Pathogen exposure leads to a transcriptional downregulation of core cellular functions that may dampen the immune response in a macroalga

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Pathogen exposure leads to a transcriptional downregulation of core cellular functions that may dampen the immune response in a macroalga

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Abstract

Diseases in marine eukaryotic organisms caused by opportunistic pathogens represent a serious threat to our oceans with potential downstream consequences for ecosystem functioning. Disease outbreaks affecting macroalgae are of particular concern due to their critical role as habitat-forming organisms. However, there is limited understanding of the molecular strategies used by macroalgae to respond to opportunistic pathogens. In this study, we used mRNA-sequencing analysis to investigate the early antipathogen response of the model macroalga *Delisea pulchra* (Rhodophyta) under the environmental conditions that are known to promote the onset of disease. Using de novo assembly methods, 27,586 unique transcripts belonging to *D. pulchra* were identified that were mostly affiliated with stress response and signal transduction processes. Differential gene expression analysis between a treatment with the known opportunistic pathogen, *Aquimarina* sp. AD1 (Bacteroidota), and a closely related benign strain (*Aquimarina* sp. AD10) revealed a downregulation of genes coding for predicted protein metabolism, stress response, energy generation and photosynthesis functions. The rapid repression of genes coding for core cellular processes is likely to interfere with the macroalgal antipathogen response, later leading to infection, tissue damage and bleaching symptoms. Overall, this study provides valuable insight into the genetic features of *D. pulchra*, highlighting potential antipathogen response mechanisms of macroalgae and contributing to an improved understanding of host–pathogen interactions in a changing environment.

KEYWORDS
macroalgae, marine disease, RNA-seq, seaweed, transcriptome

1 | INTRODUCTION

Eukaryotic organisms in the marine environment are constantly exposed to a diverse set of microorganisms ranging from beneficial symbionts to harmful pathogens. Bacterial pathogens are widely regarded as a significant threat to the health of marine organisms, having downstream consequences on ecosystem health. In recent years, there has been a rise in the number of reports of disease in

marine eukaryotic hosts and there is evidence to suggest the involvement of opportunistic bacterial pathogens as aetiological agents (Burge et al., 2013; Egan & Gardiner, 2016). Opportunistic bacterial pathogens can exist as commensal organisms in the holobiont, but under certain host or environmental conditions may rapidly colonize and exploit host resources, resulting in tissue damage and disease symptoms (Burge et al., 2013; Casadevall & Pirofski, 2000). However, there is limited understanding of how marine eukaryotic hosts respond to these opportunistic bacteria under the conditions that promote the onset of disease.

Of particular concern are disease outbreaks affecting macroalgae, which are being increasingly reported across both natural and farmed populations (Gachon et al., 2010; Ward et al., 2019). Macroalgae are critical for biodiversity where they provide food and habitat and contribute to primary production in temperate coastal ecosystems. Disease outbreaks can therefore have devastating and far-reaching consequences for community functioning, leading to the long-term decline of coastal ecosystems (Christie et al., 2009; Schiel & Lilley, 2011). To date, research surrounding diseases in macroalgae has predominantly focused on identifying pathogens and their virulence traits (Egan et al., 2014; Fernandes et al., 2011; Gardiner et al., 2015; Hudson et al., 2018, 2019; Schroeder et al., 2003). Some studies have also investigated the antipathogen response strategies of the host in relation to viruses, oomycetes and algal endophytes (Im et al., 2019; Strittmatter et al., 2016; Tang et al., 2019; Xing et al., 2021), but there remains a paucity of knowledge around the response strategies of the host towards bacterial pathogens. Where research has explored the molecular response of algae to bacterial pathogens, evidence for the role of pattern recognition receptors and intracellular signalling cascades has been found (Cosse et al., 2007; de Oliveira et al., 2017; Weinberger, 2007). These immune processes, which are similar to those in higher plants, may assist in pathogen detection and the activation of downstream immune signalling pathways. While these studies have contributed to our understanding of algal defences, we still know comparatively very little about host–pathogen interactions on the molecular level.

Bleaching disease in the red macroalga, *Delisea pulchra* (Bonnemaisonales, Rhodophyta), is one of the few well-characterized examples of disease in macroalgae. This disease is caused by a bacterial infection (Case et al., 2011; Kumar et al., 2016) leading to the loss of photosynthetic pigments along the midthallus, resulting in tissue necrosis, reduced fecundity and increased herbivory (Campbell et al., 2014). Furthermore, the occurrence of bleaching disease is highly correlated with increased seawater temperatures in the summer months, which is thought to reduce the natural chemical defences of the alga and therefore render it more susceptible to microbial infections (Campbell et al., 2011).

Previous investigations have found that members of the Flavobacteriaceae are enriched on bleached *D. pulchra* (Zozaya-Valdes et al., 2017), and the bacterium *Aquimarina* sp. AD1 (Flavobacteriaceae, Bacteroidota) has been identified as a causative agent of bleaching disease in *D. pulchra*. *Aquimarina* sp. AD1 is considered an opportunistic pathogen as it can be found on healthy individuals and only appears to cause disease symptoms on *D. pulchra* under conditions of induced thermal stress (Kumar et al., 2016). Genome analysis of *Aquimarina* sp. AD1 also identified genes involved in the nutritional acquisition of algal cell-wall components that may function as virulence traits, supporting its role as an opportunistic pathogen (Hudson et al., 2019).

The emergence of opportunistic pathogens represents a threat to marine ecosystems which is expected to intensify in the future with growing anthropogenic pressures. Thus, *D. pulchra* and *Aquimarina* sp. AD1 are an ideal model for investigating host–pathogen dynamics in macroalgae. Here we aim to characterize the response of *D. pulchra* using mRNA-sequencing (mRNA-seq) analysis after treatment with the opportunistic pathogen, *Aquimarina* sp. AD1, under the environmental conditions that are known to promote the onset of disease. We further compared this gene expression response to that of a closely related bacterial strain, *Aquimarina* sp. AD10, that does not cause disease in *D. pulchra* (Kumar et al., 2016) in order to assess the specific antipathogen response of the macroalga.

2 | MATERIALS AND METHODS

Twenty-four healthy (i.e., free from bleaching) *Delisea pulchra* individuals (~5 cm in length) were collected from Long Bay, Sydney, Australia, on July 16, 2019. Each sample was prepared and incubated under conditions that were designed to mimic the environmental conditions that have been shown to induce stress and promote the susceptibility of *D. pulchra* to bacterial infection (Campbell et al., 2011; Case et al., 2011; Gardiner et al., 2015; Hudson et al., 2018; Kumar et al., 2016; Li et al., 2021). Briefly, each sample was washed gently in sterile seawater to remove visible epiphytes and transferred to a 25-cm^2 cell culture flask with a vented cap (Nunc, Thermo Fisher Scientific) containing 50 ml sterile bromine deficient artificial seawater (Br-ASW). *Delisea pulchra* was maintained in a Climatron Plant Growth Cabinet (Thermoline Scientific) with gentle shaking at 25°C, with an irradiance of 72 μmol photons m^{-2} s^{-1} under a 14:10-h day/night cycle.

After acclimatizing for 24 h, the Br-ASW was replaced and individual *D. pulchra* were inoculated with either *Aquimarina* sp. AD1 (pathogenic strain), *Aquimarina* sp. AD10 (nonpathogenic strain) or a control (no bacterial inoculation) treatment (hereafter referred to as AD1, AD10 and control, respectively; Figure S1). To prepare the inoculum, AD1 and AD10 were cultured in marine broth (MP Bioscience) overnight to an OD_{600} of 1. Cells were harvested by centrifugation at 3000 *g* for 10 min and washed twice in Br-ASW. The cultures were resuspended to a final OD_{600} of 0.1 and 1 ml was added to the flasks containing *D. pulchra*. Washed marine broth medium was used as the control inoculum. The inoculation and incubation procedure was carried out following the method described by Kumar et al. (2016), which observed the development of bleaching symptoms after 5 days.

To characterize the transcriptional response of *D. pulchra* to pathogen exposure, four replicate samples per treatment were

collected at 24 and 48 h post-inoculation and ground in liquid nitrogen to a fine powder. The ground tissue was stored at −80°C until further use.

2.1 | RNA extraction, mRNA library preparation and sequencing

Total RNA was extracted from 100 mg of ground *D. pulchra* tissue using the Trizol Plus RNA purification kit (Invitrogen) with on-column Purelink DNase treatment (Invitrogen), following the manufacturer's instructions.

The quality and quantity of the extracted RNA were assessed on a Bioanalyzer (Agilent). RNA-seq libraries for each sample were constructed using the TruSeq Stranded mRNA kit (Illumina) and sequenced using a paired-end 100-bp kit on a single flow cell of the Illumina NovaSeq 6000 platform at the Ramaciotti Centre for Genomics at UNSW (Figure S1).

2.2 | De novo transcriptome assembly, filtering and quality assessment

2.2.1 | Assembly

Raw sequencing reads were quality trimmed with TRIM GALORE version 0.6.4 using default parameters (Krueger, 2012) and duplicate reads were removed using CLUMPIFY-DEDUPE (http://sourceforge.net/projects/bbmap/). The transcriptome was assembled de novo using TRINITY version 2.8.4 (Grabherr et al., 2011) with a minimum contig length cut-off of 500 bp. Assemblies were generated on our local HPC server across all treatments and time points and an additional clustering step for merging highly similar contigs (sequence identity threshold of 0.8) was performed using CD-HIT version 4.6.8 (Fu et al., 2012; Li & Godzik, 2006).

2.2.2 | Filtering the *Delisea pulchra* transcriptome from the TRINITY assembly

A reference list of all Rhodophyta species was generated from the NCBI taxonomy browser (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi). The assembled transcriptome was then aligned against the NCBI nonredundant protein database (version 0.8.38) using BLASTX. Only those transcripts with a BLAST bit-score > 150 and with a Rhodophyta-specific annotation were retained as *D. pulchra* transcripts. Transcripts with a BLAST hit to non-Rhodophyta species (bit-score > 150) were filtered out and retained as a "non-Rhodophyta" transcriptome. The remaining transcripts with no BLAST hits (bit-score < 150) were similarly filtered out and retained as a "no blast hit" transcriptome (Figure S1).

The *D. pulchra* transcriptome was quality assessed by calculating the N50 and Ex90N50 values and the number of full-length transcripts were examined against the Swissprot database using the TRINOTATE software (https://github.com/Trinotate/Trinotate.github.io/wiki). The completeness of the assembled transcriptome was examined by determining the proportion of conserved orthologues using the Benchmarking Universal Single Copy Ortholog (BUSCO) tool (version 3.0.2b) against the eukaryotic reference database (Seppey et al., 2019). Similar assembly metrics were calculated for the "non-Rhodophyta" and "no blast hit" assemblies for a comparative analysis.

2.2.3 | Functional annotation

The BLAST2GO (Conesa et al., 2005) annotation and analysis pipeline suite from OMICSBOX (https://www.biobam.com/omicsbox/) was used to functionally annotate and analyse the transcriptome. BLASTX searches against the nonredundant protein database (version 0.8.38) were run independently on local HPC servers and xml2 outputs were imported into the BLAST2GO environment for further analysis. Gene ontology (GO) mapping and annotations were performed within the BLAST2GO pipeline and Pfam annotations were run on local servers and integrated into BLAST2GO. KEGG terms were assigned using the BLASTKOALA online annotation tool (Kanehisa et al., 2016). Carbohydrate active enzymes (CAZymes) were identified using the DBCAN2 meta server (Yin et al., 2012; Zhang, Yohe, et al., 2018) and transcripts encoding signal peptides were identified using the SIGNALP-5.0 server (Armenteros et al., 2019; Nielsen et al., 1997). The tool TRANSDECODER was used to identify coding regions within the assembled transcript sequences (https://github.com/TransDecoder/TransDecoder/wiki).

2.3 | Read mapping and differential gene expression analysis

The quality-trimmed sequence reads were mapped back to the de novo assembled Rhodophyta-specific transcriptome using SALMON version 0.14.1 (Patro et al., 2017) with quasi mapping, and a gene expression matrix was constructed. Genes with expression levels of < 1 count per million (CPM) in fewer than half of the samples (within a specific comparison) were removed. Differences between treatments and time points were assessed using nonmetric multidimensional scaling (nMDS) ordination and permutational multivariate analysis of variance (PERMANOVA) (see Supporting Information Methods).

Differential gene expression analysis was performed in the R programming environment (version 3.6.2) using the BIOCONDUCTOR package RUVSEQ (version 1.20.0; Risso et al., 2014), which removes unwanted variation caused by batch effects using empirical control genes. Read counts were normalized using the between-lane normalization of EDASEQ (version 2.20.0; Risso et al., 2011) with upper quartile normalization. Differentially expressed genes across treatments and time points were identified using the negative binomial

generalized linear model (GLM) approach implemented in EDGER (Risso et al., 2014; Robinson et al., 2010) and genes identified with a false discovery rate (FDR) < 0.05 were considered significantly up- or downregulated.

3 | RESULTS

3.1 | Assembly and quality assessment of the *Delisea pulchra* transcriptome

To generate the *D. pulchra* transcriptome, a de novo assembly approach was required due to the absence of a complete genome from a closely related species. Illumina NovaSeq6000 sequencing yielded between 39.3 and 53.9 million paired-end reads per sample with 99.9% of reads retained after quality filtering (Table S1). TRINITY assembly followed by CD-HIT clustering resulted in an assembled transcriptome of 373,057 transcripts (Table 1). A total of 147,928 transcripts were annotated against the NCBI nonredundant protein database using BLASTX with a bit-score cut-off > 150. Within this subset of annotated transcripts, 27,586 were identified as Rhodophyta-specific and were considered *D. pulchra* sequences for downstream analysis (Table 1).

Delisea pulchra sequences ranged in length from 501 to 40,198 nucleotides (Figure 1a) with an average length of 1,992.23 nucleotides and an N50 value of 2873. A total of 3106 proteins were represented by nearly full-length transcripts having > 80% alignment coverage (Table 1). BUSCO analysis identified 59 complete single-copy orthologues and 222 complete duplicated orthologues in the transcriptome, representing 92.8% of eukaryotic single-copy orthologues. This result is consistent with, if not higher than, the number of BUSCOs reported in other de novo assembled transcriptomes of red algae such as *Lithophyllum* cf. *insipidum* (93%) and *Lithothamnion* cf. *proliferum* (87%) (Page et al., 2019) and higher than the assembled genomes of *Chondrus crispus* (77%) and *Gracilariopsis chorda* (91%) (Page et al., 2019).

The *Delisea pulchra* genes were annotated to a putative function according to their top BLASTX hit, with the majority (69%) exhibiting a best hit to *Gracilariopsis chorda* followed by *Chondrus crispus* (25%) (Figure 1b). Over 15,000 transcripts were annotated with KEGG terms (Table 2; Table S2) with 7893 transcripts assigned to the KEGG pathways for translation, folding sorting and degradation, carbohydrate metabolism, energy metabolism, and signal transduction (Figure 2).

The "non-Rhodophyta" component of the assembly comprised 120,341 transcripts derived from a range of species, predominantly from the phyla Amoebozoa, Bacillariophyta and Euglenozoa and the Opisthokonta taxonomic group (Figure S2A). The inferior values of the "non-Rhodophyta" assembly metrices when compared to the *D. pulchra* assembly (notably N50 and mean contig length) further highlighted the quality of the *D. pulchra* assembly (Table 1; Figure S2B). Furthermore, 225,130 transcripts did not receive a BLAST hit (bit-score < 150) against the nonredundant protein database (Table 1). The "no blast hit" assembly largely consisted of short contigs (Figure S2C), which could either represent transcript fragments or misassemblies displaying the lowest quality assembly metrics (Table 1).

3.2 | Differential gene expression analysis

An nMDS ordination and PERMANOVA did not identify any significant overall differences between treatments ($p > .05$) or significant differences in dispersion between samples (Betadisper $p = .099$) (Figure S3, Table S3). Although no significant GO enrichment was observed, when looking at the expression patterns of individual genes, differences between treatments were detected (FDR < 0.05). We first focused on the genes that were differentially expressed (DEGs) between treatment with either of the *Aquimarina* strains and the control treatment to determine if *D. pulchra* elicits a transcriptional response to these epiphytic bacteria. After 24 h of exposure, 70 genes were differentially expressed (FDR < 0.05) in response to AD1 relative to the control treatment (Figure 3a; Table S4). Almost half (47%) of these genes were upregulated in response to the pathogen treatment and predominantly coded for transposable elements

TABLE 1 Transcriptome assembly metrics for all assembled transcripts, *Delisea pulchra*-specific transcripts, non-Rhodophyta-specific transcripts and reads with no BLAST hi

	All transcripts	*Delisea pulchra*	Non-Rhodophyta	No BLAST hit
Total unigenes	373,057	27,586	120,341	225,130
Total assembled bases	364,151,223	54,957,684	127,096,296	182,097,243
Contig N50	989	2873	1134	782
Contig Ex90N50	1422	8852	NA	NA
Mean contig length (bp)	976.11	1992.23	1056.13	808.85
Longest contig (bp)	40,198	40,198	17,447	15,845
Shortest contig (bp)	501	501	501	501
GC%	49.76	52.02	49.01	49.61
Total complete BUSCOs (%)	96.7	92.8	NA	NA
Number of full-length transcripts	14,106	3106	11,341	2091

Abbreviation: NA, not applicable.

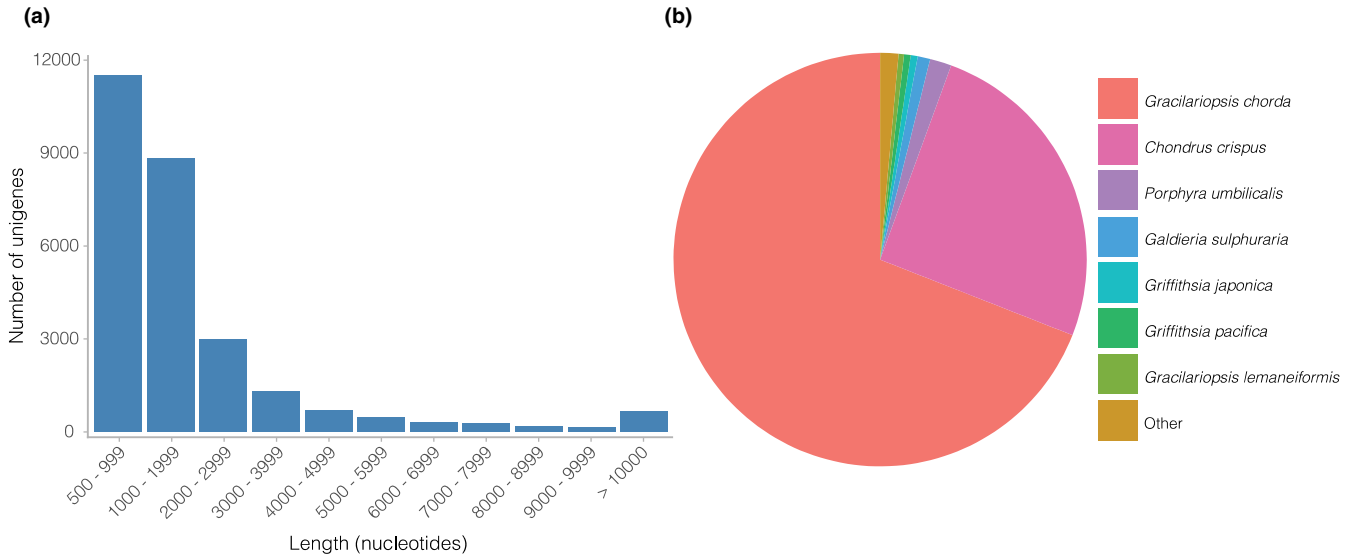

FIGURE 1 (a) Distribution of unigenes according to nucleotide length. (b) Proportion of Rhodophyta species from which unigene annotations were obtained, according to the best match BLASTX hit

TABLE 2 *Delisea pulchra* transcriptome annotation metrics

Transcripts with ORF	
Transcripts with complete 3′ and 5′	10,388
Transcripts with incomplete 5′	8136
Transcripts with incomplete 3′	2910
Transcripts with incomplete 3′ and 5′	5998
Transcripts with annotation	
NCBI nonredundant protein database	27,586
Gene ontology (GO)	14,451
KEGG	15,570
Pfam	25,682
EggNOG Mapper	4252
CAZy	294
SignalP	1215

Abbreviation: ORF, open reading frame.

in addition to a voltage-gated calcium channel and carbohydrate metabolism functions. The downregulated genes coded for proteins involved in ubiquitin-mediated protein degradation, oxidative stress response and the heat shock response (Table S4).

A different pattern in the gene expression profile was observed after 48 h of exposure to AD1. At this time point, 31 genes were differentially expressed (FDR <0.05) with 52% of these genes being upregulated in response to the pathogen (Table S4). Upregulated genes included those coding for proteins associated with the proteasome, heat shock and oxidative stress response. The downregulated genes were annotated to a range of cellular processes including two genes involved in energy generation and genes coding for two heat shock protein 70 (Hsp70) proteins.

Interestingly, the nonpathogen treatment (AD10) did not elicit any DEGs relative to the control at 24 h (Figure 3a), indicating that unlike AD1, exposure to the benign strain AD10 did not induce an early transcriptional response in *D. pulchra*. However, after 48 h of exposure, 10 genes were upregulated and 23 genes were downregulated relative to the control (Table S4). The upregulated genes included five genes annotated as hypothetical proteins, two coding for heat shock proteins (Hsp90 and Hsp70), a photosystem II extrinsic protein and a protein kinase. The downregulated genes coded for proteins associated with intracellular signalling, translation, energy generation and DEAD-box helicase functions.

To determine if *D. pulchra* can elicit a differential gene expression response that is specific to the pathogen strain, we directly compared the gene expression between the pathogen (AD1) and the nonpathogen (AD10) treatments. After 24 h of exposure, 71 DEGs were detected. Of these, 64 were downregulated and seven were upregulated in response to AD1 compared to AD10 (Figure 3b; Table S4). A high proportion of the downregulated genes coded for proteins associated with the KEGG functions for translation and protein folding (Figure 3c) including an *hsp90* gene which exhibited the highest log fold change observed in this comparison (Table 3). Three *hsp70* genes were also downregulated in addition to seven genes participating in the ubiquitin-mediated protein degradation pathway and four genes coding for translation factors. Three genes encoded proteins related to immune signalling (Table 3) and five genes were annotated with functions involved in energy generation and photosynthesis. Only seven genes were upregulated with two coding for functions related to intracellular signalling and halogen metabolism (Table 3) and other genes annotated as NAD(P)H binding proteins, NADH oxidase or tenascin. Notably, 19 of the genes differentially expressed between the AD1 and AD10 treatments were also significantly differentially expressed in response to AD1 relative to the uninoculated control (Figure 3a). These genes code for heat shock proteins, ubiquitin, protein synthesis and energy generation functions.

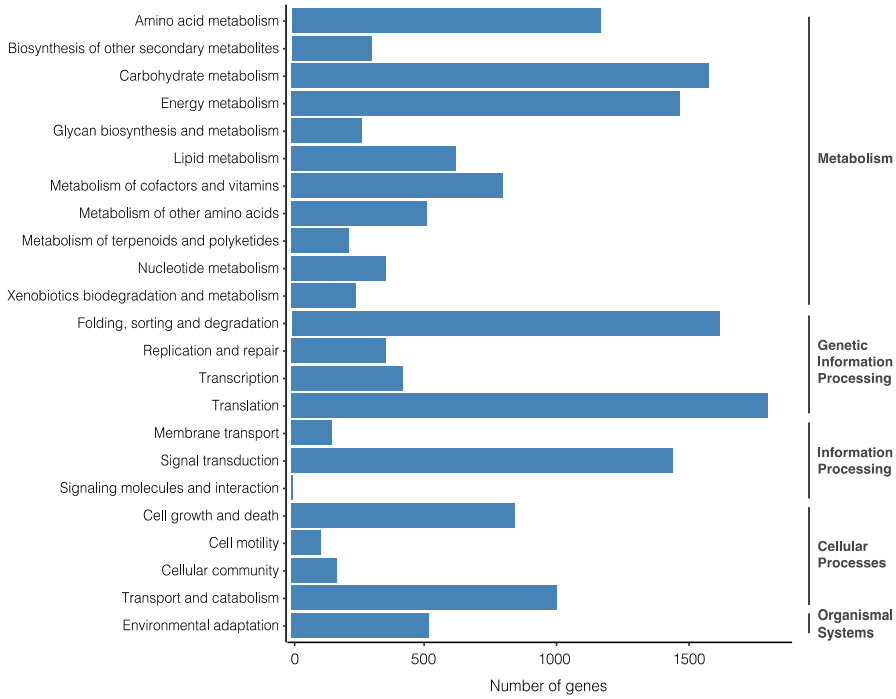

FIGURE 2 Number of genes in the *Delisea pulchra* transcriptome assembly annotated to level 2 KEGG terms and grouped under level 1 categories

At 48 h after exposure to AD1 only one gene, coding for a hypothetical protein, was significantly differentially expressed in the pathogen relative to AD10 treatment (Figure 3a; Table S4).

4 | DISCUSSION

Infectious disease outbreaks in marine organisms are being increasingly reported (Tracy et al., 2019), but few studies have assessed the antipathogen response of marine hosts against opportunistic pathogens, limiting our ability to protect important species in the future. Here, we first described the transcriptomic profile of the model macroalga *Delisea pulchra* under the environmental conditions known to promote the onset of bleaching disease. We then compared the transcriptional response of *D. pulchra* to an opportunistic pathogen (*Aquimarina* sp. AD1) relative to a benign nonpathogen (AD10) to characterize the antipathogen response mechanisms of this macroalga.

4.1 | The transcriptome of *Delisea pulchra* reveals its potential to detect and respond to pathogens

As sessile organisms macroalgae must rapidly activate cellular stress response mechanisms when subjected to pathogen stress to mitigate damage and maintain cellular functioning. We found that genes encoding cellular stress response pathways featured prominently in the *D. pulchra* transcriptome (Figure 2), including those annotated as heat shock proteins, antioxidant enzymes and intracellular signal transduction proteins (Table S2).

Heat shock proteins and antioxidant enzymes have a critical role in the algal immune response by preserving the integrity of cellular structures exposed to pathogen effector molecules and the algal oxidative defences (Cosse et al., 2009; Khan et al., 2018; Strittmatter et al., 2016). Oxidative defence in algae has also been linked to halogen metabolism, and 55 genes encoding haloperoxidase enzymes were identified in the *D. pulchra* transcriptome. Haloperoxidase enzymes remove H_2O_2 via the oxidation of halide ions to form halogenated compounds and can aid in the oxidative stress response by directly removing H_2O_2 (Cosse et al., 2007; La Barre et al., 2010). Halogenated compounds are also known to function as chemical defence molecules in macroalgae (Cosse et al., 2007; Ohsawa et al., 2001; Paul et al., 2006), including the brominated furanones produced by *D. pulchra* (de Nys et al., 1993). The observed number of transcripts for haloperoxidases suggests future studies will benefit from a closer evaluation of the role of halogenated compounds in *D. pulchra's* defence against bacterial pathogens, including *Aquimarina* sp. AD1.

The transcriptome of *D. pulchra* was also rich in transcripts associated with intracellular signal transduction pathways known to coordinate the cellular response to external stress (Figure 2). In particular, inositol phosphate metabolism, which is linked to phosphatidylinositol signalling, plays a role in heat stress in plants (Hou et al., 2016; Liu et al., 2006) and algae (Zhang et al., 2020) and has recently been shown to be positively associated with the antipathogen response of the alga *Pyropia yezoensis* (Khan et al., 2018). Although signalling pathways remain relatively uncharacterized in macroalgae, in *D. pulchra* inositol phosphate metabolism and signalling may similarly play a role in conferring tolerance to external stressors.

Genes coding for other signalling pathways, such as calcium signalling proteins and mitogen activated protein kinase (MAPK) cascades, were also expressed (Table S2). MAPK cascades are key pathways regulating the antipathogen response via pathogen receptor proteins (R-proteins) in plant systems (Pitzschke et al., 2009; Zhang et al., 2018), including red algae (de Oliveira et al., 2017; Khan

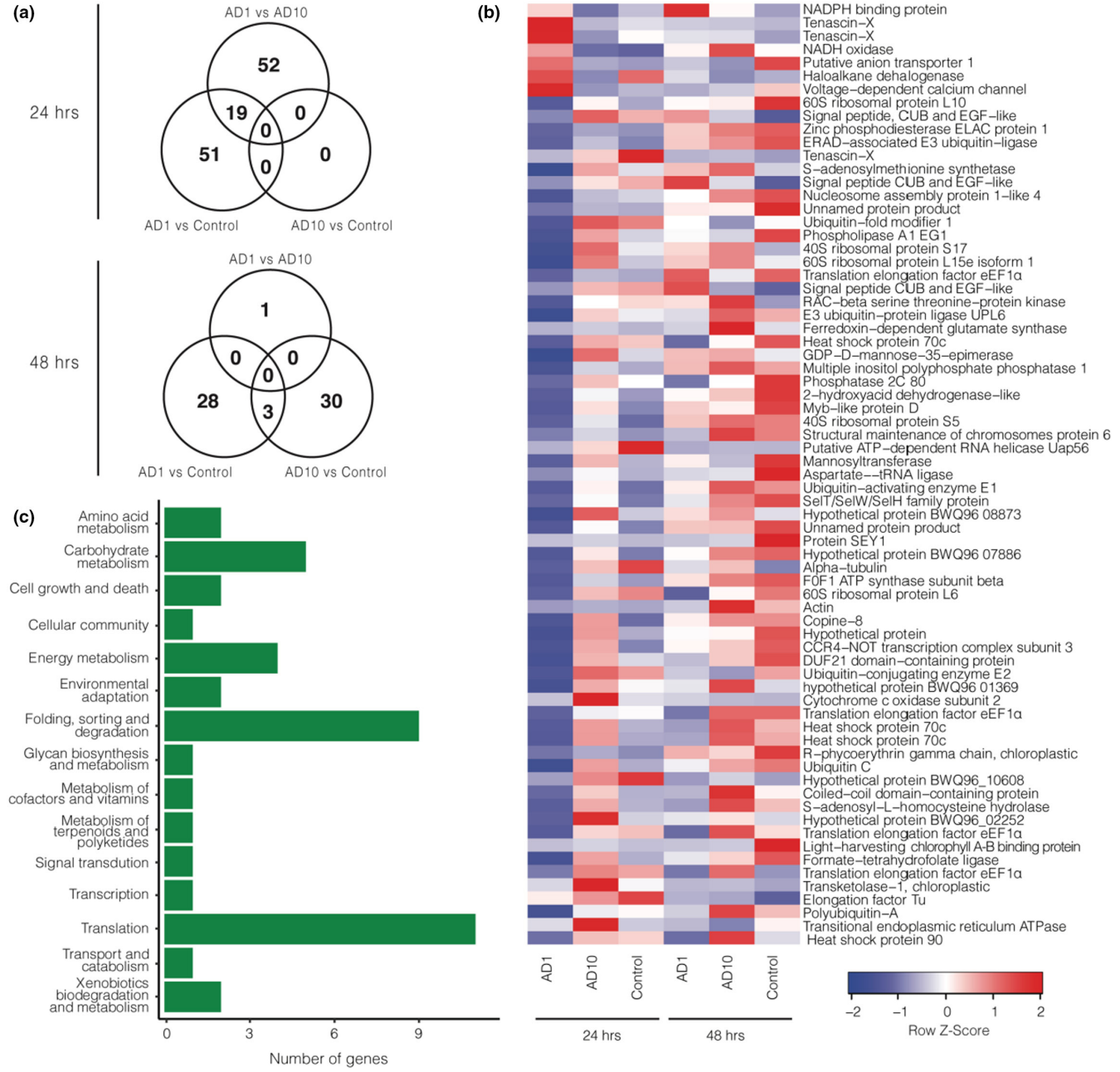

FIGURE 3 (a) Venn diagram summarizing the number of significantly differentially expressed genes in *Delisea pulchra* between treatments. (b) Heat map of significantly differentially expressed genes in *D. pulchra* treated with *Aquimarina* sp. AD1 when compared to AD10 treatment at 24 h (classified here as antipathogen response genes). The relative average expression levels of these genes across all treatments and time points are displayed. (c) The number of genes significantly differentially expressed between AD1 and AD10 at 24 h annotated according to KEGG level 2 terms

et al., 2018). Although further work is required to verify their role in pathogen recognition in algae, genes encoding domains associated with non-NBS-LRR algal R-proteins (Collén et al., 2013; de Oliveira et al., 2017; Tang et al., 2019) were identified in *D. pulchra* (Table S2). Overall, the detection of transcripts involved in stress tolerance and signalling in the transcriptome of *D. pulchra* (Figure 2) suggests that it has the capacity to detect and respond to pathogen stress under the conditions of elevated temperature in which these experiments were performed.

4.2 | Pathogen exposure leads to a repression of genes involved in core cellular functions known to dampen the immune response

We observed the highest number of DEGs occurred in response to the AD1 treatment, relative to both the control and AD10 treatments after 24 h of exposure (Figure 3a). This early transcriptional response of *D. pulchra* to a pathogen is in agreement with other studies of Rhodophyta which observed the antipathogen response to be

TABLE 3 Differentially expressed genes of interest in *Delisea pulchra* in response to *Aquimarina* sp. AD1 compared to AD10 at 24 h post-inoculation

Function	Gene annotation	LogFC
Ubiquitin-mediated protein degradation	Polyubiquitin A	−5.8
	Ubiquitin C	−4.5
	Ubiquitin-conjugating enzyme E2	−3.5
	Ubiquitin-activating enzyme E1	−2.4
	E3 ubiquitin-protein ligase UPL6	−1.8
	Ubiquitin fold modifier 1	−1.6
	ERAD-associated E3 ubiquitin-protein ligase	−1.4
Heat shock response	Heat shock protein 90	−6.3
	Heat shock protein 70c	−4.0
	Heat shock protein 70	−3.9
	Heat shock protein 70c	−2.0
Translation	Elongation factor Tu	−5.7
	Translation elongation factor eEF1, subunit alpha	−5.3
	Translation elongation factor eEF1, subunit alpha	−5.1
	Translation elongation factor eEF1, subunit alpha	−3.9
Cytoskeleton	Actin	−3.1
	Alpha-tubulin	−2.7
Immune response and intracellular signalling	Myb-like protein D	−2.2
	Multiple inositol polyphosphate phosphatase 1	−2.0
	RAC-beta serine/threonine-protein kinase	−1.8
	Voltage-dependent calcium channel subunit alpha−2/delta−4	1.0
	Haloalkane dehalogenase	1.3
Energy generation and photosynthesis	Transketolase−1	−5.6
	Photosystem II reaction centre M protein (PsbM)	−5
	Light harvesting chlorophyll A-B binding protein	−5.1
	R-phycoerythrin gamma chain, chloroplastic	−4.3
	Cytochrome c oxidase subunit 2	−3.9
	F0F1 ATP synthase subunit beta	−2.8

Abbreviation: LogFC, log fold change.

maximal after 24 h, followed by a decrease at 48 h (de Oliveira et al., 2017). At 48 h of exposure only one DEG was detected between the pathogen (AD1) and non-pathogen (AD10) treatments. These results highlight the dynamic nature of gene expression regulation in *D. pulchra* and suggest that differences in host gene expression during the initial exposure to an opportunistic pathogen are key to determining disease outcomes for the host (Kumar et al., 2016).

To understand the pathogen-response mechanisms of *D. pulchra*, we further explored the pathogen-specific differential gene expression occurring between the AD1 and AD10 treatments after 24 h. Here, seven genes were upregulated in response to AD1 when compared to AD10, including a gene coding for a voltage-gated calcium channel (Table 3) which may be important for eliciting downstream immune responses via calcium signalling pathways and MAPK cascades (Wurzinger et al., 2011). Likewise, a gene coding for a voltage-gated calcium channel was upregulated in the AD1 treatment compared to the control, suggesting that the upregulation of this gene may be in response to pathogen-specific traits of *Aquimarina* sp. AD1.

The upregulation of a gene involved in halogen metabolism (Table 3) was also observed in *D. pulchra* after exposure to AD1 and may act to degrade brominated furanones (Kunka et al., 2018). Halogen defence compounds are considered a key defence mechanism in algae (Case et al., 2011; Nylund et al., 2009; Paul et al., 2006; Strittmatter et al., 2016) and the results here suggest that *Aquimarina* sp. AD1 may target these to evade the algal immune defences. Interestingly, brominated furanones are also known to antagonize bacterial quorum sensing (cell–cell communication) systems (Manefield et al., 1999), which are known to regulate the virulence phenotype in some bacteria. Genome analysis of *Aquimarina* sp. AD1 identified genes coding for putative quorum sensing functions that were absent in *Aquimarina* sp. AD10 (Hudson et al., 2019). Therefore, suppression of the chemical-based defence responses of *D. pulchra* would not only result in an increased susceptibility of the alga to infection but may also enhance the virulence of *Aquimarina* sp. AD1.

In contrast to previous studies of plant and algal antipathogen responses, the majority of DEGs between both the AD1 and AD10

treatments and the AD1 and control treatments were downregulated at 24 h (Table S4). A large proportion of these DEGs were related to protein synthesis and turnover functions (Figure 3c) including ubiquitin-mediated protein degradation, heat shock proteins and translation (Table 3).

The ubiquitination system is widely recognized as a key component of the plant immune response (Craig et al., 2009; Delauré et al., 2008; Dielen et al., 2010; Trujillo & Shirasu, 2010) where it plays a role in tagging damaged proteins for removal (Vierstra, 2009). The repression of this system in *D. pulchra* is therefore predicted to lead to an excess of denatured proteins, impairing homeostasis and harming metabolic functioning. Moreover, in addition to their role in ubiquitination, some E3 ligases can also play a regulatory role in either initiating or repressing the plant immune response (Furniss et al., 2018; Marino et al., 2013; Ramonell et al., 2005). Together with the repression of the ubiquitination pathway, it is possible that the repression of two E3 ligases in *D. pulchra* results in an overall weakened immune response.

The expression of several heat shock protein genes was also reduced in pathogen-treated *D. pulchra* (Table 3). This observation is in contrast to studies in algae that show the upregulation of heat shock proteins in response to pathogen exposure (Khan et al., 2018; Strittmatter et al., 2016). Moreover, loss-of-function *hsp90* mutants in *Arabidopsis thaliana* have been shown to inhibit the hypersensitive response and reduce R-protein levels (Hubert et al., 2003), highlighting a specific role of Hsp90 in the maintenance of the plant immune response. In *D. pulchra*, reduced levels of *hsp90* transcripts may therefore hinder the correct initiation of immune responses, resulting in an increased susceptibility to infection by opportunistic bacteria.

Pathogen-induced changes in the expression of *D. pulchra* genes involved in protein turnover also corresponded to decreased expression of the plastid translation elongation factor Tu (EF-Tu) and three elongation factor subunit alpha (eEF1A) homologues, signifying an overall decrease in protein synthesis. The role of eEF1A has not been broadly characterized in plants or algae, but in yeast models the depletion of eEF1A resulted in the repression of protein synthesis (Kim & Coulombe, 2010). In mammalian systems, eEF1A also functions as an activator of *hsp70* gene expression (Vera et al., 2014) and may therefore account for the transcriptional repression of *hsp70* observed in this study. Moreover, the activity of eEF1A is dependent on an intact actin cytoskeleton (Gross & Kinzy, 2005), and thus the downregulation of two cytoskeleton components, actin and alpha-tubulin (Table 3), may similarly affect translation efficiency in *D. pulchra*. Interestingly, EF-Tu also acts as a chaperone and in plants it was shown to aid cellular acclimation to heat stress (Caldas et al., 2000; Fu et al., 2012; Li et al., 2018). Reduced expression of these key translation factors may result in a reduced capacity for protein turnover in *D. pulchra* and further increase its susceptibility to opportunistic pathogens such as *Aquimarina* sp. AD1. Further investigations should focus on evaluating the broader impact of translation factors and protein synthesis in the algal immune response.

A reduced capacity for *D. pulchra* to mount an effective immune response in the presence of AD1 is further supported by the reduced expression of genes encoding regulatory functions. This included the downregulation of the transcription factor MYB and the small GTPase Rac1, which are known to modulate the expression of pathogen resistance genes and co-ordinate defence following the detection of a pathogen in plants (Ambawat et al., 2013; Ono et al., 2001; Raffaele & Rivas, 2013). Previous studies in red algae have also reported the upregulation of MYB and Rac1 following pathogen exposure (de Oliveira et al., 2017), Therefore, suppression of these genes in *D. pulchra* may directly interfere with the initiation of an effective immune response. These contrasting findings between the current study and de Oliveira et al. (2017) may be due to the different environmental conditions under which these experiments were performed. Thus, investigating the effect of environmental conditions on the activity of regulatory proteins represents an avenue for future research.

In response to the pathogen, genes coding for components of the photosynthetic pathway were downregulated in *D. pulchra* including parts of the Calvin cycle (e.g., Transketolase-1) and multiple components of the photosynthetic apparatus (Table 3). These findings are consistent with what has been observed in plants and other algae, whereby photosynthesis is often repressed in response to pathogen exposure to allow for cellular energy to be re-allocated towards defence (Bilgin et al., 2010; Cohen & Leach, 2019; Khan et al., 2018). However, genes important for cellular respiration (e.g., cytochrome C oxidase subunit [Cox2] and an ATP synthase subunit beta protein) were also downregulated, potentially restricting the availability of ATP in the cell. The downregulation of both photosynthesis and cellular respiration may therefore have a global effect on the cellular energy balance in *D. pulchra*, impacting protein turnover, transcription and translation, and further hindering the algal defence mechanisms.

Collectively, the analysis of DEGs suggests that the antipathogen response of *D. pulchra* involves the repression of genes associated with a broad range of cellular functions (Figure 4). The overall downregulation in the expression of genes coding for protein and energy metabolism functions is hypothesized to compromise host cellular functioning, ultimately rendering the macroalga vulnerable to infection by opportunistic pathogens. Whilst such interactions have not been previously described in red algae, similar negative health outcomes are commonly reported in plants simultaneously exposed to heat and pathogen stress (Huot et al., 2017; Pandey et al., 2015; Prasch & Sonnewald, 2013; Wang et al., 2009). Although the effect of temperature was not specifically investigated here, this observation leads us to postulate that elevated temperature conditions may also influence host pathogen dynamics in macroalgae, promoting the onset of disease. The current study was conducted at a peak summer temperature for Sydney coastal waters (Australian Bureau of Meteorology), and such peak temperatures are predicted to become more frequent due to climate change, placing macroalgae at an increased risk of disease. Therefore, our understanding of macroalgal disease would greatly

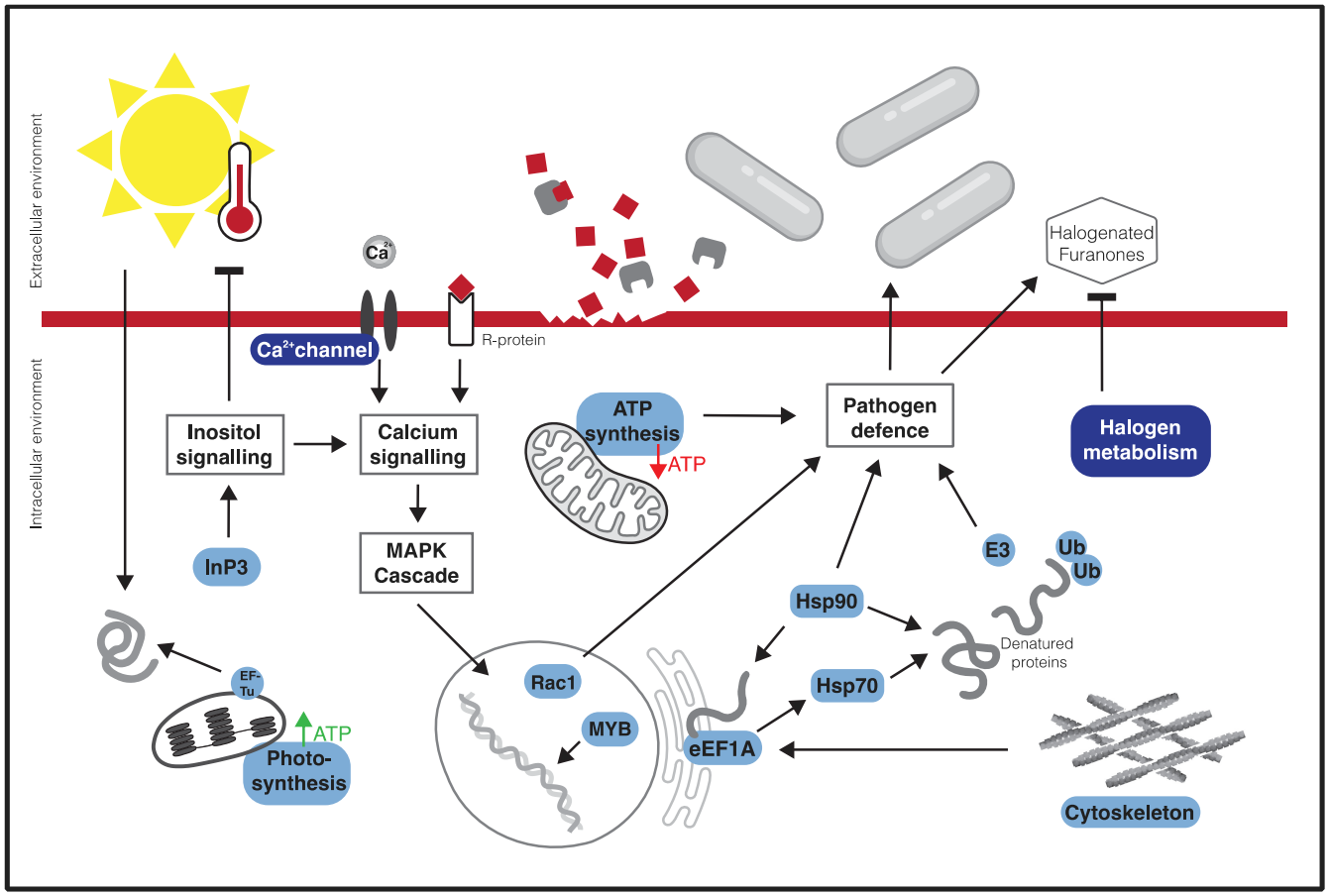

FIGURE 4 Schematic diagram of the cellular response of *Delisea pulchra* following exposure to the pathogen *Aquimarina* sp. AD1. Proteins and functions encoded by differentially expressed genes are represented in light blue, with upregulated genes shown in dark blue. Here we propose that cell-wall-degrading enzymes produced by AD1 degrade the cell wall of *D. pulchra* (see Hudson et al., 2019). Cellular damage (red squares) is detected by *D. pulchra* R-proteins, triggering an influx of calcium ions into the cell, and eliciting an antipathogen response via calcium signalling and MAPK cascades. However, reduced inositol triphosphate levels (InP3) are hypothesized to interfere with calcium signalling and downstream antipathogen responses. Downregulation of the transcription factor MYB and the small GTPase Rac1 would also probably interfere with the expression of genes involved in defence. Suppression of inositol phosphate signalling may also interfere with thermal stress resistance. Elevated temperatures would probably promote an increase in the concentration of denatured proteins (grey lines), which accumulate in the cell due to downregulation of the ubiquitin (Ub)-mediated protein degradation pathway. Likewise, suppression of heat shock proteins Hsp70 and Hsp90 would also probably contribute to the accumulation of damaged proteins and interfere with other cellular processes, including correct protein folding of newly synthesized proteins and pathogen defence. Downregulation of the translation factor eEF1A, caused by a downregulation of cytoskeleton expression, may interfere with the functioning of Hsp70 as well as prevent the synthesis of new proteins. Downregulation of photosynthesis-related proteins components would probably conserve ATP, but in the mitochondria downregulation of energy-generating functions would restrict the level of ATP available for defence. Upregulation of a gene involved in halogen metabolism is hypothesized to degrade the halogenated furanone defence molecules of *D. pulchra*, leading to an increased susceptibility to infection

benefit from further studies assessing the underlying effects of environmental conditions on the interaction between *D. pulchra* and *Aquimarina* sp. AD1.

We found the nonpathogen *Aquimarina* sp. AD10 did not elicit any DEGs at 24 h when compared to the control treatment, indicative of its role as a benign symbiont (Kumar et al., 2016). However, at 48 h an upregulation in the expression of genes encoding heat shock proteins (indicative of a stress response) was observed. Together with the reduced expression of genes coding for signal transduction and translation functions (Table S4), these results suggest that AD10 has some capacity to similarly impair the algal immune response albeit after a longer exposure time. Therefore, while *Aquimarina* sp. AD10 does not cause obvious signs of disease under the conditions tested here (Kumar et al., 2016), future work will be required to assess if prolonged exposure to AD10 results in asymptomatic impacts.

In conclusion, we hypothesize that the downregulation of genes associated with immune and defence functions in *D. pulchra* after pathogen exposure will hinder the macroalga's ability to modulate the immune response, contributing to its vulnerability to infection and leading to tissue damage and bleaching symptoms (Figure 4). Overall, this study provides valuable insight into the genetic features

of the model macroalga *D. pulchra*, highlighting potential antipathogen response mechanisms of macroalgae and has future implications for how we view host–pathogen interactions in the context of environmental change.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

J.H., S.E. and C.L. designed the study, J.H. and N.D. performed the experiments and data analyses, and J.H. and S.E. wrote the first draft of the manuscript. All authors contributed to and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

RNA-Seq data have been deposited in GenBank under the BioProject ID PRJNA610646.

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