Identification of novel differentially expressed zooxanthellal genes from *Aiptasia-Symbiodinium* endosymbiosis through SDS-based RNA purification

Short title: SDS washing is benefit to reveal novel differentially expressed genes from *Aiptasia-Symbiodinium* endosymbiosis

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Abstract

Endosymbiosis between dinoflagellates and cnidarian hosts first occurred more than 200 million years ago; however, symbiosis-specific genes and cellular processes involved in the establishment, maintenance, and breakdown of endosymbiosis remain unclear. Therefore, this study aimed to identify the zooxanthellal genes associated with the aforementioned biological processes during endosymbiosis in *Aiptasia-Symbiodinium* endosymbionts. Here, zooxanthellae isolates were treated with 0.02% SDS to decrease potential host RNA contamination and to enhance the identification of novel symbiosis/nonsymbiosis-associated differentially expressed zooxanthellal genes through suppressive subtractive hybridization (SSH) and next-generation sequencing (NGS) methods. Consequently, among 214 symbiosis-specific transcripts identified herein that displayed identity to only 5.6% of host-derived transcripts, 64% were well-known functional genes. In the nonsymbiotic stage, 181 differentially expressed transcripts were identified, of which 64.1% belonged to well-known functional genes. BLAST revealed that 8 categories of cellular processes were significantly induced in symbiotic or nonsymbiotic zooxanthellae. Together with the results of quantitative analysis, the results revealed that photosynthesis, flagellate biosynthesis and motility, stress-induced responses, cell wall biosynthesis, starch synthesis and transport, lipid biosynthesis and metabolism, host/symbiont immune response, intercellular communication, cell growth, and cell cycle regulation were the major cellular processes occurring in

symbiotic/nonsymbiotic stages. The present results provide insights into the mechanisms involved in regulating the different physiological processes in symbiotic/nonsymbiotic zooxanthellae and may guide future studies.

Key words: *Symbiodinium*, zooxanthellae, differentially expressed genes, suppressive subtractive hybridization

Introduction

Approximately one-third of all marine species reside in coral reef ecosystems and contribute to one-fourth of the produce of marine fisheries. The maintenance of coral reef ecosystems is primarily based on the association between corals and symbiotic photosynthetic dinoflagellates (*Symbiodinium* spp.). The single-cell symbiotic dinoflagellate is concealed within the organelles of the gastric dermis tissues within the host coral cells, called symbiosomes [1,2]. Symbiotic associations have numerous benefits. From symbiotic bodies, corals receive amino acids, fatty acids, and photosynthetic products including glucose, and symbionts effectively utilize nitrogen- and phosphorus-containing waste from host cells [3-7]. The maximal calcification rate of coral reefs has resulted from endosymbiosis [8-10]. Furthermore, symbiotic dinoflagellates prevent harmful ultraviolet radiation from damaging corals through mycin-like amino acids (MAAs) [11,12]. However, endosymbiosis is often disrupted owing to environmental stresses including high temperature, high CO₂ concentration, and pollution [13-16], resulting in coral bleaching [13,17]. Coral bleaching represents breakdown of the symbiotic integrity between corals, zooxanthellae, and potential microbial communities, leading to negative effects on coral health [18]. However, details regarding numerous interactions including the intracellular and molecular responses during symbiosis establishment, maintenance, and disintegration remain unclear.

Currently, successfully surviving zooxanthellae have to face the complicated host

symbiosome microenvironment, which provides resources for growth, sustenance, and reproduction [19]. Previous studies have reported the correlation between photosynthesis and carbonic anhydrase activity, indicating that sources of inorganic carbon required for zooxanthellae are actively provided by the coelenterate host [20,21]. Symbiotic dinoflagellates use phosphatase to hydrolyse the phosphate esters of the inorganic phosphates of the coelenterate host and then transport them into cells through transport proteins [22-25]. Regarding the essential nitrogen source for plants, studies have reported that ammonia waste produced by the host is transferred to the symbiosomes and then converted to ammonium ions in the acidic lumen, similar to the highly acidic inner cavity of the symbiosome, which is markedly different from seawater [26,27], or it might be transported through transport proteins into symbiosomes to facilitate the use of zooxanthellae [24,26,28]; however, the underlying mechanism is unclear. Furthermore, previous studies have reported that the TCN- β signalling pathway and Tsr protein are involved in the maintenance of coelenterate hosts and symbiotic tissues [29,30]. Furthermore, the cell cycle of symbiotic dinoflagellates is arrested in the G₁ phase for a longer period than that in the nonsymbiotic form and is not regulated by host-derived inorganic nutrients [31,32]. These associations indicate potential physiological responses and pathways of symbiotic dinoflagellates during endosymbiosis.

Recent studies have reported several potential symbiosis-related genes. Coral lectin binds to specific cell wall glycans on zooxanthellae, and their interactions serve as a potential

key mechanism for the host to obtain specific symbionts [33,34]. Corals are coated with symbiotic corpuscular membranes of symbiotic algae. Membrane-associated RAB family proteins help retain healthy zooxanthellae cells in the symbiosomes and exclude dysfunctional symbionts [35-37]. Furthermore, the genetic and molecular mechanisms in cnidarian and dinoflagellate endosymbionts have received increasing attention [38]. Next-generation sequencing (NGS) technology has revealed genomic sequences of two nonsymbiotic cnidarians, *Nematostella vectensis* and *Hydra magnipapillata* [39,40], and two symbiotic algae, *Symbiodinium kawagutii* and *S. minutum* [41,42]. Numerous studies have performed transcriptome analysis for coelenterates including *Acropora millepora*, *Acropora palmata*, *Pocillopora damicornis*, and *Aiptasia pallida* [43-47]. These data have helped understand the interactions among genes. However, observations regarding symbiosis-specific gene responses between symbiotic and nonsymbiotic states seem slightly different [48,49], potentially indicating that pure genomic analyses have not effectively revealed symbiosis-associated genes and potential cellular responses differing between the symbiotic and nonsymbiotic states. Hence, further improvement of the method is needed to obtain a molecular response truly reflecting endosymbiosis.

NGS can provide large-scale transcriptome data on various types of corals or their symbiotic algae. However, handling symbiotic zooxanthellae in host cells is difficult owing to the possibility of host RNA contamination during nucleic acid extraction. The presence of

host RNA can interfere with sequencing reactions and reduce accuracy and miscalculate minor but important symbiotic gene abundances. This study aimed to identify the zooxanthellal genes associated with the aforementioned biological processes during endosymbiosis in *Aiptasia-Symbiodinium* endosymbionts. In this study, we treated *Aiptasia-Symbiodinium* endosymbionts with SDS before nucleic acid extraction to reduce the amount of host RNA contamination and performed combinatorial suppressive subtractive hybridization (SSH)-NGS sequencing to more accurately identify potential symbiotic and nonsymbiotic gene groups. The potential molecular responses provide more accurate information for establishing and maintaining intracellular endosymbionts.

Materials and methods

Animals and dinoflagellates

Cnidarian *A. pulchella* and its symbiont, *Symbiodinium spp.* Clade B, were obtained from a private aquaculture farm in Pingtung and maintained in an aquarium of $160 \times 60 \times 75$ cm³ with 470 L of water. Salinity, temperature, and pH were maintained at 35 psu, 26–28 °C, and pH 8.0–8.5, respectively, with access to natural sunlight. Frozen shrimp was fed once a week as bait. Each month, half the volume of water was replaced with fresh sea water. To induce bleaching in the sea anemones, symbiotic sea anemones were treated with cold shock at 4 °C for 1 h and maintained in a dark aquarium until bleaching occurred. No residual

zooxanthellae were detected with the bleaching sea anemones upon microscopic analysis. Nonsymbiotic zooxanthellae were harvested from symbiotic sea anemones through homogenization, centrifugation, and resuspension. After zooxanthellae were purified, the free-living zooxanthellae were cultured in a T75 flask containing f/2 medium with additional 100, 50, and 50 µg/ml ampicillin, kanamycin, and streptomycin. Zooxanthellae were cultured at 26 °C and on a 12/12-h light/dark cycle.

RNA purification and cDNA preparation

Total RNA of *A. pulchella* and *Symbiodinium spp.* were extracted using TriZol (Thermo Fisher Scientific, USA). Additionally, before RNA extraction from *Symbiodinium spp.*, SDS treatment was performed for 10 seconds. In total, 5 µg of total RNA of both *A. pulchella* and *Symbiodinium spp.* was used to synthesize ss cDNAs using SMARTer™ PCR cDNA Synthesis Kit (Takara Bio, USA). Furthermore, smart-IV primers were used: (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3') and CDSIII primers [5'-ATTCTAGAGGCCGAGGCCCGGGGGGCC -d(T)30N-1N-3'] for reverse transcription and synthesis of ds cDNA gene libraries.

SSH analysis

Before SSH, both symbiotic and nonsymbiotic zooxanthellae were cultured in seawater

at 26 °C at a salinity of 35 psu and pH 8.0–8.5 with a light intensity of 80–100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ for 2 weeks to prevent environmental differences. After acclimatization, RNA extraction and cDNA construction were performed and 4 genes including β -actin of *A. pulchella*, and β -actin, PCP, and HSP90 of *Symbiodinium spp.* were assessed through semi-quantitative PCR analysis to evaluate potential environmental stress-induced responses. Thereafter, SSH was performed using a PCR-Select™ cDNA Subtraction Kit (Takara Bio). The differentially expressed genes thus determined were then cloned into the pCR®II-TOPO vector and transformed into Mach1™-T1R competent cells for sequencing. The sequences thus obtained were extended with a home-curated NGS-based gene database of *A. pulchella* and *Symbiodinium spp.* clade B and then were aligned with NCBI GenBank database, using BLASTX [50]. Furthermore, multiple sequence alignment and phylogenetic tree analysis were performed using DNASTAR software [51].

NGS analysis

Non-co-symbiotic symbiotic algae and albino beautiful anemones were selected for RNA extraction. Thereafter, 30-min DNase I treatment was administered at 37 °C, followed by RNA purification using TriZol. Thereafter, a Chroma spin+TE-400 column was used to eliminate small RNA. Finally, after electrophoresis with 1% agarin gum, QC analysis was outsourced, and NGS analysis was performed by BGI Genomic company (China).

Real-time quantitative PCR

The primer annealing temperature range for each gene was set at 57–63 °C, the expected product size was 50–150 bp, the final primer concentration in the reaction mix was 100 nM, and the mRNA content was 5 ng. The detail of primer sequences is all provided in supplementary information (Table S3). The real-time PCR reagent was 2× SYBR green master mix (Toyobo, Japan), and the total reaction volume was 25 µL. The cycling conditions were as follows: pre-denaturation at 95 °C for 1 min, denaturation at 95 °C for 15 s, and extension at 60 °C for 1 min, followed by melting curve analysis. The machine model used herein was the SYBR 48-well template StepOne™ system (ABi), and the analysis software was StepOne software v2.1.

Results and discussion

Host RNA contaminations was effectively reduced through SDS treatment

To reduce host RNA contamination, which may affect the accuracy and accuracy of the deduced hybridization, we compared the differentially expressed genes between symbiotic and nonsymbiotic zooxanthellae. After symbiotic/nonsymbiotic zooxanthellae were cultured and acclimated under the same conditions, RNA extraction was performed. During the

extraction of symbiotic zooxanthellae, the homogenate of the symbiotic sea anemone was treated with 0%, 0.01%, and 0.02% SDS for 30 s, followed by coupling with suspension and centrifugation until the residual host cell and tissue debris were effectively eliminated. Semi-quantitative reverse transcription PCR was performed to detect host RNA contamination levels. Consequently, 0.02% SDS treatment significantly decreased *Aiptasia* actin signalling but did not affect zooxanthellal actin, HSP90, and PCP expression (Fig 1), indicating that SDS neither causes marked stress to the zooxanthellae nor induces differential gene expression. These results indicate an effective reduction in anemone host RNA contamination, which is conducive for SSH. These results indicate that SDS effectively binds lipid molecules; thus, nucleic acids (DNAs and RNAs) become exposed in cells with damaged membranes; these nucleic acids are then exposed to the aqueous homogenate fluid and digested by endogenous RNases and DNases [52]. However, the zooxanthellal DNAs and RNAs are potentially protected by their cell wall and cannot be significantly damaged during short periods. Therefore, SDS treatment would benefit the purification of the zooxanthellae population.

Fig 1. SDS treatment-based RNA purification in zooxanthellae. When harvesting hosts harbouring zooxanthellae, 0.01% and 0.02 % SDS treatment were administered during RNA isolation to efficiently eliminate host RNA contamination. Thereafter, primers specific for

actin, PCP, and HSP90 were assessed through semi-quantitative RT-PCR analysis to reveal potential host RNA contamination. These results indicate that 0.02% SDS treatment efficiently decreased host RNA contamination without significant stress on other intracellular molecular responses. ‘ApActin’ represents *Aiptasia pulchella* actin; ‘SymPCP, SymHSP90, and SymActin’ represent zooxanthellal chloroplast soluble peridinin-chlorophyll a-binding protein precursor, heat shock protein 90, and actin, respectively. ‘Free Zoox’ indicate the cDNA derived from cultured nonsymbiotic zooxanthellae, and ‘Bleaching Ap’ was derived from bleaching *Aiptasia pulchella*.

Transcriptome assay of bleaching *A. pulchella* revealed a 5.6% reduction in host-derived SSH-harvested sequences

SSH is a potent method for analysing differences in gene expression patterns between symbiotic and nonsymbiotic zooxanthellae. Herein, SSH was coupled with an NGS-based transcriptome assay and SDS treatment to further expand the zooxanthellae gene database and effectively reduce host RNA contamination to better identify novel and unknown genetic and cellular responses. As shown in Fig 2, to confirm the reduction in host RNA contamination, the database of the bleaching anemone and nonsymbiotic zooxanthellae gene pool curated through NGS allowed for the extrapolation and identification of DNA sequences generated through SSH. After sequencing was completed, 69,858 conserved sequences of

bleaching *Aiptasia* anemones were obtained, including 17,707 contigs and 52,151 unigenes; and 69,327 conserved sequences of isolated free-living *Symbiodinium* zooxanthellae, including 6,434 contigs and 62,893 unigenes, respectively. Details regarding Clusters of Orthologous Genes (COG) are provided in Figs 3 and 4. In total, 214 differentially expressed genes were detected in the symbiotic stage and 181 genes were detected in the nonsymbiotic stage. Only 12 genes (5.6%) in the symbiotic stage expressed zooxanthellal genes and belonged to *A. pulchella* (Fig 5). Altogether, our results indicate that 0.02% SDS treatment helps effectively reduce host RNA contamination and their potential interference.

Fig 2. Schematic representation of SDS treatment, transcriptome sequencing, assembly, separation, and extension of host/symbiont sequences.

Fig 3. Clusters of Orthologous Genes classification of free-living *Symbiodinium* clade B expressing transcripts. The pathway contains: metabolism, genetic processing, environmental responses, cellular processes and organismal systems. Human diseases results were removed in the result of KOG pathways.

Fig 4. Cluster of Orthologous Genes classification of bleaching *Aiptasia pulchella* expressing transcripts. The pathway contains: metabolism, genetic processing,

environmental responses, cellular processes and organismal systems. Human diseases results were removed in the result of KOG pathways.

Fig 5. Taxonomy based on transcriptome data generated from *Aiptasia pulchella*-associated zooxanthellae. Taxonomies are provided from sequences of symbiotic and nonsymbiotic zooxanthellae through transcriptome analysis and suppressive subtractive hybridization. A strict and inclusive taxonomy is displayed. Screening revealed 214 symbiosis-specific expressed transcripts, displaying identity to only 5.6% of host-derived transcripts, 64% well-known functional genes. In the nonsymbiotic stage, the results indicated 181 differentially expressed transcripts, of which 64.1% belonged to well-known functional genes. ‘APG’ represents sequences of *Aiptasia pulchella* genes; ‘FG’ refers to genes with specific functions; ‘UKG’ represents genes of unknown function; ‘NSF’ indicates no similarity; ‘BAC’ refers to bacterial-like genes.

Screening of differentially expressed transcripts between endosymbiosis

After SSH and sequence extension, the number of transcripts corresponding with specific functional genes was identified through BLAST with the NCBI GenBank database. Overall, 202 genes were differentially expressed in the symbiotic stage, of which 137 are

genes had known functions, whereas the remaining 39 had unknown functions, 18 sequences did not display any similarity, and 8 genes were potentially bacterial genes. Otherwise, 181 genes were differentially expressed in the nonsymbiotic stage, of which 116 had known functions, 29 had unknown functions, 21 sequences did not share any similarity, and 15 genes were potentially bacterial genes (Fig 5). This study is focused on the 137 and 116 differentially expressed genes in the symbiotic and nonsymbiotic stages, respectively; they were divided into 7 important categories to elucidate the potential intracellular responses of endosymbiosis, as detailed below (Tables 1 and 2; detailed results shown in Tables S1 and S2).

276 **Table 1. List of differentially expressed transcripts in symbiotic zooxanthellae.**

Clone No.	Similar to Sequence ID	Putative gene	E-value	Processes
T1 105	OLP94869.1	Titin	2e-17	Photosynthesis
T1 187	OLP86828.1	Protochlorophyllide reductase A, chloroplastic	0.0	
T1 298	AET21370.1	chloroplast soluble peridinin-chlorophyll a-binding protein precursor	0.0	
T1 347a	OLQ03574.1	Cytochrome c	2e-74	
T1 416	OLQ14114.1	Protoporphyrinogen oxidase, chloroplastic	1e-120	
T1 085	OLP93912.1	Polycystin-2	3e-33	Flagellar construction and biosynthesis
T1 109	RXM92183.1	Tubulin beta chain	8e-07	
T1 111	XP_029221609.1	SF-assemblin/beta giardin protein	2e-36	
T1 113	PHJ21138.1	Non-muscle myosin ii heavy	7e-13	
T1 114	ABV72532.1	tubulin alpha chain	0.0	
T1 204a	OLP83016.1	Coiled-coil domain-containing protein lobo-like	0.0	
T1 347	GBG77724.1	dpy-30-like protein	1e-12	
T1 363	BAE79387.1	actin	0.0	
T1 366*	OLQ11817.1	Ankyrin-1	6e-09	
T1 402	OLP83211.1	Tubulin polyglutamylase TTLL11	9e-83	
T1 415	OLP85075.1	Developmentally-regulated GTP-binding protein 1	0.0	Stress responses
T1 415a	ADJ00012.1	tubulin alpha chain, partial	3e-49	
T1 520	XP_005774896.1	Clusterin-associated protein 1	2e-11	
T1 035	WP_007606708.1	cold-shock protein	7e-08	
T1 042	XP_013716744.1	putative activator of Hsp90 ATPase	4e-12	
T1 047*	OLQ08506.1	Chaperone protein DnaJ 2	1e-06	
T1 097	OLQ00575.1	Ubiquitin-conjugating enzyme E2 11	2e-38	
T1 098	OLQ11043.1	putative protein kinase UbiB	1e-42	
T1 108	OLQ01568.1	S-(hydroxymethyl)glutathione dehydrogenase	6e-70	

T1 124	OLP96983.1	Cyclic nucleotide-gated cation channel alpha-4	5e-100	
T1 141	OLQ02097.1	6-hydroxynicotinate 3-monooxygenase	2e-155	
T1 193	OLQ04630.1	putative E3 ubiquitin-protein ligase HERC6	2e-04	
T1 286	OLP87260.1	Mitochondrial cardiolipin hydrolase	8e-69	
T1 304	AAD09517.1	NTFP2 (chaperone protein DnaJ)	2e-10	
T1 306	OLQ04463.1	Peptidyl-prolyl cis-trans isomerase FKBP65	2e-89	
T1 319	OLQ03301.1	TPR repeat-containing thioredoxin TTL1	2e-79	
T1 370	OLP86869.1	Calmodulin-like protein 12	1e-77	
T1 386	OLP96860.1	Phosphatidylinositol 4-phosphate 5-kinase 2	0.0	
T1 411	OLP86450.1	BTB POZ and MATH domain-containing protein 2	1e-141	
T1 507a	KAA3447109.1	senescence-associated protein	2e-106	
T1 023	OLP84003.1	Ammonium transporter 1 member 1	1e-160	Nitrogen transport
T1 375	XP_003058542.1	intracellular chloride channel family	1e-115	
T1 408	OLQ11858.1	6-phosphogluconolactonase	6e-71	
T1 003	XP_009120107.1	nucleotide-sugar uncharacterized transporter 2	5e-11	
T1 010	OLP95391.1	Malonyl-CoA-acyl carrier protein transacylase, mitochondrial	9e-39	
T1 020	OLQ05486.1	Ribokinase	1e-09	Starch biosynthesis
T1 053	OLP87329.1	Pullulanase 1 chloroplastic	7e-07	
T1 308	OLP82590.1	Soluble starch synthase 1, chloroplastic/amyloplastic	0.0	
T1 351	OLP86342.1	Dual specificity protein phosphatase 9	9e-121	
T1 408	OLQ11858.1	6-phosphogluconolactonase	6e-71	
T1 045	WP_114773738.1	phytanoyl-CoA dioxygenase family protein	6e-23	
T1 046	OLP98563.1	3-oxoacyl-[acyl-carrier-protein] synthase 1	2e-42	Lipid metabolism and biosynthesis
T1 086	OLP83506.1	Pepsin A	3e-05	
T1 087*	OLP93130.1	Acyl-CoA wax alcohol acyltransferase 2	0.099	
T1 454	WP_125791499.1	phytanoyl-CoA dioxygenase family protein	1e-08	
T1 510	OLP98563.1	3-oxoacyl-[acyl-carrier-protein] synthase 1	0.0	

T1 067	AHB60958.1	Structural maintenance of chromosomes protein 1	0.0	Cell cycle and plant growth
T1 177	OLP99662.1	Calpain-D	2e-83	
T1 204	OLP94588.1	Cell division cycle protein 48-like	3e-11	
T1 294	OLP89897.1	DNA mismatch repair protein MutS	5e-25	
T1 465a	OLP80542.1	Adenine phosphoribosyltransferase 1	3e-120	
T1 005	OLP81744.1	Peptidyl-prolyl cis-trans isomerase 7	7e-109	Innate immunity and host interaction
T1 041	XP_021898429.1	PlantUBX domain-containing protein 2	6e-04	
T1 054	OLP93591.1	Kinesin-like protein	0.0	
T1 055	OLP85221.1	Dual specificity phosphatase 28	4e-36	
T1 062	OLP93124.1	Cathepsin E	9e-119	
T1 318	OLP86148.1	Reticulocyte-binding protein 2-like a	1e-31	
T1 379	WP_006970636.1	patatin-like phospholipase family protein	1e-46	
T1 398	OLP88652.1	Hypersensitive-induced response protein 3	2e-170	
T1 408	OLQ11858.1	6-phosphogluconolactonase	6e-71	
T1 410	OLP89630.1	Signal peptide, CUB and EGF-like domain-containing protein 1	5e-19	
T1 460	OLQ03573.1	Internalin-A	2e-144	

“*” refers to the sequence is not found in any transcriptome database of this study.

282 **Table 2. List of differentially expressed transcripts in nonsymbiotic zooxanthellae.**

Clone No.	Similar to Sequence ID	Putative gene	E-value	Process
T2 006	OLP97379.1	4-hydroxybutyryl-CoA dehydratasevinylacetyl-CoA-Delta-isomerase	4e-43	photosynthesis
T2 036	OLQ01971.1	chloroplast phosphoglycerate kinase precursor, partial	4e-107	
T2 037	AET21370.1	chloroplast soluble peridinin-chlorophyll a-binding protein precursor	0.0	
T2 181a	OLQ06905.1	Serine/threonine-protein phosphatase 5	2e-108	
T2 196	OLQ09445.1	Fucoxanthin-chlorophyll a-c binding protein F, chloroplastic	5e-111	
T2 229	OLQ01351.1	Cryptochrome-1	4e-165	
T2 231	OLQ11346.1	NADPH-dependent diflavin oxidoreductase 1	2e-37	
T2 105a	AFD34242.1	alpha-tubulin	0.0	Flagellar construction and motility
T2 114	OLP98069.1	Ankyrin-1	1e-38	
T2 115-1a	OLP98107.1	Prefoldin subunit 6	3e-91	
T2 120a	OLP77434.1	Kinesin-like protein KIF20B	1e-121	
T2 130a	OLQ14867.1	Kinesin-like protein KIF6	6e-15	
T2 144	OLP75066.1	33 kDa inner dynein arm light chain, axonemal	2e-121	
T2 189	OLQ10895.1	Nucleoside-diphosphatase mig-23	4e-103	
T2 002	OLP84712.1	Glutathione S-transferase class-mu 28 kDa isozyme	0.0	Stress reponses
T2 053a	OLP87708.1	putative E3 ubiquitin-protein ligase HERC1	2e-07	
T2 102a	OLP87907.1	putative formaldehyde dehydrogenase AdhA	3e-138	
T2 152	AIA81312.1	catalase peroxidase, partial	0.0	
T2 159	KOO53779.1	patellin family protein	2e-60	
T2 166	AHV78701.1	uracil phosphoribosyl transferase, partial	4e-95	
T2 167	OLP98216.1	Peptide methionine sulfoxide reductase B5	3e-85	
T2 238	OLP97743.1	MATH and LRR domain-containing protein	2e-32	Nitrogen transport
T2 055	XP_005778923.1	putative formate/nitrite transporter	2e-96	
T2 133	OLP80712.1	Ammonium transporter 2	0.0	

T2 027	OLP99206.1	Peroxisomal membrane protein PMP34	9e-42	Lipid metabolism and biosynthesis
T2 103	OLP81638.1	Acetyl-CoA carboxylase 1	0.0	
T2 121	OLP86804.1	ATP-citrate synthase	4e-143	
T2 137	ABI14266.1	acyltransferase family protein	0.0	
T2 197*	KOO29556.1	NADH-cytochrome b5 reductase 3-like protein	5e-06	
T2 205	OLQ02330.1	putative UDP-glucosyltransferase YojK	0.0	
T2 254	OLP92856.1	Sterol 3-beta-glucosyltransferase	0.0	
T2 022	OLP85495.1	putative cyclin-like protein	6e-15	
T2 023	OLQ05642.1	putative flavin-containing monooxygenase 1	2e-107	
T2 032	OLQ13627.1	putative flavin-containing monoamine oxidase A	3e-150	
T2 034	OLP98469.1	Alpha-ketoglutarate-dependent dioxygenase alkB-like 3	1e-43	Cell cycle and growth
T2 060	OLP86798.1	Protein MEI2-like 5	1e-52	
T2 060a	OLQ08499.1	Protein MEI2-like 3	5e-95	
T2 060b	OLP97847.1	Meiosis protein mei2	8e-84	
T2 127	OLP85441.1	DNA replication licensing factor mcm5-A	0.0	
T2 129	OLQ03831.1	putative serine/threonine-protein phosphatase C27B7.6	3e-180	
T2 145	XP_002767421.1	mitogen-activated protein kinase 2, putative	0.0	
T2 151	OLP97847.1	Meiosis protein mei2	6e-80	
T2 159	KOO53779.1	patellin family protein	2e-60	
T2 184	OLQ04875.1	WD repeat-containing protein 82	0.0	Cell wall biogenesis
T2 188	OLP85726.1	N-glycosylase/DNA lyase OGG1	3e-38	
T2 026a	OSG85581.1	PG1 protein, partial	3e-12	
T2 050	XP_024366151.1	galactan beta-1,4-galactosyltransferase GALS1-like isoform X2	0.004	
T2 075	SUX89036.1	Cell wall-associated hydrolase	1e-48	
T2 120	SIN12997.1	Cell wall-associated hydrolase	2e-68	
T2 348	OLP86527.1	Phosphatidylinositol 4-phosphate 5-kinase 5	0.0	
T2 047*	OLP85140.1	Novobiocin biosynthesis protein H	0.0	Innate

T2 068	OLP95205.1	Protein NLRC3	4e-116	immunity and
T2 096	OLP80406.1	Metacaspase-1	0.0	host
T2 105	OLQ11661.1	Bifunctional carbamoyltransferase TobZ	5e-120	interaction
T2 117a	OLQ10959.1	Endoribonuclease Dicer-like 2a	0.0	
T2 139	OLP88557.1	putative peptidase C1-like protein	2e-141	
T2 154	Unigene28724	Peptidyl-prolyl cis-trans isomerase A	6e-67	
T2 163	OLP90286.1	putative ABC transporter ATP-binding protein	1e-85	
T2 198	EKU21659.1	adenosine kinase	2e-28	

“*” refers to the sequence is not found in any transcriptome database of this study.

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Symbiotic zooxanthellae may produce chlorophyll to enhance photosynthesis

During photosynthesis, 5 differentially expressed genes detected in symbiotic zooxanthellae were implicated in chlorophyll biosynthesis. Table 1 shows that upregulated chloroplastic protoporphyrinogen oxidase eliminated protons from protoporphyrinogen IX to produce protoporphyrin IX, which was transformed into the pro-precursor of chlorophyll a, named protochlorophyllide (Pchlde) [53,54]. Further, light- and NADPH-dependent chloroplastic protochlorophyllide reductase A (4.8-time expression) promoted the phototransformation of protochlorophyllide to chlorophyllide (Chlide) [55], which are the biosynthetic precursors of chlorophyll a. Biosynthesis of chlorophyll a harvests solar energy from chloroplast soluble peridinin-chlorophyll a-binding protein and transport through cytochrome c-mediated electron transport [56,57], thus being essential for normal plant growth and development.

302 **Table 3. List of differentially expressed zooxanthellal genes revealed through quantitative PCR.**

Clone No.	Putative gene	Relative expression level
<i>Higher expression in symbiotic stage</i>		
T1 023	Ammonium transporter 1 member 1	3.67
T1 029	putative bifunctional methylthioribulose-1-phosphate dehydratase/enolase-phosphatase E1	2.00
T1 042	putative activator of Hsp90 ATPase	5.33
T1 051	cyclophilin 59	219.64
T1 111	SF-assemblin/beta giardin protein	1.86
T1 113	Non-muscle myosin II heavy chain	8.01
T1 114	tubulin alpha chain	11.46
T1 187	Protochlorophyllide reductase A, chloroplastic	4.80
T1 298	chloroplast soluble peridinin-chlorophyll a-binding protein precursor	1.67
T1 304	NTEP2 (chaperone protein DnaJ)	3.12
T1 306	Peptidyl-prolyl cis-trans isomerase FKBP65	3.55
T1 308	Soluble starch synthase 1, chloroplastic/amyloplastic	2.64
T1 319	TPR repeat-containing thioredoxin TTL1	3.08
<i>Higher expression in non-symbiotic stage</i>		
T2 026a	PG1 protein, partial	932109.1
T2 032	putative flavin-containing monoamine oxidase A	5.08
T2 034	Alpha-ketoglutarate-dependent dioxygenase alkB-like 3	1.19
T2 036	chloroplast phosphoglycerate kinase precursor, partial	1.68
T2 047	Novobiocin biosynthesis protein H	6.92
T2 055	putative formate/nitrite transporter	1.45

T2 060	Protein MEI2-like 5	11.76
T2 075	Cell wall-associated hydrolase	321.8
T2 102a	putative formaldehyde dehydrogenase AdhA	8.11
T2 114	Ankyrin-1	2.16
T2 120	Cell wall-associated hydrolase	1.09
T2 121	ATP-citrate synthase	2.1
T2 127	DNA replication licensing factor mcm5-A	2.51
T2 133	Ammonium transporter 2	1.27
T2 137	acyltransferase family protein	1.29
T2 144	33 kDa inner dynein arm light chain, axonemal	1.36
T2 159	patellin family protein	2.62
T2 167	Peptide methionine sulfoxide reductase B5	1.70
T2 188	N-glycosylase/DNA lyase OGG1	3.4
T2 189	Nucleoside-diphosphatase mig-23	2.99
T2 196	Fucoxanthin-chlorophyll a-c binding protein F, chloroplastic	1.18
T2 198	adenosine kinase	6.37
T2 238	MATH and LRR domain-containing protein	1.94

However, free-living zooxanthellae displayed different activities during photosynthesis. Expression of chloroplast phosphoglycerate kinase was implicated in the downregulation of photosynthetic activity during glycolysis impairment for metabolic adjustment and to optimize growth [58]. Chloroplastic fucoxanthin-chlorophyll a-c binding protein F, cryptochrome-1 and NADPH-dependent diflavin oxidoreductase 1 were differentially expressed (Table 2), suggesting that light responses of zooxanthellae were mediated by cryptochrome-1 [59], NADPH-dependent diflavin oxidoreductase 1 catalysed the NADP-dependent reduction of cytochrome c, but not cytochrome P450 *in vitro* [60] and energy was transferred from the carotenoid and chlorophyll C (or B) to chlorophyll A and the photosynthetic reaction centres where chloroplastic Fucoxanthin-chlorophyll a-c binding protein F is used to synthesize ATP and reduce solar power [61]. Altogether, the present results indicate that zooxanthellae in the symbiotic stage had an enhanced photosynthetic efficiency, which was not observed in the nonsymbiotic stage.

Zooxanthella expressed numerous genes involved in flagellar motility and construction

In the nonsymbiotic stage, zooxanthellae expressed 7 transcripts corresponding to the genes involved in the dynamics of flagellar motility (Table 2) and regulated by Kinesin II-mediated anterograde intraflagellar transport (IFT) [62]. Kinesin-like protein KIF6 and

KIF20B, the plus-end-directed motor enzyme required to complete cytokinesis and cellular motor activity, is directed toward the microtubule's plus end [63,64]. As observed, two flagella assembled on free-living zooxanthellae through the regulation of kinesin-associated protein and IFT. Axonemal 33-kDa inner dynein arm light chain (expressed at 1.36-fold; Table 3) and prefoldin subunit 6 potentially contribute to flagellar movement and the stable assembly of a subset of inner dynein arms for the binding of these arms to the outer doublet microtubules of the axoneme to facilitate swimming [65,66]. Inversely, SSH revealed 13 unique transcripts of zooxanthellae in the symbiotic stage, which were involved in flagellar function and biosynthesis (Table 1). These results are concurrent with those of a previous study wherein RNAi-mediated polycystin-2 silencing revealed defects in flagella-dependent mating in *Chlamydomonas reinhardtii* [67]. Coiled-coil domain-containing protein lobo-like is the primary component of the nexin-dynein regulatory complex (N-DRC), which is essential for N-DRC integrity and regulates flagellar motility [68]. Dpy-30-like protein markedly contributed to flagellar radial spokes, which contribute to the regulation of dynein arm activity and thus the pattern of flagellar bending [69]. Clusterin-associated protein 1 was required for ciliary biogenesis and functioned within the multiple intraflagellar transport complex B (IFT-B) [70]. Impairments of SF-assemblin-beta giardin protein in *Chlamydomonas* resulted in defects in flagellar assembly [71,72]. Developmentally regulated GTP binding protein 1 as a microtubule-binding protein can diffuse on microtubules, promote

their polymerization, drive microtubule formation into bundles, and stabilize microtubules *in vitro* [73]. Consistently, qPCR revealed that relative expression of nonmuscle myosin II heavy chain, SF-assemblin-beta giardin protein and alpha tubulin were 8.01-, 1.86-, and 11.46-fold. Interestingly, Tubulin polyglutamylase TTLL11 was also detected herein, since the well-known Polyglutamase, which preferentially modifies alpha-tubulin was required for CCSAP localization to both spindle and ciliary microtubules [74]. These results suggest that zooxanthella contained in the host cells contributed to the generation of the flagella or cilia and on membrane trafficking.

Symbiotic zooxanthellae are potentially subjected to different stresses

BLAST analysis of 17 transcripts of zooxanthellae in the symbiotic stage corresponded to various stress-induced genes (Table 1), including cold-shock protein, putative activator of Hsp90 ATPase, NTFP2, Chaperone protein DnaJ 2, Ubiquitin-conjugating enzyme E2 11, putative protein kinase UbiB, S-(hydroxymethyl) glutathione dehydrogenase, Cyclic nucleotide-gated cation channel alpha-4, 6-hydroxynicotinate 3-monooxygenase, putative E3 ubiquitin-protein ligase HERC6, mitochondrial cardiolipin hydrolase, peptidyl-prolyl cis-trans isomerase FKBP65, TPR repeat-containing thioredoxin TTL1, calmodulin-like protein 12, phosphatidylinositol 4-phosphate 5-kinase 2, BTB POZ, MATH domain-containing

protein 2, and senescence-associated protein. These genetic responses indicate that

zooxanthellae are potentially under biotic and abiotic stress including senescence, cold and

heat stress, oxidative stress, photodamage, nitrosative stress, salt stress, hyperosmotic stress,

osmotic stress, and water stress. For example, expression of cold-shock protein, putative

activator of Hsp90 ATPase, NTFP2, chaperone protein DnaJ 2, ubiquitin-conjugating enzyme

E2 11, and putative E3 ubiquitin-protein ligase HERC6 are speculated to facilitate cell

survival even at temperatures lower or higher than the optimum growth temperature [75-78].

Putative protein kinase UbiB can phosphorylate tocopherol cyclase VTE1 and help recycle

oxidated alpha-tocopherol quinone to prevent chloroplast photo damage under continuous red

light [79]. S-(hydroxymethyl) glutathione dehydrogenase modulates nitrosative stress during

plant development [80]. Mitochondrial cardiolipin hydrolase has been speculated to directly

contribute to the processing of primary piRNA transcripts to regulate mitochondria under

hyperosmotic stress [81]. TPR repeat-containing thioredoxin TTL1 potentially serves as a

positive regulator of ABA signalling during germination under osmotic stress [82]. Plant-type

phosphatidylinositol-4-phosphate 5-kinases and BTB POZ, and MATH domain-containing

protein 2 were responsive to environmental factors, thus playing an essential role in

coordinating plant growth under water stress [83]. Oxidative stress induced senescence-

associated protein to have a genetically based program leading to cell senescence and death

[84]. Furthermore, qPCR confirmed zooxanthellal NTFP2 and putative activator of Hsp90

ATPase in the symbiotic stage were upregulated (5.33-fold and 3.12-fold) rather than in the nonsymbiotic stage. However, fewer stress-induced responses were observed through differentially expressed transcripts among nonsymbiotic zooxanthellal (Table 2), suggesting that nonsymbiotic zooxanthellae may encounter phosphate limitations [85], pH stress [86], oxidative stress [87], and damage from antibiotics, which are derived from culture conditions. Overall, the results showed that symbiotic zooxanthellae are not in a completely mutually beneficial environment as we expected. To survive in the host cell, zooxanthellae may have to face a complicated microenvironment different from that of seawater; thus, they are subjected to various adverse reactions.

Starch, not lipids, may be the major product of carbohydrate biosynthesis in symbiotic zooxanthellae

The major product of photosynthesis is probably transferred to starch, and not lipids, during *A. pulchella* and *Symbiodinium* endosymbiosis. This speculation was based on the SSH findings herein, which revealed 7 differentially expressed transcripts of zooxanthellae in the symbiotic stage (Table 1), including nucleotide-sugar uncharacterized transporter 2, malonyl-CoA-acyl carrier protein transacylase, ribokinase, chloroplastic pullulanase 1, chloroplastic/amyloplastic soluble starch synthase 1 and dual-specificity protein phosphatase 9, and 6-phosphogluconolactonase. Furthermore, qPCR analysis revealed a 2.64-fold

expression level of symbiotic zooxanthellal chloroplastic/amyloplastic soluble starch synthase 1, which primarily contributes to starch synthesis [88], compared with nonsymbiotic zooxanthellae. Furthermore, symbiotic zooxanthellae expressed dual-specificity protein phosphatase 9 and chloroplastic pullulanase 1, a starch-associated phosphoglucan phosphatase and enzyme, both of which were involved in starch biosynthesis, catabolism, and degradation [89,90]. Indeed, the expression of 6-phosphogluconolactonase is potentially correlated with the requirement of sugar-dependent nitrate assimilation [91]. These results indicate that starch and starch-derived products may be the major product of carbohydrate biosynthesis in symbiotic zooxanthellae.

Interestingly, lipid biosynthesis and transport were probably the most prominent metabolic activity among nonsymbiotic zooxanthellae, instead of starch biosynthesis. Table 2 lists several differentially expressed transcripts involved in lipid biosynthesis, transport, and metabolism, including sterol 3-beta-glucosyltransferase, which catalyses the synthesis of steryl glycosides (SGs) and acyl steryl glycosides (ASGs), which are the most abundant sterol derivatives in higher plants [92]. NADH-cytochrome b5 reductase 3-like protein and acyltransferase family protein are involved in fatty acid desaturation and elongation and cholesterol biosynthesis [93,94]. Acetyl-CoA carboxylase 1 and ATP-citrate synthase couple energy metabolism with fatty acids synthesis to support cell growth [95,96]. Altogether, these results indicate that the production of lipids and lipid derivatives is potentially involved in

cell proliferation to increase the zooxanthellae population in the nonsymbiotic stage.

Symbiotic zooxanthellae potentially utilise nitrates and ammonium first, but not nitrites

Nonionic NH_3 can freely passively diffuse through biological membranes; however, it is retained within the symbiosome owing to its acidic microenvironment in the lumen [25-28]. The nitrogen transport demand between the symbiosome and zooxanthellae cells is reflected through the expression of ammonium transporter 1 and intracellular chloride channel family protein (Table 1 and 3), both of which play essential roles in ammonium and nitrate transport and their associated molecular responses [97-99]. Interestingly, zooxanthellae in the nonsymbiotic stage differentially expressed ammonium transporter 1 and putative formate nitrite transporter (1.45-fold) rather than in the symbiotic stage (Tables 1 and 3). These results indicate that nitrate and ammonium are absorbed on priority by symbiotic zooxanthellae; nonsymbiotic zooxanthellae, nitrite, and ammonium.

Nonsymbiotic zooxanthellae potentially mediate cell cycle progression and cell wall biosynthesis to accelerate cell growth

Only 6 transcripts of zooxanthellae were differentially expressed in the symbiotic stage (Table 1), and BLAST analysis revealed transcripts including structural maintenance of

chromosomes protein 1 (also known as ‘cohesion complex subunit SMC-1’), calpain-D, calpain-15 isoform X2, PBS lyase cell division cycle protein 48-like, DNA mismatch repair protein MutS, and adenine phosphoribosyltransferase 1. The cohesin complex potentially forms a large proteinaceous ring within which sister chromatids can be trapped, playing an important role in spindle pole assembly during mitosis and in chromosome migration and regulating chromosome segregation and cell division during embryogenesis [100,101]. Calpain-D and calpain-15 isoform X2, both being plant phytocalpains, are key regulators of cell proliferation and differentiation during plant organogenesis, partly acting by regulating the CycD/Rb pathway [102]. PBS lyase cell division cycle protein 48-like protein probably functions in cell division, and adenine phosphoribosyltransferase 1 converts cytokinins from free bases to their corresponding nucleotides [103,104]. DNA mismatch repair protein MutS is involved in DNA mismatch repair. Obviously, the primary cell cycle activities in symbiotic zooxanthellae were potentially correlated with the maintenance of chromosome structure and differentiation, concurrent with cell cycle arrest of symbiotic zooxanthellae in the G₁/G₀ phase.

Compared with symbiotic zooxanthellae, nonsymbiotic zooxanthellae displayed activities associated with cell cycle transition, meiosis, initiation and elongation phases of DNA replication, cell proliferation and differentiation, cytokinesis, regulation of chromatin structure, and repair of damaged DNA. The transcripts responsible for cell cycle regulation

were similar to those of putative cyclin-like protein, putative flavin-containing monooxygenase 1, alpha-ketoglutarate-dependent dioxygenase alkB-like 3, protein MEI2-like 5, protein MEI2-like 3, meiosis protein mei2, DNA replication licensing factor mcm5-A, putative serine threonine-protein phosphatase C27B7.6, mitogen-activated protein kinase 2, patellin family protein, and WD repeat-containing protein 82. As previously reported, putative cyclin-like protein and WD repeat-containing protein 82 are the potential primary regulators of cyclin-dependent kinases, playing key roles in regulating mitotic cell cycle transition [105,106]. DNA replication licensing factor mcm5-A is a putative replicative helicase essential for the initiation and elongation phases of DNA replication in plant cells [107]. Putative serine threonine-protein phosphatase C27B7.6 regulates phosphorylation involved in cell proliferation, programmed cell death, and cell differentiation [108]. Protein MEI2-like 5, protein MEI2-like 3, meiosis protein mei2, and mitogen-activated protein kinase 2 were potential RNA-binding proteins essential for commitment to meiosis [109,110]. The patellin family protein is a carrier protein potentially involved in membrane-trafficking events associated with cell plate formation during cytokinesis [111]. All differentially expressed genes in nonsymbiotic zooxanthellae increase cell division in the presence of sufficient nutrient supplements and space during culture.

Especially, five differentially expressed transcripts of nonsymbiotic zooxanthellae were implicated in cell wall biosynthesis, which is essential for cell division, thus increasing the

zooxanthellae population. In particular, PG1 protein expression levels in nonsymbiotic zooxanthellae were 93,216-fold that in symbiotic zooxanthellae (Table 3). The PG1 protein belongs to polygalacturonases (PGs), which act on the homogalacturonan chain, thus softening the cell wall [112] and potentially promoting cell division in nonsymbiotic zooxanthellae during reproduction. Several genes involved in PG modification and pectin biosynthesis were detected (Table 2), such as a cell wall-associated hydrolase with a specific role in modifying PG [113]. Galactan beta-1,4-galactosyltransferase GALS1-like isoform X2 and phosphatidylinositol 4-phosphate 5-kinase 5 both regulated pectin biosynthesis and secretion [114,115]. Robust cell wall regulation in zooxanthellae may have occurred owing to frequent cell division to increase the population of nonsymbiotic zooxanthellae; however, this effect was not observed in symbiotic zooxanthellae, and no cell-wall-related transcripts were found by the SSH assay; this result is concurrent with cell cycle arrest in the G₁/G₀ transition [31].

Symbiotic zooxanthellae expressing immune-related genes associated with host defence

Residing in the *Aiptasia* host may present challenges associated with the intracellular defence mechanism in host cells, as described previously [35-37]. As predicted, SSH screening revealed numerous transcripts corresponding to those involved in innate immunity,

inflammation, virus–host interactions, and antifungal defence, including peptidyl-prolyl cis-trans isomerase 7 (alternatively named as cyclophilin-7), which potentially regulate the responses to inflammatory diseases including atherosclerosis and arthritis and viral infections [116]. Similar to cathepsin D, cathepsin E has slightly broader specificity and potentially contributes to immune responses [117]. Kinesin-like protein and patatin-like phospholipase family protein are involved in microbial infections with fatty acid metabolism and immune regulation by apicomplexan and plants [118,119]. Dual-specificity phosphatase 28 limits the spread of the HR response after infection by necrotrophic pathogens including *Botrytis cinerea* [120]. For fungus-plant interactions, UBX domain-containing protein 2 and hypersensitive-induced response protein 3 negatively regulate the powdery mildew-plant interaction [121] and contribute to plant basal resistance via an EDS1- and SA-dependent pathway [122,123]. In particular, internalin A was differentially expressed in symbiotic zooxanthellae. As reported, internalin A mediates the entry of *L. monocytogenes* into host intestinal epithelial cells and internalin A facilitates *L. innocua* uptake by host cells [124,125]. Importantly, symbiotic zooxanthellae presented transcripts similar to gene groups associated with the immune response, suggesting that the communication between the host and symbionts is potentially associated with immune regulation, which is one of the key factors responsible for symbiosis.

Other transcripts corresponded with numerous functional genes and were distinguished

into other cellular processes (Tables 1 and 2). They may provide further evidence regarding the potential response during *A. pulchella*-*Symbiodinium* endosymbiosis.

Numerous genes of unknown function were differentially expressed between symbiotic and nonsymbiotic stages

During genetic screening, various genes of unknown function were expressed in both symbiotic and nonsymbiotic stages of zooxanthellae. In total, 57 symbiotic and 50 nonsymbiotic expressed genes with unknown function were identified, of which 18/21 sequences in symbiotic/nonsymbiotic zooxanthellae did not share any similarity with sequences in NCBI GenBank, implying the lack of information regarding the genes associated with endosymbiosis; furthermore, quantitative analysis revealed that these unknown genes even provide evidence of >10-fold expression; hence, the present results provide more information regarding the molecular regulation of endosymbiosis.

Conclusion

SSH is a potent, large-scale method for identifying differentially expressed target genes in symbiotic/nonsymbiotic zooxanthellae. This study showed that when purifying symbiotic zooxanthellae harboured in host cells, washing with 0.02% SDS effectively reduces the proportion of host RNA contamination during RNA extraction (by approximately 5.6%, Fig

5) and does not significantly influence gene expression in zooxanthellal PCP, HSP90, and actin. Therefore, SDS does not excessively affect gene expression in symbiotic zooxanthellae. Furthermore, combinatorial screening with SSH and sequence extension based on the NGS-based zooxanthellal gene database, symbiotic zooxanthellae focused on chlorophyll production during symbiosis to enhance photosynthesis. The carbohydrates produced through photosynthesis primarily include starch transported into the host cells for use. Furthermore, the symbiosome microenvironment may be subjected to stress in zooxanthellae. Numerous genes are associated with stress responses and are actively expressed herein for protection. Furthermore, the genes and channel proteins involved in immune regulation are potentially primarily implicated in the communication between the host and symbionts to the establishment and maintenance of endosymbiosis, including the cell cycle, which is arrested in the G₁/G₀ stage. Inversely, when leaving the host to live freely in seawater, photosynthesis in nonsymbiotic zooxanthellae primarily depends on the electron transport chain for photosynthesis progression, and its products are primarily converted into lipid forms for their own metabolic utilization and storage. Furthermore, genes associated with flagellar movements are expressed, thus facilitating smooth swimming. Simultaneously, to increase the zooxanthellae population, the acceleration of the cell cycle increased the rate of reproduction and population growth and is correlated with upregulated proteins including pG1, which is involved in the cell wall biosynthesis. Altogether, the present results provide

551 insights into the mechanisms underlying physiological processes and molecular responses

552 between symbiotic and nonsymbiotic zooxanthellae.

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559 **Competing Interests**

560 The authors have declared that no competing interests exist.

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References

1. Yellowlees D, Rees TA, Leggat W. Metabolic interactions between algal symbionts and invertebrate hosts. *Plant Cell Environ.* 2008;31(5):679-694. doi: 10.1111/j.1365-3040.2008.01802.x
2. Hohman TC, McNeil PL, Muscatine L. Phagosome-lysosome fusion inhibited by algal symbionts of *Hydra viridis*. *J Cell Biol.* 1982;94(1):56-63. doi: 10.1083/jcb.94.1.56
3. Muscatine L, Cernichiaro E. Assimilation of photosynthetic products of zooxanthellae by a reef coral. *Biol Bull.* 1969;137(3):506-523. doi: 10.2307/1540172
4. Muscatine L. The role of symbiotic algae in carbon and energy flux in reef corals. *Ecosyst World.* 1990;25:75-87.
5. Wang JT, Douglas AE. Essential amino acid synthesis and nitrogen recycling in an alga-invertebrate symbiosis. *Mar Biol.* 1999;135(2):219-222. doi: 10.1007/s002270050619
6. Papina M, Meziane T, van Woesik R. Acclimation effect on fatty acids of the coral *Montipora digitata* and its symbiotic algae. *Comp Biochem Physiol B Biochem Mol Biol.* 2007;147(4):583-589. doi: 10.1016/j.cbpb.2007.02.011
7. Tanaka Y, Grottoli AG, Matsui Y, Suzuki A, Sakai K. Partitioning of nitrogen sources to algal endosymbionts of corals with long-term ¹⁵N-labelling and a mixing model. *Ecol Model.* 2015;309-310:163-169. doi: 10.1016/j.ecolmodel.2015.04.017
8. Pearse VB, Muscatine L. Role of Symbiotic algae (zooxanthellae) in coral calcification.

- 581 Biol Bull. 1971;141(2):350-363. doi: 10.2307/1540123
- 582 9. Al-Hammady MAM. The effect of zooxanthellae availability on the rates of skeletal
583 growth in the red sea coral acropora hemprichii. Egypt J Aquat Res. 2013;39(3):177-183. doi:
584 10.1016/j.ejar.2013.10.005
- 585 10. Iwasaki S, Inoue M, Suzuki A, Sasaki O, Kano H, Iguchi A, et al. The role of symbiotic
586 algae in the formation of the coral polyp skeleton: 3-D morphological study based on X-ray
587 microcomputed tomography. Geochim Geophys Geosyst. 2016;17(9):3629-3637. doi:
588 10.1002/2016GC006536
- 589 11. Jokiel PL, Lesser MP, Ondrusek ME. UV-absorbing compounds in the coral Pocillopora
590 damicornis: Interactive effects of UV radiation, photosynthetically active radiation, and water
591 flow. Limnol Oceanogr. 1997;42(6):1468-1473. doi: 10.4319/lo.1997.42.6.1468
- 592 12. Rosic NN, Dove S. Mycosporine-like amino acids from coral dinoflagellates. Appl
593 Environ Microbiol. 2011;77(24):8478-8486. doi: 10.1128/AEM.05870-11
- 594 13. Glynn PW, Maté JL, Baker AC, Calderón MO. Coral bleaching and mortality in Panama
595 and Ecuador during the 1997-1998 El Niño-Southern Oscillation event: spatial/temporal
596 patterns and comparisons with the 1982-1983 event. Bull Mar Sci Miami 2001;69(1):79-109.
- 597 14. Hughes TP, Baird AH, Bellwood DR, Card M, Connolly SR, Folke C, et al. Climate
598 change, human impacts, and the resilience of coral reefs. Science. 2003;301(5635):929-933.
599 doi: 10.1126/science.1085046

- 600 15. Anthony KR, Kline DI, Diaz-Pulido G, Dove S, Hoegh-Guldberg O. Ocean acidification
601 causes bleaching and productivity loss in coral reef builders. *Proc Natl Acad Sci U S A*.
602 2008;105(45):17442-17446. doi: 10.1073/pnas.0804478105
- 603 16. Wooldridge SA. Water quality and coral bleaching thresholds: formalising the linkage
604 for the inshore reefs of the Great Barrier Reef, Australia. *Mar Pollut Bull*. 2009;58(5):745-
605 751. doi: 10.1016/j.marpolbul.2008.12.013
- 606 17. Gates RD, Baghdasarian G, Muscatine L. Temperature stress causes host cell
607 detachment in symbiotic cnidarians: implications for coral bleaching. *Biol Bull*.
608 1992;182(3):324-332. doi: 10.2307/1542252
- 609 18. Bourne DG, Garren M, Work TM, Rosenberg E, Smith GW, Harvell CD. Microbial
610 disease and the coral holobiont. *Trends Microbiol*. 2009;17(12):554-562. doi:
611 10.1016/j.tim.2009.09.004
- 612 19. Muscatine L, Pool RR, Trench RK. Symbiosis of algae and invertebrates: aspects of the
613 symbiont surface and the host-symbiont interface. *Trans Am Microsc Soc*. 1975;94(4):450-
614 469.
- 615 20. Allemand D, Furla P, Tambutté S. Mechanisms of carbon acquisition for endosymbiont
616 photosynthesis in Anthozoa. *Can J Bot*. 1998;76(6):925-941. doi: 10.1139/b98-086
- 617 21. Leggat W, Marendy EM, Baillie B, Whitney SM, Ludwig M, Badger MR, et al.
618 Dinoflagellate symbioses: strategies and adaptations for the acquisition and fixation of

- 619 inorganic carbon. *Funct Plant Biol.* 2002;29(3):309-322. doi: 10.1071/PP01202
- 620 22. Jackson AE, Miller DJ, Yellowlees D. Phosphorus metabolism in the coral-
- 621 zooxanthellae symbiosis: characterization and possible roles of two acid phosphatases in the
- 622 algal symbiont symbiodinium sp. *Proc Roy Soc Lond Ser B Biol Sci.* 1989;238(1291):193-
- 623 202.
- 624 23. Jackson AE, Yellowlees D. Phosphate uptake by zooxanthellae isolated from corals.
- 625 *Proc Roy Soc B.* 1990;242(1305):201-204. doi: 10.1098/rspb.1990.0125
- 626 24. Rands ML, Douglas AE, Loughman BC, Hawes CR. The pH of the perisymbiont space
- 627 in the green hydra-chlorella symbiosis. *Protoplasma.* 1992;170(1-2):90-93. doi:
- 628 10.1007/bf01384461
- 629 25. Rands ML, Loughman BC, Douglas AE. The symbiotic interface in an alga-invertebrate
- 630 symbiosis. *Proc Roy Soc Lond Ser B Biol Sci.* 1993;253(1337):161-165. doi:
- 631 10.1098/rspb.1993.0097
- 632 26. Venn AA, Tambutté E, Lotto S, Zoccola D, Allemand D, Tambutté S. Imaging
- 633 intracellular pH in a reef coral and symbiotic anemone. *Proc Natl Acad Sci U S A.*
- 634 2009;106(39):16574-16579. doi: 10.1073/pnas.0902894106
- 635 27. Barott KL, Venn AA, Perez SO, Tambutté S, Tresguerres M. Coral host cells acidify
- 636 symbiotic algal microenvironment to promote photosynthesis. *Proc Natl Acad Sci U S A.*
- 637 2015;112(2):607-612. doi: 10.1073/pnas.1413483112

- 638 28. Seckbach J, Hinde R, Trautman D. Symbiosis: Mechanisms and model systems (cellular
639 origin, life in extreme habitats and astrobiology). Netherlands: Springer; 2004. pp. 207-220.
- 640 29. Detournay O, Schnitzler CE, Poole A, Weis VM. Regulation of cnidarian-dinoflagellate
641 mutualisms: evidence that activation of a host TGF β innate immune pathway promotes
642 tolerance of the symbiont. Dev Comp Immunol. 2012;38(4):525-537. doi:
643 10.1016/j.dci.2012.08.008
- 644 30. Neubauer EF, Poole AZ, Neubauer P, Detournay O, Tan K, Davy SK, et al. A diverse
645 host thrombospondin-type-1 repeat protein repertoire promotes symbiont colonization during
646 establishment of cnidarian-dinoflagellate symbiosis. Elife. 2017;6:e24494. doi:
647 10.7554/eLife.24494
- 648 31. Smith GJ, Muscatine L. Cell cycle of symbiotic dinoflagellates: variation in G1 phase-
649 duration with anemone nutritional status and macronutrient supply in the *Aiptasia pulchella*-
650 *Symbiodinium pulchrorum* symbiosis. Mar Biol. 1999;134(3):405-418. doi:
651 10.1007/s002270050557
- 652 32. Davy SK, Allemand D, Weis VM. Cell biology of cnidarian-dinoflagellate symbiosis.
653 Microbiol Mol Biol Rev. 2012;76(2):229-261. doi: 10.1128/mmbr.05014-11
- 654 33. Wood-Charlson EM, Hollingsworth LL, Krupp DA, Weis VM. Lectin/glycan
655 interactions play a role in recognition in a coral/dinoflagellate symbiosis. Cell Microbiol.
656 2006;8(12):1985-1993. doi: 10.1111/j.1462-5822.2006.00765.x

- 657 34. Parkinson JE, Tivey TR, Mandelare PE, Adpressa DA, Loesgen S, Weis VM. Subtle
658 differences in symbiont cell surface glycan profiles do not explain species-specific
659 colonization rates in a model Cnidarian-Algal symbiosis. *Front Microbiol.* 2018;9:842. doi:
660 10.3389/fmicb.2018.00842
- 661 35. Chen MC, Cheng YM, Sung PJ, Kuo CE, Fang LS. Molecular identification of Rab7
662 (ApRab7) in *Aiptasia pulchella* and its exclusion from phagosomes harboring zooxanthellae.
663 *Biochem Biophys Res Commun.* 2003;308(3):586-595. doi: 10.1016/s0006-291x(03)01428-1
- 664 36. Chen MC, Cheng YM, Hong MC, Fang LS. Molecular cloning of Rab5 (ApRab5) in
665 *Aiptasia pulchella* and its retention in phagosomes harboring live zooxanthellae. *Biochem*
666 *Biophys Res Commun* 2004;324(3):1024-1033. doi: 10.1016/j.bbrc.2004.09.151
- 667 37. Chen MC, Hong MC, Huang YS, Liu MC, Cheng YM, Fang LS. ApRab11, a cnidarian
668 homologue of the recycling regulatory protein Rab11, is involved in the establishment and
669 maintenance of the *Aiptasia-Symbiodinium* endosymbiosis. *Biochem Biophys Res Commun.*
670 2005;338(3):1607-1616. doi: 10.1016/j.bbrc.2005.10.133
- 671 38. Fransolet D, Roberty S, Plumier JC. Establishment of endosymbiosis: the case of
672 cnidarians and symbiodinium. *J Exp Mar Biol Ecol.* 2012;420-421:1-7. doi:
673 10.1016/j.jembe.2012.03.015
- 674 39. Putnam NH, Srivastava M, Hellsten U, Dirks B, Chapman J, Salamov A, et al. Sea
675 anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization.

- 676 Science. 2007;317(5834):86-94. doi: 10.1126/science.1139158
- 677 40. Chapman JA, Kirkness EF, Simakov O, Hampson SE, Mitros T, Weinmaier T, et al. The
678 dynamic genome of hydra. Nature. 2010;464(7288):592-596. doi: 10.1038/nature08830
- 679 41. Shoguchi E, Shinzato C, Kawashima T, Gyoja F, Mungpakdee S, Koyanagi R, et al.
680 Draft assembly of the symbiodinium minutum nuclear genome reveals dinoflagellate gene
681 structure. Curr Biol. 2013;23(15):1399-1408. doi: 10.1016/j.cub.2013.05.062
- 682 42. Lin S, Cheng S, Song B, Zhong X, Lin X, Li W, et al. The symbiodinium kawagutii
683 genome illuminates dinoflagellate gene expression and coral symbiosis. Science.
684 2015;350(6261):691-694. doi: 10.1126/science.aad0408
- 685 43. Meyer E, Aglyamova GV, Wang S, Buchanan-Carter J, Abrego D, Colbourne JK, et al.
686 Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx. BMC
687 Genom. 2009;10(1):219. doi: 10.1186/1471-2164-10-219
- 688 44. Polato NR, Vera JC, Baums IB. Gene Discovery in the threatened elkhorn coral: 454
689 sequencing of the acropora palmata transcriptome. PLoS One. 2011;6(12):e28634. doi:
690 10.1371/journal.pone.0028634
- 691 45. Traylor-Knowles N, Granger BR, Lubinski TJ, Parikh JR, Garamszegi S, Xia Y, et al.
692 Production of a reference transcriptome and transcriptomic database (PocilloporaBase) for
693 the cauliflower coral, Pocillopora damicornis. BMC Genomics. 2011;12(1):585. doi:
694 10.1186/1471-2164-12-585

- 695 46. Lehnert EM, Burriesi MS, Pringle JR. Developing the anemone *Aiptasia* as a tractable
696 model for cnidarian-dinoflagellate symbiosis: the transcriptome of aposymbiotic *A. pallida*.
697 BMC Genomics. 2012;13:271. doi: 10.1186/1471-2164-13-271
- 698 47. Moya A, Huisman L, Ball EE, Hayward DC, Grasso LC, Chua CM, et al. Whole
699 transcriptome analysis of the coral *Acropora millepora* reveals complex responses to CO₂-
700 driven acidification during the initiation of calcification. Mol Ecol. 2012;21(10):2440-2454.
701 doi: 10.1111/j.1365-294X.2012.05554.x
- 702 48. Voolstra CR, Schwarz JA, Schnetzer J, Sunagawa S, Desalvo MK, Szmant AM, et al.
703 The host transcriptome remains unaltered during the establishment of coral–algal symbioses.
704 Mol Ecol. 2009;18(9):1823-1833. doi: 10.1111/j.1365-294X.2009.04167.x
- 705 49. Schnitzler CE, Weis VM. Coral larvae exhibit few measurable transcriptional changes
706 during the onset of coral-dinoflagellate endosymbiosis. Mar Genom. 2010;3(2):107-116. doi:
707 10.1016/j.margen.2010.08.002
- 708 50. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search
709 tool. J Mol Biol. 1990;215(3):403-410. doi: 10.1016/S0022-2836(05)80360-2
- 710 51. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing
711 phylogenetic trees. Mol Biol Evol. 1987;4(4):406-425. doi:
712 10.1093/oxfordjournals.molbev.a040454
- 713 52. Si-Qing C, Xian-Gang F, Jian-Fang Y, Jie-Hua L. Structure and activity of RNase A in

- 714 sodium dodecyl sulphate solutions. J Dispers Sci Technol. 2007;28(2):297-300. doi:
715 10.1080/01932690601062127
- 716 53. Apel K, Santel HJ, Redlinger TE, Falk H. The protochlorophyllide holochrome of barley
717 (*hordeum vulgare* L.). Eur J Biochem. 1980;111(1):251-258. doi: 10.1111/j.1432-
718 1033.1980.tb06100.x
- 719 54. Leeper FJ. The biosynthesis of porphyrins, chlorophylls, and vitamin B12. Nat Prod
720 Rep. 1989;6(2):171-203. doi: 10.1039/np9890600171
- 721 55. Su Q, Frick G, Armstrong G, Apel K. POR C of *arabidopsis thaliana*: a third light- and
722 NADPH-dependent protochlorophyllide oxidoreductase that is differentially regulated by
723 light. Plant Mol Biol. 2001;47(6):805-813. doi: 10.1023/A:1013699721301
- 724 56. Jiang J, Zhang H, Kang Y, Bina D, Lo CS, Blankenship RE. Characterization of the
725 peridinin–chlorophyll a-protein complex in the dinoflagellate *symbiodinium*. Biochim
726 Biophys Acta. 2012;1817(7):983-989. doi: 10.1016/j.bbabo.2012.03.027
- 727 57. Yoshida K, Terashima I, Noguchi K. Distinct roles of the cytochrome pathway and
728 alternative oxidase in leaf photosynthesis. Plant Cell Physiol. 2006;47(1):22-31. doi:
729 10.1093/pcp/pci219
- 730 58. Rosa-Téllez S, Anoman AD, Flores-Tornero M, Toujani W, Alseek S, Fernie AR, et al.
731 Phosphoglycerate kinases are co-regulated to adjust metabolism and to optimize growth.
732 Plant Physiol. 2018;176(2):1182. doi: 10.1104/pp.17.01227

- 733 59. Wang X, Wang Q, Nguyen P, Lin C. Cryptochrome-mediated light responses in plants.
734 Enzymes. 2014;35:167-189. doi: 10.1016/b978-0-12-801922-1.00007-5
- 735 60. Koskela MM, Dahlström KM, Goñi G, Lehtimäki N, Nurmi M, Velazquez-Campoy A,
736 et al. Arabidopsis FNRL protein is an NADPH-dependent chloroplast oxidoreductase
737 resembling bacterial ferredoxin-NADP(+) reductases. *Physiol Plant*. 2018;162(2):177-190.
738 doi: 10.1111/ppl.12621
- 739 61. Lang M, Kroth PG. Diatom fucoxanthin chlorophyll a/c-binding protein (FCP) and land
740 plant light-harvesting proteins use a similar pathway for thylakoid membrane Insertion. *J Biol*
741 *Chem*. 2001;276(11):7985-7991. doi: 10.1074/jbc.M006417200
- 742 62. Cole DG, Diener DR, Himelblau AL, Beech PL, Fuster JC, Rosenbaum JL.
743 *Chlamydomonas* kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain
744 proteins required for ciliary assembly in *Caenorhabditis elegans* sensory neurons. *J Cell Biol*.
745 1998;141(4):993-1008. doi: 10.1083/jcb.141.4.993
- 746 63. Abaza A, Soleilhac JM, Westendorf J, Piel M, Crevel I, Roux A, et al. M phase
747 phosphoprotein 1 is a human plus-end-directed kinesin-related protein required for
748 cytokinesis. *J Biol Chem*. 2003;278(30):27844-27852. doi: 10.1074/jbc.M304522200
- 749 64. Miki H, Okada Y, Hirokawa N. Analysis of the kinesin superfamily: insights into
750 structure and function. *Trends Cell Biol*. 2005;15(9):467-476. doi: 10.1016/j.tcb.2005.07.006
- 751 65. Kubo T, Hou Y, Cochran DA, Witman GB, Oda T. A microtubule-dynein tethering

- 752 complex regulates the axonemal inner dynein f (II). Mol Biol Cell. 2018;29(9):1060-1074.
- 753 doi: 10.1091/mbc.E17-11-0689
- 754 66. Gu Y, Deng Z, Paredez AR, DeBolt S, Wang ZY, Somerville C. Prefoldin 6 is required
- 755 for normal microtubule dynamics and organization in Arabidopsis. Proc Natl Acad Sci U S
- 756 A. 2008;105(46):18064-18069. doi: 10.1073/pnas.0808652105
- 757 67. Huang K, Diener DR, Mitchell A, Pazour GJ, Witman GB, Rosenbaum JL. Function and
- 758 dynamics of PKD2 in Chlamydomonas reinhardtii flagella. J Cell Biol. 2007;179(3):501-514.
- 759 doi: 10.1083/jcb.200704069
- 760 68. Yang Y, Cochran DA, Gargano MD, King I, Samhat NK, Burger BP, et al. Regulation
- 761 of flagellar motility by the conserved flagellar protein CG34110/Ccdc135/FAP50. Mol Biol
- 762 Cell. 2011;22(7):976-987. doi: 10.1091/mbc.E10-04-0331
- 763 69. Gopal R. Role of DPY-30 proteins in flagella. Dissertations. 2009.
- 764 70. Pasek RC, Berbari NF, Lewis WR, Kesterson RA, Yoder BK. Mammalian clusterin
- 765 associated protein 1 is an evolutionarily conserved protein required for ciliogenesis. Cilia.
- 766 2012;1(1):20. doi: 10.1186/2046-2530-1-20
- 767 71. Lechtreck KF, Rostmann J, Grunow A. Analysis of Chlamydomonas SF-assemblin by
- 768 GFP tagging and expression of antisense constructs. J Cell Sci. 2002;115(7):1511-1522.
- 769 72. Lechtreck KF, Silflow CD. SF-assemblin in chlamydomonas: sequence conservation
- 770 and localization during the cell cycle. Cell Motil Cytoskeleton. 1997;36(2):190-201. doi:

10.1002/(sici)1097-0169(1997)36:2<190::aid-cm8>3.0.co;2-d

73. Schellhaus AK, Moreno-Andrés D, Chugh M, Yokoyama H, Moschopoulou A, De S, et al. Developmentally regulated GTP binding protein 1 (DRG1) controls microtubule dynamics. *Sci Rep.* 2017;7(1):9996. doi: 10.1038/s41598-017-10088-5

74. van Dijk J, Rogowski K, Miro J, Lacroix B, Eddé B, Janke C. A targeted multienzyme mechanism for selective microtubule polyglutamylation. *Mol Cell.* 2007;26(3):437-448. doi: 10.1016/j.molcel.2007.04.012

75. Neutelings T, Lambert CA, Nussgens BV, Colige AC. Effects of mild cold shock (25°C) followed by warming up at 37°C on the cellular stress response. *PLoS One.* 2013;8(7):e69687. doi: 10.1371/journal.pone.0069687

76. Xu ZS, Li ZY, Chen Y, Chen M, Li LC, Ma YZ. Heat shock protein 90 in plants: molecular mechanisms and roles in stress responses. *Int J Mol Sci* 2012;13(12):15706-15723. doi: 10.3390/ijms131215706

77. Klostermeier D, Seidel R, Reinstein J. The functional cycle and regulation of the *Thermus thermophilus* DnaK chaperone system. Edited by A. R. Fersht. *J Mol Biol.* 1999;287(3):511-525. doi: 10.1006/jmbi.1999.2636

78. Gao Y, Wang Y, Xin H, Li S, Liang Z. Involvement of ubiquitin-conjugating enzyme (E2 gene family) in ripening process and response to cold and heat stress of *Vitis vinifera*. *Sci Rep.* 2017;7(1):13290. doi: 10.1038/s41598-017-13513-x

- 790 79. Huang H, Yang M, Su Y, Qu L, Deng XW. Arabidopsis atypical kinases ABC1K1 and
791 ABC1K3 act oppositely to cope with photo damage under red light. Mol Plant.
792 2015;8(7):1122-1124. doi: 10.1016/j.molp.2015.04.003
- 793 80. Lee U, Wie C, Fernandez BO, Feelisch M, Vierling E. Modulation of nitrosative stress
794 by S-Nitrosoglutathione reductase is critical for thermotolerance and plant growth in
795 Arabidopsis. Plant Cell. 2008;20(3):786-802. doi: 10.1105/tpc.107.052647
- 796 81. Pan R, Jones AD, Hu J. Cardiolipin-mediated mitochondrial dynamics and stress
797 response in Arabidopsis. Plant Cell. 2014;26(1):391-409. doi: 10.1105/tpc.113.121095
- 798 82. Rosado A, Schapire AL, Bressan RA, Harfouche AL, Hasegawa PM, Valpuesta V, et al.
799 The Arabidopsis tetratricopeptide repeat-containing protein TTL1 is required for osmotic
800 stress responses and abscisic acid sensitivity. Plant Physiol. 2006;142(3):1113-1126. doi:
801 10.1104/pp.106.085191
- 802 83. Mikami K, Katagiri T, Iuchi S, Yamaguchi-Shinozaki K, Shinozaki K. A gene encoding
803 phosphatidylinositol-4-phosphate 5-kinase is induced by water stress and abscisic acid in
804 Arabidopsis thaliana. Plant J. 1998;15(4):563-568. doi: 10.1046/j.1365-313x.1998.00227.x
- 805 84. Yap MN, Lee RH, Huang YJ, Liao CJ, Chen SC. Molecular characterization of a novel
806 senescence-associated gene SPA15 induced during leaf senescence in sweet potato. Plant Mol
807 Biol. 2003;51(4):471-481. doi: 10.1023/a:1022334820332
- 808 85. Hewitt MM, Carr JM, Williamson CL, Slocum RD. Effects of phosphate limitation on

- 809 expression of genes involved in pyrimidine synthesis and salvaging in Arabidopsis. Plant
810 Physiol Biochem. 2005;43(2):91-99. doi: 10.1016/j.plaphy.2005.01.003
- 811 86. Vidal R, López-Maury L, Guerrero MG, Florencio FJ. Characterization of an alcohol
812 dehydrogenase from the Cyanobacterium Synechocystis sp. strain PCC 6803 that responds to
813 environmental stress conditions via the Hik34-Rre1 two-component system. J Bacteriol.
814 2009;191(13):4383-4391. doi: 10.1128/JB.00183-09
- 815 87. Rey P, Tarrago L. Physiological roles of plant methionine sulfoxide reductases in redox
816 homeostasis and signaling. Antioxidants (Basel). 2018;7(9):114. doi: 10.3390/antiox7090114
- 817 88. Fujita N, Yoshida M, Asakura N, Ohdan T, Miyao A, Hirochika H, et al. Function and
818 characterization of starch synthase I using mutants in rice. Plant Physiol. 2006;140(3):1070-
819 1084. doi: 10.1104/pp.105.071845
- 820 89. Kerk D, Conley TR, Rodriguez FA, Tran HT, Nimick M, Muench DG, et al. A
821 chloroplast-localized dual-specificity protein phosphatase in Arabidopsis contains a
822 phylogenetically dispersed and ancient carbohydrate-binding domain, which binds the
823 polysaccharide starch. Plant J. 2006;46(3):400-413. doi: 10.1111/j.1365-3113.2006.02704.x
- 824 90. Delatte T, Umhang M, Trevisan M, Eicke S, Thorneycroft D, Smith SM, et al. Evidence
825 for distinct mechanisms of starch granule breakdown in plants. J Biol Chem.
826 2006;281(17):12050-12059. doi: 10.1074/jbc.M513661200
- 827 91. Bussell JD, Keech O, Fenske R, Smith SM. Requirement for the plastidial oxidative

828 pentose phosphate pathway for nitrate assimilation in Arabidopsis. Plant J. 2013;75(4):578-
829 591. doi: 10.1111/tpj.12222

830 92. Schrick K, Shiva S, Arpin JC, Delimont N, Isaac G, Tamura P, et al. Steryl glucoside
831 and acyl steryl glucoside analysis of Arabidopsis seeds by electrospray ionization tandem
832 mass spectrometry. Lipids. 2012;47(2):185-193. doi: 10.1007/s11745-011-3602-9

833 93. Wayne LL, Wallis JG, Kumar R, Markham JE, Browse J. Cytochrome b5 reductase
834 encoded by CBR1 is essential for a functional male gametophyte in Arabidopsis. Plant Cell.
835 2013;25(8):3052-3066. doi: 10.1105/tpc.113.113324

836 94. Bates PD, Fatihi A, Snapp AR, Carlsson AS, Browse J, Lu C. Acyl editing and
837 headgroup exchange are the major mechanisms that direct polyunsaturated fatty acid flux into
838 triacylglycerols. Plant Physiol. 2012;160(3):1530-1539. doi: 10.1104/pp.112.204438

839 95. Lin R, Tao R, Gao X, Li T, Zhou X, Guan K-L, et al. Acetylation stabilizes ATP-citrate
840 lyase to promote lipid biosynthesis and tumor growth. Mol Cell. 2013;51(4):506-518. doi:
841 10.1016/j.molcel.2013.07.002

842 96. Sasaki Y, Nagano Y. Plant Acetyl-CoA carboxylase: structure, biosynthesis, regulation,
843 and gene manipulation for plant breeding. Biosci Biotechnol Biochem. 2004;68(6):1175-
844 1184. doi: 10.1271/bbb.68.1175

845 97. McDonald SM, Plant JN, Worden AZ. The mixed lineage nature of nitrogen transport
846 and assimilation in marine eukaryotic phytoplankton: a case study of micromonas. Mol Biol

- 847 Evol. 2010;27(10):2268-2283. doi: 10.1093/molbev/msq113
- 848 98. Ludewig U, Neuhäuser B, Dynowski M. Molecular mechanisms of ammonium transport
849 and accumulation in plants. FEBS Lett. 2007;581(12):2301-2308. doi:
850 10.1016/j.febslet.2007.03.034
- 851 99. De Angeli A, Monachello D, Ephritikhine G, Frachisse JM, Thomine S, Gambale F, et
852 al. The nitrate/proton antiporter AtCLCa mediates nitrate accumulation in plant vacuoles.
853 Nature. 2006;442(7105):939-942. doi: 10.1038/nature05013
- 854 100. Liu Cm CM, McElver J, Tzafrir I, Joosen R, Wittich P, Patton D, et al. Condensin and
855 cohesin knockouts in Arabidopsis exhibit a titan seed phenotype. Plant J. 2002;29(4):405-
856 415. doi: 10.1046/j.1365-313x.2002.01224.x
- 857 101. Schubert V, Weissleder A, Ali H, Fuchs J, Lermontova I, Meister A, et al. Cohesin gene
858 defects may impair sister chromatid alignment and genome stability in Arabidopsis thaliana.
859 Chromosoma. 2009;118(5):591-605. doi: 10.1007/s00412-009-0220-x
- 860 102. Ahn JW, Kim M, Lim JH, Kim GT, Pai HS. Phytocalpain controls the proliferation and
861 differentiation fates of cells in plant organ development. Plant J. 2004;38(6):969-981. doi:
862 10.1111/j.1365-313X.2004.02102.x
- 863 103. Park S, Rancour DM, Bednarek SY. In planta analysis of the cell cycle-dependent
864 localization of AtCDC48A and its critical roles in cell division, expansion, and
865 differentiation. Plant Physiol. 2008;148(1):246-258. doi: 10.1104/pp.108.121897

866 104. Zhang X, Chen Y, Lin X, Hong X, Zhu Y, Li W, et al. Adenine phosphoribosyl
867 transferase 1 is a key enzyme catalyzing cytokinin conversion from nucleobases to
868 nucleotides in Arabidopsis. *Mol Plant*. 2013;6(5):1661-1672. doi: 10.1093/mp/sst071

869 105. van Nocker S, Ludwig P. The WD-repeat protein superfamily in Arabidopsis:
870 conservation and divergence in structure and function. *BMC Genom*. 2003;4(1):50. doi:
871 10.1186/1471-2164-4-50

872 106. Wang G, Kong H, Sun Y, Zhang X, Zhang W, Altman N, et al. Genome-wide analysis
873 of the cyclin family in Arabidopsis and comparative phylogenetic analysis of plant cyclin-like
874 proteins. *Plant Physiol*. 2004;135(2):1084-1099. doi: 10.1104/pp.104.040436.

875 107. Shultz RW, Lee TJ, Allen GC, Thompson WF, Hanley-Bowdoin L. Dynamic
876 localization of the DNA replication proteins MCM5 and MCM7 in plants. *Plant Physiol*.
877 2009;150(2):658-669. doi: 10.1104/pp.109.136614

878 108. Shi Y. Serine/threonine phosphatases: mechanism through structure. *Cell*.
879 2009;139(3):468-484. doi: 10.1016/j.cell.2009.10.006

880 109. Anderson GH, Hanson MR. The Arabidopsis Mei2 homologue AML1 binds
881 AtRaptor1B, the plant homologue of a major regulator of eukaryotic cell growth. *BMC Plant*
882 *Biol*. 2005;5:2. doi: 10.1186/1471-2229-5-2

883 110. Kaur J, Sebastian J, Siddiqi I. The Arabidopsis-me12-like genes play a role in meiosis
884 and vegetative growth in Arabidopsis. *Plant Cell*. 2006;18(3):545-559. doi:

885 10.1105/tpc.105.039156

886 111. Zhou H, Duan H, Liu Y, Sun X, Zhao J, Lin H. Patellin protein family functions in plant
887 development and stress response. J Plant Physiol. 2019;234-235:94-97. doi:
888 10.1016/j.jplph.2019.01.012

889 112. Babu Y, Bayer M. Plant polygalacturonases involved in cell elongation and separation-
890 the same but different? Plants (Basel). 2014;3(4):613-623. doi: 10.3390/plants3040613

891 113. Vermassen A, Leroy S, Talon R, Provot C, Popowska M, Desvaux M. Cell wall
892 hydrolases in bacteria: insight on the diversity of cell wall amidases, glycosidases and
893 peptidases toward peptidoglycan. Front Microbiol. 2019;10:331. doi:
894 10.3389/fmicb.2019.00331

895 114. Liwanag AJM, Ebert B, Verherbruggen Y, Rennie EA, Rautengarten C, Oikawa A, et
896 al. Pectin biosynthesis: GAL51 in Arabidopsis thaliana Is a β -1,4-Galactan β -1,4-
897 Galactosyltransferase. Plant Cell. 2012;24(12):5024-5036. doi: 10.1105/tpc.112.106625

898 115. Ischebeck T, Stenzel I, Heilmann I. Type B phosphatidylinositol-4-phosphate 5-kinases
899 mediate Arabidopsis and Nicotiana tabacum pollen tube growth by regulating apical pectin
900 secretion. Plant Cell. 2008;20(12):3312-3330. doi: 10.1105/tpc.108.059568

901 116. Hoffmann H, Schiene-Fischer C. Functional aspects of extracellular cyclophilins. Biol
902 Chem. 2014;395(7-8):721-735. doi: 10.1515/hsz-2014-0125

903 117. Lisón P, Rodrigo I, Conejero V. A novel function for the cathepsin D inhibitor in

904 tomato. *Plant Physiol.* 2006;142(3):1329-1339. doi: 10.1104/pp.106.086587

905 118. Abdelkhalek A, Ismail IA, Dessoky ES, El-Hallous EI, Hafez E. A tomato kinesin-like
906 protein is associated with tobacco mosaic virus infection. *Biotechnol Biotechnol Equip.*
907 2019;33(1):1424-1433. doi: 10.1080/13102818.2019.1673207

908 119. Wilson SK, Knoll LJ. Patatin-like phospholipases in microbial infections with emerging
909 roles in fatty acid metabolism and immune regulation by Apicomplexa. *Mol Microbiol.*
910 2018;107(1):34-46. doi: 10.1111/mmi.13871

911 120. Lumbreras V, Vilela B, Irar S, Solé M, Capellades M, Valls M, et al. MAPK
912 phosphatase MKP2 mediates disease responses in Arabidopsis and functionally interacts with
913 MPK3 and MPK6. *Plant J.* 2010;63(6):1017-1030. doi: 10.1111/j.1365-3113X.2010.04297.x

914 121. Chandran D, Rickert J, Cherk C, Dotson BR, Wildermuth MC. Host cell ploidy
915 underlying the fungal feeding site is a determinant of powdery mildew growth and
916 reproduction. *Mol Plant Microbe Interact.* 2013;26(5):537-545. doi: 10.1094/MPMI-10-12-
917 0254-R

918 122. Li S, Zhao J, Zhai Y, Yuan Q, Zhang H, Wu X, et al. The hypersensitive induced
919 reaction 3 (HIR3) gene contributes to plant basal resistance via an EDS1 and salicylic acid-
920 dependent pathway. *Plant J.* 2019;98(5):783-797. doi: 10.1111/tpj.14271

921 123. War AR, Paulraj MG, War MY, Ignacimuthu S. Role of salicylic acid in induction of
922 plant defense system in chickpea (*Cicer arietinum* L.). *Plant Signal Behav.* 2011;6(11):1787-

- 923 1792. doi: 10.4161/psb.6.11.17685
- 924 124. Gaillard JL, Berche P, Frehel C, Gouin E, Cossart P. Entry of *L. monocytogenes* into
- 925 cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-
- 926 positive cocci. *Cell*. 1991;65(7):1127-1141. doi: 10.1016/0092-8674(91)90009-n
- 927 125. Lecuit M, Dramsi S, Gottardi C, Fedor-Chaiken M, Gumbiner B, Cossart P. A single
- 928 amino acid in E-cadherin responsible for host specificity towards the human pathogen
- 929 *Listeria monocytogenes*. *EMBO J*. 1999;18(14):3956-3963. doi: 10.1093/emboj/18.14.3956

930

931 **Supporting information**

932 Table S1. List of all differentially expressed transcripts in symbiotic zooxanthellae.

933 Table S2. List of all differentially expressed transcripts in non-symbiotic zooxanthellae.

934

Relative amplification

Figure 1

Figure 2

Figure 3

Figure 4

Symbiotic

Non-symbiotic

■ APG ■ FG ■ UKG ■ NSF ■ BAC

Figure 5