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ORIGINAL RESEARCH

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Transcriptome and functional analyses of phenotypic plasticity in sea grape *Caulerpa okamurae*

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Abstract

Caulerpa is a marine green macroalga distinguished by a large single cell with multiple nuclei. It also exhibits remarkable morphological intraspecies variations, in response to diverse environmental types. However, the molecular mechanisms underlying this phenotypic plasticity remain poorly understood. In this work, we compare the transcriptomes of *Caulerpa okamurae* Weber Bosse, 1897 displaying altered phenotypes of cultivation and natural phenotypes and investigate significantly regulated genes and their biological functions using differential expression analyses. We observe light-harvesting complex upregulation and cellular framework stability downregulation in altered phenotypes compared to the natural phenotypes. Intertidal macrophytes reduce light capture to avoid photodamage and regulate their morphology to protect against wave damage. In contrast, the lower light conditions and the cultivation environment augment light capture and increase a morphology prioritizing light trapping. Moreover, the addition of simulated wave-sweeping stimuli induces a return to the natural morphology under high-light conditions, showing how mechanical stress affects morphological organization in *C. okamurae*. We provide detailed gene expression patterns in *C. okamurae* under varying light intensities and water conditions, suggesting a distinct influence on its morphological traits.

1 | INTRODUCTION

Caulerpa (family Caulerpaceae, order Bryopsidales) is a genus of green macrophytes distributed worldwide in tropical and subtropical oceans (Jacobs, 1994; Zuldin, 2023). *Caulerpa lentillifera*, *C. racemosa*, and other edible species of this genus are rich in soluble dietary fibers, proteins, minerals, phytochemicals, and polyunsaturated fatty acids (de Gaillande et al., 2017; Nagappan & Vairappan, 2014; Nurkolis et al., 2023; Paul et al., 2014). Additionally, they have various bioactive chemicals that produce antioxidation, antibacterial, and anticancer effects (Aroyehun et al., 2020; Nagappan & Vairappan, 2014; Nurkolis et al., 2023; Permatasari et al., 2022; Zubia et al., 2020). As such, *Caulerpa*, also known as "green caviar" or "sea grapes" is commercially cultivated, predominantly in the Philippines, Taiwan, and Japan (de Gaillande et al., 2017; Nagappan & Vairappan, 2014; Paul et al., 2014).

Caulerpa racemosa, *C. taxifolia*, and *C. cylindracea* are invasive species in the Mediterranean Sea (Cevik et al., 2007; Gennaro et al., 2015; Klein & Verlaque, 2008; Piazzi et al., 2005). *Caulerpa*'s distinctive nutrient acquisition method and environmental adaptations contribute to its invasive characteristics (Cavas et al., 2005; Gennaro et al., 2015;

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Raniello et al., 2004). Over 100 accepted *Caulerpa* species share a similar morphological structure characterized by several upright assimilators (fronds) on a long creeping stolon, with rhizoids providing support underneath (Guiry & Guiry, 2023; Malta et al., 2005). These rhizoids can attach to soft and hard substrata, such as sand, mud, pebbles, and rock (Gennaro et al., 2015; Klein & Verlaque, 2008). *Caulerpa* species absorb nutrients from these sediments via the rhizoids, unlike other marine macroalgae that solely rely on the water column (Gennaro et al., 2015; Malta et al., 2005). *Caulerpa* species also adjust the number of reaction centers in their photosystems to maintain a constant photosynthetic efficiency and flourish in both high and low light intensities (Raniello et al., 2004). Damaged or cut *Caulerpa* sections can swiftly regenerate (Matilsky & Jacobs, 1983; Shin et al., 2021). Additionally, *C. racemosa*'s antioxidant enzyme activities were higher than other marine macrophytes, indicating a potent stress tolerance (Cavas & Yurdakoc, 2005).

Phenotypic plasticity refers to an organism's ability to display variable characteristics or traits in response to fluctuating environmental conditions. This involved a broad spectrum of factors, such as changes in behavior, physiology, and/or morphology. Notably, *Caulerpa* species exhibit substantial phenotypic plasticity (Belton et al., 2014; Collado-Vides & Robledo, 1999; Klein & Verlaque, 2008). Afik et al. (2023), Calvert (1976), Cevik et al. (2007), and Ohba et al. (1992) observed that *Caulerpa* species' phenotypes shift in response to light conditions, frequently leading to misidentifications and nomenclatural problems (Belton et al., 2014; Carruthers et al., 1993).

Caulerpa okamurae is considered a climate-sensitive biological indicator species (CBIS) in Korea based on its sensitivity to environmental changes (Lee et al., 2010). In its natural habitat, adult *C. okamurae* displays several fronds surrounded by grape-like ramuli, closely resembling *C. lentillifera*. However, unlike other species in the genus *Caulerpa*, studies elucidating *C. okamurae*'s phenotypic plasticity are severely lacking. *Caulerpa okamurae* primarily inhabits the intertidal zone, an environment in which conditions rapidly change due to fluctuations between high and low tides (Kitzes & Denny, 2005), exposing macrophytes to various abiotic stresses.

This study elucidates the molecular mechanisms underlying *C. okamurae*'s phenotypic plasticity and induces a plastic response through cultivation experiments based on previous *Caulerpa* findings. We sequenced the transcriptomes (RNA-Seqs) of *C. okamurae* with altered and natural phenotypes to investigate differential gene expression patterns and the biological processes involved. These findings establish valuable insights into *C. okamurae*'s phenotypic plasticity, evidencing the ecological complexity of these commercially beneficial macroalgae.

2 | MATERIALS AND METHODS

2.1 | Algal sample collection and cultivation

Figure 1 shows an overview of our workflow. *C. okamurae* samples were collected from the lower intertidal zone along the coast of Jindo, Jeonnam, Korea (34.39° N, 126.27° E; Figure S1) on 16 June 2021 and immediately transported to the laboratory using insulated cooler boxes filled with seawater. Healthy fronds were selected and gently

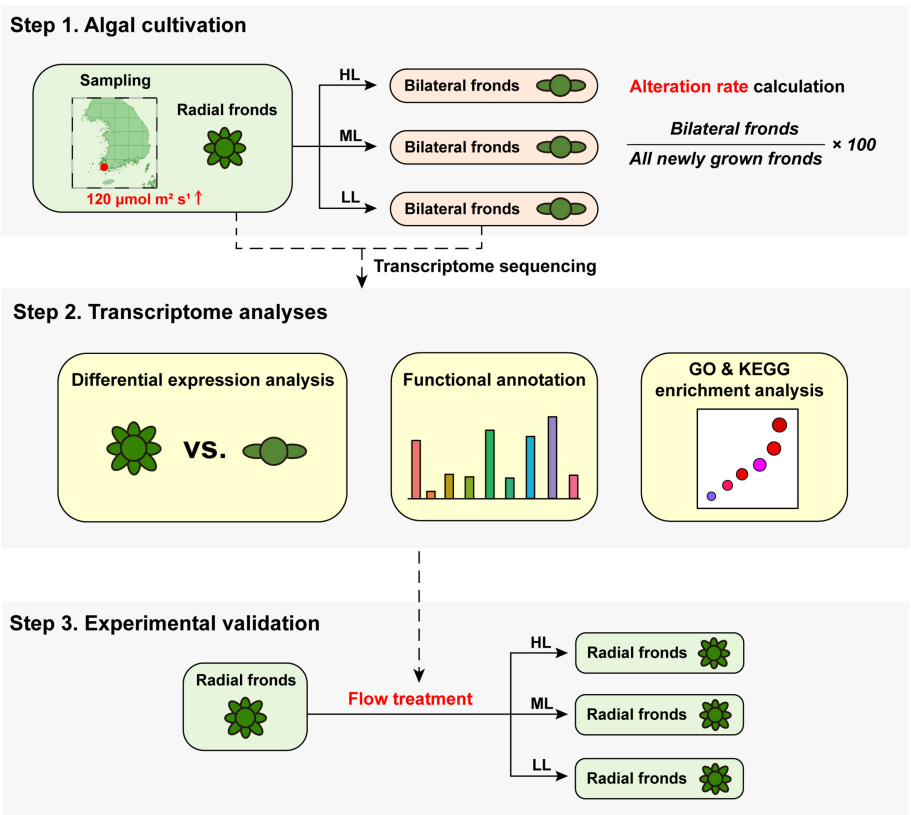

FIGURE 1 **Overview of the experimental design and analysis conducted in our study.** In step 1, we collected *Caulerpa okamurae* samples (radial fronds) from habitats with light intensity above 120 μmol m^{-2} s^{-1} and cultured them under three different light intensities to observe the plastic response of fronds (low light: LL, 30 μmol m^{-2} s^{-1}; medium light: ML, 60 μmol m^{-2} s^{-1}; high light: HL, 120 μmol m^{-2} s^{-1}). Then we performed whole transcriptome sequencing of radial and bilateral fronds. In step 2, the whole transcriptome data from each sample was employed for differential expression analysis, functional annotation, and GO & KEGG enrichment analysis. By integrating the previous reports and our transcriptome analysis results, we cultivated *C. okamurae* with flow treatment to control the phenotypic plasticity in step 3.

brushed with a smooth artist's brush to remove epiphytes from their surface. Over 180 assimilators were cut into 3-cm-long fragments and pre-cultured at room temperature for three days to minimize the wound's effect. Coral sand was sterilized by autoclaving and then dried at 60°C before being used as a substrate for fragments. The culture medium was PES30 (30% diluted Provasoli's enriched seawater medium; Table S1), which was completely changed every four days. Using pre-cultured fragments, two separate experiments were conducted: All upright fronds were attached on coral sand (20 mm in diameter) and cultured at a constant room temperature (21 ± 1°C) for ten days and under three light intensity treatments (30, 60, and 120 μmol m^{-2} s^{-1}). The light was produced by a daylight 6500 K LED light source and its intensity was measured using a digital illumination meter (DX-200, Takemura). For the second, the same procedure was followed, but a water-flow treatment with two levels (flowing or static) was added so that all possible combinations of light intensity and water flow were represented. Water flow was generated by the installation of two water pumps (3 W, HJ-311, Mondialfauna) diagonally installed on both sides of a transparent acrylic tank (530 × 325 × 200 mm) containing 10 L PES30 solution (Figure S2). Each treatment was done in triplicate, each replicate being a tank containing 10 uprights fronds. Other culture conditions were the same: a salinity of 30 psu and a 12 h: 12 h, light: dark photoperiod. After ten days of cultivation, newly grown fronds were counted, noting phenotypically abnormal bilateral fronds, to quantify the alteration rate as the percentage of phenotypically altered fronds (bilateral fronds/all newly grown fronds × 100).

2.2 | Library preparation and transcriptome sequencing

We used three biological replicates each from bilateral and radial *C. okamurae* fronds to investigate the gene expression changes associated with phenotypic plasticity. Total RNA extraction, library preparation, and RNA-seq were conducted through DNA Link, Inc. (Seoul, Korea). Libraries for each sample were generated using the TruSeq Stranded mRNA library kit (Illumina) following the manufacturer's protocol. Prepared libraries were sequenced on an Illumina Novaseq6000 platform, generating paired-end reads.

2.3 | Transcriptome analysis

Raw data were filtered to remove adaptor sequences and low-quality bases with a Phred quality score of 30 or less using Trim Galore! (ver. 0.6.6) (https://github.com/FelixKrueger/TrimGalore). After filtering, high-quality reads from all samples were assembled *de novo* using Trinity (ver. 2.12.0) under the default parameters (Grabherr et al., 2011; Jeon et al., 2023; Jung et al., 2020; Kim et al., 2024; Ranjan et al., 2015). Assembled contigs' open reading frames (ORFs) were then predicted to generate unigene sequences with a minimum protein length of 75 amino acids using TransDecoder (ver. 5.5.0) (https://github.com/TransDecoder/TransDecoder). ORF redundancies were eliminated based on a 98% sequence similarity using `cd-hit-est` (ver. 4.8.1) (Li & Godzik, 2006). Contig and unigene completeness were assessed and compared with BUSCO (ver. 5.2.2) based on the single-copy orthologs represented in the chlorophyta_odb10 database (Simão et al., 2015). Functional annotations of unigenes were analyzed using `blastx` against the National Center for Biotechnology Information (NCBI) Non-redundant (NR), UniProt, and TAIR databases. EggNOG-mapper (ver. 2.1.7) was also employed to gather additional information on unigenes using the euKaryotic Orthologous Groups of proteins (KOG), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Carbohydrate-Active enZYmes (CAZy), Biochemical Genetic and Genomic knowledgebase (BIGG), and Protein Families (PFAM) databases (Cantalapiedra et al., 2021).

2.4 | Differential expression analysis

Filtered reads for each sample were mapped onto the unigenes using Bowtie2 with the "`–very-sensitive`" setting for read count calculations (Langmead & Salzberg, 2012). Mapping results were normalized using the Cufflinks package's (ver. 2.2.1) Fragments per kilobase of transcript per million (FPKM) value to estimate gene expression levels (Trapnell et al., 2010). The criteria for significantly differentially expressed genes (DEGs) was set at a p-value <0.05 and $|\log_2(\text{fold-change})| \geq 1$ between cultivars and field samples. DEGs were visualized using the R package EnhancedVolcano (https://github.com/kevinblighe/EnhancedVolcano). Functional enrichment analysis of DEGs was conducted to elucidate significant biological information within the DEG group, employing stringent statistical criteria using a Benjamini-Hochberg adjusted p-value <0.05 on the KOBAS website (Bu et al., 2021).

3 | RESULTS

3.1 | Irradiance effects on *C. okamurae*'s phenotype

Caulerpa okamurae is characterized by a long assimilator (frond) surrounded by beaded lateral branchlets (called ramuli) on the cylindrical stolon. Previous studies noted that *Caulerpa* frond symmetry changed under different irradiances (Calvert, 1976; Cevik et al., 2007; Ohba et al., 1992). The measured light intensity in *C. okamurae*'s habitat ranged between 120 μmol m^{-2} s^{-1} and 700 μmol m^{-2} s^{-1}. The phenotypes of *C. okamurae* fronds observed in their habitats all exhibited radial symmetry (Figure 2a). Therefore, we set three light-intensity conditions, including low light (LL; 30 μmol m^{-2} s^{-1}), medium light (ML; 60 μmol m^{-2} s^{-1}), and high light (HL; 120 μmol m^{-2} s^{-1}), with three experimental replicate groups per condition to study this phenotypic plasticity. Ten days post-cultivation, all newly grown fronds under LL group displayed different symmetries compared to field fronds (Figure 2b). ML group presented 94.4%, 97.1%, and 92.3% alteration rates and the alteration rates in the HL groups were 71.1%, 67.5%, and

70.6% (Table 1 and Figure 2c). The light intensity significantly induced phenotype changes in *C. okamurae*'s frond structure ($p < 0.0001$).

3.2 | Sequencing, filtering, and *de novo* assembly

We performed the DEG analysis comparing natural and altered frond types to ascertain the regulatory mechanisms underlying *C. okamurae*'s phenotypic plasticity. Illumina paired-end RNA sequencing generated a total of 108.34 Gbp of sequence data for cultivars and field samples with 344.35 million reads (Table 2). Low-quality reads and adapter sequences were filtered, leaving 78.33 Gbp containing 252.65 million high-quality reads (Table 2). The filtered reads were assembled *de novo* into full-length transcripts and predicted ORFs were extracted. During redundancy elimination, the BUSCO assessment showed that the duplication rate decreased from 83.27 to 29.48%, resulting in a total of 57,879 unigenes obtained (Table 3 and Figure S3).

3.3 | Functional annotations of unigenes

We next conducted a `blastx` search against NCBI NR, UniProt, KOG, PFAM, KEGG, GO, BIGG, and CAZy databases to determine the unigene's functions. As a result, 37,599 (64.96%) unigenes were

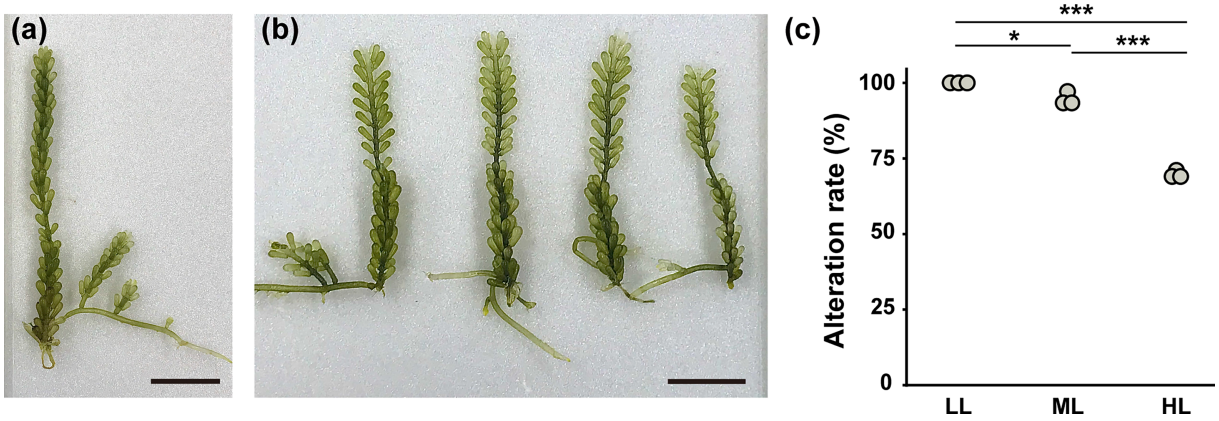

FIGURE 2 ***Caulerpa okamurae*'s phenotypic plasticity under different light intensities.** (a) Natural habitat phenotype (scale bars = 2 cm). (b) Altered phenotype ten days after growth under low light intensity (30 μmol m^{-2} s^{-1}) (scale bars = 2 cm). (c) Phenotypically altered frond percentages in cultivars. The x-axis indicates light intensity (LL, 30 μmol m^{-2} s^{-1}; ML, 60 μmol m^{-2} s^{-1}; HL, 120 μmol m^{-2} s^{-1}). The y-axis represents the average alteration rates for the three light intensities, indicating bilaterally organized frond proportions among all newly grown fronds during the cultivation. Dots correspond to the alteration rates of three replicate groups for each light intensity. The numbers of newly grown fronds are as follows: LL (20, 22, and 20), ML (36, 35, and 39), and HL (38, 40, and 34). Asterisks indicate significant differences (*, $p < 0.05$; ***, $p < 0.001$) determined by one-way ANOVA followed by Tukey's HSD (honestly significant difference) comparisons.

TABLE 1 **The effects of irradiance on *C. okamurae*'s phenotype.** Treatments consisted of three replicate groups and the altered/total number (percentage) of newly grown fronds produced during experimentation in each. The average alteration rate represents the mean value across each replicate.

Light intensity (μmol m^{-2} s^{-1})	Replicate 1	Replicate 2	Replicate 3	Avg. alt. rate[a]
30	20/20 (100%)	22/22 (100%)	20/20 (100%)	100%
60	34/36 (94.4%)	34/35 (97.1%)	36/39 (92.3%)	94.6%
120	27/38 (71.1%)	27/40 (67.5%)	24/34 (70.6%)	69.7%

[a]Average alteration rate.

TABLE 2 Information about raw and filtered data from whole transcriptome sequencing of *C. okamurae*, including three biological replicates from radial (the natural phenotype) and bilateral (the altered phenotype) frond samples.

		Yields (Gbp)		Read number (M)	
Sample ID	Read length	Raw	Filtered (F/R)	Raw	Filtered (%)
Radial 1	101 × 2	3.03 × 2	3.00 / 2.95	30.03 × 2	22.42 × 2 (74.64)
Radial 2	151 × 2	5.15 × 2	4.78 / 4.75	34.12 × 2	26.77 × 2 (78.48)
Radial 3	151 × 2	3.49 × 2	3.15 / 3.13	23.10 × 2	16.40 × 2 (70.99)
Bilateral 1	101 × 2	2.52 × 2	2.49 / 2.45	24.97 × 2	18.46 × 2 (73.93)
Bilateral 2	151 × 2	4.79 × 2	4.34 / 4.29	31.71 × 2	22.51 × 2 (70.97)
Bilateral 3	151 × 2	4.26 × 2	3.85 / 3.81	28.24 × 2	19.77 × 2 (70.00)

TABLE 3 *De novo* transcriptome assembly and *C. okamurae* annotation summary.

Contig	
Total trinity *'genes'*	52,340
Total trinity *'transcripts'*	88,982
% GC	41.39
Transcript N50	2,342
Median length	594.5
Average length (range)	1,222 (189–26765)
Total bases (Mb)	108,771,467 (108.77)
Unigene	
Total trinity *'genes'*	34,823
Total trinity *'transcripts'*	57,879
% GC	44.65
Transcript N50	807
Median length	345
Average length (range)	588.42 (222–25506)
Total bases (Mb)	34,056,948 (34.06)
Annotation	**Percent (%)**
Unigenes with NR	63.26
Unigenes with UniProt	50.58
Unigenes with TAIR	41.81
Unigenes with eggNOG	32.44
Unigenes with KOG	31.97
Unigenes with PFAM	31.62
Unigenes with KEGG	24.69
Unigenes with GO	13.46
Unigenes with BIGG	0.44
Unigenes with CAZy	0.41

annotated in at least one database (Table 3). The KOG, GO, and KEGG databases were primarily utilized to acquire a deeper insight into the biological roles of the unigenes. In particular, the KOG database classified 18,755 unigene functions (Figure S4). Excluding poorly characterized categories, the largest cluster was "Translation, ribosomal structure and biogenesis (J)" followed by "Posttranslational modification (O)", "Signal transduction mechanisms (T)", "Energy production and conversion (C)", "Intracellular trafficking (U)", "Amino acid transport and metabolism (E)", and "Cytoskeleton (Z)." The GO and KEGG databases annotated 7,791 and 14,293 unigenes, respectively (detailed in Supporting File 1).

3.4 | DEG identification and functional enrichment analysis

Approximately 71.58% of all filtered reads from the six samples were mapped to unigenes, with mapping rates of 74.29%, 72.70%, and 70.11% for the radial fronds and 71.62%, 69.88%, and 70.89% for the bilateral fronds (altered phenotype). Consequently, 2,726 DEGs were identified in the bilateral fronds, including 674 upregulated and 2,052 downregulated genes compared to the radial fronds (Figure 3a), with the KOG database classifying 1,095 (Figure 3b). The predominant cluster was "Translation, ribosomal structure and biogenesis (J)" followed by "Posttranslational modification (O)", which is similar to the results of a previous KOG classification of DEGs in *C. lentillifera* under toxic stress (Pang et al., 2022).

GO and KEGG enrichment analyses were achieved using a statistical false discovery rate <0.05 standard to elucidate the significantly regulated processes in the bilateral frond samples and upregulated and downregulated genes were analyzed separately (Supporting File 2). Among the upregulated genes, 121 terms and 23 pathways were significantly enriched (Figure 4a, b). In both databases, the photosystem's light-harvesting complex (LHC; GO:0009768, ko00196) showed the most significant enrichment among all upregulated processes. Chloroplast (GO:0009507) and general metabolic pathway (ko01100) genes were among the most significantly enriched GO terms and KEGG pathways.

Regarding the downregulated genes, 191 terms and 20 pathways were significantly enriched (Figure 4c, d). The GO enrichment analysis revealed an emphasis on cellular structure, including genes related to cytoplasm (GO:0005737), cell wall (GO:0005618), vacuole (GO:0005773), microtubule (GO:0005874), cytoskeleton organization (GO:0007010), and dynein complex (GO:0030286). Additionally, several carbon-related metabolisms were also considerably enriched, including glyceraldehyde-3-phosphate dehydrogenase activity (GO:0004365), glycolytic process (GO:0006096), and gluconeogenesis (GO:0006094). Comparatively, the KEGG database highlighted oxidative phosphorylation (ko00190), nitrogen metabolism (ko00910), carbon fixation (ko00710), photosynthesis - antenna proteins (ko00196), carbon metabolism (ko01200), and glycolysis/gluconeogenesis (ko00010). All processes achieved false discovery rate values below 0.01. In summary, bilateral fronds demonstrated the most significant induction of LHC, while also showing a decrease in investment in cellular structure and carbon metabolism compared to radial fronds.

3.5 | Experimental validation of hydrodynamic effects on phenotypic plasticity

Previous research has shown that external mechanical forces directly modulate plant cell cytoskeleton, impacting differential organ growth and altering its morphology (Hamant et al., 2019). *Caulerpa okamurae*'s natural habitat is the intertidal zone, which poses extreme environmental conditions for its inhabitants. Particularly, macrophytes on rocky shores are exposed to wave-induced mechanical stress (Carrington, 1990; Demes et al., 2021). Therefore, we conducted culture experiments to investigate the effects of artificial water flow, which generates mechanical stress on *C. okamurae*'s morphology.

Water flow had a significant impact on the alteration of *C. okamurae* fronds ($p < 0.0001$). Regardless of water flow, all samples cultivated under low light (LL) formed bilateral fronds, consistent with the morphological changes observed in the previous experiment

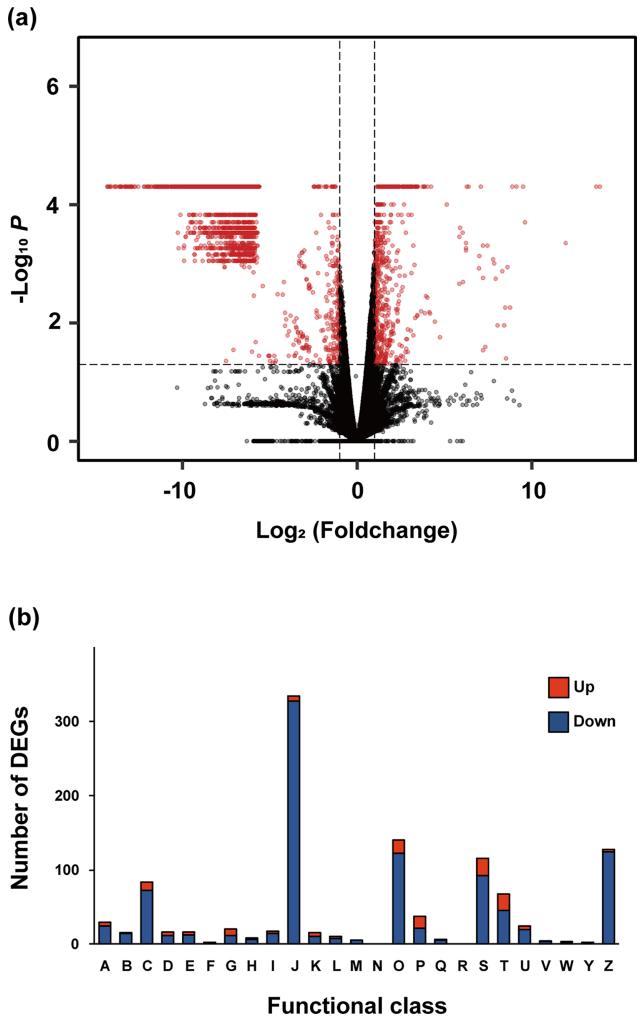

FIGURE 3 **Differentially expressed gene (DEG) analysis comparing bilateral and radial phenotype *C. okamurae* frond samples.** (a) Volcano map illustrating unigene expression levels and statistical significance. The x-axis represents the $\log_2$ (fold-change) expression level in bilateral compared to radial samples. The y-axis represents the $-\log_{10}$ (*p*-value), indicating the statistical significance of the expression level between bilateral and radial frond samples. The *p*-values ranged from 5e-5 to 1. A standard of $|\log_2$ (fold-change)| ≥ 1 and a *p*-value of <0.05 was set as the unigene threshold for significantly differential expression. DEGs are indicated by red dots. (b) DEG classifications based on the euKaryotic Orthologous Groups of proteins (KOG) database. The x-axis represents the functional classes from KOG, including (A) RNA processing and modification; (B) chromatin structure and dynamics; (C) energy production and conversion; (D) cell cycle control and mitosis; (E) amino acid metabolism and transport; (F) nucleotide metabolism and transport; (G) carbohydrate metabolism and transport; (H) coenzyme metabolism; (I) lipid metabolism; (J) translation; (K) transcription; (L) replication, recombination, and repair; (M) cell wall/membrane/envelope biogenesis; (N) cell motility; (O) posttranslational modification, protein turnover, chaperones; (P) inorganic ion transport and metabolism; (Q) secondary metabolite biosynthesis, transport, and catabolism; (R) general function prediction only; (S) function unknown; (T) signal transduction; (U) intracellular trafficking and secretion; (V) defense mechanisms; (W) extracellular structures; (Y) nuclear structure; and (Z) cytoskeleton. The y-axis represents the categorized genes in each class. Upregulated and downregulated genes are indicated by red and blue bars, respectively.

(Figure 2b). However, the flow treatment reduced plasticity in both medium light (ML) and high light (HL) groups (Table 4). Samples grown under ML revealed a 12.9% alteration rate decrease compared to LL samples and also 8.0% alteration rate decrease in flowing compared to static water. Intriguingly, the HL × flow treatment exhibited the lowest plasticity, with a 7.5% alteration rate, and thus a notable 92.5% maintained their original radial frond structure (Figure 5a). A significantly decreased alteration rate was observed between the HL × flow treatment group and both the ML × flow and HL × static treatment groups (Figure 5b). The phenotypes of all experimental groups are shown in Figure S5.

4 | DISCUSSION

Marine macrophytes commonly exhibit phenotypic plasticity, in which their morphological characteristics shift in response to environmental conditions (Crespi, 2020; Kelly et al., 2011; Stewart & Carpenter, 2003; Wells & Pigliucci, 2000; West-Eberhard, 1989). The genus *Caulerpa*, a green seaweed, has garnered attention for its striking morphological variation (Belton et al., 2014; Estrada et al., 2020; Gacia et al., 1996; Klein & Verlaque, 2008; Malta et al., 2005). In particular, Calvert (1976) and Ohba et al. (1992) reported that variation in light intensity induces morphological alterations in *Caulerpa* fronds. Based on these findings, we conducted culture experiments to investigate *Caulerpa okamurae*'s phenotypic plasticity with regard to light intensity. Calvert (1976) focused on low light intensity (about 10 and 20 μmol m^{-2} s^{-1}) and Ohba et al. (1992) experimented with light ranging from 10 to 110 μmol m^{-2} s^{-1}. Considering that the minimum light intensity in the *C. okamurae*'s habitat is 120 μmol m^{-2} s^{-1} and only radial fronds in there, we set 120 m^{-2} s^{-1} as the high light condition in our study. Then 30 μmol m^{-2} s^{-1} and 60 μmol m^{-2} s^{-1} were set as the low and medium light conditions, respectively. In our experiment, morphological alterations were primarily prevalent in frond symmetry in the form of bilateral versus radial branchlet (ramuli) arrangements. Specifically, in LL treatments, fronds exhibited bilateral symmetry, whereas in HL treatments, fronds tended to maintain radial symmetry, the typical phenotype seen in nature (Figure 2a, b). Consistent with past studies, the rates of alteration increased with decreasing light intensity.

Despite previous studies reporting morphological *Caulerpa* variations under different environmental conditions, the molecular mechanisms underlying this phenomenon remain unknown (Cevik et al., 2007; Collado-Vides & Robledo, 1999; de Senerpont Domis et al., 2003; Estrada et al., 2020; Malta et al., 2005; Ohba & Enomoto, 1987; Ohba et al., 1992; Peterson, 1972; Svedelius, 1906; Voerman et al., 2019). In the present study, we performed a comparative transcriptome analysis using high-throughput sequencing technology to investigate these mechanisms in *C. okamurae*. Differential expression analysis identified 2,726 DEGs in bilateral fronds, including 674 upregulated and 2,052 downregulated genes, compared to radial fronds. Notably, downregulated genes were three times more prevalent than upregulated genes.

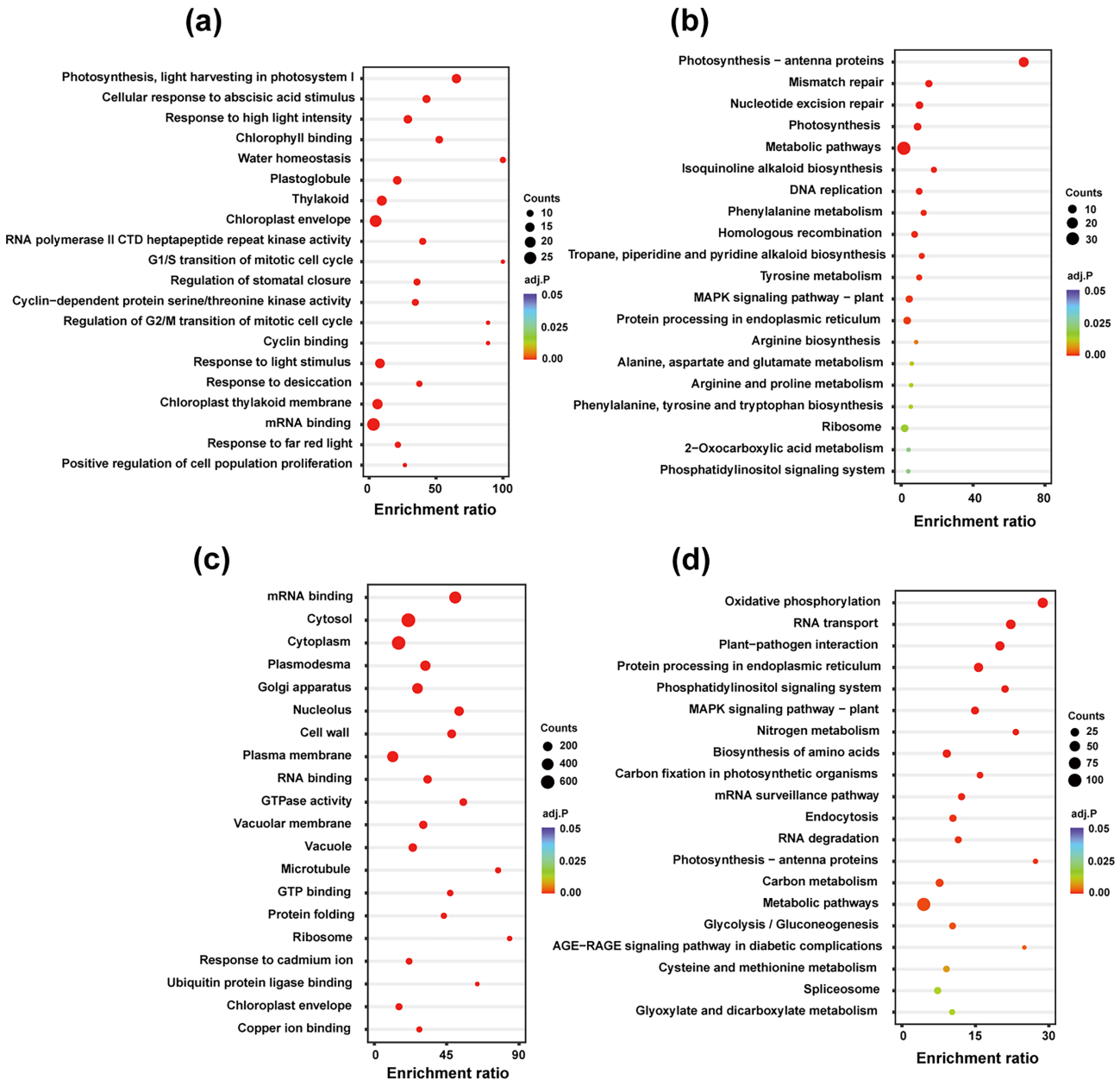

FIGURE 4 **Functional enrichment analysis of differentially expressed genes (DEGs) comparing bilateral to radial frond samples based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.** Dot size indicates the number of significant genes. Significantly enriched (a) GO terms and (b) KEGG pathways for upregulated genes and significantly enriched (c) GO terms and (d) KEGG pathways for downregulated genes. The top 20 terms and pathways are represented, while the remaining are listed in Supporting File 2. The x-axis represents the enrichment ratio, calculated by significant genes / total genes × 100. The y-axis represents the GO terms and KEGG pathways, sorted based on their significance as determined using Benjamini-Hochberg adjusted *p*-values and represented by dot colors.

Functional DEG enrichment analysis using GO and KEGG databases provided biological insights into the functions of the significantly regulated genes. The photosystem's light-harvesting complex (LHC) demonstrated the most substantial upregulation in both databases (Figure 4a, b and Supporting File 2). LHC is crucial for photosynthesis as it captures light energy and transfers this excitation energy to photosystems I and II (Formaggio et al., 2001; Yang et al., 2000). Light profoundly influences marine macrophyte growth and development, evidenced by the plasticity of responses to light condition fluctuations (Gao et al., 2019; Stewart & Carpenter, 2003). Under excessive light intensity, macrophytes undergo physiological alterations that suppress photosynthetic efficiency to prevent photodamage or photooxidation in their photosystems (Hanelt & Nultsch, 2003; Szymańska et al., 2017). Since *C. okamurae* in its natural environment is exposed to severe irradiance in the intertidal zone, lower irradiance in culture conditions may upregulate LHC.

The regulation of photosynthetic efficiency and morphological adjustments in response to varying light intensities are well-

TABLE 4 **The effects of flowing water on *C. okamurae*'s phenotypic plasticity in response to changing light intensity.** Treatments consisted of three replicate groups and the altered/total number (percentage) of newly grown fronds produced during experimentation in each. The average alteration rate indicates the mean value across each replicate.

Light intensity (μmol m^{-2} s^{-1})	Replicate 1	Replicate 2	Replicate 3	Avg. alt. rate[a]
Static water				
30	20/20 (100%)	22/22 (100%)	20/20 (100%)	100%
60	34/36 (94.4%)	34/35 (97.1%)	36/39 (92.3%)	94.6%
120	27/38 (71.1%)	27/40 (67.5%)	24/34 (70.6%)	69.7%
Flowing water				
30	39/39 (100%)	48/48 (100%)	36/36 (100%)	100%
60	38/47 (80.9%)	50/54 (92.6%)	36/41 (87.8%)	87.1%
120	11/77 (14.3%)	4/62 (6.5%)	1/53 (1.9%)	7.5%

[a]Average alteration rate.

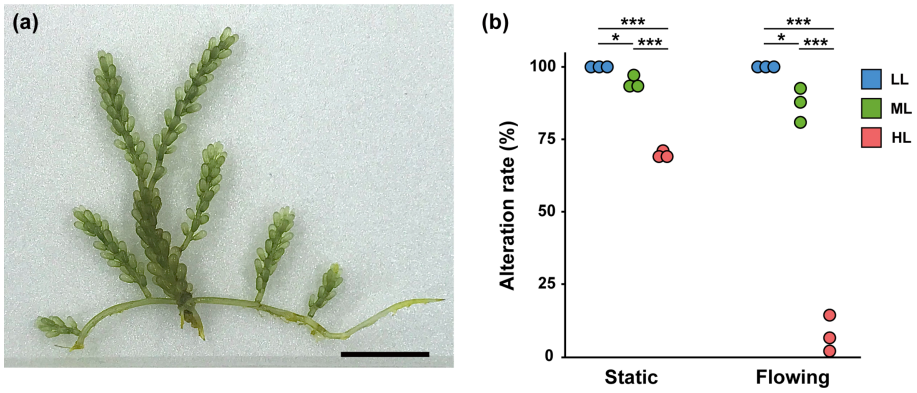

FIGURE 5 ***Caulerpa okamurae*'s phenotypic plasticity in response to water flow and light intensity.** (a) *Caulerpa okamurae*'s phenotype after ten days of growth under high light intensity (120 μmol m^{-2} s^{-1}) with a water flow treatment (scale bar = 2 cm). (b) Phenotypically altered frond percentages in *C. okamurae* grown under three light intensity treatments and hydrodynamic conditions. The x-axis indicates the hydrodynamic conditions, including static and flow treatments. The y-axis represents the alteration rate, indicating bilaterally organized frond proportions among all newly grown fronds during the experiment. Dots correspond to the alteration rates of three replicate groups for each treatment. Dot colors denote samples grown under low light (blue; 30 μmol m^{-2} s^{-1}), medium light (green; 60 μmol m^{-2} s^{-1}), and high light (red; 120 μmol m^{-2} s^{-1}). The numbers of newly grown fronds are as follows: LL × static (20, 22, and 20), ML × static (36, 35, and 39), HL × static (38, 40, and 34), LL × flow (39, 48, and 36), ML × flow (47, 54, and 41), and HL × flow (77, 62, and 53). Asterisks indicate significant differences (*, $p < 0.05$; ***, $p < 0.001$) determined by one-way ANOVA followed by Tukey's HSD (honestly significant difference) comparisons.

documented in various terrestrial and aquatic plants (Huang et al., 2019; Klein & Verlaque, 2008; Li et al., 2012; Liu et al., 2020; Sekhar et al., 2019). In *Caulerpa* species, fronds are the primary organs where most photosynthesis occurs, analogous to leaves in terrestrial plants (Chisholm & Moulin, 2003; Komatsu et al., 1997). In terrestrial plants, leaf arrangement and leaf area are not fixed (Goodwin, 2001). For example, the number of branches and the leaf area are reduced to avoid self-shading in low light conditions (Kebrom et al., 2007; Kim et al., 2005). Considering this, radial-to-bilateral alterations in low light (LL) samples were likely induced to contribute to an increase in the frond's surface area exposed to more light for photosynthesis. Glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) were involved in carbon fixation, glycolysis, gluconeogenesis, and carbon metabolism (Figure 4d and Supporting File 2). In *Arabidopsis thaliana*, the suppression of GAPDH has been shown to significantly reduce photosynthetic efficiency (Simkin et al., 2023). Among the DEGs, it was observed that 7 GAPDHs in bilateral fronds were significantly downregulated, all indicating a $\log_2$ (fold-change) under −5.9. This suggests that photosynthesis might not be sufficient in bilateral fronds at lower light levels despite LHC upregulation.

Cytosol, cell wall, vacuole, microtubule, cytoskeletal organization, and other terms related to cellular stability were significantly enriched among downregulated genes (Figure 4c and Supporting File 2). Especially, the cytoskeleton directly responds to external mechanical stimuli and influences differential growth, effectuating distinct shapes during plant organogenesis (Hamant et al., 2019; Sampathkumar et al., 2014). Tubulin-dynein and actin-myosin genes, which play a crucial role in microtubule organization for cytoskeletal support (Kimura et al., 2003; Sabnis & Jacobs, 1967), were also significantly downregulated in bilateral fronds. Aquatic macrophytes develop a robust form to tolerate drag forces generated by water movement (Bornette & Puijalon, 2011; Stewart & Carpenter, 2003). In genus *Caulerpa*,

cytoplasmic streaming affects structural stability and morphogenesis supported by microtubules (Sabnis & Jacobs, 1967) and the high velocity of cytoplasmic streaming increases the number of leaf formation in plants (Tominaga et al., 2013). Therefore, the downregulation of microtubules suggests that variations in cytoplasmic streaming between cultivation and field environments subsequently lead to the development of bilateralism with a decreased density of ramuli compared to the radial form.

The intertidal zone exposes inhabitants to challenging environmental conditions (Carrington, 1990; Demes et al., 2021; Gracey et al., 2008; Neumann, 1976). Particularly, intertidal macrophytes face excessive solar irradiance (Li et al., 2014; Unsworth et al., 2012) and mechanical stress from wave impacts (Carrington, 1990; Demes et al., 2021). Our initial experiment confirmed that irradiance could influence *C. okamurae*'s phenotype. Macrophytes also alter their cellular structure, cytoskeletal organization, and overall morphology to reduce hydrodynamic forces from continuous mechanical stress (Atapaththu et al., 2015; Bal et al., 2011; Bornette & Puijalon, 2011; Puijalon et al., 2008; Sampathkumar et al., 2014; Sand-Jensen, 2003; Starko et al., 2015). With this in mind, the absence of wave-induced stress in the initial experiment led to a significant downregulation of genes associated with mechanical stress (Figure 4c and Supporting File 2). Therefore, we hypothesized that water flow would cause the altered phenotype (bilateral symmetry) to revert to its natural phenotype (radial symmetry). The flow treatment indeed decreased alteration rates (radial-to-bilateral transition) in ML and HL conditions, impacting *C. okamurae* morphogenesis. Moreover, higher irradiance intensified this trend, affecting a statistically significant reduction in bilateralism ($p < 0.0001$). These findings show that mechanical stress from water movement influences *C. okamurae*'s phenotypic plasticity with light intensity. Furthermore, water movement not only imposes mechanical stress on macrophytes but can also be advantageous through nutrient supply (Madsen et al., 2001). Genus *Caulerpa* possesses competitive nutritional availability (Gennaro et al., 2015; Malta et al., 2005); thus, the nutrient mixing caused by water movement can enhance *C. okamurae*'s nutritional absorption. The absorbed nutrients could potentially serve as direct or indirect energy sources for *C. okamurae*'s developmental process, but the relationship between nutrient absorption and phenotypic plasticity could not be determined in this study.

In conclusion, we employed Next-Generation Sequencing (NGS) technology to further biological insights into *C. okamurae*'s phenotypic plasticity. Our results established that limited light conditions may stimulate LHC activity and then a plastic morphological response increasing the surface area exposed to photosynthetic radiation. However, despite these changes, significant GAPDH-related pathway downregulation indicates insufficient photosynthesis. Additionally, we found that the stable water conditions in the culture environment reduced the need for structural stability, allowing a phenotype prioritizing light capture in low-light conditions. This was evidenced by the formation of dense radial fronds, which serve to withstand mechanical stress, when a flow treatment imitating turbulent field conditions was applied. *Caulerpa okamurae* exhibited a tolerant phenotype over extensive light intensity and flowing water ranges, reflecting potent adaptability to diverse environments.

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DATA AVAILABILITY STATEMENT

The raw transcriptome sequencing data are available from the Sequence Read Archive (SRA) database of NCBI (accession numbers: SRR25047840-SRR25047845). All data sets used in this study are available at: http://eyunlab.cau.ac.kr/sea_grape.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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