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ORIGINAL ARTICLE

Genome-scale signatures of adaptive gene expression changes in an invasive seaweed *Gracilaria vermiculophylla*

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Abstract

Invasive species can successfully and rapidly colonize new niches and expand ranges via founder effects and enhanced tolerance towards environmental stresses. However, the underpinning molecular mechanisms (i.e., gene expression changes) facilitating rapid adaptation to harsh environments are still poorly understood. The red seaweed *Gracilaria vermiculophylla*, which is native to the northwest Pacific but invaded North American and European coastal habitats over the last 100 years, provides an excellent model to examine whether enhanced tolerance at the level of gene expression contributed to its invasion success. We collected *G. vermiculophylla* from its native range in Japan and from two non-native regions along the Delmarva Peninsula (Eastern United States) and in Germany. Thalli were reared in a common garden for 4 months at which time we performed comparative transcriptome (mRNA) and microRNA (miRNA) sequencing. MRNA-expression profiling identified 59 genes that were differently expressed between native and non-native thalli. Of these genes, most were involved in metabolic pathways, including photosynthesis, abiotic stress, and biosynthesis of products and hormones in all four non-native sites. MiRNA-based target-gene correlation analysis in native/non-native pairs revealed that some target genes are positively or negatively regulated via epigenetic mechanisms. Importantly, these genes are mostly associated with metabolism and defence capability (e.g., metal transporter Nramp5, senescence-associated protein, cell wall-associated hydrolase, ycf68 protein and cytochrome P450-like TBP). Thus, our gene expression results indicate that resource reallocation to metabolic processes is most likely a predominant mechanism contributing to the range-wide persistence and adaptation of *G. vermiculophylla* in the invaded range. This study, therefore, provides molecular insight into the speed and nature of invasion-mediated rapid adaption.

KEYWORDS

Agarophyton vermiculophyllum, environmental adaptation, metabolic pathways, miRNA-mediated epigenetic regulation, resource allocation, transcriptional profile

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1 | INTRODUCTION

Invasive marine species are a major threat to biodiversity, environmental health, food security and coastal ecosystem services (Molnar et al., 2008). Many invaders are capable of rapid colonization and expansion in novel habitats and, as a consequence, provide excellent models with which to study adaptive evolution over contemporary timescales (e.g., Le Cam et al., 2020; Sotka et al., 2018). However, exploring molecular mechanisms involved in adaptation to new habitats is still a major challenge for marine invasions (Tepolt, 2015), particularly for nonmodel species without genomic resources (e.g., Blakeslee et al., 2020).

Human-mediated macroalgal invasions have dramatically altered near shore coastal marine ecosystems (Williams & Smith, 2007). Compared to the native range, invasive macroalgae have rapidly expanded into novel environments (Sotka et al., 2018) and may be exposed to novel predators (Tamburello et al., 2014), epiphytes (Gestoso et al., 2010; Wang et al., 2017), microbial epibionts (Buschbaum et al., 2006; Saha et al., 2016), and pathogenic epibacteria (Pickett et al., 2021; Saha & Weinberger, 2019). Physiological changes observed in macroalgal thalli can be reflected in molecular responses (e.g., gene expression and its regulations; Hammann, Rempt, et al., 2016). These physiological and biochemical changes can be captured in gene expression levels when compared with the native populations using genome-scale sequencing methods (e.g., transcriptomics; Hodgins et al., 2013; Xu et al., 2019). Transcriptional changes in response to stress can be transient, short-term, or even reversible (Brilhaus et al., 2016). However, some gene expression changes and their epigenetic regulatory mechanisms can remain stable and ultimately become adaptive in time scales shorter than the several decades commonly expected under the selection of gene-sequence variants (Mounger et al., 2021; Richards et al., 2017). These molecular and epigenetic regulations may contribute to the emergence of new adaptive traits and genotypes in new habitats (Drenovsky et al., 2012; Green et al., 2020). As compared to terrestrial systems (Mounger et al., 2021; Xu et al., 2019), understanding these patterns in marine systems has not been explored to the same extent (Gao et al., 2022; Huang, Li, et al., 2017).

The red seaweed *Gracilaria vermiculophylla* (synonym: *Agarophyton vermiculophyllum*, Lyra et al., 2021) is native to the Northwest Pacific. It was inadvertently introduced throughout estuaries and marshes along North American, European, and northwestern African coastlines over the last 100 years (Krueger-Hadfield et al., 2017). Non-native *G. vermiculophylla* thalli exhibit enhanced tolerance and resistance to light intensity, thermal stress, nutrient deficiency, UV radiation, and salinity shifts (e.g., Nejrup & Pedersen, 2012; Roleda et al., 2012; Rueness, 2005; Sotka et al., 2018). In the non-native range, thalli are also less palatable to certain herbivores (Hammann, Rempt, et al., 2016) and possess a core microbial community (Bonthond et al., 2020) that further promotes the species' successful establishment and expansion. Moreover, many of the habitats to which this alga has been introduced are composed of soft sediments in which algal spores cannot recruit and as a consequence, there has been a shift from sexual to asexual reproduction and the consistent loss of the haploid gametophytic stage (Krueger-Hadfield et al., 2016, 2017, 2018). A recent study further revealed that non-native *G. vermiculophylla* tetrasporophytes were larger and stronger than their native counterparts (Murren et al., 2022). However, the molecular responses that underlie its successful colonization are still poorly known, but previous work has suggested an underlying genetic component (Sotka et al., 2018).

The identification of genes involved in successful invasion is critical to properly assess the management of current invaders and the prevention of future invasions (Guggisberg et al., 2013; Turner et al., 2017; Xu et al., 2019). However, current molecular studies related to species invasions mostly concentrate on comparative gene expression profiling between native and non-native individuals under a specific stress treatment (e.g., light and nutrient, Hodgins et al., 2013; nutrient and shading, Guggisberg et al., 2013; oxygen, Mandic et al., 2014; temperature, Hammann, Rempt, et al., 2016; salinity, Maynard et al., 2018; polyploidy, Xu et al., 2019; photosynthetic capability, Zhang et al., 2019). Responses to specific experimental treatments provide useful molecular insights to evolutionary processes underlying successful invasion, but for taxa, such as *G. vermiculophylla*, that tolerate a wide range of environmental conditions, we only obtain a glimpse into the complex network of post-invasion gene interactions. Krueger-Hadfield et al. (2017) and Flanagan et al. (2021) demonstrated that the Northern Hemisphere invasion by *G. vermiculophylla* originated overwhelmingly from sites in the Miyagi Prefecture in Japan. While there is comparable genetic diversity between the native and non-native ecotypic adaptation of *G. vermiculophylla* may have occurred during the colonization and expansion (Bock et al., 2015). Transcriptional changes, potentially under the control of epigenetic modifications, may play a central role in facilitating the adaptation of *G. vermiculophylla* to new habitats (Hu & Lopez-Bautista, 2014).

In this study, our goal was to identify potential adaptive gene expression changes that may have facilitated acclimation and adaptation of *G. vermiculophylla* and facilitated its invasion throughout the Northern Hemisphere. We used transcriptomic (mRNA) and microRNA (miRNA) sequencing to address three main questions: (i) Do populations in the non-native range show characteristic coexpression of candidate genes that discriminate them from populations in the native range? (ii) Do miRNAs adaptively regulate coexpressed functional genes in the non-native range? (iii) Are genes involved in chemical defence and metabolism specifically over-represented in terms of resource reallocation? We collected thalli from two populations in Japan (native source region, but see methods), two populations from the Delmarva Peninsula, USA (non-native), and two populations from Germany (non-native). We acclimated thalli for over 4 months in a long-term common garden before we compared mRNA and miRNA expression profiles. The expression patterns of specific gene categories in the non-native range, including the miRNA-mediated gene expression network, suggest that epigenetic regulation plays an important role in the colonization of *G. vermiculophylla* in new environments. Epigenetic changes in gene regulation

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are likely to play a role in successful invasions across taxonomic groups and biomes.

2 | MATERIALS AND METHODS

2.1 | Study species, sampling and common garden treatment

Gracilaria vermiculophylla has a haploid-diploid life cycle with morphologically similar haploid gametophytes and diploid tetrasporophytes. Krueger-Hadfield et al. (2016) found that the ecological shift from hard to soft substratum habitats, predominantly in the native versus the non-native range, respectively, was accompanied by a shift from sexual to asexual reproduction. In soft sediment habitats, algal spores are unlikely to settle and recruit. Probably driven by phenotypic differences between tetrasporophytes and gametophytes (e.g., Krueger-Hadfield, 2020; Krueger-Hadfield & Ryan, 2020; Lees et al., 2018), thallus fragmentation in soft sediment habitats results in tetrasporophytic dominance.

All thalli were sampled in June 2018 during the same low tide cycle in Japan, Virginia (USA), Maryland (USA), and northern Germany (Figure 1a). Tetrasporophytes dominate many of the non-native sites, thus we preferentially sampled reproductive tetrasporophytes at all sites to avoid stage-specific differences. When reproductive, tetrasporophytes are easily identifiable (see figure 3 in Krueger-Hadfield et al., 2018) and these diagnostic features have been used in previous studies to compare tetrasporophytes between native and non-native regions (see also Bippus et al., 2018; Bonthond et al., 2020; Murren et al., 2022; Sotka et al., 2018).

In Japan, we sampled thalli from three sites (Table S1): Hayase (hay), Futtsu-B (ftb), and Soukanzan (sou). The site at Futtsu was not the same site sampled by Krueger-Hadfield et al. (2017) in which thalli were free-floating and found buried in soft sediment. Subsequent *cox*1 barcoding and mRNA sequencing revealed ftb was *Gracilariopsis chorda* and we did not include these thalli in subsequent analysis. Hayase and Soukanzan belong to the T-mitochondrial lineage that includes the most common non-native haplotype (Haplotype 6) and was considered as the source region in previous work (see Flanagan et al., 2021; Kim et al., 2010; Krueger-Hadfield et al., 2017, 2021). Krueger-Hadfield et al. (2017) found that thalli in the non-native range were predominantly assigned to three sites along the Pacific coast of Japan: Akkeshi in Hokkaido and Soukanzan and Mangoku-ura in the Miyagi Prefecture, suggesting a very small region in the native range served as the source of the invasion. Sotka et al. (2018) used this information to document the adaptive shifts that occurred during the invasion using niche modelling and Flanagan et al. (2021) further refined the source region to Mangoku-ura over other Japanese site using ~60,000 SNPs. In this study, we considered both Hayase and Soukanzan as part of the broader T-lineage, but Soukanzan as a source population (sensu Krueger-Hadfield et al., 2017).

We sampled thalli from sites along the Delmarva Peninsula in Virginia and Maryland. Ape Hole Creek (ahc) is a site in the Chesapeake Bay near Crisfield, MD where all thalli are fixed to hard substratum and gametophytes and tetrasporophytes are common (Bonthond et al., 2020; Krueger-Hadfield et al., 2016; Krueger-Hadfield & Ryan, 2020). The upper Haul Over (wac) near Wachapreague, VA, is a free-floating site where tetrasporophytes dominate, but despite rampant thallus fragmentation, genotypic diversity is high (Krueger-Hadfield et al., 2016, 2019). In Germany, we sampled Nordstrand (nor) on the North Sea where thalli are both free-floating and fixed to hard substratum, though tetrasporophytes are still more abundant (Krueger-Hadfield et al., 2016). We also sampled free-floating tetrasporophytes at Heiligenhafen (hei). Krueger-Hadfield et al. (2017) and Flanagan et al. (2021) demonstrated that the invasions of the eastern coast of the USA and Germany were distinct though they can both be traced back to the Miyagi Prefecture in Japan.

At all sites, our sampling strategy followed the methods described by Bonthond et al. (2021), with 20 thalli sampled from each locality for the common garden (Table S1). Thalli were placed in open, polyethylene bags with a seawater moistened paper towel and shipped to GEOMAR in Kiel, Germany. Thalli arrived within 6 days of sampling (see also Bonthond et al., 2020). In a climate chamber (15°C), all thalli from each site were placed in an aquarium (20 L) containing 6 L ambient seawater (16 PSU, the Baltic Sea), and exposed to 12 h of light per day (86.0 μmol m^{-2} s^{-1} at the water surface, Bonthond et al., 2021). Separate aquaria were setup for each of the sites, but thalli from a site were pooled together. All aquaria were close to each other in the same climate room and exposed to the same regime. All thalli were acclimated from 20 June 2018 to 24 October 2018 to minimize the impact of different environmental conditions at the sampling localities, and effects of transportation stress on gene expression. Water in each aquarium was exchanged manually twice per week. After 127 days of acclimation treatment, 9 to 20 survived thalli were collected from each aquarium (Table S1) and flash frozen in liquid nitrogen and stored at −80°C.

2.2 | mRNA library construction, de novo assembly, annotation and analysis

We created three biological replicates for each locality. Each replicate was randomly composed of three independent thalli (3 × 3 = 9 thalli for each site) before RNA extraction, to reduce the effect of potential heterogeneity among individuals. Total RNA was extracted using a TRIzol reagent kit (Invitrogen). After the assessment of RNA quality based on RIN values, a total of 18 cDNA libraries were constructed by the Gene Denovo Biotechnology Company. For mRNA sequencing, the 200-bp paired-end reads were sequenced using the Illumina HiSeq 4000 platform at Guangzhou, China. High-quality filtered reads were generated using Fastp (Chen et al., 2018) by (i) removing adaptor sequences, (ii) removing reads containing the

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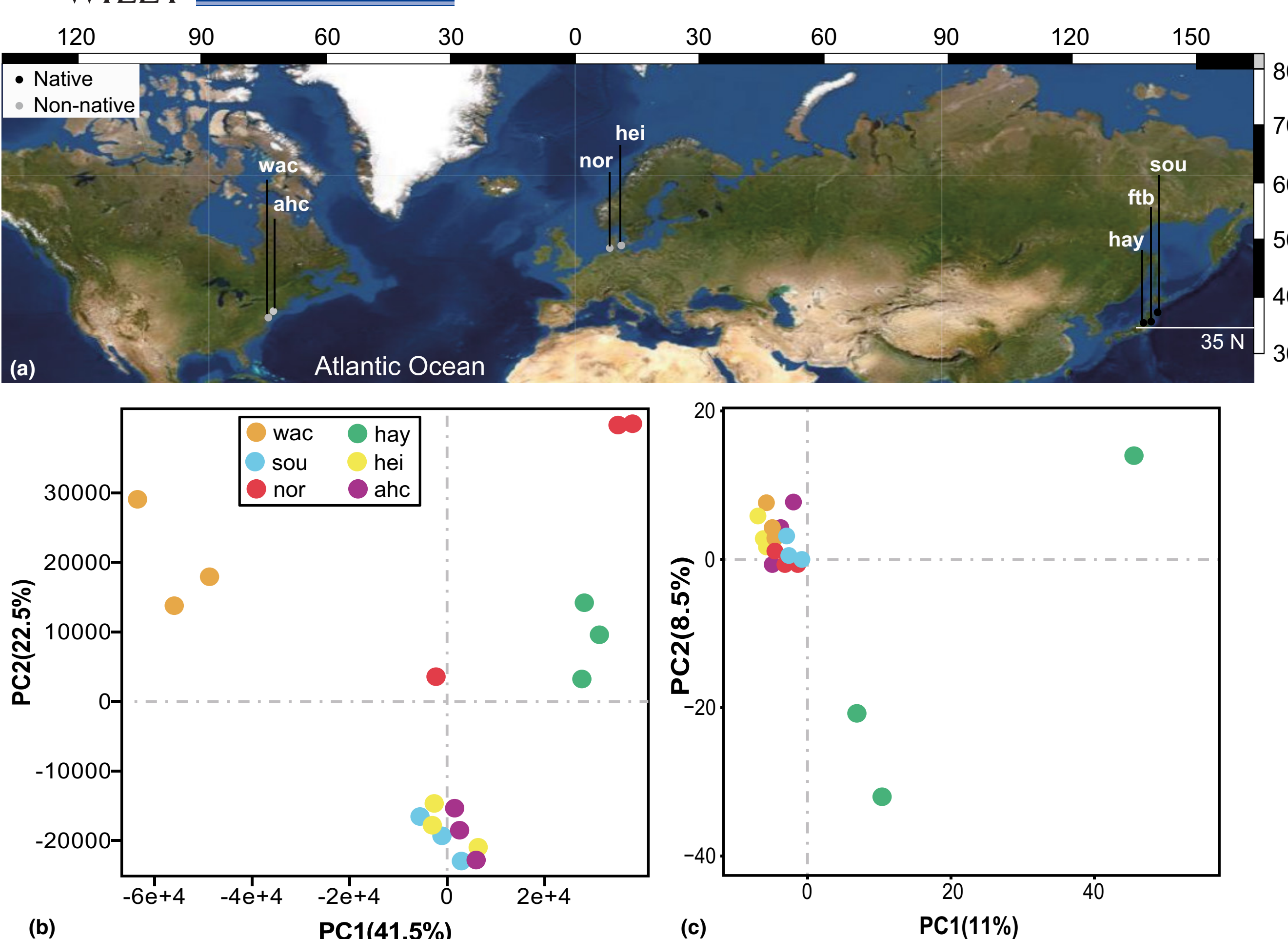

FIGURE 1 (a) Sampling locations of *Gracilaria vermiculophylla* in the native (Japan) and non-native ranges. Two localities (hay, and sou dominated by the mitochondrial T-lineage) were chosen in Japan north of c. 35°N latitude, two localities (hei, nor) were chosen in Germany and two localities were sampled along the Delmarva Peninsula in the eastern United States (wac, ahc). (b) Principal component analysis (PCA) of mRNA expression in the six sampling localities. (c) PCA of miRNA expression in the six sampling localities. [Colour figure can be viewed at wileyonlinelibrary.com]

unknown nucleotide $N > 10\%$, and (iii) removing low-quality reads containing more than 50% of bases with a Q-value ≤20.

All filtered reads were assembled in Trinity (Grabherr et al., 2011) and then mapped to ribosomal RNA (rRNA) to identify residual rRNA reads. The constructed transcripts from the three independent modules (Inchworm, Chrysalis and Butterfly) in Trinity were further clustered into nonredundant unigenes (unique genes, the longest transcript of each gene) using TGICL (Pertea et al., 2003) to eliminate the redundant Trinity-generated transcripts. The rRNA-filtered reads from 18 samples were further assembled as unigenes and pooled together to construct a uniform reference transcriptome using Bowtie2 (Langmead & Salzberg, 2012) for subsequent assembly evaluation and annotation. A Pearson correlation coefficient between biological replicates was calculated to evaluate the repeatability between samples. We used a principal component analysis (PCA) of 43,175 unigenes to reveal structure and genetic relationships among sampled individuals.

Gene function was assessed based on the following database: NCBI nonredundant protein sequences (NR), Clusters of Orthologous Groups of proteins (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), and the manually annotated and reviewed protein sequences (Swiss-Prot). The Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de) was used to identify and classify transcription factors. All nonredundant unigenes were searched against NR, COG, KEGG and Swiss-Prot using the BLAST algorithm (E-value $\leq 10^{-5}$). Based on the NR annotation, gene ontology (GO) functional annotation was achieved using Blast2GO (Conesa et al., 2005). Genes were functionally classified by GO terms using the Web Gene Ontology Annotation Plot (WEGO) (Ye et al., 2018). GO enrichment was tested with the BiNGO plugin for Cytoscape, using a hypergeometric test after false discovery rate (FDR) correction ($p < .01$, FDR ≤0.05) (Maere et al., 2005). After annotation, KEGG pathway enrichment analysis was conducted using KOBAS (Mao et al., 2005). RPKM (reads per kb per million reads, Mortazavi et al., 2008), which normalizes the influence of gene length and sequencing depth on the estimation of gene expression, was used to calculate unigene expression levels. Differentially expressed genes (DEGs) between two sampling sites were analysed using edgeR (Robinson et al., 2010), with FDR < 0.05 and absolute fold change (FC) ≥ 2.

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Transcriptional profiles can reflect the complex interactions between an individual and its habitats and usually involve thousands of up- and downregulated genes (Guggisberg et al., 2013; Maynard et al., 2018; Xu et al., 2019). First, we pooled the two sites in the native range (sou, hay) and the four non-native sites (hei, nor, ahc, and wac) as two groups, respectively, to broadly calculate DEGs between the native and non-native ranges (Figure 2a). To specifically uncover adaptive gene expressions that co-occurred in the four non-native sites, we used sou and hay from Japan, hei and nor from Germany, and ahc and wac from the USA to create eight native-vs.-non-native pairs (Figure 2b). For each pair, we calculated the up- and downregulated genes with mean $\log_2(FC) \geq 2$ ($p < .05$) and obtained the intersections of unigene profiles among eight pairs. After the elimination of unigenes that simultaneously occurred in the intersections of native and non-native sites, we kept unigenes that were codifferentiated only in the native or non-native range.

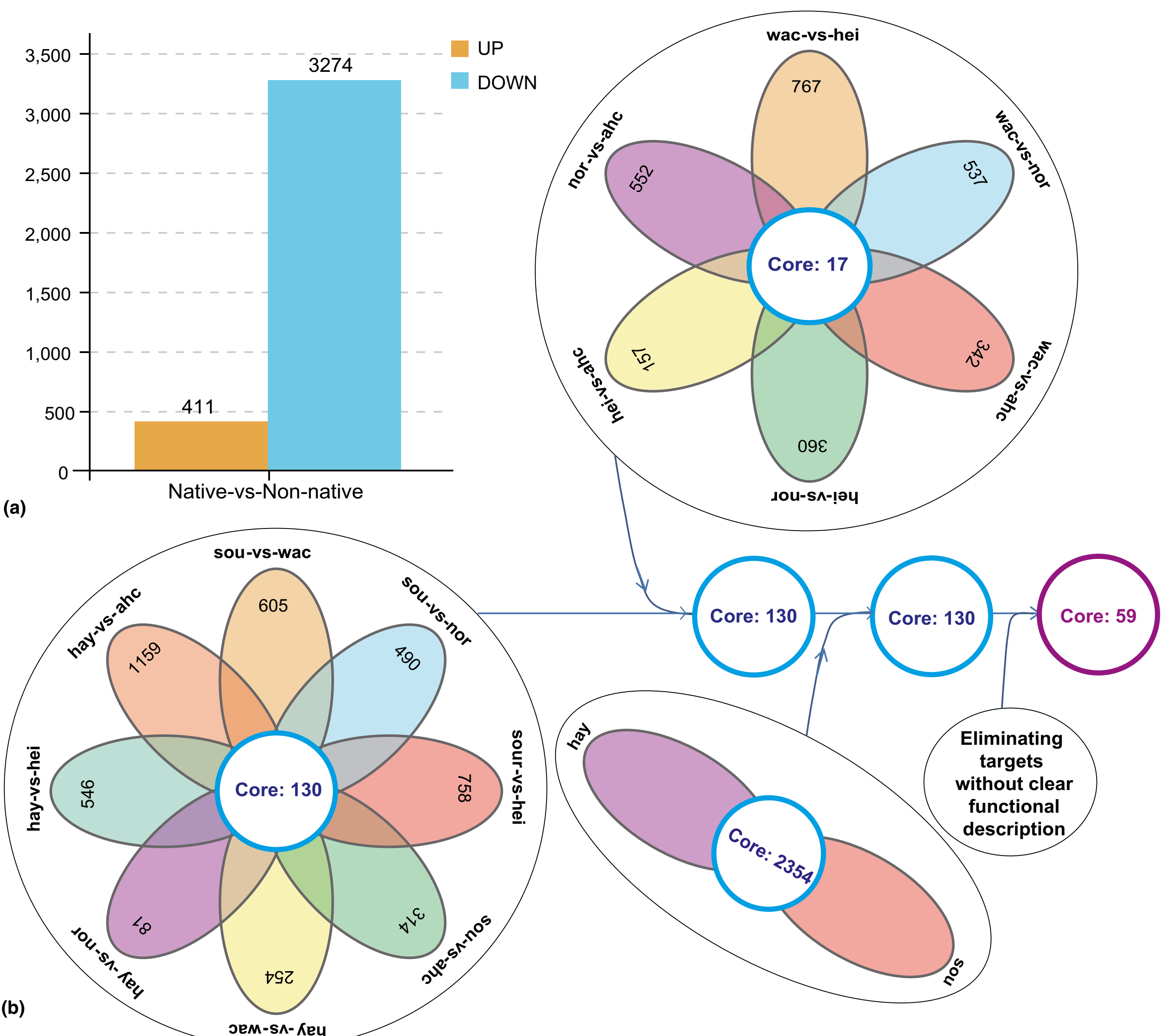

FIGURE 2 (a) Bar plot showing the number of unigenes with significant differential expression (DE) when the native (sou + hay) was compared with the non-native range (hei + nor+wac + ahc). UP/DOWN represents UP- and downregulated gene numbers in the native compared with the non-native range. (b) A schematic diagram showing how to identify the 59 unigenes that were specifically and significantly coexpressed in four different non-native sites. Numbers in the ovals (lower left) show the coexpressed unigenes (UP/DOWN) for each of the eight native vs. non-native comparison pairs, and thus totally identified 130 coexpressed unigenes that are shared between each other. Similarly, coexpressed unigenes that were shared between the six pairs in the non-native range (Core: 17, upper right) and 1 pair in the native (Core: 2354) were identified, respectively, but they were abandoned due to their asynchronous occurrence in the 130 target unigenes. [Colour figure can be viewed at wileyonlinelibrary.com]

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2.3 | miRNA library construction, de novo assembly, annotation and analysis

Eighteen miRNA libraries were also constructed using the same set of samples as for mRNA sequencing. The RNA molecules with a length of 18–30 nt were enriched by polyacrylamide gel electrophoresis (PAGE) and then the 3′ adaptors were added and the 36–44 nt RNAs were enriched via PCR. After the ligation of 5′ adaptors to the RNAs, the products were reversely transcribed by PCR amplification and cDNA libraries with a size of 140–160 bp were sequenced using Illumina HiSeq 2500. Filtered tags were obtained by filtering low-quality reads (*Q*-value ≤20), adaptors and contaminated reads, and then mapped to the *G. vermiculophylla* mRNA transcriptome by SOAP (Xie et al., 2014) using default settings. Subsequently, these tags were aligned to small RNAs in the GenBank database (Benson et al., 2008) to identify and remove the cellular structural RNAs (rRNAs, tRNAs, scRNA, snRNA and snoRNAs). After alignment to the reference draft genome of *Gracilariopsis chorda* (Lee et al., 2018), we removed filtered tags that mapped to exons or introns or repeat sequences. Mapped miRNA tags were then searched against the plant mature miRNA database (miRBase12, Griffiths-Jones et al., 2006) to identify the known miRNAs. The program miRCat2 (Paicu et al., 2017) was used to predict novel miRNA through exploring the positions of unannotated tags in the reference genome, hairpin structures and the Dicer cleavage site. The expression profiles of miRNA were calculated and normalized to transcripts per million (TPM). The PCA-based differential expression pattern of miRNAs was obtained to explore clustering relationships among samples. The program PatMatch version 1.2 (Yan et al., 2005) was used to predict target genes of miRNAs. GO and KEGG pathway enrichment were conducted using the same methods as described for mRNA.

2.4 | Visualization of DE miRNA-mediated interaction network

Because we treated thalli from Soukanzen (sou) as a source site (sensu Krueger-Hadfield et al., 2017; Sotka et al., 2018), a correlation analysis of DE miRNAs and DE mRNAs was conducted for the four pairs (sou-vs.-hei, sou-vs.-ahc, sou-vs.-nor, and sou-vs.-wac). This selective analysis can help to re-concentrate on key gene expression networks identified by mRNA-Seq analyses, despite missing some unique target unigenes from each invaded locality. GO and KEGG enrichment for each of the target pairs were analysed using the same methods as for mRNA. To identify all the possible miRNA-mRNA interactions, including positive and negative relationships between miRNA and mRNA expressions, a custom R script (R Core Team, 2012) was used to construct a miRNA-mRNA regulatory network. More details, including the normalization of all the sample-matched miRNA and mRNA sequencing data, the integration of DE miRNAs and DE mRNAs (Pearson correlation coefficient >0.7, $p<.05$), and the visualization of interaction network, can be found in the method described by Xu et al. (2019).

3 | RESULTS

3.1 | Transcriptome assembly, annotation and DEGs profiling

A total of 483.43 million (M) raw reads were produced and subjected to Seq-QC collating, which resulted in 470.84 M (97.39%) filtered reads of high-quality (Q20: 97.72%–98.65%; Q30: 93.40%–95.61%) (Table S2). The assembled unigenes were pooled together and further clustered into a reference transcriptome (43,175 "All-Unigene") with an average length of 1106 bp, N50 number of 7657 contigs, N50 value of 1695 bp, and total assembled 47,762,713 bases (Table S3). These results are comparable to other transcriptome-based studies of invasive plants (Guggisberg et al., 2013; Xu et al., 2019; Zhang et al., 2019) and red algae (Huang, Zang, et al., 2017), implying that the transcriptomic assembly in this study is of good quality. Pearson correlation coefficients (all values >0.72, Figure S1) and PCA plots (Figure 1b) revealed a high transcriptional repeatability among biological replicates in most sites (5/6), with the exception of the Nordstrand thalli from Germany. The thalli from Soukanzan in Japan were particularly closely related to the thalli from Heiligenhafen in Germany and the Ape Hole Creek in the USA (Figure 1b), in contrast to the native thalli from Hayase and two other invaded localities (wac and nor).

The BLAST against the NR, COG, KEGG and Swiss-prot databases annotated 25,415 unigenes of which 8329 were comapped to the four databases (Figure S2). Among the unigenes that had a BLAST-hit in the NR annotation, the top two best hits for *G. vermiculophylla* were *Chondrus crispus* (3985, 9.02%) (unigenes number, the percentage of the hit unigenes) and *Galdieria sulphuraria* (1810, 4.19%) (Figure S3). GO assignments showed that the biological process category was enriched with genes involved in "metabolic processes'" (3721, 29.44%) and "cellular processes" (3031, 23.98%). Under the cellular component and molecular function categories, 'cell' (2769, 25.38%) and "cell part" (2765, 25.34%) and "catalytic activity" (3302, 54.03%) were the most highly represented groups, respectively (Figure S4).

The 12,111 matched unigenes were clustered into 25 categories using the COG database. The most represented category was "general function prediction only" (12.63%); followed by the category "post-translational modification, protein turnover and chaperon" (12.02%) (Figure S5). KEGG analysis showed that 6220 unigenes mapped successfully, which accounted for 65.08% of all the five KEGG categories. In the category of metabolism, the three most represented pathways were global and overview maps (2528, 40.64%), energy metabolism (861, 13.84%) and carbohydrate metabolism (812, 13.05%) (Figure S6).

In thalli from the two native populations, 3274 unigenes were downregulated and 411 were upregulated compared with thalli from the non-native range (Figure 2a). The transcriptome of thalli from the native source site—sou—were very similar to the non-native sites, except nor. The other native site, hay, which did not contribute significantly to the invasion of the Northern Hemisphere, exhibited

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a large number of downregulated genes as compared to the non-native sites, except nor (Figure S7). Based on the intersection of eight native/non-native site pairs, 59 unigenes with gene descriptions were identified that were consistently, differentially expressed and shared among each native/non-native site pair (Figure 2b; Table S4). KEGG enrichment analyses showed that involved pathways were mainly in metabolism (Table S5). Hierarchical clustering analysis based on gene-expression profile of the 59 unigenes across six sampling sites identified two gene clusters (Figure 3). Cluster A grouped unigenes that were typically upregulated in the native, but weakly expressed in the non-native range. Cluster B grouped unigenes that were uniquely upregulated in the non-native thalli, but weakly expressed in the native range (Figure 3). Some target genes included a leucine rich protein, metal transporter Nramp5, cell wall-associated hydrolase, senescence-associated protein and dehydration responsive protein, constituted multiple orthologous unigenes and were upregulated together with >30 genes in the native range (Figure 3; Table S4). Notably, the expressions of high light

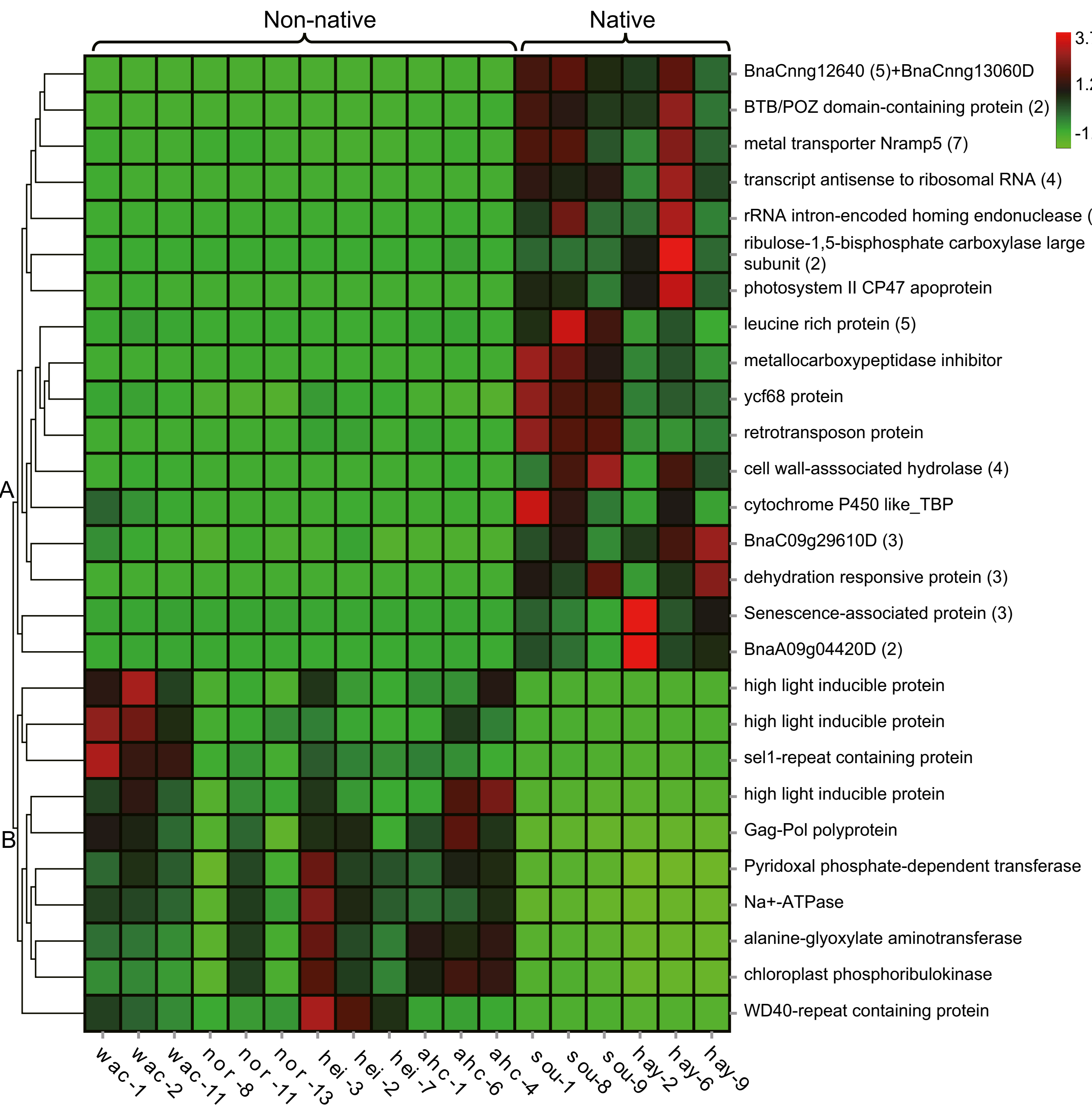

FIGURE 3 Centred, $\log_2$-transformed expression profiles and hierarchical clustering for 59 unigenes, which are consistently differentially expressed between the native and non-native sites where *Gracilaria vermiculophylla* was sampled. Cluster A and B group unigenes that are up (red) and downregulated (green) in the native range, respectively. Numbers in closed parenthesis trailing a gene name indicate the copy numbers of unigenes annotated for that function, and these unigenes share the same expression profile. [Colour figure can be viewed at wileyonlinelibrary.com]

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inducible protein (HLIP, three orthologues), Gag-Pol poly protein, Na^+-ATPase, alanine-glyoxylate transaminase, chloroplast phosphoribulokinase, and WD40-repeat containing protein were all upregulated in the non-native range (Figure 3). In addition, a total of 281 transcription factor (TF) unigenes belonging to 25 TF families were identified (Figure S8), and they displayed variable but nondistinguishable expression profiles between the native and non-native ranges (Figure S9).

3.2 | miRNA identification and expression profiling

A total of 309.35 Mb single-end raw reads were produced and subjected to quality control, which yielded >99.0% high-quality reads and >80.0% filtered tags in samples from most sampling sites (Table S6). The majority of miRNA reads ranged from 18 to 24 nt in length (Figure S10). In most cases, the most abundant miRNA sequences were 19 nt, followed by 20 nt miRNA, which is shorter than the 21 nt and 24 nt reported in land plants like *Citrus* (Xie et al., 2017) and the invasive *Solidago canadensis* (Xu et al., 2019).

Approximately 20–30 miRNAs were either down- or upregulated when the six sites were compared to each other, except for the hay thalli which exhibited 2–3-fold downregulation when compared with other localities (Figure S11). GO enrichment classified most miRNA- regulated target unigenes into the biological process (e.g., ion transport) and the molecular function (e.g., oxidoreductase activity) for the pair sou-vs.- ahc. This was similar for the pair sou-vs.-hei, although the category of molecular function dominated (Figure S12). The KEGG pathway enrichment classified all miRNA- regulated target unigenes as metabolic (e.g., photosynthesis and metabolism) and environmental information processing pathways (e.g., signal transduction) in the pair sou-vs.-ahc, whereas the target unigenes in the pair sou-vs.-hei were solely represented by metabolic pathways (e.g., nitrogen metabolism and biosynthesis of amino acids) (Figure S12).

We further attempted to identify candidate miRNAs that regulated the expression of 59 target unigenes (Figures 2b and 3) by screening the intersection of eight native/non-native site pairs (see above). Ten functionally-annotated and three novel miRNAs were identified in the pair sou-vs.-ahc to negatively regulate the expression of 30 target unigenes (Table S7). In particular, 15 target unigenes that had the same functional description (cell wall-associated hydrolase and senescence-associated protein) were included in the 59 mRNA unigenes, showing expression changes with log_2fold-change(FC) values ranging from −13.03 to 10.50 (p = [.0104, 1.19 E-11]). For the pair sou-vs.-hei, only three novel miRNAs were identified to negatively regulate the expressions of target genes with clear functional annotation (metal transporter Nramp5 and senescence-associated protein; Table S8). However, only nine miRNAs were found to positively regulate the expressions of target unigenes (e.g., senescence-associated protein and yef68 protein) in the pair sou-vs.-ahc (Table S9). Another nine miRNAs positively regulated the expressions of 40 target unigenes (e.g., metal transporter Nramp5 and PLC-like phosphodiesterase) in the pair sou-vs.-hei, with log_2FC values ranging from −16.06 to 1.274 (p = [0.0039, 3.91 E-28]) (Table S10). In addition, nine and eleven functionally-annotated miRNAs were identified to negatively regulate the expression of 16 and 42 target unigenes identified by mRNA analysis in the pairs sou-vs.-wac and sou-vs.-nor, respectively (Tables S11–S12). Fourteen and five functionally-annotated miRNAs were identified to positively regulate the expression of 24 and 36 target unigenes in the pair sou-vs.-wac and sou-vs.-nor, respectively (Tables S13–S14). Many of these target unigenes that had the same functional description (metal transporter Nramp5, senescence-associated protein, and ATP synthase subunit alpha) were included in the 59 mRNA unigenes, showing expression changes with log_2fold-change (FC) values ranging from −13.42 to 11.52 (p = [3.91 E-6, 0.0026]) (Tables S11–S14).

3.3 | Correlation of DE miRNAs and mRNAs

The miRNA-gene interactions revealed 76 and 144 interacting connections between DE miRNAs and DE mRNA in the sou-vs.-ahc and sou-vs.-hei pairs, respectively (Tables S7–S10). For annotated target unigenes in the pair sou-vs.-ahc, eight miRNAs appeared to negatively regulate gene expression, particularly for rRNA intron-encoded homing endonuclease, cell-wall associated hydrolase, and cytochrome P450-like TBP protein (Figure 4a). For example, the expression of miR10516-x was upregulated in the ahc population, but its target-gene (Unigene0024406) was downregulated. In addition, the four miRNAs (miR10516-x, miR156-y, miR5564-x and novel-m0070-5p) were correlated with multiple target unigenes, while four other miRNAs just regulated one target unigene. However, two target unigenes (metal transporter Nramp5 and senescence-associated protein) were both negatively and positively regulated by six different miRNAs (Figure 4a and b). In the pair sou-vs.-hei, miRNAs mostly increased the expressions of target-genes. In particular, the two novel miRNAs (m007-3p and m008-3p) were correlated with the expression of the target unigene (metal transport Nramp5) (Figure 5). Other target-genes (i.e., senescence-associated protein and Ycf68 protein) were positively regulated by five different miRNAs (Figure 5). Similar interacting connections between DE miRNAs and DE mRNA were observed in the sou-vs.-wac and sou-vs.-nor pairs (Figures S13–S14). In most pairs, target unigenes such as metal transporter Nramp5, senescence-associated protein, and cytochrome P450 like-TBP, were regulated by miRNAs positively or negatively.

4 | DISCUSSION

We focused on 59 candidate genes with available functional annotation using comparative mRNA sequencing that showed significantly co-differentiated expression in non-native *G. vermiculophylla* thalli as compared to their native counterparts. Some of these target genes were consistently coexpressed via miRNA probably resulting from

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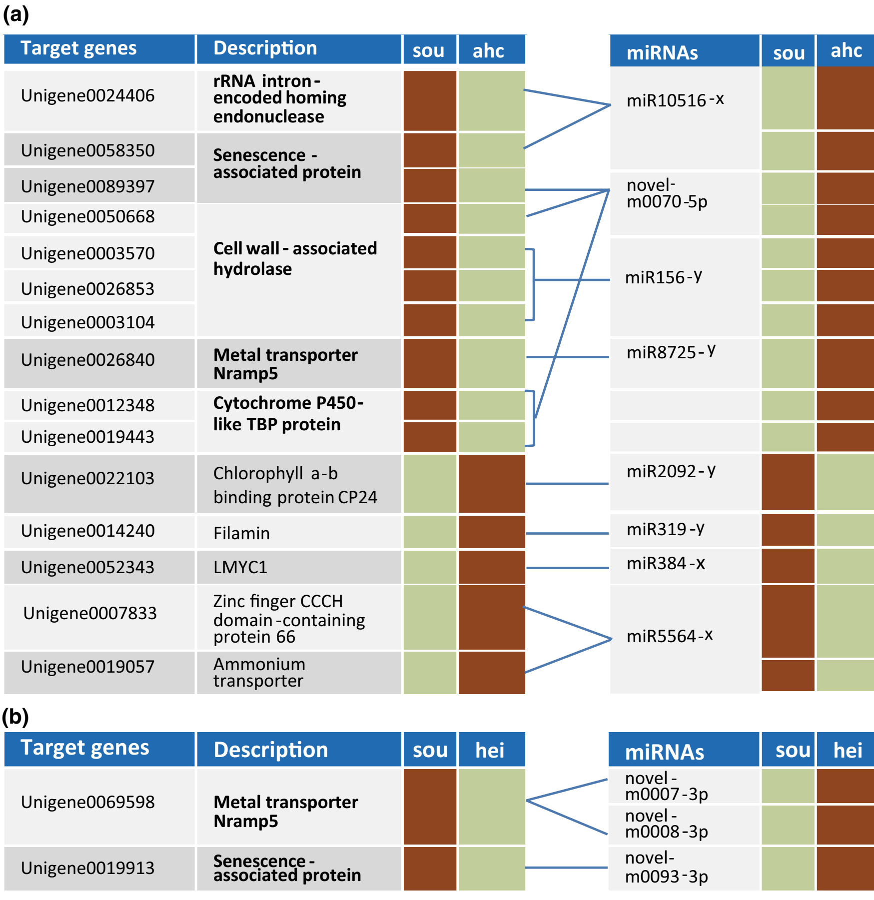

FIGURE 4 miRNA-mRNA negative correlation network. Sou-ahc (a) and sou-hei (b) represent source-to-invasion relationships. Downregulated mRNAs and miRNAs are highlighted in light green and the upregulated in brown. [Colour figure can be viewed at wileyonlinelibrary.com]

trade-offs among different metabolic pathways and may contribute to the adaptive colonization and persistence of *G. vermiculophylla* (see also reviews of plants in Bock et al., 2015; Mounger et al., 2021). Differential gene expression among populations potentially reflects the role of divergent selection and local adaptation (Hodgins et al., 2013), and invasions are no exception.

4.1 | Transcriptional alternations in invaded range

The identified 59 genes showed consistent differential expression between the native and non-native *G. vermiculophylla* thalli. However, we do not know whether these differences in expression were driven by directional selection during or after the invasion (see also Sotka et al., 2018). Flanagan et al. (2021) showed that founder effects were probably very important in driving genomic variation during the *G. vermiculophylla* invasion, despite adaptive shifts (Sotka et al., 2018) and reproductive system shifts (Krueger-Hadfield et al., 2016). It is interesting that Heiligenhafen, Germany and Ape Hole Creek, MD, USA—both non-native sites in which thalli occupy consistently low salinity environments (~15 ppt on average)—exhibit a very similar gene expression patterns (Figure 1b).

Most of the 59 genes differentially expressed between the native and non-native thalli had metabolic functions (Figure S12; Table S5). This suggests that *G. vermiculophylla* may have a similar molecular response to environmental stresses as documented for phenotypic shifts (see Sotka et al., 2018). This could account for its range-wide persistence and expansion in North America, Europe, and northwestern Africa (see also Hodgins & Rieseberg, 2011 for an example in the ragweed *Ambrosia artemisiifolia*). Some genes, such as natural resistance-associated macrophage proteins or dehydration responsive protein were downregulated in the non-native range (Figure 2). These genes have been shown to respond to pathogens and abiotic stresses (Boyle et al., 2009; Gill et al., 2017; Sasaki et al., 2012; Wang et al., 2010; Weaver et al., 1998). One of the differentially expressed genes was cytochrome P450 like_TBP which is involved in the biosynthesis of secondary products and hormones and the detoxification of xenobiotics and metabolism (Mu et al., 2015). Leucine-rich proteins are involved not only in disease resistance (Jones & Jones, 1997), but also in the formation of permeable Ca^{2+} channels that operate in plant immune responses (Huang et al., 2022). Accordingly, compared to the native thalli, non-native *G. vermiculophylla* exhibited better defence against epiphytes due to different secondary metabolites (Wang et al., 2017). Non-native thalli have also been reported to develop chemically-mediated, selective recruitment to attract the settlement of protective microbiota but reduce settlement of pathogens (Saha & Weinberger, 2019). Such evidence shows that the elevated resistance of *G. vermiculophylla* in the non-native range is probably linked to an enhanced chemical defence capacity and rapid adaptation to controlling new microbial epibionts.

The nuclear-encoded HLIP that can respond to a variety of stresses including high light, UVB (Adamska, 1995), nutrient

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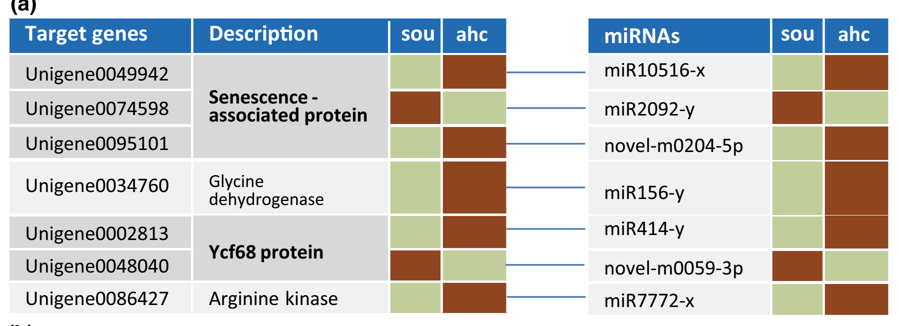

FIGURE 5 miRNA-mRNA positive correlation network. Sou-ahc (a) and sou-hei (b) represent source-to-invasion relationships. Downregulated mRNAs and miRNAs are highlighted in light green and the upregulated in brown. [Colour figure can be viewed at wileyonlinelibrary.com]

starvation (Levy et al., 1993), and senescence (Binyamin et al., 2001), was specifically upregulated in the non-native thalli with three homologues (Figure 2). The inducible HLIP gene probably can further trigger the downregulation of senescence and dehydration responsive genes in an interactive way (Sävenstrand et al., 2004). Another gene that was upregulated in the non-native range, pyridoxal 5′-phosphate (PLP), is a biologically important cofactor to metabolize cellular carbon and nitrogen (Leasure et al., 2011). Chronic exposure to ambient low UVB radiation, if applicable throughout the non-native range, may not be problematic to *G. vermiculophylla* due to its potential accumulation of PLP photoproducts. Testing whether and how the PLP works as a UVB receptor that photochemically responds to ultraviolet light will have a broad impact on our understanding of the functional adaptation of *G. vermiculophylla* to new environments.

ATPase is a proton pump that provides energy for transport of ions across the plasma membrane and tonoplast, respectively. Na^+-ATPase can form a phosphorylated intermediate during the catalytic cycle (Moller et al., 1996) and function for the heat shock protein 70 (HSP70) in an ATP-dependent manner (Mulaudzi-Masuku et al., 2015). Hammann, Wang, et al. (2016) found that the non-native *G. vermiculophylla* populations survived heat shock better and expressed HSP70 significantly higher than the native populations after acclimation to the same conditions. Therefore, our detected upregulation of HSP 90-2 (Table S11) and Na^+-ATPase (Table S13) reinforces the work of Hammann, Wang, et al. (2016) and suggests an increased activity of the transport proteins and adaptation to salinity in new habitats (see other work by Mansour et al., 2003 in plants). Alanine-glyoxylate aminotransferase (AGT) is a peroxisomal photorespiratory enzyme essential for photorespiration in plants (Liepman & Olsen, 2001). *Gracilaria vermiculophylla* can tolerate environmental variables such as CO_2, temperature, and nitrogen over wide ranges (Gorman et al., 2017; Sotka et al., 2018). The upregulation of AGT probably prevents the breach and infection of phytopathogenic fungi which can block lipid droplet mobilization in coordination of the β-oxidation and the glyoxylate cycle (Bhadauria et al., 2012). In angiosperms, CO_2 capture depends critically on the balanced expression level of the chloroplast phosphoribulokinase (PRK) that is activated by light (Zhang et al., 2018). For introduced sites with deficient nutrients, PRK can potentially allow *G. vermiculophylla* to maintain high catalytic rates by catalysing the phosphorylation of ribulose-5-phosphate into RuBisCo, particularly when the

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non-native habitats are highly variable to cause large fluctuations in metabolite content (Paul et al., 2000). Other targets, such as the Sel1 repeats-containing genes and the WD40 repeat containing protein, are either presumably involved in interactions with specific ligands during pathogenicity responses or reported in wheat as positive regulators to tolerate to salt and osmotic stresses (Kong et al., 2015).

It should be noted that some identified genes, such as photosystem II CP47 apoprotein, ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCo) or ycf68 protein (its homologue ycf30 controls the transcriptional activation of the RuBisCo operon, Minoda et al., 2010), are involved in photosynthesis and associated pathways. These downregulated genes probably did not affect the photosynthetic and respiratory efficiency because *G. vermiculophylla* has a large capacity for acclimation to prevailing environmental conditions (Phooprong et al., 2007). This capacity may enable *G. vermiculophylla* in the invaded range to be more resilient to ambient ultraviolet-B (UVB) radiation (John et al., 2001) and abiotic stresses, such as heavy metal ion pollution and extreme temperature (Hammann, Rempt, et al., 2016; Hu & Lopez-Bautista, 2014; Sotka et al., 2018). Altogether, the reduced expression of genes associated with metabolism probably cooperates with the upregulated genes to allow for invasion-mediated local adaptation of *G. vermiculophylla* in new environments (see also Sotka et al., 2018). Nevertheless, it will be of interest to further compare the metabolic differences of primary/secondary compounds between the native source and non-native thalli, including the examination of pathogens and symbiotic microbial communities (see Bonthond et al., 2020, 2021).

4.2 | MiRNA-mediated epigenetic regulation

Epigenetic phenomena can contribute to variation in gene expression and phenotype via multiple molecular mechanisms, including miRNA (Kinoshita & Jacobsen, 2012). In this study, our correlation of DE miRNAs and mRNAs show that the miRNA-mRNA regulatory network patterns involved in environmental adaptation are complex and can differ among invaded regions of *G. vermiculophylla*. Epigenetic evolutionary changes in metabolic traits, such as photosynthesis (Ycf68 protein in Figure 5) and energy consumption (cytochrome P450 in Figure 4) suggest possible rapid epigenetic adaptation in *G. vermiculophylla* within less than 100 years. Sotka et al. (2018) showed phenotypic changes occurred much quicker than currently appreciated in the literature on biological invasions. However, it is unclear whether these shifts in phenotype and genotype are exceptional in *G. vermiculophylla* or may be a feature of many successful marine invaders.

miRNA regulators can control the expressions of target-genes, and thereby impose important impacts on the phenotype, growth, and stress response (An et al., 2013). Here, we identified a subset of key candidate miRNA regulators that showed differential expression between the native source site sou and two non-native sites (ahc and hei). After comparing the expression of the miRNAs with the expression of the predicted target genes, the DEUs-based GO functional classification showed that the differentiated biological processes and molecular functions between sou and each of ahc, hei, wac, or nor were apparently correlated with miRNAs (Figures 4 and 5, Figures S12–S14). For example, specific processes were highly represented, including "transport", "localization", "metabolism", and "biology", as well as functions of "binding" and "catalytic activity". KEGG pathways further showed that photosynthesis and biosynthesis of secondary metabolities were shared between the native/non-native pairs (Figure S12c–S12d). miRNA regulation on gene expression contrasted between the native source sou and the non-native ahc and hei, with negative regulation in ahc in rRNA intron-encoding homing endonuclease, senescence-associated protein, cell wall-associated hydrolase and cytochrome P450-like TBP protein. This suggests that gene expression patterns of *G. vermiculophylla* in the non-native range probably depend on particular environments. Although there are dozens of gene expression profiles shared among populations in the non-native range, the adaptation to local environments enabled miRNAs to harbour different or opposite regulation mechanisms to different target genes. These results suggest a strong local adaptation and environment-dependent epigenetic acclimation in post-invaded *G. vermiculophylla* populations, but the use of a distantly related reference genome of *Gracilariopsis chorda* may have lowered the number of epigenetically regulated miRNAs, including the correlation analysis with target unigenes. Similar results were also found by Sotka et al. (2018) with the establishment of parallel clines following invasion. Testing for specialized changes (within the invaded-range) in different life cycle stages (i.e., gametophyte vs. tetrasporophyte) would provide a better understanding of genetic homozygosity, ecological differentiation, and reproductive shifts in an evolutionary context (Krueger-Hadfield, 2020)

4.3 | Resource reallocation towards metabolism in invaded range

Resource reallocation is context-dependent and can be detected by comparing the relation among defence, growth, and reproduction under different conditions (Lewis et al., 2006). In macroalgae, secondary metabolites, such as phlorotannins and furanones, account for the cost of metabolic defence against harsh conditions (Dworjanyn et al., 2006; Gómez et al., 2016; Jormalainen et al., 2003). In the non-native range, the feeding deterrence of some herbivores is probably attributed to the high concentration of prostaglandin E2, a toxic compound that provides protection for *G. vermiculophylla* against animal consumers (Hammann, Rempt, et al., 2016). The consistent expression of metabolism-related genes in all non-native thalli, particularly the production of carrier proteins, inhibitors, and ion pumps (Figure 2; Tables S4–S5), suggests that selection probably favours metabolic processes with increased allocation to tolerate abiotic conditions in the invaded range, along with herbivore defence (Cronin, 2001; Toth & Pavia, 2000).

Natural selection acts to optimize the allocation of resources to best suit life history and environmental shifts for invasive species

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within evolutionary and ecological constraints (Bock et al., 2015; Cronin, 2001). The upregulation of HLIP and the downregulation of photosystem II CP47 apoprotein and ycf68 protein (Figure 3), probably reduces the primary metabolic pathways of photosynthesis or respiration rate of *G. vermiculophylla* in the invaded range. This will initiate a massive degradation or biosynthesis of new macromolecules at the subcellular level (Cronin, 2001), including the recycling and reuse of nutrients and energies (e.g., Na^+-ATPase in Figure 3) at the expense of decreased photosynthesis. Downregulation of photosynthetic proteins can free resources that can be allocated to secondary metabolism, including the reduction of oxidative damage by toxic oxygen derivatives generated by the photosynthetic electron transport chain (Flöthe et al., 2014). These lines of evidence suggest that the non-native *G. vermiculophylla* thalli may have evolved a higher tolerance to biotic and abiotic stress to increase competitive ability (see also Sotka et al., 2018), and therefore perform well under competitive conditions (Hodgins & Rieseberg, 2011).

In the invaded range, *G. vermiculophylla* may be constrained in its ability to store nutrients and energy for rapid growth in contrast to the native thalli, and extending the period of active growth during summer to bestow *G. vermiculophylla* a competitive advantage in response to environmental stresses (Abreu et al., 2011). We presume that non-native populations are more efficient with respect to resource acquirement, including efficient photosynthesis, CO_2 fixation and nitrogen uptake, which enable *G. vermiculophylla* to allocate more resource into defence and stress management and expand rapidly in new environments. Elucidating mechanisms related to these processes will largely advance our understanding of how marine invaders, including all invaders, involved higher fitness traits in response to resource availability than native populations.

AUTHOR CONTRIBUTIONS

Zi-Min Hu, Florian Weinberger and Gao-Ge Wang conceived the project. Florian Weinberger, Mahasweta Saha and Stacy A. Krueger-Hadfield collected and identified algae. Mahasweta Saha, Florian Weinberger, Gaoge Wang and Zi-Min Hu conceived the experimental design. Mahasweta Saha performed common garden experiments and preserved thallus material. Kai-Le Zhong performed RNA isolation and molecular experiments. Jing-Xi Xiang, Di Zhang, and Zi-Min Hu collected and analysed molecular data. Zi-Min Hu, Mahasweta Saha, Quan-Sheng Zhang, Alexander Jueterbock, Florian Weinberger, Stacy A. Krueger-Hadfield, and Gao-Ge Wang interpreted and discussed the results. Jing-Xi Xiang, Zi-Min Hu, Mahasweta Saha, Florian Weinberger, Stacy A. Krueger-Hadfield, Gao-Ge Wang, and Alexander Jueterbock wrote and/or revised the manuscript. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The mRNA-seq and miRNA-seq raw data of *Gracilaria vermiculophylla* as fastq files have been deposited in NCBI SRA database under the accession numbers PRJNA862752 and PRJNA862878.

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