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RESEARCH NOTE

Transcriptomic landscape of the kleptoplastic sea slug *Elysia viridis*

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Certain sacoglossan sea slugs sequester photosynthetic-active chloroplasts from their algal prey. After ingestion, these chloroplasts are recognized through pattern-recognition receptors (PRRs) (Melo Clavijo *et al.*, 2020) and induce modifications in the transcriptional landscape of the host, increasing the expression of specific genes, such as reactive oxygen species (ROS) quenching genes (Chan *et al.*, 2018; Melo Clavijo *et al.*, 2020). These chloroplasts (also called kleptoplasts) are kept inside the epithelial cells of the host's digestive tubules (de Vries, Christa & Gould, 2014). The time kleptoplasts remain functional and active is species-dependent, varying from a few days (short-term retainers; StR) to over 1 month (long-term retainers; LtR) (Händeler *et al.*, 2009; Melo Clavijo *et al.*, 2020). From the beginning of their life in the host, kleptoplasts are challenged by the host's innate immune system, the first line of defence against foreign molecules or potential pathogens. The resulting immune response is triggered by recognizing evolutionarily conserved pathogen-associated molecules mediated by different types of PRRs (Cao, 2016). Two of these PRRs, the scavenger receptor (SR) and C-type lectin receptor (CTLR), may play a crucial role at the beginning of kleptoplasty (Melo Clavijo *et al.*, 2020). Once in the digestive tubules, the stolen plastids play a dual role as starch-storage devices and a nutrient source during periods of food scarcity (Cartaxana *et al.*, 2017; Laetz *et al.*, 2017), even though the plastid photosynthates are not essential for the slug's nutrition during starvation (Christa *et al.*, 2014b). During this period, damaged photosynthesis-related proteins can generate abundant ROS (Maeda *et al.*, 2021). To protect itself from ROS molecules that can damage proteins and organelles, the host triggers an autophagy signal to reduce the resulting oxidative damage (Scherz-Shouval & Elazar, 2011). Moreover, based on the ROS-quenching response of sea slugs during starvation, de Vries *et al.* (2015) proposed a new classification of the photosynthetic sea slugs based on their ability to suppress the ROS stress (i.e. classifying them as starvation-intolerant and starvation-tolerant species).

To shed more light on the mechanisms behind the kleptoplasty, we describe here for the first time the transcriptomic landscape of the photosynthetic sea slug *Elysia viridis* (Montagu, 1804) by assembling a *de novo* reference transcriptome from a pool of ten individuals. This species is a facultative Ulvophyceae-feeder LtR sacoglossan sea slug found across the limits of the European Atlantic, ranging from Scandinavia to the British Isles and the Iberian Peninsula (Jensen, 2007). Although it is commonly classified as an LtR species, *E. viridis* has a wide range of kleptoplast retention times depending on the algal plastid source (Christa *et al.*, 2014a; Rauch *et al.*, 2018). Our transcriptomic sequence data and assembly are made publicly available under the NCBI BioProject accession number PRJNA549923. Here, we address two issues: (1) the characterization of proteins involved in chloroplast recognition and (2) the characterization of proteins related to kleptoplast retention in *E. viridis* and of orthologs in StR and LtR species.

We followed a custom *de novo* transcriptome assembly and annotation pipeline based on others used for nonmodel organisms (Conesa *et al.*, 2016; Raghavan *et al.*, 2022); these are described in detail in the GitHub repository manuelsmendoza/elvira (DOI: 10.5281/zenodo.7243344). The resulting transcriptome of *E. viridis* comprised 12,884 protein-coding sequences (CDSs; lengths ranged from 261 to 8,766 bp) and had a total length of 9.3 Mbp (Supplementary Material Fig. S2 and Table S4). We analysed these CDSs and annotated 9,422 different proteins, with the best hits mainly from two genera, *Elysia* (87.2%) and *Plakobranchus* (11.0%) (Supplementary Material Fig. S2A); the remaining 2.3% comprise multiple genera of sea slugs and snails (Tectipleura) (Kano *et al.*, 2016). Our functional annotations, which were based on Gene Ontology (GO; http://geneontology.org/) corresponded to 9,333 CDSs: 4,755 associated with 2,583 biological processes; 5,466 with 683 cellular components; and 6,693 with 1,606 molecular functions.

Melo Clavijo *et al.* (2020) proposed the recognition of kleptoplasts by the SRs and CTLRs, both mediated by thrombospondin type 1 repeat (TSR) superfamily proteins; this parallels the theory proposed by Neubauer *et al.* (2017) for the recognition of algae symbionts by Cnidaria. Based on the results obtained in the analysis of the PRRs present in the transcriptome of *E. viridis*, we suggest including the Toll-like receptors (TLR) in the group of PRRs involved in the kleptoplastic process (Fig. 1). In our analysis, we identified 19 CDSs from the TSR superfamily (Supplementary Material Fig. S3) and multiple PRR families (Li & Wu, 2021) (Supplementary Material Figs S5–S7). Though we detected the presence of SR domains

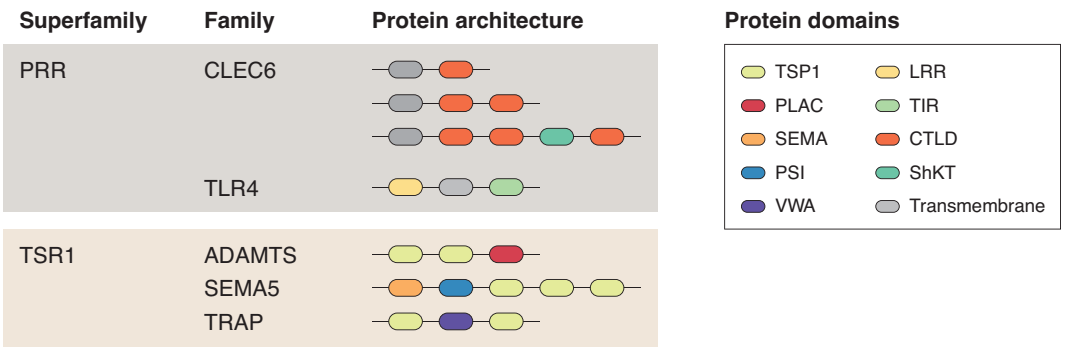

Figure 1. Graphical summary of the domain architecture of proteins found in the transcriptome of *Elysia viridis* and possibly related to the mechanism of acquiring and maintaining kleptoplasts. These include pattern-recognition receptors (CLEC6 and TLR4) and proteins involved in cell communication and tissue development, such as the TSR1 thrombospondin 1 receptor (TSR1) superfamily. The full set of sequences related to these families, as found in the *Elysia viridis* transcriptome, are reported in Supplementary Material Figures S3–S7. CLEC6 and TLR4 are two receptor families that induce a pro-inflammatory response after recognizing mannose and lipopolysaccharides, respectively. Abbreviations for other proteins: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; SEMA5, semaphorin-5; TRAP, thrombin receptor-activating peptides. Protein domains are as follows: TSP1, thrombospondin type-1 domain; PLAC, protease and lacunin domain; SEMA, semaphorin domain; PSI, plexin–semaphorin–integrin domain; VWA, von Willebrand factor type A domain; LRR, leucine-rich repeat domain; TIR, toll-interleukin-1 receptor domain; CTLD, C-type lectin-like domain; and ShKT, Stichodactyla toxin domain.

in multiple CDSs, none of them had the complete coding sequence (i.e. they lacked the transmembrane domain; Supplementary Material Fig. S5). Interestingly, we also found two CDSs of semaphorin 5 (SEMA5) (Fig. 1, Supplementary Material Figs S1 and S4), a TSR superfamily protein characteristic of vertebrates (Goodman *et al.*, 1999; Pasterkamp, 2012), but also described from different invertebrates (Supplementary Material Fig. S4) (Gerdol *et al.*, 2020; Melo Clavijo *et al.*, 2020; Maeda *et al.*, 2021). We note that a recent hypothesis suggests that the SEMA5 family originated in a common ancestor of Placozoa, Cnidaria and Bilateria (it is, however, absent in Nematodea) (Junqueira Alves *et al.*, 2019).

To reduce the damage produced by ROS, sea slugs have a repertoire of transcripts with damage control-related functions. We identified 201 CDSs related to response to stress (GO:0006950) and 10 CDSs associated with the regulation of response to stress (GO:0080134). We also found ROS-quenching-related functions: 24 CDSs related to oxidoreductase complex (GO:1990204) and 560 annotated as showing oxidoreductase activity (GO:0016491) in a large number of donors (e.g. CH-OH, CH=O, C=O, CH and CH2). In addition, we identified 39 CDSs with antioxidant activity (GO:00162099) and other CDSs with functions that reduce oxidative stress; these are superoxide dismutase (GO:0004784), peroxidase (GO:0004601), glutathione oxidoreductase (GO:0097573), glutathione peroxidase (GO:0004602) and thioredoxin peroxidase (GO:0008379). Furthermore, we found eight CDSs related to the symbiont response (GO:0140546) and nine related to the pattern recognition receptor signalling pathway (GO:0002221).

Although the kleptoplastic phenomenon has probably had multiple independent origins across the tree of life (Christa *et al.*, 2015), kleptoplastic sea slug species probably have common mechanisms for stealing the plastids from their algal prey; these mechanisms are likely to be absent in nonkleptoplastic species. Thus, we studied the orthogroups in sea slugs based on their ability to keep the plastids alive in LtR and StR species to find the mechanisms behind this classification. Information about the different species (Supplementary Material Table S5) and the assembly statistics (Supplementary Material Table S6) and analysis pipeline used are described in Supplementary Material Appendices S1 and S2. We detected that 16,997 orthogroups made up 96% of total CDSs. A total of 311 orthogroups were species-specific, while 1,947 were common to all the species (Fig. 2); 37% of orthogroups contain, on average, one or more genes per species. A total of 573 orthogroups were specific to and found in all kleptoplastic species, with 109 being exclusive to StR species and 4 being exclusive to LtR slugs (*sensu lato*) (Supplementary Material Table S7). On analysing the GO enrichment associated with the different orthogroups, we found that the different kleptoplastic slugs have developed an important burst mechanism to regulate the pH by using iron ions (GO:0006885, GO:0006879, GO:0008199 and GO:0004,322) and other enzymatic pathways (GO:0016491, GO:0016788 and GO:0016817). Focusing on the LtR species, we found enrichment of gene ontologies related to immune response (GO:0006955) mediated by the tumour necrosis factor (GO:0005164 and GO:0016021) and G proteins (GO:0004930, GO:0016021 and GO:0007186).

In summary, the longevity of kleptoplasts and sea slugs during starvation may be mediated by multiple factors, including the recognition of the plastids during feeding by multiple receptors (e.g. PRRs, CTLRs and SRs) and ROS-quenching proteins using enzymatic and nonenzymatic mechanisms. In particular, in the transcriptome of *E. viridis* we found that the presence of CDSs corresponds to multiple PRRs that may be involved in the plastid-recognition process; this is despite the fact that this species has a low receptor richness in comparison with other elysoids (Melo Clavijo *et al.*, 2020). In addition, we also detected multiple enzymatic families involved in the ROS-quenching response. In contrast, the production of antioxidant compounds may contribute in only a minor way to the control of oxidative stress. A further enriched GO category in species that sequester chloroplasts corresponded to G protein-coupled receptors, which suggests that these receptors may be required for plastid recognition in Sacoglossan sea slugs, paralleling their role in other symbioses, such as the mutualism between cnidarians and dinoflagellates (Rosset *et al.*, 2020). Sacoglossan sea slugs may also require the presence of iron ions to reduce the oxidative stress generated after plastid acquisition. All this evidence, derived from the transcriptome analysis of *E. viridis*, sheds interesting new light on the possible mechanisms used by sea slugs to recognize and establish kleptoplasts within their bodies.

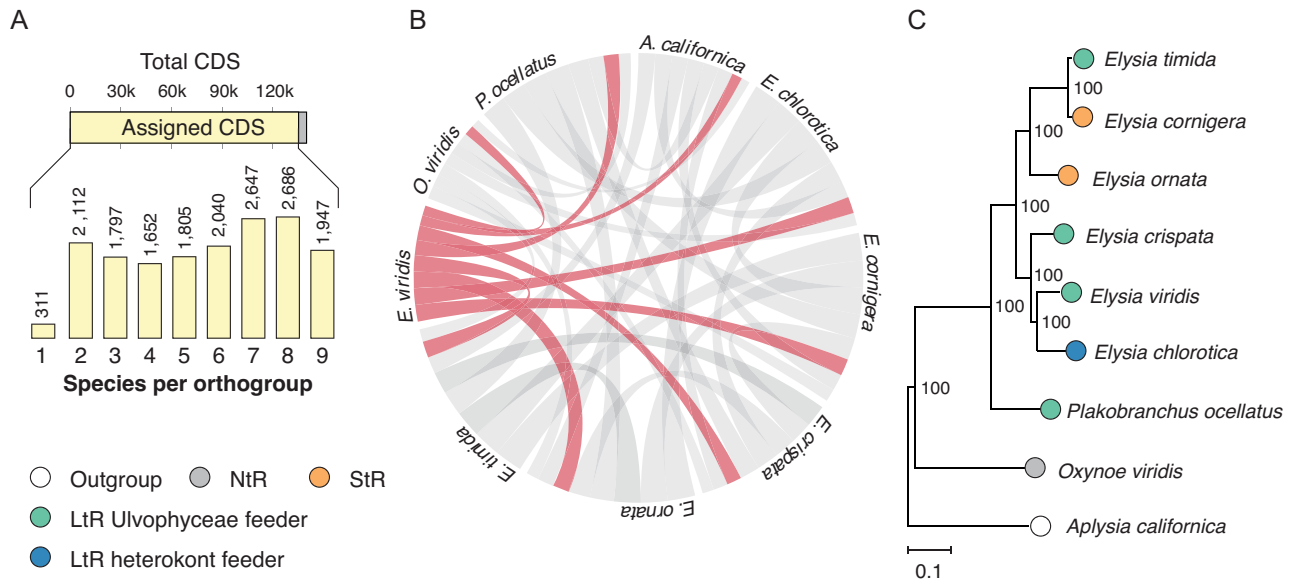

Figure 2. Orthogroup detection and phylogeny. **A.** The number of CDSs assigned to the different orthogroups and the number of species where they are present. **B.** Visual representation of the number of orthogroups shared between the different species; in red is the number of orthogroups shared between *Elysia viridis* and the other slugs. **C.** A maximum likelihood phylogenetic species tree was inferred using RAxML from the concatenated alignment of 1,380 orthologs that were predicted by OrthoFinder from the set of complete transcripts obtained after removal of potential biological contamination (Supplementary Material Table S8). The tree was inferred using the JTT + FC + I + R4 nucleotide substitution model (estimated using ProtTest3) and 10,000 bootstrap replicates (percentage bootstrap support values are shown on branches).

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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