Active Host Response to Algal Symbionts in the Sea Slug *Elysia chlorotica*

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Abstract

Sacoglossan sea slugs offer fascinating systems to study the onset and persistence of algal-plastid symbioses. *Elysia chlorotica* is particularly noteworthy because it can survive for months, relying solely on energy produced by ingested plastids of the stramenopile alga *Vaucheria litorea* that are sequestered in cells lining its digestive diverticula. How this animal can maintain the actively photosynthesizing organelles without replenishment of proteins from the lost algal nucleus remains unknown. Here, we used RNA-Seq analysis to test the idea that plastid sequestration leaves a significant signature on host gene expression during *E. chlorotica* development. Our results support this hypothesis and show that upon exposure to and ingestion of *V. litorea* plastids, genes involved in microbe-associated molecular patterns and oxidative stress-response mechanisms are significantly up-regulated. Interestingly, our results with *E. chlorotica* mirror those found with corals that maintain dinoflagellates as intact cells in symbiosomes, suggesting parallels between these animal–algal symbiotic interactions.

Key words: **symbiosis, kleptoplasty, photosynthesis, *Elysia chlorotica*, transcriptomics.**

Letter

The sacoglossan sea slug *Elysia chlorotica* is well known for its ability to sequester long-term (several months) "stolen" plastids (kleptoplasts) from the stramenopile alga *Vaucheria litorea* (Rumpho et al. 2011). In fact, after settlement, *E. chlorotica* veligers require *Vaucheria* to be present to ensure survival. The sea slugs cannot complete metamorphosis and develop into adults in the absence of the algal prey and plastid capture (Pelletreau et al. 2011). The juveniles feed on the alga for circa 1 week after which plastids alone are able to support continued growth of the animal (Rumpho et al. 2011). *Elysia chlorotica* maintains and utilizes the ingested organelles in cells lining its digestive diverticula, in the absence of both the algal nucleus (Graves et al. 1979; Rumpho et al. 2000) and algal genes derived through horizontal gene transfer in the animal genome (Bhattacharya et al. 2013). Analysis of photosynthesis demonstrates that *E. chlorotica* and its sister species *Elysia timida* rely specifically on algal energy production for development and growth (Giménez Casalduero and Muniain 2008; Rumpho et al. 2009). The metabolic connections and interdependence of the host and algal plastids in *E. chlorotica* are poorly understood. It is however known that upon plastid sequestration, this species accumulates alga-derived lipid droplets of 20:5 eicosapentaenoic acid, among others, as a possible metabolic reserve (Pelletreau et al. 2014). Analysis of lipids in *E. viridis* (Rey et al. 2017) shows that sequestered *Codium tomentosum* (green algal) chloroplasts produce the same spectrum of lipids as the free-living alga. Both of these analyses demonstrate that algal-derived kleptoplasts are capable of producing native lipids in the animal host. Furthermore, plastid activity is dynamic in the host, reflecting extant conditions. Measurement of chlorophyll *a* fluorescence in *E. viridis* shows temporal variability of photopigments, and animals maintained in the dark lose tissues when compared with sea slugs grown under low or normal light levels (Cartaxana et al. 2017). Recognizing the caveat that these data are derived from species that retain different algal plastids, the results nonetheless do not support an earlier hypothesis that kleptoplasts in *E. timida* serve solely as a food source, and that organelle photosynthetic capacity is not key to animal physiology (Christa et al. 2013). Rather, these results predict a more intimate relationship between the algal plastid and host invertebrate—which would be supported by an alga-adapted pattern of gene expression during animal development. To test this idea, we analyzed *E. chlorotica* transcriptomic data from the aposymbiotic phase (APO: prior to ingestion of plastids 1–2 days postmetamorphosis), the transient plastid sequestration phase between 1 and 5 days of feeding (5D), the transitional plastid phase at 5–7 days when the transition to permanent kleptoplasty occurs (7D), and the stable plastid phase after 10 days

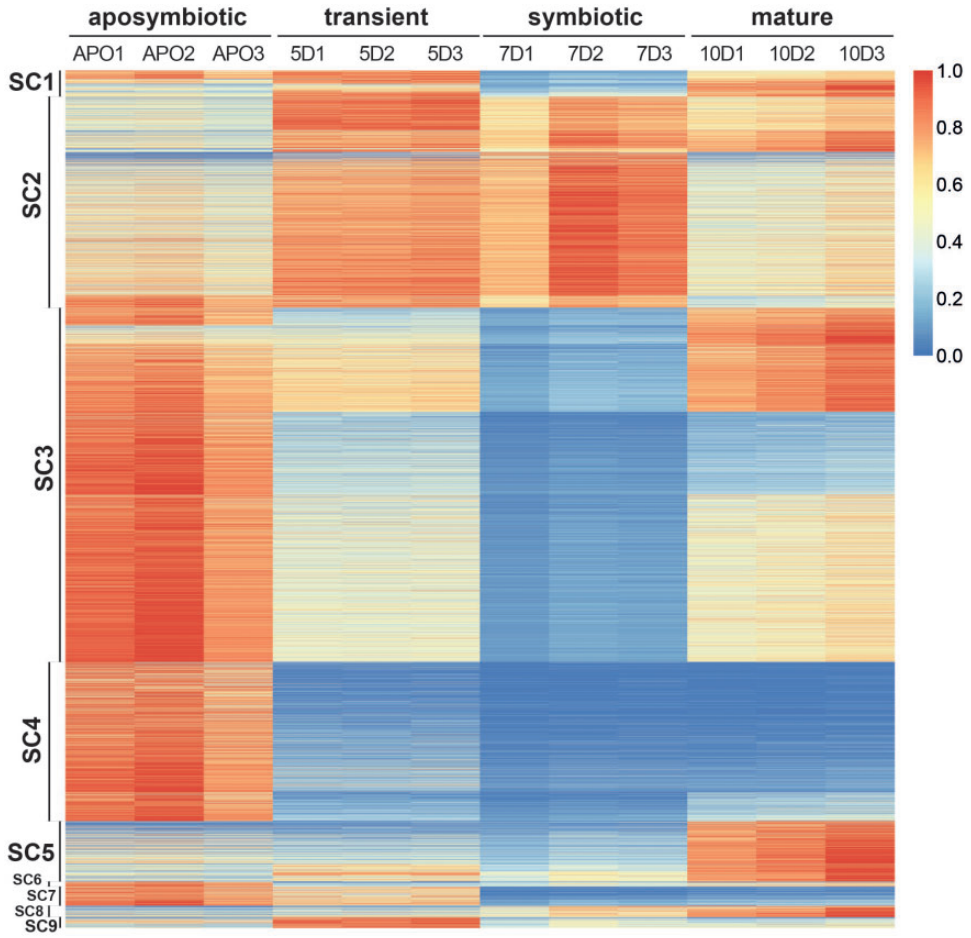

Fig. 1. Heatmap of differentially expressed transcripts in 12 samples across four stages of sea slug development upon exposure of *E. chlorotica* to *V. litorea*: aposymbiotic (APO), transient (5D), symbiotic (7D), and mature (10D). The expression level of these transcripts for each of nine superclusters (SC1–SC9) is shown.

of feeding (10D; Pelletreau et al. 2012, 2014; see Materials and Methods in the supplementary material, Supplementary Material online).

This approach identified 12,619 transcripts as being differentially expressed genes (DEGs), when comparing any two of the four conditions (supplementary table S1, Supplementary Material online). Hierarchical clustering of these DEGs yielded nine superclusters (SC1–SC9) from APO, 5D, 7D, and 10D (figs. 1 and 2; supplementary table S1, Supplementary Material online); functional annotation of these genes is shown in supplementary table S2, Supplementary Material online. Figure 3 shows the proportion of these DEGs with annotated KEGG pathways for each category (see supplementary tables S3 and S4, Supplementary Material online for detail). We also assessed the enrichment of GO terms in each of these SCs against all annotated GO terms in the entire data set (supplementary table S5, Supplementary Material online).

Functions Up-regulated Upon Exposure to *V. litorea*

We found 384 genes in SC1 (fig. 2*a*) and 161 genes in SC9 (fig. 2*i*) that exhibit up-regulation from stages APO to 5D. The expression of SC1 genes was down-regulated from stages 5D and 7D, before an increase again at 10D (fig. 2*a*), whereas the SC9 genes were continuingly down-regulated after 5D, approaching the APO level at 10D (fig. 2*i*). The prevalent functions encoded by these genes are in the KEGG categories of *glycan biosynthesis and metabolism* (i.e., a combined [SC1 + SC9] 14.2% of annotated genes are in this category; supplementary table S4, Supplementary Material online) and *membrane transport* (14.3%). Among the enriched GO terms ($P < 0.05$) in SC1 are GO: 0055085 *transmembrane transport*, GO: 0006486 *protein glycosylation*, GO: 0006006 *glucose metabolic processes*, GO: 0030334 *regulation of cell migration* (in the Biological Process category), as well as GO: 0016812 *hydrolase activity* and GO: 0016705 *oxidoreductase activity* (in the Molecular Function category).

In cnidarian (including coral)-dinoflagellate symbioses, glycans, glycoproteins, and lipopolysaccharides are signature molecules in the microbe-associated molecular patterns (MAMPs), to which pattern-recognition receptors (PPRs; e.g., scavenger receptors) from the cnidarian host bind, initiating a signaling cascade in host responses (Davy et al. 2012). Host-derived glycans from the squid *Euprymna scolopes* contribute to the stability of its mutualism with the bacterium

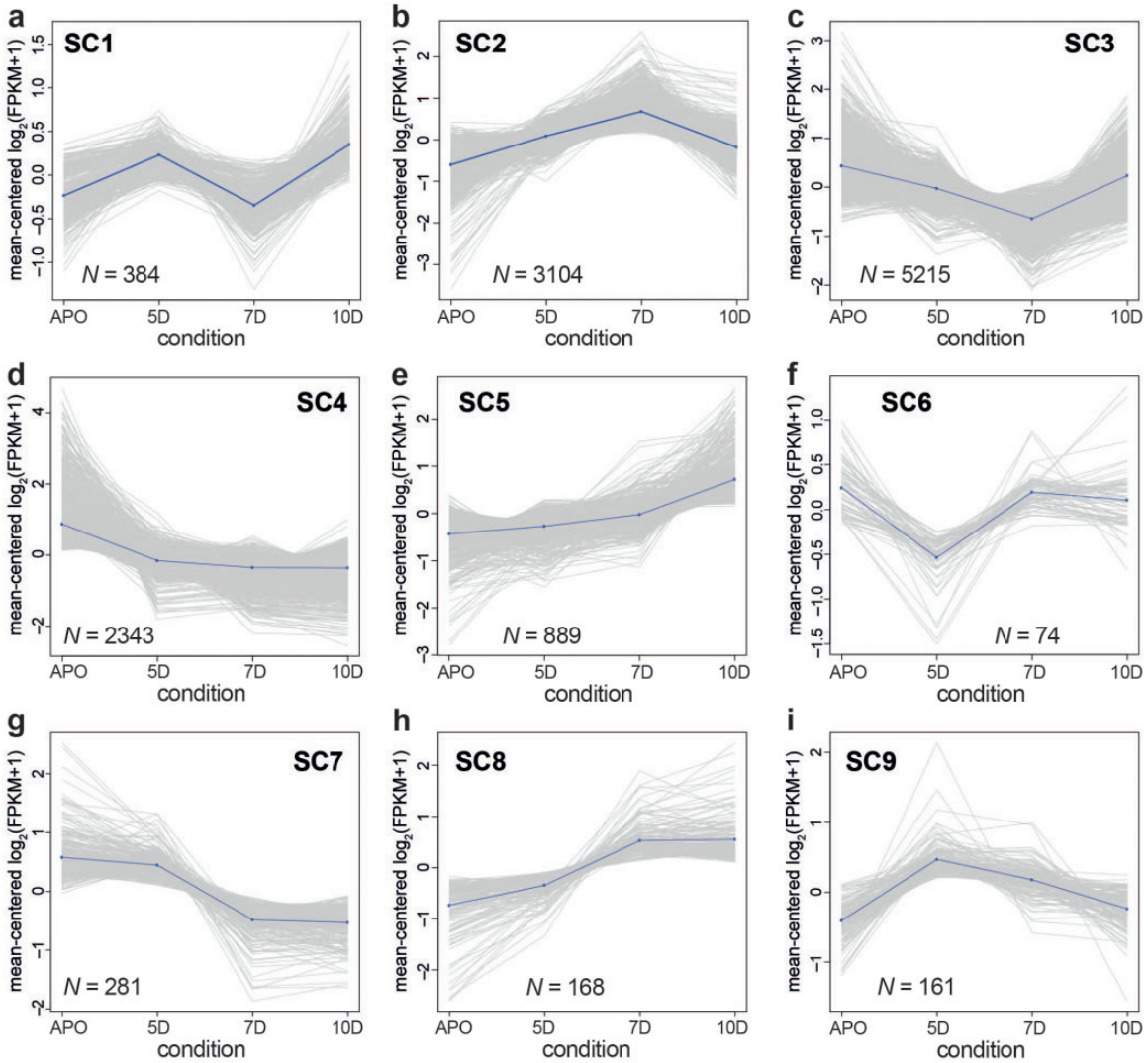

Fig. 2. Expression level of the differentially expressed transcripts shown for each of the nine superclusters, from (*a*) SC1 to (*i*) SC9, across the four stages of APO, 5D, 7D, and 10D (*x*-axis in each graph). The *y*-axis in each graph represents the mean-centered $\log_2(\text{FPKM} + 1)$ value. The line in each graph represents the average expression value.

Vibrio fischeri by modulating the pH of the symbiosis (Schwartzman et al. 2015). Among the annotated functions of *E. chlorotica* genes in SC1 (supplementary table S2, Supplementary Material online), we found scavenger receptors, toll-like receptors, enzymes involved in sugar metabolism (e.g., glucose dehydrogenase, glycoside hydrolase and glycosyltransferases), and membrane transporters of sugars (including glucose), monocarboxylate, organic cations, glycine, and phospholipids. Our results suggest the existence of a similar MAMP-PPR signaling mechanism in *E. chlorotica* upon exposure to *V. litorea*. The metabolism and transport of glycans and their derivatives are critical during this early exposure (APO, 5D), and in the subsequent maturation of the symbiosis (10D), but not during plastid stabilization (7D). Among SC9 genes, we identified a number of oxidoreductases, including ascorbate oxidase and thioredoxin, and the GO term GO: 0055114 *oxidation–reduction process* that is significantly enriched. These functions, up-regulated only during the initial exposure to *V. litorea*, suggest a potential oxidative stress-response mechanism in the sea slug.

Functions Enhanced during Symbiosis Establishment

Genes in SC2 (3,104; fig. 2*b*) are up-regulated during the early stage of symbiosis (from APO, 5D) and during the transition to permanent kleptoplasty (7D), before a decrease at 10D that approximates the APO level. The functions of these genes are likely critical to *E. chlorotica* when establishing, and less so for maintaining, the symbiosis with *V. litorea*. In addition, the expression of 168 SC8 genes were increased during the symbiosis establishment between the 5D and 7D stages.

As shown in figure 3 and supplementary table S4, Supplementary Material online, the most prevalent KEGG pathways encoded by SC2 genes are related to genetic

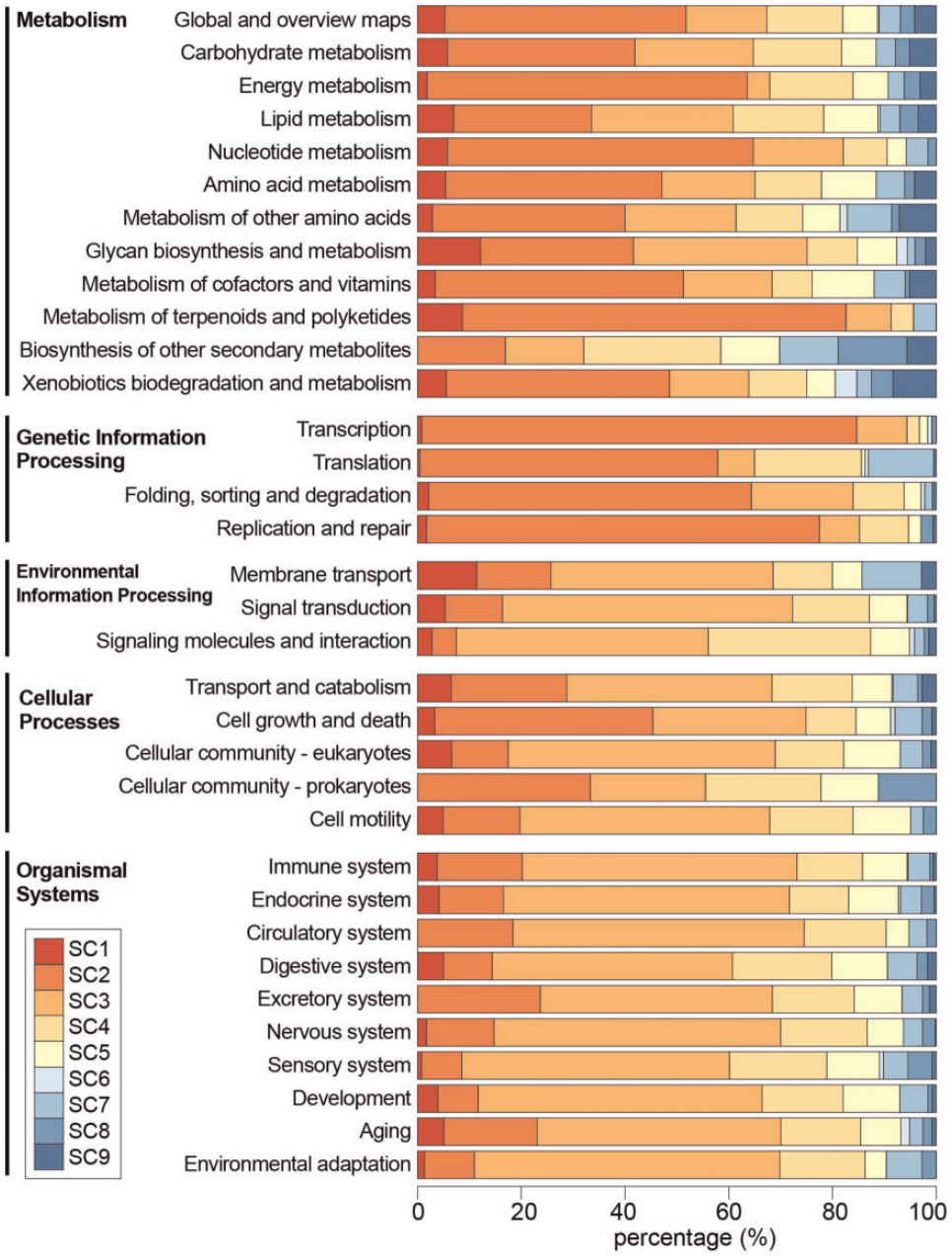

Fig. 3. Annotation of KEGG pathways encoded by differentially expressed transcripts, shown as the proportion of transcripts by supercluster in each KEGG category.

information processing; that is, *transcription* (83.9% of annotated genes in the category), *replication and repair* (75.7%), *folding, sorting, and degradation* (62.2%), and *translation* (57.4%). Among genes with functions relevant to transcription, SC2 genes encode 81 ribosomal proteins (*Ribosome*, supplementary table S3, Supplementary Material online), and 84 proteins involved in *RNA transport* (supplementary table S3, Supplementary Material online). We also observed a prevalence among all metabolic functions (705 genes in the global and overview maps). More than 25% of genes in each category (except *biosynthesis of other secondary metabolites*) are found in SC2, including *metabolism of terpenoids and polyketides* (73.9%), *energy metabolism* (61.7%), and *nucleotide metabolism* (58.9%). *Biosynthesis of antibiotics* is the most represented among SC2 genes (89 compared with ≤ 24 in the other SCs; supplementary table S3, Supplementary Material online), as are *oxidative phosphorylation* (60 compared with ≤ 12 in the others) and *glycolysis* (18 genes, ≤ 7 in the others). Enriched GO terms in SC2 include GO: 0030174 *regulation of DNA-dependent DNA replication initiation*, GO: 0006284 *base-excision repair* (in the Biological Process category), as well as GO: 0005852 *eukaryotic translation initiation factor 3 complex* in the Cellular Component category. These results indicate an increased capacity in metabolism, DNA processing including DNA repair, and phosphorylation, reflecting the host response upon exposure to *V. litorea*, and encompass molecular machineries relevant to the initial stress response and for establishing the symbiosis. Decreased

gene expression upon maturation of the symbiosis agrees with the observation of photoautotrophy in *E. chlorotica* described above, suggesting that functions related to metabolism, DNA processing, and phosphorylation are compensated for by the algal plastid.

Functions Down-Regulated during Development of the Symbiosis

The expression of 5,215 genes (SC3; fig. 2*c*) was down-regulated during early establishment but not upon maturation of the symbiosis. These genes represent 41.3% of all DEGs and show a continued decrease in expression until seven days postintroduction of *V. litorea* (i.e., from APO, 5D to 7D; fig. 2*c*), before their expression increases from 7D to 10D. The expression of an additional 74 genes (SC6; fig. 2*f*) follows a similar pattern, although the down-regulation of gene expression occurs later from 5D to 7D, during symbiosis establishment. The expression of SC3 and SC6 genes after symbiosis was established (10D) returned to the level that approximates the aposymbiotic stage (APO).

On the basis of KEGG annotations (fig. 3 and supplementary table S4, Supplementary Material online), the prevalent functions encoded by genes in SC3 and SC6 are related to environmental information processing, that is, *signaling molecules and interaction* (a combined [SC3 + SC6] 49.5% of all annotated function in this category), *signal transduction* (55.9%), and *membrane transport* (42.9%), cellular processes, for example, *cellular community—eukaryote* (51.5%), and those related to organismal systems, for example, *environmental adaptation* (58.9%), *circulatory system* (56.1%), *nervous system* (55.2%), *development* (54.7%), and *sensory system* (52.3%). Comparing annotated GO terms in SC3 against all annotated GO terms in the data set, the over-represented (false discovery rate FDR $\leq$ 0.05) terms include GO: 0035023 *regulation of Rho protein signal transduction*, GO: 0006470 *protein dephosphorylation*, GO: 0006821 *chloride transport*, and GO: 0070588 *calcium ion transmembrane transport* (in the Biological Process category), as well as GO: 0034707 *chloride channel complex* and GO: 0005891 *voltage-gated calcium channel complex* (in the Cellular Component category). This finding indicates that *E. chlorotica*, in the presence of *V. litorea* and during symbiosis establishment, undergoes an arrest in some molecular signaling and transport, movement, and neural and sensory functions (5D and 7D). These functions are subsequently up-regulated after the symbiosis is established (10D), indicating their importance in the maintenance and maturation of the interaction.

Functions Down-Regulated throughout the Symbiosis

The expression of 2,343 SC4 genes (fig. 2*d*) was down-regulated upon first introduction of *V. litorea* (from APO to 5D), and remained low at 7D and 10D after plastid exposure. The expression of 281 SC7 genes show a similar pattern (fig. 2*g*), with down-regulation occurring between 5D and 7D after plastid exposure. Of these genes, the prevalent KEGG pathways are *biosynthesis of other secondary metabolites* (a combined [SC4 + SC7] 37.7% of all annotated genes in the category; fig. 3 and supplementary table S4, Supplementary Material online), *signaling molecules and interaction* (33.2%), and *translation* (33.2%). Other prevalent functions in SC4 and SC7 genes include *digestive system* (24.8%), *sensory system* (23.4%), *development* (21.1%), and *cell motility* (18.5%). Few genes encode functions related to transcription (2.4%) and replication and repair (9.5%). Enriched GO terms in SC4 include GO: 0006414 *translational elongation*, GO: 0050765 *negative regulation of phagocytosis*, GO: 0009651 *response to salt stress*, GO: 0009414 *response to water deprivation* (in the Biological Process category), as well as GO: 0022625 *cytosolic large* (and *small*) *ribosomal subunit*, and GO: 0003735 *structural constituent of ribosome* (also overrepresented in SC7) in the Cellular Component category. These findings suggest that wide-ranging functions, in particular genes encoding signaling functions and ribosomes, are likely to be continuously suppressed as soon as *V. litorea* is introduced, through to symbiosis maturation. Some of these functions, particularly the critical function of ribosomal proteins in translation, may be compensated by *V. litorea*. In contrast, the suppression of phagocytic function may reflect the decreased dependency of *E. chlorotica* on phagocytosis. We note that this time point corresponds with observed lipid accumulation in *E. chlorotica* while the symbiosis is being established. We postulate that the suppression of phagocytosis related genes may facilitate the accumulation of plastids and their lipids in the animal cells. See the supplementary material, Supplementary Material online the description of 889 genes in SC5 that show up-regulation after symbiosis maturation.

Conclusions

Despite not encoding any algal genes in its nuclear genome, *E. chlorotica* has developed a suite of molecular machineries to respond to, cope with, and maintain its symbiosis with the stolen plastids from the alga *V. litorea*, within a high-energy environment. When first exposed to *V. litorea*, *E. chlorotica* responds by temporarily increasing its capacity in the MAMP-PPR signaling cascade relative to recognition of foreign cells, the metabolism of various metabolites and the processing of genetic information, while suppressing processes of other molecular signaling, communications, and neural activity. This response, including the up-regulation of DNA repair genes, may be attributed to an initial stress response of the animal, and progresses throughout establishment of the symbiosis. These trends revert upon maturation of the symbiosis. The marked increase in the capacity of molecular signaling and neural activity upon symbiosis maturation, and the permanent suppression of the phagosome and ribosome, may indicate the engagement of cell communication and interaction, and complementarity of gene functions between the host and the plastids of *V. litorea*.

When compared with the establishment of the symbiosome which houses dinoflagellate symbionts (i.e., whole cells, not just plastids) in corals, we find a larger impact on host cell gene expression in *E. chlorotica*. In the coral *Acropora*

digitifera, Mohamed et al. (2016) reported that only 1,073 transcripts (i.e., <3% of the total transcriptome) were differentially expressed 4 h after the exposure of coral planulae to a competent strain of *Symbiodinium*, and returned to baseline levels within 48 h. These authors proposed that the symbiosome acts as an arrested phagosome that protects the algal symbiont from host lysosomes. The current results are not directly comparable to this study due to differences in experimental design and the tools used for assessing differential expression. In addition, our work addresses many more pathways of animal development than solely algal enslavement, thereby explaining the >12K DEGs that we report. Therefore, we recognize that with these data it is impossible to clearly separate DEGs that are solely involved in animal development from those that are required for establishing and maintaining the plastid symbiosis. Nonetheless, given that *E. chlorotica* has an obligate requirement for *Vaucheria* plastids to allow development (Pelletreau et al. 2011; Rumpho et al. 2011), the DEG data must contain the signal of this process. With these considerations in mind, it is noteworthy that both coral symbiosome formation and algal kleptoplasty in *E. chlorotica* follow a similar pattern and offer complementary insights into how symbiosis impacts host gene expression in the case of organelle (sea slug) or whole algal cell (coral) capture. In summary, our findings further support the hypothesis that the plastid is not simply food stored for later as suggested by some, but rather, an energy powerhouse that supports and is integrated into animal development.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

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