Title:

Metabolic co-dependence drives the evolutionary ancient *Hydra-Chlorella* symbiosis

Authors:

Mayuko Hamada [1, 3]*, Katja Schröder [2]*, Jay Bathia [2], Ulrich Kürn [2], Sebastian Fraune [2], Mariia Khalturina [1], Konstantin Khalturin [1], Chuya Shinzato [1], Nori Satoh [1], Thomas C.G. Bosch [2]

Affiliations:

[1] Marine Genomics Unit, Okinawa Institute of Science and Technology Graduate University (OIST), 1919-1 Tancha, Onna-son, Kunigami-gun, Okinawa, 904-0495 Japan

[2] Zoological Institute and Interdisciplinary Research Center Kiel Life Science, Kiel University, Am Botanischen Garten 1-9, 24118 Kiel, Germany

[3] Ushimado Marine Institute, Okayama University, 130-17 Kashino, Ushimado, Setouchi, Okayama, 701-4303 Japan

* Authors contributed equally

Corresponding author:

Thomas C. G. Bosch

Zoological Institute, Kiel University

Am Botanischen Garten 1-9

24118 Kiel

TEL: +49 431 880 4172

tbosch@zoologie.uni-kiel.de

Abstract (148 words)

Many multicellular organisms rely on symbiotic associations for support of metabolic activity, protection, or energy. Understanding the mechanisms involved in controlling such interactions remains a major challenge. In an unbiased approach we identified key players that control the symbiosis between *Hydra viridissima* and its photobiont *Chlorella* sp. A99. We discovered significant upregulation of *Hydra* genes encoding a phosphate transporter and glutamine synthetase suggesting regulated nutrition supply between host and symbionts. Interestingly, supplementing the medium with glutamine temporarily supports in vitro growth of the otherwise obligate symbiotic *Chlorella*, indicating loss of autonomy and dependence on the host. Genome sequencing of *Chlorella* A99 revealed a large number of amino acid transporters and a degenerated nitrate assimilation pathway, presumably as consequence of the adaptation to the host environment. Our observations portray ancient symbiotic interactions as a codependent partnership in which exchange of nutrients appears to be the primary driving force.

Introduction

Symbiosis has been a prevailing force throughout the evolution of life, driving the diversification of organisms and facilitating rapid adaptation of species to divergent new niches (Moran, 2007; Joy, 2013; McFall-Ngai et al., 2013). In particular, symbiosis with photosynthetic symbiont is observed in many species of Cnidarians such as coral, jellyfish, sea anemone and hydra, contributing to the ecological success of these sessile or planktonic animals (Douglas, 1994; Davy et al., 2012b). Among the many animals dependent on algal symbionts, inter-species interactions between green hydra *Hydra viridissima* and endosymbiotic unicellular green algae of the genus *Chlorella* have been a subject of interest for decades (Muscatine and Lenhoff, 1963; Roffman and Lenhoff, 1969). Such studies not only provide insights into the basic "tool kit" necessary to establish symbiotic interactions, but are also of relevance in understanding the resulting evolutionary selective processes (Muscatine and Lenhoff, 1965a, b; Thorington and Margulis, 1981).

The interactions at play here are clearly metabolic: the algae depend on nutrients that are derived from the host or from the environment surrounding the host, while in return the host receives a significant amount of photosynthetically fixed carbon from the algae. Previous studies have provided evidence that the photosynthetic symbionts provide their host with maltose, enabling *H. viridissima* to survive periods of starvation (Muscatine and Lenhoff, 1963; Muscatine, 1965; Roffman and Lenhoff, 1969; Cook and Kelty, 1982; Huss et al., 1993/1994). *Chlorella*-to-*Hydra* translocation of photosynthates is critical for polyps to grow (Muscatine and Lenhoff, 1965b; Mews, 1980; Douglas and Smith, 1983; Douglas and Smith, 1984). Presence of symbiotic algae also has a profound impact on hydra´s fitness by promoting oogenesis (Habetha et al., 2003; Habetha and Bosch, 2005).

Pioneering studies performed in the 1980s (McAuley and Smith, 1982; Rahat and Reich, 1984) showed that there is a great deal of adaptation and specificity in this symbiotic relationship. All endosymbiotic algae found in a single host polyp are clonal and proliferation of symbiont and host is tightly correlated (Bossert and Dunn, 1986; McAuley, 1986a). Although it is not yet known how *Hydra* controls cell division in symbiotic *Chlorella*, *Chlorella* strain A99 is unable to grow outside its polyp host and is transmitted vertically to the next generation of *Hydra*, indicating loss of autonomy during establishment of its symbiotic relationship with this host (Muscatine and McAuley, 1982; Campbell, 1990; Habetha et al., 2003).

Molecular phylogenetic analyses suggest that *H. viridissima* is the most basal species in the genus *Hydra* and that symbiosis with *Chlorella* was established in the ancestral *viridissima* group after their divergence from non-symbiotic hydra groups (Martinez et al., 2010;

Schwentner and Bosch, 2015). A recent phylogenetic analysis of different strains of green hydra resulted in a phylogenetic tree that is topologically equivalent to that of their symbiotic algae (Kawaida et al., 2013), suggesting these species co-evolved as a result of their symbiotic relationship. Although our understanding of the factors that promote symbiotic relationships in cnidarians has increased (Shinzato et al., 2011; Davy et al., 2012a; Lehnert et al., 2014; Baumgarten et al., 2015; Ishikawa et al., 2016), very little is known about the molecular mechanisms allowing this partnership to persist over millions of years.

Recent advances in transcriptome and genome analysis allowed us to identify the metabolic interactions and genomic evolution involved in achieving the *Hydra-Chlorella* symbiotic relationship. We present here the first characterization, to our knowledge, of genetic complementarity between green *Hydra* and *Chlorella* algae that explains the emergence and/or maintenance of a stable symbiosis. We also provide here the first report of the complete genome sequence from an obligate intracellular *Chlorella* photobiont. Together, our results show that exchange of nutrients is the primary driving force for the symbiosis between *Chlorella* and *Hydra*. Subsequently, reduction of metabolic pathways may have further strengthened their codependency. Our findings provide a framework for understanding the evolution of a highly codependent symbiotic partnership in an early emerging metazoan.

Results

Discovery of symbiosis-dependent *Hydra* genes

As tool for our study we used the green hydra *H. viridissima* (**Figure 1A**) colonized with symbiotic *Chlorella* sp. strain A99 (abbreviated here as Hv_Sym), aposymbiotic *H. viridissima* from which the symbiotic *Chlorella* were removed (Hv_Apo), and aposymbiotic *H. viridissima* which had been artificially infected with *Chlorella variabilis* NC64A (Hv_NC64A). The latter is symbiotic to the single-cellular protist Paramecium (Karakashian and Karakashian, 1965). Although an association between *H. viridissima* and *Chlorella* NC64A can be maintained for some time, both their growth rate (**Figure 1B**) and the number of NC64A algae per *Hydra* cell (**Supplementary Figure 1**) is significantly reduced compared to the symbiosis with native symbiotic *Chlorella* A99.

H. viridissima genes involved in the symbiosis with *Chlorella* were identified by microarray based on the contigs of *Hydra viridissima* A99 transcriptome (NCBI GEO Platform ID: GPL23280). For the microarray analysis, total RNA was extracted from the polyps after light exposure for six hours. By comparing the transcriptomes of Hv_Sym and Hv_Apo, we identified

423 contigs that are upregulated and 256 contigs that are downregulated in presence of *Chlorella* A99 (**Figure 1C**). To exclude genes involved in oogenesis and embryogenesis, only contigs differently expressed with similar patterns in both sexual and asexual Hv_Sym were recorded. Interestingly, contigs whose predicted products had no discernible homologs in other organisms including other *Hydra* species were overrepresented in these differentially expressed contigs (Chi-squared test P<0.001) (**Supplementary Figure 2**). Such taxonomically restricted genes (TRGs) are thought to play important roles in the development of evolutionary novelties and morphological diversity within a given taxonomic group (Khalturin et al., 2009; Tautz and Domazet-Loso, 2011).

We further characterized functions of the differentially expressed *Hydra* genes by Gene Ontology (GO) terms (The Gene Ontology et al., 2000). This demonstrated overrepresentation of genes with GO term “localization” in upregulated contigs (Hv_Sym > Hv_Apo) and with GO term “metabolic process” in downregulated contigs (Hv_Sym < Hv_Apo) (**Figure 1D**). More specifically, the upregulated contigs include many genes related to “transmembrane transporter activity”, “transmembrane transport”, “transposition”, “cilium” and “protein binding, bridging” (**Figure 1E**). In the downregulated contig set, the GO classes “cellular amino acid metabolic process”, “cell wall organization or biogenesis” and “peptidase activity” are overrepresented (**Figure 1E**). These results suggest that the *Chlorella* photobiont affects core metabolic processes and pathways in *Hydra*. Particularly, carrier proteins and active membrane transport appears to play a prominent role in the symbiosis.

To narrow down the number of genes specifically affected by the presence of *Chlorella* A99, we identified 12 contigs that are differentially expressed in presence of *Chlorella* A99 but not in presence of *Chlorella* NC64A (**Figure 1C** A99-specific). Independent qPCR confirmed the differential expression pattern for 10 of these genes (**Supplementary Table 1**). The genes upregulated by the presence of the photobiont encode a Spot_14 protein, a glutamine synthetase (GS) and a sodium-dependent phosphate (Na/Pi) transport protein in addition to a *H. viridissima* specific gene (rc_12891: *Sym-1*) and a *Hydra* genus specific gene (rc_13570: *Sym-2*) (**Supplementary Table 1**). *Hydra* genes downregulated by the presence of *Chlorella* A99 were two *H. viridissima* specific genes and three metabolic genes encoding histidine ammonia-lyase, acetoacetyl-CoA synthetase and 2-isopropylmalate synthase (**Supplementary Table 1**). Of the upregulated genes, Spot_14 is described as thyroid hormone-responsive spot 14 protein reported to be induced by dietary carbohydrates and glucose in mammals (Tao and Towle, 1986; Brown et al., 1997). Na/Pi transport protein is a membrane transporter actively transporting phosphate into cells (Murer and Biber, 1996). GS plays an essential role in the metabolism of nitrogen by catalyzing the reaction between glutamate and ammonia to form glutamine (Liaw et al., 1995). Interestingly, out of the three

GS genes *H. viridissima* contains only *GS-1* was found to be upregulated by the presence of the photobiont (**Supplementary Figure 3**). The discovery of these transcriptional responses points to an intimate metabolic exchange between the partners in a species-specific manner..

Symbiont-dependent *Hydra* genes are upregulated by photosynthetic activity of *Chlorella A99*

To test whether photosynthetic activity of the symbiont is required for upregulation of gene expression, Hv_Sym was either cultured under a standard 12 hr light/dark alternating regime or continuously in the dark for 1 to 4 days prior to RNA extraction (**Figure 2A**). Interestingly, four (*GS1*, *Spot14*, *Na/Pi* and *Sym-1*) of five genes specifically activated by the presence of *Chlorella* A99 showed significant upregulation when exposed to light (**Figure 2B**), indicating the relevance of photosynthetic activity of *Chlorella*. This upregulation was strictly dependent on presence of the algae, as in aposymbiont Hv_Apo the response was absent (**Figure 2B**). On the other hand, symbiosis-regulated *Hydra* genes not specific for *Chlorella* A99 (**Figure 1C** Symbiosis-regulated**, Supplementary Table 2**) appear not to be upregulated in a light-dependent manner (**Supplementary Figure 4**). These genes are involved in *Hydra´*s innate immune system (e.g. proteins containing Toll/interleukin-1 receptor domain or Death domain) or in signal transduction (C-type mannose receptor, ephrin receptor, proline-rich transmembrane protein 1, “protein-kinase, interferon-inducible double stranded RNA dependent inhibitor, repressor of (p58 repressor)”). That particular transcriptional changes observed in *Hydra* rely solely on the photosynthetic activity of *Chlorella* A99 was confirmed by substituting the dark incubation with selective chemical photosynthesis inhibitor DCMU (Dichorophenyl-dimethylurea) (Vandermeulen et al., 1972), which resulted in a similar effect (**Figure 2C, D**).

Symbiont-dependent *Hydra* genes are expressed in endodermal epithelial cells and upregulated by sugars

To further characterize the photobiont induced *Hydra* genes, we performed whole mount *in situ* hybridization (**Figure 3A-F**) and quantified transcripts by qPCR using templates from isolated endoderm and ectoderm (**Supplementary Figure 5**), again comparing symbiotic and aposymbiotic polyps (**Figure 3 G-I**). The GS-1 gene and the Spot14 gene are expressed both in ectoderm and in endoderm (**Figure 3A, B**) and both genes are strongly upregulated in the presence of the photobiont (**Figure 3G, H**). In contrast, the Na/Pi gene was expressed only in the endoderm (**Figure 3C**) and there it was strongly upregulated by the photobiont (**Figure 3I**). Since *Chlorella* sp. A99 colonizes endodermal epithelial cells only, the impact of algae on symbiosis-dependent genes in both the ectodermal and the endodermal layer indicates that

photosynthetic products can be transported across these two tissue layers or some signals can be transduced by cell-cell communication.

To more closely dissect the nature of the functional interaction between *Hydra* and *Chlorella* and to explore the possibility that maltose released from the algae is involved in A99-specific gene regulation, we cultured aposymbiotic polyps (Hv_Apo) for 2 days in medium containing various concentrations of maltose (**Figure 3J**). Of the five A99 specific genes, GS-1 and the Spot14 gene were upregulated by maltose in a dose-dependent manner; the Na/Pi gene was only upregulated in 100mM maltose and the *Hydra* specific genes Sym-1 and Sym-2 did not show significant changes in expression by exposure to maltose (**Figure 3J**). This provides strong support for previous views that maltose excretion by symbiotic algae contributes to the stabilization of this symbiotic association (Cernichiari et al., 1969). When polyps were exposed to glucose instead of maltose, the genes of interest were also transcriptionally activated in a dose-dependent manner, while sucrose had no effect (**Supplementary Figure 6A-D**). Exposure to low concentrations of galactose increased transcriptional activity but at high concentration it did not, indicating a substrate inhibitor effect for this sugar. That the response to glucose is similar or even higher compared to maltose after 6 hours of treatment (**Supplementary Figure 6E**), suggests that *Hydra* cells transform maltose to glucose as a source of energy.

The *Chlorella A99* genome records a symbiotic life style

To better understand the symbiosis between *H. viridissima* and *Chlorella* and to refine our knowledge of the functions that are required in this symbiosis, we sequenced the genome of *Chlorella* sp. strain A99 and compared it to the genomes of other green algae. The genome of *Chlorella* sp. A99 was sequenced to approximately 211-fold coverage, enabling the generation of an assembly comprising a total of 40.9 Mbp (82 scaffolds, N50=1.7Mbp) (**Supplementary Table 3**). *Chlorella* sp. A99 belongs to the family *Chlorellaceae* (**Figure 4A**) and of the green algae whose genomes have been sequenced it is most closely related to *Chlorella variabilis* NC64A (NC64A) (Merchant et al., 2007; Palenik et al., 2007; Worden et al., 2009; Blanc et al., 2010; Prochnik et al., 2010; Blanc et al., 2012; Gao et al., 2014; Pombert et al., 2014). The genome size of the total assembly in strain A99 was similar to that of strain NC64A (46.2Mb) (**Figure 4B**). By k-mer analysis (k-mer = 19), the genome size of A99 was estimated to be 61 Mbp (Marcais and Kingsford, 2011). Its GC content of 68%, is the highest among the green algae species recorded (**Figure 4B**). In the A99 genome, 8298 gene models were predicted. As shown in **Figure 4C**, about 80% of these predicted genes have extensive sequence similarity to plant genes, while 13% so far have no similarity to genes of any other organisms (**Figure 4C**). It is also noteworthy that 7% of the A99 genes are similar to genes of other

kingdoms but not to *Hydra*, indicating the absence of gene transfer from *Hydra* to the photobiont genome (**Figure 4C**).

The *Chlorella* A99 genome provides evidences for extensive nitrogenous amino acid import and an incomplete nitrate assimilation pathway

Several independent lines of evidence demonstrate that nitrogen limitation and amino-acid metabolism have a key role in the *Chlorella–Hydra* symbiosis and that symbiotic *Chlorella* A99 depends on glutamine provided by its host (Rees, 1986; McAuley, 1987a, b, 1991; Rees, 1991) (Rees, 1989). To identify *Chlorella* candidate factors for the development and maintenance of the symbiotic life style, we therefore used the available genome information to assess genes potentially involved in amino acid transport and the nitrogen metabolic pathway.

When performing a search for the Pfam domain “Aa_trans” or “AA_permease” to find amino acid transporter genes in the A99 genome, we discovered numerous genes containing the Aa_trans domain (**Supplementary Table 4A**). In particular, A99 contains many orthologous genes of amino acid permease 2 and of transmembrane amino acid transporter family protein (solute carrier family 38, sodium-coupled neutral amino acid transporter), as well as NC64A (**Supplementary Table 4B, C**). Both of these gene products are known to transport neutral amino acids including glutamine. This observation is supporting the view that import of amino acids is an essential feature for the symbiotic way of life of *Chlorella*.

In nitrogen assimilation processes, plants usually take up nitrogen in the form of nitrate (NO_3^-) via nitrate transporters (NRTs) or as ammonium (NH_4^+) via ammonium transporters (AMT) (**Figure 5A**). In higher plants, two types of nitrate transporters, NRT1 and NRT2, have been identified (Krapp et al., 2014). Some NRT2 require nitrate assimilation-related component 2 (NAR2) to be functional (Quesada et al., 1994). NO_3^- is reduced to nitrite by nitrate reductase (NR), NO_2^- is transported to the chloroplast by nitrate assimilation-related component1 (NAR1), and NO_2^- is reduced to NH_4^+ by nitrite reductase (NiR). NH_4^+ is incorporated into glutamine (Gln) by glutamine synthetase (GS), and Gln is incorporated into glutamate (Glu) by NADH-dependent glutamine amide-2-oxoglutarate aminotransferase (GOGAT), also known as glutamate synthase. This pathway is highly conserved among plants. In the genomes of 10 green algae species sequenced so far, the major components of the pathway, including NRT1 and NRT2, NAR1 and NAR2, NR, NiR, AMT, GOGAT and GS, are all present, although NRT1 is absent in the *Micromonas pusilla* genome (Sanz-Luque et al., 2015).

Based on the annotation by Sanz-Luque et al. (Sanz-Luque et al., 2015), we searched these nitrogen assimilation genes in the *Chlorella* A99 genome, using ortholog grouping and a

reciprocal blast search using the protein sequences from other green algae **(Figure 5B, Supplementary Table 5**). As expected, the *Chlorella* A99 genome contains many homologues of the genes involved in nitrogen assimilation in plants including genes encoding NRT1, NAR1, NR, AMT, GS and GOGAT **(Figure 5B)**. Intriguingly, our systematic searches have failed to identify representative genes for NRT2, NAR2 and NiR in the *Chlorella* A99 genome (**Figure 5B**). We confirmed the absence of the NRT2 and NiR genes by PCR using primers designed for the conserved regions of these genes and which failed to produce a product with genomic DNA as a template **(Supplementary Figure 7).** Due to the weak sequence conservation of the NAR2 gene in the three algae genomes, PCR of that gene was not performed. Taken together, our observations indicate that *Chlorella* A99 algae appear to lack NRT2, NAR2 and NiR.

Since in many fungi, cyanobacteria and algae species, nitrate assimilation genes are known to act in concert and a gene cluster of NR and NiR genes is conserved between different green algae (Sanz-Luque et al., 2015), we next investigated the level of genomic clustering of the nitrate assimilation pathway genes in the *Chlorella* genome. Comparing the genomes of NC64A and *Coccomyxa subellipsoidea* C169 (C169) revealed the presence of a cluster of NR and NiR genes (**Figure 5C**). In NC64A, two NRT2 genes, together with genes for NAR2, NR and NiR are clustered on scaffold 21. In C169, one of NR genes and NiR are clustered together but the second NR gene is separate. Interestingly, analyzing the sequences around the NR gene in the *Chlorella* A99 genome provided no evidence for the presence of a co-localized NiR gene or any other nitrate assimilation genes, nor any conserved gene synteny to NC64A and C169 (**Figure 5C**). Our comparative genomic analyses therefore points to an incomplete as well as scattered nitrogen metabolic pathway in symbiotic *Chlorella* A99, which lacks essential transporters and enzymes for nitrate assimilation and also lacks the clustered structure of nitrate assimilation genes.

Supplementing the medium with glutamine allows temporary *in vitro* growth of symbiotic *Chlorella* A99

The absence of genes essential for nitrate assimilation in the *Chlorella* A99 genome (**Figure 5**) is consistent with its inability to grow outside the *Hydra* host cell (Habetha and Bosch, 2005) and indicates that *Chlorella* symbionts are dependent on metabolites provided by their host. We hypothesized that *Chlorella* is unable to use nitrite and ammonium as a nitrogen source, and that it relies on *Hydra* assimilating ammonium to glutamine to serve as the nitrogen source. To test this hypothesis and to examine utilization of nitrogen compounds of A99, we isolated *Chlorella* A99 from Hv_Sym and cultivated it *in vitro* using modified bold basal medium (BBM) (Nichols and Bold, 1965) containing the same amount of nitrogen in the form of NO_3^-, NH_4^+, Gln or casamino acids (**Figure 6, Supplementary Table 6**). As controls, *Chlorella variabilis*

NC64A (NC64A) isolated from Hv_NC64A and free-living C169 were used. To confirm that the cultured A99 is not contamination, we amplified and sequenced the genomic region of the 18S rRNA gene by PCR (**Supplementary Figure 8**) and checked this against the genomic sequence of A99. Kamako et al. reported that free-living algae *Chlorella vulgaris* Beijerinck var. *vulgaris* grow in media containing only inorganic nitrogen compounds as well as in media containing casamino acids as a nitrogen source, while NC64A required amino acids for growth (Kamako et al., 2005). Consistent with these observations, C169 grew in all tested media and NC64A grew in media containing casamino acids and Gln, although its growth rate was quite low in presence of NH_4^+ and NO_3^- (**Figure 6**). Remarkably, *Chlorella* A99 increased in cell number for up to 8 days in media containing casamino acids and Gln (**Figure 6**). Similar to NC64A, A99 did not grow in presence of NH_4^+ and NO_3^-. The growth rates of both A99 and NC64A were higher in medium containing a mixture of amino acids (casamino acids) than the single amino acid Gln. In contrast to NC64A, A99 could not be cultivated permanently in casamino acids or glutamine supplemented medium, indicating that additional growth factors are necessary to maintain *in vitro* growth of this obligate symbiont. Thus, although *in vitro* growth of A99 can be promoted by adding Glu and amino acids to the medium, A99 cannot be cultured permanently in this enriched medium, indicating that other host derived factors remain to be uncovered.

Discussion

Sequencing of the *Chlorella* A99 genome in combination with the transcriptome analyses of symbiotic, aposymbiotic and NC64A-infected *H. viridissima* polyps has enabled the identification of genes with specific functions in this symbiotic partnership. The *Hydra*-*Chlorella* symbiosis links carbohydrate supply from the photobiont to glutamine synthesis by the host. Characteristics of the symbiont genome obviously reflect its adaptation to this way of life, including an increase in amino acid transporters and degeneration of the nitrate assimilation pathway. This conclusion is based on six observations: **(i)** Expression of some genes including GS-1, Spot 14 and NaPi is specifically upregulated in the presence of *Chlorella* A99 (**Fig. 1C, Supplementary Table 1**), and **(ii)** they are induced by both, photosynthetic activity of *Chlorella* and by supplying exogenous maltose or glucose (**Figure 2, 3J, Supplementary Figure 6**). These results indicate that maltose release by photosynthesis of the symbiont enhances nutrition supply including glutamine by the host (**Figure 7**). **(iii)** Symbiotic *Chlorella* A99 cannot be cultivated *in vitro* in medium containing a single inorganic nitrogen source (**Figure 6**). Since medium containing glutamine supports *in vitro* growth of A99, this organism appears to depend on glutamine provided by the *Hydra* host. **(iv)** The genome of *Chlorella* A99 contains multiple amino acid transporter genes (**Supplementary Table 4)**, but lacks genes involved in nitrate assimilation (**Figure 5**), pointing to amino acids as main source of nitrogen and a degenerated

nitrate assimilation pathway. As for ammonium, which is one of the main nitrogen sources in plants, previous studies have reported the inability of symbiotic algae to take up ammonium because of the low peri-algal pH (pH 4-5) that stimulates maltose release (Douglas and Smith, 1984; Rees, 1989; McAuley, 1991; Dorling et al., 1997). Since *Chlorella* apparently cannot use nitrite and ammonium as a nitrogen source, it seems that *Hydra* has to assimilate ammonium to glutamine and provides it to *Chlorella* A99 (**Figure 7**).

(*v*) While polyps with native symbiont *Chlorella* A99 grew faster than aposymbiotic ones, symbiosis with foreign algae NC64A had no effect on the growth of polyps at all (**Figure 1B**).

(*vi*) *Hydra* endodermal epithelial cells host significantly fewer NC64A algae than A99 (**Supplementary Figure 1**) providing additional support for the view of a tightly regulated codependent partnership in which exchange of nutrients appears to be the primary driving force.

Previous studies have reported that symbiotic *Chlorella* in green hydra releases significantly larger amounts of maltose than NC64A (Mews and Smith, 1982; Rees, 1989). In addition, Rees reported that *Hydra* polyps containing high maltose releasing algae had a high GS activity, whereas aposymbiotic *Hydra* or *Hydra* with a low maltose releasing algae had lower GS activity (Rees, 1986). Although the underlying mechanism of how maltose secretion and transportation from *Chlorella* is regulated is still unclear, the amount of maltose released by the symbiont could be an important symbiont-derived driver or stabilizer of the *Hydra–Chlorella* symbiosis.

Exchange of nitrogenous compounds and photosynthetic products between host and symbiont is widely found in other symbiotic associations. For example, in marine invertebrates such as corals, sea anemones, and giant clams associated with *Symbiodinium* algae, the algae provide the photosynthate in forms of glucose, glycerol, organic acids, amino acids or lipids to their host, and in turn the symbionts receive ammonia or glutamine as nitrogen sources (Burriesci et al., 2012; Davy et al., 2012; Kellogg and Patton, 1983; Lewis and Smith, 1971; Muscatine, 1965; Muscatine and Cernichiari, 1969; 1993; Trench, 1971; Venn et al., 2008; Whitehead and Douglas, 2003; Yellowlees et al., 2008). Moreover, in corals a Na/Pi transporter is involved in the uptake of phosphate across host membranes, and the zooxanthellae contribute to the uptake of phosphate (D'Elia, 1977; Jackson et al., 1989). These observations together with the results presented here make the host-controlled supply of nitrogen and phosphorus as a response of a signal photosynthate seem the universal principle of invertebrate-algae symbiosis.

Metabolic dependence of symbionts on host supply occasionally results in genome reduction and gene loss. For example, the symbiotic *Buchnera* bacteria of insects are missing particular genes in essential amino acid pathways (Shigenobu et al., 2000; Hansen et al., 2011). The fact that the corresponding genes of the host are upregulated in the bacteriocyte, indicates complementarity and syntrophy between host and symbiont. Similarly, in *Chlorella* A99 the

nitrogen assimilation system could have been lost as result of continuous supply of nitrogenous amino acids provided by *Hydra*. On the other hand, the genome size and total gene number of *Chlorella* A99 is similar to other species in the class Trebouxiophyceae (**Figure 4B**). The apparently unchanged complexity of the Chlorella A99 genome suggests a relatively early stage of this symbiotic partnership. From these observation, we propose that the gene loss in metabolic pathway is the first step of genome reduction caused by dependency on nutrients from the host. Our study suggests metabolic-codependency is the primary driving force for the evolution of symbiosis between *Hydra* and *Chlorella*.

Materials and methods

Biological materials and procedures

Experiments were carried out with the Australian *Hydra viridissima* strain A99, which was obtained from Dr. Richard Campbell, Irvine. Polyps were maintained at 18°C on a 12 hours light/dark cycle and fed with *Artemia* two or three times a week. Aposymbiotic (algae free) polyps were obtained by photobleaching using 5 µM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) as described before (Pardy, 1976; Habetha et al., 2003). Experiments were carried out with polyps starved for 3-6 days. Isolation of endodermal layer and ectodermal layer was performed as described by Kishimoto et al. (Kishimoto et al., 1996). Symbiotic *Chlorella* were isolated as described before by Muscatine and McAuley (Muscatine, 1983; McAuley, 1986b). *Chlorella variabilis* NC64A (NIES-2541), *Coccomyxa subellipsoidea* C-169 (NIES-2166) and *Chlamydomonas reinhardtii* (NIES-2235) were obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (Tsukuba, Japan).

Nucleic acid preparation

Total RNA of *Hydra* was extracted by use of the Trizol reagent and PureLink RNA Mini Kit (Life Technology) after lysis and removal of algae by centrifugation. The genomic DNA of green algae was extracted using ISOPLANT II (Nippon Gene, Tokyo, Japan) following DNase I treatment to degrade contaminant DNA. Quantity and quality of DNA and RNA were checked by NanoDrop (Thermo Scientific Inc., Madison, USA) and BioAnalyzer (Agilent Technologies, Santa Clara, USA).

Microarray Analysis

cRNA targets labeled with cyanine-3 were synthesized from 400 ng total *Hydra* RNA using a Quick Amp Labeling Kit for one color detection (Agilent Technologies). A set of fluorescently labeled cRNA targets was employed in a hybridization reaction with 4 × 44K Custom-Made *Hydra viridissima* Microarray (Agilent Technologies) contributing a total of 43,222 transcripts that was built by mRNA-seq data (NCBI GEO Platform ID: GPL23280) (Bosch et al., 2009).

Hybridization and washing were performed using the GE Hybridization Kit and GE Wash Pack (Agilent Technologies) after which the arrays were scanned on an Agilent Technologies G2565BA microarray scanner system with SureScan technology following protocols according to the manufacturer's instructions. The intensity of probes was extracted from scanned microarray images using Feature Extraction 10.7 software (Agilent Technologies). All algorithms and parameters used in this analysis were used with default conditions. Background-subtracted signal-intensity values (gProcessedSignal) generated by the Feature Extraction software were normalized using the 75th percentile signal intensity among the microarray. Those genes differentially expressed between two samples were determined by average of fold change (cut off >2.0) and Student's t-test (P< 0.1). The data series are accessible at NCBI GEO under accession number GSE97633.

Quantitative real time RT-PCR

Total RNA was reverse transcribed using First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada). Real-time PCR was performed using GoTaq qPCR Master Mix (Promega, Madison, USA) and ABI Prism 7,300 (Applied Biosystems, Foster City, USA). All qPCR experiments were performed in duplicate with three biological replicates each. Values were normalized using the expression of the tubulin alpha gene. Primers used for these experiments are listed in **Supplementary Table 7A.**

Whole mount *in situ* hybridization

Expression patterns of specific *Hydra* genes were detected by whole mount *in situ* hybridization with digoxigenin (DIG)-labelled RNA probes. Specimens were fixed in 4% paraformaldehyde. Hybridization signal was visualized using anti-DIG antibodies conjugated to alkaline phosphatase and NBT/BCIP staining solution (Roche). DIG-labeled sense probes (targeting the same sequences as the antisense probes) were used as a control. Primers used for these experiments are listed in **Supplementary Table 7B.**

Genome sequencing and gene prediction

For genome sequencing of *Chlorella* sp. A99, *Chlorella* sp. A99 was isolated from *H. viridissima* A99 and genomic DNA was extracted. Paired-end library (insert size: 740 bp) and mate-pair libraries (insert size: 2.2 and 15.2kb) were made using Illumina TruSeq DNA LT Sample Prep Kit and Nextera Mate Pair Sample Preparation Kit respectively (Illumina Inc., San Diego, USA), following the manufacturer's protocols. Genome sequencing was performed using Illumina Miseq and Hiseq 2000 platforms. Sequence reads were assembled using Newbler Assembler version 2.8 (Roche, Penzberg, Germany) and subsequent scaffolding was performed by SSPACE (Boetzer et al., 2011). Gaps inside the scaffolds were closed with the paired-end and mate-pair data using GapCloser of Short Oligonucleotide Analysis Package (Luo et al., 2012). To

459 overcome potential assembly errors arising from tandem repeats, sequences that aligned to
460 another sequence by more than 50% of the length using blastn (1e-50) were removed from
461 the assembly. The completeness of the genome was evaluated using CEGMA v2.4 (Core
462 Eukaryotic Genes Mapping Approach) based on mapping of the 248 most highly conserved
463 core eukaryotic genes (CEGs) on the assembled genome (Parra et al., 2007). The
464 completeness of complete and partial CEGs in the A99 scaffolds was 80% and 88%,
465 respectively. The fraction of repetitive sequences was 12%. Gene models was predicted by
466 AUGUSTUS 3.0.1 using model parameters for NC64A (Stanke et al., 2006). This Whole
467 Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession
468 PCFQ00000000 (BioProject ID: PRJNA412448). The genome sequences and gene models
469 are also accessible at website of OIST Marine Genomics Unit Genome Project
470 (http://marinegenomics.oist.jp/chlorellaA99/viewer/info?project_id=65).
471
472 **Analysis of genes in *Hydra viridissima* and *Chlorella***
473 Annotation of transcriptome contigs and prediction of gene models was performed by use of
474 BLAST, Gene Ontology (The Gene Ontology et al., 2000) and blast2go (Conesa et al., 2005).
475 To examine the conservation of *H. viridissima* contigs among metazoans, homology searches
476 by blastx (evalue 1E-5) were performed using protein databases obtained from NCBI for
477 *Drosophila melanogaster* and *Homo sapiens*, from the JGI genome portal
478 (http://genome.jgi.doe.gov/) for *Branchiostoma floridae*, *Nematostella vectensis*, from
479 Echinobase (http://www.echinobase.org/EchinoBase/) for *Strongylocentrotus pupuratus*, from
480 Compagen for *Hydra magnipapillata*, and from the OIST marine genomics Genome browser
481 ver.1.1 (http://marinegenomics.oist.jp/coral/viewer/info?project_id=3) for *Acropora digitifera*.
482
483 For comparative analysis of gene models of *Chlorella* sp. A99 and other algae, domain
484 searches against the Pfam database (Pfam-A.hmm) were performed using HMMER (Eddy,
485 1998; Finn et al., 2016), and ortholog gene grouping was done using OrthoFinder (Emms and
486 Kelly, 2015). The sequences of the reference genes and genomes were obtained from the
487 database of the JGI genome portal for *Chlorella variabilis* NC64A, *Coccomyxa subellipsoidea*
488 C-169, *Volvox carteri*, *Micromonas pusilla*, and *Ostreococcus tauri*, from NCBI for
489 *Auxenochlorella protothecoides* 0710 and from Phytozome
490 (http://phytozome.jgi.doe.gov/pz/portal.html) for *Chlamydomonas reinhardtii* (Merchant et al.,
491 2007; Worden et al., 2009; Blanc et al., 2010; Prochnik et al., 2010; Blanc et al., 2012; Gao et
492 al., 2014; Pombert et al., 2014)
493
494 Nitrogen assimilation genes in *Chlorella* A99 were identified by orthologous gene groups and
495 reciprocal blast searches. The number of genes for nitrate assimilation genes, glutamine
496 synthetase and glutamate synthetase , and clustering of such genes were systematically

reported by (Sanz-Luque et al., 2015). We used these data as reference for searches of nitrogen assimilation genes, and further nitrogen assimilation genes were searched by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Kanehisa and Goto, 2000). JGI genome browsers of *Chlorella variabilis* NC64A and *Coccomyxa subellipsoidea* C-169 were also used for retrieving genes and checking gene order on the scaffolds.

Phylogenetic analysis

For a phylogenetic tree of chlorophyte green algae, the sequences of 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene were obtained from scaffold20 of *Chlorella* A99 genome sequence, and from NCBI nucleotide database entries for *Chlorella variabilis* NC64A (FM205849.1), *Auxenochlorella protothecoides* 0710 (NW_011934479.1), *Coccomyxa subellipsoidea* C169 (AGSI01000011.1), *Volvox carteri* f. nagariensis (NW_003307662.1), *Chlamydomonas reinhardtii* (FR865576.1), *Ostreococcus tauri* (GQ426340.1) and *Micromonas pusilla* (FN562452.1). Multiple alignments were produced with CLUSTALX (2.1) with gap trimming (Larkin et al., 2007). Sequences of poor quality that did not well align were deleted using BioEdit (Hall, 1999). Phylogenetic analyses were performed using the Neighbor-Joining method by CLUSTALX. Representative phylogenetic trees were drawn by using NJ plot (Perriere and Gouy, 1996).

PCR amplification of nitrate assimilation genes in green algae

Primers were designed based on the conserved region of the NRT2 gene, NiR and NR genes (positive control) identified by comparison of genes from *Chlorella variabilis* NC64A (NC64A), *Coccomyxa subellipsoidea* C169 (C169), and *Chlamydomonas reinhardtii* (Cr) which belongs to Chlorophyceae class of green algae. Primers for NAR2 could not be designed because of insufficient conservation. As positive controls, amplicons were produced for NR of all the green algae examined and of NRT2 and NiR from NC64A, C169 and Cr, after which their sequences were checked. KOD FX Neo (TOYOBO, Tokyo, Japan) was used under the following conditions: an initial denaturation phase (94 °C for 120 sec) followed by 36 cycles of (98 °C for 30 sec, 69 °C for 100 sec) for NiR, (98 °C for 30 sec, 58 °C for 30 sec and 68 °C for 210 sec) for NRT2 and (98 °C for 30 sec, 59 °C for 30 sec and 68 °C for 60 sec) for NR. In each case, 10 ng gDNA was used as a template. The primers used are described in **Supplementary Table 7C**. PCR products were sequenced to confirm amplification of the target genes using ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific Inc., Madison, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

***In vitro* culture of algae**

To isolate symbiotic algae, polyps were quickly homogenized in 0.25% sodium dodecyl sulfate (SDS) solution and centrifuged at 3000g for 1 min. The pellet was resuspended in 0.05% SDS

and centrifuged at 500g for 5min. Isolated A99, NC64A and C169 were washed by sterilized Bold Basal Medium (Bischoff and Bold, 1963) modified by the addition of 0.5% glucose, 1.2mg/L vitamine B1 (Thiaminhydrochloride), 0.01mg/L vitamine B12 (Cyanocobalamin) (**Supplementary Table 6**) and incubated for two days in modified Bold Basal Medium with 50mg/l ampicillin and streptomycin. The algae were cultivated in 5 ml of modified Bold Basal Medium (BBM) with the same amount of nitrogen (2.9 mM $NaNO_3$, NH_4Cl, glutamine or 426 mg/l casamino acids) and 5mg/l Carbendazim (anti-fungal) with fluorescent illumination (12 hour light, 12 hour dark) at 20˚C. Mean numbers of algae per ml were calculated from three tubes enumerated at 4, 8, and 12 days after inoculation with 10^6 cell/sml using a hemocytometer. After cultivation, gDNA was isolated from the A99 cultured in Gln-containing BBM and casamino acid-containing BBM and A99 was isolated from green hydra directly. A partial genomic region of the 18S rRNA gene was amplified by PCR and sequenced to confirm absence of contamination by other algae. PCR was performed using AmpliTaq Gold (Thermo Fisher Scientific). Sequencing was performed as described above. The primers used are described in **Supplementary Table 7D**.

Acknowledgement

We thank Trudy Wassenaar for critical reading the text and for discussion. We also thank the DNA Sequencing Section in OIST for genome sequencing. This work was supported in part by JSPS KAKENHI Grant-in-Aid for Young Scientists (B) 25840132 and Scientific Research (C) 15K07173 to M.H. and by the Deutsche Forschungsgemeinschaft (DFG) (CRC1182 “Origin and Function of Metaorganisms”). T.C.G.B. gratefully appreciates support from the Canadian Institute for Advanced Research (CIFAR).

Competing interests

The authors declare that no competing interests exist.

References

Baumgarten, S., Simakov, O., Esherick, L.Y., Liew, Y.J., Lehnert, E.M., Michell, C.T., Li, Y., Hambleton, E.A., Guse, A., Oates, M.E., Gough, J., Weis, V.M., Aranda, M., Pringle, J.R., Voolstra, C.R., 2015. The genome of Aiptasia, a sea anemone model for coral symbiosis. Proceedings of the National Academy of Sciences 112, 11893-11898. 10.1073/pnas.1513318112

Bischoff, H.W., Bold, H.C., 1963. Some soil algae from enchanted rock and related Algal species. [s.n.], Austin.

Blanc, G., Agarkova, I., Grimwood, J., Kuo, A., Brueggeman, A., Dunigan, D.D., Gurnon, J.,

573 Ladunga, I., Lindquist, E., Lucas, S., Pangilinan, J., Proschold, T., Salamov, A., Schmutz, J., Weeks,
574 D., Yamada, T., Lomsadze, A., Borodovsky, M., Claverie, J.M., Grigoriev, I.V., Van Etten, J.L., 2012.
575 The genome of the polar eukaryotic microalga Coccomyxa subellipsoidea reveals traits of cold
576 adaptation. Genome Biol. 13, R39. 10.1186/gb-2012-13-5-r39
577 Blanc, G., Duncan, G., Agarkova, I., Borodovsky, M., Gurnon, J., Kuo, A., Lindquist, E., Lucas, S.,
578 Pangilinan, J., Polle, J., Salamov, A., Terry, A., Yamada, T., Dunigan, D.D., Grigoriev, I.V., Claverie,
579 J.M., Van Etten, J.L., 2010. The Chlorella variabilis NC64A genome reveals adaptation to
580 photosymbiosis, coevolution with viruses, and cryptic sex. Plant Cell 22, 2943-2955.
581 10.1105/tpc.110.076406
582 Boetzer, M., Henkel, C.V., Jansen, H.J., Butler, D., Pirovano, W., 2011. Scaffolding pre-assembled
583 contigs using SSPACE. Bioinformatics 27, 578-579. 10.1093/bioinformatics/btq683
584 Bosch, T.C., Augustin, R., Anton-Erxleben, F., Fraune, S., Hemmrich, G., Zill, H., Rosenstiel, P.,
585 Jacobs, G., Schreiber, S., Leippe, M., Stanisak, M., Grotzinger, J., Jung, S., Podschun, R., Bartels,
586 J., Harder, J., Schroder, J.M., 2009. Uncovering the evolutionary history of innate immunity: the
587 simple metazoan Hydra uses epithelial cells for host defence. Dev. Comp. Immunol. 33, 559-569.
588 10.1016/j.dci.2008.10.004
589 Bossert, P., Dunn, K.W., 1986. Regulation of intracellular algae by various strains of the symbiotic
590 Hydra viridissima. J. Cell Sci. 85, 187-195
591 Brown, S.B., Maloney, M., Kinlaw, W.B., 1997. "Spot 14" protein functions at the pretranslational
592 level in the regulation of hepatic metabolism by thyroid hormone and glucose. J. Biol. Chem. 272,
593 2163-2166
594 Campbell, R.D., 1990. Transmission of symbiotic algae through sexual reproduction in hydra:
595 movement of algae into the oocyte. Tissue Cell 22, 137-147
596 Cernichiari, E., Muscatine, L., Smith, D.C., 1969. Maltose Excretion by the Symbiotic Algae of
597 Hydra viridis. Proc. R. Soc. Lond., Ser. B: Biol. Sci. 173, 557-576. 10.1098/rspb.1969.0077
598 Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., Robles, M., 2005. Blast2GO: a
599 universal tool for annotation, visualization and analysis in functional genomics research.
600 Bioinformatics 21, 3674-3676. 10.1093/bioinformatics/bti610
601 Cook, C.B., Kelty, M.O., 1982. Glycogen, protein, and lipid content of green, aposymbiotic, and
602 nonsymbiotic hydra during starvation. J. Exp. Zool. 222, 1-9. 10.1002/jez.1402220102
603 Davy, S.K., Allemand, D., Weis, V.M., 2012a. Cell Biology of Cnidarian-Dinoflagellate Symbiosis.
604 Microbiology and Molecular Biology Reviews : MMBR 76, 229-261. 10.1128/MMBR.05014-11
605 Davy, S.K., Allemand, D., Weis, V.M., 2012b. Cell biology of cnidarian-dinoflagellate symbiosis.
606 Microbiol. Mol. Biol. Rev. 76, 229-261. 10.1128/MMBR.05014-11
607 Dorling, M., McAuley, P.J., Hodge, H., 1997. Effect of pH on growth and carbon metabolism of
608 maltose-releasingChlorella(Chlorophyta). Eur. J. Phycol. 32, 19-24.
609 10.1080/09541449710001719335
610 Douglas, A., Smith, D.C., 1984. The Green Hydra Symbiosis. VIII. Mechanisms in Symbiont

611 Regulation. Proceedings of the Royal Society of London. Series B. Biological Sciences 221, 291-319.
612 10.1098/rspb.1984.0035
613 Douglas, A.E., 1994. Symbiotic interactions / by A.E. Douglas. Oxford University Press, Oxford ;
614 New York.
615 Douglas, A.E., Smith, D.C., 1983. The cost of symbionts to the host in the green hydra symbiosis.
616 W. DeGriiyter and Co. Berlin.
617 Eddy, S.R., 1998. Profile hidden Markov models. Bioinformatics 14, 755-763
618 Emms, D.M., Kelly, S., 2015. OrthoFinder: solving fundamental biases in whole genome
619 comparisons dramatically improves orthogroup inference accuracy. Genome Biol. 16, 157.
620 10.1186/s13059-015-0721-2
621 Finn, R.D., Coggill, P., Eberhardt, R.Y., Eddy, S.R., Mistry, J., Mitchell, A.L., Potter, S.C., Punta,
622 M., Qureshi, M., Sangrador-Vegas, A., Salazar, G.A., Tate, J., Bateman, A., 2016. The Pfam protein
623 families database: towards a more sustainable future. Nucleic Acids Res. 44, D279-285.
624 10.1093/nar/gkv1344
625 Gao, C., Wang, Y., Shen, Y., Yan, D., He, X., Dai, J., Wu, Q., 2014. Oil accumulation mechanisms of
626 the oleaginous microalga Chlorella protothecoides revealed through its genome, transcriptomes,
627 and proteomes. BMC Genomics 15, 582. 10.1186/1471-2164-15-582
628 Habetha, M., Anton-Erxleben, F., Neumann, K., Bosch, T.C., 2003. The Hydra viridis/Chlorella
629 symbiosis. Growth and sexual differentiation in polyps without symbionts. Zoology 106, 101-108.
630 10.1078/0944-2006-00104
631 Habetha, M., Bosch, T.C., 2005. Symbiotic Hydra express a plant-like peroxidase gene during
632 oogenesis. J. Exp. Biol. 208, 2157-2165. 10.1242/jeb.01571
633 Hall, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for
634 Windows 95/98/NT. Nucleic Acid Symposium Series 41, 95-98
635 Huss, V.A.R., Holweg, C., Seidel, B., Reich, V., Rahat, M., Kessler, E., 1993/1994. There is an
636 ecological basis for host/symbiont specificity in Chlorella/Hydra symbioses. Endocytobiosis and Cell
637 Research 10, 35-46
638 Ishikawa, M., Yuyama, I., Shimizu, H., Nozawa, M., Ikeo, K., Gojobori, T., 2016. Different
639 Endosymbiotic Interactions in Two Hydra Species Reflect the Evolutionary History of
640 Endosymbiosis. Genome Biol. Evol. 8, 2155-2163. 10.1093/gbe/evw142
641 Joy, J.B., 2013. Symbiosis catalyses niche expansion and diversification. Proceedings of the Royal
642 Society B: Biological Sciences 280. 10.1098/rspb.2012.2820
643 Kamako, S.-i., Hoshina, R., Ueno, S., Imamura, N., 2005. Establishment of axenic endosymbiotic
644 strains of Japanese Paramecium bursaria and the utilization of carbohydrate and nitrogen
645 compounds by the isolated algae. Eur. J. Protistol. 41, 193-202. 10.1016/j.ejop.2005.04.001
646 Kanehisa, M., Goto, S., 2000. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res.
647 28, 27-30
648 Karakashian, S.J., Karakashian, M.W., 1965. Evolution and Symbiosis in the Genus Chlorella and

649 Related Algae. Evolution 19, 368-377
650 Kawaida, H., Ohba, K., Koutake, Y., Shimizu, H., Tachida, H., Kobayakawa, Y., 2013. Symbiosis
651 between hydra and chlorella: molecular phylogenetic analysis and experimental study provide
652 insight into its origin and evolution. Mol. Phylogenet. Evol. 66, 906-914.
653 10.1016/j.ympev.2012.11.018
654 Khalturin, K., Hemmrich, G., Fraune, S., Augustin, R., Bosch, T.C., 2009. More than just orphans:
655 are taxonomically-restricted genes important in evolution? Trends Genet. 25, 404-413.
656 10.1016/j.tig.2009.07.006
657 Kishimoto, Y., Murate, M., Sugiyama, T., 1996. Hydra regeneration from recombined ectodermal
658 and endodermal tissue. I. Epibolic ectodermal spreading is driven by cell intercalation. J. Cell Sci.
659 109, 763-772
660 Krapp, A., David, L.C., Chardin, C., Girin, T., Marmagne, A., Leprince, A.-S., Chaillou, S., Ferrario-
661 Méry, S., Meyer, C., Daniel-Vedele, F., 2014. Nitrate transport and signalling in Arabidopsis. J. Exp.
662 Bot. 65, 789-798. 10.1093/jxb/eru001
663 Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin,
664 F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W
665 and Clustal X version 2.0. Bioinformatics 23, 2947-2948. 10.1093/bioinformatics/btm404
666 Lehnert, E.M., Mouchka, M.E., Burriesci, M.S., Gallo, N.D., Schwarz, J.A., Pringle, J.R., 2014.
667 Extensive Differences in Gene Expression Between Symbiotic and Aposymbiotic Cnidarians. G3:
668 Genes|Genomes|Genetics 4, 277-295. 10.1534/g3.113.009084
669 Liaw, S.-H., Kuo, I., Eisenberg, D., 1995. Discovery of the ammonium substrate site on glutamine
670 synthetase, A third cation binding site. Protein Sci. 4, 2358-2365. 10.1002/pro.5560041114
671 Luo, R., Liu, B., Xie, Y., Li, Z., Huang, W., Yuan, J., He, G., Chen, Y., Pan, Q., Liu, Y., Tang, J., Wu,
672 G., Zhang, H., Shi, Y., Liu, Y., Yu, C., Wang, B., Lu, Y., Han, C., Cheung, D.W., Yiu, S.M., Peng, S.,
673 Xiaoqian, Z., Liu, G., Liao, X., Li, Y., Yang, H., Wang, J., Lam, T.W., Wang, J., 2012. SOAPdenovo2:
674 an empirically improved memory-efficient short-read de novo assembler. Gigascience 1, 18.
675 10.1186/2047-217X-1-18
676 Marcais, G., Kingsford, C., 2011. A fast, lock-free approach for efficient parallel counting of
677 occurrences of k-mers. Bioinformatics 27, 764-770. 10.1093/bioinformatics/btr011
678 Martinez, D.E., Iniguez, A.R., Percell, K.M., Willner, J.B., Signorovitch, J., Campbell, R.D., 2010.
679 Phylogeny and biogeography of Hydra (Cnidaria: Hydridae) using mitochondrial and nuclear DNA
680 sequences. Mol. Phylogenet. Evol. 57, 403-410. 10.1016/j.ympev.2010.06.016
681 McAuley, P.J., 1986a. The cell cycle of symbiotic Chlorella. III. Numbers of algae in green hydra
682 digestive cells are regulated at digestive cell division. J. Cell Sci. 85, 63-71
683 McAuley, P.J., 1986b. Isolation of viable uncontaminated Chlorella from green hydra. Limnol.
684 Oceanogr. 31, 222–224. 10.4319/lo.1986.31.1.0222
685 McAuley, P.J., 1987a. Nitrogen limitation and amino-acid metabolism of Chlorella symbiotic with
686 green hydra. Planta 171, 532-538. 10.1007/bf00392303

McAuley, P.J., 1987b. Quantitative Estimation of Movement of an Amino Acid from Host to Chlorella Symbionts in Green Hydra. Biol. Bull. 173, 504-512. 10.2307/1541696

McAuley, P.J., 1991. Amino acids as a nitrogen source for Chlorella symbiotic with green hydra, in: Williams, R.B., Cornelius, P.F.S., Hughes, R.G., Robson, E.A. (Eds.), Coelenterate Biology: Recent Research on Cnidaria and Ctenophora: Proceedings of the Fifth International Conference on Coelenterate Biology, 1989. Springer Netherlands, Dordrecht, pp. 369-376. 10.1007/978-94-011-3240-4_53

McAuley, P.J., Smith, D.C., 1982. The Green Hydra Symbiosis. V. Stages in the Intracellular Recognition of Algal Symbionts by Digestive Cells. Proc. R. Soc. Lond., Ser. B: Biol. Sci. 216, 7-23

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C.G., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S.F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A.H., Kremer, N., Mazmanian, S.K., Metcalf, J.L., Nealson, K., Pierce, N.E., Rawls, J.F., Reid, A., Ruby, E.G., Rumpho, M., Sanders, J.G., Tautz, D., Wernegreen, J.J., 2013. Animals in a bacterial world, a new imperative for the life sciences. Proceedings of the National Academy of Sciences 110, 3229-3236. 10.1073/pnas.1218525110

Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., Terry, A., Salamov, A., Fritz-Laylin, L.K., Marechal-Drouard, L., Marshall, W.F., Qu, L.H., Nelson, D.R., Sanderfoot, A.A., Spalding, M.H., Kapitonov, V.V., Ren, Q., Ferris, P., Lindquist, E., Shapiro, H., Lucas, S.M., Grimwood, J., Schmutz, J., Cardol, P., Cerutti, H., Chanfreau, G., Chen, C.L., Cognat, V., Croft, M.T., Dent, R., Dutcher, S., Fernandez, E., Fukuzawa, H., Gonzalez-Ballester, D., Gonzalez-Halphen, D., Hallmann, A., Hanikenne, M., Hippler, M., Inwood, W., Jabbari, K., Kalanon, M., Kuras, R., Lefebvre, P.A., Lemaire, S.D., Lobanov, A.V., Lohr, M., Manuell, A., Meier, I., Mets, L., Mittag, M., Mittelmeier, T., Moroney, J.V., Moseley, J., Napoli, C., Nedelcu, A.M., Niyogi, K., Novoselov, S.V., Paulsen, I.T., Pazour, G., Purton, S., Ral, J.P., Riano-Pachon, D.M., Riekhof, W., Rymarquis, L., Schroda, M., Stern, D., Umen, J., Willows, R., Wilson, N., Zimmer, S.L., Allmer, J., Balk, J., Bisova, K., Chen, C.J., Elias, M., Gendler, K., Hauser, C., Lamb, M.R., Ledford, H., Long, J.C., Minagawa, J., Page, M.D., Pan, J., Pootakham, W., Roje, S., Rose, A., Stahlberg, E., Terauchi, A.M., Yang, P., Ball, S., Bowler, C., Dieckmann, C.L., Gladyshev, V.N., Green, P., Jorgensen, R., Mayfield, S., Mueller-Roeber, B., Rajamani, S., Sayre, R.T., Brokstein, P., Dubchak, I., Goodstein, D., Hornick, L., Huang, Y.W., Jhaveri, J., Luo, Y., Martinez, D., Ngau, W.C., Otillar, B., Poliakov, A., Porter, A., Szajkowski, L., Werner, G., Zhou, K., Grigoriev, I.V., Rokhsar, D.S., Grossman, A.R., 2007. The Chlamydomonas genome reveals the evolution of key animal and plant functions. Science 318, 245-250. 10.1126/science.1143609

Mews, L.K., 1980. The Green Hydra Symbiosis. III. The Biotrophic transport of Carbohydrate from Alga to Animal. Proc. R. Soc. Lond., Ser. B: Biol. Sci. 209, 377-401. 10.1098/rspb.1980.0101

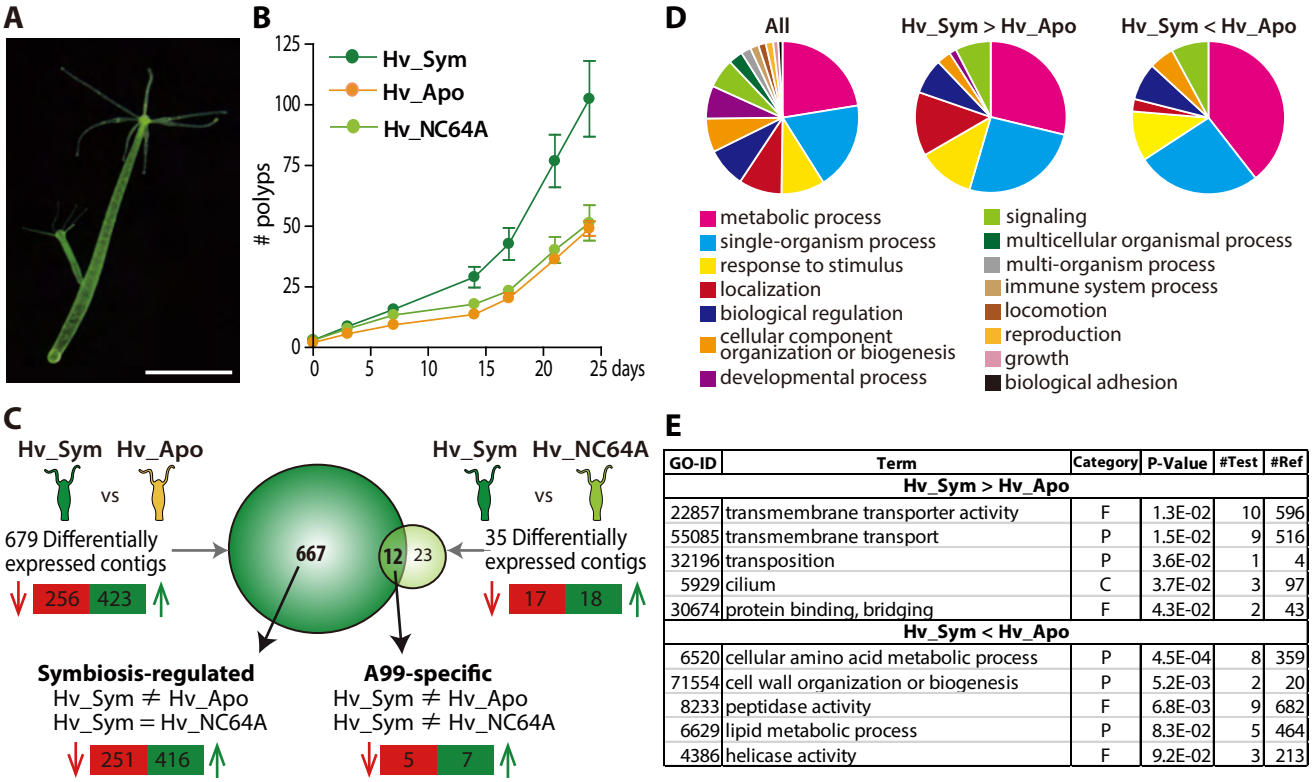
Mews, L.K., Smith, D.C., 1982. The Green Hydra Symbiosis. VI. What is the Role of Maltose Transfer from Alga to Animal? Proceedings of the Royal Society of London. Series B. Biological Sciences 216, 397-413. 10.1098/rspb.1982.0083

725 Moran, N.A., 2007. Symbiosis as an adaptive process and source of phenotypic complexity. Proc.
726 Natl. Acad. Sci. U. S. A. 104, 8627-8633. 10.1073/pnas.0611659104
727 Murer, H., Biber, J., 1996. Molecular mechanisms of renal apical Na/phosphate cotransport. Annu.
728 Rev. Physiol. 58, 607-618. 10.1146/annurev.ph.58.030196.003135
729 Muscatine, L., 1965. Symbiosis of hydra and algae. 3. Extracellular products of the algae. Comp.
730 Biochem. Physiol. 16, 77-92
731 Muscatine, L., 1983. Hydra: Research Methods. Plenum Press, New York.
732 Muscatine, L., Lenhoff, H.M., 1963. Symbiosis: On the Role of Algae Symbiotic with Hydra. Science
733 142, 956-958. 10.1126/science.142.3594.956
734 Muscatine, L., Lenhoff, H.M., 1965a. Symbiosis of Hydra and Algae. I. Effects of Some
735 Environmental Cations on Growth of Symbiotic and Aposymbiotic Hydra. Biol. Bull. 128, 415-424.
736 10.2307/1539903
737 Muscatine, L., Lenhoff, H.M., 1965b. Symbiosis of Hydra and Algae. II. Effects of Limited Food and
738 Starvation on Growth of Symbiotic and Aposymbiotic Hydra. Biol. Bull. 129, 316-328
739 Muscatine, L., McAuley, P.J., 1982. Transmission of symbiotic algae to eggs of green hydra. Cytobios
740 33, 111-124
741 Nichols, H.W., Bold, H.C., 1965. Trichosacinapolymorpha Gen. et sp. NOV. Journal of Phycology 1,
742 34-38
743 Palenik, B., Grimwood, J., Aerts, A., Rouze, P., Salamov, A., Putnam, N., Dupont, C., Jorgensen, R.,
744 Derelle, E., Rombauts, S., Zhou, K., Otillar, R., Merchant, S.S., Podell, S., Gaasterland, T., Napoli,
745 C., Gendler, K., Manuell, A., Tai, V., Vallon, O., Piganeau, G., Jancek, S., Heijde, M., Jabbari, K.,
746 Bowler, C., Lohr, M., Robbens, S., Werner, G., Dubchak, I., Pazour, G.J., Ren, Q., Paulsen, I.,
747 Delwiche, C., Schmutz, J., Rokhsar, D., Van de Peer, Y., Moreau, H., Grigoriev, I.V., 2007. The tiny
748 eukaryote Ostreococcus provides genomic insights into the paradox of plankton speciation. Proc.
749 Natl. Acad. Sci. U. S. A. 104, 7705-7710. 10.1073/pnas.0611046104
750 Pardy, R.L., 1976. The morphology of green hydra endosymbionts as influenced by host strain and
751 host environment. J. Cell Sci. 20
752 Parra, G., Bradnam, K., Korf, I., 2007. CEGMA: a pipeline to accurately annotate core genes in
753 eukaryotic genomes. Bioinformatics 23. 10.1093/bioinformatics/btm071
754 Perriere, G., Gouy, M., 1996. WWW-query: an on-line retrieval system for biological sequence banks.
755 Biochimie 78, 364-369
756 Pombert, J.F., Blouin, N.A., Lane, C., Boucias, D., Keeling, P.J., 2014. A lack of parasitic reduction
757 in the obligate parasitic green alga Helicosporidium. PLoS Genet. 10, e1004355.
758 10.1371/journal.pgen.1004355
759 Prochnik, S.E., Umen, J., Nedelcu, A.M., Hallmann, A., Miller, S.M., Nishii, I., Ferris, P., Kuo, A.,
760 Mitros, T., Fritz-Laylin, L.K., Hellsten, U., Chapman, J., Simakov, O., Rensing, S.A., Terry, A.,
761 Pangilinan, J., Kapitonov, V., Jurka, J., Salamov, A., Shapiro, H., Schmutz, J., Grimwood, J.,
762 Lindquist, E., Lucas, S., Grigoriev, I.V., Schmitt, R., Kirk, D., Rokhsar, D.S., 2010. Genomic analysis

763 of organismal complexity in the multicellular green alga Volvox carteri. Science 329, 223-226.
764 10.1126/science.1188800
765 Quesada, A., Galvan, A., Fernandez, E., 1994. Identification of nitrate transporter genes in
766 Chlamydomonas reinhardtii. Plant J. 5, 407-419
767 Rahat, M., Reich, V., 1984. Intracellular infection of aposymbiotic Hydra viridis by a foreign free-
768 living Chlorella sp.: initiation of a stable symbiosis. J. Cell Sci. 65, 265-277
769 Rees, T.A.V., 1986. The Green Hydra Symbiosis and Ammonium I. The Role of the Host in
770 Ammonium Assimilation and its Possible Regulatory Significance. Proc. R. Soc. Lond., Ser. B: Biol.
771 Sci. 229, 299-314. 10.1098/rspb.1986.0087
772 Rees, T.A.V., 1989. The Green Hydra Symbiosis and Ammonium II. Ammonium Assimilation and
773 Release by Freshly Isolated Symbionts and Cultured Algae. Proc. R. Soc. Lond., Ser. B: Biol. Sci.
774 235
775 Rees, T.A.V., 1991. Are Symbiotic Algae Nutrient Deficient? Proc. R. Soc. Lond. B. Biol. Sci. 243,
776 227-233. 10.1098/rspb.1991.0036
777 Roffman, B., Lenhoff, H.M., 1969. Formation of Polysaccharides by Hydra from Substrates
778 produced by their Endosymbiotic Algae. Nature 221, 381-382
779 Sanz-Luque, E., Chamizo-Ampudia, A., Llamas, A., Galvan, A., Fernandez, E., 2015.
780 Understanding nitrate assimilation and its regulation in microalgae. Front Plant Sci 6, 899.
781 10.3389/fpls.2015.00899
782 Schwentner, M., Bosch, T.C., 2015. Revisiting the age, evolutionary history and species level
783 diversity of the genus Hydra (Cnidaria: Hydrozoa). Mol. Phylogenet. Evol. 91, 41-55.
784 10.1016/j.ympev.2015.05.013
785 Shinzato, C., Shoguchi, E., Kawashima, T., Hamada, M., Hisata, K., Tanaka, M., Fujie, M.,
786 Fujiwara, M., Koyanagi, R., Ikuta, T., Fujiyama, A., Miller, D.J., Satoh, N., 2011. Using the
787 Acropora digitifera genome to understand coral responses to environmental change. Nature 476,
788 320-323. 10.1038/nature10249
789 Stanke, M., Keller, O., Gunduz, I., Hayes, A., Waack, S., Morgenstern, B., 2006. AUGUSTUS: ab
790 initio prediction of alternative transcripts. Nucleic Acids Res. 34. 10.1093/nar/gkl200
791 Tao, T.Y., Towle, H.C., 1986. Coordinate regulation of rat liver genes by thyroid hormone and dietary
792 carbohydrate. Ann. N. Y. Acad. Sci. 478, 20-30
793 Tautz, D., Domazet-Loso, T., 2011. The evolutionary origin of orphan genes. Nat. Rev. Genet. 12,
794 692-702. 10.1038/nrg3053
795 The Gene Ontology, C., Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M.,
796 Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L.,
797 Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., Sherlock, G.,
798 2000. Gene Ontology: tool for the unification of biology. Nat. Genet. 25, 25-29. 10.1038/75556
799 Thorington, G., Margulis, L., 1981. Hydra viridis: transfer of metabolites between Hydra and
800 symbiotic algae. The Biological Bulletin 160, 175-188. 10.2307/1540911

801 Vandermeulen, J.H., Davis, N.D., Muscatine, L., 1972. The effect of inhibitors of photosynthesis on
802 zooxanthellae in corals and other marine invertebrates. Mar. Biol. 16, 185-191. 10.1007/bf00346940
803 Worden, A.Z., Lee, J.H., Mock, T., Rouze, P., Simmons, M.P., Aerts, A.L., Allen, A.E., Cuvelier, M.L.,
804 Derelle, E., Everett, M.V., Foulon, E., Grimwood, J., Gundlach, H., Henrissat, B., Napoli, C.,
805 McDonald, S.M., Parker, M.S., Rombauts, S., Salamov, A., Von Dassow, P., Badger, J.H., Coutinho,
806 P.M., Demir, E., Dubchak, I., Gentemann, C., Eikrem, W., Gready, J.E., John, U., Lanier, W.,
807 Lindquist, E.A., Lucas, S., Mayer, K.F., Moreau, H., Not, F., Otillar, R., Panaud, O., Pangilinan, J.,
808 Paulsen, I., Piegu, B., Poliakov, A., Robbens, S., Schmutz, J., Toulza, E., Wyss, T., Zelensky, A., Zhou,
809 K., Armbrust, E.V., Bhattacharya, D., Goodenough, U.W., Van de Peer, Y., Grigoriev, I.V., 2009.
810 Green evolution and dynamic adaptations revealed by genomes of the marine picoeukaryotes
811 Micromonas. Science 324, 268-272. 10.1126/science.1167222
812

Figures



E

GO-ID	Term	Category	P-Value	#Test	#Ref
	Hv_Sym > Hv_Apo				
22857	transmembrane transporter activity	F	1.3E-02	10	596
55085	transmembrane transport	P	1.5E-02	9	516
32196	transposition	P	3.6E-02	1	4
5929	cilium	C	3.7E-02	3	97
30674	protein binding, bridging	F	4.3E-02	2	43
	Hv_Sym < Hv_Apo				
6520	cellular amino acid metabolic process	P	4.5E-04	8	359
71554	cell wall organization or biogenesis	P	5.2E-03	2	20
8233	peptidase activity	F	6.8E-03	9	682
6629	lipid metabolic process	P	8.3E-02	5	464
4386	helicase activity	F	9.2E-02	3	213

Figure 1. *Hydra* growth and differential expression of Hydra genes resulting from symbiosis

(A) *Hydra viridissima* strain A99 used for this study. Scale bar, 2 mm. (B) Growth rates of polyps grown with native symbiotic Chlorella A99 (Hv_Sym, dark green), Aposymbiotic polyps from which Chlorella were removed (Hv_Apo, orange) and aposymbiotic polyps reinfected with Chlorella variabilis NC64A (Hv_NC64A, light green). (C) Graphic representation of differentially expressed genes identified by microarray. The transcriptome of Hv_Sym is compared with that of Hv_Apo and Hv_NC64A with the number of down-regulated contigs in Hv_Sym shown in red and those up-regulated in green. Genes differentially expressed in Hv_Sym compared to both Hv_Apo and Hv_NC64A are given as “A99-specific”, those differentially expressed between Hv_A99 and Hv_Apo but not Hv_NC64A as “Symbiosis-regulated”. (D) GO distribution of Biological Process at level 2 in all contigs (All), up-regulated contigs (Hv_Sym > Hv_Apo) and down-regulated contigs (Hv_Sym < Hv_Apo) in Hv_Sym. (E) Overrepresented GO terms in up-regulated contigs (Hv_Sym > Hv_Apo) and down-regulated contigs (Hv_Sym < Hv_Apo). Category, F: molecular function, C: cellular component, P: biological process. P-values, probability of Fisher’s exact test. #Test, number of corresponding contigs in differentially expressed contigs. #Ref, number of corresponding contigs in all contigs.

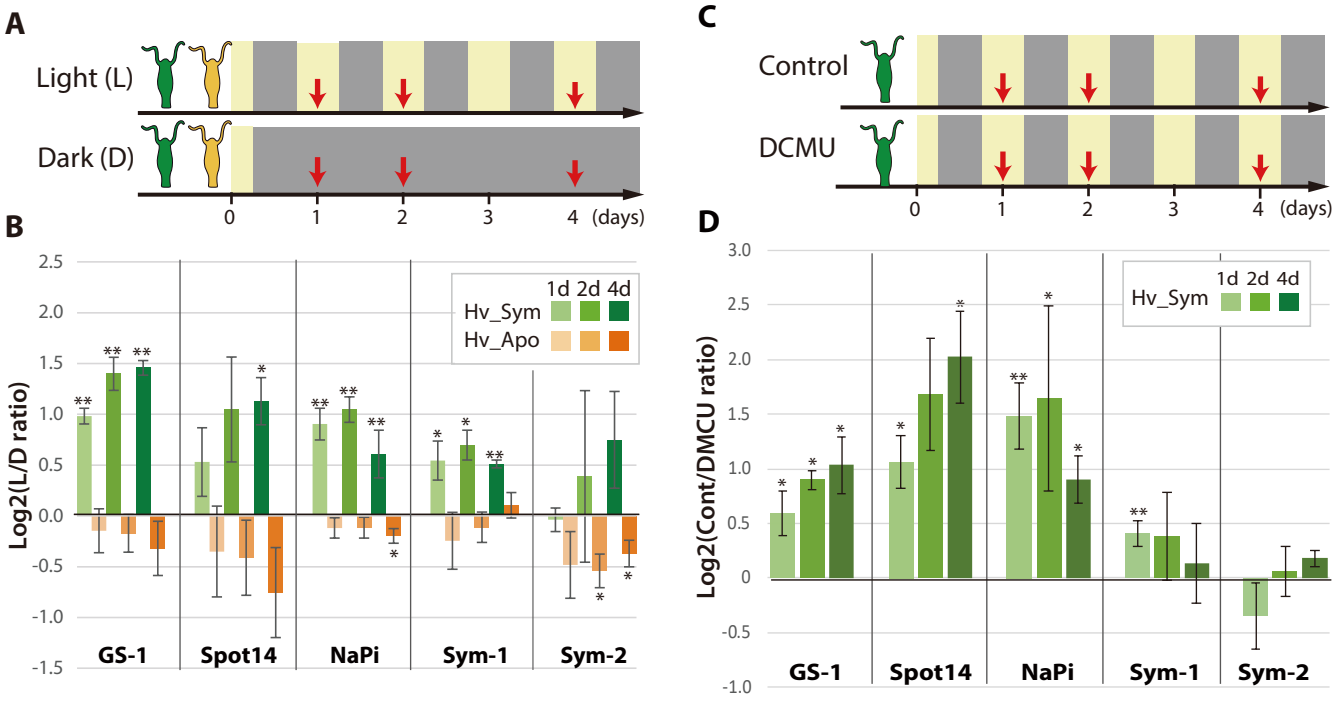


Figure 2. Differential expression of *Hydra* genes under influence of *Chlorella* photosynthesis

(A) Sampling scheme. Hv_Sym (green) and Hv_Apo (orange) were cultured under a standard light-dark regime (Light: L) and in continuous darkness (Dark: D), and RNA was extracted from the polyps at the days indicated by red arrows. (B) Expression difference of five A99-specific genes in Hv_Sym (green bars) and Hv_Apo (orange bars) between the light-dark condition and darkness. The vertical axis shows log scale (log2) fold changes of relative expression level in Light over Dark. (C) Sampling scheme of inhibiting photosynthesis. (D) Differential expression of the five A99-specific genes under conditions allowing (Control) or inhibiting photosynthesis (DCMU). The vertical axis shows log scale (log2) fold changes of relative expression level in Control over DCMU treated. T-tests were performed between Light and Dark

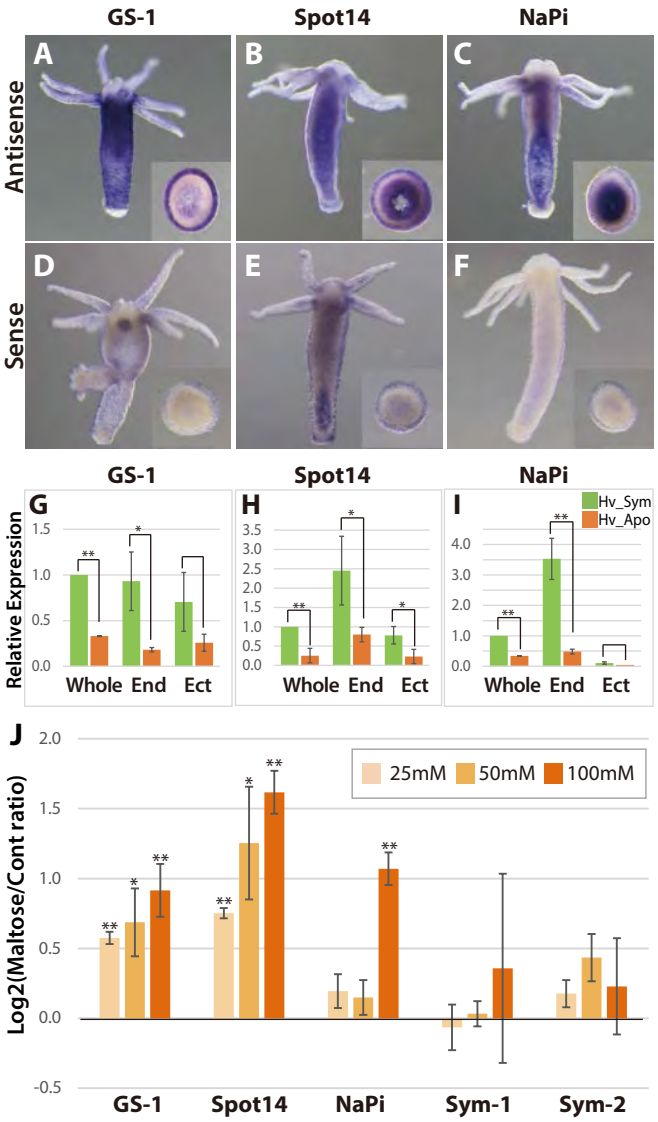
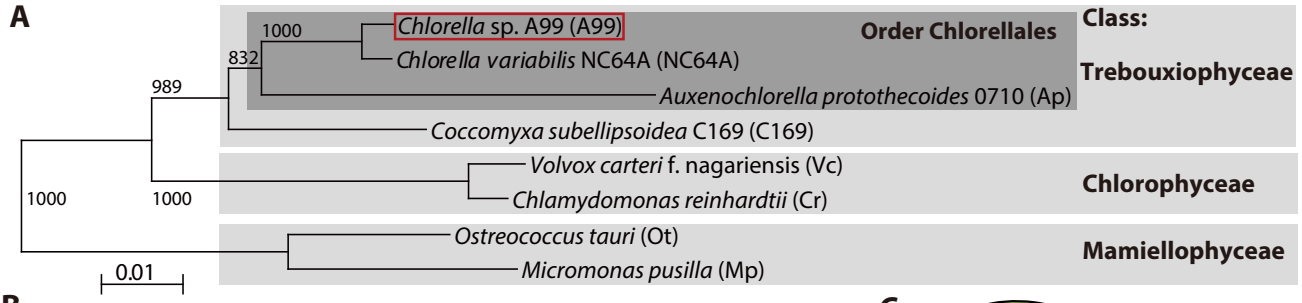


Figure 3. Spatial expression patterns of genes coding for glutamine synthetase, Spot 14 and Na/Pi-transporter.

A-F; Whole mount in situ hybridization using antisense (A-C) and sense probes (D-F; negative controls) for glutamine synthetase-1 (GS-1; left), Spot 14 (center) and Na/Pi-transporter (NaPi; right). Inserts show cross sections of the polyp' s body. (G-I) Relative expression levels of whole animal (whole), isolated endoderm (End) and isolated ectoderm (Ect) tissue of Hv_Sym (green bars) and Hv_Apo (orange bars). T-test was performed between Hv_Sym and Hv_apo. Pvalue, * <0.05, ** <0.01. (J) Expression change of genes GS-1, Spot14, NaPi, Sym-1 and Sym-2 following exposure to 25mM, 50mM and 100mM maltose in Hv_Apo. The vertical axis shows log scale (log2) fold changes of relative expression level of maltose-treated over the untreated Hv_Apo control. T-test was performed between maltose-treated and control. Pvalue, * <0.05, ** <0.01.



B

Phylum	Chlorophyta								
Class	Trebouxiophyceae					Chlorophyceae		Mamiellophyceae	
Order	Chlorellales								
	Symbiotic		Parasitic	Free-living					
Species	**A99**	**NC64A**	**Hel**	**Ap**	**C169**	**Cr**	**Vc**	**Mp**	**Ot**
Assembly length (Mb)	40.9	46.2	12.4	22.9	48.8	121	138	21.9	12.6
Number of gene	8298	9791	6035	7039	9851	15143	14520	10575	8166
GC%	68	67	62	63	53	64	56	65	58

C

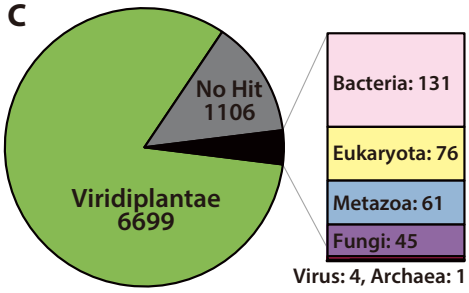
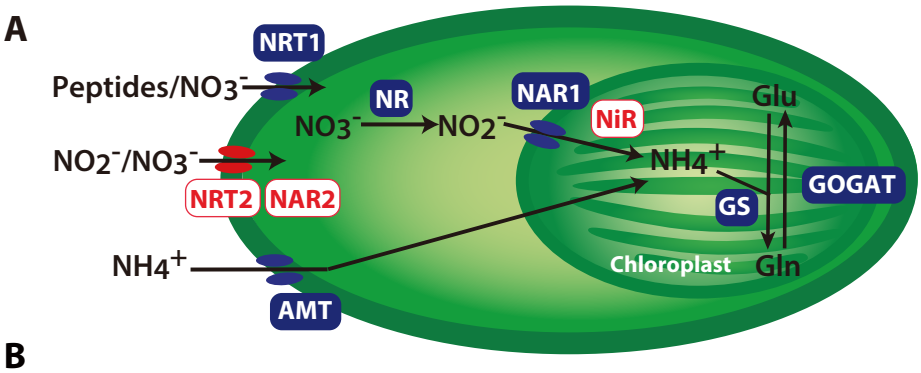


Figure 4. Comparison of key features deduced from the *Chlorella* A99 genome with other green algae

(A) Phylogenetic tree of eight genome sequenced chlorophyte green algae including ***Chlorella*** sp. A99. The NJ tree is based on sequences of the 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene. (B) Genomic features and taxonomy of the sequenced chlorophyte green algae. Hel: *Helicosporidium* sp. ATCC50920. (C) The proportion of similarity of Chlorella A99 gene models to those of other organisms.



B

GeneName		Gene Description	A99	NC64A	c169	Cr	Vc	Mp	Ot
nitrate/nitrite transporter	NRT1	nitrate/peptide transporter family, low affinity nitrate transporter	1	2	2	1	1	0	1
	NRT2	high affnity nitrate/nitrite transporter	0	2	1	6	3	1	1
nitrate assimilation-related components	NAR1	formate-nitrite transporter family	4	3	2	6	5	1	1
	NAR2	nitrate high-affinity transporter accessory	0	1	1	1	1	1	1
nitrate reductase	NR	nitrate reductase (NAD(P)H)	1	1	2	1	1	2	1
nitrite reductase	NiR	ferredoxin-nitrite reductase	0	1	1	2	1	1	1
ammonium transporter	AMT	ammonium transporter, Rh	0	1	2	3	0	0	0
		ammonium transporter, Amt family	3	5	2	8	10	5	4
glutamate synthase	GOGAT	glutamate synthase	1	2	2	2	2	2	1
glutamine synthetase	GS	glutamine synthetase	2	2	4	4	4	1	1

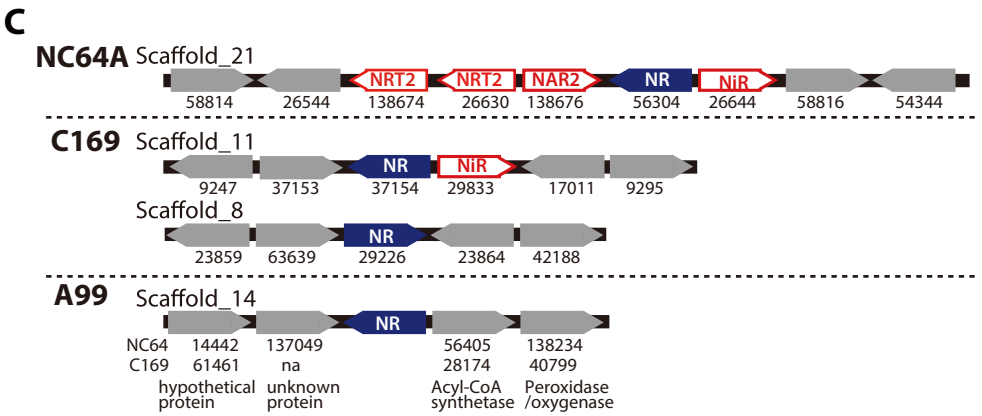


Figure 5. Nitrogen assimilation pathways in *Chlorella* A99

(A) Schematic diagram of the nitrogen assimilation pathway in plants showing the function of nitrate transporters NRT1 (peptides/nitrate transporter) and NRT2 (nitrate/nitrite transporter), nitrate assimilation-related components NAR1 and NAR2, nitrate reductase NR, nitrite reductase NiR, ammonium transporter AMT, glutamate synthetase GOGAT and glutamine synthetase GS. Genes shown in red boxes (NRT2, NAR2 and NiR) were not found in the *Chlorella* sp. A99 genome. (B) Table showing the number of nitrogen assimilation genes in *Chlorella* sp. A99 (A99), *Chlorella variabilis* NC64A (NC64A), *Coccomyxa subellipsoidea* C169 (C169), *Volvox carteri* f. nagariensis (Vc), *Chlamydomonas reinhardtii* (Cr), *Ostreococcus tauri* (Ot) and *Micromonas pusilla* (Mp). (C) Gene clusters of nitrate assimilation genes around the shared NR genes (blue) in the genomes of NC64A, C169 and A99. Red boxes show nitrate assimilation genes absent in A99 and gray boxes depict other genes. Numbers below the boxes are JGI protein IDs of NC64A and C169. Numbers below the genes of A99 are JGI protein IDs of the best hit genes in NC64A and C169 and their gene name.

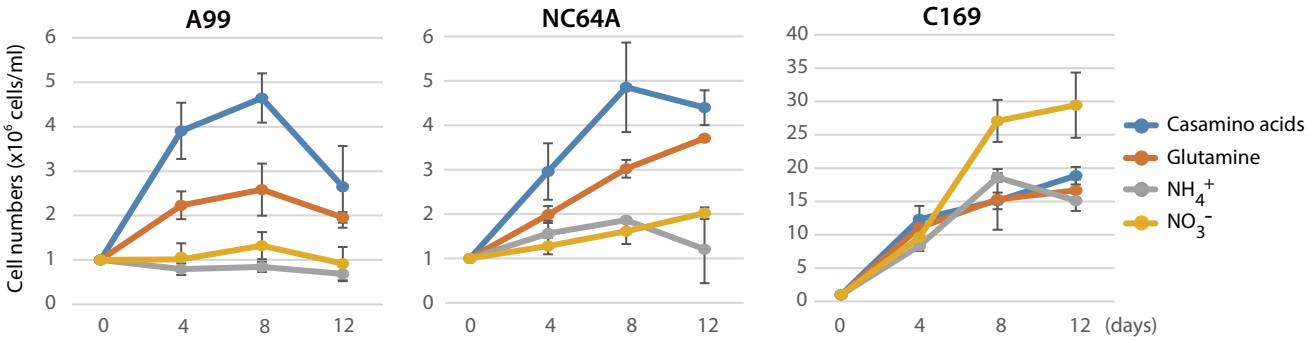


Figure 6. Growth of green algae in presence of various nitrogen sources

The growth rate of *Chlorella* A99 (A99), *Chlorella variabilis* NC64A (NC64A) and *Coccomyxa subellipsoidea* C-169 (C169) by in vitro culture was assessed for different nitrogen sources with casamino acids (blue), glutamine (orange), ammonium (gray) and nitrate (yellow). Mean number of algae per ml were determined at 4, 8, 12 days after inoculation with 106 cell/ml.

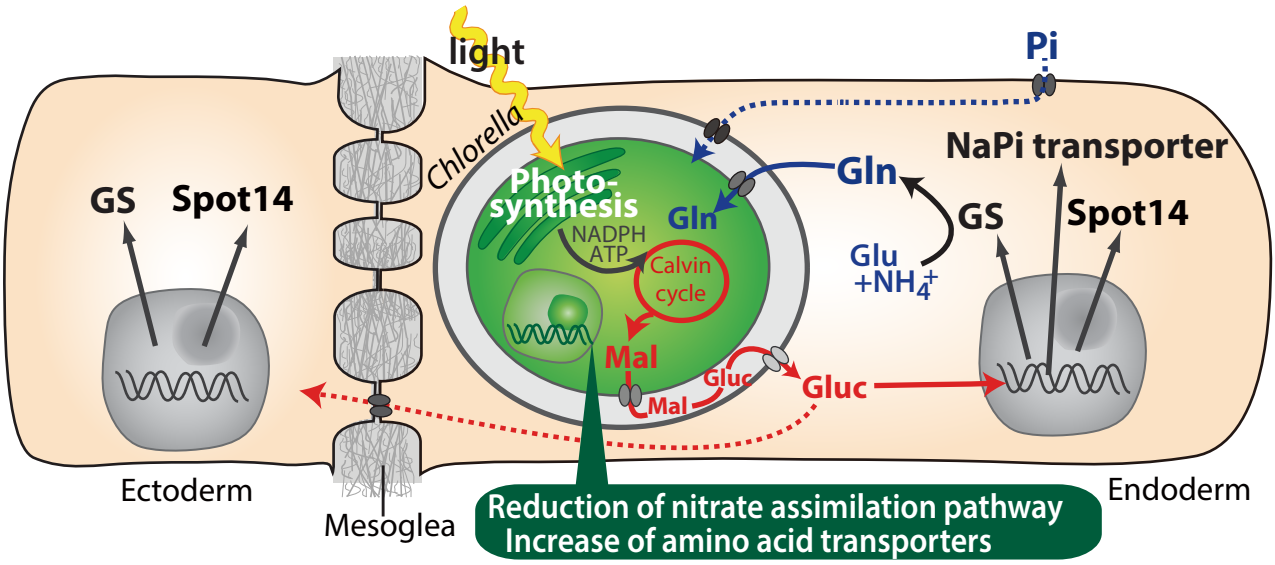


Figure. 7. Molecular interactions in the symbiosis between green hydra and Chlorella A99

During light conditions, *Chlorella* performs photosynthesis and produces maltose (Mal) which is secreted into the Hydra symbiosome where it is possibly digested to glucose (Gluc), shown in red. The sugar induces expression of hydra genes encoding glutamine synthetase (GS), Na/Pi transporter (NaPi) and Spot14. GS synthesizes glutamine (Gln) from glutamate (Glu) and ammonium (NH4+). Gln is used by *Chlorella* as a nitrogen source. Since the sugar also upregulates the gene for NaPi which controls intracellular phosphate levels, it might be involved in the supply of phosphorus to *Chlorella* as well (blue broken line). The sugar is transmitted or defused to the ectoderm (red broken line) and there induces the expression of GS and Spot14. In the *Chlorella* A99 genome, degeneration of the nitrate assimilation system and an increase of amino acid transporters was observed (green balloon).

Supplemental Tables

Supplemental Table 1.

List of the A99 specific genes differentially expressed in Hv_Sym compared to both Hv_Apo and Hv_NC64A and fold changes of expression level examined by microarray and qPCR.

A99>Apo, NC64A

Probe name (gene ID)	Microarray		qPCR		Seq Description (1)	InterProScan
	A99/Apo	A99/NC64A	A99/Apo	A99/NC64A		
rc_13579	12.8	4.0	11.2	4.0	uncharacterized protein LOC101238438 [Hydra vulgaris]	
rc_12891	9.0	2.9	14.6	6.9	(Hydra viridis specific)	
27417	4.5	4.8	3.0	3.0		IPR009786 Spot_14
rc_26218	3.3	2.4	2.5	2.3		PTHR10010 Sodium-dependent phosphate transport protein 2C
1046	3.1	2.1	2.2	1.6	glutamine synthetase-like	

A99<Apo, NC64A

Probe name (gene ID)	Microarray		qPCR		Seq Description	InterProScan
	Apo/A99	NC64A/A99	Apo/A99	NC64A/A99		
NPNHRC_26859	83.2	9.7	∞	∞	(Hydra viridis specific)	
RC_FVQRUGK01AXSJ	13.7	2.6	2.1	1.5	acetoacetyl-CoA synthetase-like	
rc_14793	7.2	4.1	9.4	4.8	2-isopropylmalate synthase	IPR013785 Aldolase_TIM,
FV81RT002HT2FL	2.8	2.0	3.1	1.8	histidine ammonia-lyase-like	IPR001106 Aromatic_Lyase IPR008948 L-Aspartase-like
NPNHRC_12201	2.7	2.3	2.6	2.5	(Hydra viridis specific)	

(1) Gene annotations by blast2go.

Supplemental Table 2.
List of the genes differentially expressed between Hv_Sym and Hv_Apo and fold changes of expression level examined by microarray and qPCR.

A99>Apo

Probe name (gene ID)	Microarray	qPCR	HS_Best Hit (1)	InterProScan (2)
	A99/Apo	A99/Apo		
5168	9.3	7.4		IPR000157 TIR_dom PTHR23097 Tumor necrosis factor receptor superfamily member
6508	6.7	2.9		IPR011029:DEATH-like_dom
11411	2.9	2.0	C-type mannose receptor 2	IPR000742 EG-like_dom IPR001304 C-type_lectin
26108	7.2	7.2	ephrin type-A receptor 6 isoform a	no IPS match
rc_2417	5.4	3.5		IPR000488 Death_domain
rc_24563	6.1	6.7	Proline-rich transmembrane protein 1	IPR007593 CD225/Dispanin_fam PTHR14948 NG5
rc_9398	6.2	5.4	protein-kinase, interferon-inducible double stranded RNA dependent inhibitor, repressor of (P58 repressor)	PTHR11697 general transcription factor 2-related zinc finger protein

A99<Apo

Probe name (gene ID)	Microarray	qPCR	HS_best hit	InterProScan
	Apo/A99	Apo/A99		
rc_10789	2.5	3.7	endoribonuclease Dicer	IPR000999 RNase_III_dom PTHR1495 helicase-related
rc_12826	3.0	2.3	interferon regulatory factor 1	IPR001346 Interferon_reg_fact_DNA-bd_dom; IPR011991 WHTH_DNA-bd_dom PTHR11949 interferon regulatory factor
rc_8898	6.1	4.1	leucine-rich repeat-containing protein 15 isoform b	IPR001611 Leu-rich_rp PTHR24373 Toll-like receptor 9
FV81RT001CSTY	3.2	2.0	astrocytic phosphoprotein PEA-15	IPR001875 DED, IPR011029 DEATH-like_dom
RSASM_17752	4.0	2.1	CD97 antigen isoform 2 precursor	IPR000832 GPCR_2_secretin-like PTHR12011 vasoactive intestinal polypeptide receptor 2

(1) Best hit human genes. (2) Results of domain search.

Supplemental Table 3
Summary of sequence data for assembling Chlorella sp. A99 genome sequences

Number of reads	85469010	
Number of reads assembled	61838513	
Number of bases	17398635102	
	Scaffolds	**Contigs**
Total length of sequence	40934037	40687875
Total number of sequences	82	7455
Maximum length of sequence	4003385	171868
N50	1727419	12747
GC contents (%)	68.07 %	69.95 %

Supplemental Table 4

A. The number of Pfam domains related to amino acids transport

Pfam Domain Name	A99	NC64A	c169	Cr	Vc	Mp	Ot
Aa_trans	30	38	21	9	7	9	8
AA_permease	4	6	15	5	6	1	1

B. Ortholog groups including Aa_trans containing genes

Ortholog Group ID	A99	NC64A	c169	Cr	Vc	Mp	Ot
OG0000040	12	12	6	3	1	0	0
OG0000324	6	7	1	2	1	0	0
OG0001336	2	1	1	1	1	1	1
OG0004053	1	2	2	1	0	0	0
OG0006517	1	1	0	0	0	0	0
OG0001069	1	2	3	1	1	1	1
OG0000830	1	4	1	2	1	2	1
OG0002190	1	1	1	1	1	1	1
OG0011340	1	0	0	0	0	0	0
OG0004863	1	5	0	0	0	0	0
OG0000468	2	2	2	2	2	2	2
OG0003354	2	1	1	1	1	1	1
OG0003801	1	1	0	1	1	1	1

C. Blast best hit genes of Arabidopsis thaliana of genes belonging to OG0000040 and OG0000324

OG0000040	Best Hit gene ID	Best Hit gene of Arabidopsis thaliana	e-value
scaffold1.g5447.t1	NP_196484.1	amino acid permease 2	4E-62
scaffold1.g5579.t1	NP_196484.1	amino acid permease 2	7E-32
scaffold12.g8277.t1	NP_196484.1	amino acid permease 2	1E-34
scaffold13.g380.t1	NP_196484.1	amino acid permease 2	6E-12
scaffold14.g1284.t1	NP_196484.1	amino acid permease 2	2E-33
scaffold2.g7119.t1	NP_001318716.1	lysine histidine transporter 1	9E-73
scaffold2.g7251.t1	NP_196484.1	amino acid permease 2	3E-59
scaffold21.g2221.t1	NP_175076.2	amino acid permease 5	9E-60
scaffold40.g5168.t1	NP_196484.1	amino acid permease 2	5E-38
scaffold5.g864.t1	NP_196484.1	amino acid permease 2	2E-60
scaffold6.g2644.t1	NP_196484.1	amino acid permease 2	2E-58
scaffold6.g2815.t1	NP_186825.2	Transmembrane amino acid transporter family protein	3E-43

OG0000324	**Best Hit gene ID**	**Best Hit gene of Arabidopsis thaliana**	**e-value**
scaffold10.g2481.t1	NP_001330273.1	Transmembrane amino acid transporter family protein	7E-06
scaffold11.g3916.t1*	NP_172258.1	Histone superfamily protein	1E-47
	NP_565239.1	Transmembrane amino acid transporter family protein	7E-40
scaffold15.g4364.t1	NP_566854.1	Transmembrane amino acid transporter family protein	8E-31
scaffold2.g7197.t1	NP_566854.1	Transmembrane amino acid transporter family protein	2E-24
scaffold21.g2185.t1	NP_565239.1	Transmembrane amino acid transporter family protein	5E-14
scaffold3.g6325.t1	NP_566854.1	Transmembrane amino acid transporter family protein	6E-34

* This sequence consists from a region similar to NP_172258.1 and a region similar to NP_565239.1.

Supplementary Table 5

Sequence ID of nitrogen assimilation genes

A99	NC64A	c169	Cr	Vol	Mp	Ot
GOGAT (glutamate synthase)*						
scaffold18.g1969.t1	33619	22625	Cre12.g514050	Vocar.0006s0290	57115	29431
	142154	53183	Cre13.g592200	Vocar.0064s0005	70244	
GS (Glutamine synthetase)*						
scaffold6.g2861.t1	56005	23194	Cre02.g113200	Vocar.0001s1591	4228	15060
scaffold7.g3389.t1	143431	23517	Cre03.g207250	Vocar.0011s0254		
		30043	Cre12.g530600	Vocar.0028s0089		
		31742	Cre12.g530650	Vocar.0028s0090		
AMT (ammonium transporter, Rh)						
na	21763	65570	Cre06.g284100	na	na	na
		65572	Cre06.g284150			
AMT (ammonium transporter, Amt family)						
scaffold2.g7405.t1	36096	47532	Cre02.g111050	Vocar.0001s1695	29536	29863
scaffold2.g7406.t1	56592	52218	Cre03.g159254	Vocar.0008s0224	45964	18135
scaffold3.g6262.t1	58614		Cre06.g293051	Vocar.0019s0251	48406	25714
	136742		Cre07.g355650	Vocar.0022s0058	50351	29181
	141357		Cre09.g400750	Vocar.0028s0082	59331	
			Cre12.g531000	Vocar.0049s0040		
			Cre13.g569850	Vocar.0051s0018		
			Cre14.g629920	Vocar.0054s0046		
				Vocar.0063s0027		
				Vocar.0069s0013		
NRT2 (high affnity nitrate/nitrite transporter)*						
na	26630	28993	Cre02.g110800	Vocar.0008s0137	49583	24168
	138674		Cre03.g150101	Vocar.0008s0138		
			Cre03.g150151	Vocar.0008s0200		
			Cre09.g396000			
			Cre09.g410800			
			Cre09.g410850			
NRT1 (nitrate/peptide transporter family, low affinity nitrate transporter)*						
scaffold14.g1044.t1	23105	19259	Cre04.g224700	Vocar.0049s0020	na	2706
	144528	48785				
NAR1 (formate-nitrite transporter family)*						

scaffold12.g8267.t1	34412	20872	Cre01.g012050	Vocar.0007s0061	70731	19784
scaffold18.g1993.t1	25301	46302	Cre04.g217915	Vocar.0008s0135		
scaffold4.g4586.t1	53335		Cre06.g309000	Vocar.0011s0110		
scaffold6.g2788.t1			Cre07.g335600	Vocar.0030s0055		
			Cre12.g541200	Vocar.0046s0010		
			Cre12.g541250			
NAR2 (nitrate high-affinity transporter accessory)*						
na	138676	47957	Cre09.g410900	Vocar.0008s0139	70904	24167
NiR (ferredoxin-nitrite reductase)*						
na	26644	29833	Cre09.g410750	Vocar.0008s0136	70828	26396
NR (nitrate reductase (NAD(P)H))*						
scaffold14.g1111.t1	56304	37154	Cre09.g410950	Vocar.0008s0140	39565	19576
		29226			57689	

Sequences were obtained from the database of JGI genome portal in *Chlorella variabilis NC64A* (NC64A), *Coccomyxa subellipsoidea* C-169 (C169), *Volvox carteri* (Vc), *Micromonas pusilla* (Mp) and *Ostreococcus tauri* (Ot) and of Phytozome in *Chlamydomonas reinhardtii*.

* The number of genes in NC64A, C169, Vc, Cr, Mp, Ot were based on Sanz-Luque et al. (2015)

Supplemental Table 6

Composition of modified Bold's Basal Medium for 1 liter (pH. 7)

KH_2PO_4	175 mg
K_2HPO_4	100 mg
$MgSO_4 \cdot 7H2O$	75 mg
$CaCl_2 \cdot 2H_2O$	25 mg
NaCl	25 mg
$FeSO_4 \cdot 7H_2O$, H_2SO_4	5.0 mg, 1ul
Na_2EDTA, KOH	50 mg, 31 mg
H_3BO_3	11.4 mg
$ZnSO_4 \cdot 7H_2O$	8.8 mg
$MnCl_2 \cdot 7H_2O$	1.4 mg
MoO_3	0.7 mg
$CuSO_4 \cdot 5H_2O$	1.6 mg
$Co(NO_3)_2 \cdot 6H_2O$	0.5 mg
glucose	5 g
Vitamine B1 (Thiaminhydrochloride)	1.2 mg
Vitamine B12 (Cyanocobalamin)	0.01 mg

Nitrogen Components

$NaNO_3$	250 mg
NH_4Cl	155 mg
Glutamine	426 mg
Casamino acids	426 mg

Supplemental Table 7

A. Primer sequences for quantitative real time RT-PCR

Gene ID	Forward Primers	Reverse Primers
5168	ACTTTTCGGATATCAAACCCATTC	AATTGAACCTATTCCTCGAACGTC
6508	GCATCAAATGCGTCCAAATAAC	TTACCGAATATTCAGGCCTTTCTC
rc_2417	CTTATTGCTCATGACCGTAAAGATG	TCGATTTTCACCCTTGATGG
24563	TGCGCCTTAGTTATATCTCCTCTC	TCTCTTTCTTGTGTTGTTTCTTTCC
rc_9398	GATGTTTGTAGAACACGTTGGATTG	TTCAAGACAGGAGACCACAGG
11411	TCTTGCTCATGCAACACTGG	CGGTTTACTGCCAATCACATAC
26108	AATTCCTGTCCGACTGATTTCC	CCAAATCGACCCTTACTTGTTTG
rc_10789	TTGCAAGAATATCTGCTGCTAAG	AGAAATCAACGGAGATCGTGTAG
rc_12826	TTTATTCAAGCAATGGGCAATC	CGTTGCGTTTGTCCCTTTC
rc_8898	TTAAGCATCAACGAAATATCCACTC	ACTTGTTTTGTTGCAAGTGTAGAGC
FV81RT001CSTY	TTAGAAATGCATGGTGTTGTTGG	CGGGTCTGTCAAGCATAAGAAG
RSASM_17752	AGAATTGCTTGGGGTGTTCC	GCATATCCACGAATGAGACAAAG
rc_13579	ACGGAGGTTTGGGGAAATAG	TTTGGTCTTAGGAGTGCTCGTC
27417	TGTACCTGTCCATGGAATTAAAGC	TACCTTGTCCGAATAGCAGCTC
rc_26218	TTAAACTTCGAAGCTGGAAATGG	TTAGCGAAGACTTTGTCGTATGG
1046	GTGGGTTGCTCGTTATCTACTTG	CACCAGGGATGGGTTTAGG
rc_12891	GTCGGTATGGGAGGTGGAG	CCCAATATACCGCCGACAG
NPNHRC_26859	TGATGAACAAAAGAGCCGTATCTC	GCACGAACCGATACGTCAAC
RC_FVQRUGK01AXSJ	TCCCTTATGCACAGGTACGG	GGATCAATAACTGGTGGCACTG
rc_14793	CACCCTTGGGCTGGTAAG	GGGATCTATGGGCAAATAAGG
FV81RT002HT2FL	CCAGCAAAAGCCCTTGATTAC	CCTGAATTCACCCCTCCATC
NPNHRC_12201	GTCGGTATGGGAGGTGGAG	CCCAATATACCGCCGACAG
finalASM_15403	AATAGGTGATGCTGGAGAGAATC	AGTATATATGGCTCTCGAGAGTG
finalASM_344	ATGTGAGCCATGTCCAATTGGA	CACTTCTATTGGCAGCTTTCTC
tubulin alpha	TTCCTTGCTCATGGAACGAC	AGCAGGGTAAACTGCAAACTCC
ef1-alpha	ACCAACATTGTCACCTGGGAG	GGAACAGTACCTGTTGGTCGT

B. Primer sequences for *in situ* hybridization probes

Gene Name	Forward Primers	Reverse Primers
GS-1	TTGCTGACCCATTCAGAGGA	CCGAACCCAAAAGACCAAAG
NaPi	TTGGGAACACAACTGCTGAT	AAAGTTTAGCGAAGACTTTGTCG
Spot14	GAGAAATTGATTAAGCAAGTAAGAG	GGTCAATTGCTCGGTTTC

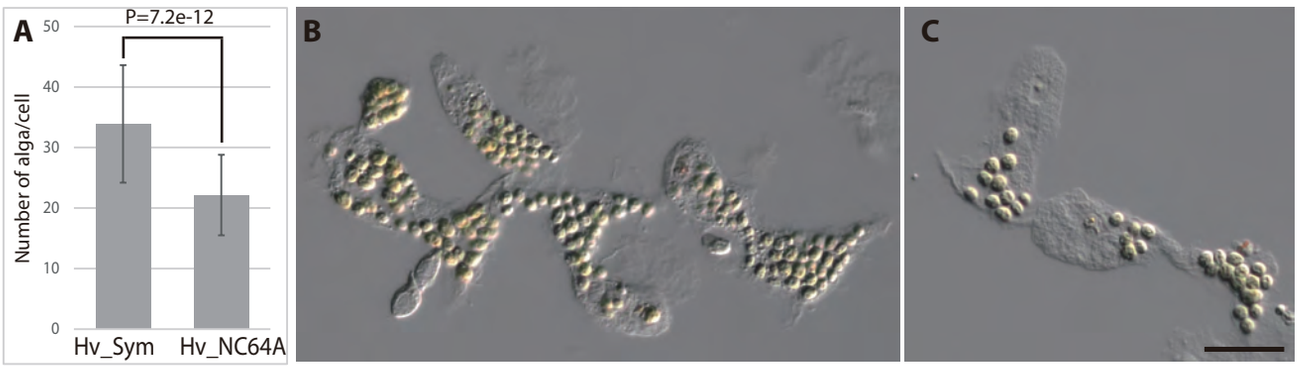
C. Primer sequences for PCR amplification of nitrogen assimilation genes in green algae

Gene	Forward Primers	Reverse Primers
NRT2	YCAGTTCTGGTSCKSBRYSMTGTTC	CCCACATGGGRAASYRRATG
NiR	ACATCACCACVCGCGCCAACATC	TYGWRKCCMACGTCGTTGATGTG
NR	CTGGTGGTACMRSCCSGASTT	SAKCATSCCMATSASRTTCC

D. Primer sequences for PCR amplification of 18S ribosomal DNA gene in green algae

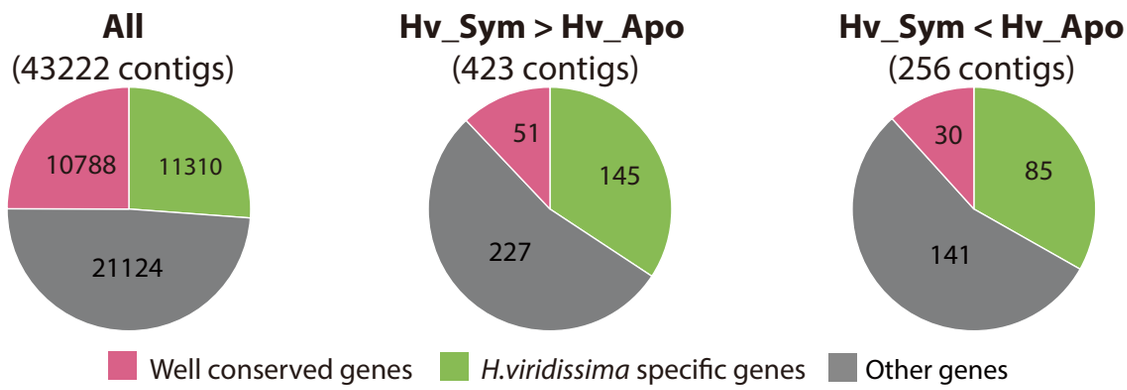
Forward Primers	Reverse Primers
GGAATAACACGATAGGACTCTGG	GACGGGCGGTGTGTACAAAG

Supplementary Figures



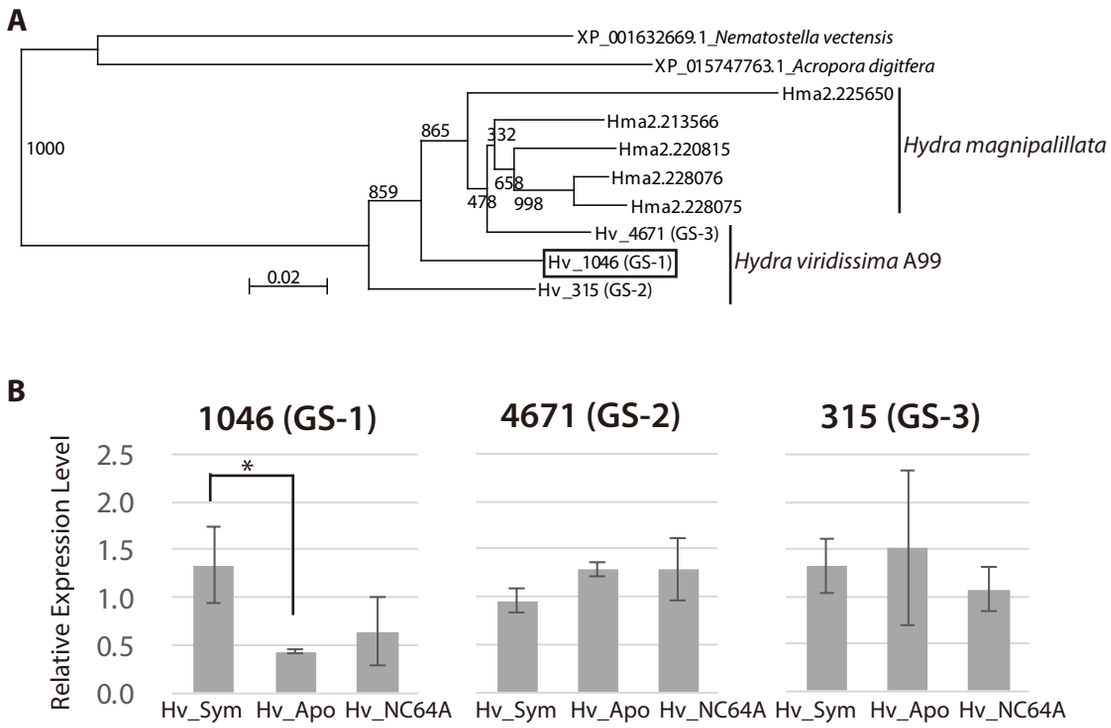
Supplementary Figure 1. *Chlorella* sp. A99 and *Chlorella variabilis* NC64A in *Hydra viridissima* A99

(A) Average number of algae per *Hydra* cell, for native *Chlorella* sp. A99 (Hv_Sym) and aposymbiont *Hydra* re-infected with *Chlorella variabilis* NC64A (Hv_NC64A). (B) Endodermal epithelial cells of Hv_Sym showing intracellular algae (C) Endodermal epithelial cells of Hv_NC64A. Scale bar, 20 µm



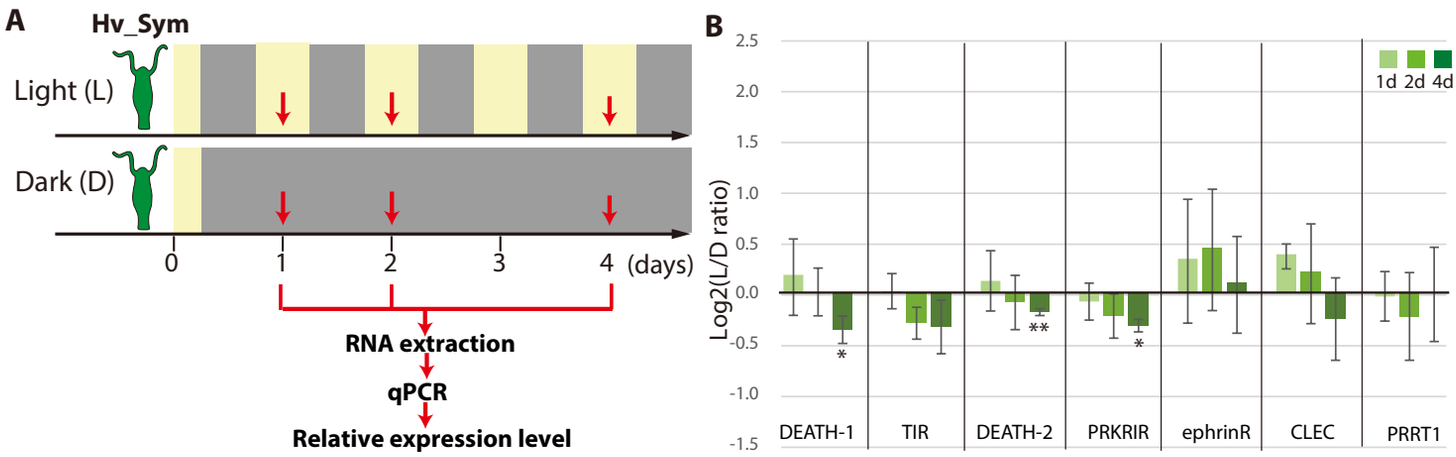
Supplementary Figure 2. Conserved genes and species-specific genes differentially expressed in symbiotic *Hydra*

Distribution of well-conserved *Hydra viridissma* genes (pink), *Hydra viridissima*-specific genes (green) and other genes (shared by some but not all metazoans, gray) among eight metazoans: *Hydra magnipapillata*, *Acropora digitifera*, *Nematostella vectensis*, *Strongylocentrotus pupuratus*, *Branchiostoma floridae*, *Homo sapiens* and *Drosophila melanogaster* and *Hydra viridissima*. Pie charts are shown for all contigs (All), up-regulated contigs (Hv_Sym > Hv_Apo) and down-regulated contigs (Hv_Sym < Hv_Apo).



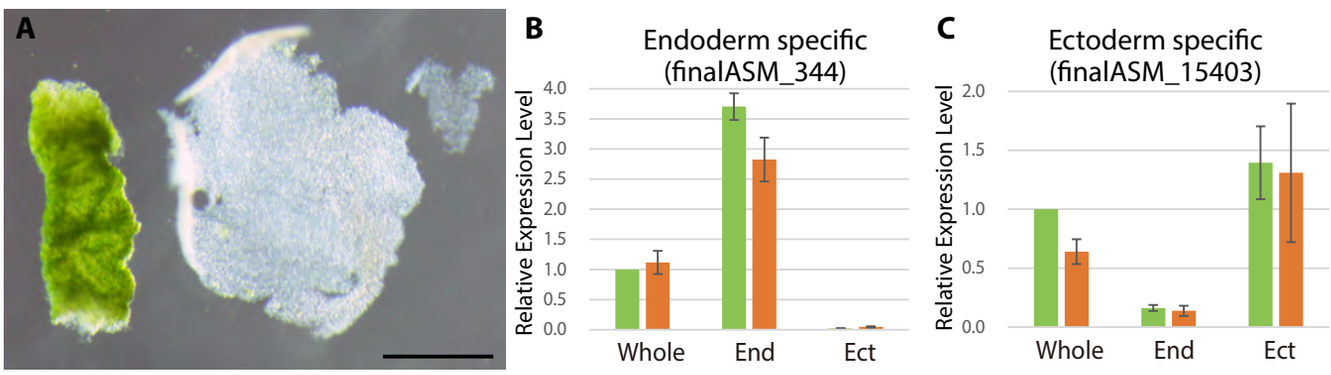
Supplementary Figure 3. Glutamine synthetase (GS) genes in Cnidarians.

(A) Phylogenetic tree of the GS gene of four species in Cnidarians. While anthozoans (*Nematostella vectensis*, *Acropora digitifera*) have a single GS gene, *Hydra magnipappilata* (Hma) has five genes and *Hydra viridissima* A99 has three genes, Hv_1046 (GS-1), Hv_315 (GS-2) and Hv_4671 (GS-3). (B) Relative expression level of the three GS genes in Hv_Sym, Hv_NC64A and Hv_Apo as determined by microarray analysis.



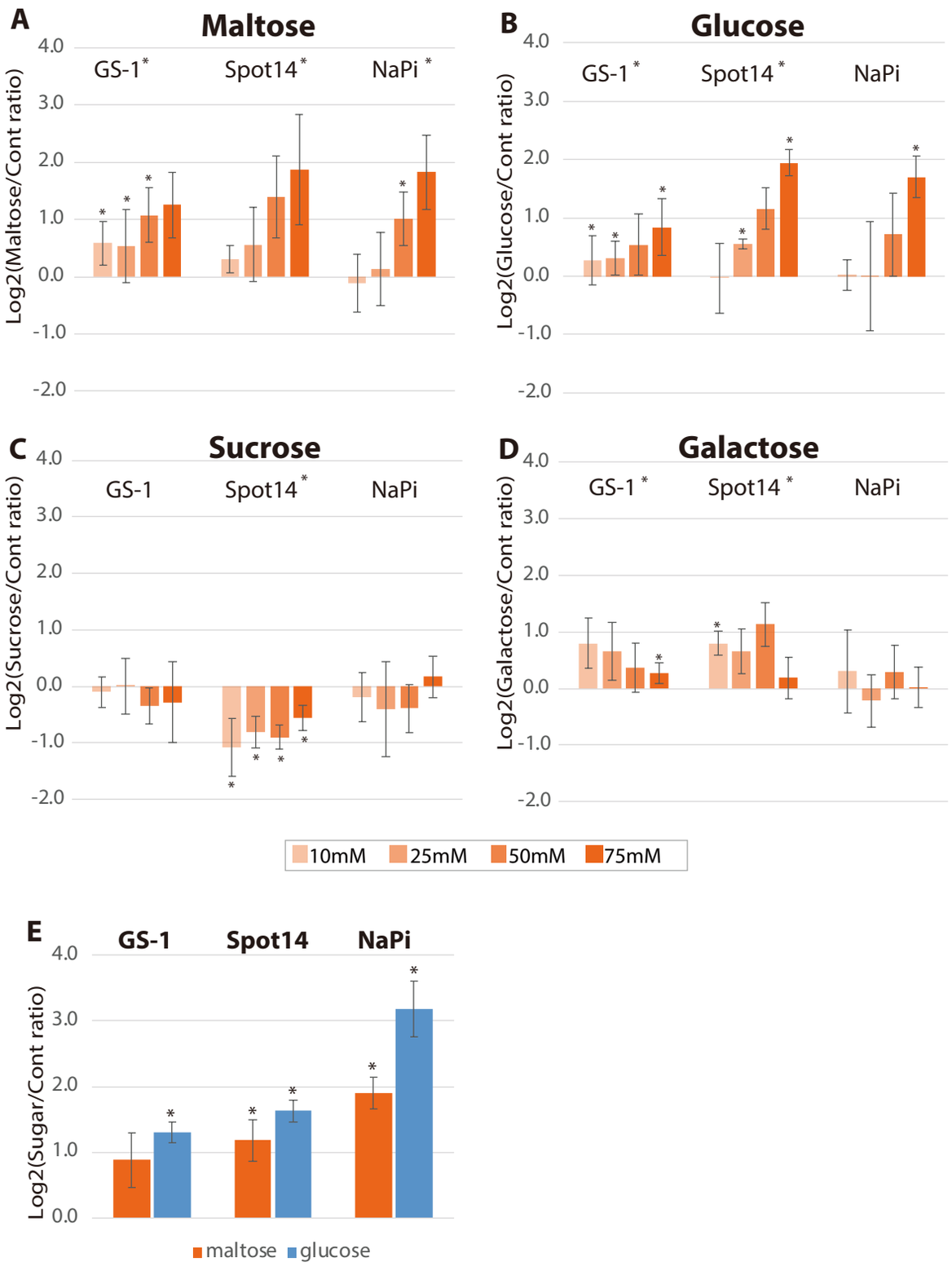
Supplementary Figure 4. Differential expression of symbiosis-dependent *Hydra* genes grown under light/dark condition and in darkness.

(A) Sampling scheme. Hv_Sym was cultured in the light-dark condition (Light: L) and in the continuous dark (Dark: D). Gene expression levels were examined by qPCR at 1, 2, 4 days for each condition (red arrows). (B) Expression difference of the genes in Hv_A99 between the two conditions. DEATH-1 and DEATH-2: Death domain containing proteins (gene ID: 6508 and rc_2417), TIR: Toll/interleukin-1 receptor domain containing protein (gene ID: 5168), PRKRIR: protein-kinase interferon-inducible double stranded RNA dependent inhibitor, repressor of (p58 repressor) (gene ID: rc_9398), ephrinR: ephrin receptor (gene ID: 26108), CLEC: C-type mannose receptor (gene ID: 11411), PRRT1: proline-rich transmembrane protein 1 (gene ID: rc_24563). The vertical axis shows log scale (log2) fold change of relative expression levels in the light condition over the dark condition. T-test evalue, * <0.05, ** <0.01.



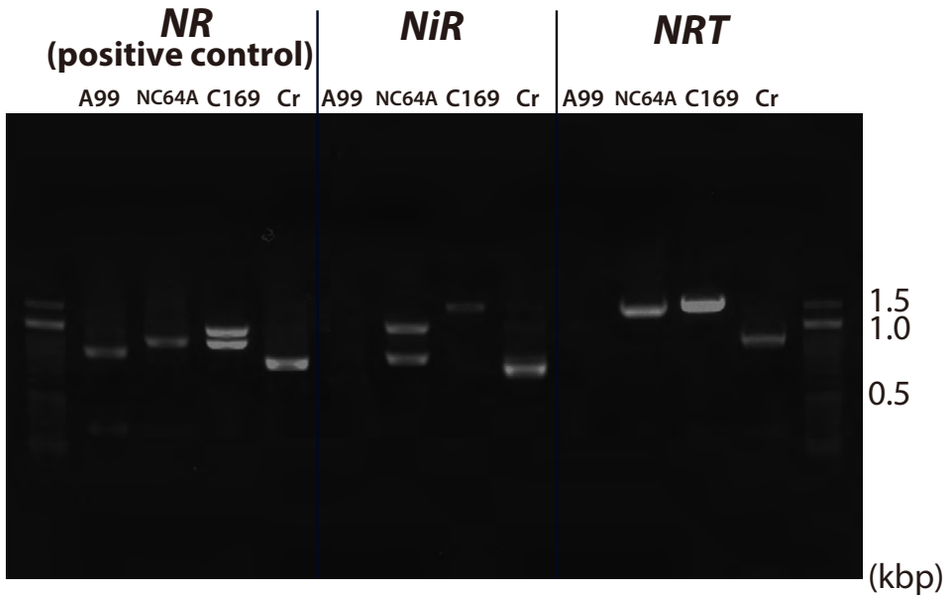
Supplementary Figure 5. Tissue isolation of green hydra.

(A) Isolated endoderm (left) and isolated ectoderm (right). Scale bar, 1 mm. Expression levels of an endoderm specific gene finalASM_15403 (B) and that of an ectoderm specific gene finalASM_344 (C) in whole hydra (Whole) and isolated endoderm (End) and ectoderm (Ect) were examined to confirm whether tissue isolation had performed properly.



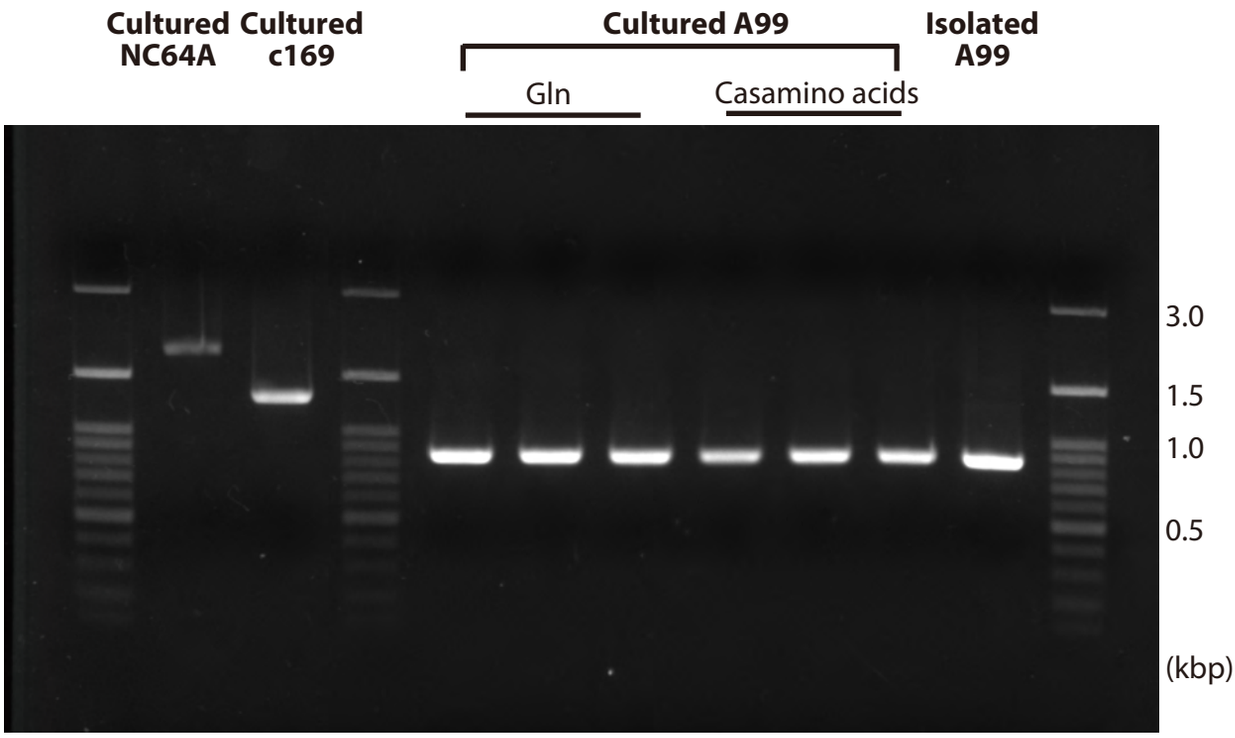
Supplementary Figure 6. Effects of sugars on *Hydra* growth

Effects of growth in presence of maltose (A), glucose (B), sucrose (C) and galactose (D) on gene expression of GS-1, Spot14 and NaPi. Hv_Apo were cultured in medium containing 10 mM, 25 mM, 50 mM or 75 mM of each sugar for 48 hours, and 75 mM maltose (orange) and glucose (blue) for 6 hours (E). RNA was extracted from the polyps in the light condition. Expression difference of the genes was examined by qPCR. The vertical axis is log scale (log2) fold change of relative expression level of sugar-treated hydras over controls. Error bars indicate standard deviation. T-test in each concentration and Kruskal-Wallis test in the series of 48 hours treatment were performed. * p-value <0.05



Supplementary Figure 7. PCR of nitrate assimilation genes

PCR amplification of genomic DNA corresponding to the genes *NRT2*, *NiR* and *NR* (positive control) was performed in *Chlorella* sp. A99 (A99), *Chlorella variabilis* NC64A (NC64A), *Coccomyxa subellipsoidea* C169 (C169) and *Chlamydomonas reinhardtii* (Cr).



Supplementary Figure 8. PCR of 18S rRNA genes in cultured algae

PCR amplification of genomic DNA of the 18S rRNA gene was performed in *Chlorella* A99 shortly after isolation from *H. viridissima* A99 (Isolated A99), cultured in medium containing glutamine (Glu) and in medium with casamino acids for 12 days, with cultured NC64A and C169 added for comparison.