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1 **Title:**

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

3

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25 **Abstract (148 words)**

26

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

40

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41 **Introduction**

42

43 Symbiosis has been a prevailing force throughout the evolution of life, driving the diversification
44 of organisms and facilitating rapid adaptation of species to divergent new niches (Moran, 2007;
45 Joy, 2013; McFall-Ngai et al., 2013). In particular, symbiosis with photosynthetic symbiont is
46 observed in many species of Cnidarians such as coral, jellyfish, sea anemone and hydra,
47 contributing to the ecological success of these sessile or planktonic animals (Douglas, 1994;
48 Davy et al., 2012b). Among the many animals dependent on algal symbionts, inter-species
49 interactions between green hydra *Hydra viridissima* and endosymbiotic unicellular green algae
50 of the genus *Chlorella* have been a subject of interest for decades (Muscatine and Lenhoff,
51 1963; Roffman and Lenhoff, 1969). Such studies not only provide insights into the basic "tool
52 kit" necessary to establish symbiotic interactions, but are also of relevance in understanding
53 the resulting evolutionary selective processes (Muscatine and Lenhoff, 1965a, b; Thorington
54 and Margulis, 1981).

55

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

66

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

75

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

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79 Schwentner and Bosch, 2015). A recent phylogenetic analysis of different strains of green
80 hydra resulted in a phylogenetic tree that is topologically equivalent to that of their symbiotic
81 algae (Kawaida et al., 2013), suggesting these species co-evolved as a result of their symbiotic
82 relationship. Although our understanding of the factors that promote symbiotic relationships in
83 cnidarians has increased (Shinzato et al., 2011; Davy et al., 2012a; Lehnert et al., 2014;
84 Baumgarten et al., 2015; Ishikawa et al., 2016), very little is known about the molecular
85 mechanisms allowing this partnership to persist over millions of years.
86
87 Recent advances in transcriptome and genome analysis allowed us to identify the metabolic
88 interactions and genomic evolution involved in achieving the *Hydra-Chlorella* symbiotic
89 relationship. We present here the first characterization, to our knowledge, of genetic
90 complementarity between green *Hydra* and *Chlorella* algae that explains the emergence
91 and/or maintenance of a stable symbiosis. We also provide here the first report of the complete
92 genome sequence from an obligate intracellular *Chlorella* photobiont. Together, our results
93 show that exchange of nutrients is the primary driving force for the symbiosis between *Chlorella*
94 and *Hydra*. Subsequently, reduction of metabolic pathways may have further strengthened
95 their codependency. Our findings provide a framework for understanding the evolution of a
96 highly codependent symbiotic partnership in an early emerging metazoan.
97
98
99 **Results**
100
101 **Discovery of symbiosis-dependent *Hydra* genes**
102
103 As tool for our study we used the green hydra *H. viridissima* (**Figure 1A**) colonized with
104 symbiotic *Chlorella* sp. strain A99 (abbreviated here as Hv_Sym), aposymbiotic *H. viridissima*
105 from which the symbiotic *Chlorella* were removed (Hv_Apo), and aposymbiotic *H. viridissima*
106 which had been artificially infected with *Chlorella variabilis* NC64A (Hv_NC64A). The latter is
107 symbiotic to the single-cellular protist Paramecium (Karakashian and Karakashian, 1965).
108 Although an association between *H. viridissima* and *Chlorella* NC64A can be maintained for
109 some time, both their growth rate (**Figure 1B**) and the number of NC64A algae per *Hydra* cell
110 (**Supplementary Figure 1**) is significantly reduced compared to the symbiosis with native
111 symbiotic *Chlorella* A99.
112
113 *H. viridissima* genes involved in the symbiosis with *Chlorella* were identified by microarray
114 based on the contigs of *Hydra viridissima* A99 transcriptome (NCBI GEO Platform ID:
115 GPL23280). For the microarray analysis, total RNA was extracted from the polyps after light
116 exposure for six hours. By comparing the transcriptomes of Hv_Sym and Hv_Apo, we identified

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117 423 contigs that are upregulated and 256 contigs that are downregulated in presence of
118 *Chlorella* A99 (**Figure 1C**). To exclude genes involved in oogenesis and embryogenesis, only
119 contigs differently expressed with similar patterns in both sexual and asexual Hv_Sym were
120 recorded. Interestingly, contigs whose predicted products had no discernible homologs in other
121 organisms including other *Hydra* species were overrepresented in these differentially
122 expressed contigs (Chi-squared test P<0.001) (**Supplementary Figure 2**). Such
123 taxonomically restricted genes (TRGs) are thought to play important roles in the development
124 of evolutionary novelties and morphological diversity within a given taxonomic group (Khalturin
125 et al., 2009; Tautz and Domazet-Loso, 2011).
126
127 We further characterized functions of the differentially expressed *Hydra* genes by Gene
128 Ontology (GO) terms (The Gene Ontology et al., 2000). This demonstrated overrepresentation
129 of genes with GO term “localization” in upregulated contigs (Hv_Sym > Hv_Apo) and with GO
130 term “metabolic process” in downregulated contigs (Hv_Sym < Hv_Apo) (**Figure 1D**). More
131 specifically, the upregulated contigs include many genes related to “transmembrane
132 transporter activity”, “transmembrane transport”, “transposition”, “cilium” and “protein binding,
133 bridging” (**Figure 1E**). In the downregulated contig set, the GO classes “cellular amino acid
134 metabolic process”, “cell wall organization or biogenesis” and “peptidase activity” are
135 overrepresented (**Figure 1E**). These results suggest that the *Chlorella* photobiont affects core
136 metabolic processes and pathways in *Hydra*. Particularly, carrier proteins and active
137 membrane transport appears to play a prominent role in the symbiosis.
138
139 To narrow down the number of genes specifically affected by the presence of *Chlorella* A99,
140 we identified 12 contigs that are differentially expressed in presence of *Chlorella* A99 but not
141 in presence of *Chlorella* NC64A (**Figure 1C** A99-specific). Independent qPCR confirmed the
142 differential expression pattern for 10 of these genes (**Supplementary Table 1**). The genes
143 upregulated by the presence of the photobiont encode a Spot_14 protein, a glutamine
144 synthetase (GS) and a sodium-dependent phosphate (Na/Pi) transport protein in addition to a
145 *H. viridissima* specific gene (rc_12891: *Sym-1*) and a *Hydra* genus specific gene (rc_13570:
146 *Sym-2*) (**Supplementary Table 1**). *Hydra* genes downregulated by the presence of *Chlorella*
147 A99 were two *H. viridissima* specific genes and three metabolic genes encoding histidine
148 ammonia-lyase, acetoacetyl-CoA synthetase and 2-isopropylmalate synthase
149 (**Supplementary Table 1**). Of the upregulated genes, Spot_14 is described as thyroid
150 hormone-responsive spot 14 protein reported to be induced by dietary carbohydrates and
151 glucose in mammals (Tao and Towle, 1986; Brown et al., 1997). Na/Pi transport protein is a
152 membrane transporter actively transporting phosphate into cells (Murer and Biber, 1996). GS
153 plays an essential role in the metabolism of nitrogen by catalyzing the reaction between
154 glutamate and ammonia to form glutamine (Liaw et al., 1995). Interestingly, out of the three

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155 GS genes *H. viridissima* contains only *GS-1* was found to be upregulated by the presence of
156 the photobiont (**Supplementary Figure 3**). The discovery of these transcriptional responses
157 points to an intimate metabolic exchange between the partners in a species-specific manner..
158

159 **Symbiont-dependent *Hydra* genes are upregulated by photosynthetic activity of**
160 ***Chlorella A99***
161

162 To test whether photosynthetic activity of the symbiont is required for upregulation of gene
163 expression, Hv_Sym was either cultured under a standard 12 hr light/dark alternating regime
164 or continuously in the dark for 1 to 4 days prior to RNA extraction (**Figure 2A**). Interestingly,
165 four (*GS1*, *Spot14*, *Na/Pi* and *Sym-1*) of five genes specifically activated by the presence of
166 *Chlorella* A99 showed significant upregulation when exposed to light (**Figure 2B**), indicating
167 the relevance of photosynthetic activity of *Chlorella*. This upregulation was strictly dependent
168 on presence of the algae, as in aposymbiont Hv_Apo the response was absent (**Figure 2B**).
169 On the other hand, symbiosis-regulated *Hydra* genes not specific for *Chlorella* A99 (**Figure 1C**
170 Symbiosis-regulated**, Supplementary Table 2**) appear not to be upregulated in a light-
171 dependent manner (**Supplementary Figure 4**). These genes are involved in *Hydra´*s innate
172 immune system (e.g. proteins containing Toll/interleukin-1 receptor domain or Death domain)
173 or in signal transduction (C-type mannose receptor, ephrin receptor, proline-rich
174 transmembrane protein 1, "protein-kinase, interferon-inducible double stranded RNA
175 dependent inhibitor, repressor of (p58 repressor)"). That particular transcriptional changes
176 observed in *Hydra* rely solely on the photosynthetic activity of *Chlorella* A99 was confirmed by
177 substituting the dark incubation with selective chemical photosynthesis inhibitor DCMU
178 (Dichorophenyl-dimethylurea) (Vandermeulen et al., 1972), which resulted in a similar effect
179 (**Figure 2C, D**).
180

181 **Symbiont-dependent *Hydra* genes are expressed in endodermal epithelial cells and**
182 **upregulated by sugars**
183

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

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193 photosynthetic products can be transported across these two tissue layers or some signals
194 can be transduced by cell-cell communication.
195
196 To more closely dissect the nature of the functional interaction between *Hydra* and *Chlorella*
197 and to explore the possibility that maltose released from the algae is involved in A99-specific
198 gene regulation, we cultured aposymbiotic polyps (Hv_Apo) for 2 days in medium containing
199 various concentrations of maltose (**Figure 3J**). Of the five A99 specific genes, GS-1 and the
200 Spot14 gene were upregulated by maltose in a dose-dependent manner; the Na/Pi gene was
201 only upregulated in 100mM maltose and the *Hydra* specific genes Sym-1 and Sym-2 did not
202 show significant changes in expression by exposure to maltose (**Figure 3J**). This provides
203 strong support for previous views that maltose excretion by symbiotic algae contributes to the
204 stabilization of this symbiotic association (Cernichiari et al., 1969). When polyps were exposed
205 to glucose instead of maltose, the genes of interest were also transcriptionally activated in a
206 dose-dependent manner, while sucrose had no effect (**Supplementary Figure 6A-D**).
207 Exposure to low concentrations of galactose increased transcriptional activity but at high
208 concentration it did not, indicating a substrate inhibitor effect for this sugar. That the response
209 to glucose is similar or even higher compared to maltose after 6 hours of treatment
210 (**Supplementary Figure 6E**), suggests that *Hydra* cells transform maltose to glucose as a
211 source of energy.
212

213 The *Chlorella A99* genome records a symbiotic life style

214
215 To better understand the symbiosis between *H. viridissima* and *Chlorella* and to refine our
216 knowledge of the functions that are required in this symbiosis, we sequenced the genome of
217 *Chlorella* sp. strain A99 and compared it to the genomes of other green algae. The genome of
218 *Chlorella* sp. A99 was sequenced to approximately 211-fold coverage, enabling the generation
219 of an assembly comprising a total of 40.9 Mbp (82 scaffolds, N50=1.7Mbp) (**Supplementary**
220 **Table 3**). *Chlorella* sp. A99 belongs to the family *Chlorellaceae* (**Figure 4A**) and of the green
221 algae whose genomes have been sequenced it is most closely related to *Chlorella variabilis*
222 NC64A (NC64A) (Merchant et al., 2007; Palenik et al., 2007; Worden et al., 2009; Blanc et al.,
223 2010; Prochnik et al., 2010; Blanc et al., 2012; Gao et al., 2014; Pombert et al., 2014). The
224 genome size of the total assembly in strain A99 was similar to that of strain NC64A (46.2Mb)
225 (**Figure 4B**). By k-mer analysis (k-mer = 19), the genome size of A99 was estimated to be 61
226 Mbp (Marcais and Kingsford, 2011). Its GC content of 68%, is the highest among the green
227 algae species recorded (**Figure 4B**). In the A99 genome, 8298 gene models were predicted.
228 As shown in **Figure 4C**, about 80% of these predicted genes have extensive sequence
229 similarity to plant genes, while 13% so far have no similarity to genes of any other organisms
230 (**Figure 4C**). It is also noteworthy that 7% of the A99 genes are similar to genes of other

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231 kingdoms but not to *Hydra*, indicating the absence of gene transfer from *Hydra* to the
232 photobiont genome (**Figure 4C**).
233
234 **The *Chlorella* A99 genome provides evidences for extensive nitrogenous amino acid**
235 **import and an incomplete nitrate assimilation pathway**
236
237 Several independent lines of evidence demonstrate that nitrogen limitation and amino-acid
238 metabolism have a key role in the *Chlorella–Hydra* symbiosis and that symbiotic *Chlorella* A99
239 depends on glutamine provided by its host (Rees, 1986; McAuley, 1987a, b, 1991; Rees, 1991)
240 (Rees, 1989). To identify *Chlorella* candidate factors for the development and maintenance of
241 the symbiotic life style, we therefore used the available genome information to assess genes
242 potentially involved in amino acid transport and the nitrogen metabolic pathway.
243
244 When performing a search for the Pfam domain "Aa_trans" or "AA_permease" to find amino
245 acid transporter genes in the A99 genome, we discovered numerous genes containing the
246 Aa_trans domain (**Supplementary Table 4A**). In particular, A99 contains many orthologous
247 genes of amino acid permease 2 and of transmembrane amino acid transporter family protein
248 (solute carrier family 38, sodium-coupled neutral amino acid transporter), as well as NC64A
249 (**Supplementary Table 4B, C**). Both of these gene products are known to transport neutral
250 amino acids including glutamine. This observation is supporting the view that import of amino
251 acids is an essential feature for the symbiotic way of life of *Chlorella*.
252
253 In nitrogen assimilation processes, plants usually take up nitrogen in the form of nitrate (NO_3^-)
254 via nitrate transporters (NRTs) or as ammonium (NH_4^+) via ammonium transporters (AMT)
255 (**Figure 5A**). In higher plants, two types of nitrate transporters, NRT1 and NRT2, have been
256 identified (Krapp et al., 2014). Some NRT2 require nitrate assimilation-related component 2
257 (NAR2) to be functional (Quesada et al., 1994). NO_3^- is reduced to nitrite by nitrate reductase
258 (NR), NO_2^- is transported to the chloroplast by nitrate assimilation-related component1 (NAR1),
259 and NO_2^- is reduced to NH_4^+ by nitrite reductase (NiR). NH_4^+ is incorporated into glutamine
260 (Gln) by glutamine synthetase (GS), and Gln is incorporated into glutamate (Glu) by NADH-
261 dependent glutamine amide-2-oxoglutarate aminotransferase (GOGAT), also known as
262 glutamate synthase. This pathway is highly conserved among plants. In the genomes of 10
263 green algae species sequenced so far, the major components of the pathway, including NRT1
264 and NRT2, NAR1 and NAR2, NR, NiR, AMT, GOGAT and GS, are all present, although NRT1
265 is absent in the *Micromonas pusilla* genome (Sanz-Luque et al., 2015).
266
267 Based on the annotation by Sanz-Luque et al. (Sanz-Luque et al., 2015), we searched these
268 nitrogen assimilation genes in the *Chlorella* A99 genome, using ortholog grouping and a

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269 reciprocal blast search using the protein sequences from other green algae **(Figure 5B,**
270 **Supplementary Table 5**). As expected, the *Chlorella* A99 genome contains many homologues
271 of the genes involved in nitrogen assimilation in plants including genes encoding NRT1, NAR1,
272 NR, AMT, GS and GOGAT **(Figure 5B)**. Intriguingly, our systematic searches have failed to
273 identify representative genes for NRT2, NAR2 and NiR in the *Chlorella* A99 genome (**Figure**
274 **5B**). We confirmed the absence of the NRT2 and NiR genes by PCR using primers designed
275 for the conserved regions of these genes and which failed to produce a product with genomic
276 DNA as a template **(Supplementary Figure 7).** Due to the weak sequence conservation of the
277 NAR2 gene in the three algae genomes, PCR of that gene was not performed. Taken together,
278 our observations indicate that *Chlorella* A99 algae appear to lack NRT2, NAR2 and NiR.
279
280 Since in many fungi, cyanobacteria and algae species, nitrate assimilation genes are known
281 to act in concert and a gene cluster of NR and NiR genes is conserved between different green
282 algae (Sanz-Luque et al., 2015), we next investigated the level of genomic clustering of the
283 nitrate assimilation pathway genes in the *Chlorella* genome. Comparing the genomes of
284 NC64A and *Coccomyxa subellipsoidea* C169 (C169) revealed the presence of a cluster of NR
285 and NiR genes (**Figure 5C**). In NC64A, two NRT2 genes, together with genes for NAR2, NR
286 and NiR are clustered on scaffold 21. In C169, one of NR genes and NiR are clustered together
287 but the second NR gene is separate. Interestingly, analyzing the sequences around the NR
288 gene in the *Chlorella* A99 genome provided no evidence for the presence of a co-localized NiR
289 gene or any other nitrate assimilation genes, nor any conserved gene synteny to NC64A and
290 C169 (**Figure 5C**). Our comparative genomic analyses therefore points to an incomplete as
291 well as scattered nitrogen metabolic pathway in symbiotic *Chlorella* A99, which lacks essential
292 transporters and enzymes for nitrate assimilation and also lacks the clustered structure of
293 nitrate assimilation genes.
294
295 **Supplementing the medium with glutamine allows temporary *in vitro* growth of**
296 **symbiotic *Chlorella* A99**
297
298 The absence of genes essential for nitrate assimilation in the *Chlorella* A99 genome (**Figure**
299 **5**) is consistent with its inability to grow outside the *Hydra* host cell (Habetha and Bosch, 2005)
300 and indicates that *Chlorella* symbionts are dependent on metabolites provided by their host.
301 We hypothesized that *Chlorella* is unable to use nitrite and ammonium as a nitrogen source,
302 and that it relies on *Hydra* assimilating ammonium to glutamine to serve as the nitrogen source.
303 To test this hypothesis and to examine utilization of nitrogen compounds of A99, we isolated
304 *Chlorella* A99 from Hv_Sym and cultivated it *in vitro* using modified bold basal medium (BBM)
305 (Nichols and Bold, 1965) containing the same amount of nitrogen in the form of NO_3^-, NH_4^+,
306 Gln or casamino acids (**Figure 6, Supplementary Table 6**). As controls, *Chlorella variabilis*

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307 NC64A (NC64A) isolated from Hv_NC64A and free-living C169 were used. To confirm that the
308 cultured A99 is not contamination, we amplified and sequenced the genomic region of the 18S
309 rRNA gene by PCR (**Supplementary Figure 8**) and checked this against the genomic
310 sequence of A99. Kamako et al. reported that free-living algae *Chlorella vulgaris* Beijerinck var.
311 *vulgaris* grow in media containing only inorganic nitrogen compounds as well as in media
312 containing casamino acids as a nitrogen source, while NC64A required amino acids for growth
313 (Kamako et al., 2005). Consistent with these observations, C169 grew in all tested media and
314 NC64A grew in media containing casamino acids and Gln, although its growth rate was quite
315 low in presence of NH_4^+ and NO_3^- (**Figure 6**). Remarkably, *Chlorella* A99 increased in cell
316 number for up to 8 days in media containing casamino acids and Gln (**Figure 6**). Similar to
317 NC64A, A99 did not grow in presence of NH_4^+ and NO_3^-. The growth rates of both A99 and
318 NC64A were higher in medium containing a mixture of amino acids (casamino acids) than the
319 single amino acid Gln. In contrast to NC64A, A99 could not be cultivated permanently in
320 casamino acids or glutamine supplemented medium, indicating that additional growth factors
321 are necessary to maintain *in vitro* growth of this obligate symbiont. Thus, although *in vitro*
322 growth of A99 can be promoted by adding Glu and amino acids to the medium, A99 cannot be
323 cultured permanently in this enriched medium, indicating that other host derived factors remain
324 to be uncovered.
325
326 ## Discussion
327
328 Sequencing of the *Chlorella* A99 genome in combination with the transcriptome analyses of
329 symbiotic, aposymbiotic and NC64A-infected *H. viridissima* polyps has enabled the
330 identification of genes with specific functions in this symbiotic partnership. The *Hydra*-*Chlorella*
331 symbiosis links carbohydrate supply from the photobiont to glutamine synthesis by the host.
332 Characteristics of the symbiont genome obviously reflect its adaptation to this way of life,
333 including an increase in amino acid transporters and degeneration of the nitrate assimilation
334 pathway. This conclusion is based on six observations: **(i)** Expression of some genes including
335 GS-1, Spot 14 and NaPi is specifically upregulated in the presence of *Chlorella* A99 (**Fig. 1C,**
336 **Supplementary Table 1**), and **(ii)** they are induced by both, photosynthetic activity of *Chlorella*
337 and by supplying exogenous maltose or glucose (**Figure 2, 3J, Supplementary Figure 6**).
338 These results indicate that maltose release by photosynthesis of the symbiont enhances
339 nutrition supply including glutamine by the host (**Figure 7**). **(iii)** Symbiotic *Chlorella* A99 cannot
340 be cultivated *in vitro* in medium containing a single inorganic nitrogen source (**Figure 6**). Since
341 medium containing glutamine supports *in vitro* growth of A99, this organism appears to depend
342 on glutamine provided by the *Hydra* host. **(i*v*)** The genome of *Chlorella* A99 contains multiple
343 amino acid transporter genes (**Supplementary Table 4)**, but lacks genes involved in nitrate
344 assimilation (**Figure 5**), pointing to amino acids as main source of nitrogen and a degenerated

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345 nitrate assimilation pathway. As for ammonium, which is one of the main nitrogen sources in
346 plants, previous studies have reported the inability of symbiotic algae to take up ammonium
347 because of the low peri-algal pH (pH 4-5) that stimulates maltose release (Douglas and Smith,
348 1984; Rees, 1989; McAuley, 1991; Dorling et al., 1997). Since *Chlorella* apparently cannot use
349 nitrite and ammonium as a nitrogen source, it seems that *Hydra* has to assimilate ammonium
350 to glutamine and provides it to *Chlorella* A99 (**Figure 7**).
351 **(*v*)** While polyps with native symbiont *Chlorella* A99 grew faster than aposymbiotic ones,
352 symbiosis with foreign algae NC64A had no effect on the growth of polyps at all (**Figure 1B**).
353 **(*vi*)** *Hydra* endodermal epithelial cells host significantly fewer NC64A algae than A99
354 (**Supplementary Figure 1**) providing additional support for the view of a tightly regulated
355 codependent partnership in which exchange of nutrients appears to be the primary driving
356 force.
357 Previous studies have reported that symbiotic *Chlorella* in green hydra releases significantly
358 larger amounts of maltose than NC64A (Mews and Smith, 1982; Rees, 1989). In addition, Rees
359 reported that *Hydra* polyps containing high maltose releasing algae had a high GS activity,
360 whereas aposymbiotic *Hydra* or *Hydra* with a low maltose releasing algae had lower GS activity
361 (Rees, 1986). Although the underlying mechanism of how maltose secretion and transportation
362 from *Chlorella* is regulated is still unclear, the amount of maltose released by the symbiont
363 could be an important symbiont-derived driver or stabilizer of the *Hydra*–*Chlorella* symbiosis.
364
365 Exchange of nitrogenous compounds and photosynthetic products between host and symbiont
366 is widely found in other symbiotic associations. For example, in marine invertebrates such as
367 corals, sea anemones, and giant clams associated with *Symbiodinium* algae, the algae provide
368 the photosynthate in forms of glucose, glycerol, organic acids, amino acids or lipids to their
369 host, and in turn the symbionts receive ammonia or glutamine as nitrogen sources (Burriesci
370 et al., 2012; Davy et al., 2012; Kellogg and Patton, 1983; Lewis and Smith, 1971; Muscatine,
371 1965; Muscatine and Cernichiari, 1969; 1993; Trench, 1971; Venn et al., 2008; Whitehead and
372 Douglas, 2003; Yellowlees et al., 2008). Moreover, in corals a Na/Pi transporter is involved in
373 the uptake of phosphate across host membranes, and the zooxanthellae contribute to the
374 uptake of phosphate (D'Elia, 1977; Jackson et al., 1989). These observations together with the
375 results presented here make the host-controlled supply of nitrogen and phosphorus as a
376 response of a signal photosynthate seem the universal principle of invertebrate-algae
377 symbiosis.
378 Metabolic dependence of symbionts on host supply occasionally results in genome reduction
379 and gene loss. For example, the symbiotic *Buchnera* bacteria of insects are missing particular
380 genes in essential amino acid pathways (Shigenobu et al., 2000; Hansen et al., 2011). The fact
381 that the corresponding genes of the host are upregulated in the bacteriocyte, indicates
382 complementarity and syntrophy between host and symbiont. Similarly, in *Chlorella* A99 the

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383 nitrogen assimilation system could have been lost as result of continuous supply of nitrogenous
384 amino acids provided by *Hydra*. On the other hand, the genome size and total gene number
385 of *Chlorella* A99 is similar to other species in the class Trebouxiophyceae (**Figure 4B**). The
386 apparently unchanged complexity of the Chlorella A99 genome suggests a relatively early
387 stage of this symbiotic partnership. From these observation, we propose that the gene loss in
388 metabolic pathway is the first step of genome reduction caused by dependency on nutrients
389 from the host. Our study suggests metabolic-codependency is the primary driving force for the
390 evolution of symbiosis between *Hydra* and *Chlorella*.
391

392 ## Materials and methods
393

394 ### Biological materials and procedures
395 Experiments were carried out with the Australian *Hydra viridissima* strain A99, which was
396 obtained from Dr. Richard Campbell, Irvine. Polyps were maintained at 18°C on a 12 hours
397 light/dark cycle and fed with *Artemia* two or three times a week. Aposymbiotic (algae free)
398 polyps were obtained by photobleaching using 5 μM DCMU (3-(3,4-dichlorophenyl)-1,1-
399 dimethylurea) as described before (Pardy, 1976; Habetha et al., 2003). Experiments were
400 carried out with polyps starved for 3-6 days. Isolation of endodermal layer and ectodermal layer
401 was performed as described by Kishimoto et al. (Kishimoto et al., 1996). Symbiotic *Chlorella*
402 were isolated as described before by Muscatine and McAuley (Muscatine, 1983; McAuley,
403 1986b). *Chlorella variabilis* NC64A (NIES-2541), *Coccomyxa subellipsoidea* C-169 (NIES-
404 2166) and *Chlamydomonas reinhardtii* (NIES-2235) were obtained from the Microbial Culture
405 Collection at the National Institute for Environmental Studies (Tsukuba, Japan).
406

407 ### Nucleic acid preparation
408 Total RNA of *Hydra* was extracted by use of the Trizol reagent and PureLink RNA Mini Kit (Life
409 Technology) after lysis and removal of algae by centrifugation. The genomic DNA of green
410 algae was extracted using ISOPLANT II (Nippon Gene, Tokyo, Japan) following DNase I
411 treatment to degrade contaminant DNA. Quantity and quality of DNA and RNA were checked by
412 NanoDrop (Thermo Scientific Inc., Madison, USA) and BioAnalyzer (Agilent Technologies,
413 Santa Clara, USA).
414

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

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421 Hybridization and washing were performed using the GE Hybridization Kit and GE Wash Pack
422 (Agilent Technologies) after which the arrays were scanned on an Agilent Technologies
423 G2565BA microarray scanner system with SureScan technology following protocols according
424 to the manufacturer's instructions. The intensity of probes was extracted from scanned
425 microarray images using Feature Extraction 10.7 software (Agilent Technologies). All
426 algorithms and parameters used in this analysis were used with default conditions.
427 Background-subtracted signal-intensity values (gProcessedSignal) generated by the Feature
428 Extraction software were normalized using the 75th percentile signal intensity among the
429 microarray. Those genes differentially expressed between two samples were determined by
430 average of fold change (cut off >2.0) and Student's t-test (P< 0.1). The data series are
431 accessible at NCBI GEO under accession number GSE97633.
432
433 **Quantitative real time RT-PCR**
434 Total RNA was reverse transcribed using First Strand cDNA Synthesis Kit (Fermentas, Ontario,
435 Canada). Real-time PCR was performed using GoTaq qPCR Master Mix (Promega, Madison,
436 USA) and ABI Prism 7,300 (Applied Biosystems, Foster City, USA). All qPCR experiments
437 were performed in duplicate with three biological replicates each. Values were normalized
438 using the expression of the tubulin alpha gene. Primers used for these experiments are listed
439 in **Supplementary Table 7A.**
440
441 **Whole mount *in situ* hybridization**
442 Expression patterns of specific *Hydra* genes were detected by whole mount *in situ*
443 hybridization with digoxigenin (DIG)-labelled RNA probes. Specimens were fixed in 4%
444 paraformaldehyde. Hybridization signal was visualized using anti-DIG antibodies conjugated
445 to alkaline phosphatase and NBT/BCIP staining solution (Roche). DIG-labeled sense probes
446 (targeting the same sequences as the antisense probes) were used as a control. Primers used
447 for these experiments are listed in **Supplementary Table 7B.**
448
449 **Genome sequencing and gene prediction**
450 For genome sequencing of *Chlorella* sp. A99, *Chlorella* sp. A99 was isolated from *H. viridissima*
451 A99 and genomic DNA was extracted. Paired-end library (insert size: 740 bp) and mate-pair
452 libraries (insert size: 2.2 and 15.2kb) were made using Illumina TruSeq DNA LT Sample Prep Kit
453 and Nextera Mate Pair Sample Preparation Kit respectively (Illumina Inc., San Diego, USA),
454 following the manufacturer's protocols. Genome sequencing was performed using Illumina Miseq
455 and Hiseq 2000 platforms. Sequence reads were assembled using Newbler Assembler version
456 2.8 (Roche, Penzberg, Germany) and subsequent scaffolding was performed by SSPACE
457 (Boetzer et al., 2011). Gaps inside the scaffolds were closed with the paired-end and mate-
458 pair data using GapCloser of Short Oligonucleotide Analysis Package (Luo et al., 2012). To

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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

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497 reported by (Sanz-Luque et al., 2015). We used these data as reference for searches of
498 nitrogen assimilation genes, and further nitrogen assimilation genes were searched by Kyoto
499 Encyclopedia of Genes and Genomes (KEGG) pathway (Kanehisa and Goto, 2000). JGI
500 genome browsers of *Chlorella variabilis* NC64A and *Coccomyxa subellipsoidea* C-169 were
501 also used for retrieving genes and checking gene order on the scaffolds.
502

503 **Phylogenetic analysis**

504 For a phylogenetic tree of chlorophyte green algae, the sequences of 18S rRNA gene, ITS1,
505 5.8S rRNA gene, ITS2 and 28S rRNA gene were obtained from scaffold20 of *Chlorella* A99
506 genome sequence, and from NCBI nucleotide database entries for *Chlorella variabilis* NC64A
507 (FM205849.1), *Auxenochlorella protothecoides* 0710 (NW_011934479.1), *Coccomyxa*
508 *subellipsoidea* C169 (AGSI01000011.1), *Volvox carteri* f. nagariensis (NW_003307662.1),
509 *Chlamydomonas reinhardtii* (FR865576.1), *Ostreococcus tauri* (GQ426340.1) and
510 *Micromonas pusilla* (FN562452.1). Multiple alignments were produced with CLUSTALX (2.1)
511 with gap trimming (Larkin et al., 2007). Sequences of poor quality that did not well align were
512 deleted using BioEdit (Hall, 1999). Phylogenetic analyses were performed using the Neighbor-
513 Joining method by CLUSTALX. Representative phylogenetic trees were drawn by using NJ
514 plot (Perriere and Gouy, 1996).
515

516 **PCR amplification of nitrate assimilation genes in green algae**

517 Primers were designed based on the conserved region of the NRT2 gene, NiR and NR genes
518 (positive control) identified by comparison of genes from *Chlorella variabilis* NC64A (NC64A),
519 *Coccomyxa subellipsoidea* C169 (C169), and *Chlamydomonas reinhardtii* (Cr) which belongs
520 to Chlorophyceae class of green algae. Primers for NAR2 could not be designed because of
521 insufficient conservation. As positive controls, amplicons were produced for NR of all the green
522 algae examined and of NRT2 and NiR from NC64A, C169 and Cr, after which their sequences
523 were checked. KOD FX Neo (TOYOBO, Tokyo, Japan) was used under the following
524 conditions: an initial denaturation phase (94 °C for 120 sec) followed by 36 cycles of (98 °C for
525 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)
526 for NRT2 and (98 °C for 30 sec, 59 °C for 30 sec and 68 °C for 60 sec) for NR. In each case,
527 10 ng gDNA was used as a template. The primers used are described in **Supplementary Table**
528 **7C**. PCR products were sequenced to confirm amplification of the target genes using ABI
529 PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific Inc., Madison, USA) using BigDye
530 Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).
531

532 ***In vitro* culture of algae**

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

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535 and centrifuged at 500g for 5min. Isolated A99, NC64A and C169 were washed by sterilized
536 Bold Basal Medium (Bischoff and Bold, 1963) modified by the addition of 0.5% glucose,
537 1.2mg/L vitamine B1 (Thiaminhydrochloride), 0.01mg/L vitamine B12 (Cyanocobalamin)
538 (**Supplementary Table 6**) and incubated for two days in modified Bold Basal Medium with
539 50mg/l ampicillin and streptomycin. The algae were cultivated in 5 ml of modified Bold Basal
540 Medium (BBM) with the same amount of nitrogen (2.9 mM $NaNO_3$, NH_4Cl, glutamine or 426
541 mg/l casamino acids) and 5mg/l Carbendazim (anti-fungal) with fluorescent illumination (12
542 hour light, 12 hour dark) at 20˚C. Mean numbers of algae per ml were calculated from three
543 tubes enumerated at 4, 8, and 12 days after inoculation with 10^6 cell/sml using a
544 hemocytometer. After cultivation, gDNA was isolated from the A99 cultured in Gln-containing
545 BBM and casamino acid-containing BBM and A99 was isolated from green hydra directly. A
546 partial genomic region of the 18S rRNA gene was amplified by PCR and sequenced to confirm
547 absence of contamination by other algae. PCR was performed using AmpliTaq Gold (Thermo
548 Fisher Scientific). Sequencing was performed as described above. The primers used are
549 described in **Supplementary Table 7D**.
550
551
552 **Acknowledgement**
553 We thank Trudy Wassenaar for critical reading the text and for discussion. We also thank the
554 DNA Sequencing Section in OIST for genome sequencing. This work was supported in part by
555 JSPS KAKENHI Grant-in-Aid for Young Scientists (B) 25840132 and Scientific Research (C)
556 15K07173 to M.H. and by the Deutsche Forschungsgemeinschaft (DFG) (CRC1182 "Origin
557 and Function of Metaorganisms"). T.C.G.B. gratefully appreciates support from the Canadian
558 Institute for Advanced Research (CIFAR).
559
560 **Competing interests**
561 The authors declare that no competing interests exist.
562
563
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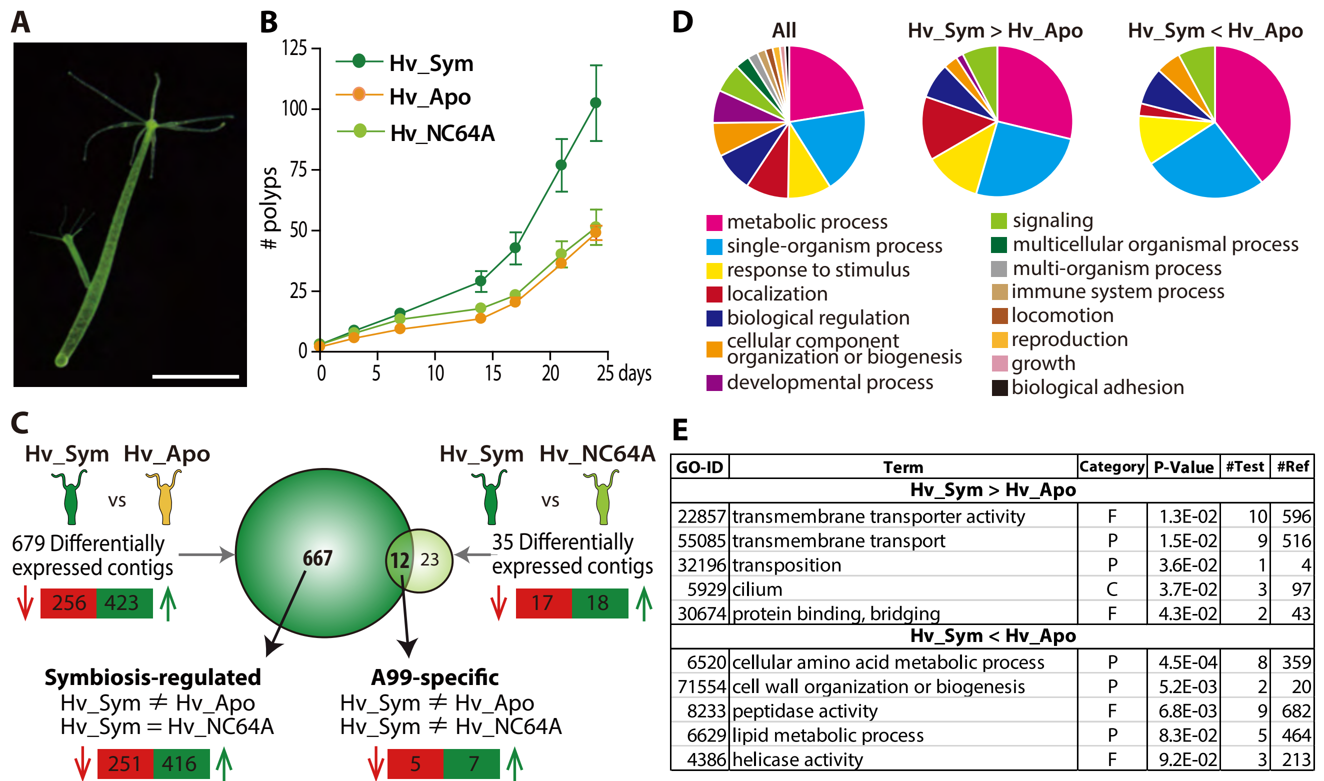
Figures



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.

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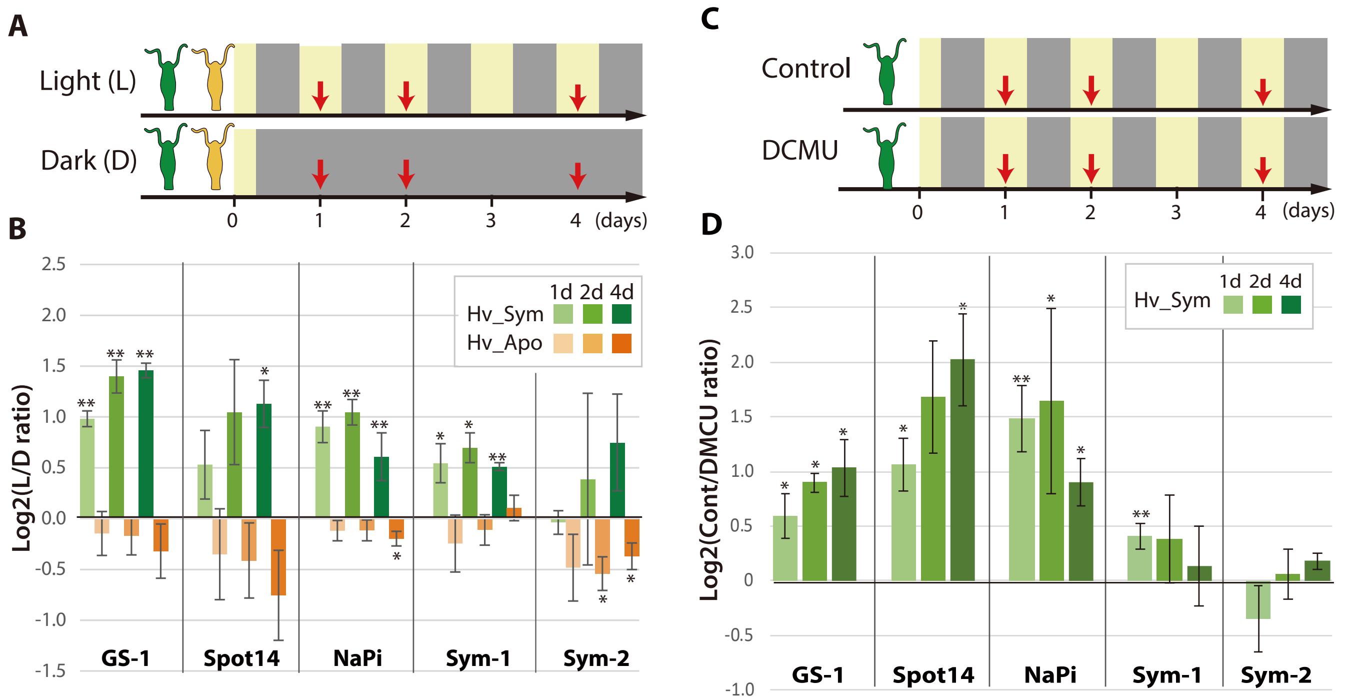

Figure 2. Differential expression of *Hydra* genes under influence of *Chlorella* photosynthesis

(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

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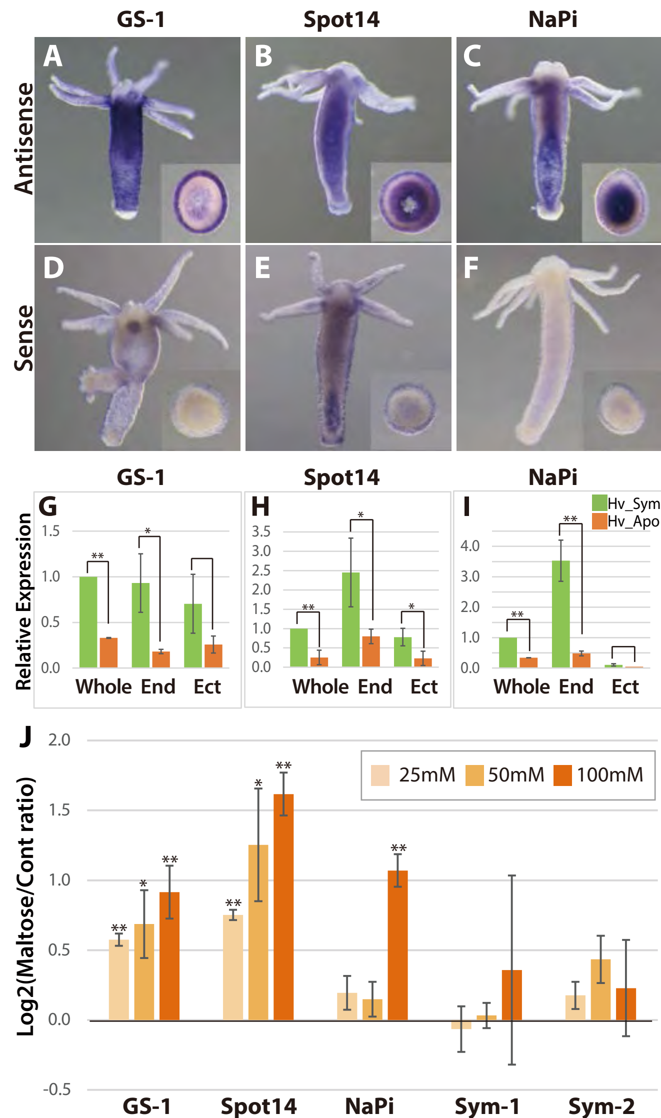

Figure 3. Spatial expression patterns of genes coding for glutamine synthetase, Spot 14 and Na/Pi-transporter.

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.

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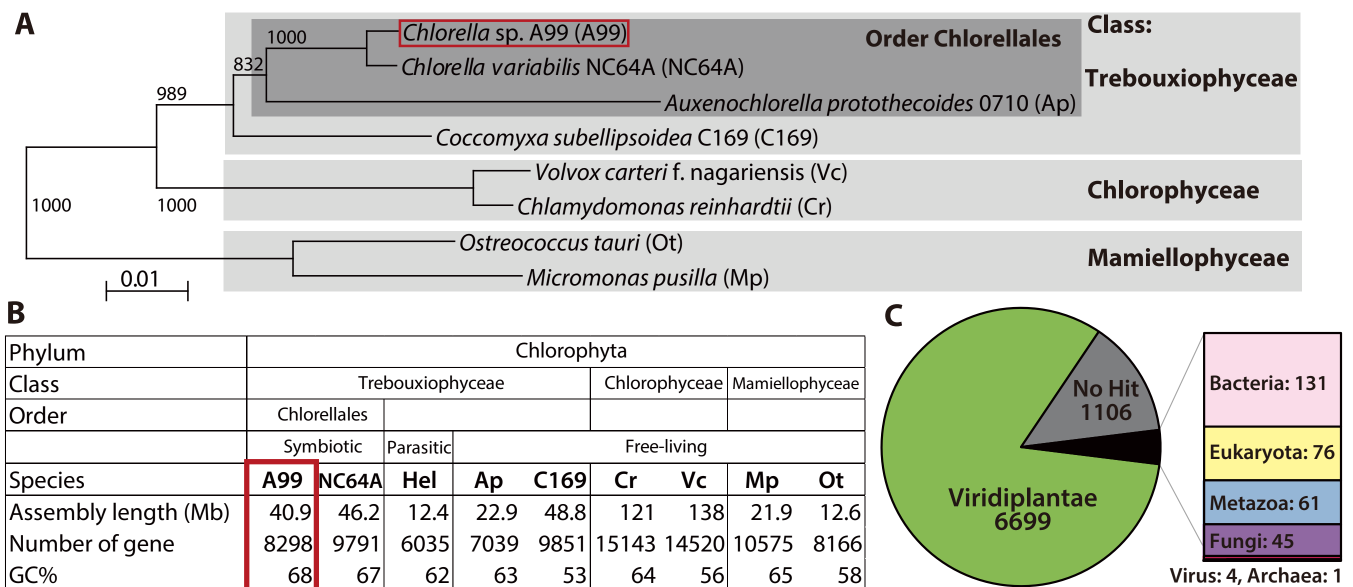

Phylum	Chlorophyta								
Class	Trebouxiophyceae					Chlorophyceae		Mamiellophyceae	
Order	Chlorellales								
	Symbiotic		Parasitic	Free-living					
Species	A99	NC64A	Hel	Ap	C169	Cr	Vc	Mp	Ot
Assembly length (Mb)	40.9	46.2	12.4	22.9	48.8	121	138	21.9	12.6
Number of gene	8298	9791	6035	7039	9851	15143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

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.

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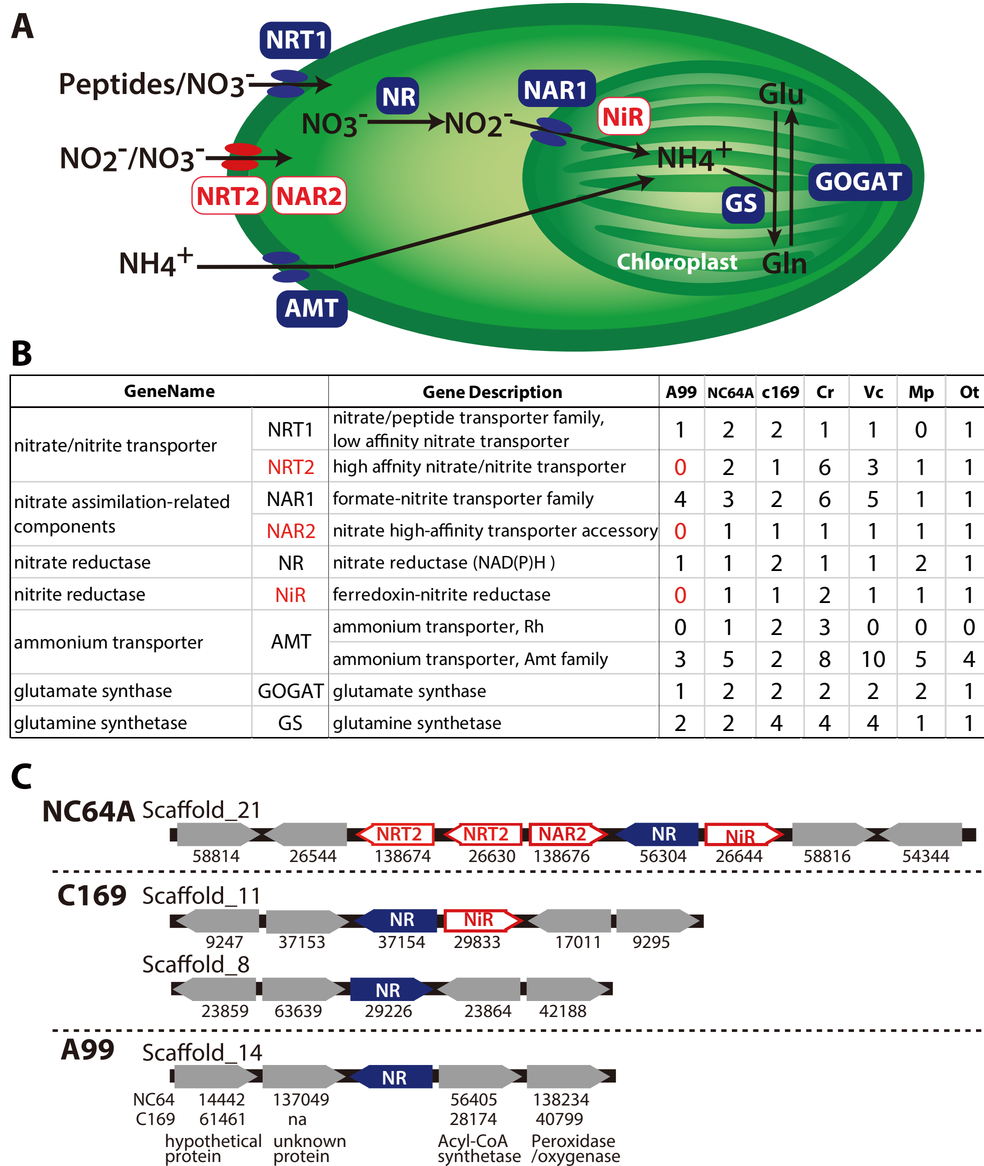

GeneName		Gene Description	A99	NC64A	c169	Cr	Vc	Mp	Ot
nitrate/nitrite transporter	NRT1	nitrate/peptide transporter family, low affinity nitrate transporter	1	2	2	1	1	0	1
	NRT2	high affinity nitrate/nitrite transporter	0	2	1	6	3	1	1
nitrate assimilation-related components	NAR1	formate-nitrite transporter 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.

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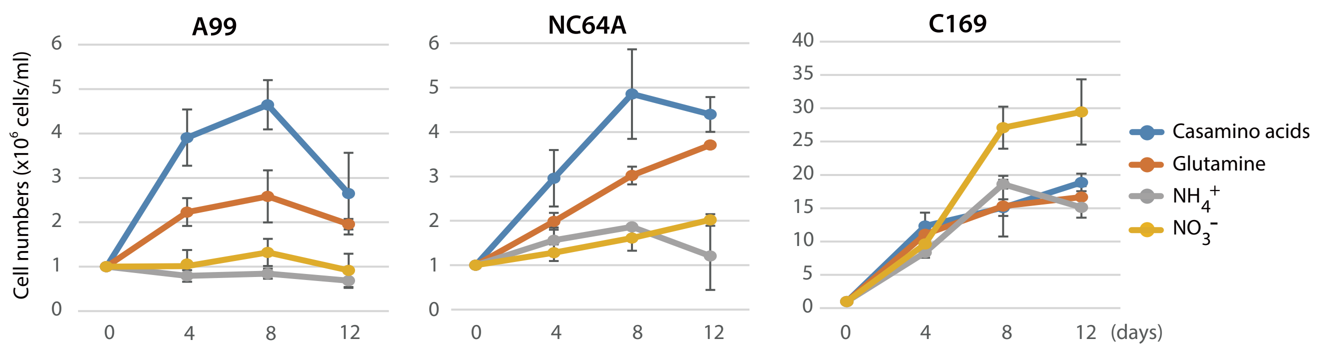

Figure 6. Growth of green algae in presence of various nitrogen sources

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.

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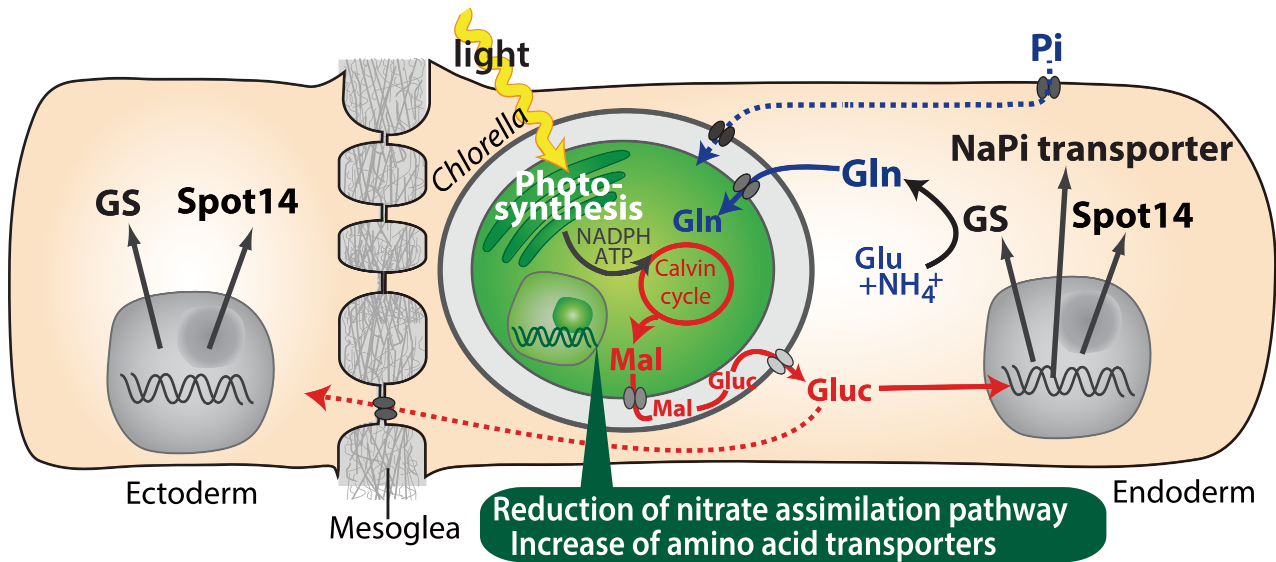

Figure. 7. Molecular interactions in the symbiosis between green hydra and Chlorella A99

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).

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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.

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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.

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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 %

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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

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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.

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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)*						

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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)

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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

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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

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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

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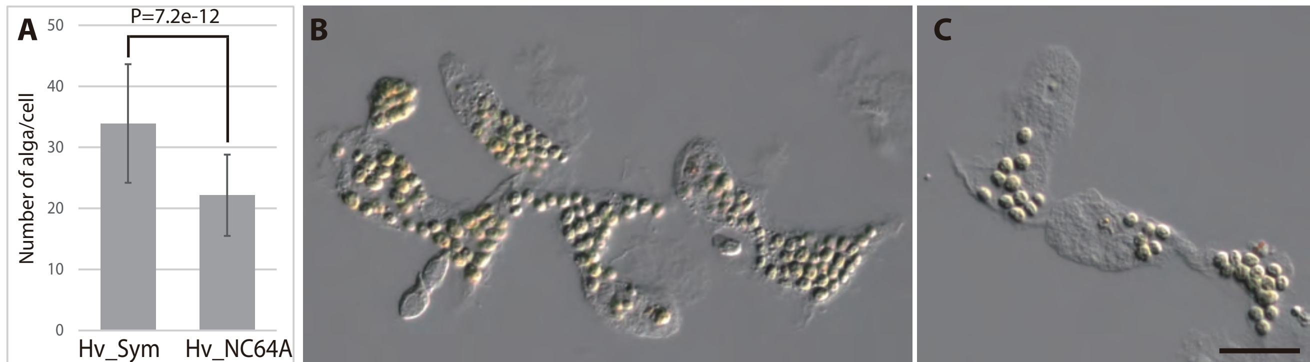
Supplementary Figures



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

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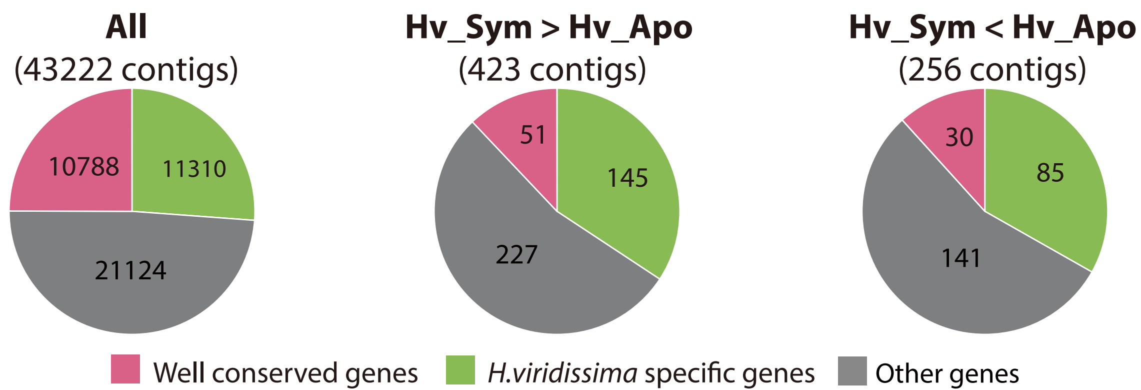

Supplementary Figure 2. Conserved genes and species-specific genes differentially expressed in symbiotic *Hydra*
Distribution of well-conserved *Hydra viridissma* genes (pink), *Hydra viridissima*-specific genes (green) and other genes (shared by some but not all metazoans, gray) among eight metazoans: *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).

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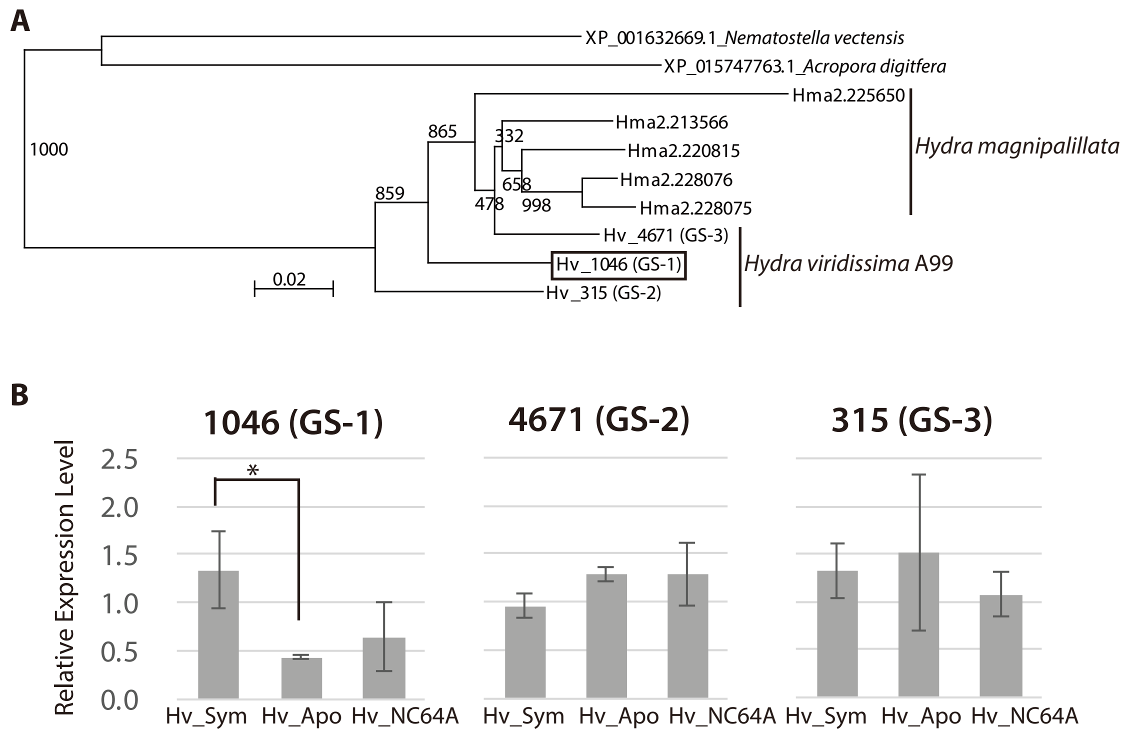

Supplementary Figure 3. Glutamine synthetase (GS) genes in Cnidarians.

(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.

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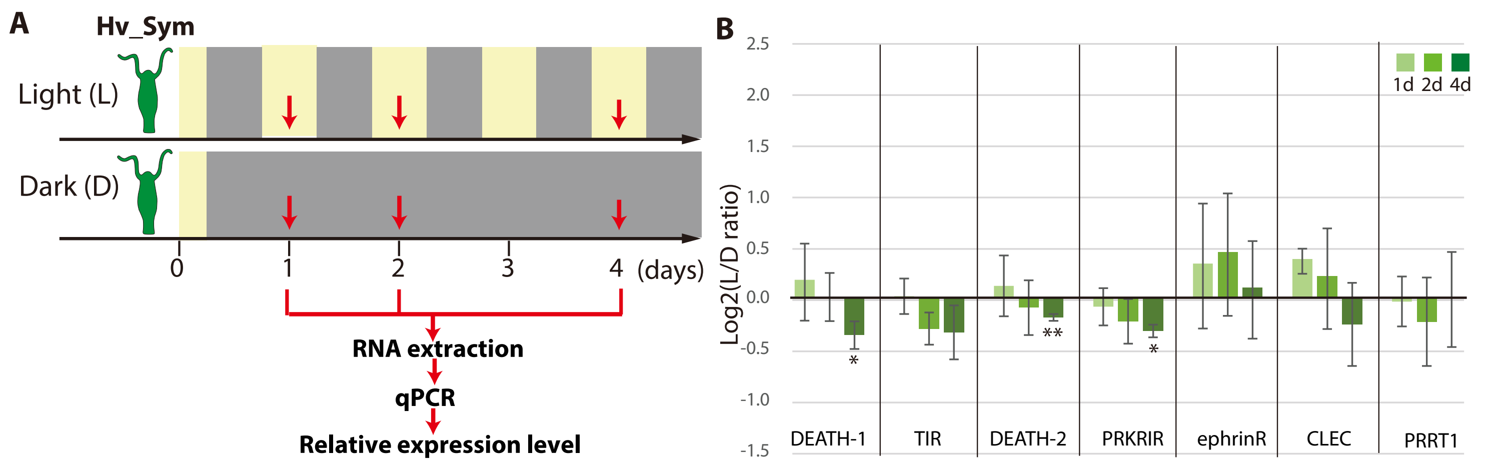

Supplementary Figure 4. Differential expression of symbiosis-dependent *Hydra* genes grown under light/dark condition and in darkness.

(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.

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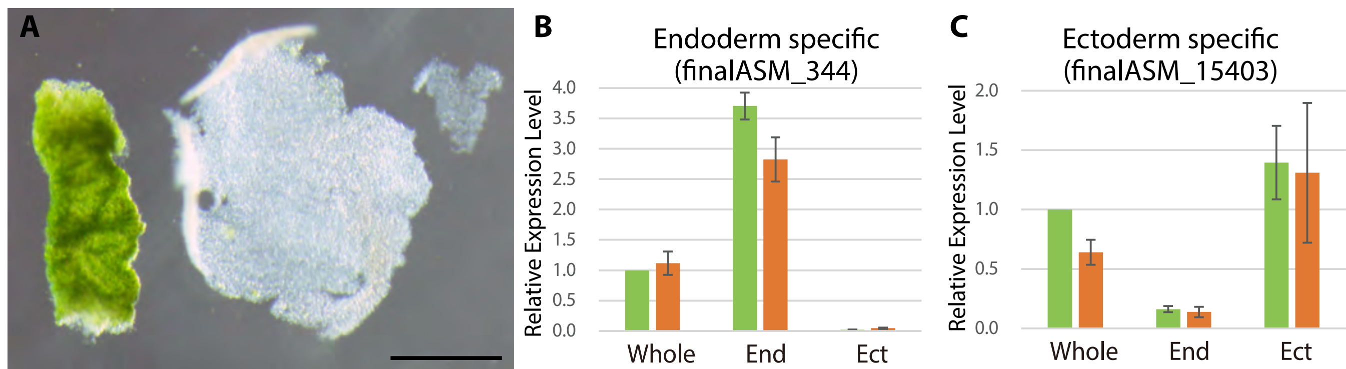

Supplementary Figure 5. Tissue isolation of green hydra.

(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.

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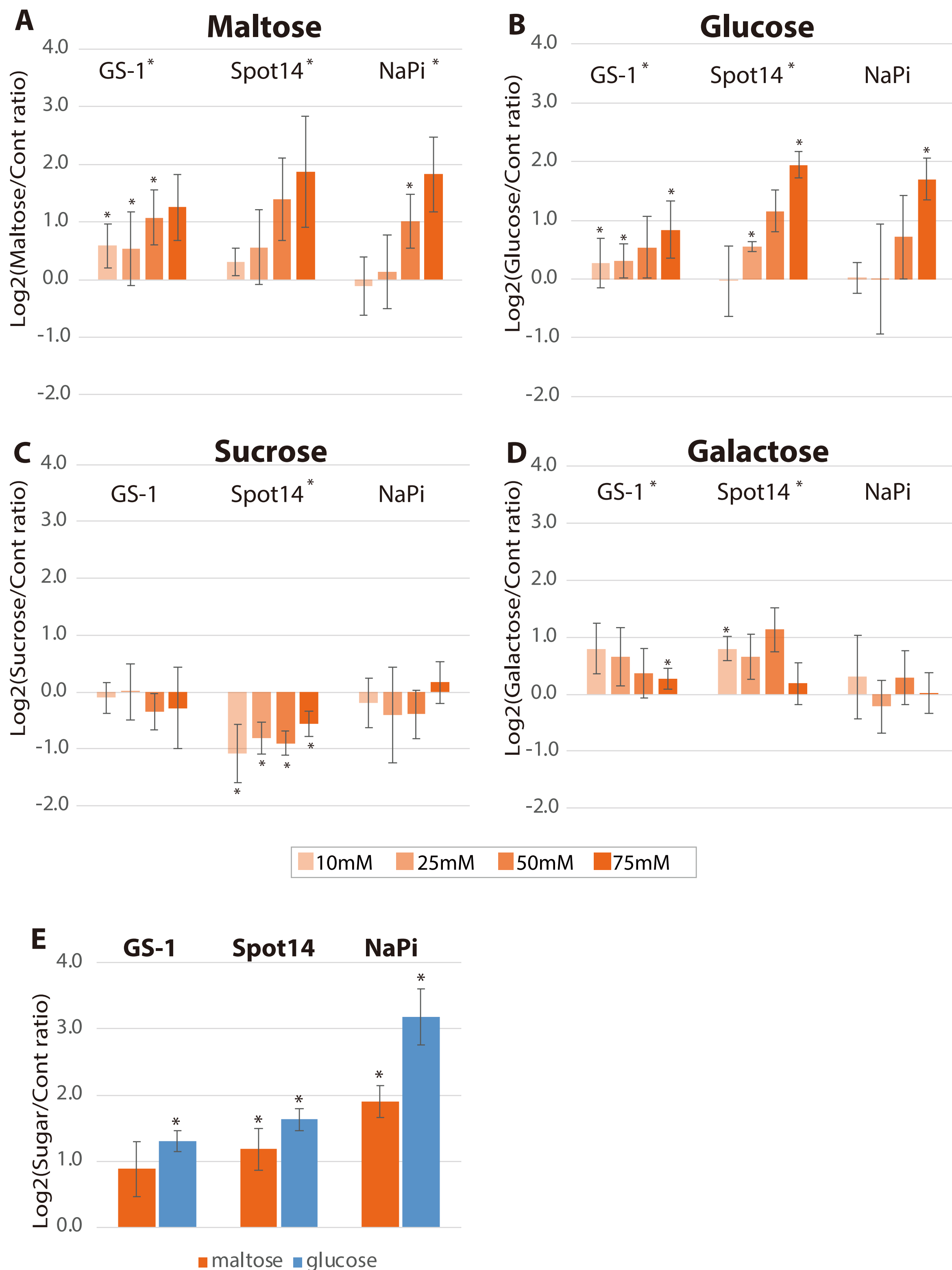

Supplementary Figure 6. Effects of sugars on *Hydra* growth

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

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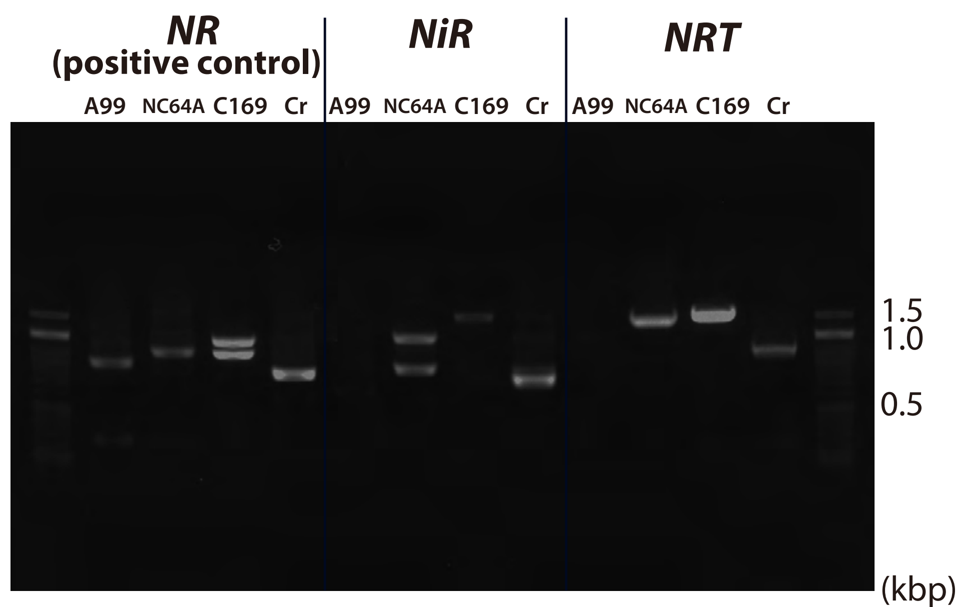

Supplementary Figure 7. PCR of nitrate assimilation genes

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).

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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.