source of terpenes in the marine environment: They biosynthesize diverse monoterpenes, diterpenes, sesquiterpenes, and triterpenes with relevant ecological and biotechnological potential [89]. Notwithstanding the high number of studies in the past 50 years regarding the discovery and chemical characterization of new terpenoid compounds from *Laurencia* species [4,5], this is the first survey of the genes coding terpene synthases in this genus. In fact, to our knowledge this is the first molecular study addressing this relevant family of enzymes in seaweeds. In this work we report the occurrence of 21 terpene synthases in *L. dendroidea* based on Hidden Markov Models profiles and sequence similarity to genes previously described, mainly in plants. The blast e-values for all these genes were equal to or below e-04, and the similarity with the corresponding genes in the available databases was above 40%, conferring reliability to the sequence annotation (Table 2) [90]. Additionally, we provide in the Supplementary File a comparison between the domain composition of these sequences from *L. dendroidea* and the corresponding reference genes available at SwissProt/UniProt and PlantCycDB databases.

We also detected the expression of the gene-encoding, vanadium-dependent bromoperoxidase. This enzyme is involved in the halogenation and cyclization of terpenes in red seaweeds [60], and is probably related to the chemical defense of Rhodophyta in response to infection signals [91]. Taking into account the high evolutionary divergence between plants and seaweeds and the ecological and

biotechnological relevance of the algal terpenes, this study provides essential information and points to the need to extend molecular research on seaweeds, especially concerning genes of ecological and biotechnological interest associated with secondary metabolism.

Monoterpene Synthase Genes

The expression of the monoterpene synthase genes (3*R*)-linalool synthase (EC 4.2.3.26), (+)-trans-carveol dehydrogenase (EC 1.1.1.275), (+)-pulegone reductase (EC 1.3.1.81), (−)-isopiperitenone reductase (EC 1.3.1.82), and secologanin synthase (EC 1.3.3.9) was detected in the transcriptome of *L. dendroidea* (Table 2). The linalool is an acyclic monoterpene possibly involved in signaling pathways related to defense, since the expression of (3*R*)-linalool synthase in plants is induced by wounding and jasmonic acid (JA) [40], and linalool-accumulating transgenic rice plants show an upregulation of defense-related genes [41]. The monoterpene linalool was detected in the red seaweed *Portieria hornemannii* by GC-MS [92], but its role in algae has not yet been demonstrated. Considering that the treatment of the seaweeds *Fucus vesiculosus* and *Chondrus crispus* with methyl jasmonate results in an increase in the biosynthesis of defense compounds and in the transcription of stress-related genes [93,94], we could propose a role for linalool in seaweeds as a signal molecule involved in defense, similarly to that observed in plants, although further studies are necessary to test this hypothesis. This metabolite also displays a pharmacological potential due to its antibiotic [37], antifungal [38], anticonvulsant [39], and antitumor [95] activities.

The enzyme (+)-trans-carveol dehydrogenase is responsible for the conversion of (+)-trans-carveol to (+)-(*S*)-carvone. This secondary metabolite acts as a feeding deterrent and antifungal compound in plants [44,45], and was also detected in *P. hornemannii* by GC-MS [92], despite the missing information about its role in seaweeds. Some biotechnologically interesting activities of (+)-(*S*)-carvone include its anticonvulsant [42], antibiotic, and cytotoxic roles; it also acts as an anti-sprouting agent in potatoes [43].

The enzymes (+)-pulegone reductase and (−)-isopiperitenone reductase are involved in the biosynthesis of (+)-pulegone and (−)-menthone, which act as defense compounds in plants [50,51]. In addition, (−)-menthone has acaricidal [49] and antibiotic activities [52], and (+)-pulegone presents analgesic [46], antibacterial,

antifungal [47] insecticidal [48], and acaricidal activities [49]. Despite the high similarity of some sequences from *L. dendroidea* with these plant genes (e-value up to e-39), the enzymes and their metabolic products were not previously detected in seaweeds, possibly suggesting the presence of homologous genes in *L. dendroidea* that could be responsible for a similar reaction in this seaweed, since these enzymes act as monoterpene double-bond reductases.

The gene for secologanin synthase, identified in this transcriptome, participates in the biosynthesis of secologanin, a precursor for the production of indole alkaloids. In the marine environment, most of the indole group alkaloids are concentrated in red seaweeds [96]. Several brominated indoles were previously isolated from *Laurencia brongniartii* [53], *L. decumbens*, and *L. similis* [54], some of them with an antimicrobial activity.

Triterpene Synthase Genes

The genus *Laurencia* is a prolific source of secondary metabolites derived from squalene [97], some of them with a relevant pharmacological activity, such as cytotoxicity against cancer cell lines [98]. The genes coding farnesyl-diphosphate farnesyltransferase (EC 2.5.1.21) and squalene monooxygenase (EC 1.14.13.132), encountered in the transcriptome of *L. dendroidea* are essential to the biosynthesis of triterpenoid precursors [55] (Figure 3). Moreover, we detected genes involved in the biosynthesis of two different types of triterpenes in the transcriptome of *L. dendroidea*. The gene encoding squalene-hopene/tetraprenyl-beta-curcumene cyclase (EC 4.2.1.129) is involved in the biosynthesis of triterpenes with a chair-chair-chair-chair conformation [56], and the gene coding lupeol synthase 1 (EC 5.4.99.41) is related to the biosynthesis of triterpenes with a chair-chair-chair-boat conformation. The enzyme lupeol synthase 1 is involved in the biosynthesis of pentacyclic triterpenes, and in *Arabidopsis thaliana* it catalyzes the production of not only lupeol, but some other metabolites, such as β-amyrin. Some squalene-derived pentacyclic triterpenes were previously isolated from *Laurencia* species [99], including the β-amyrin, which is known for its medically relevant antibacterial [57], anti-fungal, and anti-inflammatory activities [58]. Further, lupeol displays antineoplastic, anti-inflammatory, antihypertensive, and antiurolithiatic activities [59].

Sesquiterpene Synthase Genes

The nerolidol synthase gene (EC 4.2.3.48) detected in this transcriptome profile is responsible for the biosynthesis of nerolidol, an acyclic sesquiterpene that through a bromonium-ion-induced cyclization by vanadium-dependent bromoperoxidase, generates α- and β-snyderols [60], secondary metabolites isolated from *L. obtusa* [14] (Figure 4). Moreover, nerolidol presents antileishmanial [100], antischistosomal [101], and antiulcer [102] activities.

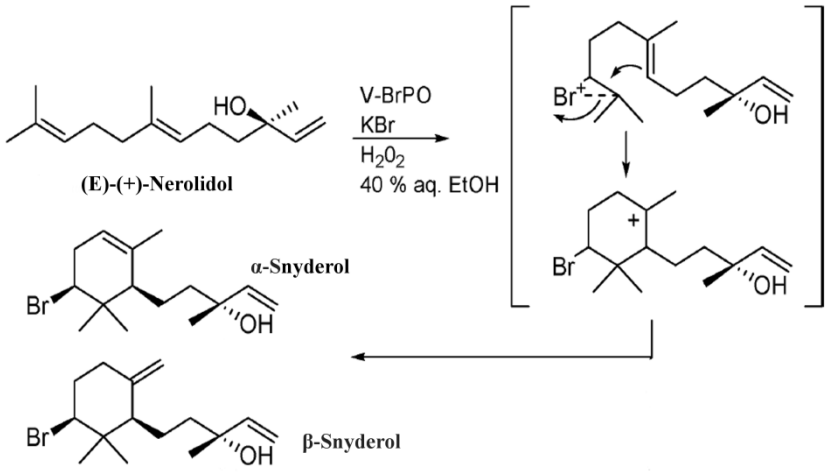


Figure 4. Proposed mechanism for the biosynthesis of α- and β-snyderols from (*E*)-(+)-nerolidol (adapted from [60]).

Moreover, we detected the gene coding alpha-bisabolene synthase (EC 4.2.3.38), which is responsible for the biosynthesis of sesquiterpenes of the bisabolene-type. The biosynthesis of (−)-elatol, one of the major secondary metabolites from *L. dendroidea*, and of some other relevant sesquiterpenes is suggested to involve the enzymatic addition of bromochloride at a bisaboloniun ion stage before the cyclization to the chamigrene derivative [103]. In addition, some other bisabolene-type sesquiterpenes, such as caespitol, filiformin, and β-bisabolene sesquiterpenoids, were previously reported for the genus *Laurencia* [104–106]. The sesquiterpene (−)-elatol plays important ecological roles defending *Laurencia* against biofoulers [7] herbivores [6], and marine pathogenic bacteria [61]. In addition, this compound has important pharmacological potential due to its antileishmanial [15], anti-trypanosomal [16], antibiotic [62], and anti-tumor effects [63]. Also, caespitol displays a relevant cytotoxic activity against three human tumor cell lines [20].

The enzymes germacrene-A synthase (EC 4.2.3.23), germacrene A oxidase (EC 1.1.1.314), aristolochene synthase (EC 4.2.3.9), and 5-epiaristolochene 1,3-dihydroxylase (EC 1.14.13.119) are involved in the biosynthesis of germacrene-type sesquiterpenes [64,65]. Germacrene is the biosynthetic precursor of the eudesmane class and some other secondary metabolites previously isolated from *L. microcladia* and *L. filiformis* [107–109]. The 5-epiaristolochene 1,3-dihydroxylase is responsible for the biosynthesis of capsidiol, which is involved in plant defense [66,67], and has a pharmacological potential derived from its antibiotic [68] and prostaglandin inhibition activities [69].

Moreover, we detected the expression of the genes coding pentalenene synthase (EC 4.2.3.7) and (+)-delta-cadinene synthase (EC 4.2.3.13), which are involved in the biosynthesis of humulene-type and canydil-type sesquiterpenes, respectively. There are few reports on the occurrence of sesquiterpenes from these skeletal classes in *Laurencia*, except for dactylol, previously isolated from *Laurencia poitei*, which is assumed to be biosynthetically derived from humulene [70] and (−)-δ-cadinene and (+)-α-cadinol, which are canydil-type sesquiterpenes isolated from *L. microcladia* [108]. Despite the few reports of canydil-type sesquiterpenes in seaweeds, including *Laurencia* and some other red and brown seaweeds [92,110], these types of metabolites are widespread in terrestrial vascular plants. The fungal-elicited production of δ-cadinene synthase in cotton suggests a role for this enzyme in plant defense [71], and the antibiotic activity against *Streptococcus pneumoniae* resistant to conventional antibiotics imply a pharmacological potential for δ-cadinene [72].

Additionally, the sequence annotation revealed a gene coding zerumbone synthase (EC 1.1.1.326), which is related to the biosynthesis of zerumbone, a plant humulane sesquiterpenoid with antitumor activity [73] and a potential candidate for the developmental of anti-Alzheimer's disease treatment [74]. There is no report on the occurrence of this secondary metabolite in seaweeds. However, our findings possibly indicate the existence of a homologous gene in *L. dendroidea* that could be involved in a similar chemical reaction.

Diterpene Synthase Genes

Laurencia species biosynthesize diterpenoid compounds that can be involved in the defense of these seaweeds [111] and also show some pharmacologically relevant

antibiotic [112], cytotoxic [113], and anti-inflammatory activities [17]. Through the analysis of the transcriptome of *L. dendroidea*, we detected the expression of three genes involved in the biosynthesis of diterpenes: the gibberellin 20-oxidase (EC 1.14.11.12), the gibberellin 2-oxidase (EC 1.14.11.13), and the abietadienol/abietadienal oxidase genes (EC 1.14.13.109) (Table 2 and Supplementary Material). Notwithstanding the absence of information regarding the biosynthesis of abietic acid and derivatives in seaweeds, the enzyme abietadienol/abietadienal oxidase is a multifunctional and multisubstrate cytochrome P450 enzyme [77], and could be involved in the biosynthesis of diverse diterpenes in *L. dendroidea* through oxidation steps.

The gibberellins are important endogenous growth regulators, well recognized in vascular plants. Diverse studies point to a gibberellin-like activity in extracts of seaweeds, which led to the broad utilization of seaweeds in the formulation of commercial plant fertilizers [75]. Recently, a chemical analysis of the extract of the brown seaweed *Ecklonia maxima* was able to reliably demonstrate for the first time the presence of gibberellins in seaweed [76]. The expression of candidate genes for gibberellin 20-oxidase and gibberellin 2-oxidase in *L. dendroidea* is the first evidence for the occurrence of gibberellins in a red seaweed, although a more detailed chemical evaluation is necessary. A comparison between the conserved domain hits found for some candidate genes from *L. dendroidea* and the sequences coding for gibberellin 20-oxidase and gibberellin 2-oxidase from *Arabidopsis thaliana* showed the presence of typical gibberellin synthase domains (available in the Supplementary Information).

Conclusions

Laurencia dendroidea expresses a suite of genes encoding terpene synthases that catalyze the chemical modifications of precursors resulting in the high diversity of terpenoid compounds known for this species. The unveiling of genes associated to the chemical defense against natural enemies and the cytochemical evidence for the occurrence of the mevalonate pathway in *L. dendroidea* provided a better understanding of the molecular and biochemical processes and the cell structures involved in the biosynthesis of these secondary compounds that affect the biological interactions of this

seaweed in the marine environment. The present work offered valuable information toward future sustainable production of biotechnologically relevant terpenes from *L. dendroidea* using genetically modified organisms in fermentation processes.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Louisi Souza de Oliveira carried out the sample collection, RNA extraction, and sequencing, and participated in the bioinformatic analysis and drafting of the manuscript. Diogo Antonio Tschoeke participated in the bioinformatic analysis and in the discussions and drafting of the manuscript. Aline Santos de Oliveira participated in the sample collection, production of clones of *Laurencia dendroidea*, and in the discussions and drafting of the manuscript. Lilian Jorge Hill performed the cytochemical labeling assay, and participated in discussion of the results and drafting of the manuscript. Wladimir Costa Paradas participated in cytochemical labeling assay and drafting of the manuscript. Leonardo Tavares Salgado participated in the acquisition of funding, work planning, discussion of the results, and drafting of the manuscript. Cristiane Carneiro Thompson participated in the acquisition of funding, discussion of the results, and drafting of the manuscript. Renato Crespo Pereira participated in the acquisition of funding, work planning, discussion of the results, and drafting of the manuscript. Fabiano L. Thompson participated in the acquisition of funding, work planning, data interpretation, and drafting of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We acknowledge the financial support of CAPES, CNPq, and FAPERJ. This paper is part of the DSc. requirements of Louisi Souza de Oliveira at the Biodiversity and Evolutionary Biology Graduate Program of the Federal University of Rio de Janeiro. Fabiano L. Thompson, Leonardo Tavares Salgado, and Renato Crespo Pereira thank CNPq for their Research Productivity Fellowships.

Author details
[1] Institute of Biology, Federal University of Rio de Janeiro (UFRJ) Av. Carlos Chagas Filho, 373-CCS-IB-BLOCO A (ANEXO) A3-202, SAGE-COPPE, Rio de Janeiro, 21941-599, Brazil;
E-Mails: louisioliveira@gmail.com (L.S.O.); diogoat@gmail.com (D.A.T.); thompsoncristiane@gmail.com (C.C.T)
[2] Federal Institute of Education, Science and Technology of Rio de Janeiro (IFRJ), Campus Duque de Caxias, Avenida República do Paraguai, 120, Sarapuí, Duque de Caxias, 25050-100, Brazil;
E-Mail: linesoliveira@gmail.com
[3] Research Institute of the Botanical Garden of Rio de Janeiro, Rua Pacheco Leão, 915, Jardim Botânico, Rio de Janeiro, 22460-030, Brazil; E-Mails: lilianhill.bio@gmail.com (L.J.H.); wladimirparadas@gmail.com (W.C.P.); lsalgado.jbrj@gmail.com (L.T.S.)
[4] Departament of Marine Biology, Federal Fluminense University (UFF), Morro do Valonguinho, s/n, Centro, Niterói, 24001-970, Brazil; E-Mail: rcrespo@id.uff.br

***** Author to whom correspondence should be addressed; E-Mail: fabianothompson1@gmail.com; Tel.: +55-21-39386567.

Academic Editor: RuAngelie Edrada-Ebel

Received: 14 September 2014 / Accepted: 12 January 2015 / Published: 10 February 2015

References

1. Hay, M.E. Marine chemical ecology: Chemical signals and cues structure marine populations, communities, and ecosystems. *Ann. Rev. Mar. Sci.* **2009**, *1*, 193–212.
2. Amsler, C.D. Chemical ecology of seaweeds. In *Seaweed Biology*; Wiencke, C., Bischof, K., Eds.; Ecological Studies; Springer Berlin Heidelberg: Berlin, Heidelberg, 2012; Volume 219, pp. 177–188.
3. Rasher, D.B.; Hay, M.E. Chemically rich seaweeds poison corals when not controlled by herbivores. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 9683–9688.
4. Irie, T.; Suzuki, M.; Masamune, T. Laurencin, a constituent from *Laurencia* species. *Tetrahedron Lett.* **1965**, *6*, 1091–1099.
5. Liang, Y.; Li, X.-M.; Li, C.-S.; Sun, H.; Wang, B.-G. Laurane-, cyclolaurane-, and cuparane-type sesquiterpenes from the marine red alga *Laurencia okamurai*. *Nat. Prod. Commun.* **2014**, *9*, 323–324.
6. Pereira, R.C.; da Gama, B.AP.; Teixeira, V.L.; Yoneshigue-Valentin, Y. Ecological roles of natural products of the Brazilian red seaweed *Laurencia obtusa*. *Braz. J. Biol.* **2003**, *63*, 665–72.

7. Da Gama, B.A; Pereira, R.C.; Soares, A.R.; Teixeira, V.L.; Yoneshigue-Valentin, Y. Is the mussel test a good indicator of antifouling activity? A comparison between laboratory and field assays. *Biofouling* **2003**, *19* (Suppl.), 161–169.
8. Sudatti, D.B.; Rodrigues, S.V.; Pereira, R.C. Quantitative GC-ECD analysis of halogenated metabolites: Determination of surface and within-thallus elatol of *Laurencia obtusa*. *J. Chem. Ecol.* **2006**, *32*, 835–843.
9. Oliveira, A.S.; Sudatti, D.B.; Fujii, M.T.; Rodrigues, S.V.; Pereira, R.C. Inter- and intrapopulation variation in the defensive chemistry of the red seaweed *Laurencia dendroidea* (Ceramiales, Rhodophyta). *Phycologia* **2013**, *52*, 130–136.
10. Sudatti, D.B.; Fujii, M.T.; Rodrigues, S.V.; Turra, A.; Pereira, R.C. Effects of abiotic factors on growth and chemical defenses in cultivated clones of *Laurencia dendroidea* J. Agardh (Ceramiales, Rhodophyta). *Mar. Biol.* **2011**, *158*, 1439–1446.
11. Sakemi, S.; Higa, T.; Jefford, C.W.; Bernardinelli, G. Venustatriol. A new, anti-viral, triterpene tetracyclic ether from *Laurencia venusta*. *Tetrahedron Lett.* **1986**, *27*, 4287–4290.
12. Vairappan, C.S.; Daitoh, M.; Suzuki, M.; Abe, T.; Masuda, M. Antibacterial halogenated metabolites from the Malaysian *Laurencia* species. *Phytochemistry* **2001**, *58*, 291–297.
13. Vairappan, C.S.; Kawamoto, T.; Miwa, H.; Suzuki, M. Potent antibacterial activity of halogenated compounds against antibiotic-resistant bacteria. *Planta Med.* **2004**, *70*, 1087–1090.
14. Topcu, G.; Aydogmus, Z.; Imre, S.; Gören, A.C.; Pezzuto, J.M.; Clement, J.A.; Kingston, D.G.I. Brominated sesquiterpenes from the red alga *Laurencia obtusa*. *J. Nat. Prod.* **2003**, *66*, 1505–1508.
15. Dos Santos, A.O.; Veiga-Santos, P.; Ueda-Nakamura, T.; Filho, B.P.D.; Sudatti, D.B.; Bianco, E.M.; Pereira, R.C.; Nakamura, C.V. Effect of elatol, isolated from red seaweed *Laurencia dendroidea*, on *Leishmania amazonensis*. *Mar. Drugs* **2010**, *8*, 2733–2743.
16. Veiga-Santos, P.; Pelizzaro-Rocha, K.J.; Santos, A.O.; Ueda-Nakamura, T.; Dias Filho, B.P.; Silva, S.O.; Sudatti, D.B.; Bianco, E.M.; Pereira, R.C.; Nakamura, C.V. *In vitro* anti-trypanosomal activity of elatol isolated from red seaweed *Laurencia dendroidea*. *Parasitology* **2010**, *137*, 1661–1670.
17. Chatter, R.; Ben Othman, R.; Rabhi, S.; Kladi, M.; Tarhouni, S.; Vagias, C.; Roussis, V.; Guizani-Tabbane, L.; Kharrat, R. *In vivo* and *in vitro* anti-inflammatory activity of neorogioltriol, a new diterpene extracted from the red algae *Laurencia glandulifera*. *Mar. Drugs* **2011**, *9*, 1293–1306.
18. Kladi, M.; Xenaki, H.; Vagias, C.; Papazafiri, P.; Roussis, V. New cytotoxic sesquiterpenes from the red algae *Laurencia obtusa* and *Laurencia microcladia*. *Tetrahedron* **2006**, *62*, 182–189.

19. Kim, M.-M.; Mendis, E.; Kim, S.-K. *Laurencia okamurai* extract containing laurinterol induces apoptosis in melanoma cells. *J. Med. Food* **2008**, *11*, 260–266.
20. Lhullier, C.; Falkenberg, M.; Ioannou, E.; Quesada, A.; Papazafiri, P.; Horta, P.A.; Schenkel, E.P.; Vagias, C.; Roussis, V. Cytotoxic halogenated metabolites from the Brazilian red alga *Laurencia catarinensis*. *J. Nat. Prod.* **2010**, *73*, 27–32.
21. Da Gama, B.A.P.; Pereira, R.C.; Carvalho, A.G.V; Coutinho, R.; Yoneshigue-Valentin, Y. The effects of seaweed secondary metabolites on biofouling. *Biofouling* **2002**, *18*, 13–20.
22. White, D.E.; Stewart, I.C.; Grubbs, R.H.; Stoltz, B.M. The catalytic asymmetric total synthesis of elatol. *J. Am. Chem. Soc.* **2008**, *130*, 810–811.
23. Pereira, R.C.; Costa-Lotufo, L.V. Bioprospecting for bioactives from seaweeds: Potential, obstacles and alternatives. *Rev. Bras. Farmacogn.* **2012**, *22*, 894–905.
24. Ignea, C.; Cvetkovic, I.; Loupassaki, S.; Kefalas, P.; Johnson, C.B.; Kampranis, S.C.; Makris, A.M. Improving yeast strains using recyclable integration cassettes, for the production of plant terpenoids. *Microb. Cell Fact.* **2011**, *10*, 4.
25. Martin, V.J.J.; Pitera, D.J.; Withers, S.T.; Newman, J.D.; Keasling, J.D. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* **2003**, *21*, 796–802.
26. De Oliveira, L.S.; Gregoracci, G.B.; Silva, G.G.Z.; Salgado, L.T.; Filho, G.A.; Alves-Ferreira, M.; Pereira, R.C.; Thompson, F.L. Transcriptomic analysis of the red seaweed *Laurencia dendroidea* (Florideophyceae, Rhodophyta) and its microbiome. *BMC Genomics* **2012**, *13*, 487.
27. Lohr, M.; Schwender, J.; Polle, J.E. W. Isoprenoid biosynthesis in eukaryotic phototrophs: A spotlight on algae. *Plant Sci.* **2012**, *185–186*, 9–22.
28. Fenical, W. Halogenation in the Rhodophyta, a Review. *J. Phycol.* **1975**, *11*, 245–259.
29. Cabrita, M.T.; Vale, C.; Rauter, A.P. Halogenated compounds from marine algae. *Mar. Drugs* **2010**, *8*, 2301–2317.
30. Young, D.N.; Howard, B.M.; Fenical, W. Subcellular localization of brominated secondarymetabolites in the red alga *Laurencia snyderae*. *J. Phycol.* **1980**, *16*, 182–185.
31. Salgado, L.T.; Viana, N.B.; Andrade, L.R.; Leal, R.N.; da Gama, B.A.P.; Attias, M.; Pereira, R.C.; Amado Filho, G.M. Intra-cellular storage, transport and exocytosis of halogenated compounds in marine red alga *Laurencia obtusa*. *J. Struct. Biol.* **2008**, *162*, 345–355.
32. Reis, V.M.; Oliveira, L.S.; Passos, R.M.F.; Viana, N.B.; Mermelstein, C.; Sant'anna, C.; Pereira, R.C.; Paradas, W.C.; Thompson, F.L.; Amado-Filho, G.M.; Salgado, L.T. Traffic of secondary metabolites to cell surface in the red alga *Laurencia dendroidea* depends on a two-step transport by the cytoskeleton. *PLoS One* **2013**, *8*, e63929.

33. Paradas, W.C.; Salgado, L.T.; Sudatti, D.B.; Crapez, M.A; Fujii, M.T.; Coutinho, R.; Pereira, R.C.; Amado Filho, G.M. Induction of halogenated vesicle transport in cells of the red seaweed *Laurencia obtusa*. *Biofouling* **2010**, *26*, 277–286.
34. Nakamura, Y.; Sasaki, N.; Kobayashi, M.; Ojima, N.; Yasuike, M.; Shigenobu, Y.; Satomi, M.; Fukuma, Y.; Shiwaku, K.; Tsujimoto, A.; *et al*. The first symbiont-free genome sequence of marine red alga, Susabi-nori (*Pyropia yezoensis*). *PLoS One* **2013**, *8*, e57122.
35. Collén, J.; Porcel, B.; Carré, W.; Ball, S.G.; Chaparro, C.; Tonon, T.; Barbeyron, T.; Michel, G.; Noel, B.; Valentin, K.; *et al*. Genome structure and metabolic features in the red seaweed *Chondrus crispus* shed light on evolution of the Archaeplastida. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 5247–5252.
36. Kapraun, D.F. Nuclear DNA content estimates in multicellular green, red and brown algae: Phylogenetic considerations. *Ann. Bot.* **2005**, *95*, 7–44.
37. Salehi, P.; Sonboli, A.; Asghari, B. Chemical composition of the essential oil of *Stachys acerosa* and its antibacterial and antioxidant activities. *Chem. Nat. Compd.* **2007**, *43*, 339–341.
38. Khan, A.; Ahmad, A.; Manzoor, N.; Khan, L.A. Antifungal activities of *Ocimum sanctum* essential oil and its lead molecules. *Nat. Prod. Commun.* **2010**, *5*, 345–349.
39. Elisabetsky, E.; Brum, L.F.; Souza, D.O. Anticonvulsant properties of linalool in glutamate-related seizure models. *Phytomedicine* **1999**, *6*, 107–113.
40. Jia, J.W.; Crock, J.; Lu, S.; Croteau, R.; Chen, X.Y. (3R)-Linalool synthase from *Artemisia annua* L.: cDNA isolation, characterization, and wound induction. *Arch. Biochem. Biophys.* **1999**, *372*, 143–149.
41. Taniguchi, S.; Hosokawa-Shinonaga, Y.; Tamaoki, D.; Yamada, S.; Akimitsu, K.; Gomi, K. Jasmonate induction of the monoterpene linalool confers resistance to rice bacterial blight and its biosynthesis is regulated by JAZ protein in rice. *Plant. Cell Environ.* **2014**, *37*, 451–461.
42. De Sousa, D.P.; de Farias Nóbrega, F.F.; de Almeida, R.N. Influence of the chirality of (*R*)-(−)- and (*S*)-(+)-carvone in the central nervous system: A comparative study. *Chirality* **2007**, *19*, 264–268.
43. De Carvalho, C.; da Fonseca, M. Carvone: Why and how should one bother to produce this terpene. *Food Chem.* **2006**, *95*, 413–422.
44. Frank, T.; Biert, K.; Speiser, B. Feeding deterrent effect of carvone, a compound from caraway seeds, on the slug *Arion lusitanicus*. *Ann. Appl. Biol.* **2002**, *141*, 93–100.
45. Smid, E.J.; de Witte, Y.; Gorris, L.G.M. Secondary plant metabolites as control agents of postharvest *Penicillium* rot on tulip bulbs. *Postharvest Biol. Technol.* **1995**, *6*, 303–312.

46. De Sousa, D.P.; Nóbrega, F.F.F.; de Lima, M.R.V; de Almeida, R.N. Pharmacological activity of (*R*)-(+)-pulegone, a chemical constituent of essential oils. *Z. Naturforsch. C.* **2011**, *66*, 353–359.
47. Arruda, T.A.; Antunes, R.M.P.; Catão, R.M.R.; Lima, E.O.; Sousa, D.P.; Nunes, X.P.; Pereira, M.S.V.; Barbosa-Filho, J.M.; da Cunha, E.V.L. Preliminary study of the antimicrobial activity of *Mentha x villosa* Hudson essential oil, rotundifolone and its analogues. *Rev. Bras. Farmacogn.* **2006**, *16*, 307–311.
48. Franzios, G.; Mirotsou, M.; Hatziapostolou, E.; Kral, J.; Scouras, Z.G.; Mavragani-Tsipidou, P. Insecticidal and genotoxic activities of mint essential oils. *J. Agric. Food Chem.* **1997**, *45*, 2690–2694.
49. Sánchez-Ramos, I.; Castañera, P. Acaricidal activity of natural monoterpenes on *Tyrophagus putrescentiae* (Schrank), a mite of stored food. *J. Stored Prod. Res.* **2000**, *37*, 93–101.
50. Eisner, T.; McCormick, K.D.; Sakaino, M.; Eisner, M.; Smedley, S.R.; Aneshansley, D.J.; Deyrup, M.; Myers, R.L.; Meinwald, J. Chemical defense of a rare mint plant. *Chemoecology* **1990**, *1*, 30–37.
51. Banchio, E.; Zygadlo, J.; Valladares, G.R. Effects of mechanical wounding on essential oil composition and emission of volatiles from *Minthostachys mollis*. *J. Chem. Ecol.* **2005**, *31*, 719–727.
52. Vimal, M.; Vijaya, P.P.; Mumtaj, P.; Farhath, M.S. Antibacterial activity of selected compounds of essential oils from indigenous plants. *J. Chem. Pharm. Res.* **2013**, *5*, 248–253.
53. Carter, G.T.; Rinehart, K.L.; Li, L.H.; Kuentzel, S.L.; Connor, J.L. Brominated indoles from *Laurencia brongniartii*. *Tetrahedron Lett.* **1978**, *19*, 4479–4482.
54. Güven, K.C.; Percot, A.; Sezik, E. Alkaloids in marine algae. *Mar. Drugs* **2010**, *8*, 269–284.
55. Phillips, D.R.; Rasbery, J.M.; Bartel, B.; Matsuda, S.P. Biosynthetic diversity in plant triterpene cyclization. *Curr. Opin. Plant Biol.* **2006**, *9*, 305–314.
56. Nakano, S.-I.; Ohashi, S.; Hoshino, T. Squalene-hopene cyclase: Insight into the role of the methyl group on the squalene backbone upon the polycyclization cascade. Enzymatic cyclization products of squalene analogs lacking a 26-methyl group and possessing a methyl group at C7 or C11. *Org. Biomol. Chem.* **2004**, *2*, 2012–2022.
57. Abdel-Raouf, N.; Al-Enazi, N.M.; Al-Homaidan, A.A.; Ibraheem, I.B. M.; Al-Othman, M.R.; Hatamleh, A.A. Antibacterial β-amyrin isolated from *Laurencia microcladia*. *Arab. J. Chem.* **2013**, *8*, 32–37.
58. Vázquez, L. H.; Palazon, J.; Navarro-Ocaña, A. The pentacyclic triterpenes α-, β-amyrins: a review of sources and biological activities. In *Phytochemicals-A Global Perspective of Their Role in Nutrition and Health*; Rao, V., Ed.; InTech: Rijeka, Croatia, 2012.

59. Gallo, M.; Sarachine, M. Biological activities of lupeol. *Int. J. Biomed. Pharm. Sci.* **2009**, *3*, 46–66.
60. Carter-Franklin, J.N.; Butler, A. Vanadium bromoperoxidase-catalyzed biosynthesis of halogenated marine natural products. *J. Am. Chem. Soc.* **2004**, *126*, 15060–15066.
61. Vairappan, C.S.; Anangdan, S.P.; Tan, K.L.; Matsunaga, S. Role of secondary metabolites as defense chemicals against ice-ice disease bacteria in biofouler at carrageenophyte farms. *J. Appl. Phycol.* **2009**, *22*, 305–311.
62. Vairappan, C.S. Potent antibacterial activity of halogenated metabolites from Malaysian red algae, *Laurencia majuscula* (Rhodomelaceae, Ceramiales). *Biomol. Eng.* **2003**, *20*, 255–259.
63. Vairappan, C.S.; Kamada, T.; Lee, W.-W.; Jeon, Y.-J. Anti-inflammatory activity of halogenated secondary metabolites of *Laurencia snackeyi* (Weber-van Bosse) Masuda in LPS-stimulated RAW 264.7 macrophages. *J. Appl. Phycol.* **2013**, *25*, 1805–1813.
64. Cane, D.E. Enzymic formation of sesquiterpenes. *Chem. Rev.* **1990**, *90*, 1089–1103.
65. Rising, K.A.; Starks, C.M.; Noel, J.P.; Chappell, J. Demonstration of germacrene A as an intermediate in 5-epi-aristolochene synthase catalysis. *J. Am. Chem. Soc.* **2000**, *122*, 1861–1866.
66. Bailey, J.A.; Burden, R.S.; Vincent, G.G. Capsidiol: An antifungal compound produced in *Nicotiana tabacum* and *Nicotiana clevelandii* following infection with tobacco necrosis virus. *Phytochemistry* **1975**, *14*, 597.
67. Bohlmann, J.; Stauber, E.J.; Krock, B.; Oldham, N.J.; Gershenzon, J.; Baldwin, I.T. Gene expression of 5-epi-aristolochene synthase and formation of capsidiol in roots of *Nicotiana attenuata* and *N. sylvestris*. *Phytochemistry* **2002**, *60*, 109–116.
68. De Marino, S.; Borbone, N.; Gala, F.; Zollo, F.; Fico, G.; Pagiotti, R.; Iorizzi, M. New constituents of sweet *Capsicum annuum* L. fruits and evaluation of their biological activity. *J. Agric. Food Chem.* **2006**, *54*, 7508–7516.
69. Nasiri, A.; Holth, A.; Björk, L. Effects of the sesquiterpene capsidiol on isolated guinea-pig ileum and trachea, and on prostaglandin synthesis *in vitro*. *Planta Med.* **1993**, *59*, 203–206.
70. Kelecom, A. Synthesis of marine natural products in Brazil. *J. Braz. Chem. Soc.* **1998**, *9*, 101–118.
71. Chen, X.Y.; Chen, Y.; Heinstein, P.; Davisson, V.J. Cloning, expression, and characterization of (+)-delta-cadinene synthase: A catalyst for cotton phytoalexin biosynthesis. *Arch. Biochem. Biophys.* **1995**, *324*, 255–66.
72. Pérez-López, A.; Cirio, A.T.; Rivas-Galindo, V.M.; Aranda, R.S.; de Torres, N.W. Activity against *Streptococcus pneumoniae* of the Essential Oil and δ-Cadinene Isolated from *Schinus molle* fruit. *J. Essent. Oil Res.* **2011**, *23*, 25–28.

73. Kirana, C.; McIntosh, G.H.; Record, I.R.; Jones, G.P. Antitumor activity of extract of *Zingiber aromaticum* and its bioactive sesquiterpenoid zerumbone. *Nutr. Cancer* **2003**, *45*, 218–225.
74. Bustamam, A.; Ibrahim, S.; Al-Zubairi, A.S. Zerumbone: A natural compound with anti-cholinesterase activity. *Am. J. Pharmacol. Toxicol.* **2008**, *3*, 209–211.
75. Crouch, I.J.; Staden, J. Evidence for the presence of plant growth regulators in commercial seaweed products. *Plant Growth Regul.* **1993**, *13*, 21–29.
76. Stirk, W.A.; Tarkowská, D.; Turečová, V.; Strnad, M.; van Staden, J. Abscisic acid, gibberellins and brassinosteroids in Kelpak®, a commercial seaweed extract made from *Ecklonia maxima*. *J. Appl. Phycol.* **2014**, *26*, 561–567.
77. Ro, D.-K.; Arimura, G.-I.; Lau, S.Y.W.; Piers, E.; Bohlmann, J. Loblolly pine abietadienol/abietadienal oxidase PtAO (CYP720B1) is a multifunctional, multisubstrate cytochrome P450 monooxygenase. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 8060–8065.
78. McGarvey, D.J.; Croteau, R. Terpenoid metabolism. *Plant Cell* **1995**, *7*, 1015–1026.
79. Leivar, P.; González, V.M.; Castel, S.; Trelease, R.N.; López-Iglesias, C.; Arró, M.; Boronat, A.; Campos, N.; Ferrer, A.; Fernàndez-Busquets, X. Subcellular localization of *Arabidopsis* 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Plant Physiol.* **2005**, *137*, 57–69.
80. Brooker, J.D.; Russell, D.W. Subcellular localization of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Pisum sativum* seedlings. *Arch. Biochem. Biophys.* **1975**, *167*, 730–737.
81. Arebalo, R.E.; Mitchell, E.D. Cellular distribution of 3-hydroxy-3-methylglutaryl coenzyme a reductase and mevalonate kinase in leaves of *Nepeta cataria*. *Phytochemistry* **1984**, *23*, 13–18.
82. Kuhn, D.N.; Knauf, M.; Stumpf, P.K. Subcellular localization of acetyl-CoA synthetase in leaf protoplasts of *Spinacia oleracea*. *Arch. Biochem. Biophys.* **1981**, *209*, 441–450.
83. Lombard, J.; Moreira, D. Origins and early evolution of the mevalonate pathway of isoprenoid biosynthesis in the three domains of life. *Mol. Biol. Evol.* **2011**, *28*, 87–99.
84. Lange, B.M.; Rujan, T.; Martin, W.; Croteau, R. Isoprenoid biosynthesis: The evolution of two ancient and distinct pathways across genomes. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13172–13177.
85. Laule, O.; Fürholz, A.; Chang, H.-S.; Zhu, T.; Wang, X.; Heifetz, P.B.; Gruissem, W.; Lange, M. Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6866–6871.
86. Adam, K.P.; Thiel, R.; Zapp, J. Incorporation of 1-[1-(13)C]Deoxy-D-xylulose in chamomile sesquiterpenes. *Arch. Biochem. Biophys.* **1999**, *369*, 127–132.

87. Chen, F.; Tholl, D.; Bohlmann, J.; Pichersky, E. The family of terpene synthases in plants: A mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. *Plant J.* **2011**, *66*, 212–229.
88. Degenhardt, J.; Köllner, T.G.; Gershenzon, J. Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry* **2009**, *70*, 1621–1637.
89. Blunt, J.W.; Copp, B.R.; Keyzers, R.A.; Munro, M.H. G.; Prinsep, M.R. Marine natural products. *Nat. Prod. Rep.* **2012**, *29*, 144–222.
90. Pearson, W.R. An introduction to sequence similarity ("homology") searching. *Curr. Protoc. Bioinform.* **2013**. Available online: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3820096/pdf/nihms519883.pdf (accessed on 15 september 2014).
91. Weinberger, F.; Coquempot, B.; Forner, S.; Morin, P.; Kloareg, B.; Potin, P. Different regulation of haloperoxidation during agar oligosaccharide-activated defence mechanisms in two related red algae, *Gracilaria* sp. and *Gracilaria chilensis*. *J. Exp. Bot.* **2007**, *58*, 4365–4372.
92. Payo, D.A.; Colo, J.; Calumpong, H.; de Clerck, O. Variability of non-polar secondary metabolites in the red alga *Portieria*. *Mar. Drugs* **2011**, *9*, 2438–2468.
93. Arnold, T.M.; Targett, N.M.; Tanner, C.E.; Hatch, W.I.; Ferrari, K.E. Evidence for methyl jasmonate-induced phlorotannin production in *Fucus vesiculosus* (Phaeophyceae). *J. Phycol.* **2001**, *37*, 1026–1029.
94. Collén, J.; Hervé, C.; Guisle-Marsollier, I.; Léger, J.J.; Boyen, C. Expression profiling of *Chondrus crispus* (Rhodophyta) after exposure to methyl jasmonate. *J. Exp. Bot.* **2006**, *57*, 3869–3881.
95. Chang, M.-Y.; Shen, Y.-L. Linalool exhibits cytotoxic effects by activating antitumor immunity. *Molecules* **2014**, *19*, 6694–6706.
96. Kasım Cemal Güven, Burak Coban, Ekrem Sezik, Hüseyin Erdugan, F.K. Alkaloids of marine macroalgae. In *Natural Products. Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*; Ramawat, K.G., Mérillon, J.-M., Eds.; Springer Berlin Heidelberg: Berlin, Heidelberg, Germany, 2013; pp. 25–37.
97. Manríquez, C.P.; Souto, M.L.; Gavín, J.A.; Norte, M.; Fernández, J.J. Several new squalene-derived triterpenes from *Laurencia*. *Tetrahedron* **2001**, *57*, 3117–3123.
98. Pacheco, F.C.; Villa-Pulgarin, J.A.; Mollinedo, F.; Martín, M.N.; Fernández, J.J.; Daranas, A.H. New polyether triterpenoids from *Laurencia viridis* and their biological evaluation. *Mar. Drugs* **2011**, *9*, 2220–2235.
99. Matsuo, Y.; Suzuki, M.; Masuda, M. Enshuol, a novel squalene-derived pentacyclic triterpene alcohol from a new species of the red algal genus *Laurencia*. *Chem. Lett.* **1995**, 1043–1044.

100. Arruda, D.C.; D'Alexandri, F.L.; Katzin, A.M.; Uliana, S.R. B. Antileishmanial activity of the terpene nerolidol. *Antimicrob. Agents Chemother.* **2005**, *49*, 1679–1687.
101. Silva, M.P.N.; Oliveira, G.L.S.; de Carvalho, R.B.F.; de Sousa, D.P.; Freitas, R.M.; Pinto, P.L.S.; de Moraes, J. Antischistosomal activity of the terpene nerolidol. *Molecules* **2014**, *19*, 3793–3803.
102. Klopell, F.C.; Lemos, M.; Sousa, J.P.B.; Comunello, E.; Maistro, E.L.; Bastos, J.K.; de Andrade, S.F. Nerolidol, an antiulcer constituent from the essential oil of *Baccharis dracunculifolia* DC (Asteraceae). *Z. Naturforsch. C.* **2007**, *62*, 537–542.
103. Schever, P. *Marine Natural Products V1: Chemical and Biological Perspectives*; Academic Press, New York, USA, 2012; Volume 1, p. 318.
104. Fujii, M.T.; Cassano, V.; Senties, A.; Diaz-Larrea, J.; Machin-Sanchez, M.; Candelaria Gil-Rodriguez, M. Comparative analysis of the corps en cerise in several species of *Laurencia* (Ceramiales, Rhodophyta) from the Atlantic Ocean. *Rev. Bras. Farm. J. Pharmacogn.* **2012**, *22*, 795.
105. Davyt, D.; Fernandez, R.; Suescun, L.; Mombrú, A.W.; Saldaña, J.; Domínguez, L.; Fujii, M.T.; Manta, E. Bisabolanes from the red alga *Laurencia scoparia*. *J. Nat. Prod.* **2006**, *69*, 1113–1116.
106. Yu, X.-Q.; He, W.-F.; Liu, D.-Q.; Feng, M.-T.; Fang, Y.; Wang, B.; Feng, L.-H.; Guo, Y.-W.; Mao, S.-C. A seco-laurane sesquiterpene and related laurane derivatives from the red alga *Laurencia okamurai* Yamada. *Phytochemistry* **2014**, *103*, 162–170.
107. Brennan, M.R.; Erickson, K.L. Austradiol acetate and austradiol diacetate, 4,6-dihydroxy-(+)-selinane derivatives from an Australian *Laurencia* sp. *J. Org. Chem.* **1982**, *47*, 3917–3921.
108. Guellaa, G., Skropetaa, D., Mancinia, I., Pietrab, F. The First 6,8-Cycloeudesmane sesquiterpene from a amrine organism: The red seaweed *Laurencia microcladia* from the Baia di Calenzana, Elba Island. *Z. Naturforsch* **2001**, *57B*, 1147–1151.
109. Guella, G.; Skropeta, D.; Mancini, I.; Pietra, F. Calenzanane sesquiterpenes from the red seaweed *Laurencia microcladia* from the Bay of Calenzana, Elba Island: Acid-Catalyzed stereospecific conversion of calenzanol into indene- and guaiazulene-type sesquiterpenes. *Chemistry* **2003**, *9*, 5770–5777.
110. Qiao, Y.-Y.; Ji, N.-Y.; Wen, W.; Yin, X.-L.; Xue, Q.-Z. A new epoxy-cadinane sesquiterpene from the marine brown alga *Dictyopteris divaricata*. *Mar. Drugs* **2009**, *7*, 600–604.
111. Takahashi, Y.; Daitoh, M.; Suzuki, M.; Abe, T.; Masuda, M. Halogenated metabolites from the new Okinawan red alga *Laurencia yonaguniensis*. *J. Nat. Prod.* **2002**, *65*, 395–398.

112. Vairappan, C.S.; Ishii, T.; Lee, T.K.; Suzuki, M.; Zhaoqi, Z. Antibacterial activities of a new brominated diterpene from Borneon *Laurencia* spp. *Mar. Drugs* **2010**, *8*, 1743–1749.
113. Iliopoulou, D.; Mihopoulos, N.; Vagias, C.; Papazafiri, P.; Roussis, V. Novel cytotoxic brominated diterpenes from the red alga *Laurencia obtusa*. *J. Org. Chem.* **2003**, *68*, 7667–7674.
114. Oliveira, E.C.; Paula, E.J.; Plastino, E.M.; Petti, R. Metodologias para cultivo de algas em laboratório. In *Macroalgas de Interes Económico: Cultivo, Manejo y Industrialización*; Ferrario, M.E., Sar, E., Eds.; Universidad Nacional de La Plata: La Plata, Argentina, 1996; pp. 175–198.
115. Schmieder, R.; Edwards, R. Quality control and preprocessing of metagenomic datasets. *Bioinformatics* **2011**, *27*, 863–864.
116. Grabherr, M.G.; Haas, B.J.; Yassour, M.; Levin, J.Z.; Thompson, D.A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q.; *et al*. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **2011**, *29*, 644–652.
117. Finn, R.D.; Clements, J.; Eddy, S.R. HMMER web server: Interactive sequence similarity searching. *Nucleic Acids Res.* **2011**, *39*, W29–W37.
118. Cane, D.E.; Ikeda, H. Exploration and mining of the bacterial terpenome. *Acc. Chem. Res.* **2012**, *45*, 463–472.
119. Von Stosch, H.A. Wirkung von Arsenit auf Meeresalgen in Kultur. In Proceedings of the 4th International Seaweed Symposium, Biarritz, France, September 1963; pp. 142–150.
120. Curry, K. Initiation of terpenoid synthesis in osmophores of *Stanhopea anfracta* (Orchidaceae): A cytochemical study. *Am. J. Bot.* **1987**, *74*, 1332–1338.
121. Croteau, R.; Kutchan, T.M.; Lewis, N.G. Natural Products (Secondary Metabolites). In *Biochemistry & Molecular Biology of Plants*; Buchanan, B.B., Gruissem, W., Jones, R.L., Eds.; American Society of Plant physiologists: Maryland, MD, USA, 2000; pp. 1251–1318.
122. Kanehisa, M. and Goto, S. Kegg Pathway Database: Terpenoid backbone biosynthesis-reference pathway. Available online : http://www.genome.jp/kegg-bin/show_pathway?map00900 (accessed on 15 September 2014).
123. Kanehisa, M. and Goto, S. Kegg Pathway Database: Sesquiterpenoid and triterpenoid biosynthesis—reference pathway. Available online: http://www.genome.jp/kegg-bin/show_pathway?map00909 (accessed on 15 September 2014).

CHAPTER 4 - Transcriptomic reprogramming of the red seaweed *Laurencia dendroidea* in response to the potentially pathogenic bacteria *Vibrio madracius* (In preparation)

Louisi Souza de Oliveira[1], Diogo Antonio Tschoeke[1], Ana Carolina Rubem Magalhães Lopes[2], Daniela Bueno Sudatti[2], Pedro Milet Meirelles[1], Renato Crespo Pereira[2], Fabiano Thompson[1*].

Abstract

The ability to defend against pathogens is essential for seaweeds to survive in the marine environment and requires a multi-level strategy. We report for the first time the large-scale transcriptomic changes involved in seaweed-bacteria interactions, by analyzing the transcriptomic profile of *Laurencia dendroidea* in response to the potentially pathogenic bacteria *Vibrio madracius* during a three days period. Genes related to transcription and translation activators were up-regulated in inoculated samples, reflecting the recruitment of the cell machinery necessary to promote defense–related metabolic shifts in *L. dendroidea*. Reactive oxygen species metabolism, intracellular signaling and vesicle trafficking appear to be relevant for defense in *L. dendroidea*. Besides, genes involved in energy conversion pathways, such as glycolysis, gluconeogenesis, pentose phosphate pathway, tricarboxylic acid cycle and mitochondrial electron transport chain were up-regulated in *L. dendroidea* in response to *V. madracius*, suggesting that seaweed defense strategies implies a metabolic cost. This study contributes significantly to the current knowledge of the molecular mechanisms involved in the highly dynamic defense process in seaweeds.

Keywords: algae, pathogen, defense, differential expression, reactive oxygen species, vesicle traffic, energy metabolism.

Introduction

Seaweeds are extremely susceptible to microbial colonization due to the release of large amounts of carbon compounds acting as chemical attractants and nutrient

sources to bacteria [1]. The microbial community associated to macroalgae tends to be species-specific and different from seawater [2]. In a previous study, based on transcriptomic analysis, we verified the predominance of a beneficial microbiota associated to the red seaweed *Laurencia dendroidea*, which can fix nitrogen and provide relevant amino acids and vitamins to the seaweed [3]. The tight relationship between macroalgae and epiphytic microbes led to the establishment of a holobiont concept, analogous to the relationship well described in corals [4].

However, potential pathogens were previously detected on seaweed thalli and include microorganisms capable of degrading cell wall polysaccharides [5–7]. Diseases can significantly impact host populations by promoting a fitness cost for resistance against pathogens and affecting the seaweeds ability to defend against herbivores [8]. Besides, the occurrence of disease outbreaks in valuable seaweed farmings, such as *Porphyra* (nori) cultures, causes significant economical losses due to a reduction on annual production [9].

Bacteria have been recognized as relevant causative agents of seaweeds diseases [10, 11]. Indeed, *Vibrio* species were associated to ice-ice disease in several red seaweeds such as *Kappaphycus alvarezii* and *Eucheuma denticulatum* [5] and also in hole-rotten disease in the brown seaweed *Laminaria japonica* [12]. *V. madracius* is phylogenetically close to *V. mediterranei* species [13], which were previously reported to cause bleaching in corals by inhibiting zooxantellae photosynthesis [14–16].

Laurencia is a widely distributed red seaweed genus, recognized for the synthesis of diverse halogenated secondary metabolites, especially terpenes and acetogenins with relevant ecological [17, 18] and pharmacological activities [19–23]. Some of these halogenated compounds are able to prevent the growth of marine bacteria [24, 25], including potentially pathogenic species [26]. The halogenated metabolites in *L. dendroidea* are stored in vacuolar cell structures named *corps en cerise* (CC) [27] and released to cell surface through a regulated vesicle trafficking [28] that can be induced by microbes [29].

The expression of a large array of genes responsible for the biosynthesis of terpenes in *L. dendroidea* was recently reported [30]. However, the molecular mechanisms involved in the defense of *Laurencia* species in response to pathogens are still largely unknown.

Seaweeds defense against pathogens involves a multi-level mechanism that starts with the recognition of microbe-associated molecular patterns (MAMPs) or pathogen-induced molecular patterns (PIMPs). Overall, MAMPs include conserved molecules that are characteristic of microbes but are absent in hosts, e.g. bacterial cell wall components (peptidoglycans, lipoteichoic acid and lipopolysaccharides) or flagellin [31]. PIMPs are the products of the microbial degradation of seaweeds cell wall matrix, including oligoagars and oligoguluronates [32].

After pathogen perception an oxidative burst is frequently induced [33]. The release of reactive oxygen species (ROS) reduce the microbial growth [34] and act as a signal to activate other levels of response, such as the expression of genes related to the biosynthesis of secondary metabolites [35]. However, the presence of ROS can damage the seaweed cell structures, such as membrane, proteins and nucleic acids, so the oxidative burst needs to be tightly regulated through the activation of antioxidant enzymes [36].

The current knowledge about macroalgal defense strategies is still limited. In fact, most studies regarding seaweed resistance to pathogens are based on indirect stimulus through the application of MAMPs [37], PIMPS [38, 39] and signaling molecules (e.g. arachidonic acid, linolenic acid and methyl jasmonate) in the culture medium [40]. The direct or indirect effect of pathogens on seaweeds gene expression was rarely evaluated and basically relayed on microarray, suppression subtractive hybridization (SSH) and real-time PCR techniques, monitoring a limited number of genes [35, 41–44].

The dynamic nature of seaweed response to pathogens implicates in temporal complexity and major metabolic shifts. In the present study, we explore for the first time the molecular mechanisms involved in the response of a red seaweed to the presence of a potentially pathogenic bacteria. Here, we describe a large-scale trascriptomic reprogramming of *L. dendroidea* during a three days induction by *Vibrio madracius*.

Methods

Laurencia dendroidea was sampled at Castelhanos beach in Anchieta municipality, Espírito Santo State (20°51'40"S, 40°37'00"W) and maintained in laboratory. The unialgal culture of this seaweed was established through successive excision of the apices. In order to reduce the bacteria in the culture, these algal clones were treated with 100 µg/ml ampicillin, 120 µg/ml streptomycin and 60 µg/ml gentamicin. After that, the clones were grown in sterile seawater with germanium dioxide (1 mg/l) and 50% Provasoli solution (ESW) for 2 days before the experiment. The culture and experimental conditions were temperature 22 ± 1°C, salinity 32 ± 1, irradiance 80 ± 5 µmol photons.m^{-2}.s^{-1} and 14 h light/ 10 h dark.

Vibrio madracius was isolated from the coral *Madracis dedactis* sampled in Saint Peter and Saint Paul archipelagae [13]. The bacteria were grown at 30^{o}C in sterile Marine broth to the OD_{600} 0.8, corresponding to 10^{8} CFU.ml^{-1} and precipitated for 5 minutes at 3000 rpm (Centrifuge 5415R, Eppendorf). The supernatant was discarded and the pellet was resuspended in sterile seawater and inoculated in falcon tubes containing 250 mg of *L. dendroidea* and 40 ml of ESW (n=2). The final concentration of *V. madracius* in the treatment was 10^{7} CFU.ml^{-1} (T1 and T2). Besides, the same quantity of bacteria was inoculated in falcon tubes containing 40 ml of ESW (n=2) in the absence of *L. dendroidea* (CV1 and CV2). The culture medium was plated in TCBS media (n=3) right after bacterial inoculation and 24, 48, 72, 96 and 144 hours after bacterial inoculation in the presence and absence of the seaweed. Also, 144 h after bacterial inoculation, the seaweed thalli were homogenized in sterile NaCl 3% solution for 1 h using the vortex and this tissue homogenate was plated in TCBS. The Petri dishes were incubated overnight at 30^{o}C and the colonies were counted when present.

To evaluate the transcriptomic profile of *L. dendroidea* in the presence and absence of *V. madracius*, the control tubes were set with 250 mg of *L. dendroidea* and 40 ml of ESW (n=3) and the inoculated tubes contained 250 mg of *L. dendroidea*, 40 ml of ESW and *V. madracius* at 10^{7} CFU.ml^{-1} (n=3). After 24, 48 and 72 h, the seaweeds in the control and in the inoculated tubes were frozen and separately ground in liquid nitrogen using a mortar and pestle. The total RNA was extracted using the TRIzol (Life Technologies) protocol. The ds cDNA libraries were prepared using the TruSeq stranded mRNA LT Sample Preparation Kit (Illumina). Library size distribution was

accessed using the 2100 Bioanalyzer (Agilent) and the High Sensitivity DNA Kit (Agilent). The accurate quantification of the libraries was accomplished using the 7500 Real Time PCR (Applied Biosystems) and the KAPA Library Quantification Kit (Kapa Biosystems). Paired-end sequencing (2 × 250 bp) was performed on a MiSeq (Illumina).

The sequences were preprocessed to trim poly-A/T tails at least 20 bp long, to remove reads shorter than 35 bp, and to trim sequences with quality score lower than 30, using the software Prinseq [45]. Then the sequences from all the samples were assembled using the software Trinity and both contigs and singlets, larger than 199 bp were used in the downstream analysis. Sequences from each sample were mapped against the assembled reads using Bowtie 2 [46] and clustered into genes using the Corset software (minimum read count = 5) [47]. Differentially expressed genes between the control and the inoculated samples were identified using the edgeR software package associated to Exact Fisher Test and Bonferroni multiple correction (p-value $\leq$ 0.001, logFC $\geq$ 2.0) [48]. The differentially expressed sequences were annotated through BLAST search against the NCBI-nr database (e-value < 10^{-5}) and GO terms were assigned using the Blast2go tool [49].

Results

The concentration of *V. madracius* in the culture medium reduced progressively after 72 hours in the presence of *L. dendroidea* (Figure 1). Plating the seaweed tissue homogenate on TCBS media did not result in bacterial growth, suggesting that this reduction was not due to bacterial attachment to *L. dendroidea* thalli.

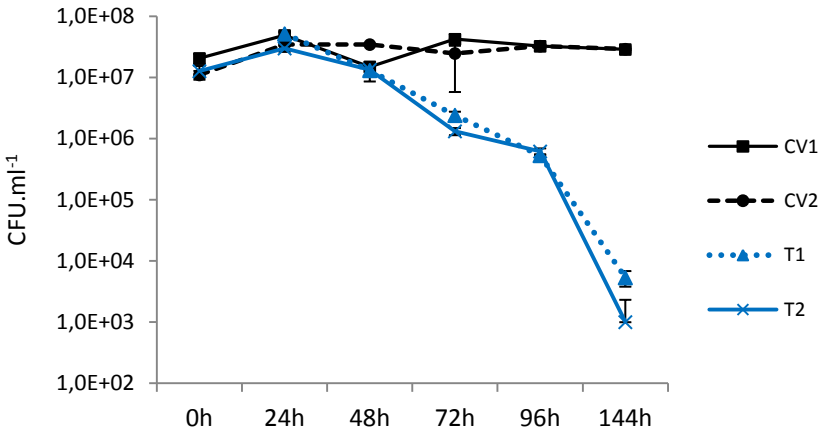


Figure 1. Concentration of *V. madracius* in the culture medium in the presence (T1 and T2) and absence (CV1 and CV2) of *L. dendroidea*. The concentration of *V. madracius* is presented as colony-forming unit per ml of culture medium (CFU.ml^{-1}) and measured for 144 hours after bacterial inoculation (average ± standard error).

The transcriptomic profile of *L. dendroidea* was analyzed 24 h, 48 h and 72 h after *V. madracius* inoculation in the culture medium. A total of 15 cDNA libraries were sequenced, resulting in 12.58 Gbp. After the pre-processing step the sequences were de novo assembled resulting on 151,740 contigs that were compiled in 53,677 clusters, which are referred to hereinafter as genes (Table 1). A total of 19,476 clusters (36.28%) were shared among all the control samples regardless of the time elapsed since the beginning of the experiment, while 2,034 clusters (3.79%) were shared among all the inoculated samples (Supplementary File 1). We detected in both, control and inoculated samples the expression of genes coding for leucine-rich repeat receptor-like serine/threonine-protein kinase (LRR-RLK) (Supplementary file 2).

Table1. Characteristics of the cDNA sequences from *L. dendroidea* after pre-processing and assembly. Uninoculated samples (ctrl.) and inoculated samples (inoc.) 24 h, 48 h and 72 h after inoculation with *V. madracius* (SD = standard deviation).

	Ctrl. 24 h	Inoc.24 h	Ctrl. 48 h	Inoc. 48 h	Ctrl. 72 h	Inoc. 72 h	Assembled sequences
Total nucleotides	1,981	3,856	2,606	2,492	2,177	2,328	91.46
N. sequences	6,016,980	12,040,124	8,266,332	7,941,106	12,588,310	13,655,346	151,740
Average size (bp) ± SD	172.5 ± 63.4	168.8 ± 59.1	164.1 ± 59.5	163.2 ± 58.3	172.6 ± 49.4	170.1 ± 49.9	602 ± 674

The comparative analysis between control and inoculated specimens of *L. dendroidea* revealed the change in the gene expression profile of this seaweed in

response to *V. madracius*. Overall, 24 hours post inoculation (hpi) we observed the up-regulation of 675 genes, of which 75.8% were annotated using the Blast2go tool, and the down-regulation of 6 genes (16.7% annotated) in *L. dendroidea* (Figure 2a). Besides, 48 hours after *V. madracius* inoculation, 299 genes were up-regulated, of which 82.3% were annotated, and 4 genes were down-regulated, all of them annotated only as hypothetical proteins (Figure 2b). Finally, 72 h after the introduction of *V. madracius* in the culture medium, the expression level of 5 genes increased, but none of them were identified through Blast, and 5 genes were repressed, of which 60% were annotated at least in a protein family level (Figure 2c).

Most of the genes differentially expressed were up-regulated in the inoculated samples, especially 24 and 48 hpi. We verified a significant reduction in the number of genes differentially expressed 72 h after *V. madracius* inoculation. Among the genes up-regulated 72 hpi, we were able to identify two bestrophin related genes and one cbbX homolog.

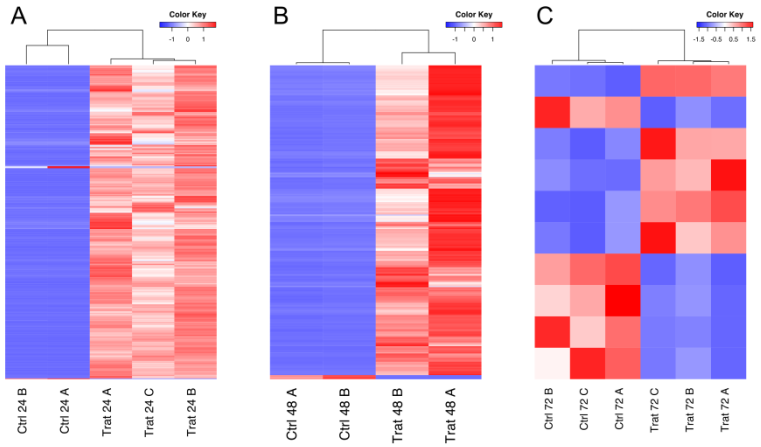


Figure 2. Heatmap of expression values (logFC) for differentially expressed genes in *L. dendroidea* 24 hours (a), 48 hours (b) and 72 hours (c) after *V. madracius* inoculation. Both annotated and not annotated genes are represented.

Among the genes induced 24 h and 48 hpi, stand out the ones associated to "regulation of transcription DNA-templated", "ribosome biogenesis", "translation" and "regulation of translational initiation", suggesting the recruitment of the cell machinery to increase the transcription and translation of genes relevant for seaweeds defense (Figure 3). The over-expression of genes coding for WRKY, MYB, ethylene-responsive

transcription factor (ERF), heat stress transcription factor (HSF) and histone acetytransferase HAC1-like were detected 24 h after infection and represent relevant transcriptional activators. The gene coding for a lysine-specific histone demethylase (LSD) was down-regulated 24 hpi. Besides, we detected the over-expression of paired amphipathic helix protein sin3a, a transcriptional repressor, 24 h after infection. The genes coding for the translation initiation factors ATP-dependent RNA helicase (eIF4a) and eukaryotic initiation factor 3 (eIF3) were also up-regulated 24 and 48 h after *V. madracius* inoculation (Figure 4a).

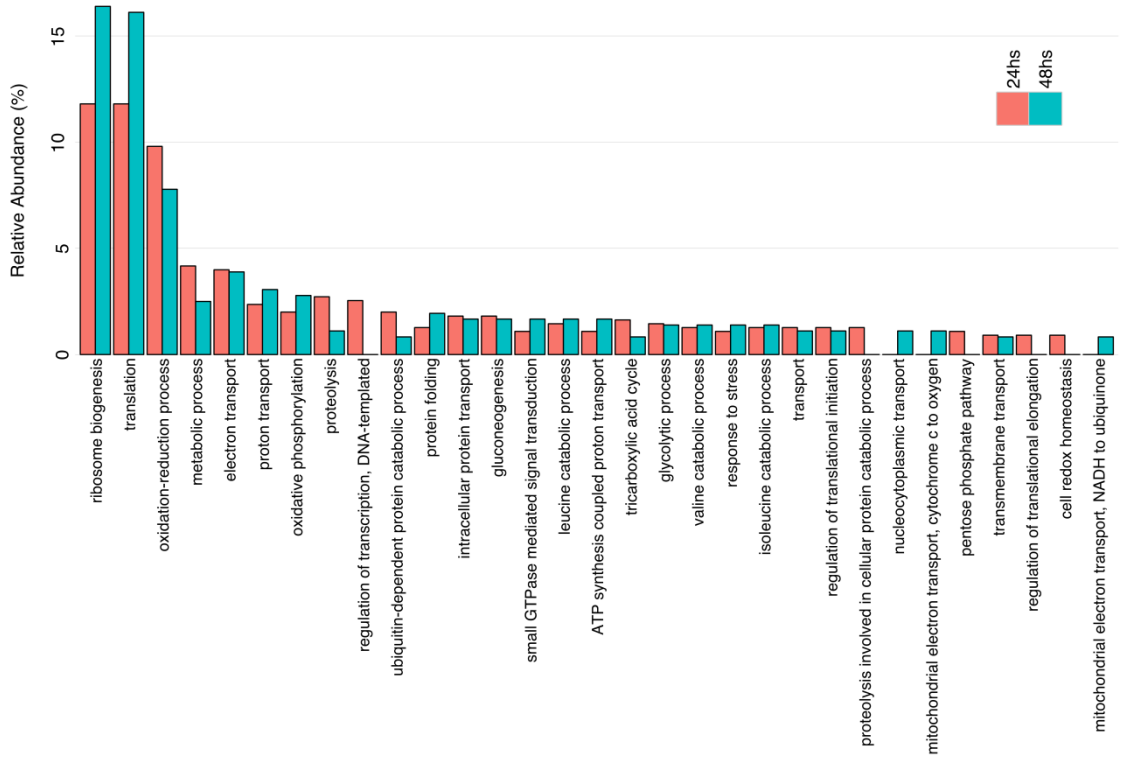


Figure 3. Most representative overrepresented COG biological process categories in *L. dendroidea* 24 and 48 h after *V. madracius* inoculation.

Further, we verified the up-regulation of a gene coding for NADPH oxidase (NADPH ox) in *L. dendroidea* 24 hpi (Figure 4b), which is responsible for a transient production of reactive oxygen species (ROS). The "oxidation-reduction processes" were overrepresented both 24 and 48 h after *V. madracius* infection (Figure 3) and cover diverse cell reactions that involve the transfer of electrons between chemical compounds, especially relevant to cell redox homeostasis, highlighting the up-

regulation of the genes coding for antioxidant enzymes such as thioredoxin (TRX), peroxiredoxin (PRX), glutathione S-transferase (GST) and superoxide dismutase (SOD) (Figure 4b).

During stress, the ability to maintain proteins in their functional conformation and remove potentially harmful polypeptides is especially relevant for cell survival. Indeed, 24 and 48 hpi, we observed the up-regulation of several genes under the category "response to stress" (1.1% and 1.4%, respectively) associated to protein folding (Figure 3), such as 10 kDa chaperonin (Cpn10), 60 kDa chaperonin (Cpn60), heat shock protein 70 (Hsp70), heat shock protein 90 (Hsp90), calreticulin (CALR), peptidyl-prolyl cis-trans isomerase (PPCTI) and protein disulfide-isomerase (PDI) (Figure 4c). Moreover, the genes related to "proteolysis" (2.7% after 24 h and 1.1% after 48 h), "ubiquitin-dependent protein catabolic process" (2.0% after 24 h and 0.8% after 48 h) and "proteolysis involved in cellular protein catabolic process" (1.3% after 24h) were also up-regulated after *V. madracius* inoculation (Figure 3). Particularly, the genes coding for lysosomal proteases (e.g. lysosomal aspartic protease (LAP), cathepsin z (CATZ) and tripeptidyl-peptidase (TPP)), 26s proteasome (26S) and ubiquitin-protein ligase (UPL) (Figure 4d) were up-regulated following *V. madracius* inoculation. Although genes related to proteolysis were verified in the inoculated samples both 24 and 48 hpi, the number of genes activated 48 h after infection was considerably lower.

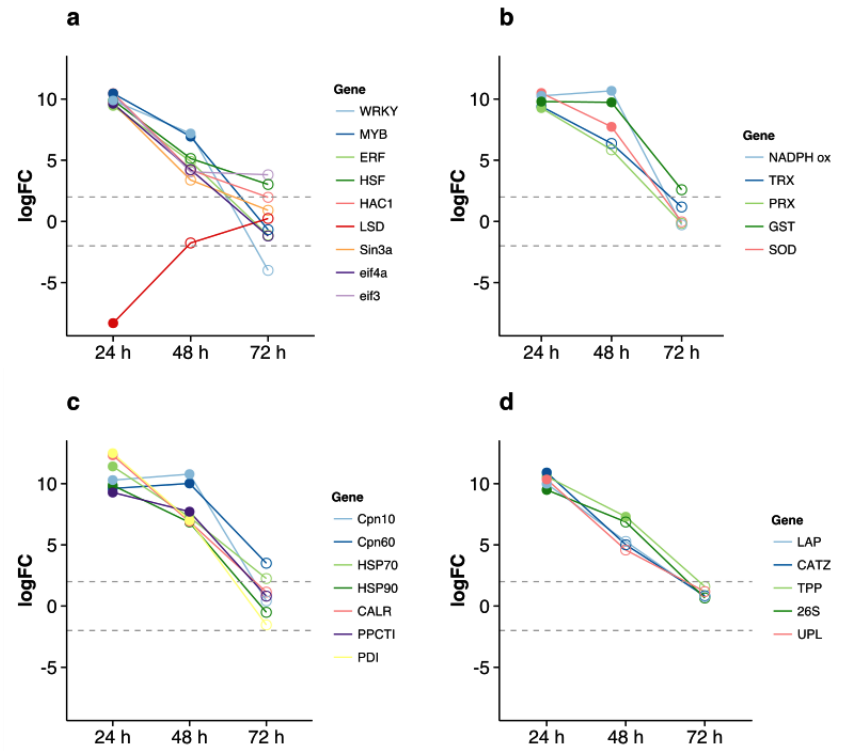


Figure 4. Relevant differentially expressed genes in *L. dendroidea* 24, 48 and 72 h after inoculation with *V. madracius*. A. Regulators of transcription and translation; B. Genes involved in the oxidative burst and antioxidant mechanisms; C. Genes involved in protein folding; D. Genes related to protein degradation. Open circles indicate values of logFC that were not statistically significant (p-value > 0.001, logFC < |2.0|).

Another relevant biological process overrepresented 24 and 48 h after *V. madracius* inoculation was the "small GTPase mediated signal transduction" (1.1% and 1.7%, respectively, Figure 3), encompassing the genes that code for Rho-related protein rac1, Ras-related protein Rab, ADP-ribosylation factor (Arf), and GTP-binding nuclear protein Ran (Figure 5a). These genes, together with coatomer (coat α-2), clathrin (CLT), vacuolar H^+-ATPase (V-ATPase), vacuolar pyrophosphatase (V-PPase), ATP-binding cassette (ABC) and Sec14, are relevant for vesicle trafficking and were distributed in the categories "transport" (1.3% after 24 h and 1.1% after 48 h) and "intracellular protein transport" (1.8% after 24 h and 1.7% after 48 h) (Figure 3 and Figure 5b). Genes coding for actin and tubulin were also up-regulated, mainly 24 hpi and could be involved in vesicle transport (Figure 5b). Additionally, genes coding for phosphatidylinositol 4-phosphate 5-kinase (PIP5K), myo-inositol 1-phosphate synthase (MIPS), type II inositol 1,4,5-trisphosphate 5-phosphatase (5PTase), phosphatidylinositol 4-kinase (P4K), calmodulin (CaM), calcium calmodulin-dependent

protein kinase (CDPK) and Snf1-related protein kinase (SnRK) were up-regulated mainly 24 hpi and are involved in pathogenesis-related cell signaling (Figure 5c).

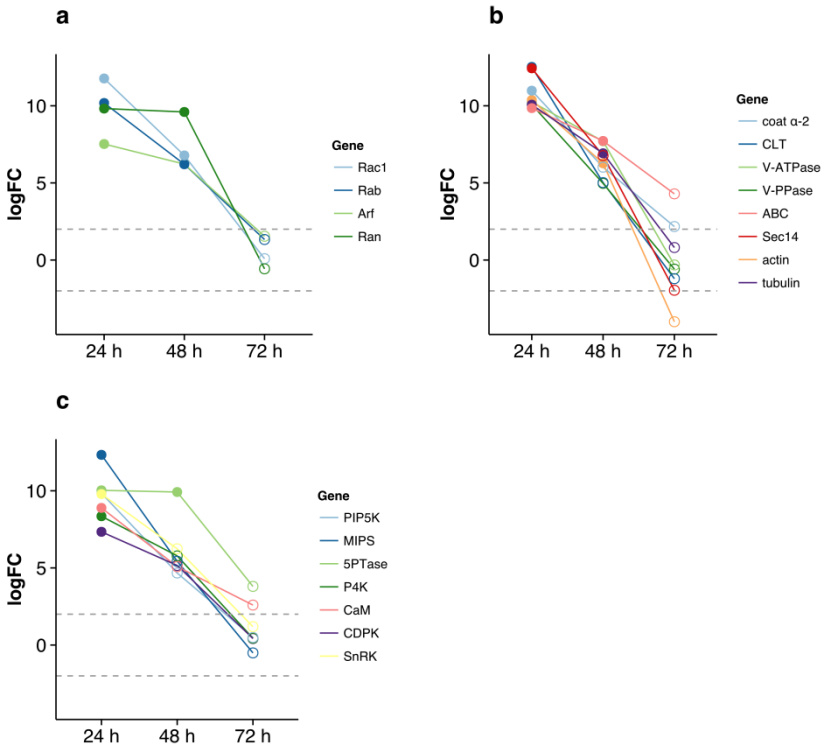


Figure 5. Representative differentially expressed genes of *L. dendroidea* in response to *V. madracius*, 24, 48 and 72 h after bacterial inoculation. A. Genes involved in small GTPase mediated signaling; B. Genes involved in vesicle trafficking; C. Genes related to phosphoinositide and calcium-dependent signaling. Open circles indicate values of logFC that were not statistically significant (p-value > 0.001, logFC < |2.0|).

Moreover, functional categories directly or indirectly associated to energy production were overrepresented in the transcriptome of *L. dendroidea* 24 h and 48 h after the introduction of *V. madracius* in the culture medium, such as "glycolytic process" (1.4% after both 24 and 48 h) and "gluconeogenesis" (1.8% and 1.7%, respectively) (Figure 3). Among the up-regulated transcripts, we detected several involved in glycolysis or gluconeogenesis, such as glucose-6-phosphate isomerase (G6PI), fructose-bisphosphate aldolase (FBA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphopyruvate hydratase (PPH) (Figure 6a). The gene coding for phosphoenolpyruvate carboxykinase (PEPCK), which participates in gluconeogenesis, was also up-regulated 24 and 48 hpi (Figure 6a).

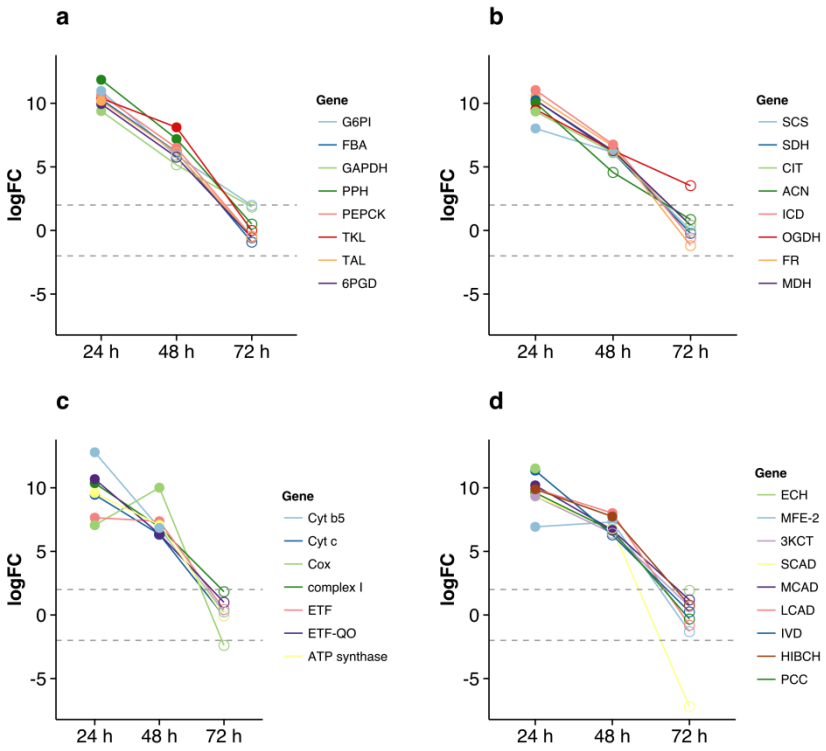


Figure 6. Representative genes related to energy metabolism that were differentially expressed in *L. dendroidea* 24, 48 and 72 h after *V. madracius* inoculation. A. Genes involved in glycolysis and/or gluconeogenesis and genes related to pentose phosphate pathway. B. Genes involved in tricarboxylic acid cycle. C. Genes involved in mitochondrial electron transport chain and ATP synthesis. D. Genes related to fatty acid oxidation and branched-chain amino acids catabolism. Open circles indicate values of logFC that were not statistically significant (p-value > 0.001, logFC < |2.0|).

Further, genes related to "pentose phosphate pathway" were up-regulated 24 h after *V. madracius* inoculation (1.1%, Figure 3), such as transketolase (TKL), transaldolase (TAL) and 6-phosphogluconate dehydrogenase (decarboxylating) (6PGD) (Figure 6a). We also observed the up-regulation of genes related to "tricarboxylic acid cycle" in *L. dendroidea* 24 and 48 hpi (1.6% and 0.83%, respectively), such as succinyl-CoA ligase (SCS), succinate dehydrogenase (SDH), citrate synthase (CIT), aconitate hydratase (ACN), isocitrate dehydrogenase (ICD), 2-oxoglutarate dehydrogenase (OGDH), fumarate reductase (FR) and malate dehydrogenase (MDH) (Figure 6b).

Besides, 24 and 48 hpi, we verified the up-regulation of genes involved in "oxidative phosphorylation" (2.0% and 2.8%, respectively), "ATP synthesis coupled proton transport" (1.1% and 1.7%, respectively) and mitochondrial electron transport chain, which were distributed in the categories "electron transport" (4.0% and 3.9%, respectively) and "proton transport"(2.4% and 3.1%, respectively) (Figure 3), and included cytochrome b5 (Cyt b5), cytochrome c (Cyt c), cytochrome c oxidase (Cox),

NADH-ubiquinone oxidoreductase (complex I), electron transfer flavoprotein (ETF), electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) and ATP synthase (Figure 6c)

Finally, we detected 24 and 48 hpi the up-regulation of genes related to fatty acid oxidation, such as enoyl-CoA hydratase (ECH), peroxisomal multifunctional enzyme 2 (MFE-2), 3-ketoacyl-CoA thiolase (3KCT), short/branched chain specific acyl-CoA dehydrogenase (SCAD), medium-chain specific acyl-CoA dehydrogenase (MCAD) and long-chain specific acyl-CoA dehydrogenase (LCAD), which were grouped in the category "metabolic process" (4.2% and 2.5%, respectively) (Figure 3 and Figure 6d). Also, 24 and 48 hpi we observed the up-regulation of genes involved in the catabolism of leucine (1.4% and 1.7%, respectively), isoleucine (1.3% and 1.4%, respectively) and valine (1.3% and 1.4%, respectively), such as isovaleryl-CoA dehydrogenase (IVD), 3-hydroxyisobutyryl-CoA hydrolase (HIBCH) and propionyl-CoA carboxylase (PCC) (Figure 3 and Figure 6d).

Discussion

The large-scale transcriptional reprogramming is an important part of the response to a pathogen infection. In this study, the concentration of *V. madracius* reduced progressively after 72 hours in the presence of *L. dendroidea*. Since this reduction could not be attributed to a biofilm formation, we hypothesized that it should be due to defense strategies activated in *L. dendroidea* in the first 72 hours after *V. madracius* inoculation.

The recognition of potentially pathogenic bacteria requires the activity of specific membrane receptors. Here we detected the expression of genes coding for leucine-rich repeat receptor-like serine/threonine-protein kinases (LRR-RLKs) both in control and inoculated *L. dendroidea* samples, suggesting that these genes are constitutively expressed. In plants, LRR-RLK is the major class of receptors involved in microbe detection, especially through the recognition of MAMPs, such as bacterial flagellin and elongation factor (EF-Tu) [50]. Further studies are necessary to prove the involvement of LRR-RLKs in pathogen recognition by *L. dendroidea*.

The number of differentially expressed genes in *L. dendroidea* was maximal 24 h after *V. madracius* inoculation (Figure 2a) and the seaweed transcriptomic profile tends to be similar to the control condition 72 hpi (Figure 2c). Clearly most of the transcriptomic reprogramming of *L. dendroidea* in response to *V. madracius* involved the up-regulation of genes, mainly 24 and 48 h after bacterial inoculation.

Transcription and translation activation is essential in *L. dendroidea* defense response to *V. madracius* inoculation

In order to allow the induction of relevant genes and the metabolic modifications necessary to defense, the cell components involved in transcription and translation, need to be recruited (Figure 7). Some transcriptional activators up-regulated in *L. dendroidea* 24 h after *V. madracius* inoculation, such as WRKY and MYB (Figure 4a), are known to be relevant for plant defense against pathogenic microorganisms by positively regulating genes related to plant immunity [51, 52]. Accordingly, both transcription factors were up-regulated 12 h after peach leaves were inoculated with a bacterial pathogen [53] and MYB expression was induced 24 hpi of *Arabidopsis* with a pathogenic fungus [54].

Moreover the up-regulation of an ethylene-responsive transcription factor (ERF) was detected 24 hpi. The ERFs are a large family of transcription factors that respond to both jasmonic acid (JA) and ethylene (ET) in plants and have diverse functions in plant defense [55, 56]. Although the role of ethylene inducing defense mechanisms in seaweeds was not demonstrated yet, the ability to synthesize and respond to this plant hormone was previously detected in *Enteromorpha intestinalis* [57] and *Pterocladiella capillacea* [58]. Further, there are evidences that jasmonic acid, or structurally similar compounds, are also involved in defense signals in macroalgae, since this substance induced the expression of stress-related genes in *Chondrus crispus* [59], induced an increase in the biosynthesis of phlorotannins in *Fucus vesiculosus* [60] and activated oxidative cascades, potentially related to defense against pathogens in *Laminaria digitata* [40] and *C. crispus* [61].

Another important transcriptional regulatory element up-regulated in *L. dendroidea* 24 hpi was the heat stress transcription factor (Hsf), that have been associated mainly with responses to abiotic stress [62], defense gene activation and

pathogen-induced systemic acquired resistance in plants [63]. The Hsf is also important for the transcriptional reprogramming in plants redirecting energy resources from growth to defense mechanisms [64].

The epigenetic modifications of chromatin structure also affect gene expression. In this study we observed the up-regulation of the histone acetytransferase HAC1-like gene 24 h after bacterial inoculation (Figure 4a). This enzyme is responsible for histone acetylation that weakens the interaction between histones and DNA, turning the condensed chromatin into a more relaxed structure that is often associated with an increased level of gene transcription [65]. Similarly, HAC1 proved to be necessary for *Arabidopsis* resistance against bacteria, especially after repetitive exposure to stress [66]. Further, we verified the down-regulation of the gene coding for a lysine-specific histone demethylase (LSD) 24 hpi (Figure 4a). LSD removes methyl groups from methylated histone lysine residues and usually act as a transcription co-repressor [67]. The down-regulation of LSD in *L. dendroidea* could be a relevant mechanism to allow the transcription of defense-related genes.

In contrast, we verified the up-regulation of the paired amphipathic helix protein Sin3a 24 h after *V. madracius* inoculation (Figure 4a). This protein form a complex that recruits histone deacetylases and in many cases represses transcription [68]. The up-regulation of Sin3a 24 hpi possibly reflects the balance of induction and repression of specific genes in *L. dendroidea* defense process. The specific targets and the expression patterns for Sin3a in plants and seaweeds are largely unknown, except for its role regulating seed dormancy in *Arabidopsis* [69].

Apart from the expected up-regulation of genes related to ribosome biogenesis, the genes coding for ATP-dependent RNA helicase (eIF4a) and eukaryotic initiation factor 3 (eIF3) were also overrepresented in the transcriptome of *L. dendroidea* 24 and 48 hpi and are important to promote translation initiation (Figure 4a). The eIF4a is a DEAD box protein that is responsible for the melting of secondary structure in the 5' untranslated region of eukaryotic mRNAs, facilitating the attachment of the 40S ribosomal subunit [70]. The eIF3 is a complex that promotes the binding of mRNA to 40S ribosomal subunit and the dissociation of 40S and 60S subunits [71]. These findings indicate that translation activation is a relevant part of the metabolic shift induced in *L. dendroidea* in response to *V. madracius*.

Reactive oxygen species release is an important mechanism in *L. dendroidea* innate immune response

Most of the researches regarding seaweed defense strategies have focused on the constitutive biosynthesis of secondary metabolites. However, evidence has emerged for the occurrence and significant role of innate immunity processes as the first line of defense in seaweeds, similar to the observed in vascular plants and metazoans [31–33, 61]. After pathogen recognition, one of the first responses of the seaweeds involves an oxidative burst, based on a transient production of reactive oxygen species (ROS) [39, 72, 73]. Besides being directly toxic to microbes [74], ROS participate in intracellular signaling mechanisms leading to the activation of other defense responses [75] (Figure 7). The major site of ROS production in seaweeds in response to microbes are membrane-located NADPH oxidases [40, 72, 76]. Here we verified the up-regulation of the gene coding for NADPH oxidase in *L. dendroidea* 24 hpi, reinforcing the occurrence of a transient oxidative burst in response to *V. madracius* (Figure 4b).

Nevertheless, ROS can react with essential host molecules, destroying or altering the function of proteins, lipids and nucleic acids. Hence, the activity of antioxidant enzymes is important to limit the oxidative burst and prevent cellular damage. In this work, we report the up-regulation of several antioxidant enzymes, especially thioredoxin (TRX), peroxiredoxin (PRX), glutathione s-transferase (GST) and superoxide dismutase (SOD) 24 and 48 hpi (Figure 4b). Accordingly the expression of TRX, PRX and GST increased in *Laminaria digitata* in response to oligoguluronates [35, 77]. Besides, the activity of SOD increased in *Saccharina japonica* elicited with flg22, a microbe-associated molecular pattern (MAMP) [78].

Despite the activation of antioxidant mechanisms, the enhanced production of ROS during oxidative burst can cause cellular damage, for example by altering protein structure. In this sense, we verified the up-regulation of genes coding for chaperonin (Cpn) and heat shock proteins (Hsp) in *L. dendroidea* 24 and 48 h after bacterial inoculation. Hsp and Cpn are commonly induced during plant biotic [79] or abiotic stress [80] and act promoting the correct folding of damaged proteins (Figure 4c).

The up-regulation of Hsp was verified in *C. crispus* in response to methyl jasmonate exposure [41] and in *Laminaria digitata* in response to oligoguluronates [35], both related to pathogenesis in seaweed. The up-regulation of Hsp70 was also detected during the resistance of *Ectocarpus siliculosus* against an oomycete pathogen

and after treatment with H_2O_2 [43]. Other genes induced in *L. dendroidea* 24 and 48 hpi code for calreticulin (CALR), peptidyl-prolyl cis-trans isomerase (PPCTI) and protein disulfide-isomerase (PDI) (Figure 4c), which are located in the endoplasmic reticulum (ER) and are especially relevant to promote the folding and trafficking of proteins during plant stress conditions [81]. Similarly, the genes coding for Hsp, PDI and PPCTI were up-regulated in *C. crispus* protoplasts, reinforcing their involvement in algae stress responses [82].

Hsp and molecular chaperones also play an important role targeting damaged proteins for degradation in lysosomes or proteasomes [81, 83]. We detected the up-regulation of *L. dendroidea* genes related to proteolysis, including lysosomal and ubiquitin-26S proteasome system, mainly 24 hpi (Figure 4d). Accordingly an indirect evidence for the activation of the ubiquitin-26S proteasome system was detected during *Ectocarpus siliculosus* infection by an oomycete [42]. Beyond acting in protein degradation, ubiquitin-26S proteasome system appears to be involved in plant immune signaling [84].

Intracellular signaling and vesicle trafficking are involved in the defense of *L. dendroidea* against *V. madracius*

After pathogen recognition, several intracellular signaling cascades are activated to modulate defense responses. Here we detected the up-regulation of genes in *L. dendroidea* coding for small GTPase proteins from the families Rho, Ran, Rab and Arf, 24 and 48 hpi (Figure 5a). Small GTPase proteins are important molecular switches activated by GTP and inactivated by the hydrolysis of GTP to GDP. We also detected 24 h after *V. madracius* inoculation the induction of genes in *L. dendroidea* coding for guanine nucleotide exchange factor and Rho GTPase-activating protein that work, respectively, as a positive and a negative regulator for Rho GTPases [85].

Rac is a member of Rho family in plants and is considered a key regulator of immunity, controlling the transmission of extracellular signals to intracellular signaling pathways [85]. Rac1 homolog of rice is a regulator of ROS production and alters the expression of defense-related genes promoting resistance against pathogenic bacteria [86] (Figure 7). Ran regulates nucleocytoplasmic transport of RNA and proteins across

the nuclear membrane [87] (Figure 7). The Ran promoter activity is induced by MeJA, what could indicate the participation of this GTPase in plant defense mechanisms [88].

Arf and Rab function in distinct steps of membrane trafficking (Figure 7). Rab is the largest family of small GTPases and participates in intracellular membrane trafficking providing specificity for membrane fusion events by regulating the movement of vesicles along cytoskeletal filaments [89]. The Arf subfamily is necessary for vesicle budding in the secretory system and is required for activating the assembly of coatomers and clathrins in the donor membranes [90]. Besides, Arf is involved in the resistance against fungi penetration in barley plants [91].

We detected the up-regulation of genes coding for coatomer and clathrin in *L. dendroidea* 24 and 48 hpi (Figure 5b). Clathrin-coated vesicles are mainly involved in endocytosis and in the transport from trans-Golgi network to endosomes [92]. The ligand-induced receptor endocytosis is a essential component of plant signaling in defense reactions [93] and the clathrin endocytic pathway is activated by PIMPs in plant species [94, 95]. Coatomer complex (COPI) is required for vesicle traffic between the endoplasmic reticulum (ER) and the Golgi complex and between Golgi cisternae [92].

Vesicle trafficking is required for defense in *L. dedroidea*, since the halogenated secondary metabolites are stored into vacuole-like specialized structures, the *corps en cerise* (CC) [27]. These compounds are released to the cell surface in a regulated manner through vesicle transport induced by abiotic stress and bacterial fouling [29]. Moreover, we observed in *L. dendroidea* the up-regulation of genes coding for actin, which compose the structure of the connections linking the CC to the cell periphery and genes coding for tubulin which are responsible for the positioning of the vesicles along the cell periphery towards exocytosis sites [28]. Thus, the up-regulation of genes related to vesicle trafficking in *L. dendroidea* after *V. madracius* inoculation might indicate an increase in the transport and release of secondary metabolites to fight bacterial infection (Figure 7).

The compartmentation of secondary metabolites in vacuoles was previously observed in plants and other seaweeds, possibly to avoid autotoxicity [96, 97]. In the plant species *Catharanthus roseus*, the uptake of these metabolites to the vacuoles can occur through mechanisms that depend on a H^+-electrochemical potential difference across the vacuolar membrane, which is created by vacuolar H^+-ATPase (V-ATPase) and vacuolar pyrophosphatase (V-PPase) [98]. Instead, this transport can occur through

an ATP-binding cassette (ABC) transporter [98]. In *L. dendroidea*, we observed the up-regulation of V-ATPase, V-PPase and ABC transporters 24 and 48 hpi (Figure 5b and Figure 7). Further studies are necessary to unravel the mechanisms used by this seaweed to accumulate secondary metabolites in the CC.

Besides, we detected the up-regulation of genes coding for Sec14 proteins in *L. dendroidea* 24 and 48 hpi. This protein regulates both the transport of vesicles from the trans-Golgi network to the plasma membrane and vacuole, and also the phosphatidylinositol signaling pathway [99].

In fact, several genes involved in phosphatidylinositol signaling were up-regulated in *L. dendroidea* 24 hpi (Figure 5c). Phosphoinositide-mediated signaling affects Ca^{2+} release and the expression of defense related genes in plants [100] (Figure 7). Cellular calcium signaling is another important mechanism involved in plant immunity since elicitor-induced Ca^{2+} release act as a master messenger for downstream defense reactions [101]. Indeed, we detected the up-regulation of genes coding for calmodulin (CAM) and calcium calmodulin-dependent protein kinase (CDPK) 24 and 48 hpi, which are required for sensing and decoding Ca^{2+} signals (Figure 5c and Figure 7). Accordingly, a pathogenesis related activation of CDPK was detected [102] and this protein kinase regulates the production of ROS by NADPH oxidase in plants [103].

Therewithal, we detected the up-regulation of genes conding for bestrophin in *L. dendroidea* 72 hpi. Although absent in plants, bestrophin genes were detected and differentially expressed in the microalgae *Chlamydomonas reinhardtii* and *Thalassiosira pseudonana* in response to variable CO_2 availability [104, 105]. Bestrophin function as transmembrane chloride channels in humans and may also serve as regulators of intracellular calcium signaling [106]. Further studies are necessary to clarify the function of bestrophins in seaweeds and their possible involvement in the resistance to pathogens.

Another gene coding for a protein kinase up-regulated in *L. dendroidea* 24 hpi was the Snf1-related protein kinase. The expression of this gene in plants is induced by *Pseudomonas syringae [107]*. Besides, a Snf1-related protein kinase regulates NPR1 (Nonexpressor of Pathogenesis-Related gene 1), a major co-activator of plant defense, and mediate expression of the pathogenesis-related transcription factor WRKY [107]. Further, a relevant role was attributed to Snf1-related protein kinases as global regulators of gene expression involved in primary and secondary metabolism during

plant stress, inducing catabolic pathways that provide alternative sources of energy and controlling genes that encode transcription regulators and signal transduction components [108].

Interestingly, an up-regulation of genes related to the biosynthesis of secondary metabolites was not verified in *L. dendroidea* in response to *V. madracius* in the time course of this experiment. We hypothesize that due to the storage of secondary metabolites in the CC and the regulated release of these compounds to cell surface, the activation of biosynthetic pathways would only be necessary after the storage was depleted. Long-term experiments are necessary to validate this hypothesis.

Energy production is necessary to allow the defense of *L. dendroidea* against *V. madracius*

Respiration is the most important process that provides energy in the form of ATP to eukaryotic cells, and can be divided in three main pathways: glycolysis, TCA cycle, and mitochondrial electron transport. In this work we verified the transient up-regulation of *L. dendroidea* genes involved in energy conversion 24 and 48 hpi, suggesting the need for cellular energy to resist to a potential infection of *V. madracius* (Figure 6). Among the up-regulated transcripts, we detected several genes involved in glycolysis or gluconeogenesis. Glycolysis is a metabolic process essential to convert glucose into pyruvate, generating chemical energy in the form of ATP and NADH [109] (Figure 7).

Moreover, the gene coding for phosphoenolpyruvate carboxykinase was up-regulated 24 and 48 hpi and is considered as the rate-controlling step of gluconeogenesis, being responsible for the conversion of oxaloacetate into phosphoenolpyruvate (PEP) [109]. Alternatively, PEP can be converted to pyruvate. In this study, we observed the up-regulation 24 hpi of the gene coding for pyruvate carboxylase, which catalyzes the conversion of pyruvate in oxaloacetate, and also of the gene coding for pyruvate dehydrogenase that is important to convert pyruvate in acetyl-CoA, both necessary to the TCA cycle.

Therewithal, we detected the up-regulation of genes involved in the pentose phosphate pathway in *L. dendroidea* 24 hpi. This pathway can be an alternative route to glycolysis for energy production, besides generating reducing power, as NADPH, and

intermediates for biosynthetic processes [109]. NADPH is an important molecule in maintaining the redox balance state of the cell [110]. This is especially relevant during seaweed-pathogen interactions since the production of ROS is one of the first defensive responses after pathogen recognition. The up-regulation of genes involved in the pentose phosphate pathway was also observed in *Laminaria digitata* elicited with oligoguluronates [35].

Furthermore, we detected 24 and 48 h after *V. madracius* inoculation the up-regulation of several genes that are essential to the occurrence of TCA cycle in *L. dendroidea* (Figure 6b and Figure 7). The TCA cycle is responsible for the oxidation of carbohydrates, fatty acids and amino acids, producing GTP, NADH and $FADH_2$ [111]. The molecules NADH and $FADH_2$ are reoxidized through the oxidative phosphorylation involving the mitochondrial electron transport chain to generate ATP. Accordingly, we verified the up-regulation of genes involved in the mitochondrial electron transport chain and of the gene coding for ATP synthase 24 and 48 hpi (Figure 6c and Figure 7).

Beyond that, we observed the up-regulation of genes involved in the catabolism of branched-chain amino acids and in the β-oxidation of fatty acids (Figure 6d and Figure 7). These pathways provide alternative sources of respiratory substrates for the TCA cycle, especially during severe plant stress and in response to infection [112, 113].

Photosynthetic metabolism is usually reduced in plants upon infection, possibly as a mechanism to redirect metabolic resources and energy to defense related pathways [114, 115]. In contrast, we observed the up-regulation of a cbbX homolog in *L. dendroidea* 72 hpi, a red-type Rubisco activase specific to red algae, bacteria and phytoplankton [116]. Nevertheless, together with the decreased number of differentially expressed genes, this could indicate a tendency of *L. dendroidea* to return to a cellular metabolic state similar to the control samples 72 h after *V. madracius* inoculation.

Diverse evidences suggest that fighting against pathogens is energetically demanding in vascular plants [113, 117–121]. Although most previous work suggest the same pattern in seaweeds [121, 122] the results of experiments intending to evaluate the potential costs involved in seaweeds defense had mixed results [123]. Most of these experiments used correlations between the concentration of specific secondary metabolites (or the defense capability) and variations in fitness, measured as growth or fertility levels. The problem with this approach is that these correlations may reflect co-

variance responses to environmental factors instead of indicating causality. Moreover, the response to pathogens involves complex multilayered defense strategies that are not considered during most experiments.

The researches concerning the effect of pathogenesis-related signals on the transcriptomic profile of seaweeds are rare and present contradictory results. Using the suppression subtractive hybridization (SSH) technique, an increase in the expression of genes involved in cellular energy was detected following the exposure of *Laminaria digitata* to oligoguluronates [35]. In contrast, the down-regulation of genes involved in energy conversion was detected, through microarray, after the exposure of *Chondrus crispus* to methyl jasmonate [59]. Since both studies were based in a relatively small number of sequences, the conflicting results could be attributed to intrinsic biological differences between the two seaweed species or to the low sequencing coverage. In this study it was clearly demonstrated the transient requirement of metabolic energy for *L. dendroidea* to fight against *V. madracius*, as evidenced by the massive up-regulation of genes involved in aerobic respiration 24 and 48 h after bacterial inoculation.

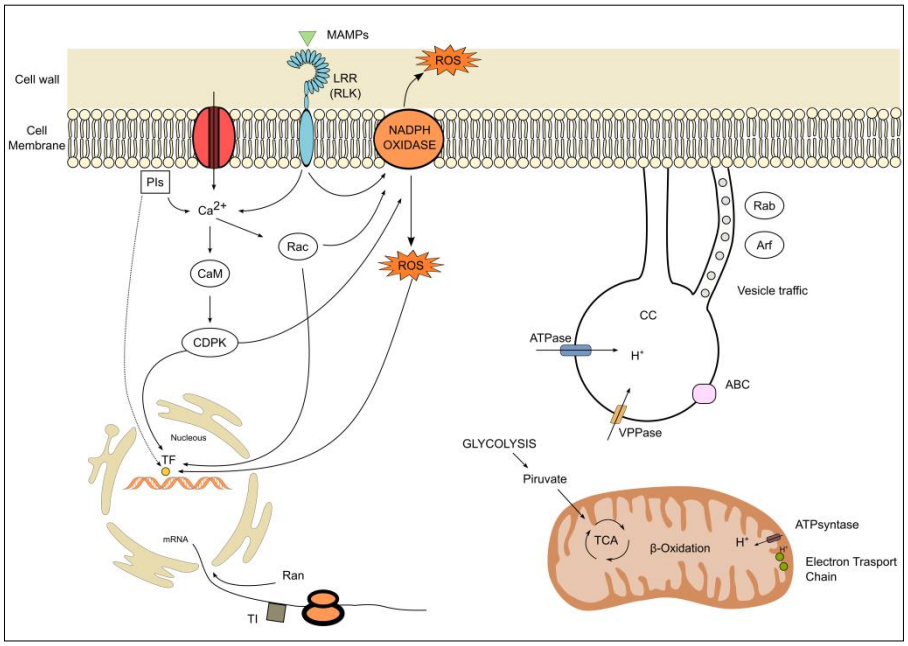


Figure 7. Hypothetical model representing bacteria recognition (through MAMP) and some relevant metabolic process over-represented in the transcriptomic profile of *L. dendroidea* in response to *V. madracius*. Note: this figure is not drawn to scale.

Conclusion

The defense reactions of *L. dendrodea* in response to *V. madracius* involve a large-scale transcriptomic reprogramming, especially 24 and 48 h after bacterial inoculation. The up-regulation of genes coding for NADPH oxidase and antioxidant enzymes suggests the occurrence of an oxidative burst, frequently observed in plant-pathogen interactions. Intracellular signaling mediated by small GTPases, phosphatidylinositol and calcium calmodulin-dependent protein kinase were also observed in the first 48 hours of bacterial inoculation. Besides, we detected the up-regulation of genes involved in the traffic of vesicles, which could be related to the transport of halogenated secondary metabolites to the cell surface, as previously reported in *L. dendroidea*. Finally, we verified the up-regulation of genes associated to energy metabolism, indicating that the defense mechanisms in *L. dendroidea* might implicate an energy cost, as previously noted for vascular plants. The present study provided novel insights into the complexity of seaweed-microbe interactions and the defense strategies of *L. dendroidea*.

Author details

[1] Instituto de Biologia, Universidade Federal do Rio de Janeiro (UFRJ) Av. Carlos Chagas Filho, 373-CCS - IB - BLOCO A (ANEXO) A3- 202, SAGE-COPPE, Rio de Janeiro, 21941-599, Brazil; E-Mails: louisioliveira@gmail.com (LSO); diogoat@gmail.com (DAT); pedrommeirelles@gmail.com (PMM).

[2] Departamento de Biologia Marinha, Universidade Federal Fluminense (UFF). Morro do Valonguinho, s/n. Centro, Niteroi, 24001-970, RJ, Brazil; E-Mail: rcrespo@id.uff.br (RCP); anacarolrubem@gmail.com (ACL); dbsudatti@gmail.com (DBS).

* Fabiano Thompson; E-Mail: fabianothompson1@gmail.com;
Tel.: +55-21-39386567.

References

1. Haas AF, Nelson CE, Wegley Kelly L, Carlson CA, Rohwer F, Leichter JJ, Wyatt A, Smith JE: **Effects of coral reef benthic primary producers on dissolved organic carbon and microbial activity.** *PLoS One* 2011, **6**:e27973.
2. Burke C, Steinberg P, Rusch D, Kjelleberg S, Thomas T: **Bacterial community assembly based on functional genes rather than species.** *Proc Natl Acad Sci U S A* 2011, **108**:14288–93.

3. De Oliveira LS, Gregoracci GB, Silva GGZ, Salgado LT, Filho GA, Alves-Ferreira M, Pereira RC, Thompson FL: **Transcriptomic analysis of the red seaweed *Laurencia dendroidea* (Florideophyceae, Rhodophyta) and its microbiome.** *BMC Genomics* 2012, **13**:487.

4. Egan S, Harder T, Burke C, Steinberg P, Kjelleberg S, Thomas T: **The seaweed holobiont: understanding seaweed-bacteria interactions.** *FEMS Microbiol Rev* 2013, **37**:462–76.

5. Largo DB, Fukami K, Nishijima T: **Occasional pathogenic bacteria promoting ice-ice disease in the carrageenan-producing red algae *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Solieriaceae, Gigartinales, Rhodophyta)**. *J Appl Phycol* 1995, **7**:545–554.

6. Jaffray AE, Anderson RJ, Coyne VE: **Investigation of bacterial epiphytes of the agar-producing red seaweed *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine et Farnham from Saldanha Bay, South Africa and Lüderitz, Namibia**. *Bot Mar* 1997, **40**.

7. Sakai T, Ishizuka K, Kato I: **Isolation and characterization of a fucoidan-degrading marine bacterium**. *Mar Biotechnol* 2003, **5**:409–416.

8. Campbell AH, Vergés A, Steinberg PD: **Demographic consequences of disease in a habitat-forming seaweed and impacts on interactions between natural enemies**. *Ecology* 2014, **95**:142–152.

9. Ding H, Ma J: **Simultaneous infection by red rot and chytrid diseases in *Porphyra yezoensis* Ueda**. *J Appl Phycol* 2005, **17**:51–56.

10. Gachon CMM, Sime-Ngando T, Strittmatter M, Chambouvet A, Kim GH: **Algal diseases: spotlight on a black box.** *Trends Plant Sci* 2010, **15**:633–40.

11. Fernandes N, Case RJ, Longford SR, Seyedsayamdost MR, Steinberg PD, Kjelleberg S, Thomas T: **Genomes and virulence factors of novel bacterial pathogens causing bleaching disease in the marine red alga *Delisea pulchra*.** *PLoS One* 2011, **6**:e27387.

12. Wang G, Shuai L, Li Y, Lin W, Zhao X, Duan D: **Phylogenetic analysis of epiphytic marine bacteria on Hole-Rotten diseased sporophytes of *Laminaria japonica***. *J Appl Phycol* 2008, **20**:403–409.

13. Moreira APB, Duytschaever G, Tonon LAC, Dias GM, Mesquita M, Cnockaert M, Francini-Filho RB, De Vos P, Thompson CC, Thompson FL: ***Vibrio madracius* sp. nov. isolated from *Madracis decactis* (Scleractinia) in St Peter & St Paul Archipelago, Mid-Atlantic Ridge, Brazil.** *Curr Microbiol* 2014, **69**:405–11.

14. Ben-Haim Y, Banim E, Kushmaro A, Loya Y, Rosenberg E: **Inhibition of photosynthesis and bleaching of zooxanthellae by the coral pathogen *Vibrio shiloi***. *Environ Microbiol* 1999, **1**:223–229.

15. Kushmaro A, Banin E, Loya Y, Stackebrandt E, Rosenberg E: ***Vibrio shiloi* sp. nov., the causative agent of bleaching of the coral *Oculina patagonica*.** *Int J Syst Evol Microbiol* 2001, **51**(Pt 4):1383–8.

16. Thompson FL, Hoste B, Thompson CC, Huys G, Swings J: **The coral bleaching *Vibrio shiloi* Kushmaro et al. 2001 is a later synonym of *Vibrio mediterranei* Pujalte and Garay 1986.** *Syst Appl Microbiol* 2001, **24**:516–9.
17. Da Gama BAP, Pereira RC, Carvalho AG V, Coutinho R, Yoneshigue-Valentin Y: **The effects of seaweed secondary metabolites on biofouling**. *Biofouling* 2002, **18**:13–20.
18. Pereira RC, Da Gama B a P, Teixeira VL, Yoneshigue-Valentin Y: **Ecological roles of natural products of the Brazilian red seaweed *Laurencia obtusa* .** *Braz J Biol* 2003, **63**:665–72.
19. Vairappan CS, Kawamoto T, Miwa H, Suzuki M: **Potent antibacterial activity of halogenated compounds against antibiotic-resistant bacteria.** *Planta Med* 2004, **70**:1087–90.
20. Dos Santos AO, Veiga-Santos P, Ueda-Nakamura T, Filho BPD, Sudatti DB, Bianco EM, Pereira RC, Nakamura CV: **Effect of elatol, isolated from red seaweed *Laurencia dendroidea* , on *Leishmania amazonensis* .** *Mar Drugs* 2010, **8**:2733–43.
21. Veiga-Santos P, Pelizzaro-Rocha KJ, Santos a O, Ueda-Nakamura T, Dias Filho BP, Silva SO, Sudatti DB, Bianco EM, Pereira RC, Nakamura C V.: **In vitro anti-trypanosomal activity of elatol isolated from red seaweed *Laurencia dendroidea* .** *Parasitology* 2010, **137**:1661–70.
22. Chatter R, Ben Othman R, Rabhi S, Kladi M, Tarhouni S, Vagias C, Roussis V, Guizani-Tabbane L, Kharrat R: **In vivo and in vitro anti-inflammatory activity of neorogioltriol, a new diterpene extracted from the red algae *Laurencia glandulifera* .** *Mar Drugs* 2011, **9**:1293–306.
23. Lhullier C, Falkenberg M, Ioannou E, Quesada A, Papazafiri P, Horta PA, Schenkel EP, Vagias C, Roussis V: **Cytotoxic halogenated metabolites from the Brazilian red alga *Laurencia catarinensis* .** *J Nat Prod* 2010, **73**:27–32.
24. Vairappan CS, Daitoh M, Suzuki M, Abe T, Masuda M: **Antibacterial halogenated metabolites from the Malaysian *Laurencia* species**. *Phytochemistry* 2001, **58**:291–297.
25. Vairappan CS: **Potent antibacterial activity of halogenated metabolites from Malaysian red algae, *Laurencia majuscula* (Rhodomelaceae, Ceramiales).** *Biomol Eng* 2003, **20**:255–9.
26. Vairappan CS, Anangdan SP, Tan KL, Matsunaga S: **Role of secondary metabolites as defense chemicals against ice-ice disease bacteria in biofouler at carrageenophyte farms**. *J Appl Phycol* 2010, **22**:305–311.
27. Salgado LT, Viana NB, Andrade LR, Leal RN, da Gama BAP, Attias M, Pereira RC, Amado Filho GM: **Intra-cellular storage, transport and exocytosis of halogenated compounds in marine red alga *Laurencia obtusa* .** *J Struct Biol* 2008, **162**:345–55.

28. Reis VM, Oliveira LS, Passos RMF, Viana NB, Mermelstein C, Sant'anna C, Pereira RC, Paradas WC, Thompson FL, Amado-Filho GM, Salgado LT: **Traffic of secondary metabolites to cell surface in the red alga *Laurencia dendroidea* depends on a two-step transport by the cytoskeleton.** *PLoS One* 2013, **8**:e63929.

29. Paradas WC, Salgado LT, Sudatti DB, Crapez M a, Fujii MT, Coutinho R, Pereira RC, Amado Filho GM: **Induction of halogenated vesicle transport in cells of the red seaweed *Laurencia obtusa* .** *Biofouling* 2010, **26**:277–86.

30. De Oliveira LS, Tschoeke DA, de Oliveira AS, Hill LJ, Paradas WC, Salgado LT, Thompson CC, Pereira RC, Thompson FL: **New Insights on the terpenome of the red seaweed *Laurencia dendroidea* (Florideophyceae, Rhodophyta).** *Mar Drugs* 2015, **13**:879–902.

31. Nurnberger T, Brunner F, Kemmerling B, Piater L: **Innate immunity in plants and animals: striking similarities and obvious differences**. *Immunol Rev* 2004, **198**:249–266.

32. Weinberger F: **Pathogen-induced defense and innate immunity in macroalgae.** *Biol Bull* 2007, **213**:290–302.

33. Egan S, Fernandes ND, Kumar V, Gardiner M, Thomas T: **Bacterial pathogens, virulence mechanism and host defence in marine macroalgae.** *Environ Microbiol* 2014, **16**:925–38.

34. Weinberger F, Friedlander M: **Response of *Gracilaria conferta* (Rhodophyta) to oligoagars results in defense against agar-degrading epiphytes**. *J Phycol* 2000, **36**:1079–1086.

35. Cosse A, Potin P, Leblanc C: **Patterns of gene expression induced by oligoguluronates reveal conserved and environment-specific molecular defense responses in the brown alga *Laminaria digitata*.** *New Phytol* 2009, **182**:239–50.

36. Dring MJ: **Stress resistance and disease resistance in seaweeds: the role of reactive oxygen metabolism**. *Adv Bot Res* 2005, **43**:175–207.

37. Küpper FC, Gaquerel E, Boneberg E-M, Morath S, Salaün J-P, Potin P: **Early events in the perception of lipopolysaccharides in the brown alga *Laminaria digitata* include an oxidative burst and activation of fatty acid oxidation cascades.** *J Exp Bot* 2006, **57**:1991–9.

38. Küpper FC: **Oligoguluronates elicit an oxidative burst in the brown algal kelp *Laminaria digitata***. *PLANT Physiol* 2001, **125**:278–291.

39. Küpper FC, Müller DG, Peters AF, Kloareg B, Potin P: **Oligoalginate recognition and oxidative burst play a key role in natural and induced resistance of sporophytes of Laminariales**. *J Chem Ecol* 2002, **28**:2057–2081.

40. Küpper FC, Gaquerel E, Cosse A, Adas F, Peters AF, Müller DG, Kloareg B, Salaün J-P, Potin P: **Free fatty acids and methyl jasmonate trigger defense reactions in *Laminaria digitata*.** *Plant Cell Physiol* 2009, **50**:789–800.

41. Collén J, Hervé C, Guisle-Marsollier I, Léger JJ, Boyen C: **Expression profiling of *Chondrus crispus* (Rhodophyta) after exposure to methyl jasmonate.** *J Exp Bot* 2006, **57**:3869–81.
42. Strittmatter M, Grenville-Briggs LJ, Breithut L, VAN West P, Gachon CMM, Küpper FC: **Infection of the brown alga *Ectocarpus siliculosus* by the oomycete *Eurychasma dicksonii* induces oxidative stress and halogen metabolism.** *Plant Cell Environ* 2015.
43. Zambounis A, Strittmatter M, Gachon CMM: **Chronic stress and disease resistance in the genome model marine seaweed *Ectocarpus siliculosus***. *Aquat Bot* 2013, **104**:147–152.
44. Tonon T, Rousvoal S, Roeder V, Boyen C: **Expression profiling of the mannuronan C5-epimerase multigenic family in the brown alga *Laminaria digitata* (Phaeophyceae) under biotic stress conditions**. *J Phycol* 2008, **44**:1250–1256.
45. Schmieder R, Edwards R: **Quality control and preprocessing of metagenomic datasets.** *Bioinformatics* 2011, **27**:863–4.
46. Langmead B, Salzberg SL: **Fast gapped-read alignment with Bowtie 2.** *Nat Methods* 2012, **9**:357–9.
47. Davidson NM, Oshlack A: **Corset: enabling differential gene expression analysis for de novo assembled transcriptomes**. *Genome Biol* 2014, **15**:410.
48. Robinson MD, McCarthy DJ, Smyth GK: **edgeR: a Bioconductor package for differential expression analysis of digital gene expression data.** *Bioinformatics* 2010, **26**:139–40.
49. Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M: **Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research.** *Bioinformatics* 2005, **21**:3674–6.
50. Nürnberger T, Kemmerling B: **Receptor protein kinases--pattern recognition receptors in plant immunity.** *Trends Plant Sci* 2006, **11**:519–22.
51. Meng Y, Wise RP: **HvWRKY10, HvWRKY19, and HvWRKY28 regulate Mla-triggered immunity and basal defense to barley powdery mildew.** *Mol Plant Microbe Interact* 2012, **25**:1492–505.
52. Raffaele S, Rivas S: **Regulate and be regulated: integration of defense and other signals by the AtMYB30 transcription factor.** *Front Plant Sci* 2013, **4**:98.
53. Socquet-Juglard D, Kamber T, Pothier JF, Christen D, Gessler C, Duffy B, Patocchi A: **Comparative RNA-seq analysis of early-infected peach leaves by the invasive phytopathogen *Xanthomonas arboricola* pv. pruni.** *PLoS One* 2013, **8**:e54196.
54. Mengiste T, Chen X, Salmeron J, Dietrich R: **The BOTRYTIS SUSCEPTIBLE1 gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*.** *Plant Cell* 2003, **15**:2551–65.

55. Oñate-Sánchez L, Singh KB: **Identification of *Arabidopsis* ethylene-responsive element binding factors with distinct induction kinetics after pathogen infection.** *Plant Physiol* 2002, **128**:1313–22.

56. Oñate-Sánchez L, Anderson JP, Young J, Singh KB: **AtERF14, a member of the ERF family of transcription factors, plays a nonredundant role in plant defense.** *Plant Physiol* 2007, **143**:400–9.

57. Plettner I, Steinke M, Malin G: **Ethene (ethylene) production in the marine macroalga *Ulva* (*Enteromorpha*) intestinalis L. (Chlorophyta, Ulvophyceae): effect of light-stress and co-production with dimethyl sulphide**. *Plant, Cell Environ* 2005, **28**:1136–1145.

58. García-Jiménez P, Robaina RR: **Effects of ethylene on tetrasporogenesis in *Pterocladiella capillacea* (Rhodophyta)1**. *J Phycol* 2012, **48**:710–715.

59. Collén J, Hervé C, Guisle-Marsollier I, Léger JJ, Boyen C: **Expression profiling of *Chondrus crispus* (Rhodophyta) after exposure to methyl jasmonate.** *J Exp Bot* 2006, **57**:3869–81.

60. Arnold TM, Targett NM, Tanner CE, Hatch WI, Ferrari KE: **Evidence for methyl jasmonate-induced phlorotannin production in *Fucus vesiculosus* (Phaeophyceae)**. *J Phycol* 2001, **37**:1026–1029.

61. Bouarab K, Adas F, Gaquerel E, Kloareg B, Salaün J-P, Potin P: **The innate immunity of a marine red alga involves oxylipins from both the eicosanoid and octadecanoid pathways.** *Plant Physiol* 2004, **135**:1838–48.

62. Von Koskull-Döring P, Scharf K-D, Nover L: **The diversity of plant heat stress transcription factors.** *Trends Plant Sci* 2007, **12**:452–7.

63. Pick T, Jaskiewicz M, Peterhänsel C, Conrath U: **Heat shock factor HsfB1 primes gene transcription and systemic acquired resistance in *Arabidopsis*.** *Plant Physiol* 2012, **159**:52–5.

64. Pajerowska-Mukhtar KM, Wang W, Tada Y, Oka N, Tucker CL, Fonseca JP, Dong X: **The HSF-like transcription factor TBF1 is a major molecular switch for plant growth-to-defense transition.** *Curr Biol* 2012, **22**:103–12.

65. Struhl K: **Histone acetylation and transcriptional regulatory mechanisms**. *Genes Dev* 1998, **12**:599–606.

66. Singh P, Yekondi S, Chen P-W, Tsai C-H, Yu C-W, Wu K, Zimmerli L: **Environmental history modulates *Arabidopsis* pattern-triggered immunity in a HISTONE ACETYLTRANSFERASE1-dependent manner.** *Plant Cell* 2014, **26**:2676–2688.

67. Liu C, Lu F, Cui X, Cao X: **Histone methylation in higher plants.** *Annu Rev Plant Biol* 2010, **61**:395–420.

68. Viiri KM, Korkeamäki H, Kukkonen MK, Nieminen LK, Lindfors K, Peterson P, Mäki M, Kainulainen H, Lohi O: **SAP30L interacts with members of the Sin3A corepressor complex and targets Sin3A to the nucleolus.** *Nucleic Acids Res* 2006, **34**:3288–98.

69. Wang Z, Cao H, Sun Y, Li X, Chen F, Carles A, Li Y, Ding M, Zhang C, Deng X, Soppe WJJ, Liu Y-X: ***Arabidopsis*** **paired amphipathic helix proteins SNL1 and SNL2 redundantly regulate primary seed dormancy via abscisic acid-ethylene antagonism mediated by histone deacetylation.** *Plant Cell* 2013, **25**:149–66.

70. Aubourg S, Kreis M, Lecharny A: **The DEAD box RNA helicase family in *Arabidopsis thaliana***. *Nucleic Acids Res* 1999, **27**:628–636.

71. Burks EA, Bezerra PP, Le H, Gallie DR, Browning KS: **Plant initiation factor 3 subunit composition resembles mammalian initiation factor 3 and has a novel subunit.** *J Biol Chem* 2001, **276**:2122–31.

72. Weinberger F, Leonardi P, Miravalles A, Correa JA, Lion U, Kloareg B, Potin P: **Dissection of two distinct defense-related responses to agar oligosaccharides in *Gracilaria chilensis* (Rhodophyta) and *Gracilaria conferta* (Rhodophyta)1**. *J Phycol* 2005, **41**:863–873.

73. Küpper FC, Gaquerel E, Boneberg E-M, Morath S, Salaün J-P, Potin P: **Early events in the perception of lipopolysaccharides in the brown alga *Laminaria digitata* include an oxidative burst and activation of fatty acid oxidation cascades.** *J Exp Bot* 2006, **57**:1991–9.

74. Mellersh DG, Foulds I V., Higgins VJ, Heath MC: **H2O2 plays different roles in determining penetration failure in three diverse plant-fungal interactions**. *Plant J* 2002, **29**:257–268.

75. Schmitt F-J, Renger G, Friedrich T, Kreslavski VD, Zharmukhamedov SK, Los DA, Kuznetsov V V, Allakhverdiev SI: **Reactive oxygen species: re-evaluation of generation, monitoring and role in stress-signaling in phototrophic organisms.** *Biochim Biophys Acta* 2014, **1837**:835–48.

76. Luo Q, Zhu Z, Yang R, Qian F, Yan X, Chen H: **Characterization of a respiratory burst oxidase homologue from *Pyropia haitanensis* with unique molecular phylogeny and rapid stress response**. *J Appl Phycol* 2014, **27**:945–955.

77. Thomas F, Cosse A, Le Panse S, Kloareg B, Potin P, Leblanc C: **Kelps feature systemic defense responses: insights into the evolution of innate immunity in multicellular eukaryotes.** *New Phytol* 2014, **204**:567–76.

78. Wang S, Zhao F, Wei X, Lu B, Duan D, Wang G: **Preliminary study on flg22-induced defense responses in female gametophytes of *Saccharina japonica* (Phaeophyta)**. *J Appl Phycol* 2012, **25**:1215–1223.

79. Wang Y, Kim SG, Kim ST, Agrawal GK, Rakwal R, Kang KY: **Biotic stress-responsive rice proteome: an overview**. *J Plant Biol* 2011, **54**:219–226.

80. Kosová K, Vítámvás P, Prášil IT, Renaut J: **Plant proteome changes under abiotic stress--contribution of proteomics studies to understanding plant stress response.** *J Proteomics* 2011, **74**:1301–22.

81. Gupta D, Tuteja N: **Chaperones and foldases in endoplasmic reticulum stress signaling in plants**. *Plant Signal Behav* 2014, **6**:232–236.

82. Collen J, Roeder V, Rousvoal S, Collin O, Kloareg B, Boyen C: **An expressed sequence tag analysis of thallus and regenerating protoplasts of *Chondrus crispus* (Gigartinales, Rhodophyceae)**. *J Phycol* 2006, **42**:104–112.
83. Wang W, Vinocur B, Shoseyov O, Altman A: **Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response.** *Trends Plant Sci* 2004, **9**:244–52.
84. Marino D, Peeters N, Rivas S: **Ubiquitination during plant immune signaling.** *Plant Physiol* 2012, **160**:15–27.
85. Kawano Y, Kaneko-Kawano T, Shimamoto K: **Rho family GTPase-dependent immunity in plants and animals.** *Front Plant Sci* 2014, **5**:522.
86. Ono E, Wong HL, Kawasaki T, Hasegawa M, Kodama O, Shimamoto K: **Essential role of the small GTPase Rac in disease resistance of rice**. *Proc Natl Acad Sci* 2001, **98**:759–764.
87. Ma Q-H: **Small GTP-binding proteins and their functions in plants**. *J Plant Growth Regul* 2007, **26**:369–388.
88. Tian Q, Lin Y, Yang M, Zhang D, Lai R, Lai Z: **DlRan3A is involved in hormone, light, and abiotic stress responses in embryogenic callus of *Dimocarpus longan* Lour.** *Gene* 2015.
89. Stenmark H, Olkkonen V: **The Rab GTPase family**. *Genome Biol* 2001, **2**:reviews3007.1–reviews3007.7.
90. Pandey GK, Sharma M, Pandey A, Shanmugam T: **Overview of Small GTPase Signaling Proteins in Plants**. In *GTPases: Versatile Regulators of Signal Transduction in Plants*. Cham: Springer International Publishing; 2015:9–14. [*SpringerBriefs in Plant Science*]
91. Böhlenius H, Mørch SM, Godfrey D, Nielsen ME, Thordal-Christensen H: **The multivesicular body-localized GTPase ARFA1b/1c is important for callose deposition and ROR2 syntaxin-dependent preinvasive basal defense in barley.** *Plant Cell* 2010, **22**:3831–44.
92. Kirchhausen T: **Three ways to make a vesicle.** *Nat Rev Mol Cell Biol* 2000, **1**:187–98.
93. Sharfman M, Bar M, Ehrlich M, Schuster S, Melech-Bonfil S, Ezer R, Sessa G, Avni A: **Endosomal signaling of the tomato leucine-rich repeat receptor-like protein LeEix2.** *Plant J* 2011, **68**:413–23.
94. Leborgne-Castel N, Lherminier J, Der C, Fromentin J, Houot V, Simon-Plas F: **The plant defense elicitor cryptogein stimulates clathrin-mediated endocytosis correlated with reactive oxygen species production in bright yellow-2 tobacco cells.** *Plant Physiol* 2008, **146**:1255–66.
95. Bar M, Avni A: **EHD2 inhibits signaling of leucine rich repeat receptor-like proteins.** *Plant Signal Behav* 2009, **4**:682–4.

96. Paul NA, Cole L, de Nys R, Steinberg PD: **Ultrastructure of the gland cells of the red alga *Asparagopsis armata* (Bonnemaisoniaceae)**. *Journal of Phycology* 2006.

97. Dworjanyn SA, De Nys R, Steinberg PD: **Localisation and surface quantification of secondary metabolites in the red alga *Delisea pulchra***. *Mar Biol* 1999, **133**:727–736.

98. Roytrakul S, Verpoorte R: **Role of vacuolar transporter proteins in plant secondary metabolism: *Catharanthus roseus* cell culture**. *Phytochem Rev* 2007, **6**:383–396.

99. Curwin AJ, Fairn GD, McMaster CR: **Phospholipid transfer protein Sec14 is required for trafficking from endosomes and regulates distinct trans-Golgi export pathways.** *J Biol Chem* 2009, **284**:7364–75.

100. Hung C-Y, Aspesi P, Hunter MR, Lomax AW, Perera IY: **Phosphoinositide-signaling is one component of a robust plant defense response.** *Front Plant Sci* 2014, **5**:267.

101. Batistič O, Kudla J: **Analysis of calcium signaling pathways in plants.** *Biochim Biophys Acta* 2012, **1820**:1283–93.

102. Romeis T, Ludwig AA, Martin R, Jones JD: **Calcium-dependent protein kinases play an essential role in a plant defence response.** *EMBO J* 2001, **20**:5556–67.

103. Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, Doke N, Yoshioka H: **Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase.** *Plant Cell* 2007, **19**:1065–80.

104. Hennon GMM, Ashworth J, Groussman RD, Berthiaume C, Morales RL, Baliga NS, Orellana M V., Armbrust E V.: **Diatom acclimation to elevated CO2 via cAMP signalling and coordinated gene expression**. *Nat Clim Chang* 2015.

105. Jungnick N, Ma Y, Mukherjee B, Cronan JC, Speed DJ, Laborde SM, Longstreth DJ, Moroney J V: **The carbon concentrating mechanism in *Chlamydomonas reinhardtii*: finding the missing pieces.** *Photosynth Res* 2014, **121**:159–73.

106. Stauber T, Jentsch TJ: **Chloride in vesicular trafficking and function.** *Annu Rev Physiol* 2013, **75**:453–77.

107. Xie C, Zhou X, Deng X, Guo Y: **PKS5, a SNF1-related kinase, interacts with and phosphorylates NPR1, and modulates expression of WRKY38 and WRKY62**. *J Genet Genomics* 2010, **37**:359–369.

108. Baena-González E, Rolland F, Thevelein JM, Sheen J: **A central integrator of transcription networks in plant stress and energy signalling.** *Nature* 2007, **448**:938–42.

109. Nelson DL, Cox M.: **Glycolysis, gluconeogenesis, and the pentos phosphate pathway**. In *Lehninger Principles of Biochemistry*. 5th edition. Edited by Freeman W. New York: Sara Tenney; 2008:527–568.

110. Das P, Nutan KK, Singla-Pareek SL, Pareek A: **Oxidative environment and redox homeostasis in plants: dissecting out significant contribution of major cellular organelles**. *Front Environ Sci* 2015, **2**.
111. Fernie AR, Carrari F, Sweetlove LJ: **Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport.** *Curr Opin Plant Biol* 2004, **7**:254–61.
112. Taylor NL: **Lipoic acid-dependent oxidative catabolism of keto acids in mitochondria provides evidence for branched-chain amino acid catabolism in *Arabidopsis***. *PLANT Physiol* 2004, **134**:838–848.
113. Bolton MD: **Primary metabolism and plant defense--fuel for the fire.** *Mol Plant Microbe Interact* 2009, **22**:487–97.
114. Doehlemann G, Wahl R, Horst RJ, Voll LM, Usadel B, Poree F, Stitt M, Pons-Kühnemann J, Sonnewald U, Kahmann R, Kämper J: **Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph *Ustilago maydis*.** *Plant J* 2008, **56**:181–95.
115. Manter DK, Kelsey RG, Karchesy JJ: **Photosynthetic declines in phytophthora ramorum-infected plants develop prior to water stress and in response to exogenous application of elicitins.** *Phytopathology* 2007, **97**:850–6.
116. Mueller-Cajar O, Stotz M, Wendler P, Hartl FU, Bracher A, Hayer-Hartl M: **Structure and function of the AAA+ protein CbbX, a red-type Rubisco activase.** *Nature* 2011, **479**:194–9.
117. Heil M: **Fitness costs of induced resistance: emerging experimental support for a slippery concept**. *Trends Plant Sci* 2002, **7**:61–67.
118. Denancé N, Sánchez-Vallet A, Goffner D, Molina A: **Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs.** *Front Plant Sci* 2013, **4**:155.
119. Steinberg PD: **Seasonal variation in the relationship between growth rate and phlorotannin production in the kelp *Ecklonia radiata***. *Oecologia* , **102**:169–173.
120. Rojas CM, Senthil-Kumar M, Tzin V, Mysore KS: **Regulation of primary plant metabolism during plant-pathogen interactions and its contribution to plant defense.** *Front Plant Sci* 2014, **5**:17.
121. Nylund GM, Enge S, Pavia H: **Costs and benefits of chemical defence in the Red Alga *Bonnemaisonia hamifera*.** *PLoS One* 2013, **8**:e61291.
122. A. Dworjanyn S, T. Wright J, A. Paul N, de Nys R, D. Steinberg P: **Cost of chemical defence in the red alga *Delisea pulchra***. *Oikos* 2006, **113**:13–22.
123. PFISTER CA: **Costs of reproduction in an intertidal kelp: patterns of allocation and life history consequences**. *Ecology* , **73**:1586–1596.

CHAPTER 5 - Discussão Geral

O gênero *Laurencia* compreende algas vermelhas com ampla distribuição geográfica [1] e reconhecidas por ser uma fonte prolífica de metabólitos secundários, especialmente terpenos halogenados [2]. Esses produtos naturais intermediam importantes interações ecológicas, uma vez que inibem o consumo de *Laurencia* por herbívoros generalistas e impedem a fixação de organismos incrustantes em seu talo [3, 4].

A maior parte dos estudos acerca desse gênero de macroalgas se concentra nos aspectos químicos, estando o conhecimento molecular restrito a marcadores taxonômicos. A presente tese descreve pela primeira vez o transcriptoma de *Laurencia dendroidea* e oferece informações valiosas para a compreensão dos mecanismos essenciais à sobrevivência de algas vermelhas no ambiente marinho, levando em consideração as interações com a microbiota associada (Capítulo 2).

Laurencia dendroidea se destaca como a principal responsável pela produtividade primária do holobionte, expressando genes relacionados à fotossíntese e à síntese de carboidratos de reserva. Os principais grupos taxonômicos ativos na microbiota associada a essa alga foram Cyanobacteria e Proteobacteria. Entre as cianobactérias, encontramos várias ordens caracterizadas pela capacidade de fixar nitrogênio atmosférico, uma atividade especialmente relevante em ambientes costeiros, onde o nitrogênio tende a ser limitante [5]. Já as proteobactérias, em especial as classes Gammaproteobacteria e Alphaproteobacteria são predominantemente heterotróficas e estariam se beneficiando desse ambiente oxigenado e produtivo. Outra possível forma de interação apontada nesse trabalho é a troca de compostos nitrogenados entre *L. dendroidea* e as bactérias associadas ao seu talo, de forma análoga à observada em plantas terrestres, embora estudos mais aprofundados sejam necessários para comprovar tais evidências.

As bactérias associadas ao talo de *L. dendroidea* expressam genes importantes para a quimiotaxia e o estabelecimento de biofilmes microbianos. As algas são especialmente suscetíveis a epibiose por liberarem compostos orgânicos que servem como atrativo químico e fonte de nutrientes para as bactérias [6]. Porém, algumas bactérias marinhas têm o potencial de causar doenças em macroalgas, provocando danos irreversíveis, o que evidencia a necessidade de defesa contra esses possíveis patógenos

[7]. Apesar da forte atividade antibiótica descrita para terpenos produzidos por *Laurencia* [8, 9], os mecanismos moleculares envolvidos na defesa dessa alga, bem como os genes relacionados à síntese desses metabólitos secundários permaneciam desconhecidos.

A biossíntese de terpenos depende da combinação das unidades isoprênicas (C5) isopentenil pirofosfato (IPP) e dimetilalil pirofosfato (DMAPP) pela ação de preniltransferases. Em algas vermelhas, o IPP pode ser sintetizado através das vias do mevalonato (MVA) e do metileritrolfosfato (MEP) [10, 11]. Nesse estudo, foram detectados todos os genes envolvidos na síntese de esqueletos terpênicos pela via MEP em *L. dendroidea* (Capítulos 2 e 3). Além disso, dois dos genes relacionados à via MVA foram identificados (acetil-CoA C-acetiltransferase e mevalonato quinase) e a atividade das enzimas 3-hidroxi-3-metilglutaril-CoA sintase (HMGS, EC 2.3.3.10) e/ou 3-hidroxi-3-metilglutaril-CoA redutase (HMGR, EC 1.1.1.34) foi detectada através de um ensaio citoquímico (Capítulo 3) [12].

A diversidade natural surpreendente de terpenos em *Laurencia* decorre de modificações químicas do esqueleto terpênico que são catalisadas por terpeno sintases, basicamente não caracterizadas em macroalgas. Através do presente estudo, foi possível caracterizar 21 genes que codificam terpeno sintases, bem como o gene da bromoperoxidase dependente de vanádio, uma enzima fundamental para a halogenação e ciclização de terpenos em algas vermelhas [13] (Capítulo 3). Em conjunto, essas enzimas são responsáveis pelas modificações químicas dos precursores terpênicos que resulta na grande diversidade de monoterpenos, diterpenos e sesquiterpenos halogenados, tipicamente encontrada em *L. dendroidea* [12].

A capacidade de *L. dendroidea* para responder à presença de inimigos naturais foi previamente sugerida pelo aumento na concentração de (-)-elatol quando essa alga foi submetida a uma simulação de herbivoria [14]. Além disso, foi verificado um aumento no tráfego de vesículas contendo metabólitos halogenados a partir dos *corps en cerise* (CC) em direção à periferia celular, em resposta à colonização bacteriana do talo dessas algas [15]. Entretanto, os mecanismos moleculares envolvidos na percepção e no reconhecimento de patógenos em potencial por *L. dendroidea* não haviam até então sido investigados.

A resposta de *L. dendroidea* à presença da bactéria potencialmente patogênica *Vibrio madracius* foi avaliada ao longo de três dias através do sequenciamento de RNA

e da análise de expressão diferencial dessa alga (Capítulo 4). Nesse estudo, foram identificados receptores de membrana do tipo “proteínas receptoras quinases com repetições ricas em leucina” (LRR-RLK), possivelmente associadas à percepção de patógenos. Além disso, observou-se uma mudança geral no perfil do transcriptoma de *L. dendroidea*, envolvendo principalmente o aumento na expressão de genes específicos durante as primeiras 48 horas em contato com *V. madracius*.

Os principais genes de *L. dendroidea* induzidos na presença de *V. madracius* estão relacionados à produção de espécies reativas de oxigênio (ROS), à sinalização intracelular mediada por GTPases, fosfatidilinositol e calmodulina e ao tráfego de vesículas, possivelmente associado ao transporte de metabólitos halogenados dos CC para a periferia celular. As vias metabólicas envolvidas na obtenção de energia, como glicólise, ciclo tricarboxílico e a oxidação de lipídeos e aminoácidos de cadeia ramificada também foram induzidas na presença de *V. madracius*, sugerindo que os mecanismos de defesa em *L. dendroidea* impõe um custo metabólico. Esse estudo foi o primeiro a investigar o efeito direto de bactérias sobre o perfil do transcriptoma de macroalgas, contribuindo significativamente para a compreensão dos mecanismos moleculares envolvidos na resistência contra patógenos no ambiente marinho.

Além da importância ecológica dos metabólitos secundários sintetizados por *Laurencia*, o potencial biotecnológico dessas substâncias vem atraindo a atenção de vários grupos de pesquisa. Os terpenos isolados de *Laurencia* apresentam forte ação antibiótica, antitumoral e antiparasitária [16–18]. Além disso, a forte ação anti-incrustante de compostos como o (-)-elatol, um dos metabólitos majoritários em *Laurencia dendroidea*, levou ao estabelecimento de uma patente nacional para o uso dessa substância em tintas navais antiincrustantes. Nesse contexto, o presente estudo fornece informações essenciais para uma possível síntese heteróloga de terpenos de *Laurencia* com o uso de organismos geneticamente modificados. Este avanço tecnológico permitirá superar o obstáculo para obtenção em grande quantidade desses compostos de interesse biotecnológico, produzidos naturalmente em concentrações tão baixas quanto 0.001% a 2.2% do peso seco da alga.

Conclusão

O estudo do transcriptoma de *L. dendroidea* permitiu ampliar significativamente o conhecimento molecular da classe Florideophyceae e conhecer os genes envolvidos nas principais vias metabólicas essenciais à sobrevivência das macroalgas vermelhas no ambiente marinho. Além disso, o conhecimento de importantes interações a nível molecular entre *L. dendroidea* e as bactérias associados ao seu talo reforça a necessidade de estudar as macroalgas como holobiontes. Através de análises de expressão gênica foi possível detectar os mecanismos moleculares envolvidos na resposta de *L. dendroidea* à bactéria potencialmente patogênica *V. madracius*, contribuindo para a compreensão das estratégias de defesa em macroalgas. Por fim, através dessa tese foi possível identificar grande parte dos genes envolvidos na síntese de terpenos em *L. dendroidea*. As perspectivas futuras incluem aprofundar a caracterização de receptores de membrana relacionados à percepção de patógenos, bem como clonar e expressar genes de *L. dendroidea* em leveduras para viabilizar a síntese heteróloga de terpenos com interesse biotecnológico.

Referencias

1. **AlgaeBase. World-wide electronic publication, National University of Ireland, Galway.** [http://www.algaebase.org]
2. Irie T, Suzuki M, Kurosawa E, Masamune T: **Laurinterol, debromolaurinterol and isolaurinterol, constituents of *Laurencia* intermedia Yamada**. *Tetrahedron* 1970, **26**:3271–3277.
3. Pereira RC, Da Gama B a P, Teixeira VL, Yoneshigue-Valentin Y: **Ecological roles of natural products of the Brazilian red seaweed *Laurencia obtusa* .** *Braz J Biol* 2003, **63**:665–72.
4. Da Gama B a, Pereira RC, Soares AR, Teixeira VL, Yoneshigue-Valentin Y: **Is the mussel test a good indicator of antifouling activity? A comparison between laboratory and field assays.** *Biofouling* 2003, **19 Suppl**:161–9.
5. Hoffman L: **Marine cyanobacteria in tropical regions: diversity and ecology**. *Eur J Phycol* 1999, **34**:371–379.
6. Haas AF, Nelson CE, Wegley Kelly L, Carlson CA, Rohwer F, Leichter JJ, Wyatt A, Smith JE: **Effects of coral reef benthic primary producers on dissolved organic carbon and microbial activity.** *PLoS One* 2011, **6**:e27973.

7. Largo DB, Fukami K, Nishijima T: **Occasional pathogenic bacteria promoting ice-ice disease in the carrageenan-producing red algae *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Solieriaceae, Gigartinales, Rhodophyta)**. *J Appl Phycol* 1995, **7**:545–554.

8. Vairappan CS: **Potent antibacterial activity of halogenated metabolites from Malaysian red algae, *Laurencia majuscula* (Rhodomelaceae, Ceramiales).** *Biomol Eng* 2003, **20**:255–9.

9. Vairappan CS, Anangdan SP, Tan KL, Matsunaga S: **Role of secondary metabolites as defense chemicals against ice-ice disease bacteria in biofouler at carrageenophyte farms**. *J Appl Phycol* 2010, **22**:305–311.

10. Schwender J, Zeidler J, Gröner R, Müller C, Focke M, Braun S, Lichtenthaler FW, Lichtenthaler HK: **Incorporation of 1-deoxy-D-xylulose into isoprene and phytol by higher plants and algae**. *FEBS Lett* 1997, **414**:129–134.

11. Wanke M, Skorupinska-Tudek K, Swiezewska E: **Isoprenoid biosynthesis via 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway.** *Acta Biochim Pol* 2001, **48**:663–72.

12. De Oliveira LS, Tschoeke DA, de Oliveira AS, Hill LJ, Paradas WC, Salgado LT, Thompson CC, Pereira RC, Thompson FL: **New Insights on the terpenome of the red seaweed *Laurencia dendroidea* (Florideophyceae, Rhodophyta).** *Mar Drugs* 2015, **13**:879–902.

13. Carter-Franklin JN, Butler A: **Vanadium bromoperoxidase-catalyzed biosynthesis of halogenated marine natural products.** *J Am Chem Soc* 2004, **126**:15060–6.

14. Sudatti DB: **Influência de fatores abióticos e bióticos na química defensiva da macroalga marinha *Laurencia dendroidea* J. Agardh (Ceramiales: Rhodophyta)**. Universidade Federal Fluminense; 2010.

15. Paradas WC, Salgado LT, Sudatti DB, Crapez M a, Fujii MT, Coutinho R, Pereira RC, Amado Filho GM: **Induction of halogenated vesicle transport in cells of the red seaweed *Laurencia obtusa* .** *Biofouling* 2010, **26**:277–86.

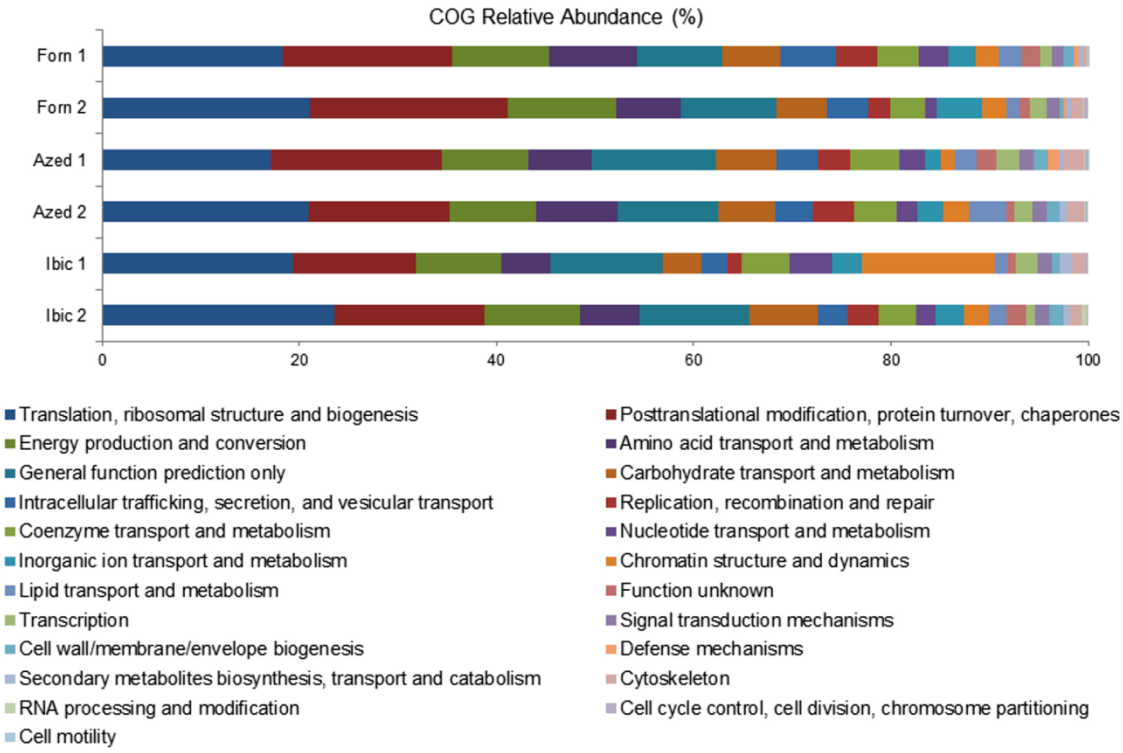
16. Vairappan CS, Kawamoto T, Miwa H, Suzuki M: **Potent antibacterial activity of halogenated compounds against antibiotic-resistant bacteria.** *Planta Med* 2004, **70**:1087–90.

17. Kladi M, Xenaki H, Vagias C, Papazafiri P, Roussis V: **New cytotoxic sesquiterpenes from the red algae *Laurencia obtusa* and *Laurencia microcladia* .** *Tetrahedron* 2006, **62**:182–189.

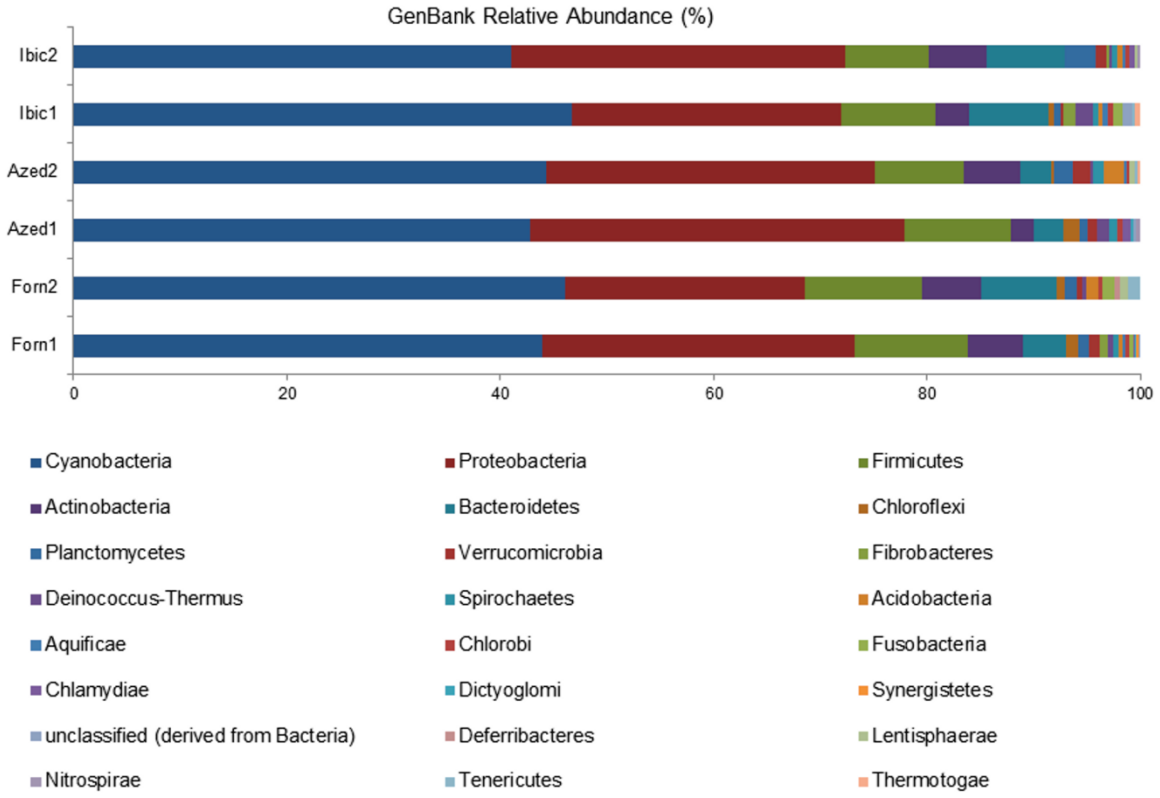
18. Veiga-Santos P, Pelizzaro-Rocha KJ, Santos a O, Ueda-Nakamura T, Dias Filho BP, Silva SO, Sudatti DB, Bianco EM, Pereira RC, Nakamura C V.: **In vitro anti-trypanosomal activity of elatol isolated from red seaweed *Laurencia dendroidea* .** *Parasitology* 2010, **137**:1661–70.

Suplementary Material

Chapter 2



Additional file 1 - COG functional profile of the transcriptome of *L. dendroidea* (separate samples).



Additional file 2 - Bacterial phyla recognized on the transcriptome of *L. dendroidea* (separate samples).

Additional file 3 - Relevant functions for the interaction between Bacteria and Eukarya in the transcriptomic profile of the holobiont.

Function	Eukarya	Bacteria	p-value
Heat shock protein 60 family chaperone GroEL	**0**	**3.171**	**4.89E-06**
Chaperone protein DnaJ	**0**	**1.057**	**0.019**
Chaperone protein DnaK	0	0.846	0.057
Manganese superoxide dismutase	0	0.528	0.172
Glutaredoxins	0	0.423	0.305
Alkyl hydroperoxide reductase	0	0.210	0.548
Photosynthesis	**3.179**	**1.578**	**0.011**
starch and sucrose metabolism (synthesis)	**0.663**	**0**	**0.0257**
Carbohydrate transport and metabolism	**3.480**	**5.626**	**0.0307**
Lipid transport and metabolism	**1.408**	**3.580**	**0.002**
Energy production and conversion	**5.799**	**11.381**	**1.42E-05**
Amino acid transport and metabolism	**3.645**	**11.509**	**2.84E-11**
Glutamate synthase domain 2	**0.580**	**0**	**0.047**

Chapter 3

Supplementary Information

The domain composition of sequences from *Laurencia dendroidea* that were annotated as terpene synthases and presented in Table 2 of the main manuscript were obtained through search for conserved domains using the NCBI Conserved Domain Database (CDD, [1]) and compared with the domain composition of corresponding sequences available in the SwissProt/UniProt and PlantCycDB databases. Analyzing the tables and figures below, we reinforce the Blast annotations presented in Table 2 of the main manuscript text, although biochemical and gene cloning approaches are necessary to prove these *in silico* identifications. For two of the 21 terpene synthase sequences from *Laurencia* (identified as nerolidol synthase and (+)-delta-cadinene synthase), we were not able to identify conserved domains, possibly because they were partial sequences. In the case of alpha-bisabolene synthase, we provide the domain composition of a reference sequence and the alignment of the sequence from *L. dendroidea* with this reference, showing a high similarity between them.

Table S1. List of domain hits for a putative (3*R*)-linalool synthase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
rad1	TIGR00596	DNA repair protein (rad1); All proteins in this family for which functions are known are ...	1–51	1.04e-15

Figure S1. Conserved domains detected in a putative (3R)-linalool synthase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S2. List of domain hits for the (3R)-linalool synthase gene from *Zea mays mays* (GDQC-116173-MONOMER—PlantCycDB).

Name	Accession	Description	Interval	E-Value
Terpene_cyclase_plant_C1	cd00684	Plant Terpene Cyclases, Class 1; This CD includes a diverse group of monomeric plant terpene ...	1377–1833	9.57e-107
ERCC4	pfam02732	ERCC4 domain; This domain is a family of nucleases. The family includes EME1 which is an ...	732–859	2.10e-28
rad1	TIGR00596	DNA repair protein (rad1); All proteins in this family for which functions are known are ...	88–933	0e+00

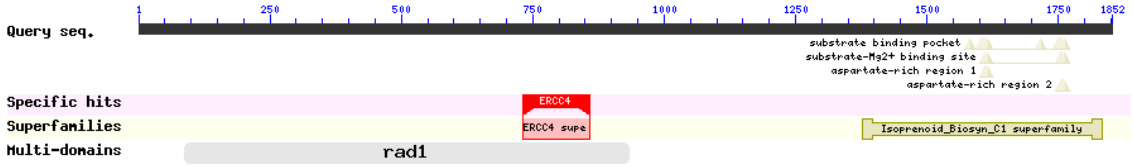


Figure S2. Conserved domains detected in the (3R)-linalool synthase gene from *Zea mays mays* (GDQC-116173-MONOMER—PlantCycDB) (edited from the NCBI CDD database, [1]).

Table S3. List of domain hits for a putative (+)-trans-carveol dehydrogenase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-value
CR_SDR_c	cd08936	Porcine peroxisomal carbonyl reductase like, classical (c) SDR; This subgroup contains porcine ...	17–272	1.05e-91
PRK05875	PRK05875	short chain dehydrogenase; Provisional	11–267	2.12e-39
adh_short_C2	pfam13561	Enoyl-(Acyl carrier protein) reductase;	22–268	1.19e-35
PLN02730	PLN02730	enoyl-[acyl-carrier-protein] reductase	85–262	3.56e-14
sepiapter_red	TIGR01500	sepiapterin reductase; This model describes sepiapterin reductase, a member of the short chain ...	17–201	5.40e-06
PKS_KR	smart00822	This enzymatic domain is part of bacterial polyketide synthases; It catalyses the first step ...	19–167	2.88e-04
fabG	PRK07231	3-ketoacyl-(acyl-carrier-protein) reductase; Provisional	17–267	2.43e-77
FabG	COG1028	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases) ...	14–267	4.59e-61
3oxo_ACP_reduc	TIGR01830	3-oxoacyl-(acyl-carrier-protein) reductase; This model represents 3-oxoacyl-[ACP] reductase, ...	18–267	3.80e-55
adh_short	pfam00106	short chain dehydrogenase; This family contains a wide variety of dehydrogenases.	17–185	4.24e-25
PLN02253	PLN02253	xanthoxin dehydrogenase	10–267	3.65e-18
Se_ygfK	TIGR03315	putative selenate reductase, YgfK subunit; Members of this protein family are YgfK, predicted ...	6–63	5.85e-03

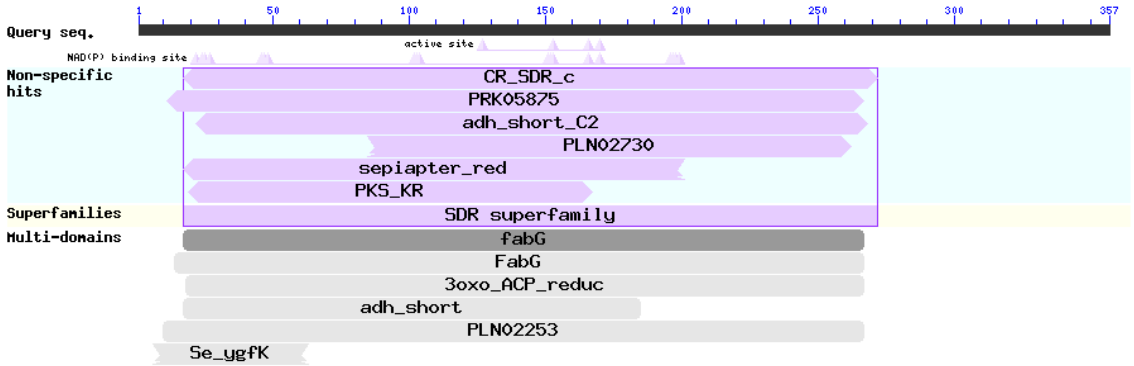


Figure S3. Conserved domains detected in a putative (+)-trans-carveol dehydrogenase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S4. List of domain hits for the (+)-trans-carveol dehydrogenase gene from *Mentha piperita* (sp|Q5C9I9|ISPD_MENPI).

Name	Accession	Description	Interval	E-Value
secoisolariciresinol-DH_like_SDR_c	cd05326	secoisolariciresinol dehydrogenase (secoisolariciresinol-DH)-like, classical (c) SDRs;	6–255	4.43e-70
PRK06947	PRK06947	glucose-1-dehydrogenase; Provisional	10–254	1.34e-28
adh_short_C2	pfam13561	Enoyl-(Acyl carrier protein) reductase;	16–255	1.75e-19
PLN00015	PLN00015	protochlorophyllide reductase	13–102	3.87e-10
PKS_KR	smart00822	This enzymatic domain is part of bacterial polyketide synthases; It catalyses the first step ...	10–124	5.58e-09
LPOR	TIGR01289	light-dependent protochlorophyllide reductase; This model represents the light-dependent,	12–101	3.14e-06
PLN02253	PLN02253	xanthoxin dehydrogenase	3–257	2.86e-63
PRK08324	PRK08324	short chain dehydrogenase; Validated	1–255	4.79e-61
FabG	COG1028	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases) ...	6–255	1.64e-50
RhaD_aldol-ADH	TIGR02632	rhamnulose-1-phosphate aldolase/alcohol dehydrogenase;	1–255	3.70e-45
adh_short	pfam00106	short chain dehydrogenase; This family contains a wide variety of dehydrogenases.	10–178	4.18e-20

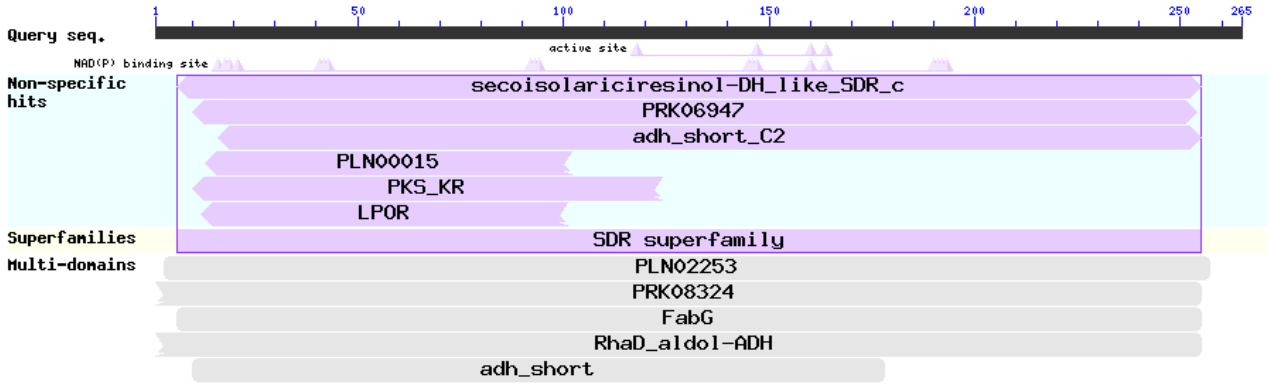


Figure S4. Conserved domains detected in the (+)-trans-carveol dehydrogenase gene from *Mentha piperita* (sp|Q5C9I9|ISPD_MENPI) (edited from the NCBI CDD database, [1]).

Table S5. List of domain hits for a putative (+)-pulegone reductase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
PGDH	cd05288	Prostaglandin dehydrogenases; Prostaglandins and related eicosanoids are metabolized by the ...	1–293	9.21e-128
leukotriene_B4_DH_like	cd08294	13-PGR is a bifunctional enzyme with delta-13 15-prostaglandin reductase and leukotriene B4 12 ...	14–294	1.49e-74
double_bond_reductase_like	cd08295	Arabidopsis alkenal double bond reductase and leukotriene B4 12-hydroxydehydrogenase; This ...	1–295	7.02e-73
PTGR2	cd08293	Prostaglandin reductase; Prostaglandins and related eicosanoids are metabolized by the ...	1–294	3.04e-65

Mgc45594_like	cd08250	Mgc45594 gene product and other MDR family members; Includes Human Mgc45594 gene product of ...	28–294	1.18e-48
QOR1	cd08241	Quinone oxidoreductase (QOR); QOR catalyzes the conversion of a quinone + NAD(P)H to a ...	29–294	1.89e-39
p53_inducible_oxidoreductase	cd05276	PIG3 p53-inducible quinone oxidoreductase; PIG3 p53-inducible quinone oxidoreductase, a medium ...	53–293	9.65e-29
zeta_crystallin	cd08253	Zeta-crystallin with NADP-dependent quinone reductase activity (QOR); Zeta-crystallin is a eye ...	65–295	6.42e-27
MDR_like_2	cd05289	alcohol dehydrogenase and quinone reductase-like medium chain degydrogenases/reductases; ...	45–293	5.28e-26
Zn_ADH_like1	cd08266	Alcohol dehydrogenases of the MDR family; This group contains proteins related to the ...	85–295	9.64e-24

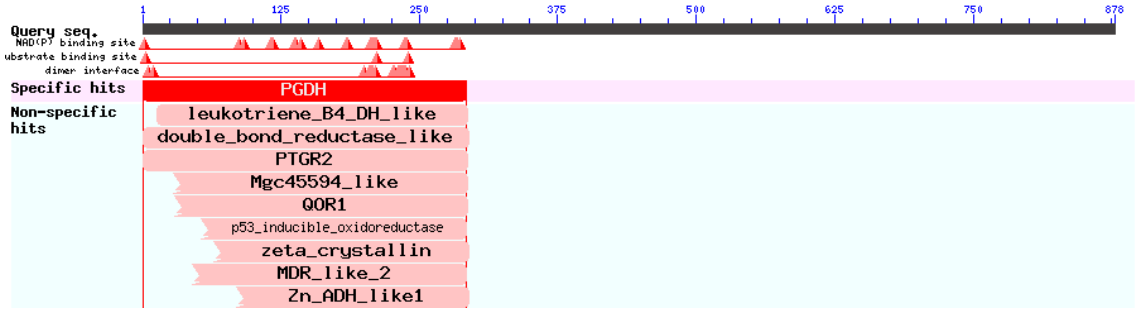


Figure S5. Conserved domains detected in a putative (+)-pulegone reductase gene from *L. dendroidea* (edited from the NCBI CDD database [1]).

Table S6. List of domain hits for the (+)-pulegone reductase gene from *Mentha piperita* (sp|Q6WAU0|PULR_MENPI).

Name	Accession	Description	Interval	E-Value
double_bond_reductase_like	cd08295	Arabidopsis alkenal double bond reductase and leukotriene B4 12-hydroxydehydrogenase; This ...	2–339	0e+00
PGDH	cd05288	Prostaglandin dehydrogenases; Prostaglandins and related eicosanoids are metabolized by the ...	3–337	1.06e-144
leukotriene_B4_DH_like	cd08294	13-PGR is a bifunctional enzyme with delta-13 15-prostaglandin reductase and leukotriene B4 12 ...	2–339	1.23e-104
PTGR2	cd08293	Prostaglandin reductase; Prostaglandins and related eicosanoids are metabolized by the ...	3–339	3.91e-65
Mgc45594_like	cd08250	Mgc45594 gene product and other MDR family members; Includes Human Mgc45594 gene product of ...	137–337	7.60e-47
zeta_crystallin	cd08253	Zeta-crystallin with NADP-dependent quinone reductase activity (QOR); Zeta-crystallin is a eye ...	29–283	3.04e-28
MDR	cd05188	Medium chain reductase/dehydrogenase (MDR)/zinc-dependent alcohol dehydrogenase-like family; ...	94–297	9.69e-27
p53_inducible_oxidoreductase	cd05276	PIG3 p53-inducible quinone oxidoreductase; PIG3 p53-inducible quinone oxidoreductase, a medium ...	138–307	1.44e-25

MDR_like_2	cd05289	alcohol dehydrogenase and quinone reductase-like medium chain degydrogenases/reductases; ...	31–323	3.42e-25
ADH_zinc_N	pfam00107	Zinc-binding dehydrogenase;	16–298	3.91e-21

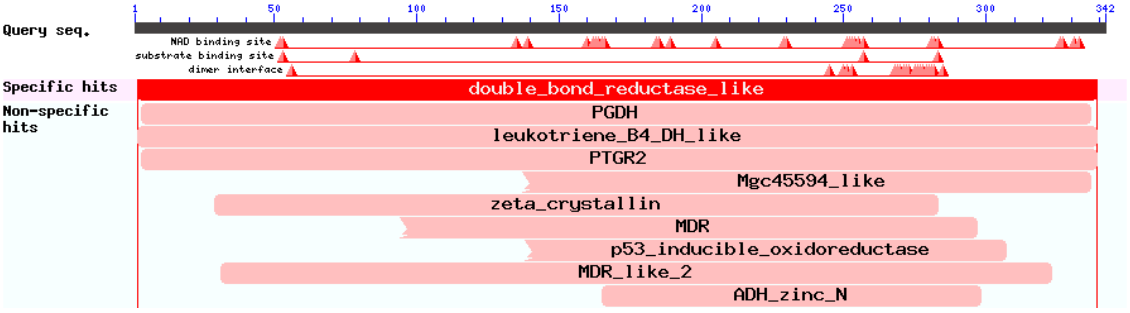


Figure S6. Conserved domains detected in the (+)-pulegone reductase gene from *Mentha piperita* (sp|Q6WAU0|PULR_MENPI) (edited from the NCBI CDD database, [1]).

Table S7. List of domain hits for a putative (−)-isopiperitenone reductase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
17beta-HSD1_like_SDR_c	cd05356	17-beta-hydroxysteroid dehydrogenases (17beta-HSDs) types -1, -3, and -12, -like, classical (c) ...	34–146	3.56e-31
PRK08339	PRK08339	short chain dehydrogenase; Provisional	27–112	1.20e-09
sepiapter_red	TIGR01500	sepiapterin reductase; This model describes sepiapterin reductase, a member of the short chain ...	37–119	3.85e-06
DltE	COG0300	Short-chain dehydrogenases of various substrate specificities [General function prediction ...	32–146	7.97e-29
PLN02780	PLN02780	ketoreductase/oxidoreductase	14–146	7.50e-23
fabG	PRK07666	3-ketoacyl-(acyl-carrier-protein) reductase; Provisional	37–145	3.61e-17
23BDH	TIGR02415	acetoin reductases; One member of this family, as characterized in Klebsiella terrigena, is ...	37–145	3.62e-12
adh_short	pfam00106	short chain dehydrogenase; This family contains a wide variety of dehydrogenases.	36–145	1.50e-09

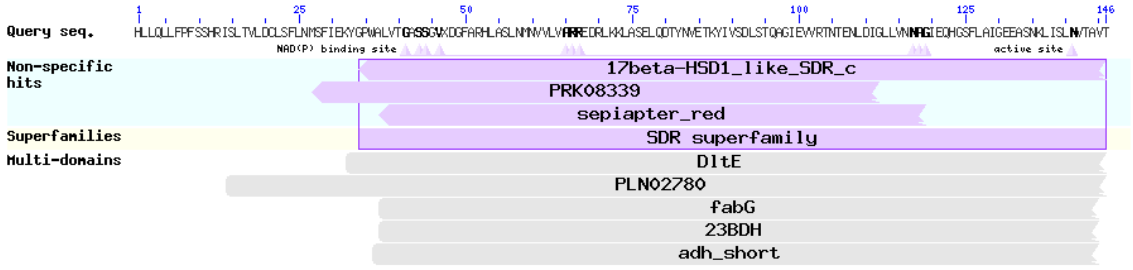


Figure S7. Conserved domains detected in a putative (−)-isopiperitenone reductase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S8. List of domain hits for the (−)-isopiperitenone reductase gene from *Mentha piperita* (sp|Q6WAU1|IPIPR_MENPI).

Name	Accession	Description	Interval	E-Value
carb_red_PTCR-like_SDR_c	cd05324	Porcine testicular carbonyl reductase (PTCR)-like, classical (c) SDRs; PTCR is a classical SDR ...	6–311	1.99e-74
PRK05854	PRK05854	short chain dehydrogenase; Provisional	6–96	4.90e-15
adh_short_C2	pfam13561	Enoyl-(Acyl carrier protein) reductase;	15–126	4.28e-11
PKS_KR	smart00822	This enzymatic domain is part of bacterial polyketide synthases; It catalyses the first step ...	9–96	8.02e-11
sepiapter_red	TIGR01500	sepiapterin reductase; This model describes sepiapterin reductase, a member of the short chain ...	7–120	1.78e-06
PLN00015	PLN00015	protochlorophyllide reductase	9–96	1.60e-03
PRK12939	PRK12939	short chain dehydrogenase; Provisional	6–299	2.99e-15
FabG	COG1028	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases) .	8–300	4.86e-27
adh_short	pfam00106	short chain dehydrogenase; This family contains a wide variety of dehydrogenases.	8–138	3.61e-19

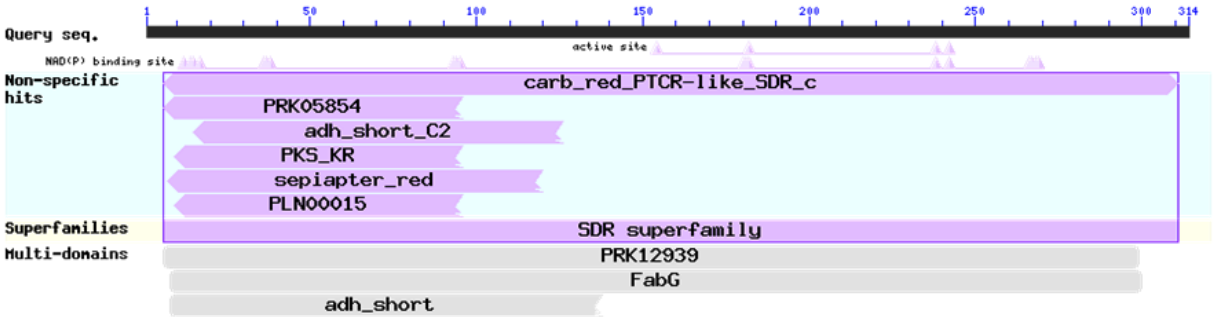


Figure S8. Conserved domains detected in the (−)-isopiperitenone reductase gene from *Mentha piperita* (sp|Q6WAU1|IPIPR_MENPI) (edited from the NCBI CDD database, [1]).

Table S9. List of domain hits for a putative secologanin synthase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
p450	pfam00067	Cytochrome P450; Cytochrome P450s are haem-thiolate proteins involved in the oxidative	160–384	2.32e-29
CypX	COG2124	Cytochrome P450 [Secondary metabolites biosynthesis, transport, and catabolism]	13–378	2.81e-25
PLN02290	PLN02290	cytokinin trans-hydroxylase	170–351	8.00e-17
PLN02169	PLN02169	fatty acid (omega-1)-hydroxylase/midchain alkane hydroxylase	168–356	8.21e-17
PLN03195	PLN03195	fatty acid omega-hydroxylase; Provisional	167–378	9.71e-15
PLN02426	PLN02426	cytochrome P450, family 94, subfamily C protein	168–370	6.50e-14
PLN00168	PLN00168	Cytochrome P450; Provisional	160–356	7.30e-14
PLN02936	PLN02936	epsilon-ring hydroxylase	162–380	1.74e-13
PLN02687	PLN02687	flavonoid 3′-monooxygenase	159–344	6.33e-09
PLN02655	PLN02655	ent-kaurene oxidase	160–378	1.04e-07
PLN00110	PLN00110	flavonoid 3′,5′-hydroxylase (F3′5′H); Provisional	154–300	6.62e-07
PTZ00404	PTZ00404	cytochrome P450; Provisional	164–359	7.19e-07
PLN02183	PLN02183	ferulate 5-hydroxylase	160–298	1.08e-06
PLN03018	PLN03018	homomethionine *N*-hydroxylase	155–349	1.21e-06
PLN03112	PLN03112	cytochrome P450 family protein; Provisional	172–300	3.83e-06
PLN02971	PLN02971	tryptophan *N*-hydroxylase	157–357	5.06e-06

PLN02966	PLN02966	cytochrome P450 83A1	157–338	5.44e-06
PLN03234	PLN03234	cytochrome P450 83B1; Provisional	144–345	1.74e-05
PLN02394	PLN02394	trans-cinnamate 4-monooxygenase	178–380	2.89e-04
PLN02302	PLN02302	ent-kaurenoic acid oxidase	152–359	4.88e-04
PLN02774	PLN02774	brassinosteroid-6-oxidase	159–361	6.03e-03

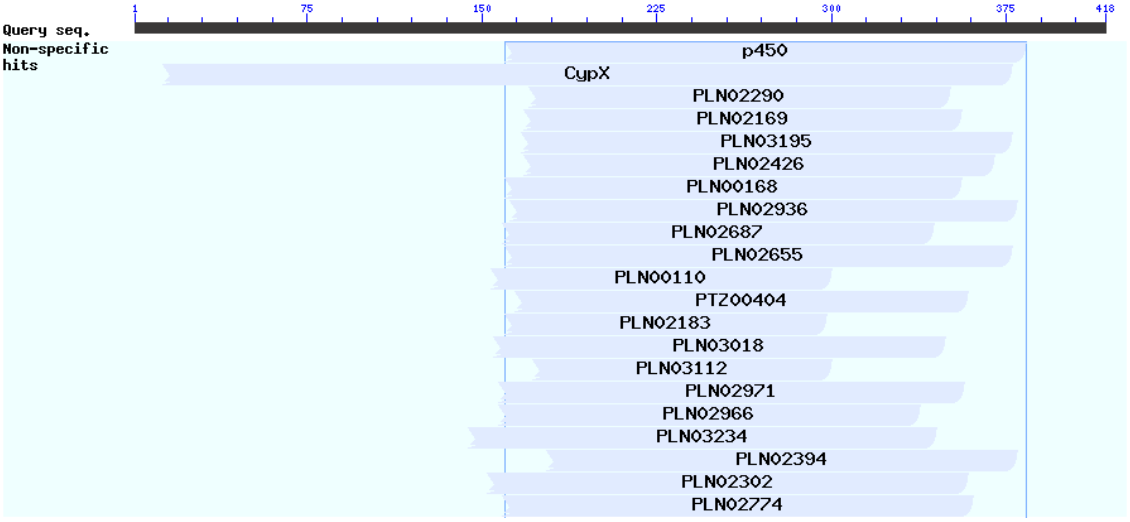


Figure S9. Conserved domains detected in a putative secologanin synthase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S10. List of domain hits for the secologanin synthase gene from *Catharanthus roseus* (sp|Q05047|C72A1_CATRO).

Name	Accession	Description	Interval	E-Value
PLN02290	PLN02290	cytokinin trans-hydroxylase	5–524	2.05e-129
p450	pfam00067	Cytochrome P450; Cytochrome P450s are haem-thiolate proteins involved in the oxidative ...	102–497	4.56e-82
CypX	COG2124	Cytochrome P450 [Secondary metabolites biosynthesis, transport, and catabolism]	114–521	3.87e-50
PLN02936	PLN02936	epsilon-ring hydroxylase	100–497	3.71e-41
PLN02687	PLN02687	flavonoid 3′-monooxygenase	190–496	2.09e-26
PLN02426	PLN02426	cytochrome P450, family 94, subfamily C protein	260–503	2.27e-26
PTZ00404	PTZ00404	cytochrome P450; Provisional	64–493	7.42e-24
PLN03195	PLN03195	fatty acid omega-hydroxylase; Provisional	110–497	8.36e-24
PLN02302	PLN02302	ent-kaurenoic acid oxidase	88–507	3.89e-23
PLN02183	PLN02183	ferulate 5-hydroxylase	208–495	8.15e-23
PLN03112	PLN03112	cytochrome P450 family protein; Provisional	93–497	1.07e-21
PLN02169	PLN02169	fatty acid (omega-1)-hydroxylase/midchain alkane hydroxylase	260–521	1.98e-21
PLN02655	PLN02655	ent-kaurene oxidase	228–491	2.30e-21
PLN02966	PLN02966	cytochrome P450 83A1	183–495	8.06e-19
PLN03234	PLN03234	cytochrome P450 83B1; Provisional	164–495	2.81e-18
PLN00168	PLN00168	Cytochrome P450; Provisional	314–497	3.95e-16
PLN02987	PLN02987	Cytochrome P450, family 90, subfamily A	223–493	2.87e-15
PLN02971	PLN02971	tryptophan *N*-hydroxylase	242–498	3.03e-15
PLN02394	PLN02394	trans-cinnamate 4-monooxygenase	332–491	1.33e-14
PLN00110	PLN00110	flavonoid 3′,5′-hydroxylase (F3′5′H); Provisional	247–491	6.65e-12
PLN02500	PLN02500	cytochrome P450 90B1	272–496	1.01e-11
PLN03018	PLN03018	homomethionine *N*-hydroxylase	150–522	3.32e-11
PLN02774	PLN02774	brassinosteroid-6-oxidase	107–494	4.36e-11

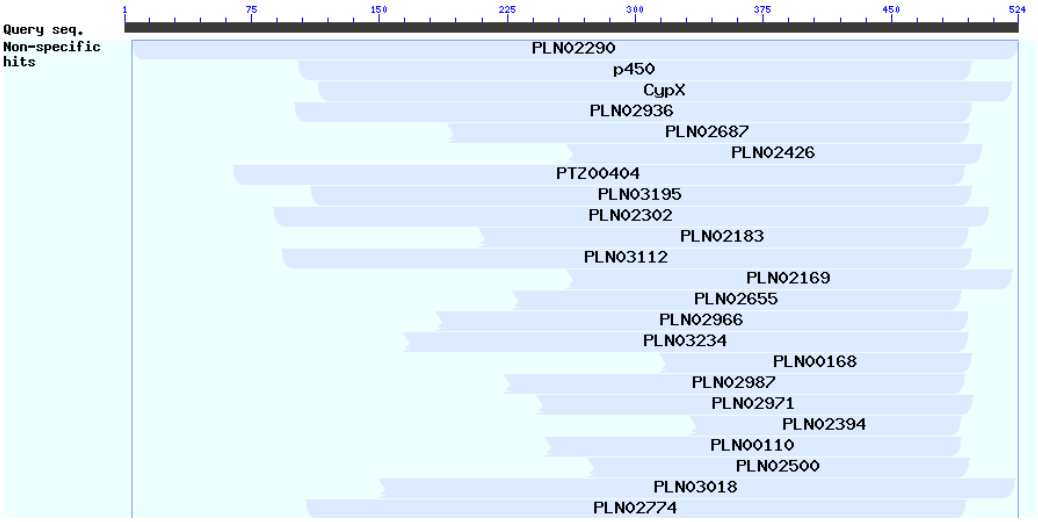


Figure S10. Conserved domains detected in the secologanin synthase gene from *Catharanthus roseus* (sp|Q05047|C72A1_CATRO) (edited from the NCBI CDD database, [1]).

Table S11. List of domain hits for a putative farnesyl-diphosphate farnesyltransferase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
squal_synth	TIGR01559	farnesyl-diphosphate farnesyltransferase; This model describes farnesyl-diphosphate ...	139–411	1.00e-104
Trans_IPPS_HH	cd00683	Trans-Isoprenyl Diphosphate Synthases, head-to-head; These trans-Isoprenyl Diphosphate ...	165–364	2.49e-47
SQS_PSY	pfam00494	Squalene/phytoene synthase;	158–361	1.01e-37
ERG9	COG1562	Phytoene/squalene synthetase [Lipid metabolism]	180–370	2.86e-35
Trans_IPPS	cd00867	Trans-Isoprenyl Diphosphate Synthases; Trans-Isoprenyl Diphosphate Synthases (Trans_IPPS) of ...	179–286	1.13e-17
Isoprenoid_Biosyn_C1	cd00385	Isoprenoid Biosynthesis enzymes, Class 1; Superfamily of trans-isoprenyl diphosphate synthases ...	174–325	4.43e-17
HpnD	TIGR03465	squalene synthase HpnD; The genes of this family are often found in the same genetic locus ...	193–275	1.41e-08
PLN02632	PLN02632	phytoene synthase	185–275	4.93e-07

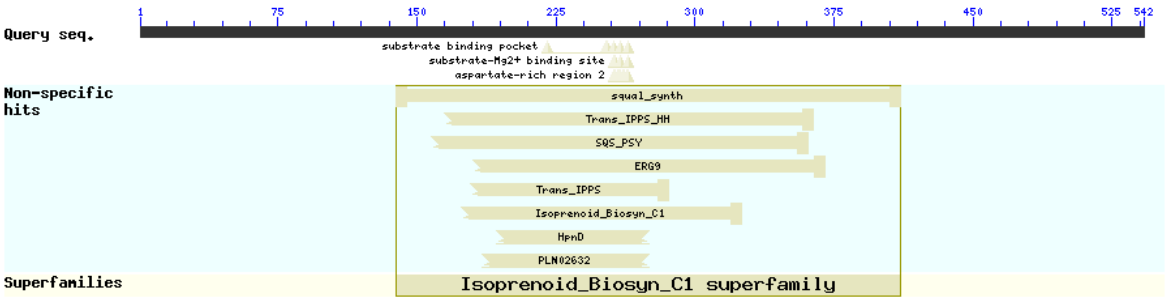


Figure S11. Conserved domains detected in a putative farnesyl-diphosphate farnesyltransferase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S12. List of domain hits for the farnesyl-diphosphate farnesyltransferase gene from *Scheffersomyces stipitis* (tr|A3LTQ5|A3LTQ5_PICST).

Name	Accession	Description	Interval	E-value
Trans_IPPS_HH	cd00683	Trans-Isoprenyl Diphosphate Synthases, head-to-head; These trans-Isoprenyl Diphosphate ...	40–333	2.34e-74
squal_synth	TIGR01559	farnesyl-diphosphate farnesyltransferase; This model describes farnesyl-diphosphate ...	39–381	0e+00
ERG9	COG1562	Phytoene/squalene synthetase [Lipid metabolism]	39–342	1.08e-57
SQS_PSY	pfam00494	Squalene/phytoene synthase;	48–329	5.08e-48
PLN02632	PLN02632	phytoene synthase	170–242	9.10e-07

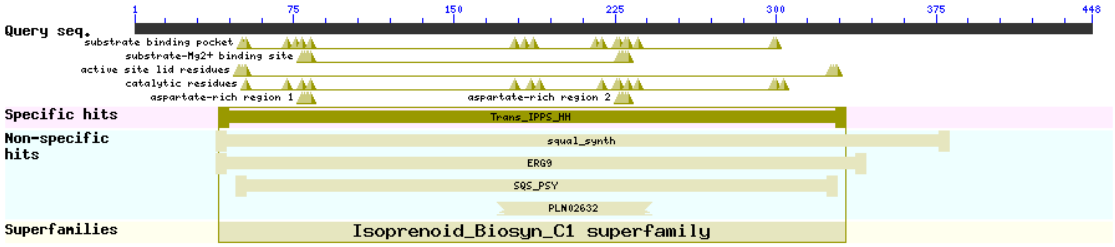


Figure S12. Conserved domains detected in the farnesyl-diphosphate farnesyltransferase gene from *Scheffersomyces stipitis* (tr|A3LTQ5|A3LTQ5_PICST) (edited from the NCBI CDD database, [1]).

Table S13. List of domain hits for a putative squalene monooxygenase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
SE	pfam08491	Squalene epoxidase; This domain is found in squalene epoxidase (SE) and related proteins which ...	11–258	2.16e-101
PRK07608	PRK07608	ubiquinone biosynthesis hydroxylase family protein; Provisional	100–200	3.03e-04
COQ6	TIGR01989	ubiquinone biosynthesis monooxygenase COQ6; This model represents the monooxygenase ...	110–198	7.59e-03
PTZ00367	PTZ00367	squalene epoxidase; Provisional	15–275	2.05e-68
PLN02985	PLN02985	squalene monooxygenase	30–275	6.46e-56
UbiH	COG0654	2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases [Coenzyme ...	16–205	7.72e-12
PRK07045	PRK07045	putative monooxygenase; Reviewed	114–185	1.84e-04

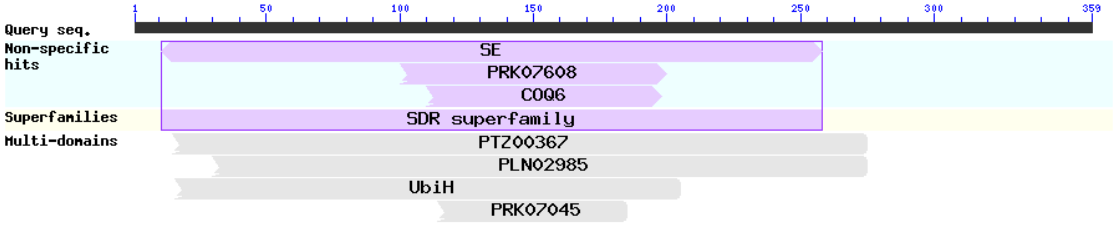


Figure S13. Conserved domains detected in a putative squalene monooxygenase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S14. List of domain hits for the squalene monooxygenase gene from *Arabidopsis thaliana* (sp|O65403|ERG13_ARATH).

Name	Accession	Description	Interval	E-value
SE	pfam08491	Squalene epoxidase; This domain is found in squalene	195–472	2.27e-124

		epoxidase (SE) and related proteins which ...		
PRK08773	PRK08773	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase; Validated	40–364	3.79e-09
NAD_binding_8	pfam13450	NAD(P)-binding Rossmann-like domain;	48–74	5.95e-07
PRK12834	PRK12834	putative FAD-binding dehydrogenase; Reviewed	44–105	1.76e-06
THI4	COG1635	Ribulose 1,5-bisphosphate synthetase, converts PRPP to RuBP, flavoprotein [Carbohydrate ...	44–76	2.60e-05
PLN02661	PLN02661	Putative thiazole synthesis	44–74	3.21e-03
PTZ00383	PTZ00383	malate:quinone oxidoreductase; Provisional	25–74	5.01e-03
PLN02985	PLN02985	squalene monooxygenase	1–515	0e+00
PTZ00367	PTZ00367	squalene epoxidase; Provisional	6–499	3.11e-86
UbiH	COG0654	2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases [Coenzyme ...	44–439	6.61e-36

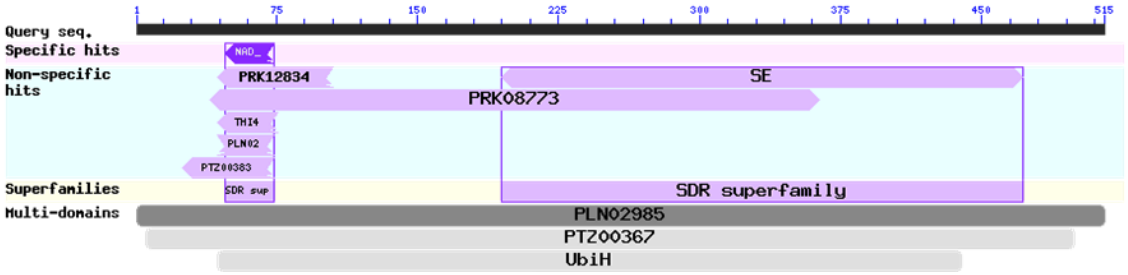


Figure S14. Conserved domains detected in the squalene monooxygenase gene from *Arabidopsis thaliana* (sp|O65403|ERG13_ARATH) (edited from the NCBI CDD database, [1]).

Table S15. List of domain hits for a putative squalene-hopene/tetraprenyl-beta-curcumene cyclase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-value
SQCY_1	cd02892	Squalene cyclase (SQCY) domain subgroup 1; found in class II terpene cyclases that have an ...	1–363	5.98e-143
SQCY	cd02889	Squalene cyclase (SQCY) domain; found in class II terpene cyclases that have an alpha 6— ...	24–363	8.74e-126
ISOPREN_C2_like	cd00688	This group contains class II terpene cyclases, protein prenyltransferases beta subunit, two ...	1–363	6.47e-39
Prenyltrans	pfam00432	Prenyltransferase and squalene oxidase repeat;	207–240	3.26e-04
SQCY_1	cd02892	Squalene cyclase (SQCY) domain subgroup 1; found in class II terpene cyclases that have an ...	207–274	4.34e-03
Prenyltrans_2	pfam13249	Prenyltransferase-like;	211–331	7.97e-09
Prenyltrans_2	pfam13249	Prenyltransferase-like;	134–275	5.74e-08
squalene_cyclas	TIGR01787	squalene/oxidosqualene cyclases; This family of enzymes catalyzes the cyclization of the ...	3–363	1.19e-117
PLN03012	PLN03012	Camelliol C synthase	3–363	1.41e-106
PLN02993	PLN02993	lupeol synthase	3–369	4.48e-97
osq_cycl	TIGR03463	2,3-oxidosqualene cyclase; This model identifies 2,3-oxidosqualene cyclases from Stigmatella ...	1–361	1.37e-91
SqhC	COG1657	Squalene cyclase [Lipid metabolism]	3–367	4.95e-56
hopene_cyclase	TIGR01507	squalene-hopene cyclase; SHC is an essential prokaryotic gene in hopanoid (triterpenoid) ...	5–369	9.84e-30

squa_tetra_cyc	TIGR04277	squalene--tetrahymanol cyclase; This enzyme, also called squalene--tetrahymanol cyclase, ...	126–363	2.76e-10
Prenyltrans_1	pfam13243	Prenyltransferase-like;	207–274	1.62e-08
Prenyltrans_2	pfam13249	Prenyltransferase-like;	83–224	1.02e-03
Prenyltrans_1	pfam13243	Prenyltransferase-like;	129–270	1.31e-03

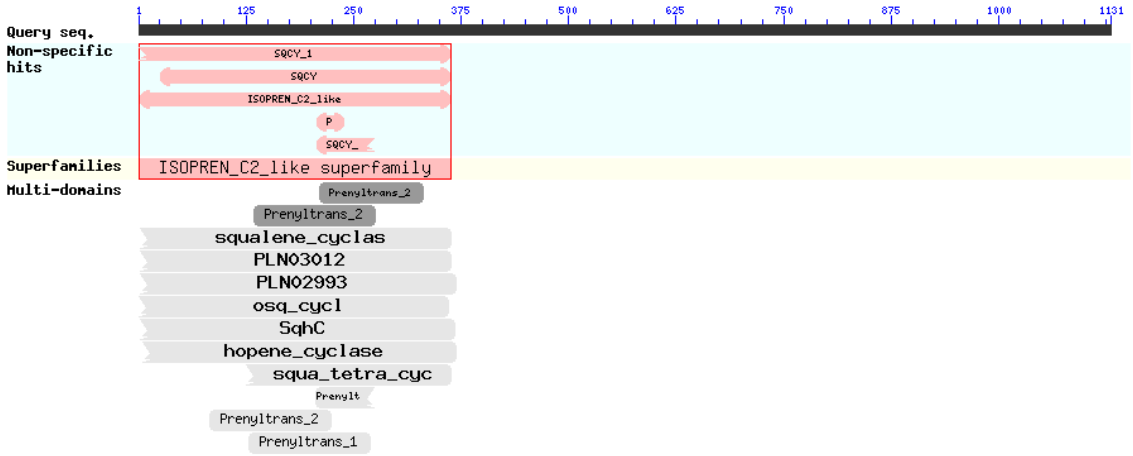


Figure S15. Conserved domains detected in a putative squalene-hopene/tetraprenyl-beta-curcumene cyclase gene from *L. dendroidea*, (edited from the NCBI CDD database, [1]).

Table S16. List of domain hits for the squalene-hopene/tetraprenyl-beta-curcumene cyclase gene from *Bradyrhizobium* sp. (tr|A4Z167 A4Z167_BRASO).

Name	Accession	Description	Interval	E-Value
SQCY_1	cd02892	Squalene cyclase (SQCY) domain subgroup 1; found in class II terpene cyclases that have an ...	26–677	0e+00
SQCY	cd02889	Squalene cyclase (SQCY) domain; found in class II terpene cyclases that have an alpha 6—...	332–676	8.79e-130
ISOPREN_C2_like	cd00688	This group contains class II terpene cyclases, protein prenyltransferases beta subunit, two ...	326–677	9.84e-39
SQCY	cd02889	Squalene cyclase (SQCY) domain; found in class II terpene cyclases that have an alpha 6— ...	26–375	3.52e-08
ISOPREN_C2_like	cd00688	This group contains class II terpene cyclases, protein prenyltransferases beta subunit, two ...	26–410	7.38e-08
SQCY	cd02889	Squalene cyclase (SQCY) domain; found in class II terpene cyclases that have an alpha 6— ...	18–97	1.79e-07
SQCY_1	cd02892	Squalene cyclase (SQCY) domain subgroup 1; found in class II terpene cyclases that have an ...	23–96	9.72e-07
ISOPREN_C2_like	cd00688	This group contains class II terpene cyclases, protein prenyltransferases beta subunit, two ...	17–132	4.24e-05
SQCY	cd02889	Squalene cyclase (SQCY) domain; found in class II terpene cyclases that have an alpha 6— ...	17–129	8.21e-05
SQCY_1	cd02892	Squalene cyclase (SQCY) domain subgroup 1; found in class II terpene cyclases that have an ...	17–129	1.16e-04
Prenyltrans	pfam00432	Prenyltransferase and squalene oxidase repeat;	493–535	3.52e-04
Prenyltrans	pfam00432	Prenyltransferase and squalene oxidase repeat;	541–583	5.36e-04
hopene_cyclase	TIGR01507	squalene-hopene cyclase; SHC is an essential prokaryotic gene in hopanoid (triterpenoid) ...	26–676	0e+00
squalene_cyclas	TIGR01787	squalene/oxidosqualene cyclases; This family of enzymes catalyzes the cyclization of the ...	25–676	1.99e-157
SqhC	COG1657	Squalene cyclase [Lipid metabolism]	110–683	9.16e-74
squa_tetra_cyc	TIGR04277	squalene—tetrahymanol cyclase; This enzyme, also called squalene—tetrahymanol cyclase, ...	77–681	6.52e-61

osq_cycl	TIGR03463	2,3-oxidosqualene cyclase; This model identifies 2,3-oxidosqualene cyclases from Stigmatella ...	33–676	1.09e-49
PLN02993	PLN02993	lupeol synthase	18–566	6.57e-33
PLN03012	PLN03012	Camelliol C synthase	24–675	9.97e-33
Prenyltrans_2	pfam13249	Prenyltransferase-like;	431–562	4.07e-16
Prenyltrans_1	pfam13243	Prenyltransferase-like;	495–614	2.09e-14

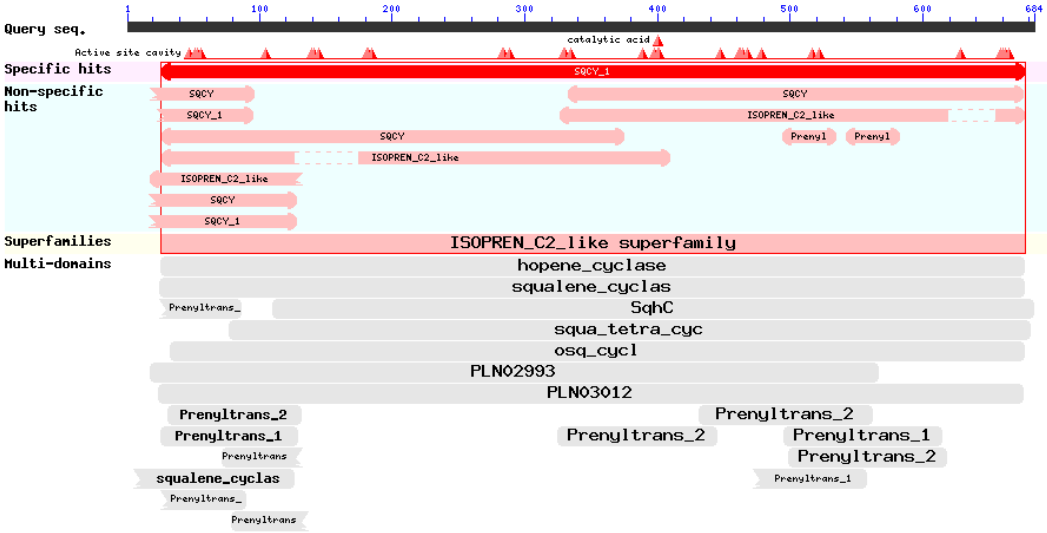


Figure S16. Conserved domains detected in the squalene-hopene/tetraprenyl-beta-curcumene cyclase gene from *Bradyrhizobium sp* (tr|A4Z167 A4Z167_BRASO) (edited from the NCBI CDD database, [1]).

Table S17. List of domain hits for a putative lupeol synthase 1 gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
SQCY_1	cd02892	Squalene cyclase (SQCY) domain subgroup 1; found in class II terpene cyclases that have an ...	1363	5.98e-143
SQCY	cd02889	Squalene cyclase (SQCY) domain; found in class II terpene cyclases that have an alpha 6 - ...	24–363	8.74e-126
ISOPREN_C2_like	cd00688	This group contains class II terpene cyclases, protein prenyltransferases beta subunit, two ...	1–363	6.47e-39
Prenyltrans	pfam00432	Prenyltransferase and squalene oxidase repeat;	207–240	3.26e-04
SQCY_1	cd02892	Squalene cyclase (SQCY) domain subgroup 1; found in class II terpene cyclases that have an ...	207–274	4.34e-03
Prenyltrans_2	pfam13249	Prenyltransferase-like;	211–331	7.97e-09
Prenyltrans_2	pfam13249	Prenyltransferase-like;	134–275	5.74e-08
squalene_cyclas	TIGR01787	squalene/oxidosqualene cyclases; This family of enzymes catalyzes the cyclization of the ...	3–363	1.19e-117
PLN03012	PLN03012	Camelliol C synthase	3–363	1.41e-106
PLN02993	PLN02993	lupeol synthase	3–369	4.48e-97
osq_cycl	TIGR03463	2,3-oxidosqualene cyclase; This model identifies 2,3-oxidosqualene cyclases from Stigmatella ...	1–361	1.37e-91
SqhC	COG1657	Squalene cyclase [Lipid metabolism]	3–367	4.95e-56

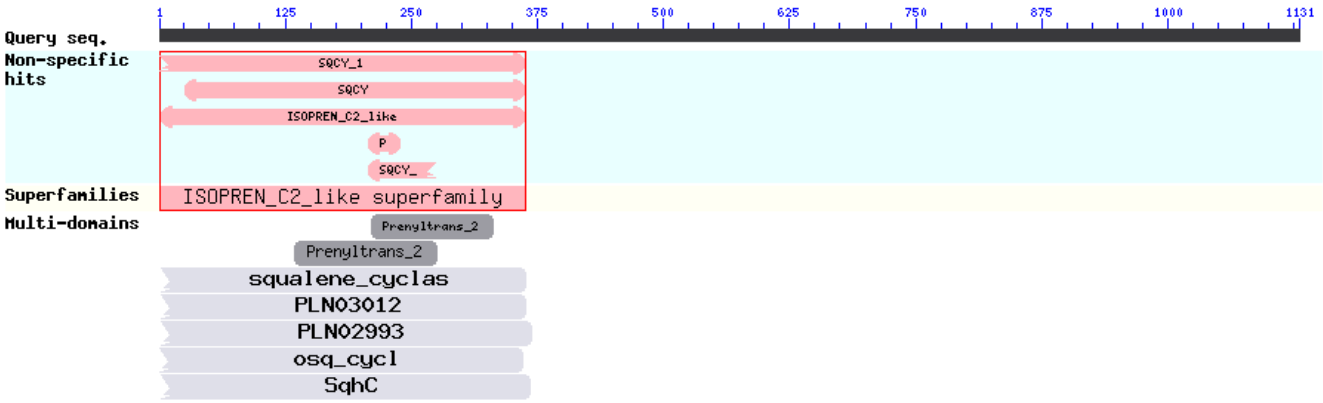


Figure S17. Conserved domains detected in a putative lupeol synthase 1 gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S18. List of domain hits for the lupeol synthase 1 gene from *Arabidopsis thaliana* (sp|Q9C5M3|LUP1_ARATH).

Name	Accession	Description	Interval	E-Value
SQCY_1	cd02892	Squalene cyclase (SQCY) domain subgroup 1; found in class II terpene cyclases that have an ...	99–749	0e+00
Prenyltrans	pfam00432	Prenyltransferase and squalene oxidase repeat;	589–629	1.24e-07
PLN02993	PLN02993	lupeol synthase	1–757	0e+00
squalene_cyclas	TIGR01787	squalene/oxidosqualene cyclases; This family of enzymes catalyzes the cyclization of the ...	98–751	0e+00
SqhC	COG1657	Squalene cyclase [Lipid metabolism]	186–749	3.47e-59
Prenyltrans_2	pfam13249	Prenyltransferase-like;	593–717	5.37e-15

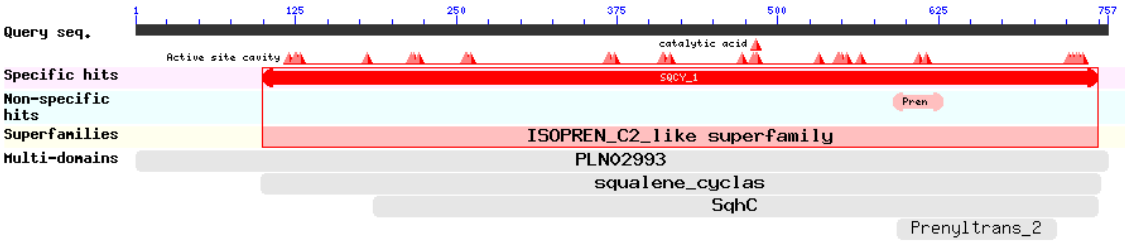


Figure S18. Conserved domains detected in the lupeol synthase 1 gene from *Arabidopsis thaliana* (sp|Q9C5M3|LUP1_ARATH) (edited from the NCBI CDD database, [1]).

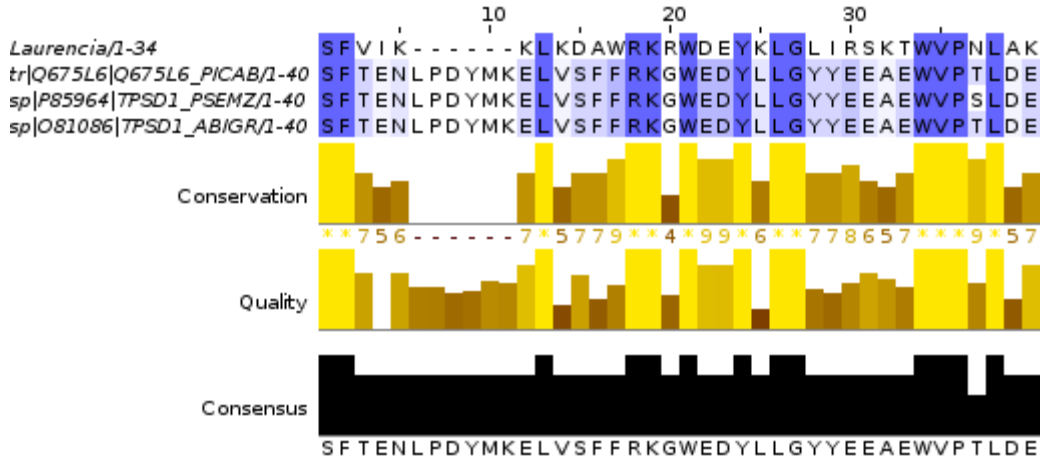


Figure S19. Multiple alignment analysis between a putative alpha-bisabolene synthase from *L. dendroidea* and alpha-bisabolene synthase reference sequences from *Picea abies*, *Pseudotsuga menziesii* and *Abies grandis* downloaded from Uniprot. The alignment was done using Muscle software [2] and visualized with Jalview [3].

Table S19. List of domain hits for the alpha-bisabolene synthase gene from *Picea abies* (tr|Q675L6 Q675L6_PICAB).

Name	Accession	Description	Interval	E-Value
Terpene_cyclase_plant_C1	cd00684	Plant Terpene Cyclases, Class 1; This CD includes a diverse group of monomeric plant terpene ...	271–804	0e+00
Terpene_synth_C	pfam03936	Terpene synthase family, metal binding domain; It has been suggested that this gene family be ...	481–751	1.09e-97
Terpene_cyclase_C1	cd00868	Terpene cyclases, Class 1; Terpene cyclases, Class 1 (C1) of the class 1 family of isoprenoid ...	495–775	3.12e-89
Terpene_synth	pfam01397	Terpene synthase, *N*-terminal domain; It has been suggested that this gene family be designated ...	246–439	6.37e-66
Isoprenoid_Biosyn_C1	cd00385	Isoprenoid Biosynthesis enzymes, Class 1; Superfamily of trans-isoprenyl diphosphate synthases ...	534–771	1.28e-29
PLN02150	PLN02150	terpene synthase/cyclase family protein	714–807	1.15e-07
ISOPREN_C2_like	cd00688	This group contains class II terpene cyclases, protein prenyltransferases beta subunit, two ...	73–156	3.94e-03
PLN02279	PLN02279	ent-kaur-16-ene synthase	60–807	5.06e-148
PLN02592	PLN02592	ent-copalyl diphosphate synthase	57–713	4.31e-165
Prenyltrans_1	pfam13243	Prenyltransferase-like;	99–157	9.07e-04
Prenyltrans_1	pfam13243	Prenyltransferase-like;	78-154	1.33e-03

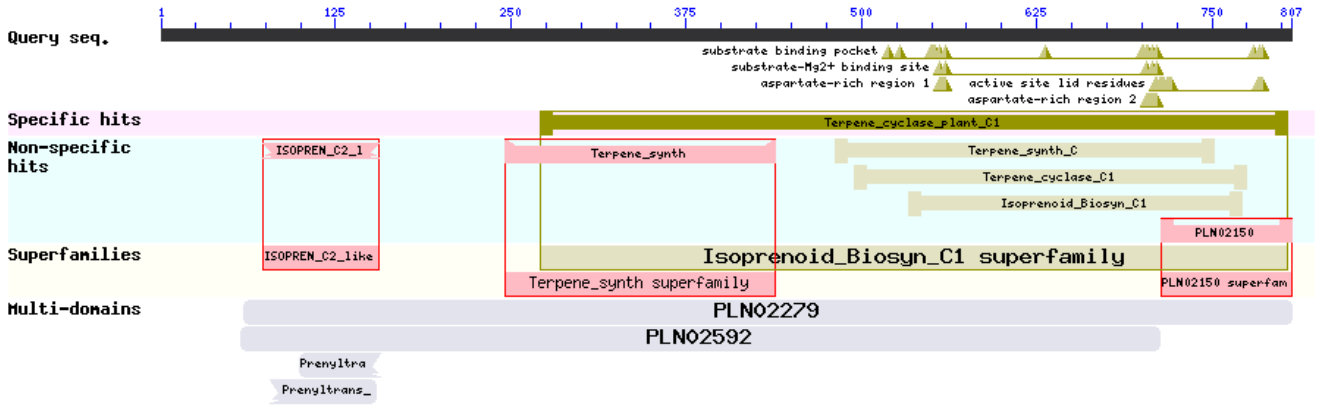


Figure S20. Conserved domains detected in the alpha-bisabolene synthase gene from *Picea abies* (tr|Q675L6 Q675L6_PICAB) (edited from the NCBI CDD database, [1]).

Table S20. List of domain hits for a putative germacrene-*A* synthase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
Terpene_cyclase_nonplant_C1	cd00687	Non-plant Terpene Cyclases, Class 1; This CD includes terpenoid cyclases such as pentalenene ...	56-353	8.36e-25
Terpene_cyclase_C1	cd00868	Terpene cyclases, Class 1; Terpene cyclases, Class 1 (C1) of the class 1 family of isoprenoid ...	60-349	2.45e-11
Terpene_synth_C	pfam03936	Terpene synthase family, metal binding domain; It has been suggested that this gene family be ...	56-314	1.07e-04

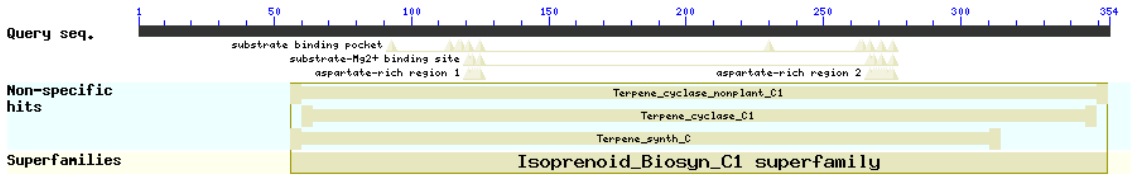


Figure S21. Conserved domains detected in a putative germacrene-*A* synthase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S21. List of domain hits for the germacrene-*A* synthase gene from *Nostoc punctiforme* (sp|B2J4A4 GERAS_NOSP7).

Name	Accession	Description	Interval	E-Value
Terpene_cyclase_nonplant_C1	cd00687	Non-plant Terpene Cyclases, Class 1; This CD includes terpenoid cyclases such as pentalenene ...	10–309	3.27e-123
Terpene_cyclase_C1	cd00868	Terpene cyclases, Class 1; Terpene cyclases, Class 1 (C1) of the class 1 family of isoprenoid ...	23–305	7.93e-51
Terpene_synth_C	pfam03936	Terpene synthase family, metal binding domain; It has been suggested that this gene family be ...	31–264	1.87e-20

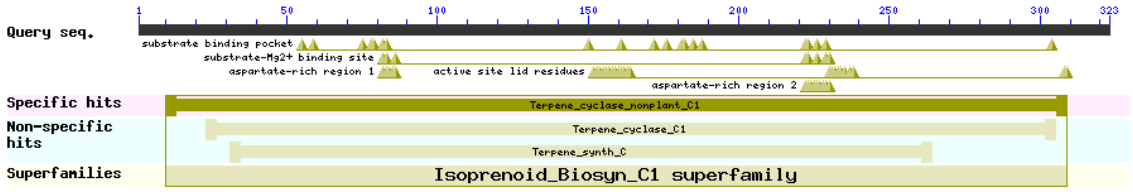


Figure S22. Conserved domains detected in the germacrene-A synthase gene from *Nostoc punctiforme* (sp|B2J4A4 GERAS_NOSP7) (edited from the NCBI CDD database, [1]).

Table S22. List of domain hits for a putative germacrene *A* oxidase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
p450	pfam00067	Cytochrome P450; Cytochrome P450s are haem-thiolate proteins involved in the oxidative ...	367–564	1.03e-44
CypX	COG2124	Cytochrome P450 [Secondary metabolites biosynthesis, transport, and catabolism]	367–535	3.07e-31
PLN02687	PLN02687	flavonoid 3′-monooxygenase	370–531	6.05e-21
PTZ00404	PTZ00404	cytochrome P450; Provisional	370–539	3.35e-18
PLN02738	PLN02738	carotene beta-ring hydroxylase	370–534	1.21e-17

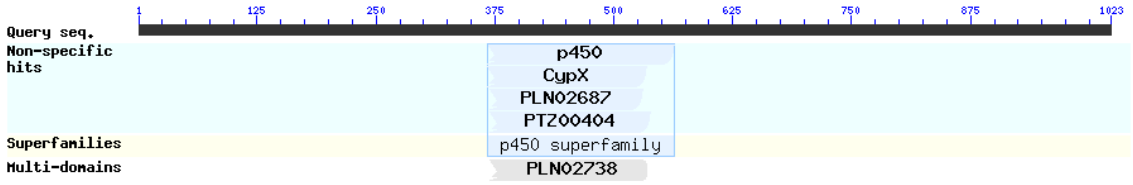


Figure S23. Conserved domains detected in a putative germacrene *A* oxidase gene from *L. dendroidea* (edited from the NCBI CDD database [1]).

Table S23. List of domain hits for the germacrene *A* oxidase gene from *Cichorium intybus* (sp|D5JBW8|GAO_CICIN).

Name	Accession	Description	Interval	E-value
PLN02687	PLN02687	flavonoid 3′-monooxygenase	5–486	4.19e-134
p450	pfam00067	Cytochrome P450; Cytochrome P450s are haem-thiolate proteins involved in the oxidative ...	33–460	7.76e-99
PTZ00404	PTZ00404	cytochrome P450; Provisional	5–477	1.31e-56
CypX	COG2124	Cytochrome P450 [Secondary metabolites biosynthesis, transport, and catabolism]	67–460	1.34e-38
PLN02738	PLN02738	carotene beta-ring hydroxylase	31–487	6.67e-29

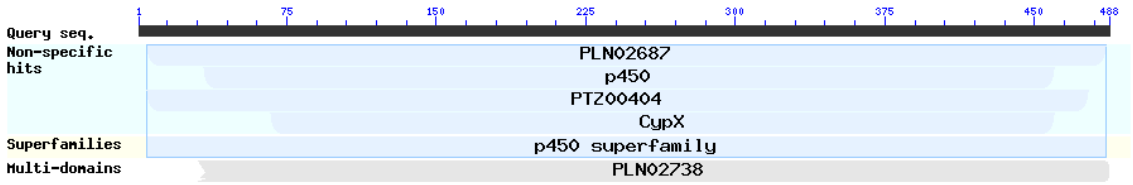


Figure S24. Conserved domains detected in the germacrene *A* oxidase gene from *Cichorium intybus* (sp|D5JBW8|GAO_CICIN) (edited from the NCBI CDD database, [1].

Table S24. List of domain hits for a putative aristolochene synthase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
Terpene_cyclase_nonplant_C1	cd00687	Non-plant Terpene Cyclases, Class 1; This CD includes terpenoid cyclases such as pentalenene	56–353	8.36e-25
Terpene_cyclase_C1	cd00868	Terpene cyclases, Class 1; Terpene cyclases, Class 1 (C1) of the class 1 family of isoprenoid	60–349	2.45e-11
Terpene_synth_C	pfam03936	Terpene synthase family, metal binding domain; It has been suggested that this gene family be	56–314	1.07e-04

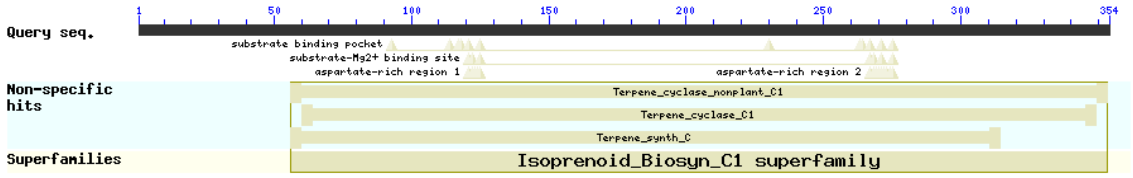


Figure S25. Conserved domains detected in a putative aristolochene synthase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S25. List of domain hits for the aristolochene synthase gene from *Aspergillus terreus* (sp|Q9UR08 ARIS_ASPTE).

Name	Accession	Description	Interval	E-Value
Terpene_cyclase_nonplant_C1	cd00687	Non-plant Terpene Cyclases, Class 1; This CD includes terpenoid cyclases such as pentalenene	18–312	1.26e-98
Terpene_cyclase_C1	cd00868	Terpene cyclases, Class 1; Terpene cyclases, Class 1 (C1) of the class 1 family of isoprenoid	28–308	6.00e-32
Terpene_synth_C	pfam03936	Terpene synthase family, metal binding domain; It has been suggested that this gene family be	21–269	2.55e-30
Isoprenoid_Biosyn_C1	cd00385	Isoprenoid Biosynthesis enzymes, Class 1; Superfamily of trans-isoprenyl diphosphate synthases	63–280	6.29e-09

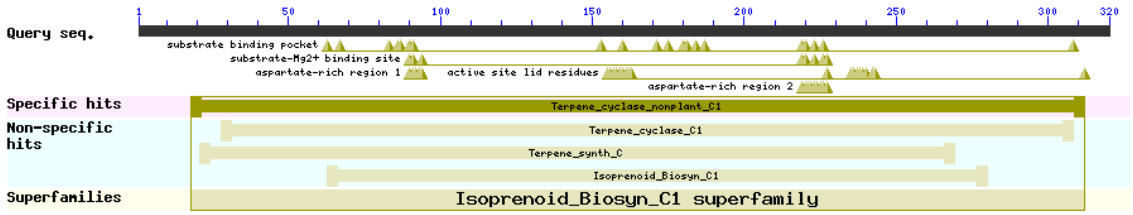


Figure S26. Conserved domains detected in the aristolochene synthase gene from *Aspergillus terreus* (sp|Q9UR08 ARIS_ASPTE) (edited from the NCBI CDD database, [1]).

Table S26. List of domain hits for a putative 5-epiaristolochene 1,3-dihydroxylase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
p450	pfam00067	Cytochrome P450; Cytochrome P450s are haem-thiolate proteins involved in the oxidative ...	367–564	1.10e-44
CypX	COG2124	Cytochrome P450 [Secondary metabolites biosynthesis, transport, and catabolism]	367–535	3.30e-31
PLN02687	PLN02687	flavonoid 3′-monooxygenase	370–531	5.92e-21

PLN02183	PLN02183	ferulate 5-hydroxylase	367–530	2.79e-20
PLN02302	PLN02302	ent-kaurenoic acid oxidase	384–551	1.27e-19
PLN02966	PLN02966	cytochrome P450 83A1	367–548	3.18e-19
PLN02394	PLN02394	trans-cinnamate 4-monooxygenase	373–521	8.60e-19
PLN02655	PLN02655	ent-kaurene oxidase	371–517	2.94e-18
PTZ00404	PTZ00404	cytochrome P450; Provisional	370–539	3.54e-18
PLN03195	PLN03195	fatty acid omega-hydroxylase; Provisional	367–529	1.14e-17
PLN03234	PLN03234	cytochrome P450 83B1; Provisional	367–526	1.40e-16
PLN02290	PLN02290	cytokinin trans-hydroxylase	373–540	5.60e-16
PLN02936	PLN02936	epsilon-ring hydroxylase	370–529	6.75e-16
PLN02196	PLN02196	abscisic acid 8′-hydroxylase	367–538	1.04e-15
PLN00110	PLN00110	flavonoid 3′,5′-hydroxylase (F3′5′H); Provisional	367–521	1.60e-15
PLN02169	PLN02169	fatty acid (omega-1)-hydroxylase/midchain alkane hydroxylase	367–537	4.63e-15
PLN02426	PLN02426	cytochrome P450, family 94, subfamily C protein	367–545	6.97e-15
PLN02500	PLN02500	cytochrome P450 90B1	413–536	3.43e-14
PLN03112	PLN03112	cytochrome P450 family protein; Provisional	370–524	9.26e-13
PLN02987	PLN02987	Cytochrome P450, family 90, subfamily A	367–536	1.95e-12
PLN03141	PLN03141	3-epi-6-deoxocathasterone 23-monooxygenase; Provisional	365–535	4.81e-09
PLN02774	PLN02774	brassinosteroid-6-oxidase	367–524	5.38e-09
PLN00168	PLN00168	Cytochrome P450; Provisional	371–525	2.91e-08
PLN03018	PLN03018	homomethionine *N*-hydroxylase	384–520	4.02e-08
PLN02971	PLN02971	tryptophan *N*-hydroxylase	370–522	4.17e-08
PLN02648	PLN02648	allene oxide synthase	412–489	8.49e-08
PLN02738	PLN02738	carotene beta-ring hydroxylase	370–534	1.28e-17

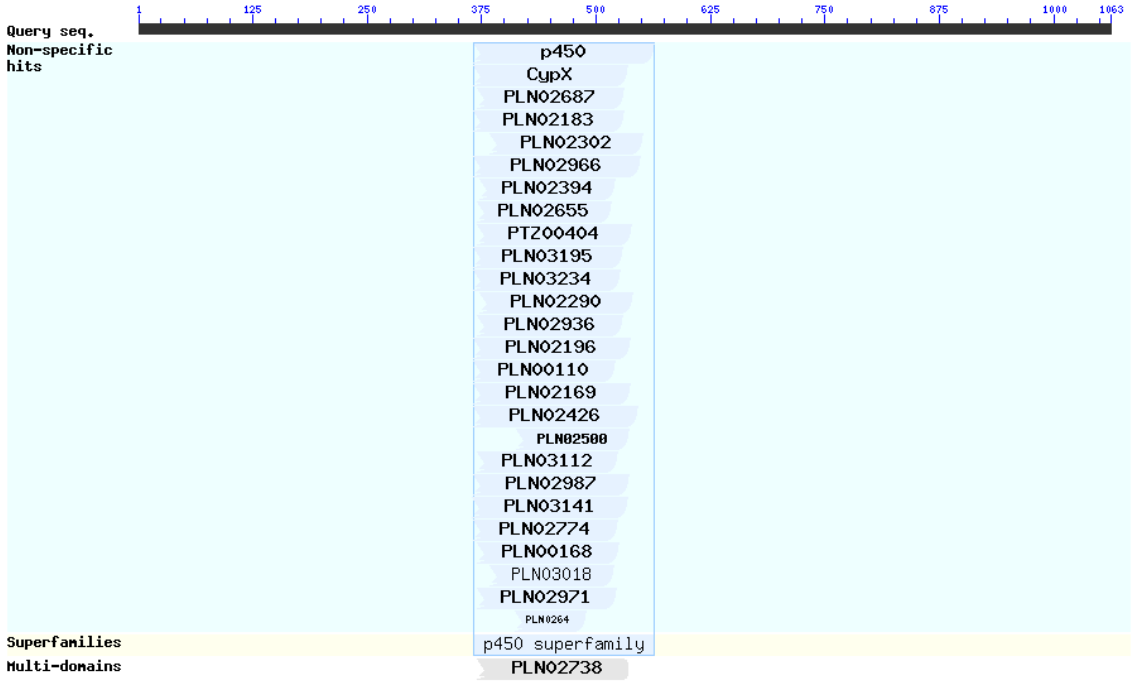


Figure S27. Conserved domains detected in a putative 5-epiaristolochene 1,3-dihydroxylase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S27. List of domain hits for the 5-epiaristolochene 1,3-dihydroxylase gene from *Nicotiana tabacum* (sp|Q94FM7|C71DK_TOBAC).

Name	Accession	Description	Interval	E-Value
PLN02183	PLN02183	ferulate 5-hydroxylase	33–488	9.91e-128
PLN02687	PLN02687	flavonoid 3′-monooxygenase	30–498	5.87e-123
PLN03112	PLN03112	cytochrome P450 family protein; Provisional	4–498	6.11e-114

p450	pfam00067	Cytochrome P450; Cytochrome P450s are haem-thiolate proteins involved in the oxidative ...	33–494	2.34e-109
PLN03234	PLN03234	cytochrome P450 83B1; Provisional	23–499	4.35e-109
PLN02966	PLN02966	cytochrome P450 83A1	22–494	2.38e-103
PLN00110	PLN00110	flavonoid 3′,5′-hydroxylase (F3′5′H); Provisional	29–498	2.76e-102
PLN02394	PLN02394	trans-cinnamate 4-monooxygenase	31–483	1.57e-81
PLN02971	PLN02971	tryptophan *N*-hydroxylase	25–468	5.45e-60
PLN02655	PLN02655	ent-kaurene oxidase	34–498	8.16e-59
PLN00168	PLN00168	Cytochrome P450; Provisional	20–466	4.94e-57
PLN03018	PLN03018	homomethionine *N*-hydroxylase	25–477	8.67e-54
PTZ00404	PTZ00404	cytochrome P450; Provisional	24–486	1.12e-53
CypX	COG2124	Cytochrome P450 [Secondary metabolites biosynthesis, transport, and catabolism]	33–470	4.01e-43
PLN02936	PLN02936	epsilon-ring hydroxylase	62–497	5.84e-36
PLN02302	PLN02302	ent-kaurenoic acid oxidase	234–463	4.10e-28
PLN03195	PLN03195	fatty acid omega-hydroxylase; Provisional	27–463	5.47e-27
PLN02290	PLN02290	cytokinin trans-hydroxylase	219–463	1.48e-25
PLN02987	PLN02987	Cytochrome P450, family 90, subfamily A	31–465	2.75e-23
PLN02196	PLN02196	abscisic acid 8′-hydroxylase	3–467	2.78e-21
PLN02774	PLN02774	brassinosteroid-6-oxidase	233–463	1.42e-20
PLN02169	PLN02169	fatty acid (omega-1)-hydroxylase/midchain alkane hydroxylase	230–470	3.80e-20
PLN03141	PLN03141	3-epi-6-deoxocathasterone 23-monooxygenase; Provisional	234–465	1.83e-18
PLN02426	PLN02426	cytochrome P450, family 94, subfamily C protein	122–444	3.98e-15
PLN02500	PLN02500	cytochrome P450 90B1	233–467	2.22e-14
PLN02648	PLN02648	allene oxide synthase	331–418	7.72e-06
PLN02738	PLN02738	carotene beta-ring hydroxylase	269–497	3.46e-34

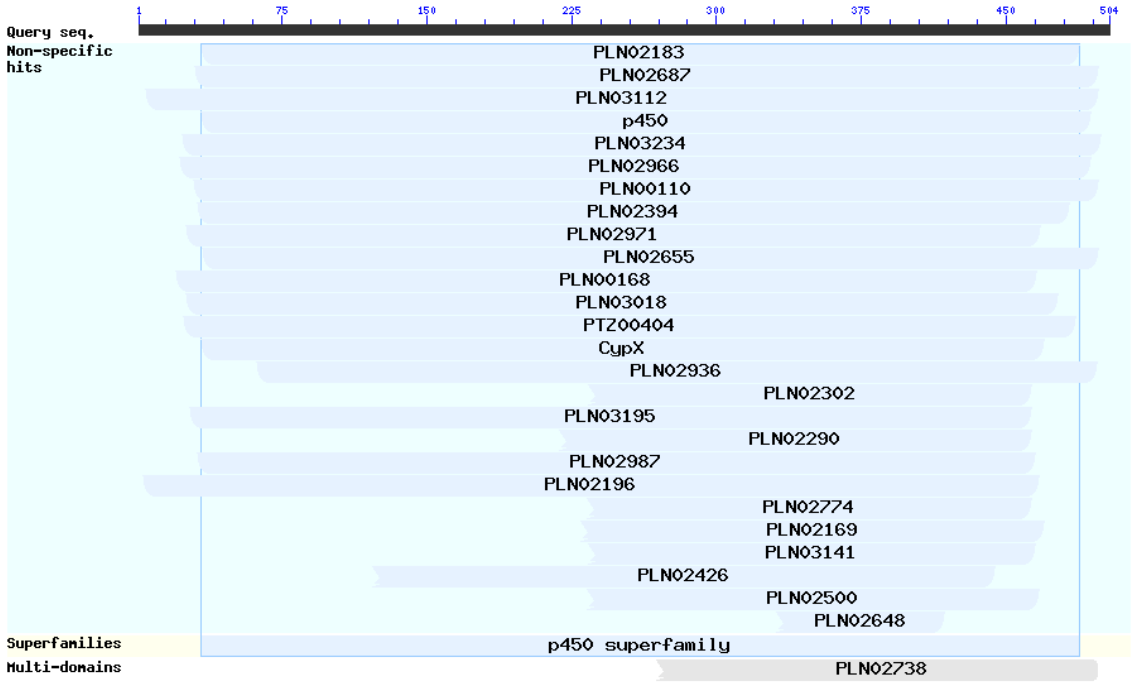


Figure S28. Conserved domains detected in the 5-epiaristolochene 1,3-dihydroxylase gene from *Nicotiana tabacum* (sp|Q94FM7|C71DK_TOBAC) (edited from the NCBI CDD database, [1]).

Table S28. List of domain hits for a putative pentalenene synthase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-value
Terpene_cyclase_nonplant_C1	cd00687	Non-plant Terpene Cyclases, Class 1; This CD includes terpenoid cyclases such as pentalenene ...	11–298	3.90e-21
Terpene_cyclase_C1	cd00868	Terpene cyclases, Class 1; Terpene cyclases, Class 1 (C1) of the class 1 family of isoprenoid ...	15–282	4.15e-09
Terpene_synth_C	pfam03936	Terpene synthase family, metal binding domain; It has been suggested that this gene family be ...	15–262	2.96e-04

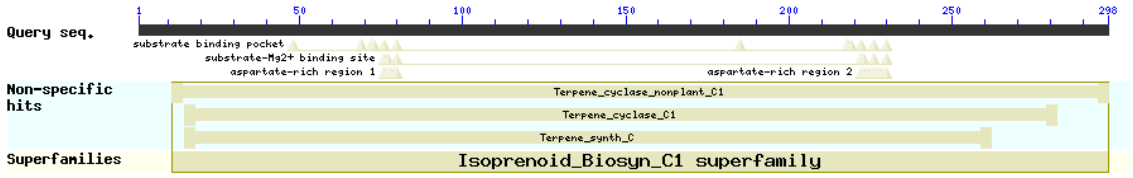


Figure S29. Conserved domains detected in a putative pentalenene synthase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S29. List of domain hits for the pentalenene synthase gene from *Streptomyces exfoliatus* (sp|Q55012 PENA_STREX).

Name	Accession	Description	Interval	E-Value
Terpene_cyclase_nonplant_C1	cd00687	Non-plant Terpene Cyclases, Class 1; This CD includes terpenoid cyclases such as pentalenene ...	8–313	2.37e-108
Terpene_cyclase_C1	cd00868	Terpene cyclases, Class 1; Terpene cyclases, Class 1 (C1) of the class 1 family of isoprenoid ...	19–309	4.49e-41
Terpene_synth_C	pfam03936	Terpene synthase family, metal binding domain; It has been suggested that this gene family be ...	29–265	1.57e-34
Isoprenoid_Biosyn_C1	cd00385	Isoprenoid Biosynthesis enzymes, Class 1; Superfamily of trans-isoprenyl diphosphate synthases ...	72–292	9.90e-10

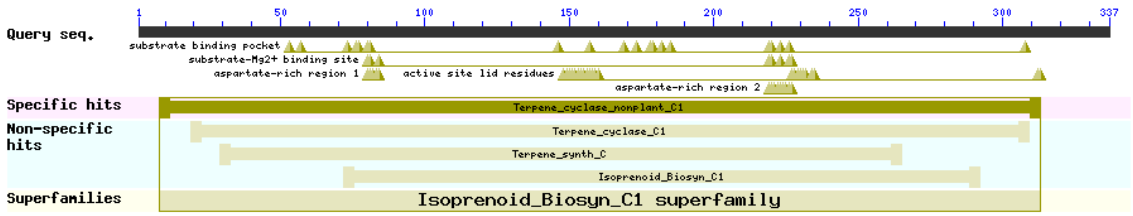


Figure S30. Conserved domains detected in the pentalenene synthase gene from *Streptomyces exfoliatus* (sp|Q55012 PENA_STREX) (edited from the NCBI CDD database, [1]).

Table S30. List of domain hits for a putative zerumbone synthase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
SDR_c	cd05233	classical (c) SDRs; SDRs are a functionally diverse family of oxidoreductases that have a ...	44–275	1.22e-32
fabG	PRK06550	3-ketoacyl-(acyl-carrier-protein) reductase; Provisional	41–233	4.23e-17
adh_short_C2	pfam13561	Enoyl-(Acyl carrier protein) reductase;	51–278	1.00e-07
PKS_KR	smart00822	This enzymatic domain is part of bacterial polyketide synthases; It catalyses the first step ...	42–132	1.88e-05
FabG	COG1028	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases) ...	39–278	2.19e-34
fabG	PRK05565	3-ketoacyl-(acyl-carrier-protein) reductase; Provisional	38–242	7.99e-26
PHB_DH	TIGR01963	3-hydroxybutyrate dehydrogenase; This model represents a subfamily of the short chain ...	41–279	1.08e-22
adh_short	pfam00106	short chain dehydrogenase; This family contains a wide variety of dehydrogenases.	42–209	1.95e-16
PLN02253	PLN02253	xanthoxin dehydrogenase	36–224	2.44e-13
secoisolariciresinol-DH_like_SDR_c	cd05326	secoisolariciresinol dehydrogenase (secoisolariciresinol-DH)-like, classical (c) SDRs;	38–225	1.86e-15

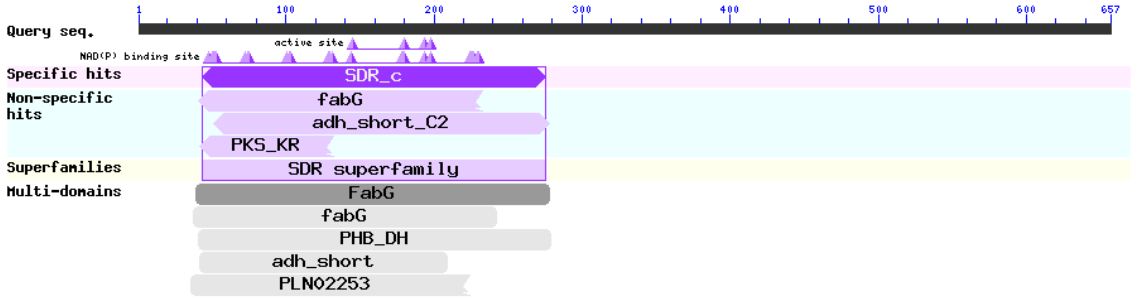


Figure S31. Conserved domains detected in a putative zerumbone synthase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S31. List of domain hits for the zerumbone synthase gene from *Zingiber zerumbet* (sp|F1SWA0|ZERSY_ZINZE).

Name	Accession	Description	Interval	E-Value
secoisolariciresinol-DH_like_SDR_c	cd05326	secoisolariciresinol dehydrogenase (secoisolariciresinol-DH)-like, classical (c) SDRs; ...	2–257	5.98e-118
PRK07069	PRK07069	short chain dehydrogenase; Validated	8–257	5.07e-52
adh_short_C2	pfam13561	Enoyl-(Acyl carrier protein) reductase;	12–257	2.29e-27
PKS_KR	smart00822	This enzymatic domain is part of bacterial polyketide synthases; It catalyses the first step ...	6–151	2.29e-08
PLN02730	PLN02730	enoyl-[acyl-carrier-protein] reductase	122–255	1.58e-04
PLN02253	PLN02253	xanthoxin dehydrogenase	2–267	1.11e-165
fabG	PRK07231	3-ketoacyl-(acyl-carrier-protein) reductase; Provisional	1–258	4.45e-76

FabG	COG1028	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases) ...	1–257	9.62e-59
3oxo_ACP_reduc	TIGR01830	3-oxoacyl-(acyl-carrier-protein) reductase; This model represents 3-oxoacyl-[ACP] reductase, ...	8–255	2.40e-55
adh_short	pfam00106	short chain dehydrogenase; This family contains a wide variety of dehydrogenases.	6–173	4.49e-30

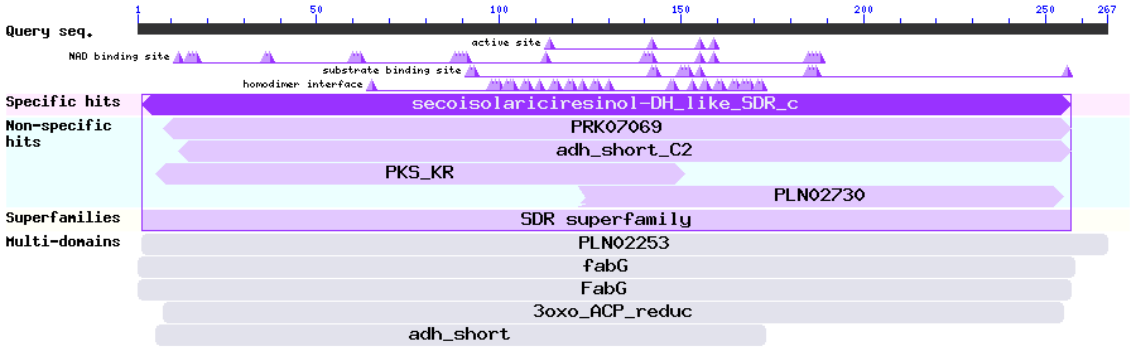


Figure S32. Conserved domains detected in the zerumbone synthase gene from *Zingiber zerumbet* (sp|F1SWA0|ZERSY_ZINZE) (edited from the NCBI CDD database, [1]).

Table S32. List of domain hits for a putative gibberelin 20-oxidase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
DIOX_N	pfam14226	non-haem dioxygenase in morphine synthesis *N*-terminal; This is the highly conserved *N*-terminal ...	2129–2401	2.84e-12
2OG-FeII_Oxy	pfam03171	2OG-Fe(II) oxygenase superfamily; This family contains members of the 2-oxoglutarate (2OG) and ...	1616–1954	7.56e-09
PTZ00273	PTZ00273	oxidase reductase; Provisional	1508–2443	8.96e-24
PcbC	COG3491	Isopenicillin N synthase and related dioxygenases [General function prediction only]	1595–2419	5.94e-29
PLN02485	PLN02485	Oxidoreductase	1511–2497	2.50e-19
PLN03002	PLN03002	oxidoreductase, 2OG-Fe(II) oxygenase family protein	1508–2362	6.26e-18
PLN02750	PLN02750	oxidoreductase, 2OG-Fe(II) oxygenase family protein	1517–2443	1.70e-12
PLN02393	PLN02393	leucoanthocyanidin dioxygenase like protein	1529–2521	1.25e-11
PLN02276	PLN02276	gibberellin 20-oxidase	1598–2497	1.94e-10

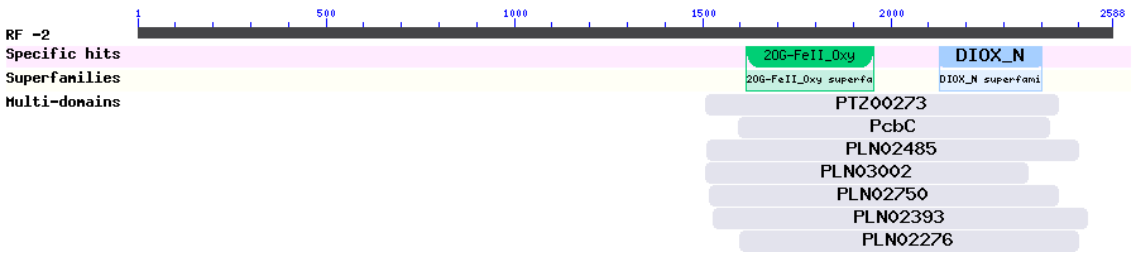


Figure S33. Conserved domains detected in a putative gibberelin 20-oxidase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S33. List of domain hits for the gibberelin 20-oxidase gene from *Arabidopsis thaliana* (gi|60390168|sp|Q39112.1|GAOX3_ARATH).

Name	Accession	Description	Interval	E-value
2OG-FeII_Oxy	pfam03171	2OG-Fe(II) oxygenase superfamily; This family contains members of the 2-oxoglutarate (2OG) and ...	222–321	3.06e-39
DIOX_N	pfam14226	non-haem dioxygenase in morphine synthesis *N*-terminal; This is the highly conserved *N*-terminal ...	58–160	3.86e-21
PLN03176	PLN03176	flavanone-3-hydroxylase; Provisional	41–137	1.49e-05
PLN02276	PLN02276	gibberellin 20-oxidase	17–380	0e+00
PLN02393	PLN02393	leucoanthocyanidin dioxygenase like protein	37–334	9.01e-65
PLN02254	PLN02254	gibberellin 3-beta-dioxygenase	22–360	7.45e-62
PLN03178	PLN03178	leucoanthocyanidin dioxygenase; Provisional	37–354	5.59e-61
PLN02912	PLN02912	oxidoreductase, 2OG-Fe(II) oxygenase family protein	36–370	4.36e-60
PLN02639	PLN02639	oxidoreductase, 2OG-Fe(II) oxygenase family protein	26–350	4.80e-58
PLN02758	PLN02758	oxidoreductase, 2OG-Fe(II) oxygenase family protein	37–360	6.27e-57
PLN02750	PLN02750	oxidoreductase, 2OG-Fe(II) oxygenase family protein	37–353	3.35e-53
PcbC	COG3491	Isopenicillin *N* synthase and related dioxygenases [General function prediction only]	54–361	5.21e-52

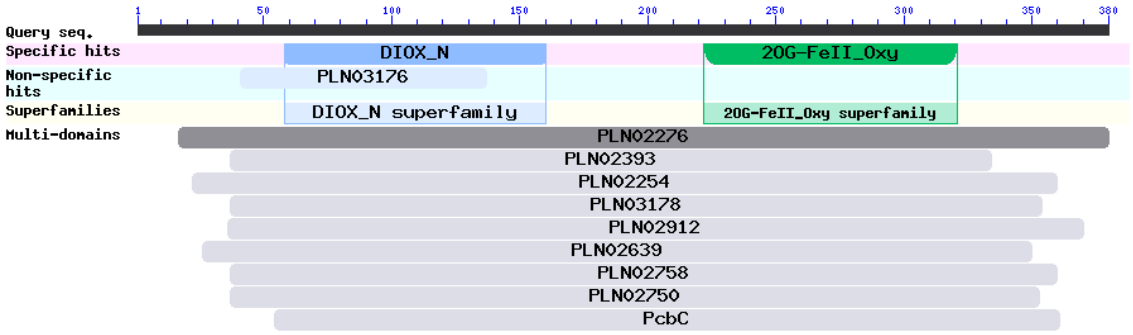


Figure S34. Conserved domains detected in the gibberelin 20-oxidase gene from *Arabidopsis thaliana* (gi|60390168|sp|Q39112.1|GAOX3_ARATH) (edited from the NCBI CDD database, [1]).

Table S34. List of domain hits for a putative gibberelin 2-oxidase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
DIOX_N	pfam14226	non-haem dioxygenase in morphine synthesis *N*-terminal; This is the highly conserved *N*-terminal ...	97–471	4.75e-09
2OG-FeII_Oxy	pfam03171	2OG-Fe(II) oxygenase superfamily; This family contains members of the 2-oxoglutarate (2OG) and ...	610–927	4.13e-07
PTZ00273	PTZ00273	oxidase reductase; Provisional	94–1035	1.91e-32
PcbC	COG3491	Isopenicillin *N* synthase and related dioxygenases [General function prediction only]	85–948	1.05e-32
PLN02485	PLN02485	oxidoreductase	91–873	1.63e-15
PLN03002	PLN03002	oxidoreductase, 2OG-Fe(II) oxygenase family protein	214–1056	2.60e-09

PLN02156	PLN02156	gibberellin 2-beta-dioxygenase	79–873	3.20e-08

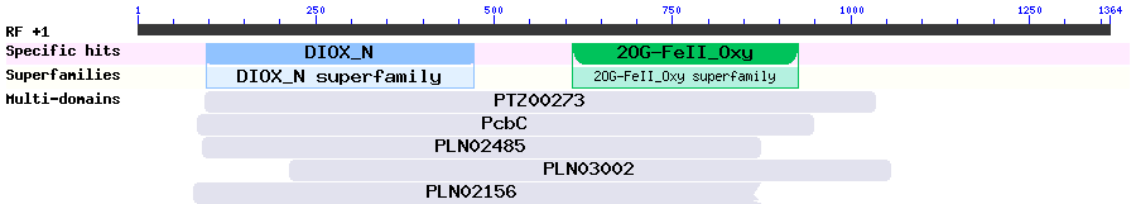


Figure S35. Conserved domains detected in a putative gibberelin 2-oxidase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S35. List of domain hits for the the gibberelin 2-oxidase gene from *Arabidopsis thaliana*.

Name	Accession	Description	Interval	E-Value
2OG-FeII_Oxy	pfam03171	2OG-Fe(II) oxygenase superfamily; This family contains members of the 2-oxoglutarate (2OG) and ...	157–269	3.98e-28
DIOX_N	pfam14226	non-haem dioxygenase in morphine synthesis *N*-terminal; This is the highly conserved *N*-terminal ...	15–108	9.31e-20
PLN03176	PLN03176	flavanone-3-hydroxylase; Provisional	9–71	3.40e-09
PcbC	COG3491	Isopenicillin *N* synthase and related dioxygenases [General function prediction only]	13–309	1.14e-49
PLN02156	PLN02156	gibberellin 2-beta-dioxygenase	15–316	7.18e-98

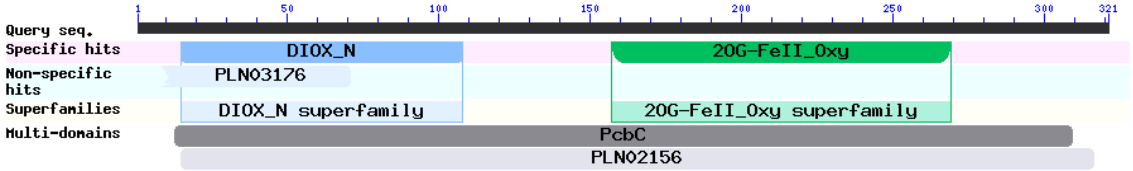


Figure S36. Conserved domains detected in the gibberelin 2-oxidase gene from *Arabidopsis thaliana* (gi|75308865|sp|Q9C7Z1.1|G2OX4_ARATH) (edited from the NCBI CDD database, [1]).

Table S36. List of domain hits for a putative abietadienol/ abietadienal oxidase gene from *L. dendroidea*.

Name	Accession	Description	Interval	E-Value
PLN02936	PLN02936	epsilon-ring hydroxylase	135–620	5.82e-141
p450	pfam00067	Cytochrome P450; Cytochrome P450s are haem-thiolate proteins involved in the oxidative ...	139–613	6.45e-78
CypX	COG2124	Cytochrome P450 [Secondary metabolites biosynthesis, transport, and catabolism]	154–620	2.27e-42
PLN02290	PLN02290	cytokinin trans-hydroxylase	207–618	1.36e-32
PLN03195	PLN03195	fatty acid omega-hydroxylase; Provisional	179–620	3.59e-31
PLN02302	PLN02302	ent-kaurenoic acid oxidase	381–579	1.91e-27
PLN02183	PLN02183	ferulate 5-hydroxylase	394–594	1.27e-24
PLN00110	PLN00110	flavonoid 3′,5′-hydroxylase (F3′5′H); Provisional	382–594	1.17e-23
PLN02655	PLN02655	ent-kaurene oxidase	370–623	1.76e-23
PLN02426	PLN02426	cytochrome P450, family 94, subfamily C protein	387–620	1.04e-22
PLN02687	PLN02687	flavonoid 3′-monooxygenase	326–621	1.89e-22

PLN02394	PLN02394	trans-cinnamate 4-monooxygenase	399–589	3.07e-22
PTZ00404	PTZ00404	cytochrome P450; Provisional	139–597	1.17e-21
PLN03112	PLN03112	cytochrome P450 family protein; Provisional	359–624	1.27e-21
PLN03234	PLN03234	cytochrome P450 83B1; Provisional	129–615	3.95e-21
PLN02169	PLN02169	fatty acid (omega-1)-hydroxylase/midchain alkane hydroxylase	179–621	1.62e-19
PLN02966	PLN02966	cytochrome P450 83A1	372–624	2.70e-18
PLN02987	PLN02987	Cytochrome P450, family 90, subfamily A	382–598	3.61e-18
PLN02500	PLN02500	cytochrome P450 90B1	380–597	2.47e-17
PLN02196	PLN02196	abscisic acid 8'-hydroxylase	358–620	1.48e-16
PLN00168	PLN00168	Cytochrome P450; Provisional	145–591	5.93e-16
PLN02774	PLN02774	brassinosteroid-6-oxidase	380–588	1.93e-12
PLN03018	PLN03018	homomethionine N-hydroxylase	386–588	4.15e-11
PLN03141	PLN03141	3-epi-6-deoxocathasterone 23-monooxygenase; Provisional	386–622	1.97e-10
PLN02971	PLN02971	tryptophan *N*-hydroxylase	386–624	1.99e-10
PLN02738	PLN02738	carotene beta-ring hydroxylase	135–633	2.48e-167

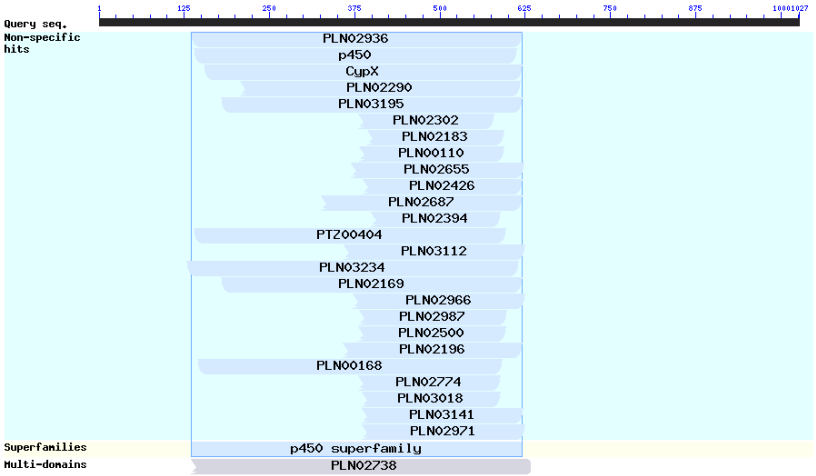


Figure S37. Conserved domains detected in a putative abietadienol/abietadienal oxidase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Table S37. List of domain hits for the abietadienol/abietadienal oxidase gene from *Pinus taeda* (sp|Q50EK6] C72B1_PINTA).

Name	Accession	Description	Interval	E-Value
PLN03141	PLN03141	3-epi-6-deoxocathasterone 23-monooxygenase; Provisional	45–481	4.09e-132
PLN02987	PLN02987	Cytochrome P450, family 90, subfamily A	5–481	1.47e-130
PLN02500	PLN02500	cytochrome P450 90B1	1–477	9.23e-117
PLN02774	PLN02774	brassinosteroid-6-oxidase	5–477	5.51e-104
PLN02302	PLN02302	ent-kaurenoic acid oxidase	5–477	6.93e-82
PLN02196	PLN02196	abscisic acid 8′-hydroxylase	3–477	9.81e-71
p450	pfam00067	Cytochrome P450; Cytochrome P450s are haem-thiolate proteins involved in the oxidative ...	46–454	7.80e-49
CypX	COG2124	Cytochrome P450 [Secondary metabolites biosynthesis, transport, and catabolism]	60–477	3.30e-46
PTZ00404	PTZ00404	cytochrome P450; Provisional	18–452	1.33e-17
PLN02936	PLN02936	epsilon-ring hydroxylase	283–459	3.81e-17
PLN02183	PLN02183	ferulate 5-hydroxylase	46–454	8.12e-17
PLN02290	PLN02290	cytokinin trans-hydroxylase	229–460	1.01e-15
PLN02687	PLN02687	flavonoid 3′-monooxygenase	248–458	5.51e-14
PLN03112	PLN03112	cytochrome P450 family protein; Provisional	8–447	8.67e-13
PLN02966	PLN02966	cytochrome P450 83A1	39–436	1.26e-11
PLN00168	PLN00168	Cytochrome P450; Provisional	239–459	6.04e-09

PLN02426	PLN02426	cytochrome P450, family 94, subfamily C protein	251–481	1.59e-08
PLN03234	PLN03234	cytochrome P450 83B1; Provisional	265–453	2.81e-08
PLN03195	PLN03195	fatty acid omega-hydroxylase; Provisional	3–480	1.77e-07
PLN00110	PLN00110	flavonoid 3′,5′-hydroxylase (F3′5′H); Provisional	254–454	4.65e-07
PLN02655	PLN02655	ent-kaurene oxidase	330–453	5.28e-06
PLN02971	PLN02971	tryptophan *N*-hydroxylase	32–459	6.10e-06
PLN02169	PLN02169	fatty acid (omega-1)-hydroxylase/midchain alkane hydroxylase	264–442	1.52e-05
PLN02394	PLN02394	trans-cinnamate 4-monooxygenase	279–451	2.37e-05
PLN03018	PLN03018	homomethionine *N*-hydroxylase	4–100	2.90e-04
PLN02738	PLN02738	carotene beta-ring hydroxylase	256–461	4.83e-23

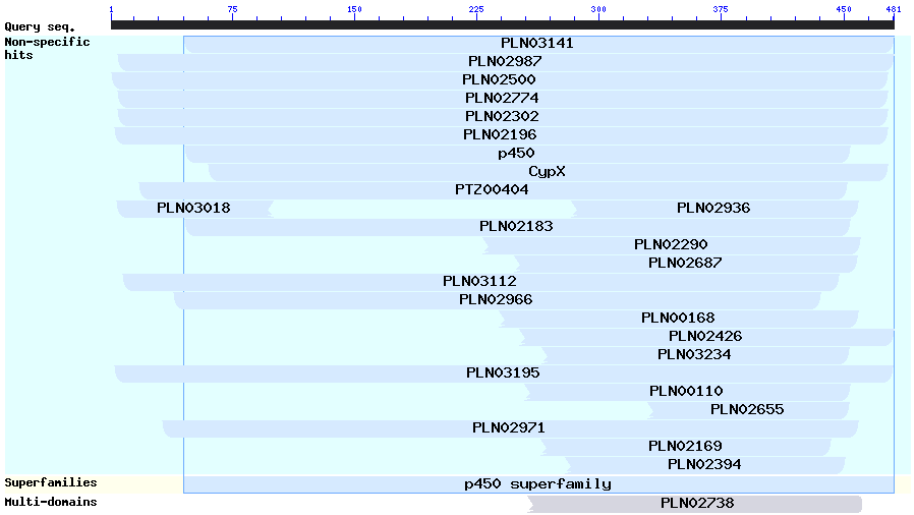
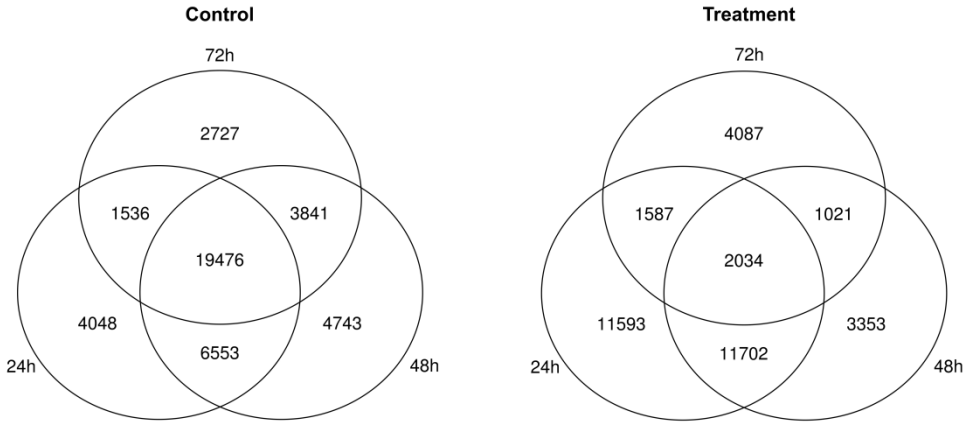


Figure S38. Conserved domains detected in the abietadienol/abietadienal oxidase gene from *Pinus taeda* (sp|Q50EK6] C72B1_PINTA) (edited from the NCBI CDD database, [1]).

References

1. Conserved Domain Database (CDD) Website: http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
2. Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, *32*, 1792c1797.
3. Waterhouse, A.M.; Procter, J.B.; Martin, D.M.A.; Clamp, M.; Barton, G.J. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **2009**, *25*, 1189–1191.

Chapter 4



Supplementary File 1. Number of genes shared among control (left) and inoculated (right) samples of *L. dendroidea*.

Supplementary File 2

The domain composition of sequences from *Laurencia dendroidea* annotated as leucine-rich repeat receptor-like serine/threonine-protein kinase (LRR-RLK) was obtained through search for conserved domains using the NCBI Conserved Domain Database (CDD, [1]). The sequence annotation through Blast against the SwissProt database is also provided. Biochemical and gene cloning approaches are necessary to prove these *in silico* identifications.

Cluster-20798.0 – LRR receptor-like serine/threonine-protein kinase (Query cover 73%; e-value 4e-15; identity 34%; similarity 52%).

Table S1. List of domain hits for a putative LRR receptor-like serine/threonine-protein kinase gene from *L. dendroidea*

Name	Accession	Description	Interval	E-value
LRR_RI	cd00116	Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily. LRRs are 20-29 ...	205-834	1.16e-07
LRR_4	pfam12799	Leucine Rich repeats (2 copies); Leucine rich repeats are short sequence motifs present in a ...	721-858	1.06e-05
LRR_8	pfam13855	Leucine rich repeat;	238-408	2.72e-09
LRR_8	pfam13855	Leucine rich repeat;	664-831	1.70e-05
LRR_8	pfam13855	Leucine rich repeat;	376-549	3.95e-03
PLN00113	PLN00113	leucine-rich repeat receptor-like protein kinase; Provisional	7-849	1.54e-36
LRR	COG4886	Leucine-rich repeat (LRR) protein [Transcription]; linked to 3D-structure	190-852	3.18e-21
PLN00113	PLN00113	leucine-rich repeat receptor-like protein kinase; Provisional	325-876	2.49e-13
PRK15370	PRK15370	E3 ubiquitin-protein ligase SlrP; Provisional	163-870	3.94e-06
PLN03150	PLN03150	hypothetical protein; Provisional	376-705	2.07e-03

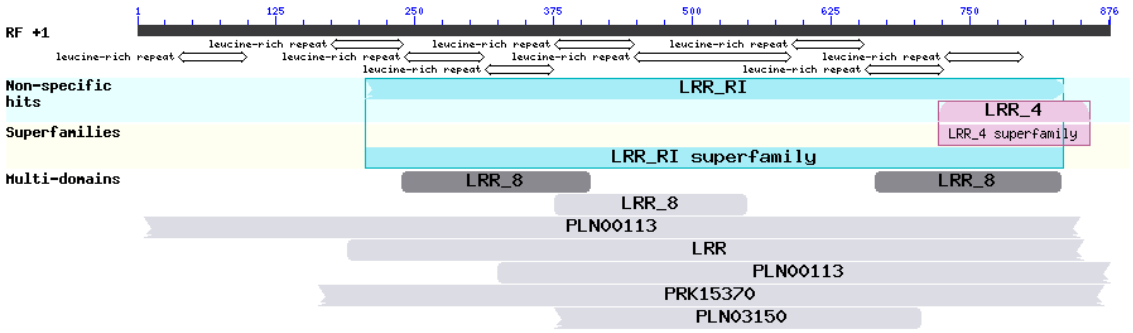


Figure S1. Conserved domains detected in a putative LRR receptor-like serine/threonine-protein kinase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Cluster-7101.0 - LRR receptor-like serine/threonine-protein kinase (Query cover 33%; e-value 2e-18; identity 35%; similarity 53%).

Table S2. List of domain hits for a putative LRR receptor-like serine/threonine-protein kinase gene from *L. dendroidea*

Name	Accession	Description	Interval	E-value
LRR_RI	cd00116	Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily. LRRs are 20-29 ...	458-868	3.92e-05
LRR_8	pfam13855	Leucine rich repeat;	452-637	1.35e-06
LRR_8	pfam13855	Leucine rich repeat;	689-868	5.20e-04
PLN00113	PLN00113	leucine-rich repeat receptor-like protein kinase; Provisional	425-928	4.84e-24
PLN00113	PLN00113	leucine-rich repeat receptor-like protein kinase; Provisional	536-898	3.23e-17
LRR	COG4886	Leucine-rich repeat (LRR) protein [Transcription]; linked to 3D-structure	416-898	2.18e-11

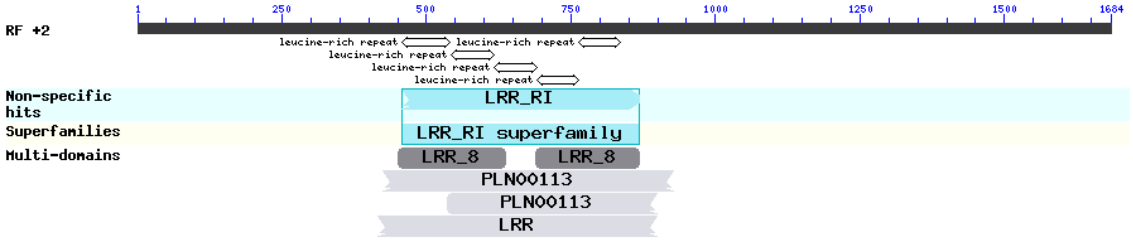


Figure S2. Conserved domains detected in a putative LRR receptor-like serine/threonine-protein kinase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Cluster-3564.0 - LRR receptor-like serine/threonine-protein kinase (Query cover 94%; e-value 9e-23; identity 30%; similarity 51%)

Table S3. List of domain hits for a putative LRR receptor-like serine/threonine-protein kinase gene from *L. dendroidea*

Name	Accession	Description	Interval	E-value
LRR_4	pfam12799	Leucine Rich repeats (2 copies); Leucine rich repeats are short sequence motifs present in a ...	487-594	2.60e-03
LRR_4	pfam12799	Leucine Rich repeats (2 copies); Leucine rich repeats are short sequence motifs present in a ...	415-525	2.62e-03
LRR_8	pfam13855	Leucine rich repeat;	274-522	1.46e-05
LRR_8	pfam13855	Leucine rich repeat;	487-663	9.65e-03
PLN00113	PLN00113	leucine-rich repeat receptor-like protein kinase; Provisional	274-756	3.39e-25
LRR	COG4886	Leucine-rich repeat (LRR) protein [Transcription]; linked to 3D-structure	274-792	1.85e-12
PLN00113	PLN00113	leucine-rich repeat receptor-like protein kinase; Provisional	394-786	5.55e-11

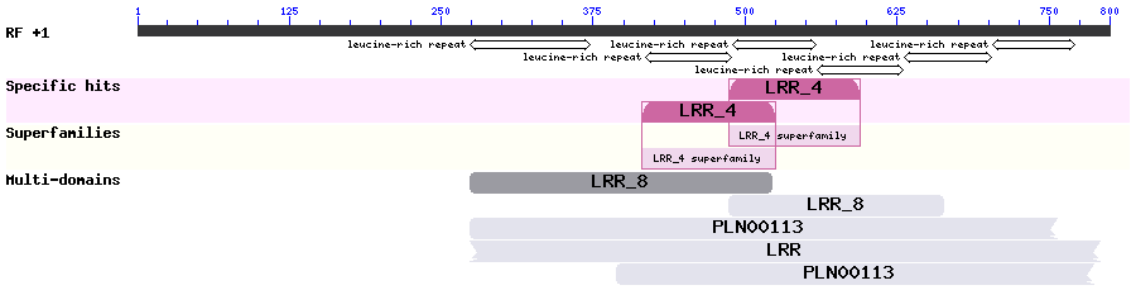


Figure S3. Conserved domains detected in a putative LRR receptor-like serine/threonine-protein kinase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Cluster-18188.0 - LRR receptor-like serine/threonine-protein kinase (Query cover 65%; e-value 2e-07; identity 35%; similarity 50%)

Table S4. List of domain hits for a putative LRR receptor-like serine/threonine-protein kinase gene from *L. dendroidea*

Name	Accession	Description	Interval	E-value
LRR_RI	cd00116	Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily. LRRs are 20-29 ...	3-464	9.31e-05
LRR_4	pfam12799	Leucine Rich repeats (2 copies); Leucine rich repeats are short sequence motifs present in a ...	171-260	1.92e-03
LRR_8	pfam13855	Leucine rich repeat;	219-392	1.06e-05
LRR_8	pfam13855	Leucine rich repeat;	357-527	9.84e-03
PLN00113	PLN00113	leucine-rich repeat receptor-like protein kinase; Provisional	3-497	3.54e-18
LRR	COG4886	Leucine-rich repeat (LRR) protein [Transcription]; linked to 3D-structure	3-527	1.12e-11

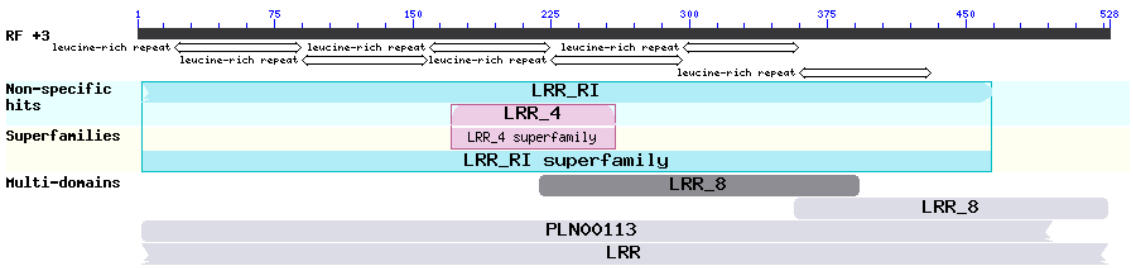


Figure S4. Conserved domains detected in a putative LRR receptor-like serine/threonine-protein kinase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Cluster-6725.0 - LRR receptor-like serine/threonine-protein kinase (Query cover 100%; e-value 6e-21; identity 42%; similarity 58%)

Table S5. List of domain hits for a putative LRR receptor-like serine/threonine-protein kinase gene from *L. dendroidea*

Name	Accession	Description	Interval	E-value
LRR_4	pfam12799	Leucine Rich repeats (2 copies); Leucine rich repeats are short sequence motifs present in a ...	196-309	1.46e-07
LRR_RI	cd00116	Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily. LRRs are 20-29 ...	1-387	4.49e-06
LRR_4	pfam12799	Leucine Rich repeats (2 copies); Leucine rich repeats are short sequence motifs present in a ...	130-243	3.11e-05
LRR	smart00370	Leucine-rich repeats, outliers;	193-258	9.90e-03
LRR_8	pfam13855	Leucine rich repeat;	199-378	1.62e-11
LRR_8	pfam13855	Leucine rich repeat;	55-234	2.84e-06
PLN00113	PLN00113	leucine-rich repeat receptor-like protein kinase; Provisional	1-432	1.42e-31
PLN03150	PLN03150	hypothetical protein; Provisional	22-303	7.02e-19
LRR	COG4886	Leucine-rich repeat (LRR) protein [Transcription]; linked to 3D-structure	1-378	1.43e-12
LRR	COG4886	Leucine-rich repeat (LRR) protein [Transcription]; linked to 3D-structure	1-309	1.62e-07
PLN03150	PLN03150	hypothetical protein; Provisional	1-117	1.12e-03
LRR_8	pfam13855	Leucine rich repeat;	1-90	2.88e-03

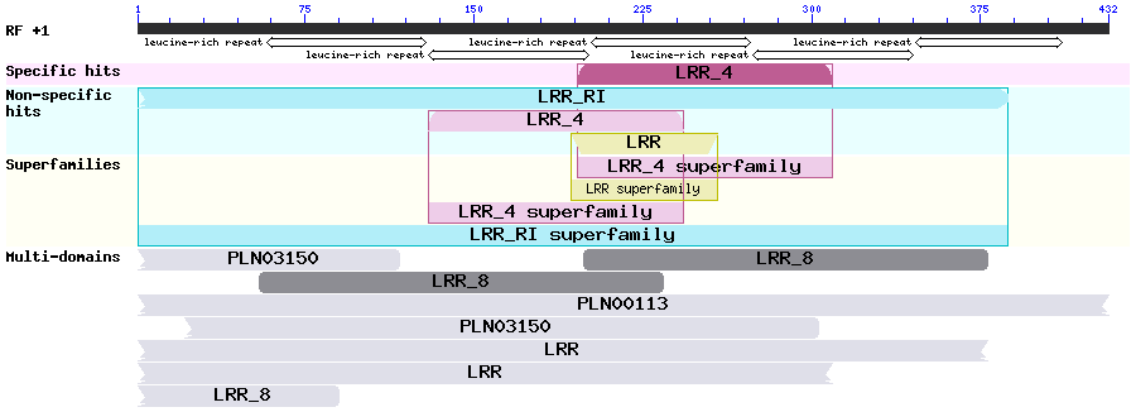


Figure S5. Conserved domains detected in a putative LRR receptor-like serine/threonine-protein kinase gene from *L. dendroidea* (edited from the NCBI CDD database, [1]).

Reference

1. Conserved Domain Database (CDD) Website: http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi