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探討草履蟲與綠藻的內共生建立

Investigating the establishment of endosymbiosis between

the ciliate *Paramecium bursaria* and the green algae

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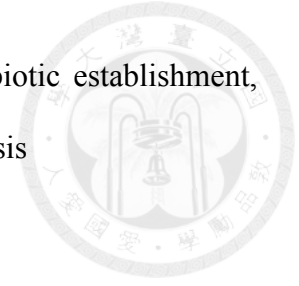
摘要

草履蟲 *Paramecium bursaria* 與其內共生的綠藻是常被用來探討內共生關係的模式生物，然而，*P. bursaria* 及其內共生藻建立的機制尚不清楚。因此，本計畫將探討影響 *P. bursaria* 與內共生藻 *Chlorella variabilis* 建立內共生的因素。透過感染實驗發現無綠藻共生的 *P. bursaria* 相較於長期實驗室環境培養的綠藻 *C. variabilis* ，其能夠更有效地與從與新鮮分離的內共生藻狀態的 *C. variabilis* 建立內共生關係。此外，從草履蟲剛分離的 *C. variabilis* 在宿主外進行短期培養後，綠藻與 *P. bursaria* 內共生建立的效率會逐漸降低，並在短期培養 4 天後降低到與長期實驗室環境培養的綠藻相近的內共生建立效率。此現象推測為分離的內共生藻 *C. variabilis* 於生理或形態上有所改變，使其與長期實驗室環境培養的綠藻特徵相似。因此，我們進行連續時間點的轉錄體分析，以進一步瞭解從宿主分離後的內共生藻變化，結果顯示與光合作用、抗氧化能力和細胞壁合成相關的基因有所改變，這些變化與建立能力有潛在的相關性。此外，我們也會將綠藻以不同的條件處理，進而操作感染實驗，以探討各種因素如何影響內共生的建立以及誘發這些因素的宿主環境。我們的研究結果發現，較低的葉綠素含量和內共生藻分泌的因子可能影響內共生的建立，而內共生藻的變化與宿主衍生因子的誘導有關鍵的影響。此研究將有助於更深入地瞭解內共生建立的過程。

關鍵字：草履蟲、綠藻、內共生建立、感染實驗、時間點轉錄體分析、光合作用

Abstract

The ciliate *Paramecium bursaria* engages in a facultative mutualistic interaction with hundreds of green algal endosymbionts in freshwater, providing an excellent model for studying endosymbiotic relationships. However, the mechanisms underlying the establishment of *P. bursaria* and its algal endosymbionts remain unclear. This study aims to elucidate the factors influencing the establishment of *P. bursaria* and its algal endosymbionts *Chlorella variabilis*. Through re-infection experiments, we demonstrated that *P. bursaria* establishes endosymbiosis more efficiently with freshly isolated endosymbiotic *C. variabilis* from symbiotic *P. bursaria* than with long-term free-living algal cultures of the same strain. Moreover, when isolated endosymbiotic *C. variabilis* were cultured outside the host, their establishment efficiency gradually diminished, reaching the level of free-living algal cultures within a few cell divisions. This phenomenon is primarily attributed to non-genetic alterations in the isolated endosymbionts, aligning them with the characteristics of free-living algal cultures. To investigate these alterations, we performed time-course transcriptome analysis, revealing changes in gene expression associated with photosynthesis, oxidative stress response, and cell wall synthesis, which correlated with the establishment ability. Additionally, we conducted physiological measurements and re-infection experiments with algae subjected to different treatments to explore how various factors influence the establishment ability and the host environments that induce these factors. Our findings suggest that lower chlorophyll content and factors secreted from freshly isolated *C. variabilis* potentially influence the establishment of endosymbiosis, with specific host-derived factors playing a crucial role in inducing algal changes. This comprehensive investigation contributes to a deeper understanding of endosymbiotic establishment mechanisms.

Keywords: *Paramecium bursaria*, *Chlorella variabilis*, endosymbiotic establishment, re-infection assay, time-course transcriptomic analysis, photosynthesis
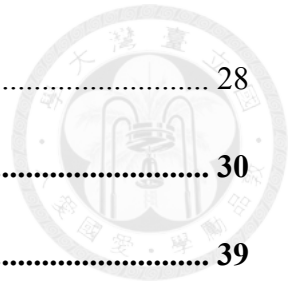
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1. Introduction

Endosymbiosis is a symbiotic relationship in which one organism resides within another driving the original evolution of eukaryotes (Archibald 2015). A notable example of this is the establishment of plastids in eukaryotic cells, a well-known photo-endosymbiosis event where an ancestral eukaryotic cell partnered with cyanobacteria as phototrophic endosymbionts through phagocytosis. This process, known as primary endosymbiosis, involves a non-photosynthetic eukaryotic cell engulfing a phototrophic prokaryotic cell. The lineages that acquired plastids from this initial photo-endosymbiosis event are grouped under the kingdom Archaeplastida, which includes Glaucophyta, red algae, green algae, and land plants (Reyes-Prieto, Weber et al. 2007, Oborník 2019). When these eukaryotic cells harboring primary plastids are engulfed and partnered with non-photosynthetic eukaryotic cells, this process is defined as secondary endosymbiosis (Archibald 2015). This concept extends to tertiary endosymbiosis, where a similar mechanism occurs (Bhattacharya, Yoon et al. 2004). Moreover, other independent instances of primary, secondary, and tertiary photo-endosymbiosis frequently occur in diverse environments worldwide. Examples include the relationships between Cnidaria and Symbiodiniaceae, *Hydra* and Chlorellaceae, and Ciliophora and Chlorellaceae (Yellowlees, Rees et al. 2008, Esteban, Fenchel et al. 2010, Nowack and Melkonian 2010, Quevarec, Brasseur et al. 2023). However, the underlying mechanisms by which eukaryotic cells and chloroplasts initiate and maintain photo-endosymbiotic relationships remain unknown. Investigating ongoing independent instances of photo-endosymbiosis might provide crucial insights into the evolutionary characteristics of chloroplast photo-endosymbiosis.

The ciliate *Paramecium bursaria* is one of two *Paramecium* species capable of forming endosymbiosis with algae, harboring hundreds of algal endosymbionts,

primarily identified as Chlorellaceae (Hoshina and Imamura 2008), within its cytoplasm in freshwater environments. During asexual reproduction, *P. bursaria* ensures the division and equal transmission of its algal endosymbionts to the daughter cells. This mutualistic interaction between *P. bursaria* and its algal endosymbionts includes bidirectional nutrient fluxes and physical protection (Reisser 1976, KAWAKAMI and KAWAKAMI 1978, Albers, Reisser et al. 1982, Kato, Ueno et al. 2006, Kato and Imamura 2009, Summerer, Sonntag et al. 2009, Kodama and Miyazaki 2021). From the perspective of the algal endosymbionts, *P. bursaria* provides carbon dioxide, nitrogen compounds, and a shelter to prevent predatory attacks. In return, the algal endosymbionts supply the host with photosynthetic products, including organic carbon sources and oxygen, which help the host overcome nutrient depletion (Kodama and Miyazaki 2021) and thrive in anoxic environments (Berninger, Finlay et al. 1986, Finlay, Maberly et al. 1996). Moreover, these endosymbionts play a crucial role in providing photoprotection for the host.
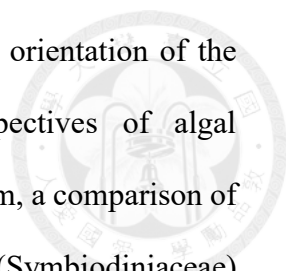
The levels of dependency between different strains of *P. bursaria* and their algal endosymbionts vary (Minter, Lowe et al. 2018). If the host and the algal endosymbionts have a facultative endosymbiotic relationship, we can artificially isolate and culture them independently under laboratory conditions (Weis 1969, Gerashchenko, Nishihara et al. 2000, Kodama and Fujishima 2008). For instance, the algal endosymbionts are usually isolated using physical methods to lyse the host and release them, such as sonication and high-speed centrifugation. Alternatively, chemical methods can remove the algal endosymbionts from the host, using agents like 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 1,1'-Dimethyl-4,4'-bipyridinium dichloride (also known as paraquat), which block electron flow in photosystem II and I, respectively, and cycloheximide, which interferes with algal endosymbiont translation. Aposymbiotic *P. bursaria*, which has eliminated its algal endosymbionts, and symbiotic *P. bursaria*,

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which retains them, can both be heterotrophic and mixotrophic by feeding on microorganisms such as bacteria, yeast, or non-symbiotic algae. The growth fitness of symbiotic *P. bursaria* is superior to that of aposymbiotic individuals due to the benefits conferred by the algal endosymbionts. However, aposymbiotic *P. bursaria* and the symbiotic algae can re-establish endosymbiosis through a re-infection assay by mixing them (Karakashian 1975), making *P. bursaria* a suitable model for studying the establishment and maintenance of endosymbiosis.

Previous studies (Kodama and Fujishima 2009, Kodama and Fujishima 2009) have provided a basic cytological understanding of the re-establishment of endosymbiosis in *P. bursaria*. When aposymbiotic *P. bursaria* engulfs algal cells, the engulfed algal cells are clustered in the digestive vacuoles (DVs). Within minutes, acidosomes and lysosomes begin fusing with the DV membrane to acidify the DVs and facilitate digestion, turning some digested algal cells yellow or brown. However, some algal cells escape lysosomal degradation and bud out as single cells enclosed within DVs approximately 30 minutes post-engulfment. The translocation of these algal cells, enclosed within the DV membrane and differentiated into the perialgal vacuole (PV) membrane, occurs beneath the cortex of *P. bursaria* to initiate the establishment of endosymbiosis and maintain a stable endosymbiotic relationship. The compatibility between *P. bursaria* and algal cells within Chlorellaceae depends on various factors, including the specific algal species and the source of the algae, such as other host species and the natural environment (Takeda, Sekiguchi et al. 1998, Flemming, Potekhin et al. 2020). Comparisons from reinfection assays reveal that *P. bursaria* exhibits better compatibility with algal species isolated from other ciliate species compared to those from symbiotic *Hydra* (*Hydra viridissima*) or freely found in the natural environment. Additionally, algal species isolated from trumpet animalcules (*Stentor polymorphus*) and freshwater sponge (*Spongilla fluviatilis*) fail to establish an

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endosymbiotic relationship with *P. bursaria* (Summerer, Sonntag et al. 2007). However, the fundamental mechanism by which algal cells evade digestion or how *P. bursaria* recognizes features of different algal species remains unclear.

To better understand the genetic foundation of the evolution of endosymbiosis establishment in hosts, most studies have focused on using comparative genomics between symbiotic hosts and non-symbiotic relatives to identify endosymbiosis-specific genome evolution. Previous studies revealed protein expansion and the duplication of six gene families in the *P. bursaria* genome (Cheng, Liu et al. 2020). Another study found that the differentially enriched genes of *P. bursaria* are involved in multiple molecular functions and biological pathways, such as oxygen binding, mineral absorption, nitrogen metabolism, and N-glycan biosynthesis, when compared with its sister clade *P. caudatum* (He, Wang et al. 2019). This suggests that the genomic divergence of *P. bursaria* may play a crucial role in the evolutionary process involving algal endosymbionts. *P. bursaria* might provide minerals and nitrogen sources to its algal endosymbionts, while simultaneously obtaining sufficient oxygen from them. Moreover, another common approach is to compare gene expression between the symbiotic and aposymbiotic stages of the host. Transcriptomic comparisons of *P. bursaria* have revealed that life stage changes correlate with the expression of the heat shock 70 kDa protein, which may be related to the organism's ability to adapt to environmental changes, and glutathione S-transferase, an enzyme that protects against oxidative stress (Veal, Toone et al. 2002, Kodama, Suzuki et al. 2014). Additionally, transcriptomic comparisons of life stage changes in two ongoing and relatively stable *Hydra* species have shown that antioxidative-related genes are dramatically upregulated in the relatively stable symbiotic *Hydra* species. This indicates that the reactive oxygen species (ROS) scavenging ability plays an important role in establishing a mature photo-endosymbiotic relationship (Ishikawa, Yuyama et al. 2016).

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Currently, numerous studies have extensively reported on the orientation of the symbiotic host. In contrast, information regarding the perspectives of algal endosymbionts is relatively scarce. In the coral–dinoflagellate system, a comparison of gene expression in the algal endosymbiont *Breviolum minutum* (Symbiodiniaceae) between the endosymbiotic and free-living stages revealed that transporter-related genes are predominantly upregulated (Maor–Landaw, van Oppen et al. 2020). This suggests that these candidate transporters may play a crucial role in nutrient exchange between the host and the algal endosymbionts. Furthermore, cell surface carbohydrates of Symbiodiniaceae have been identified as essential factors for the specific recognition of the host (Tivey, Parkinson et al. 2020, Tortorelli, Rautengarten et al. 2022). However, the molecular or physiological basis for algal endosymbionts to establish endosymbiosis in *P. bursaria* remains unclear. Physiological and morphological changes in algae have been observed between the endosymbiotic and free-living life stages in *P. bursaria*. The cell wall thickness of endosymbiotic algae is thinner than that of free-living algae, with glycosaminoglycans potentially contributing to these differences in cell wall composition (Higuchi, Song et al. 2018). Moreover, the cell and chloroplast volumes of endosymbiotic algae are significantly larger than those of their free-living counterparts. Additionally, carbon fixation ability is higher in isolated endosymbiotic algae, as evidenced by pyrenoid observations and carbon fixation quantification (Catacora Grundy, Chevalier et al. 2023).

To test whether the changes in endosymbiotic and free-living algae affect their ability to establish symbiosis, we isolated fresh endosymbiotic algae, *Chlorella variabilis* NIES 2540, from symbiotic *P. bursaria*. We then compared the establishment efficiency with our long-term free-living *C. variabilis* cultures using a reinfection assay. Our findings revealed that the freshly isolated endosymbiotic algae had significantly better establishment efficiency than the long-term free-living algae. Therefore, the

objectives of this study are to understand the factors in green algae involved in the establishment of endosymbiosis and to identify the environmental conditions that induce these factors. This extensive study aims to provide greater insight into the establishment of photo-endosymbiotic relationships.

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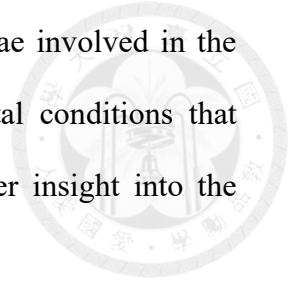
2. Materials and Methods

2.1 *Paramecium bursaria* DK2, *Chlorella variabilis* NIES 2540, and culture conditions

P. bursaria DK2 undergoes sexual reproduction derived from the Japanese strains Dd1 and Km2. It is nourished with *Klebsiella pneumoniae* (NBRC 100048 strain) as its food source and cultivated in Lettuce medium, comprising Boston lettuce juice and modified Dryl's solution. Feeding occurs every 3-4 days. Furthermore, aposymbiotic *P. bursaria* is derived from symbiotic *P. bursaria* treated with Paraquat, which disrupts the electron flow of photosystem I, maintained for one week. Conversely, *C. variabilis* NIES 2540 was isolated from another Japanese *P. bursaria* strain and has been cultured in CA cultural medium for the past 50 years. Symbiotic *P. bursaria* hosting *C. variabilis* NIES 2540 is produced through reinfection of aposymbiotic *P. bursaria* and long-term free-living *C. variabilis* NIES 2540, with maintenance durations of 4 months. Regarding cultural conditions, aposymbiotic *P. bursaria* is maintained on the bench, while symbiotic *P. bursaria* and long-term free-living *C. variabilis* NIES 2540 are housed in a light incubator following a 12-hour light/12-hour dark cycle. All environments are maintained at 23°C.

2.2 Re-infection assay

To prepare both the white-cell and symbiotics of *P. bursaria*, start by filtering out biofilm and debris from the culture medium using tissue paper. Then, wash away bacteria with 1X modified Dryl's solution three times, using an 11 μm nylon membrane, followed by concentrating *P. bursaria* in a 2 mL microcentrifuge tube.

For freshly isolated endosymbiotic *C. variabilis*, utilize a high-speed centrifuge set at $21,000 \times g$ for 10 minutes to lyse the symbiotic *P. bursaria*. Subsequently, wash

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with 1X phosphate buffered saline (PBS) buffer and centrifuge at 5,000 rpm for 1 minute and 30 seconds, followed by 20 seconds, 15 seconds, and 10 seconds to remove *P. bursaria* debris and bacteria. Regarding the long-term free-living *C. variabilis*, harvest the algal cells during the exponential phase. Algal cells can be harvested using a centrifuge at 3,000 rpm for 5 minutes to remove the culture medium.

Following this, mix the aposymbiotic *P. bursaria* and the algal cells at fixed cell densities of 5x10^3 and 5x10^7 respectively for 1 hour. Then, wash out the algal cells from outside of *P. bursaria*. Follow the aforementioned conditions to culture the reinfected *P. bursaria*.

2.3 Microscopic observation for reinfected *P. bursaria*

After reinfection, observe the number of algal cells inside *P. bursaria* on days 2 and 6. Two methods were employed for calculation. First, for computational analysis, reinfected *P. bursaria* samples were obtained from the culture, fixed with 8% formaldehyde, and then prepared on slides sealed with nail polish. Subsequently, images of *P. bursaria* and the algal cells were captured using an ZEISS Axioscan 7 fluorescent microscope. Algal cells were detected using two channels: A488 (excitation: 499 nm/emission: 520 nm) and A680 (excitation: 530 nm/emission: 680 nm) respectively. Next, computational calculation for the number of algal cells inside *P. bursaria* was performed using CellProfiler v4.2.6 (https://cellprofiler.org/). A total of 90 *P. bursaria* cells were sampled for this analysis.

To obtain the actual number of algal cells in time-course reinfection experiment, 50 *P. bursaria* were manually broken using a cover slide placed on a slide. The autofluorescence of the algal cells in each *P. bursaria* was detected using the Cy5 channel (excitation: 530 nm/emission: 680 nm) with a Zeiss AxioImager Z1 fluorescent microscope. Subsequently, the number of algal cells was counted using ImageJ v 13.0.6

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(https://imagej.net/ij/index.html).

2.4 Time-course short-term free-living *C. variabilis* culture and collection

To culture the freshly isolated endosymbiotic *C. variabilis* outside of the host, begin by lysing the symbiotic *P. bursaria* following the procedures outlined above. Next, resuspend the freshly isolated endosymbionts in CA medium supplemented with 100 ug/mL Ampicillin (CA+AMP medium) at a concentration of $3x10^6$ algal cells/ mL. Subsequently, filter the short-term free-living culture using a 60 μm nylon membrane to remove debris and contaminants. Concentrate the filtered culture using a centrifuge at 3,000 rpm for 5 minutes, followed by washing with CA+AMP medium using a centrifuge at 5,000 rpm for 1 minute, repeated three times. Finally, resuspend the culture in fresh CA+AMP medium every two days until day 6. For the time-course short-term free-living *C. variabilis* collection, adhere to the same procedures outlined for collecting the long-term one, as described in the "Reinfection assay" section. In addition to collecting the freshly isolated endosymbionts on day 0, collect the short-term free-living samples on days 1, 2, 4, and 6.

2.5 *C. variabilis* genome assembly and annotation

For the assembly of the reference genome, Illumina HiSeq 4000 paired-end sequencing and MinION sequencing read data of the *C. variabilis* NC64A (ATCC 50528) genome were obtained from NCBI BioProject PRJDB7392 (Minei, Hoshina et al. 2018). The quality of the short and long read data was evaluated using FastQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and NanoQC v0.9.4 (De Coster, D'hert et al. 2018), respectively. Low-quality reads were trimmed using Trimmomatic v0.39 (Bolger and Giorgi 2014) for short reads and NanoFilt v2.8.0 (De Coster, D'hert et al. 2018) for long reads. Subsequently, a hybrid assembly of the

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C. variabilis genome was performed using MaSuRCA v4.1.0 (Zimin, Marçais et al. 2013) with default parameters. The assembly quality was assessed using Quast v5.2.0 (Gurevich, Saveliev et al. 2013) by comparing the indices with previous reports. The genome assembly was further improved using Redundans v2.0.1 (Pryszcz and Gabaldón 2016) under the scaffolding with long reads condition. The assembled genome, containing 260 contigs, has a total size of 44.2 Mb and a GC content of 67.1%. The N50 value of the genome is 296,995 bp. The mapping rate of RNA raw reads aligned with the assembled genome is around 88% using HISAT2 v2.2.1 (Kim, Paggi et al. 2019). Additionally, the gene structure of the genome was predicted using Braker3 v3.0.8 (Gabriel, Brůna et al. 2024) , resulting in 12,785 predicted genes. The completeness of the gene predictions was evaluated using BUSCO v 5.7.1 (Manni, Berkeley et al. 2021) with the Eukaryota odb10 dataset (255 single-copy orthologs), with approximately 90.58% of the complete genes matching the BUSCO profile. Furthermore, 7,615 genes were functionally annotated with GO terms using InterProScan v5.67-99.0 (Jones, Binns et al. 2014). Thus, this draft genome was used as the reference for subsequent analyses.

2.6 RNA isolation and sequencing

To prepare the algal samples for RNA isolation, 3×10^8 algal cells from freshly isolated endosymbiotic algae (D0), short-term (D1, D2, D4, and D6), and long-term (FL) free-living algal cultures were collected according to the procedures for time-course re-infection assays. The algal cells were washed twice with 1X PBS buffer and resuspended in nuclease-free water to a total volume of 200 µL. All algal samples were processed in triplicate. For RNA isolation, 0.8 mL Tri reagent and 0.5 mL 0.01 mm glass beads were added to each sample, and the algal cells were homogenized at room temperature for 80 seconds using a bead beater, followed by immediate cooling on ice.

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The supernatants of the algal samples were transferred and centrifuged at 12,000 x g for 10 minutes at 4°C to eliminate algal debris and polysaccharides. Subsequently, the supernatants were transferred again, mixed with 50 µL of 0.5 M potassium acetate, incubated for 5 minutes at room temperature, and then 300 µL of chloroform was added and mixed vigorously using a vortex mixer for 3 minutes at room temperature. The samples were centrifuged at 21,000 x g for 20 minutes at 4°C to separate the phases, and the aqueous phase was carefully transferred into 0.6 mL of ice-cold 100% ethanol (EtOH). For the subsequent steps, the RNA was cleaned and collected using the RNeasy Mini Kit (Qiagen).

After evaluating the RNA quality of all samples, library preparation and paired-end read sequencing of total RNA for the algal samples were performed using the SureSelect Strand-Specific RNA Library and NovaSeq X Plus. For each sample, 20 million reads were generated.

2.7 Transcriptome analysis

The quality of raw reads from all algal samples was evaluated using FastQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adapter contamination and low-quality reads were trimmed using Trimmomatic v0.39 (Bolger and Giorgi 2014). Filtered reads were quantified for gene expression in the different algal samples using Salmon v0.14.1 (Patro, Duggal et al. 2017), and the mean TPM (Transcripts Per Million) value for each gene was calculated from triplicates for each algal sample condition. To identify potential gene expression changes correlated with the establishment ability of endosymbiosis, we performed Pearson correlation analyses between the mean TPM values for each gene and the median algal cell numbers at two or six days post-reinfection from the time-course re-infection assays. The correlation values were classified into two groups: positive and negative. Candidate genes were

selected based on correlation values greater than |0.9| and |0.95| within each group at two or six days post-reinfection. These candidate genes were then subjected to Gene Ontology (GO) enrichment analysis (Ashburner, Ball et al. 2000) to identify enriched molecular functions or biological processes. Heatmaps and enrichment analyses were generated using the ggplot2 v3.5.1 (https://ggplot2.tidyverse.org/) and clusterProfiler v4.12.0 (Wu, Hu et al. 2021) packages in R v4.4.1.

2.8 Identification of physiological factors

2.8.1 Measurement of cell size and chlorophyll fluorescence

To compare the cell size, cell structure, and chlorophyll fluorescence of different states or treatments of *C. variabilis*, 1×10^7 algal cells were harvested and resuspended in 1 mL of 1X PBS buffer. The signals of forward scatter (FSC), side scatter (SSC), and chlorophyll fluorescence were measured using a BD FACSymphony™ A1 Cell Analyzer. The signals from 30,000 algal cells were recorded for each condition. Cells without chlorophyll fluorescence were gated out to eliminate machine noise and debris from the culture. The signals from 5,000 algal cells were sampled for display in the results.

2.8.2 Transmission electron microscopy (TEM)

The freshly isolated endosymbiotic algae and long-term free-living algae, resuspended in 500 µL of 1X modified Dryl's solution, were chemically fixed by immersing them in 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M sodium cacodylate buffer. This process involved 500 W microwave heating for 1 minute, repeated eight times, and then the samples were kept at 4°C overnight. Following this, the samples were rinsed with 0.1 M sodium cacodylate buffer. The algal samples were then post-fixed in 1% osmium tetroxide in cacodylate buffer with 450 W microwave heating for 1 minute, repeated eight times, and rinsed with deionized water.

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Dehydration was performed through a graded ethanol series, followed by infiltration with resin. The samples were embedded in resin and polymerized at 60°C for 24-48 hours. Ultrathin sections (80 nm) were obtained using an ultramicrotome, stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (TEM).

2.8.3 Measurement of calcofluor white (CFW) intensity

To compare the cell wall composition of different states of *C. variabilis*, 1×10^7 algal cells were harvested and stained with 1 µg/mL Calcofluor White Stain in a total volume of 100 µL for 30 minutes in the dark. Subsequently, the stained algal cells were washed with 1X modified Dryl's solution using centrifugation at 5,000 rpm for 1 minute, repeated three times. The signals of forward scatter (FSC) and Calcofluor White (CFW) intensity for the stained algal cells were detected using a BD FACSymphony™ A1 Cell Analyzer, with the following analyses conducted as previously described. The normalized CFW intensity was calculated by dividing the CFW intensity signal by the FSC value for each algal cell.

2.8.4 Secretion: supernatant collection and process

Supernatants from the freshly isolated endosymbiotic algae and the long-term free-living algal cultures were prepared by harvesting 2×10^7 algal cells and resuspending them in 2 mL of 1X modified Dryl's solution for 24 hours. After the 24-hour secretion period, the supernatants from the algal cells at the two life stages were collected by centrifugation at 13,000 rpm for 10 minutes. For the re-infection assays, the original suspension of 1X modified Dryl's solution used for *P. bursaria* and the algal cells was replaced with the supernatants obtained from the two conditions.

2.8.5 Measurement of chlorophyll content

To quantify the chlorophyll content of the different algal states, 1×10^7 algal cells were harvested for each condition using centrifugation at 3,000 rpm for 5 minutes to

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remove the supernatant. This process was repeated for three replicates in each condition. Subsequently, 1 mL of methanol was added, and the algal samples were heated at 65°C for 10 minutes to efficiently extract the chlorophyll. The bleached algal cells were then removed by centrifugation at 13,000 rpm for 10 minutes. The supernatant was collected for chlorophyll measurements using spectrophotometry, with wavelengths set at A470, A652.4, and A665.2 nm. The values for chlorophyll a, chlorophyll b, and carotene were calculated using the following equations(Sumanta, Haque et al. 2014):

$$\text{Chlorophyll a (Ca)} = 16.72*A665.2 - 9.16*A652.4$$

$$\text{Chlorophyll b (Cb)} = 34.09*A652.4 - 15.28*A665.2$$

$$\text{Carotene} = (1000*A470 - 1.63*Ca - 104.96*Cb)/221$$

2.8.6   Measurement of photosystem II efficiency

To measure the efficiency of photosystem II (PSII), 7.5×10^6 algal cells were harvested from isolated endosymbiotic algae and long-term free-living algae and resuspended in 1 mL of 1X modified Dryl's solution in a 24-well plate. This process was repeated for five replicates under each condition. The algal samples were pre-incubated in the dark for 1 hour, and the efficiency of PSII between the two life stages was measured using a PAM instrument. Subsequently, the value of Fv/Fm, which indicates the quantum efficiency of open PSII centers as an indicator of PSII efficiency, was calculated for each algal sample using the software ImagingWin v2.41a. (https://www.walz.com/products/chl_p700/imaging-pam_ms/downloads.html).

2.8.7   Adjustment of light conditions and DCMU pretreatment for re-infection assays

To verify whether the ability to establish endosymbiosis is affected by the light source within 24 hours after re-infection, we incubated the reinfected *P. bursaria*, which had engulfed freshly isolated endosymbiotic algae for 1 hour, under both 12-hour light/12-hour dark and 24-hour dark conditions for 24 hours. Following this period, the

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reinfected *P. bursaria* initially incubated in the dark environment were transferred back to the normal 12-hour light/12-hour dark environment for subsequent observations.

For the pretreatment with DCMU of algal cells from two life stages, 2×10^7 algal cells of each condition were harvested and resuspended in 1X modified Dryl's solution. The cells were then pretreated with final concentrations of DCMU at 10^{-3}, 10^{-4}, and 10^{-5} M in a total volume of 2 mL for 24 hours. After pretreatment, the algal cells were washed with 1X modified Dryl's solution using centrifugation at 5,000 rpm for 1 minute, repeated three times before re-infection.

2.9 Environmental condition reinfection

2.9.1 Pretreatment of pH

To pretreat the long-term free-living algal cultures under different pH conditions, CA medium containing 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer was adjusted to pH 5, 6, or 7 using 5N NaOH. The long-term free-living algal cultures were harvested and resuspended at concentrations of 3×10^6 and 1×10^6 algal cells/mL for two and seven days of pre-incubation, respectively. After incubation, the pretreated algal cells were washed with 1X modified Dryl's solution using centrifugation at 5,000 rpm for 1 minute, repeated three times before re-infection.

2.9.2 Pretreatment of nitrogen source pretreatment

The different nitrogenous conditions for the pretreatment of the long-term free-living algal cultures included glutamine-supplemented and nitrogen-reduced media. For the preparation of the conditional medium, 10 mM glutamine was added to the standard CA+AMP medium, and the final concentration of 0.6 mM NH_4NO_3 in the CA+AMP medium was reduced to 0.3 mM for the nitrogen-reduced condition. The long-term free-living algal cultures were pre-cultured in these two nitrogenous conditions for 7 days before re-infection.

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2.9.3　　Host extracts collection and process
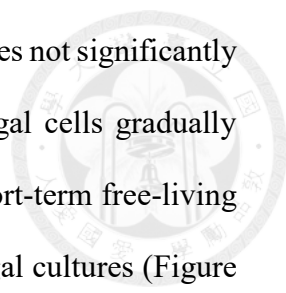
To obtain extracts from symbiotic *P. bursaria*, we first washed and concentrated 2 mL of the symbiotic *P. bursaria* in 1X modified Dryl's solution. The cell density of the symbiotic *P. bursaria* was then estimated by counting. Subsequently, the concentrated *P. bursaria* cells were filtered using a 0.2 μm filter to eliminate the algal endosymbionts and host debris. Following the estimation of cell density, the extracts suspended in 1X modified Dryl's solution were diluted to concentrations of 5×10^3 and 1×10^4 symbiotic *P. bursaria* cells/mL in CA+AMP medium. The long-term free-living algal cultures were harvested and resuspended to a concentration of 3×10^6 algal cells/mL in different treatment concentrations for 3 days before re-infection.

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3. Results

3.1 Enhanced Endosymbiosis Establishment by Fresh Algal Endosymbionts Compared to Reduced Efficiency in Short- and Long-Term Free-Living Algae

To examine the establishment ability of algae in different life stages (Figure 1), endosymbiotic *C. variabilis* isolated from symbiotic *P. bursaria* and long-term free-living algal cultures were respectively re-infected into aposymbiotic *P. bursaria*. The results showed that algae from different conditions were engulfed in similar numbers into the *P. bursaria* cytoplasm an hour post-reinfection, indicating the number of algal cells engulfed by *P. bursaria* within this time frame. Additionally, the number of algal cells inside the host was measured after reinfection, with observations recorded after two- and six- day. These time points indicate the number of algal cells that were not digested and had initiated the endosymbiotic relationship. After two days of digestion, the endosymbiotic *C. variabilis* (Figure 2: D0) was significantly more maintained inside the host compared to the long-term free-living algae (Figure 2: FL). This implies that the endosymbiotic algae have a better ability to escape from or are more resistant to host digestion. After six days of establishment, the number of endosymbiotic algae per *P. bursaria* was much higher than that of the long-term free-living algae (Figure 2). Summarizing the six-day observation, the results reveal that the freshly isolated endosymbiotic *C. variabilis* exhibits significantly enhanced establishment efficiency. However, the changes in the endosymbiotic *C. variabilis* might result from either non-genetic or genetic influences as it co-evolves with the symbiotic *P. bursaria*.

To further confirm if the endosymbiotic algae can rearrange its behaviors and align their establishment ability with long-term free-living algae cultures (Figure 1), the endosymbiotic algae are isolated and cultured as short-term free-living algae cultures lasting 6 days in the same condition as long-term ones using the time-course re-

infection assays. It was observed that the engulfment of algal cells does not significantly differ among the various algal states. However, the number of algal cells gradually decreases from the endosymbiotic algae (Figure 2: D0) through short-term free-living cultures (Figure 2: D1, D2, D4 and D6) to long-term free-living algal cultures (Figure 2: FL). This decline was measured as the median number of algal cells per *P. bursaria* two days after re-infection. Furthermore, it was found that although the number of algal cells also shows a decreasing pattern six days after re-infection, the number of algal cells in six-day short-term free-living cultures is still significantly higher than that of long-term cultures (Figure 2). This implies that the behaviors of short-term algal cultures have not completely reverted to those of long-term cultures. The results revealed that the causal influences in the freshly isolated endosymbiotic algae might be due to non-genetic changes in the host environment. This is suggested by the fact that the establishment ability of the short-term algal cultures can nearly align back to that of long-term cultures when the endosymbiotic algae are cultured outside of the host.

3.2 Changes in Gene Expression Associated with Establishment Ability Involve Various Molecular Functions and Biological Processes

To identify potential gene expression changes associated with establishment ability, we conducted transcriptomic analyses on different algal states under conditions identical to those in the time-course re-infection assays (Figure 1). The mean transcript per million (TMP) of each gene from the time-course transcriptomic data was correlated with the median values of algal cells from observations at two- and six- day post-reinfection in time-course re-infection assays, which we defined as the establishment ability of endosymbiosis (Figure 2). The results of the correlation distribution indicated that the changes in gene expression differed between the observations at two- and six-day post-reinfection among the algal states. At two days post-reinfection, 7.0% and

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1.9% of genes showed positive and negative correlations, respectively, with correlation values greater than 0.9. In contrast, at six days post-reinfection, 13.4% and 8.2% of genes exhibited positive and negative correlations, respectively, with correlation values greater than 0.9 (Figures 3B, 3D).

Additionally, genes with correlation values greater than |0.9| and |0.95| were selected for both positive and negative Pearson correlations with observations at two- and six- day post-reinfection. The candidate genes positively or negatively correlated with the establishment ability of endosymbiosis were further subjected to Gene Ontology (GO) enrichment analysis. Although these candidate genes were not statistically enriched in specific GO terms, we manually examined their cellular component (CC), molecular function (MF), and biological process (BP) based on a p-value threshold of <0.05 without adjustment in the GO enrichment analysis. Among the positively correlated genes at two- and six-day post-reinfection, gene expression was highest in freshly isolated endosymbiotic algae. These genes were associated with functions such as L-ascorbic acid binding, vesicle-mediated transport, and transporter activity, suggesting roles in reducing oxidative stress and enhancing substance transportation in freshly isolated endosymbiotic algae (Table 1). Conversely, the negatively correlated genes at two- and six-day post-reinfection showed the highest expression in long-term free-living algal cultures. These genes were linked to functions such as extracellular space and 2 iron-2 sulfur (2Fe-2S) cluster binding, indicating possible changes in cell wall synthesis and photosynthesis in long-term free-living algal cultures (Figure 3, Table 2).

In summary, the expression of genes correlated with establishment ability altered rapidly when freshly isolated endosymbiotic algae were removed from the host. These genes are involved in photosynthesis, secretion, and antioxidative functions, potentially influencing the establishment ability of endosymbiosis.

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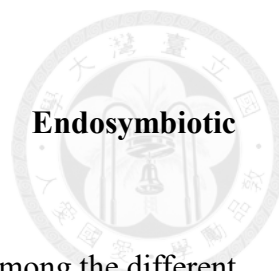
3.3 Characterization of Physiological Factors Affecting Endosymbiotic Establishment

To characterize the physiological and morphological changes among the different algal states and to identify the contributing factors for the establishment of endosymbiosis, several measurements were performed. These measurements included cell size, cell wall composition, secretion activity, and photosynthetic ability (Figure 1).

3.3.1 Algal Cell Size and Structure Alterations Without Correlation to Endosymbiotic Integration

The larger cell size observed in freshly isolated endosymbiotic algae compared to free-living algal cultures has been reported (Catacora Grundy, Chevalier et al. 2023). Therefore, it is important to monitor whether the cell size and structure undergo changes and restructure to align with those typical of long-term free-living forms when the endosymbiotic algae are outside of the host. The cell size and structure of the different algal states were measured using flow cytometry, with forward scatter (FSC) and side scatter (SSC) providing the respective values. FSC provides information about cell size, while SSC specifically offers insights into internal cellular complexity. Results indicate that the values of cell size and structure in freshly isolated endosymbiotic algae are higher than those in long-term free-living algal cultures, consistent with previous observations. However, the variations in cell size and structure among the different algal states are mild. The pattern of cell size and structure from freshly isolated endosymbiotic algae through short-term free-living algal cultures to long-term ones decreased one day after isolation and then increased four days after isolation (Figures 4A and 4B).

To further verify the association of cell size and cell structure with establishment ability, a Spearman correlation test and linear regression analysis were performed using the

median values from the flow cytometry analysis and the median number of algal cells from the time-course re-infection among the different algal states. The results showed a correlation value of 0.37 (p-value = 0.5) for cell size and 0.12 (p-value = 0.83) for cell structure from the Spearman correlation test, and R^2 value of 0.21 (p-value = 0.34) for cell size and 0.05 (p-value = 0.66) for cell structure from linear regression analysis (Figures 4C and 4D). These findings indicate that there is no significant association between establishment ability and either cell size or cell structure, despite the mild variations observed among the different algal states.

3.3.2 Changes in Cell Wall Composition and Their Lack of Correlation with Establishment in Algal Cultures

The thinner cell wall thickness and the lower intensity of Calcofluor White staining, which binds to several polysaccharides on the cell wall, have been observed in endosymbiotic algae compared to long-term free-living algal cultures (Higuchi, Song et al. 2018). Thus, it is worth characterizing whether the cell wall features in our study align with previous observations. The thickness of the cell wall between endosymbiotic algae and long-term free-living algal cultures was compared using TEM. The figures illustrated that the cell wall thickness of endosymbiotic algae is uneven in a complete whole cell compared to long-term free-living algal cultures (Figure 5). This implies that the cell wall structure of endosymbiotic algae might change in the host environment.

To analyze if the compositions of the cell wall are altered across different algal states and reorganize to correspond with the cell wall features of long-term free-living algal cultures, different algal states were stained with Calcofluor White (CFW) and detected using flow cytometry. This approach, following previous reports, aimed to simply identify changes in the polysaccharide composition of the cell wall. The results showed that the median values of CFW intensity normalized to cell size (FSC) are

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similar from freshly isolated endosymbiotic algae to four days post-isolation. However, the median value of normalized CFW intensity began to increase from the algal sample on the sixth day post-isolation to that of long-term free-living algal cultures (Figure 6A). This indicates that the cell wall features of endosymbiotic algae can realign with those of long-term free-living algal cultures a few days after being isolated from the host environment.

Moreover, a Spearman correlation test and linear regression analysis were conducted to investigate the association of cell wall composition with establishment ability, using the median values of normalized Calcofluor White (CFW) intensity from the flow cytometry analysis and the median number of algal cells from the time-course re-infection among different algal states. The statistical analysis showed correlation value of -0.14 (p-value = 0.8) and R^2 value of 0.33 (p-value = 0.23) for the normalized CFW intensity (Figure 6B). These results suggest that changes in cell wall composition and establishment ability have no significant association among the different algal states.

In summary, although changes in the cell wall structure and composition among different algal states were observed, the connection between cell wall features and establishment ability is not strong. Therefore, the perspective of the cell wall's role in establishment ability still requires further verification.

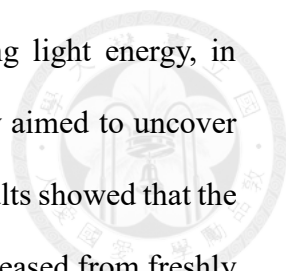### 3.3.3 The secretion of endosymbionts containing key factors for endosymbiotic establishment

To validate whether factors secreted by freshly isolated endosymbiotic algae can influence and improve the establishment efficiency of long-term free-living algal cultures, 24-hour supernatants from both endosymbiotic algae and long-term free-living algal cultures were collected for use in re-infection assays. The results revealed that

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when the re-infection samples (aposymbiotic *P. bursaria* and long-term free-living algae) were respectively resuspended in the supernatant of endosymbiotic algae (ES), long-term free-living algal cultures (FS), and the original Dryl's solution (D), the number of algal cells engulfed and maintained per *P. bursaria* did not differ among the treatments at 1 hour and 2 days after re-infection. However, the median number of algal cells per *P. bursaria* was highest in the ES treatment, although the difference between ES and FS was not statistically significant (Figure 7).

These findings suggest that substances from the 24-hour supernatants of both life stage algae can increase the establishment efficiency of long-term free-living algae compared to the original operation. Nevertheless, the supernatant of freshly isolated endosymbiotic algae potentially contains distinct factors that greatly enhance the establishment ability of the algae.

3.3.4   The capacity for photosynthesis is essential for the initiation of endosymbiosis

Photosynthesis is a crucial process for algae, providing photosynthates to the host and consisting of light reactions for photochemical energy transformation and the Calvin cycle for carbon fixation. It has been demonstrated that endosymbiotic algae exhibit a larger pyrenoid—a structure within the chloroplast crucial for carbon fixation—and a higher rate of carbon fixation compared to long-term free-living algal cultures (Catacora Grundy, Chevalier et al. 2023). Additionally, we observed a higher production of starch grains within the chloroplasts of freshly isolated endosymbiotic algae compared to long-term free-living algae (Figures 5 and 8), consistent with previous observations (Catacora Grundy, Chevalier et al. 2023). However, whether algae can reprogram their physiological status during the light reactions of photosynthesis remains unknown. Previous studies have demonstrated that algae can

adjust their chlorophyll content, which is important for harvesting light energy, in response to various environmental conditions. Therefore, this study aimed to uncover changes in chlorophyll content among different algal states. The results showed that the contents of chlorophyll a, chlorophyll b, and carotene gradually increased from freshly isolated algae to algal samples on the second day post-isolation. Notably, chlorophyll contents significantly increased on the four- and six-day post-isolation, approaching those of long-term free-living algal cultures (Figure 9A). This suggests that when fresh endosymbiotic algae are released from the host environment, short-term free-living algal cultures adjust their chlorophyll features to resemble those of long-term cultures.

To further investigate the relationship between chlorophyll content and establishment ability, a Spearman correlation test and linear regression analysis were performed to investigate the mean values of each chlorophyll content with the median number of algal cells over time during re-infection across different algal states. The results indicated correlation coefficients of -0.66 (p-value = 0.18), -0.77 (p-value = 0.1), and -0.54 (p-value = 0.3) and R^2 values of 0.55 (p-value = 0.09), 0.57 (p-value = 0.08), and 0.49 (p-value = 0.11) for chlorophyll a, chlorophyll b, and carotene with establishment ability, respectively (Figure 9B). These findings suggest a weak negative association between chlorophyll content and the establishment ability of endosymbiosis.

Furthermore, we examined the efficiency of photosystem II (PSII) between freshly isolated endosymbiotic algae and long-term free-living algae. The results showed that the efficiency of PSII was lower in freshly isolated endosymbiotic algae. Taken together, these findings suggest that freshly isolated endosymbiotic algae may need to balance chlorophyll content and PSII efficiency within the host environment to mitigate oxidative stress during these processes.

Following the above observations, it was essential to examine whether the adjustment of photosynthetic ability affects the establishment of endosymbiosis. To

investigate if establishment ability is influenced by the light reactions in photosynthesis, the light source was completely eliminated during the initial establishment of endosymbiosis. One hour after re-infection, the reinfected *P. bursaria* with freshly isolated endosymbiotic algae were incubated under both 12-hour light/12-hour dark and 24-hour dark conditions for 24 hours. The initial engulfment of algal cells was higher under 24-hour dark conditions. However, the number of algal cells maintained in *P. bursaria* two- and six- day after re-infection was higher under 12-hour light/12-hour dark conditions (Figure 11). This indicates that light is crucial in the photosynthetic process for algae, preventing digestion by the host.

Subsequently, to verify if the inhibition of PSII efficiency, a partial light reaction, affects establishment ability, freshly isolated endosymbiotic algae and long-term free-living algal cultures were treated with different concentrations of DCMU, an inhibitor of electron flow in photosystem II, for 24 hours. Chlorophyll fluorescence, which is correlated with chlorophyll content, was measured for different algal samples using flow cytometry. Chlorophyll fluorescence was lower in 10^{-3} M DCMU treatment for both conditions (Figure 12). Re-infection results showed that the average median number of algal cells engulfed was around 26-46 cells per host one hour after re-infection across all conditions. The effect of photosynthesis inhibition was significant only in freshly isolated endosymbiotic algae treated with 10^{-3} M DCMU. The number of algal cells was significantly lower than in other treatments two days after re-infection, with no observable changes among other treatments or in long-term free-living conditions. However, in both freshly isolated endosymbiotic algae and long-term free-living algal cultures, the median number of maintained algal cells increased from no treatment through 10^{-5} M to 10^{-4} M DCMU six days post-infection. Both conditions treated with 10^{-3} M DCMU showed a sudden decrease compared to 10^{-4} M DCMU (Figure 13). Comparing the data sets, the effects of 10^{-3} M DCMU caused decreased

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chlorophyll fluorescence and establishment efficiency of endosymbiosis, suggesting that reduced photosynthetic ability affects establishment ability. The unexpected unchanged pattern among the control, 10^{-5} M, and 10^{-4} M DCMU treatments of algal samples may be due to essential pathways being induced or inhibited by DCMU, maintaining or enhancing establishment efficiency.
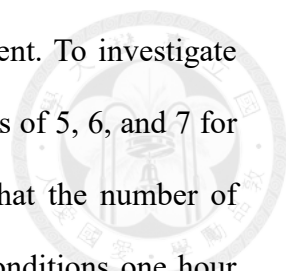
In summary, photosynthetic ability is critical for the establishment of endosymbiosis. Therefore, differences in the photosynthetic process between freshly isolated endosymbiotic algae and long-term free-living algal cultures might potentially influence the establishment efficiency of endosymbiosis.

3.4 Identification of Environmental Conditions Inducing Factors for Endosymbiotic Establishment

The freshly isolated endosymbiotic algae maintained their endosymbiotic relationship with *P. bursaria* for four months, suggesting that the factors contributing to their enhanced establishment ability may be induced by the host environment. To identify the potential host environmental conditions that induce these factors for efficient establishment of endosymbiosis, we examined the effects of pH, nitrogen source, and host extract (Figure 1). Long-term free-living algal cultures were pretreated with these conditions before undergoing re-infection assays to determine if the factors could be induced in long-term free-living algal cultures and enhance their establishment ability in endosymbiosis.

3.4.1 Possibly Minor Influence of pH on Inducing Factors in Fresh Endosymbionts

The presence of algal endosymbionts in an acidic environment within the host has been reported in various photosymbioses (Reisser 1981, Venn, Tambutte et al. 2009, Barott, Venn et al. 2015). Therefore, it is possible that the critical features of freshly

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isolated endosymbiotic algae are triggered by a low pH environment. To investigate this, long-term free-living algal cultures were pretreated at pH levels of 5, 6, and 7 for 2 and 7 days. The results of the two-day pretreatment revealed that the number of engulfed algal cells did not differ significantly among the three conditions one hour after re-infection, with a slightly higher number observed in the pH 7 condition two days after re-infection. By the sixth day, the highest number of algal cells was found in the pH 6 condition compared to the other two conditions, which showed similar results (Figure 14A). Additionally, the seven-day pretreatment at pH 5 was too severe to maintain the normal physiology and morphology of the long-term free-living algal cultures, resulting in poorly maintained algal cells in *P. bursaria* throughout the re-infection assay (Figure 14B).

In summary, the results suggest that a low pH environment may not be the sole factor responsible for the feature changes of algal endosymbionts that enhance their establishment ability.

3.4.2 Possibly Minor Influence of Nitrogen Source on Activation Factors in Fresh Endosymbionts

Nitrogen plays a crucial role in algal metabolism. It has been reported that the cell number of algal endosymbionts is controlled by nitrogen limitation within the host in the cnidarian-dinoflagellate symbiosis (Xiang, Lehnert et al. 2020, Cui, Liew et al. 2022, Cui, Mi et al. 2023). Additionally, a comparative genomic study has verified that the nitrogen source provided by *P. bursaria* to its algal endosymbionts might be glutamine, facilitating the endosymbiotic relationship(He, Wang et al. 2019). To determine whether the nitrogen control strategy is similar in *P. bursaria* and *C. variabilis*, and whether the feature changes of freshly isolated endosymbiotic algae are triggered in a glutamine-supplemented environment, long-term free-living algal cultures were pre-cultured in

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nitrogen-reduced and glutamine-supplemented CA medium for 7 days before re-infection assays.
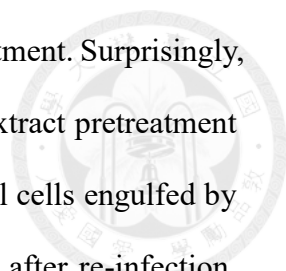
The results showed that the median number of algal cells were around 36-51 cells per host among the three conditions one hour after re-infection. After two- and six- day of observations, there was no significant difference in the number of algal cells in the glutamine-supplemented condition compared to the control condition, while the algal cells pre-cultured in the nitrogen-reduced condition exhibited the lowest establishment efficiency (Figure 15). These findings suggest that long-term free-living algal cultures pre-cultured under modified nitrogen conditions did not show significant improvements compared to the control group.

This indicates that these two nitrogenous adjustments might not be the primary environmental factors required to activate the establishment ability, or the most similar nitrogenous condition of the host environment was not accurately mimicked. Further fine-tuning of the experimental conditions is needed.

3.4.3 Exploring the Role of Symbiotic *P. bursaria* Factors in Inducing Physiological Changes for Endosymbiosis Establishment

The co-cultivation of algal cells with extracts from *P. bursaria* hosts has been reported to enhance carbon fixation efficiency (KAMAKO and Imamura 2006). We hypothesized that the phenotypic changes in the algal endosymbionts, which contribute to their improved establishment ability, may be stimulated by specific factors from the *P. bursaria* host. To investigate this, long-term free-living algal cultures were pretreated with two different concentrations of extracts filtered from symbiotic *P. bursaria* for three days prior to the re-infection assay. The concentrations used were 5×10^3 and 1×10^4 symbiotic *P. bursaria* cells/ mL.

After three days of pretreatment, the growth rate of the algal cells pretreated with

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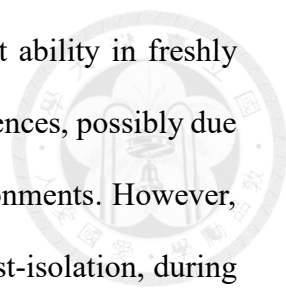
the host extracts was faster than that of the algal cells without pretreatment. Surprisingly, however, the chlorophyll fluorescence decreased under both host-extract pretreatment conditions (Figure 16). In the re-infection assay, the number of algal cells engulfed by each *P. bursaria* was approximately 19-30 cells per host one hour after re-infection, with no observable differences in the number of maintained algal cells between treatments two days after re-infection. Nevertheless, after six days, the algal cells pretreated with the symbiotic host extracts exhibited a significantly higher establishment efficiency compared to the control group without pretreatment (Figure 17). These findings indicate that although the chlorophyll fluorescence of the algal cells decreased under host-extract pretreatment, the establishment efficiency increased. Therefore, it is suggested that the decreased chlorophyll content or other functional changes in the freshly isolated endosymbiotic algae might be triggered by the host-specific extracts, enhancing the initial establishment ability of endosymbiosis.

4. Discussion

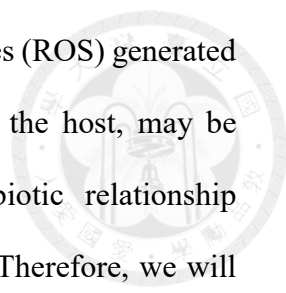The establishment of endosymbiosis is crucial for the evolution of eukaryotic cells, yet the underlying molecular basis of this process remains unclear. Despite this, endosymbiosis continues to occur throughout the tree of life. *Paramecium bursaria*, a ciliate species, establishes an endosymbiotic relationship with green algae in freshwater environments. This obligate endosymbiotic relationship provides an excellent model for studying endosymbiosis using re-infection assays. In this study, we found that algal endosymbionts *C. variabilis* isolated from symbiotic *P. bursaria* hosts demonstrated significantly higher establishment efficiency with aposymbiotic *P. bursaria* compared to long-term free-living *C. variabilis* (Figure 2), corroborating previous findings (Kodama and Fujishima 2016).

Genetic and non-genetic (epigenetic/physiological) changes in endosymbionts are prevalent across various endosymbiotic relationships. The genetic patterns of re-isolated endosymbionts differ from those of their initially introduced counterparts (Belda-Baillie, Sison et al. 1999, Bayliss, Scott et al. 2019, Stoy, Gibson et al. 2020). Additionally, non-genetic (epigenetic/physiological) changes in endosymbionts regulate gene expression (Asgari 2014). These changes occur in response to coordination with the host or variable environmental conditions, as reported in numerous endosymbiotic systems. To determine whether differences in algal endosymbionts result from non-genetic or genetic changes, we isolated and cultured endosymbiotic algal cells under conditions identical to long-term free-living algal cultures. This approach aimed to assess whether the establishment ability of endosymbiotic algae could be consistently maintained outside of the host environment. The results indicated a loss of establishment ability in endosymbiotic algae across short-term free-living cultures, aligning with the characteristics of long-term free-living algal

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cultures (Figure 2). This suggests that differences in establishment ability in freshly isolated endosymbiotic algae primarily stem from non-genetic influences, possibly due to adjustments in physiological status upon exposure to new environments. However, it should be noted that our observations were limited to six days post-isolation, during which the establishment ability remained higher than that of long-term free-living algal cultures. This suggests the possibility of minor genetic changes or a longer-term regulation of gene expression linked to the physiological status of long-term free-living algal cultures.

To further elucidate the causal factors influencing the establishment ability of endosymbiosis, we employed both non-targeted and targeted analyses. Initially, non-targeted analysis involved time-course transcriptomic profiling of different algal states, with subsequent Gene Ontology (GO) enrichment analysis of genes highly correlated with establishment ability (Figure 3). However, no GO terms were enriched among these genes, and this result was consistent when comparing differentially expressed genes between freshly isolated endosymbiotic algae (D0) and long-term free-living algal cultures (FL). Several hypotheses emerged from these findings. It is plausible that changes in gene expression in endosymbiotic algae are not concentrated within specific pathways but rather occur globally across various pathways. Additionally, given that only 7615 out of 12785 genes were functionally annotated in our reference genome, there may be biases in our transcriptomic analysis due to annotation limitations. Future efforts should therefore focus on enhancing functional annotations of our reference genome, potentially integrating information from well-studied plant or algal species. Despite these challenges, we manually investigated GO terms associated with highly correlated genes that passed a p-value <0.05 threshold without adjustment. We found that genes positively correlated with establishment ability were enriched in molecular functions such as L-ascorbic acid binding (Tóth 2023) (Table 1). This suggests that

antioxidative functions, crucial for mitigating reactive oxygen species (ROS) generated during photosynthesis and thereby preventing oxidative stress in the host, may be essential for establishing and maintaining the algal endosymbiotic relationship (Hörtnagl and Sommaruga 2007, Sommaruga and Sonntag 2009). Therefore, we will focus on quantifying ROS production among different algal states. Additionally, genes related to vesicle-mediated transport and transporter activity were highly expressed (Table 1), indicating their potential role in enhancing substance transportation, which is crucial for delivering or obtaining substances to/from the host. Conversely, negatively correlated genes were associated with cellular components such as the extracellular space and molecular functions like 2 iron-2 sulfur (2Fe-2S) cluster binding (Table 2). These associations suggest possible links between these genes and processes like cell wall synthesis or photosynthesis, which may affect the establishment ability of endosymbiosis. Moving forward, we plan to validate the association of these genes with establishment ability and identify additional potential causal genes using various analytical approaches and parameters applied to our time-course transcriptomic data.

Furthermore, we hypothesized several physiological measurements and re-infection assays for targeted analysis, including cell size, structure, cell wall composition, secretion, and photosynthetic ability. These parameters were assessed to examine changes among different algal states and their correlation with the establishment ability of endosymbiosis. The larger cell and plastid volumes of endosymbiotic algae compared to free-living algae have been observed in *Phaeocystis cordata* and *Micractinium condutrix* using the FIB-SEM technique (Uwizeye, Mars Brisbin et al. 2021, Catacora Grundy, Chevalier et al. 2023). We hypothesized that larger cell size in algae increases the likelihood of recognition and maintenance within *P. bursaria*, as beads larger than 3 µm are more easily budded out to form single beads from the digestive vacuole (Kodama and Fujishima 2012). Therefore, we measured the
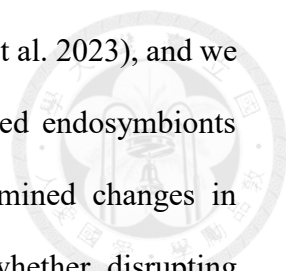
cell size and structure of different *C. variabilis* states using flow cytometry analysis. However, the results showed only mild increases in cell size and structure in freshly isolated endosymbiotic algae compared to short-term and long-term free-living cultures. Importantly, the correlation of cell size and structure with the establishment ability of endosymbiosis was found to be nonexistent (Figure 4). These findings suggest that while larger cell size may facilitate certain aspects of recognition and maintenance, it may not be a primary determinant of endosymbiotic establishment.

Previous studies have reported potential influences of thinner cell walls and changes in glycosaminoglycans on cell wall composition between life stages in *C. variabilis*(Higuchi, Song et al. 2018). Additionally, the role of glycans on the cell wall of Symbiodiniaceae in recognizing lectins from Cnidarians for establishing endosymbiotic relationships is well-documented (Tivey, Parkinson et al. 2020, Tortorelli, Rautengarten et al. 2022). Given these observations, we investigated whether changes in algal cell wall features contribute to differences in establishment efficiency using approaches from previous study. Our observations revealed that the thickness of freshly isolated endosymbiotic algae was not consistently thinner than that of long-term free-living algae, and exhibited uneven thickness within single algal cells (Figure 5). Possible reasons include ambiguities in TEM results due to chemical techniques affecting the distinction between cell membrane and cell wall, or incomplete acclimation of endosymbiotic algae maintained in the host environment for only four months, particularly in cell wall synthesis. Furthermore, we assessed cell wall composition in different algal states using CFW staining, which reacts with polysaccharides such as sialic acid, chitin, cellulose, and glycosaminoglycans. The results indicated an increase in CFW intensity six days post-isolation in both short-term and long-term free-living algal cultures. Although differences in CFW intensity between endosymbiotic algae and long-term free-living algae were consistent with

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previous findings, no association was found between changes in CFW intensity and establishment ability (Figure 6). Nevertheless, genes identified in our transcriptomic data potentially related to cell wall synthesis suggest further investigation into differences across algal states and their impacts on establishment ability in endosymbiosis. Future research may include specific digestion of cell walls for re-infection assays or further profiling of cell wall glycome to deepen our understanding.
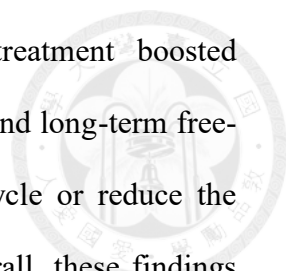
In other symbiotic systems, *Cladonia grayi* detects algal secretion of ribitol before and after cell-cell contact, facilitating lichen symbiosis (Joneson, Armaleo et al. 2011). Additionally, many bacterial species produce and secrete indole acetic acid (IAA) to promote algal growth and establish symbiotic interactions with algae (Dao, Wu et al. 2018). Conversely, pathogen-secreted effector proteins for host infection have been widely reported (Rovenich, Boshoven et al. 2014, Asai and Shirasu 2015, Cui, Tsuda et al. 2015). We hypothesized that freshly isolated endosymbiotic algae secrete specific factors or nutrients that *P. bursaria* can easily recognize, preventing the algae from digestion compared to long-term free-living cultures. To test this, we collected supernatants from endosymbiotic algae and long-term free-living cultures for re-infection assays. The results indicated that the supernatants from freshly isolated endosymbiotic algae potentially improved establishment efficiency (Figure 7). Our time-course transcriptomic data also identified highly correlated genes associated with the endoplasmic reticulum and vesicle-mediated transport (Table 1), suggesting that secretion factors from freshly isolated endosymbiotic algae may be crucial for establishing and maintaining the endosymbiotic relationship. Therefore, it is worth conducting a more detailed investigation into algal secretion using secretome analysis.

Photosynthesis is crucial for algal growth, and algal endosymbionts can provide photosynthetic products such as carbohydrates and oxygen for the host. Furthermore, increased carbon fixation efficiency and larger plastid and pyrenoid volumes have been
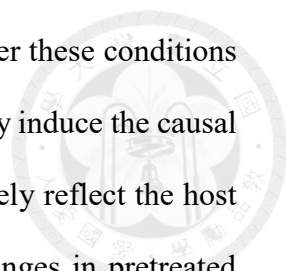
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demonstrated in endosymbiotic algae (Catacora Grundy, Chevalier et al. 2023), and we also observed higher production of starch grains in freshly isolated endosymbionts (Figure 8), consistent with previous studies. Therefore, we examined changes in chlorophyll content among different algal states and assessed whether disrupting photosynthesis affects establishment ability. The results showed increasing chlorophyll content from freshly isolated endosymbiotic algae through short-term to long-term free-living algal cultures, with a negative association between chlorophyll content and the establishment ability of endosymbiosis (Figure 9). Moreover, measurements of PSII efficiency revealed lower efficiency in freshly isolated endosymbiotic algae (Figure 10). Therefore, we hypothesized that high chlorophyll levels and PSII efficiency might induce oxidative stress in the host, leading to the reprogramming of the photosynthetic processes in the host environment. Correspondingly, endosymbiotic algae may increase carbon fixation efficiency to compensate for the reduction in chlorophyll content and PSII efficiency, as supported by TEM observations (Figure 5) and previous findings (Catacora Grundy, Chevalier et al. 2023). In rice (Oryza sativa L.), low chlorophyll content correlates with high electron transport rates and elevated Rubisco content for carboxylation (Gu, Zhou et al. 2017).

Moreover, previous studies have demonstrated that when endosymbiotic *C. variabilis* are pre-incubated in a 24-hour dark environment, they exhibit a reduced ability to prevent digestion by *P. bursaria* (Kodama and Fujishima 2014). In the symbiosis between Cnidarians and Symbiodiniaceae, photosynthetic mutants of *B. minutum* influence the efficiency of the initial establishment of endosymbiosis with the Cnidarian host (Jinkerson, Russo et al. 2022). To validate the influence of photosynthetic ability on the establishment in our system, we conducted re-infection assays under different light conditions and pretreated the algae with DCMU. The results revealed that light conditions and high concentrations of DCMU affected establishment

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efficiency (Figures 11 and 13). However, mild DCMU pretreatment boosted establishment ability in both freshly isolated endosymbiotic algae and long-term free-living cultures. This suggests that DCMU may arrest the cell cycle or reduce the efficiency of PSII, which enhances establishment efficiency. Overall, these findings indicate that the regulation of photosynthetic machinery is a crucial factor for the establishment and maintenance of endosymbiotic algae in *P. bursaria*. Future research should focus on the effects of high chlorophyll content and PSII efficiency on endosymbiotic relationships, and explore the changes in other photosynthetic processes that affect the establishment of endosymbiosis.

The physiological and gene expression changes in freshly isolated endosymbiotic algae might be activated by the host environment, enhancing the establishment ability of endosymbiosis. Algal endosymbionts residing in the acidic vacuole of the host have been observed in *P. bursaria* and other Cnidarian symbioses (Reisser 1981, Venn, Tambutte et al. 2009, Barott, Venn et al. 2015). When free-living *C. variabilis* is pretreated in a pH 5 condition, it secretes maltose, a carbon source it cannot utilize for its own growth, potentially providing it to the host (Shibata, Takahashi et al. 2016, Shibata, Takahashi et al. 2021). Moreover, the cell number of algal endosymbionts within the host is controlled by the limitation of nitrogenous nutrients from the host. Gene expression under nitrogen-depleted conditions in free-living algae is similar to that in the host environment, a phenomenon frequently observed in Cnidaria and Symbiodiniaceae (Xiang, Lehnert et al. 2020, Cui, Liew et al. 2022, Cui, Mi et al. 2023). For *P. bursaria* and *C. variabilis*, glutamine provided by *P. bursaria* is essential for maintaining a stable endosymbiotic relationship (He, Wang et al. 2019). To test this, we pretreated long-term free-living algal cultures with adjusted pH and nitrogenous conditions. However, the results showed no significant enhancement in the establishment ability of long-term free-living algal cultures (Figures 14 and 15).

Despite significant physiological changes in algae pre-cultured under these conditions compared to untreated algae, the mimic environments might not fully induce the causal factors of establishment ability, or the conditions might not accurately reflect the host environment. Therefore, monitoring whether the physiological changes in pretreated algal cells resemble those of freshly isolated endosymbiotic algae using the aforementioned approaches is necessary. Additionally, when free-living algae were treated with host extracts filtered from *P. bursaria*, they showed enhanced carbon fixation efficiency, as previously reported (KAMAKO and Imamura 2006). Accordingly, we pre-cultured long-term free-living algal cultures with host extracts. The results indicated that pre-incubation with host extracts improved the establishment ability of endosymbiosis (Figure 17), while chlorophyll fluorescence decreased compared to untreated algae (Figure 16). This connection was also observed in time-course re-infection assays and chlorophyll content characterization. Thus, chlorophyll content might be regulated by specific factors in the host environment and is an important causal factor in endosymbiotic establishment. Other potential physiological factors not identified and simulated by the host extracts might also play essential roles in the endosymbiotic relationship. Therefore, further investigation into the impacts of host extracts is required. We plan to fine-tune different conditions for host extracts to assess their influence on establishment ability and explore specific host factors of interest.

In this study, we investigated the non-genetic changes in endosymbiotic algae isolated from symbiotic *P. bursaria* and their role in enhancing the establishment ability of endosymbiosis. Using time-course transcriptomics, physiological measurements, and re-infection assays with various treatments for different algal states, we identified potential factors contributing to this establishment. Our findings suggest that cell wall synthesis, secreted substances, photosynthetic ability, and antioxidative functions in

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freshly isolated endosymbionts are complex causal factors for successful endosymbiosis, with some feature changes potentially induced by host-specific factors (Figure 18). This study provides a deeper understanding of the mechanisms underlying endosymbiotic establishment from the perspective of algal endosymbionts.

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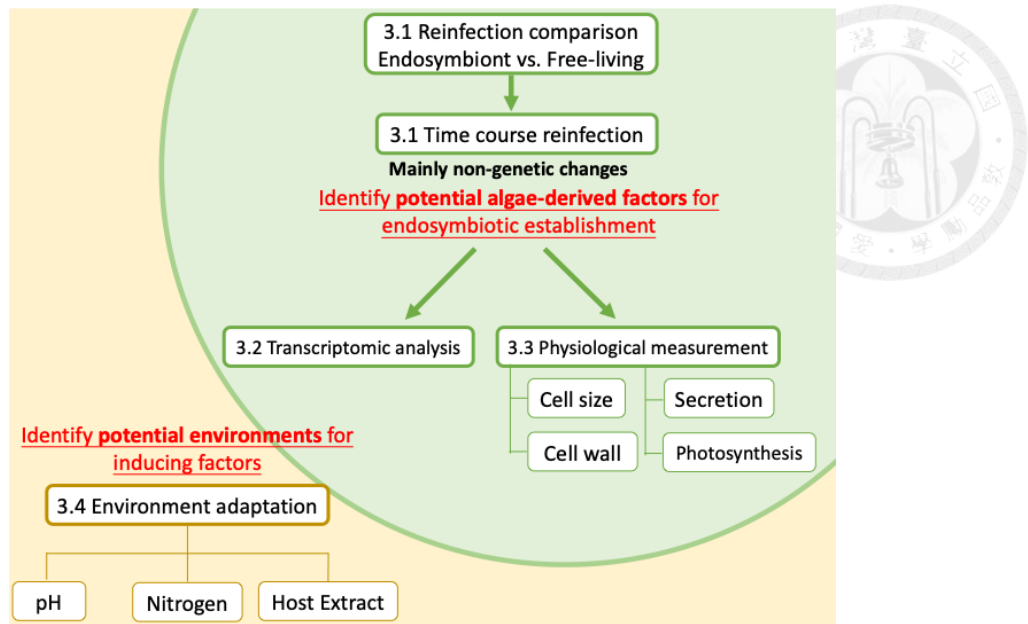
Figure 1. The work flow of the study.

At the beginning (3.1), we used a reinfection assay to verify that the establishment ability of freshly isolated algae is greater than that of long-term free-living algae. We further demonstrated that the main differences between these two algal life stages result from non-genetic changes using time-course reinfection assays (3.1). We observed that endosymbiotic algae isolated outside of the host as short-term free-living algae lose their ability to establish endosymbiosis over time. To identify potential algae-derived causal factors for the improved establishment ability, we performed time-course transcriptomic analysis (3.2) to identify gene expression changes correlated with the establishment ability of endosymbiosis among different algal states. Additionally, we conducted several physiological measurements and reinfection assays with treatments (3.3) to verify phenotypic changes among different algal states, such as cell size (3.3.1), cell wall (3.3.2), secretion (3.3.3), and photosynthesis (3.3.4). Moreover, we identified the environmental conditions (3.4) within the host environment that can induce algae-derived factors to boost the establishment ability of endosymbiosis, including pH (3.4.1), nitrogen (3.4.2), and host-derived extracts (3.4.3), using long-term free-living algae pretreated with different conditions for various reinfection assays.
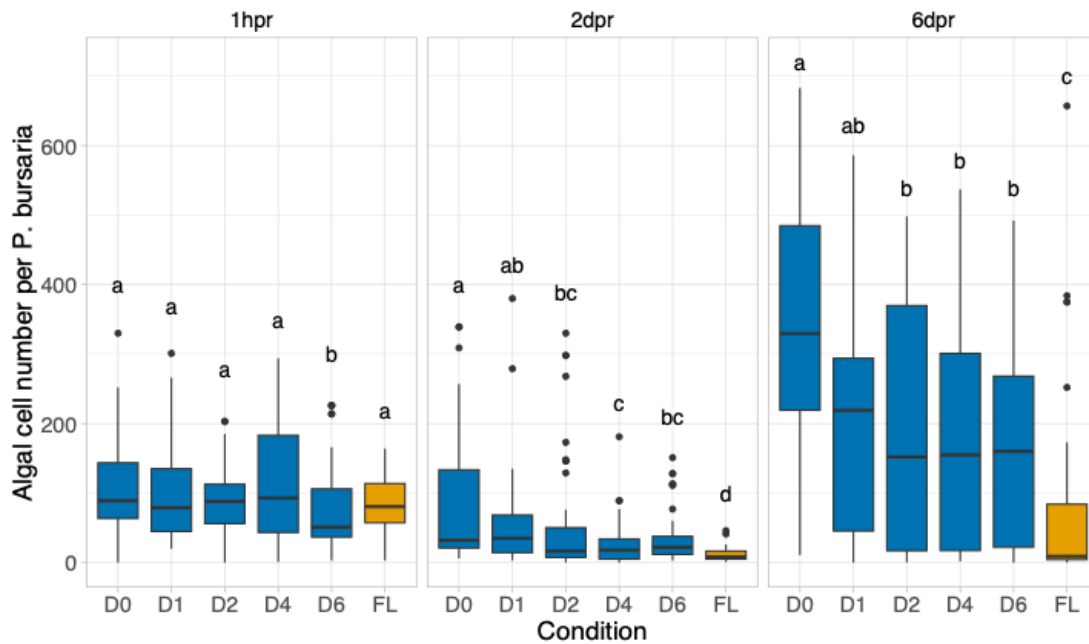
Figure 2. More efficient endosymbiosis establishment by the fresh algal endosymbionts compared to that in short-term and long-term free-living algae.

At the top of the figure, the time points indicate observations at 1-hour post-reinfection (1hpr), 2 days post-reinfection (2dpr), and 6 days post-reinfection (6dpr). At the bottom of the figure, the conditions for the time-course re-infection assay are shown, including freshly isolated endosymbiotic algae (D0), short-term free-living algal cultures post-isolation (D1, D2, D4, D6), and long-term free-living cultures (FL). The y-axis represents the number of algal cells per re-infected *P. bursaria*. Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (50 *P. bursaria* per sample).
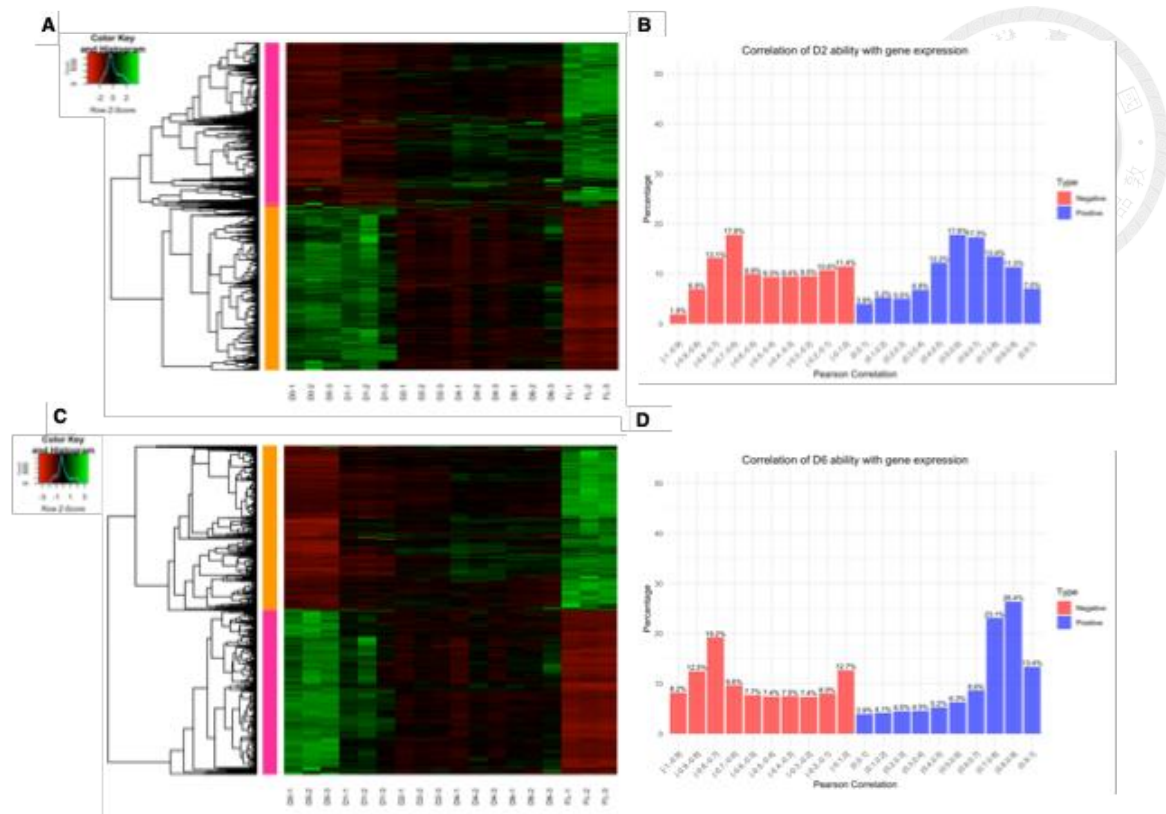
Figure 3. Correlation of gene expression changes with the establishment ability of endosymbiosis observed in time-course reinfection assays.

(A, C) Heat maps of the top 5% of genes highly correlated with positive and negative groups. (A) Represents data at two days post-reinfection, and (C) at six days post-reinfection. Columns represent algal states progressing from freshly isolated endosymbiotic algae through short-term to long-term free-living algal cultures. Rows depict hierarchical and K-means clustering of the top 5% selected genes, with colors indicating TPM scaled across all algal samples and generating row z-scores from high expression (red) to low expression (green). (B, D) Distribution of gene expression correlations with (B) observations at two days post-reinfection and (D) at six days post-reinfection. The x-axis shows correlation values from Pearson correlation tests, the y-axis indicates the percentage of correlation values, and colors denote positive and negative correlations.
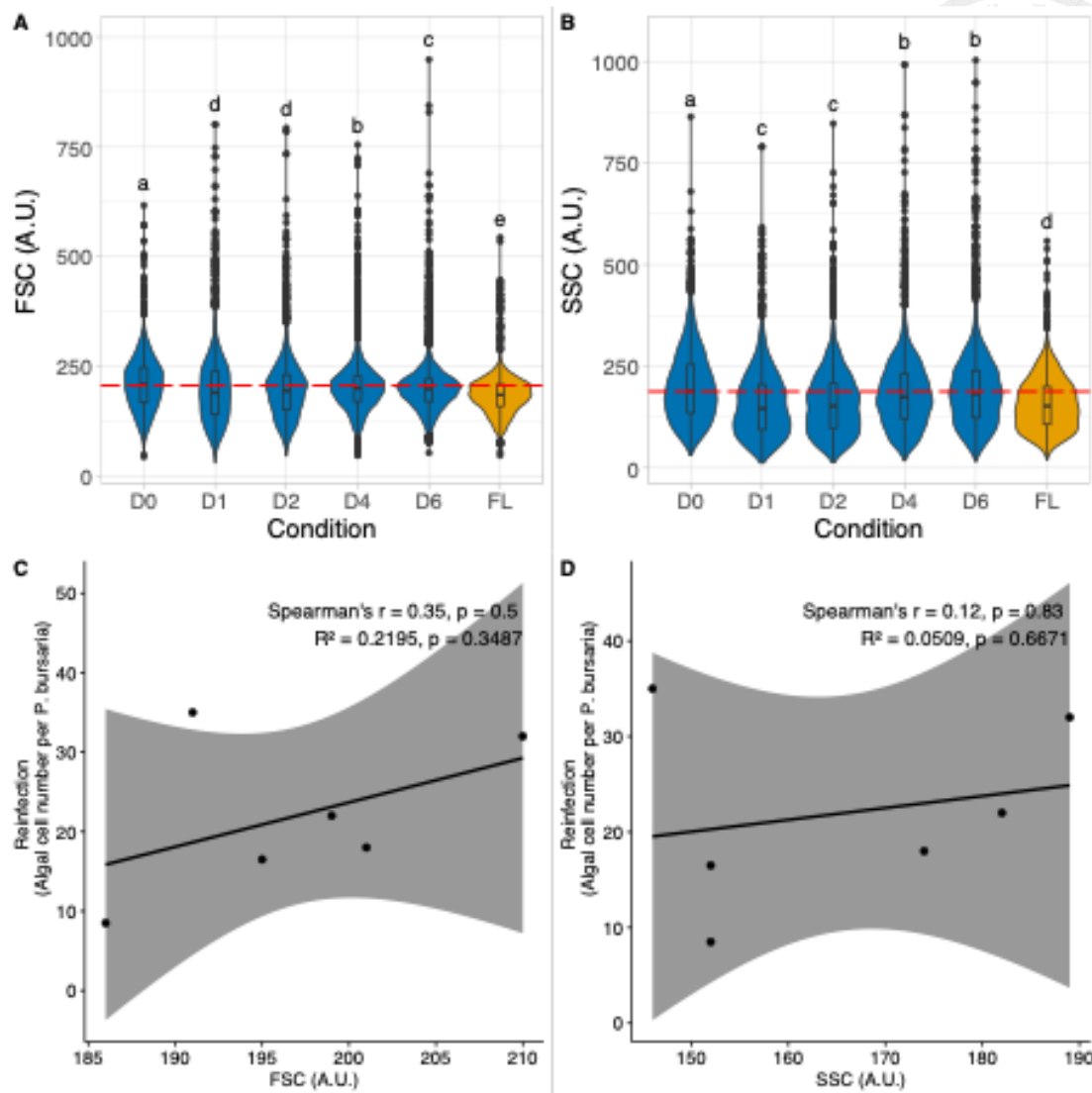
Figure 4. Mild decrease in cell size and structure observed in algal endosymbiont-exposed free-living algae cultures, without a strong association with establishment efficiency.

(A) Changes in FSC and (B) SSC among different algal states (Unit: A.U.). Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (5000 algal cells per sample). Correlation and linear regression analysis of the median number of algal cells from the time-course re-infection assays with (C) median values of FSC and (D) SSC among different algal states. Statistical analysis was performed using the Spearman correlation test and linear regression model.
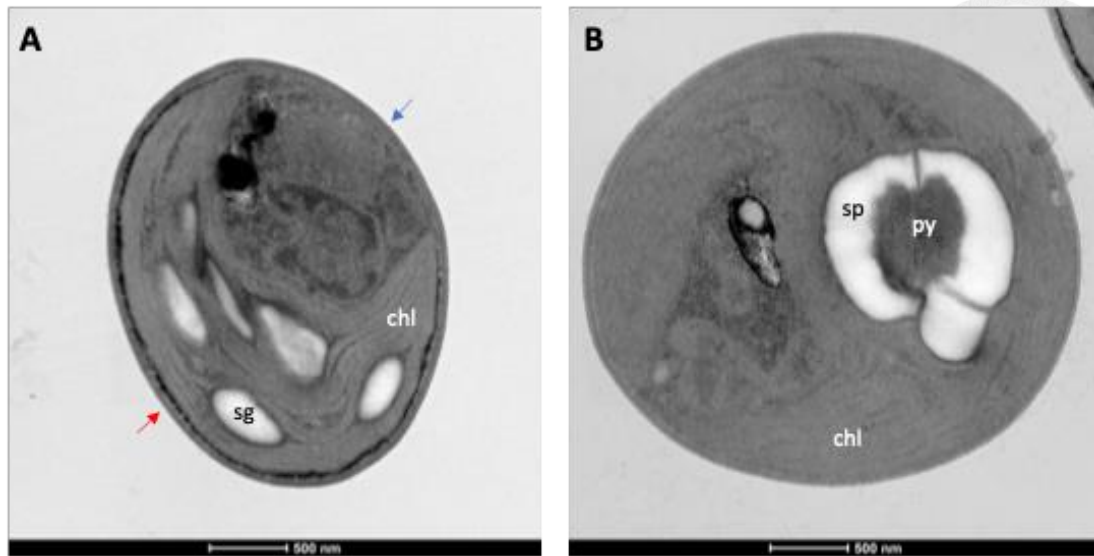
Figure 5. Uneven cell wall thickness and higher starch production in isolated endosymbionts compared to long-term free-living algae

TEM results of (A) freshly isolated endosymbiotic algae and (B) long-term free-living algal cultures. (Red arrow: thicker part of the cell wall; blue arrow: thinner part of the cell wall; chl: chloroplast; py: pyrenoid; sp: starch plate; sg: starch grain. Scale bar: 500 nm)
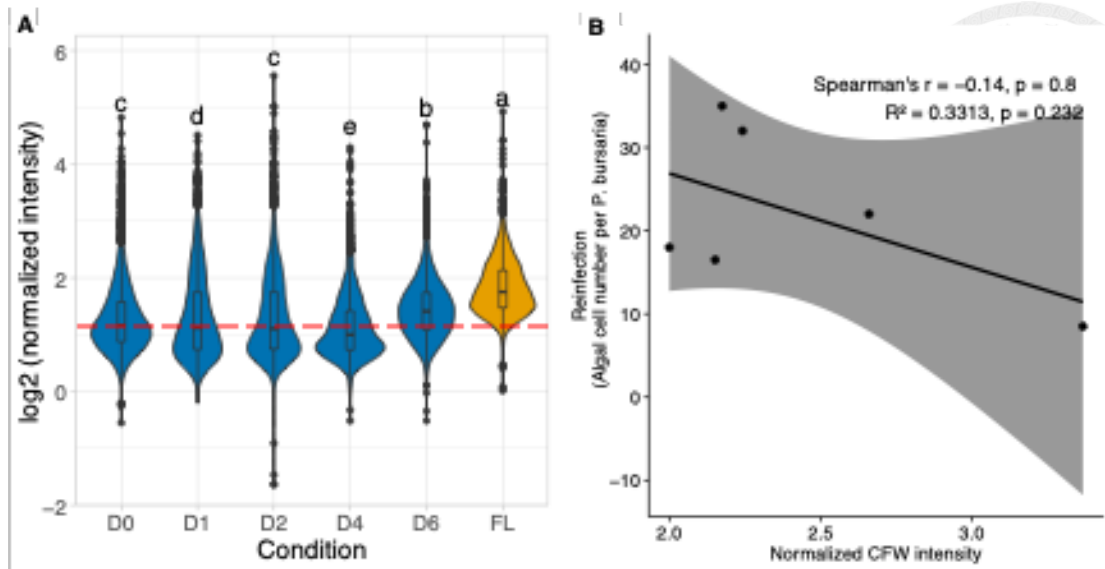
Figure 6. Changes in cell wall components from algal endosymbiont through short-term to long-term free-living cultures, without association with establishment.

(A) Changes in normalized CFW intensity among different algal states (y-axis: log2 normalized CFW intensity). Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (5000 algal cells per sample). (B) Correlation and linear regression analysis of the median number of algal cells from the time-course re-infection assays with the median values of normalized CFW intensity among different algal states. Statistical analysis was performed using the Spearman correlation test and linear regression model.
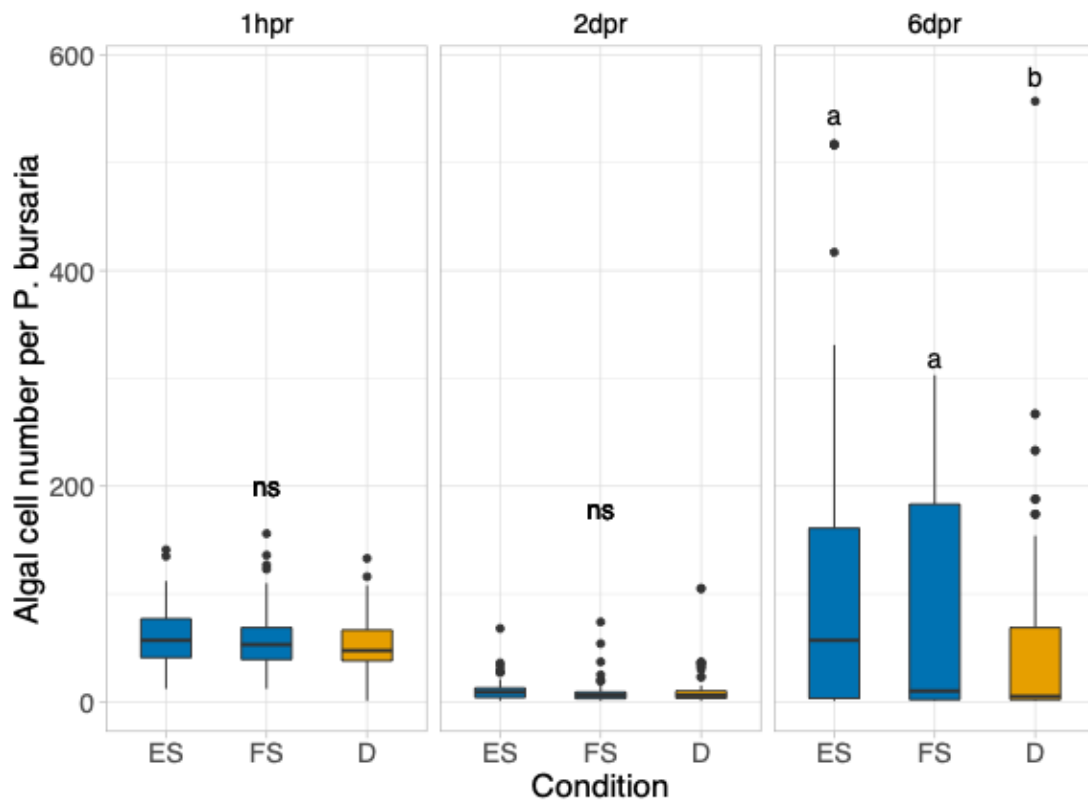
Figure 7. The secretion of algal endosymbionts containing key factors for endosymbiotic establishment.

At the top of the figure, the time points indicate observations at 1 hour post-reinfection (1hpr), 2 days post-reinfection (2dpr), and 6 days post-reinfection (6dpr). At the bottom of the figure, conditions for the re-infection assays are shown, including supernatants of freshly isolated endosymbiotic algae (ES), long-term free-living cultures (FS), and 1X modified Dryl's solution (D). The y-axis represents the number of algal cells per re-infected *P. bursaria*. Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (90 *P. bursaria* cells per sample).
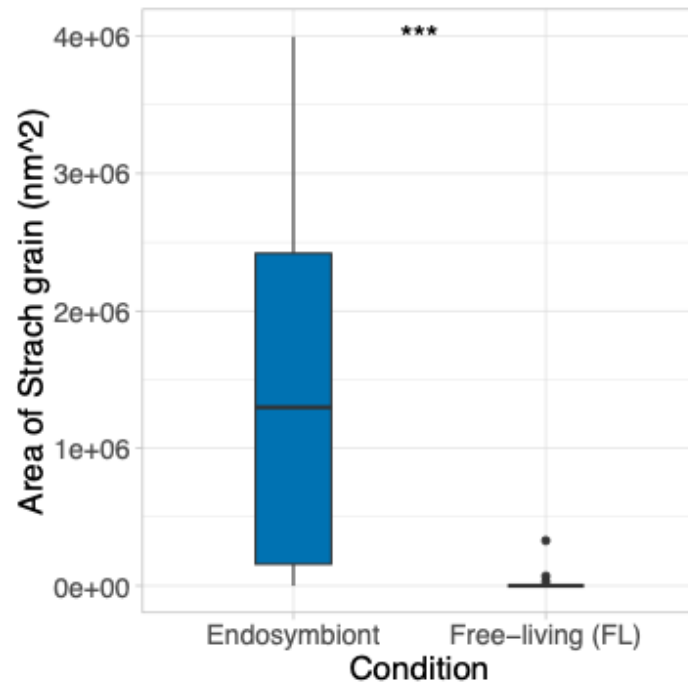
Figure 8. Production of starch grains is higher in the chloroplasts of freshly isolated endosymbionts compared to long-term free-living algae.

The x-axis and colors represent freshly isolated endosymbiotic algae (blue) and long-term free-living algae (yellow). The y-axis shows the starch grain area (Unit: nm²) for each condition. Statistical analysis was conducted using the Wilcoxon signed-rank test with Bonferroni correction, based on 19 endosymbiont cells and 13 free-living algae cells (*** p-value < 0.001).
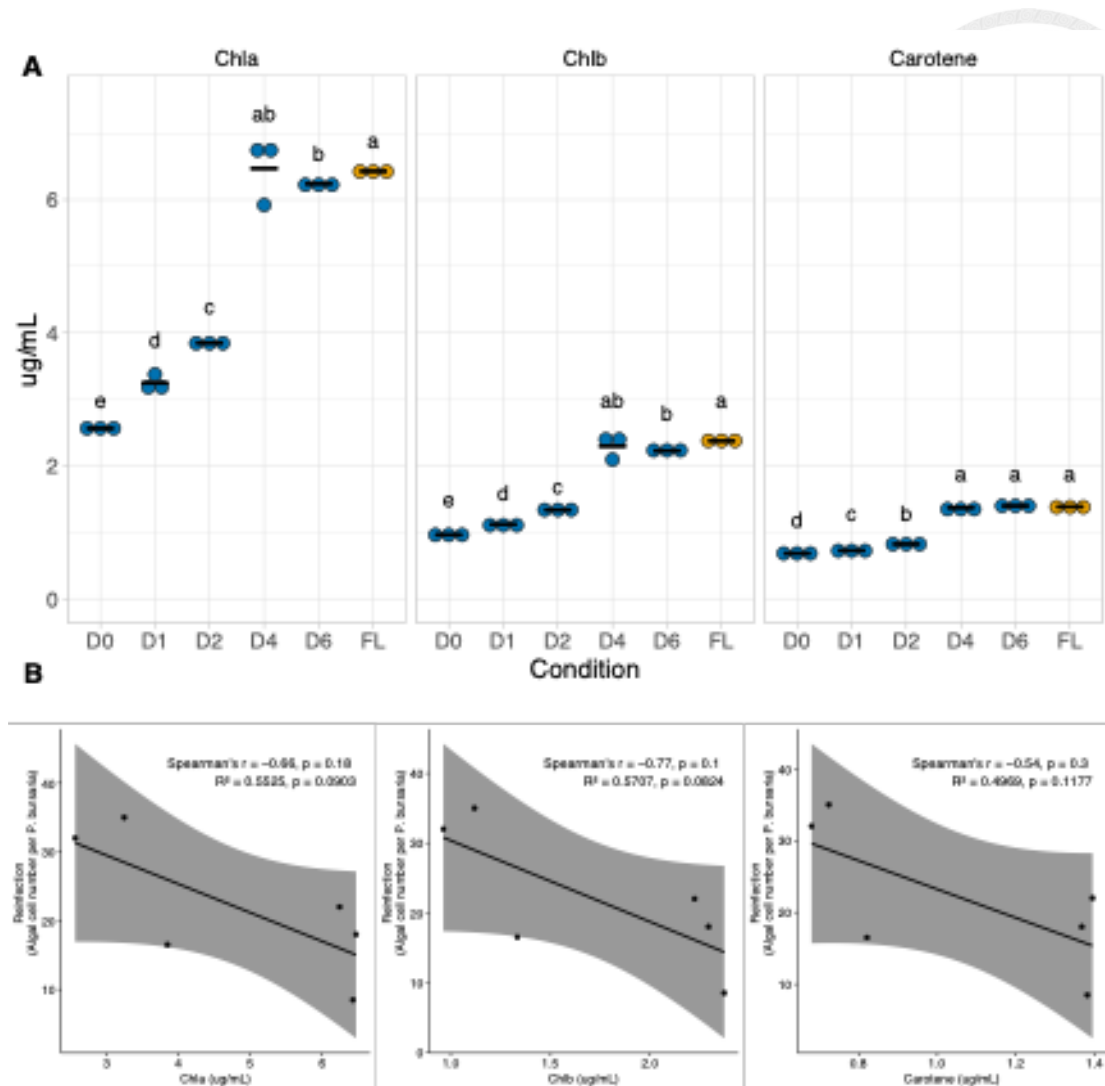
Figure 9. Increasing chlorophyll content from isolated algal endosymbionts across short-term to long-term free-living algal cultures and its negative correlation with establishment efficiency.

(A) Changes in the concentration of chlorophyll a, b, and carotene among different algal states (Unit: µg/mL). Statistical analysis was performed using the two-sample t-test with Bonferroni correction (10^7 algal cells per replicate, and triplicate per sample). (B) Correlation and linear regression analysis of the median number of algal cells from time-course reinfection assays with the mean chlorophyll content across different algal states. Statistical analysis was conducted using the Spearman correlation test and a linear regression model.
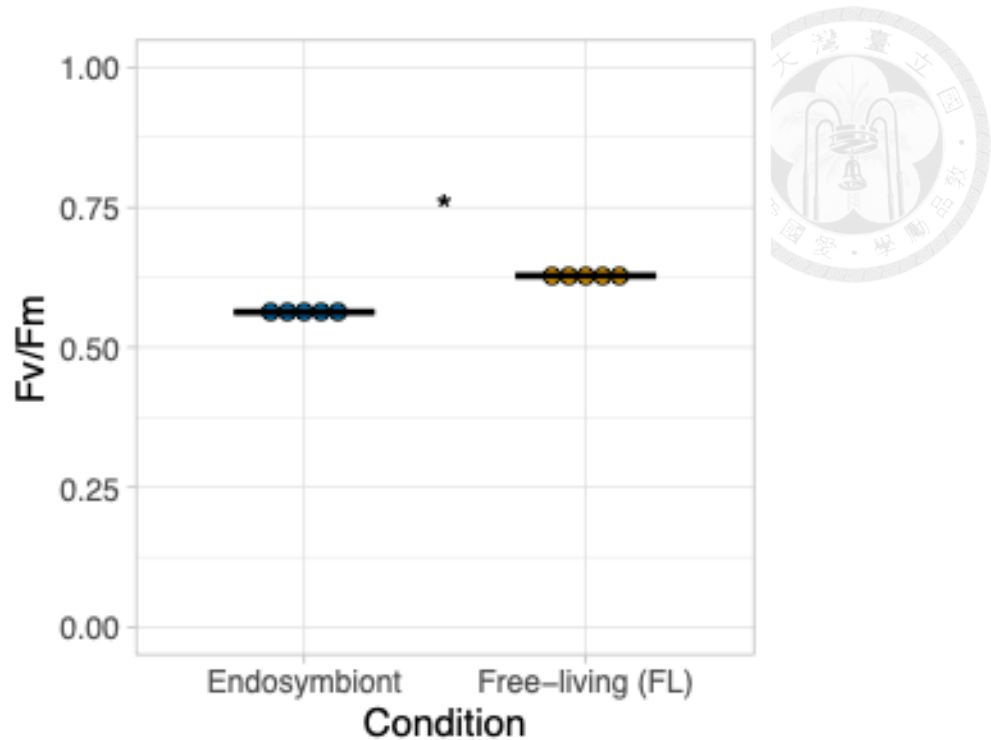
Figure 10. PSII efficiency is lower in freshly isolated endosymbiotic algae compared to the long-term free-living algae.

The x-axis and colors represent the freshly isolated endosymbiotic algae (blue) and long-term free-living algae (yellow). The y-axis represents the value of PSII efficiency (Fv/Fm) between the conditions. Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (7.5×10^6 algal cells per replicate, and five replicates per sample, * p-value < 0.05).
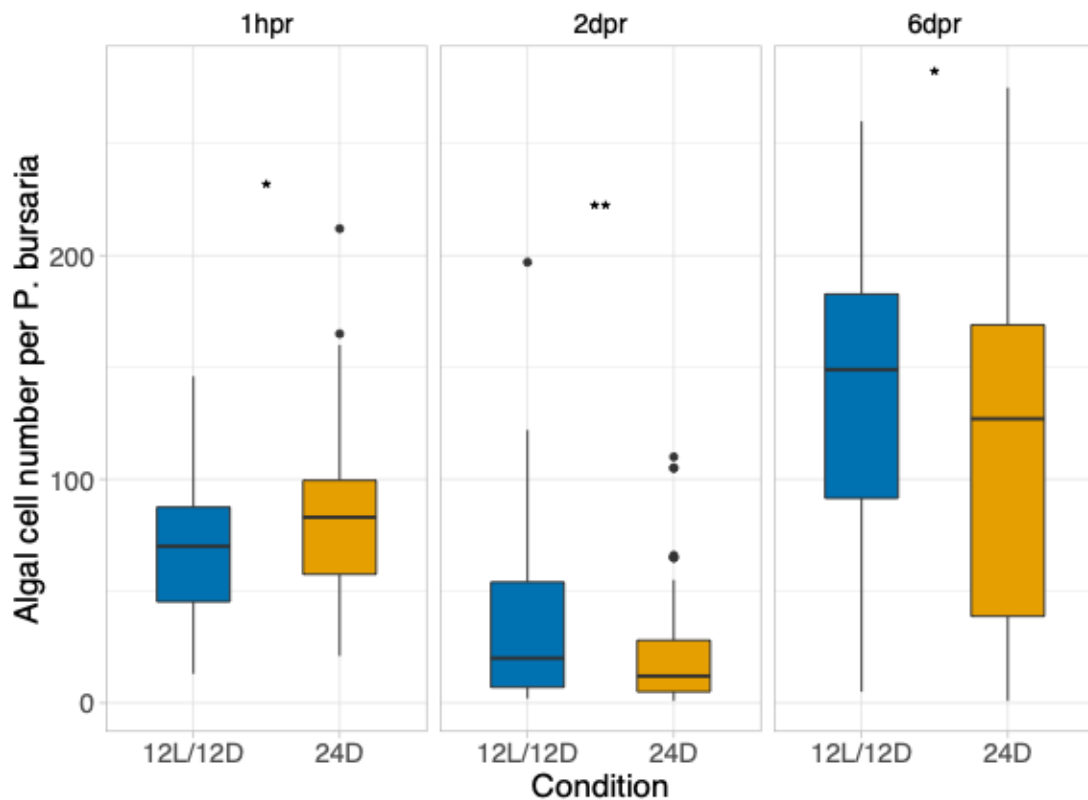
Figure 11. Light is crucial for algae to perform photosynthesis during the initial establishment of endosymbiosis.

At the top of the figure, the time points indicate observations at 1 hour post-reinfection (1hpr), 2 days post-reinfection (2dpr), and 6 days post-reinfection (6dpr). At the bottom of the figure, conditions for the re-infection assays are shown, including re-infected *P. bursaria* cultured under both 12-hour light/12-hour dark (12L/12D) and 24-hour dark (24D) conditions for 24 hours. The y-axis represents the number of algal cells per re-infected *P. bursaria*. Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (90 *P. bursaria* cells per sample, * p-value < 0.05, and ** p-value < 0.01).
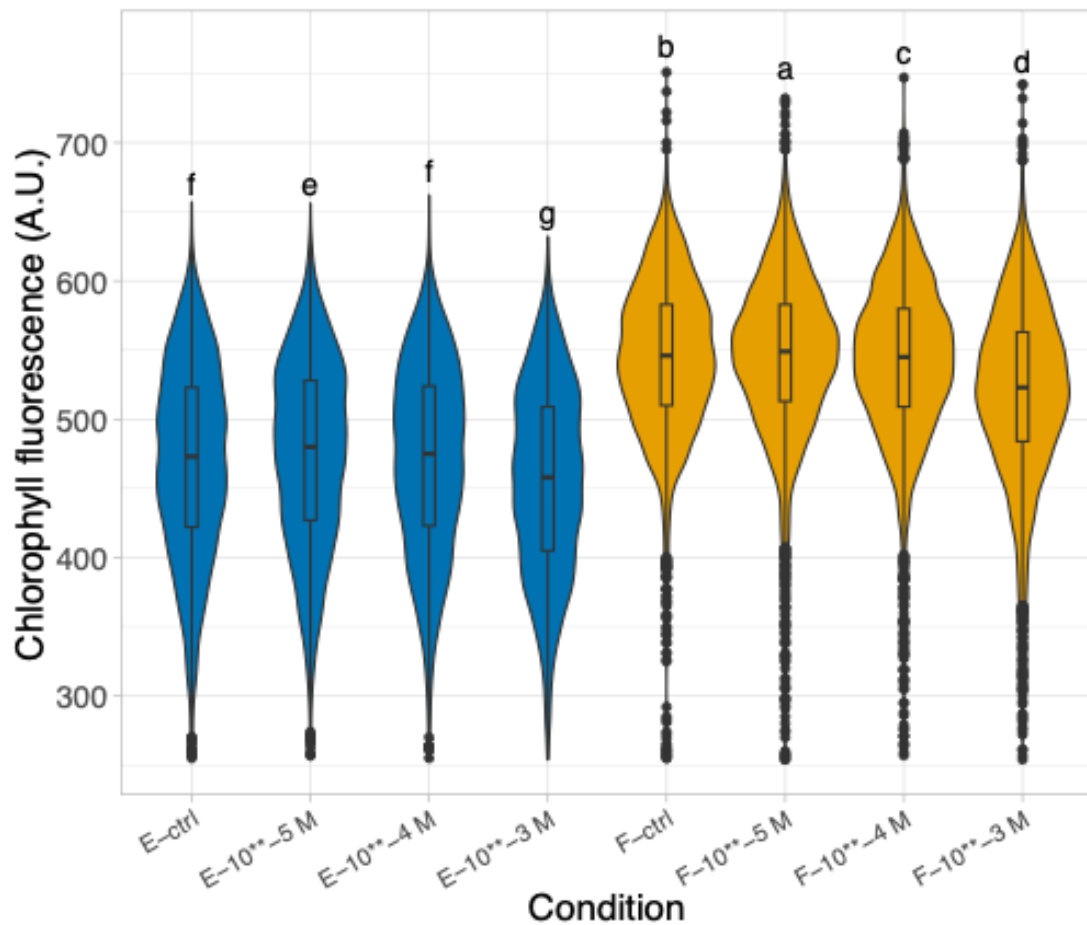
Figure 12. Decrease in chlorophyll fluorescence in freshly isolated algal endosymbionts and free-living algal cultures pretreated with 10^{-4} M DCMU.

Changes in chlorophyll fluorescence of freshly isolated endosymbiotic algae and long-term free-living algal cultures pretreated with 10^{-3}, 10^{-4}, and 10^{-5} (10**-3, 10**-4, and 10**-5) M DCMU for 24 hours, respectively. (Unit: A.U.) Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (5000 algal cells per sample).
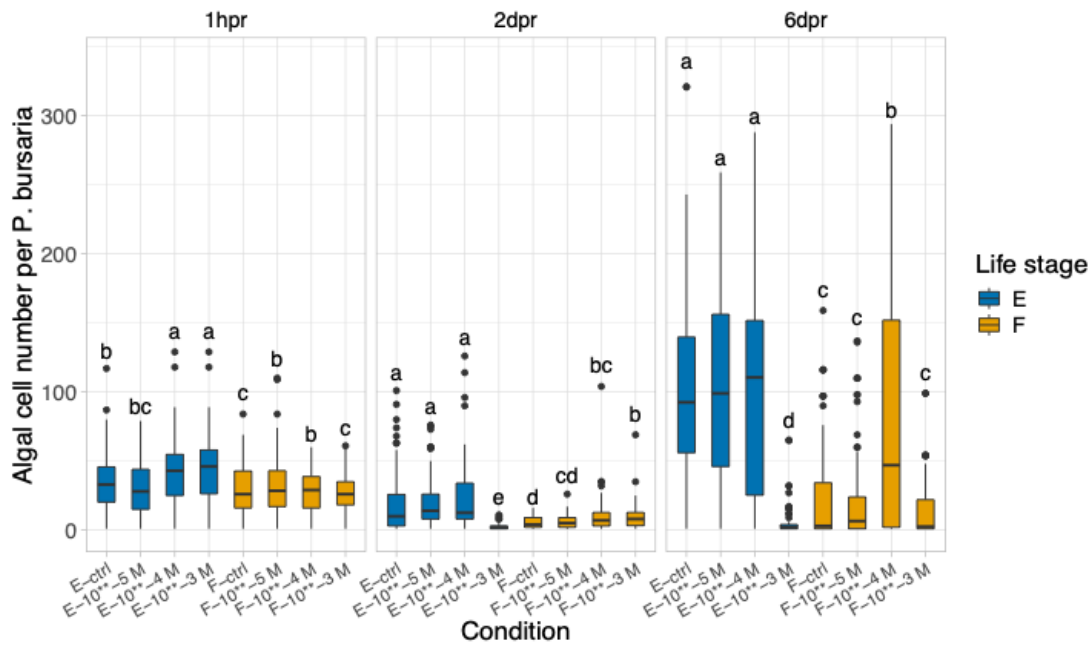
Figure 13. Pretreatment with DCMU may arrest algal physiology or impair photosynthetic ability, thereby affecting establishment efficiency.

At the top of the figure, the time points indicate observations at 1 hour post-reinfection (1hpr), 2 days post-reinfection (2dpr), and 6 days post-reinfection (6dpr). At the bottom of the figure, conditions for the re-infection assays are shown, including freshly isolated endosymbiotic algae and long-term free-living algal cultures pretreated with 10^{-3}, 10^{-4}, and 10^{-5} M DCMU, and a control (10**-3, 10**-4, 10**-5, and ctrl) for 24 hours, respectively. The y-axis represents the number of algal cells per re-infected *P. bursaria*. Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (90 *P. bursaria* cells per sample).

Figure 14. Possibly minor influence of pH on inducing factors in freshly isolated algal endosymbionts.

At the top of the figure, the time points indicate observations at 1 hour post-reinfection (1hpr), 2 days post-reinfection (2dpr), and 6 days post-reinfection (6dpr). At the bottom of the figure, the conditions for the re-infection assays are shown, including long-term free-living algal cultures pretreated in pH 5, pH 6, and pH 7 conditions for (A) 2 days and (B) 7 days. The y-axis represents the number of algal cells per re-infected *P. bursaria*. Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (90 *P. bursaria* cells per sample).
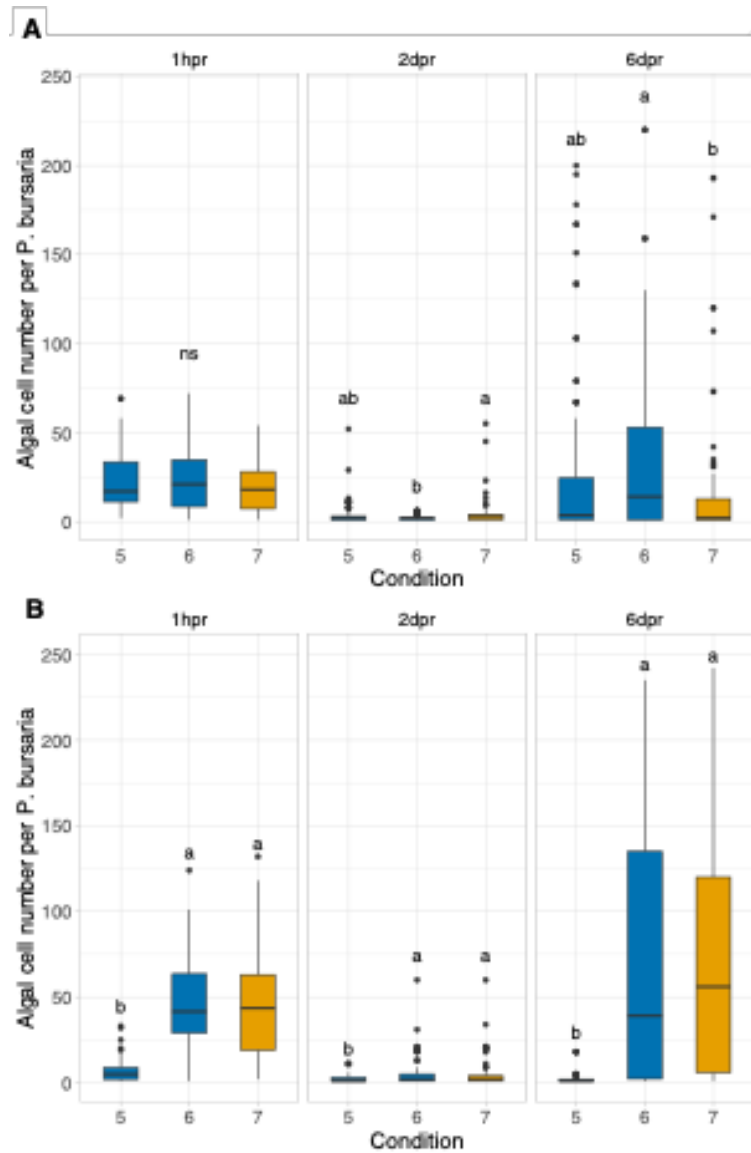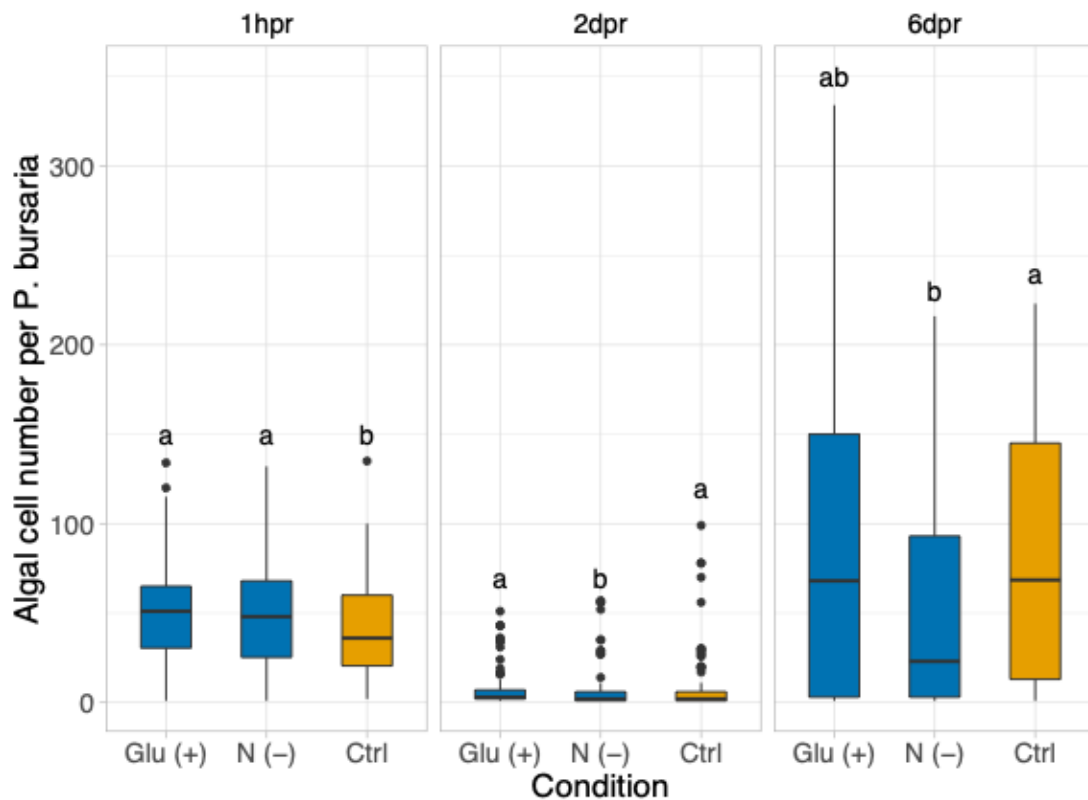
Figure 15. Potential slight impact of nitrogen source on inducing factors in freshly isolated algal endosymbionts.

At the top of the figure, the time points indicate observations at 1 hour post-reinfection (1hpr), 2 days post-reinfection (2dpr), and 6 days post-reinfection (6dpr). At the bottom of the figure, the conditions for the re-infection assays are shown, including long-term free-living algal cultures precultured in glutamine-supplemented (Glu (+)), nitrogen-reduced (N (-)), and control (ctrl) conditions for 7 days, respectively. The y-axis represents the number of algal cells per re-infected *P. bursaria*. Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (90 *P. bursaria* cells per sample).
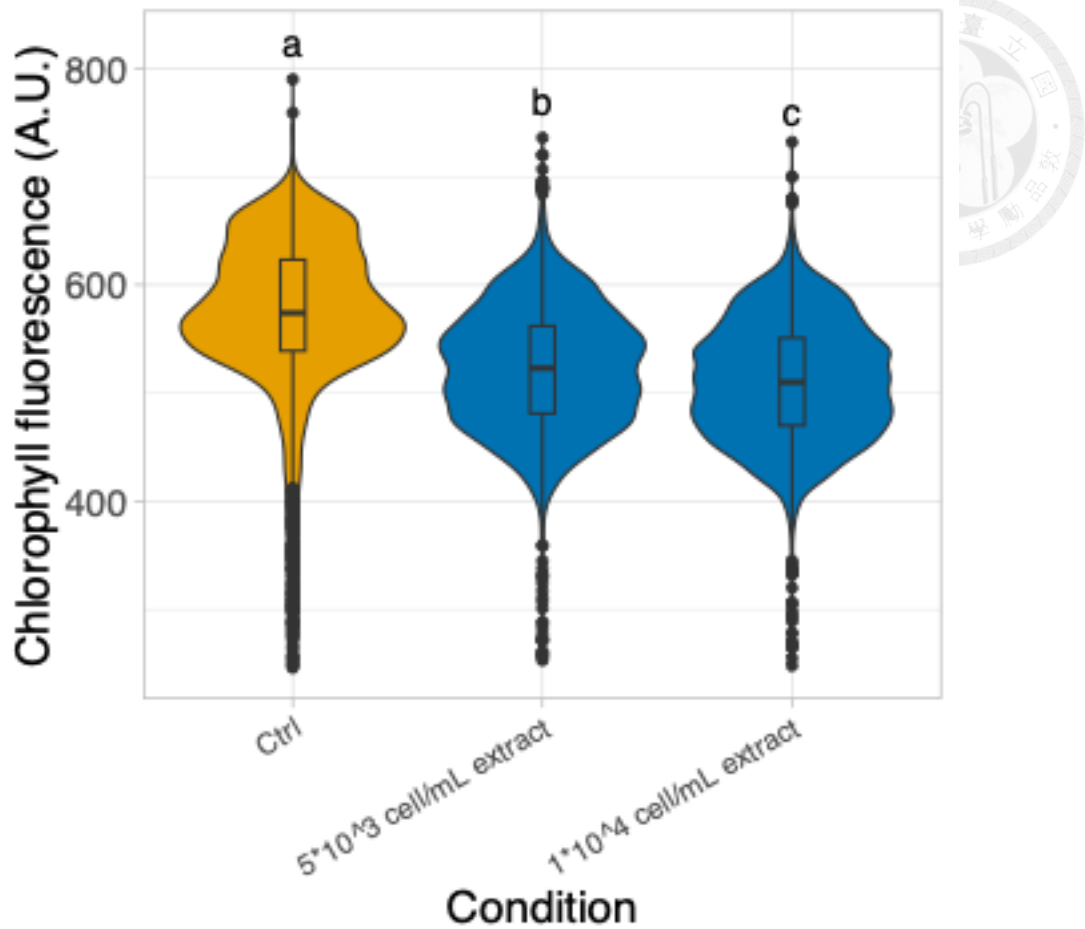
Figure 16. Pretreatments of the host extract for long-term free-living algal cultures decreased chlorophyll fluorescence.

Changes in chlorophyll fluorescence of long-term free-living algal cultures precultured with 5×10^{-3} (5*10^3 cells/mL extract) and 10^{-4} *P. bursaria* cells/mL (1*10^4 cells/mL extract) for 3 days, respectively. (Unit: A.U.) Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (5000 algal cells per sample).
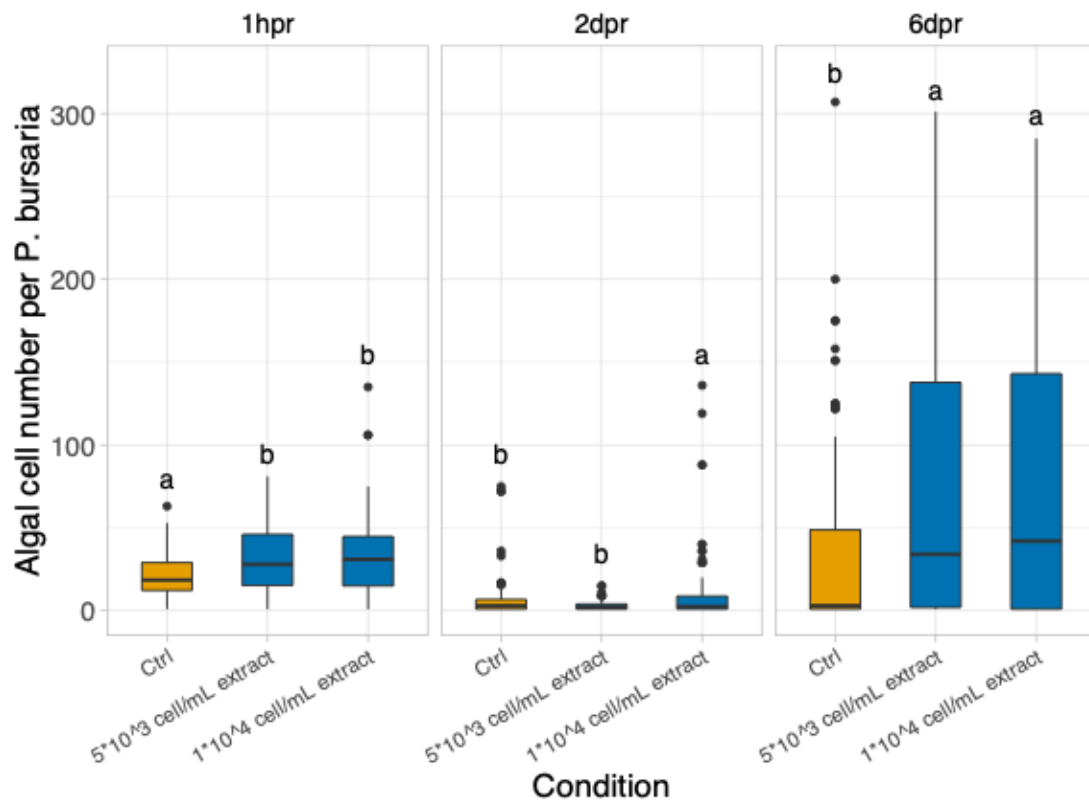
Figure 17. The factors from the symbiotic type *P. bursaria* may play a crucial role in inducing physiological changes for the establishment of endosymbiosis.

At the top of the figure, the time points indicate observations at 1 hour post-reinfection (1hpr), 2 days post-reinfection (2dpr), and 6 days post-reinfection (6dpr). At the bottom of the figure, the conditions for the re-infection assays are shown, including long-term free-living algal cultures precultured with 5×10^{-3} (5*10^3 cells/mL extract) and 10^{-4} *P. bursaria* cells/mL (1*10^4 cells/mL extract) extracts and control (Ctrl) for 3 days. The y-axis represents the number of algal cells per re-infected *P. bursaria*. Statistical analysis was performed using the Wilcoxon signed-rank test with Bonferroni correction (90 *P. bursaria* cells per sample).
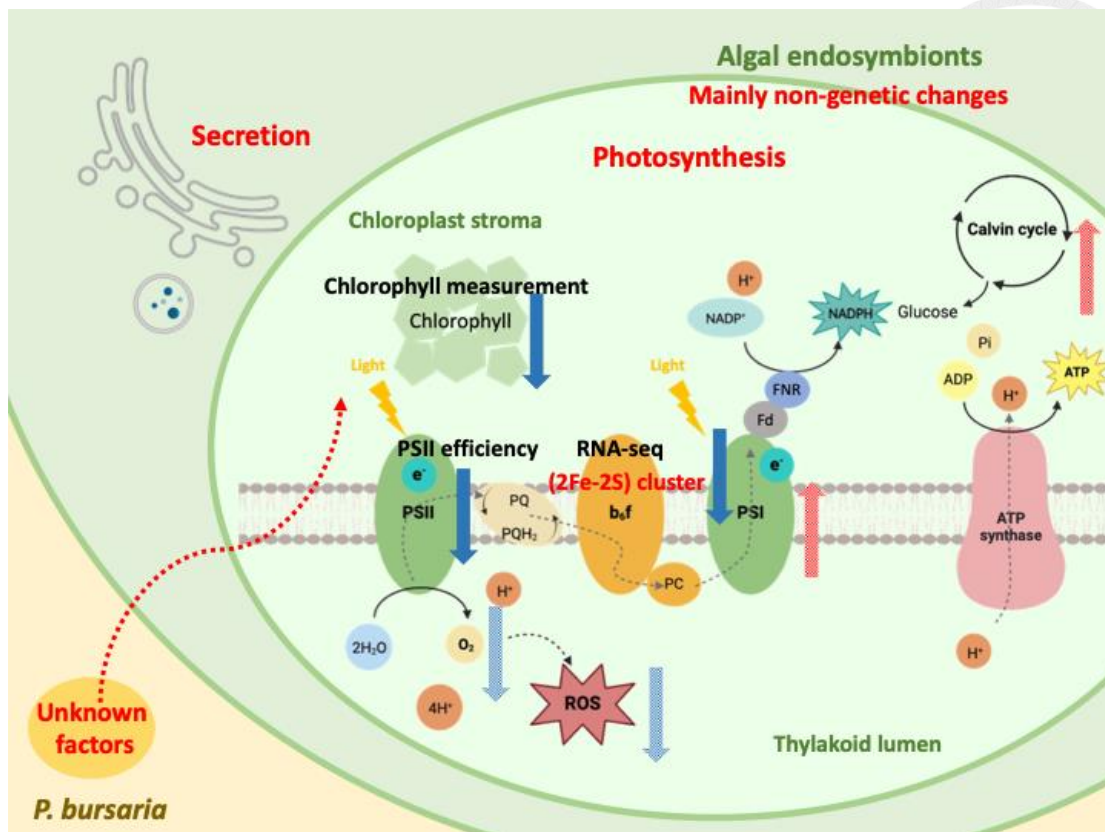
Figure 18. The schematic model of the status adjustment of algal endosymbiont *C. variabilis* within the host *P. bursaria*.

When the endosymbiotic algae *C. variabilis* reside within the host *P. bursaria*, host-derived factors may induce the algae to undergo non-genetic changes, including reprogramming of the photosynthesis process, reduction of oxidative stress, and secretion of specific substances. These non-genetic alterations enable the endosymbiotic algae, when freshly isolated from the host *P. bursaria*, to efficiently establish an endosymbiotic relationship with a new host *P. bursaria*.

Table 1 GO terms highly positively correlated with the establishment ability of endosymbiosis.

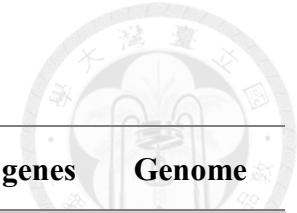| Criteria	GO	Description	Ontology	p-value	Candidate genes	Genome
		Correlation with the observations of **two** days after re-infection				
	GO:0008289	lipid binding	MF	0.013	4/309	23/7615
	GO:0005783	endoplasmic reticulum	CC	0.022	5/309	40/7615
>0.9	GO:0016192	vesicle-mediated transport	BP	0.039	6/309	62/7615
	GO:0051087	protein-folding chaperone binding	MF	0.045	3/309	20/7615
	GO:0006412	translation	BP	0.049	12/309	173/7615
	GO:0015935	small ribosomal subunit	CC	0.016	2/108	14/7615
>0.95	GO:0022627	cytosolic small ribosomal subunit	CC	0.032	2/108	20/7615
	GO:0031418	L-ascorbic acid binding	MF	0.042	2/108	23/7615
		Correlation with the observations of **six** days after re-infection				
	GO:0000387	spliceosomal snRNP assembly	BP	0.005	4/601	10/7615
	GO:0006470	protein dephosphorylation	BP	0.008	9/601	45/7615
>0.9	GO:0005525	GTP binding	MF	0.008	16/601	105/7615
	GO:0008168	methyltransferase activity	MF	0.017	11/601	68/7615
	GO:0016192	vesicle-mediated transport	BP	0.022	10/601	62/7615

|	GO:0003677	DNA binding	MF	0.025	35/601	316/7615
	GO:0003723	RNA binding	MF	0.027	38/601	351/7615
>0.9	GO:0005839	proteasome core complex	CC	0.033	4/601	16/7615
	GO:0000470	maturation of LSU-rRNA	BP	0.039	3/601	10/7615
	GO:0003724	RNA helicase activity	MF	0.048	6/601	34/7615
	GO:0005525	GTP binding	MF	0.002	9/200	105/7615
	GO:0051287	NAD binding	MF	0.017	4/200	38/7615
	GO:0005215	transporter activity	MF	0.024	3/200	24/7615
>0.95	GO:0000387	spliceosomal snRNP assembly	BP	0.027	2/200	10/7615
	GO:0006334	nucleosome assembly	BP	0.027	2/200	10/7615
	GO:0000105	L-histidine biosynthetic process	BP	0.038	2/200	12/7615
	GO:0003924	GTPase activity	MF	0.042	5/200	73/7615
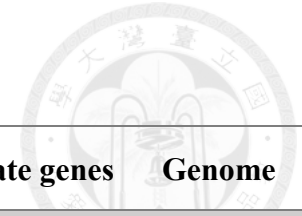
Table 2 GO terms highly negatively correlated with the establishment ability of endosymbiosis.

Criteria	GO	Description	Ontology	p-value	Candidate genes	Genome
		Correlation with the observations of **two** days after re-infection				
>0.9	GO:0009058	biosynthetic process	BP	0.022	2/29	60/7615
	GO:0008168	methyltransferase activity	MF	0.027	2/29	68/7615
	GO:0015144	carbohydrate transmembrane transporter activity	MF	0.037	1/29	10/7615
	GO:0006629	lipid metabolic process	BP	0.041	2/29	85/7615
	GO:0005643	nuclear pore	CC	0.045	1/29	12/7615
	GO:0008408	3'-5' exonuclease activity	MF	0.045	1/29	12/7615
>0.95	GO:0008408	3'-5' exonuclease activity	MF	0.013	1/8	12/7615
	GO:0006139	nucleobase-containing compound metabolic process	BP	0.023	1/8	22/7615
	GO:0051537	2 iron, 2 sulfur cluster binding	MF	0.035	1/8	34/7615
		Correlation with the observations of **six** days after re-infection				
>0.9	GO:0003899	DNA-directed 5'-3' RNA polymerase activity	MF	0.009	3/142	23/7615
	GO:0015144	carbohydrate transmembrane transporter activity	MF	0.014	2/142	10/7615
	GO:0035556	intracellular signal transduction	BP	0.014	2/142	10/7615

	GO:0002161	aminoacyl-tRNA editing activity	MF	0.017	2/142	11/7615
	GO:0005615	extracellular space	CC	0.018	3/142	30/7615
	GO:0006418	tRNA aminoacylation for protein translation	BP	0.029	3/142	36/7615
>0.9	GO:0004812	aminoacyl-tRNA ligase activity	MF	0.038	3/142	40/7615
	GO:0006351	DNA-templated transcription	BP	0.038	3/142	40/7615
	GO:0004252	serine-type endopeptidase activity	MF	0.042	5/142	102/7615
	GO:0016592	mediator complex	CC	0.043	2/142	18/7615
	GO:0002161	aminoacyl-tRNA editing activity	MF	0.002	2/48	11/7615
>0.95	GO:0006418	tRNA aminoacylation for protein translation	BP	0.021	2/48	36/7615
	GO:0004812	aminoacyl-tRNA ligase activity	MF	0.026	2/48	40/7615
	GO:0000398	mRNA splicing, via spliceosome	BP	0.048	2/48	56/7615

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