# Transcriptome landscape of kleptoplastic sea slug *Elysia viridis*

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Certain sacoglossan sea slugs can sequester and maintain photosynthetically active chloroplasts through algae feeding, a phenomenon called kleptoplasty (Rumpho et al., 2001; Cruz et al., 2013). The amount of time these kleptoplasts remain active in the slug is species- and environment-dependent (Curtis et al., 2015; Laetz & Wägele, 2018; Donohoo et al., 2020). This period can span from a few days in short-term retaining species (StR) to more than three weeks in long-term retaining species (LtR) (de Vries et al., 2014). Since Trench described kleptoplasty by studying the green sea slug *Elysia crispata* (Trench, 1969), multiple hypotheses have been proposed to explain the mechanism behind this extraordinary phenomenon (Rumpho et al., 2001). One of the most intriguing hypotheses was the possibility of lateral gene transfer (LGT) of chloroplast-maintenance genes from the algae nuclei to the slug's genome. Although early results supported the LGT hypothesis (Rumpho et al., 2008), late experiments based on high-throughput sequencing at transcriptomic and genomic level provided enough evidence to dismantle this hypothesis (Wägele et al., 2011; Bhattacharya et al., 2013; Maeda et al., 2021), making necessary to explore other potential mechanisms underlying kleptoplasts maintenance i.e., photoprotective behaviour to reduce light-induced oxidative stress (Cruz et al., 2015; Chan et al., 2018; Cartaxana et al., 2019).

Adding to the available data to shed some light on these processes, here we report for the first time the transcriptome of the LtR sea slug *Elysia viridis* (Montagu, 1804). This species is an Ulvophyceae-feeder sea slug found across the European Atlantic border, from Scandinavia to the British Isles and the Iberian Peninsula (Jensen, 2007). To build this reference transcriptome, we sampled wild *E. viridis* individuals from a subtidal *Codium* spp. meadow settled on a granite outcrop (Cabo Estai, Spain, $42°10'5''N 8°48'48''W$). After starving for two weeks, ten individuals were pooled before preservation in RNAlater at room temperature. This pooled sample was sent for RNA extraction, library preparation and Illumina sequencing to the BGI Genomics (China).

We used 27 million 90bp reads for *de novo* transcriptome assembly with Trinity (Haas et al., 2013). From this assembly, we extracted the protein-coding sequences (CDSs) using TransDecoder and annotated them through BLASTX alignment (Camacho et al., 2009; Gish & States, 1993) against the UniProtKB protein database (release 2021_04). In addition, we identified protein domains using HMMER (Eddy, 2011). Finally, we performed a gene ontology (GO) enrichment analysis by using topGO (Alexa & Rahnenführer, 2009). The subgroups for GO-enrichment analysis were established based on transcript expression estimated using Salmon (Patro et al., 2017). The reads and assembled transcriptome were deposited in the NCBI database under the BioProject accession number PRJNA549923.

The obtained *E. viridis* reference transcriptome consisted of 16,977 CDSs, most of them partial sequences (Fig. 1A). After aligning the CDSs to the Uniprot database, we identified 15,525 matches (91.4% of the CDS) corresponding to 10,708 proteins. All these entries are molluscan-related proteins, mainly from the *Elysia* genus (78%) (Fig. 1B). Almost half of the proteins annotated by homology corresponded to uncharacterised protein products (49.7%). From the rest, the highest number of matches corresponded to protein kinase (74), G_PROTEIN_RECEP_F1_2 (58), and MFS (major facilitator superfamily) (55) domain-containing proteins. Furthermore, we detected 13,021 CDSs (76.7%) with at least one protein domain. From all the CDSs, 75% of them had four or fewer protein domains and four CDSs had more than 100 domains (Fig. 1C, right tail). In summary, we annotated 12,539 CDSs with GO terms by combining the information obtained from the homology and protein domain predictions.

Using the latter annotation, we did not find any GO term related to plastids or photosynthesis. All the enriched GO terms reflected homeostasis management and energy production mitochondrial functions. In contrast, Maeda et al. detected multiple photosynthesis-related terms associated with the chloroplast (chloroplast, GO:0009507; and chloroplast envelope, GO:0009941) in the genome of *Plakobranchus ocellatus* (Maeda et al., 2021). In the transcriptome of *E. viridis* we found nine CDSs related to response to oxidative stress (GO:0006979). These CDSs correspond to multiple enzymatic families (e.g. catalases, dual oxidases and glutathione peroxidases) that prevent the oxidative damage caused by the reactive oxygen species (ROS) (Chan et al., 2018; Foyer & Shigeoka, 2011; Foyer, 2018). We also detected other CDSs with putative protective roles from oxidative stress: methionine sulfoxide reductases and sestrin. Although these results agree with previous studies Chan et al. 2018, it is hard to distinguish functions corresponding to the regular metabolism of the sea slug from the mechanisms involved in the kleptoplastic processes. Increasing the number of sequenced samples and obtaining more information about sacoglossan transcriptional landscapes at different developmental and metabolic stages may help to improve the identification of transcripts involved in kleptoplasty mechanisms and chloroplast maintenance in sea slugs in further studies.

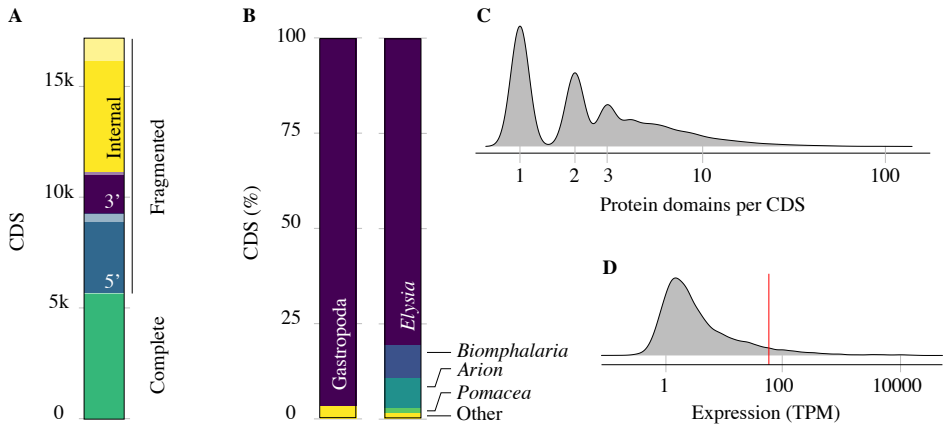


Figure 1: Summary of the *Elysia viridis* transcriptome. **A** CDSs in the final transcriptome are classified by their completeness: complete (containing the start and stop codons), containing only the start codon (5'), containing only the stop (3') codon, and internal fragments (without the start and stop codons). The different colour depths reflect the number of CDSs: with any match in the database (dark) and without (light). **B** Taxonomic classification by best BLASTX match. **C** Number of protein domains per sequence. **D** Distribution of CDS expression in the transcriptome of *E. viridis* expressed in transcripts per million (TPM). Red line represents the $P_{95}$.

For comparative analysis, we fetched reads from the NCBI SRA database to assemble and annotate the individual transcriptomes for other sea slug species: *Aplysia californica* J. G. Cooper, 1863 (outgroup, carnivorous sea hare from American Pacific Ocean; SRR6871219), *Oxynoe viridis* (Pease, 1861) (non-retainer Ulvophyceae-feeder from Oceania; SRR1505125), *Elysia ornata* (Swainson, 1840) (StR Ulvophyceae-feeder from the Antilles; DRR029459), *Elysia cornigera* Nuttall, 1989 (StR Ulvophyceaefeeder from the Gulf of Mexico; SRR1582561), *Elysia timida* (Risso, 1818) (LtR Ulvophyceae-feeder from the Mediterranean Sea; SRR1582571), *Elysia crispata* Mörch, 1863 (LtR Ulvophyceae-feeder from the Antilles; SRR11015444), *Elysia chlorotica* Gould, 1870 (LtR Heterokonts-feeder from American Atlantic Ocean; SRR8282417), and *Plakobranchus ocellatus* van Hasselt, 1824 (LtR Heterokonts-feeder from Indonesia; DRR236388) (Fig. 2) following the pipeline described above. We used the complete CDSs from these transcriptomes, together with the *E. viridis* one, to extract orthologous genes with OrthoFinder (Emms & Kelly, 2019). We found 12,254 orthogroups from 89,047 completely assembled CDSs in the nine studied species; only 481 (3.9%) CDSs were species-specific, and 553 (4.5%) orthogroups were shared by all the species. We detected 388 orthogroups, conformed by 5,122 CDSs, common to all the species that can feed on and retain plastids (Fig. 2E). In those species with active kleptoplasts, LtR species shared nine orthogroups, and StR ones had in common 44 orthogroups. The LtR elysoids, excluding *P. ocellatus*, shared only two orthogroups that consisted of 10 CDSs (*E. chlorotica*, 2; *E. crispata*, 3; *E. timida*, 3; and *E. viridis*, 2) (Fig.2E).

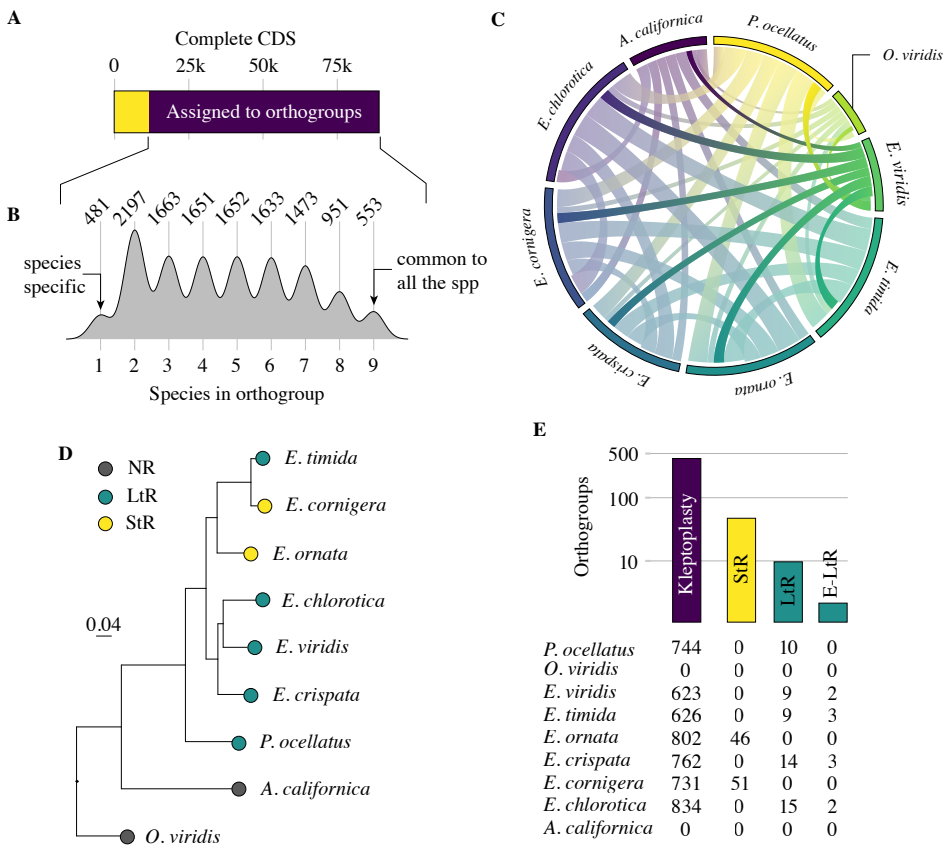


Figure 2: Comparative transcriptome analyses. **A** Number of CDSs completely assembled. **B** Number of complete CDSs shared among species. **C** Number of complete CDSs shared between different species, the thickness of the links between the different sea slugs are proportional to the number of orthologs shared. **D** Ortholog clustering obtained with Orthofinder. **E** Orthogroups shared by all different species and classified attending to their capability to sequester functional chloroplasts and time remaining active (E-LtR: Long-term retaining *Elysia* spp.).

The GO enrichment analyses of StR, LtR and E-LtR species did not show any shared term of interest that could be related to plastid maintenance. Even if the structure of the clustering resulted from our analysis (Fig. 2E) disagrees with the topology of sea slugs' phylogenetic tree (Maeda et al., 2010; Klochkova et al., 2013; Christa et al., 2014), in both methods StR and LtR do not conform to a uniform group, suggesting multiple origins for kleptoplast maintenance (Christa et al., 2015).

In conclusion, we did not detect any transcript associated with photosynthesis or plastid-related functions or components, nor found any transcript with possible algal origin by BLAST. Our results are in agreement with those latest obtained from genomic data (Maeda et al., 2021; Rauch et al., 2015), in that there is no evidence of LGT from algae to slugs. Therefore, our results add to the

bulk of evidence that it is time to look behind the search of algal genes in sea slugs and focus on the search of mechanisms involved in the reduction of oxidative stress that could explain the mechanisms behind kleptoplasty and plastid longevity.

## Acknowledgements

This work was supported by the Consellería de Educación e Ordenación Universitaria, Xunta de Galicia (GPC2014/067) and the Universidade de Vigo to D.P.; and from the European Social Fund and the Government of Xunta de Galicia (ED481A-2018/305) to M.M. We thank the Supercomputing Centre of Galicia (CESGA) for providing computational resources and technical support.

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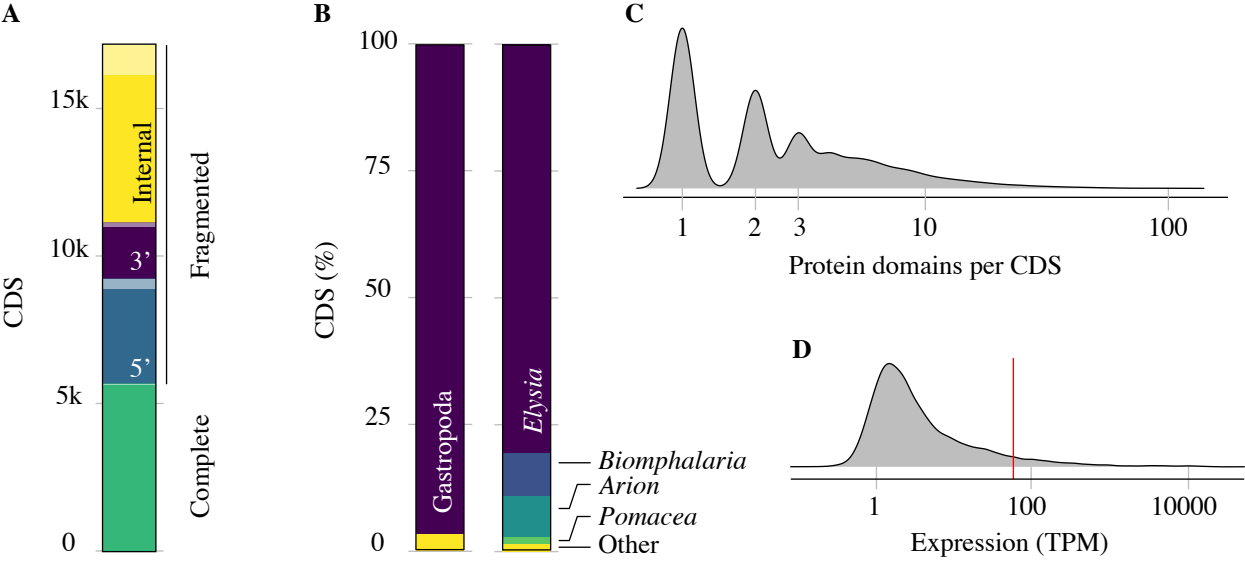
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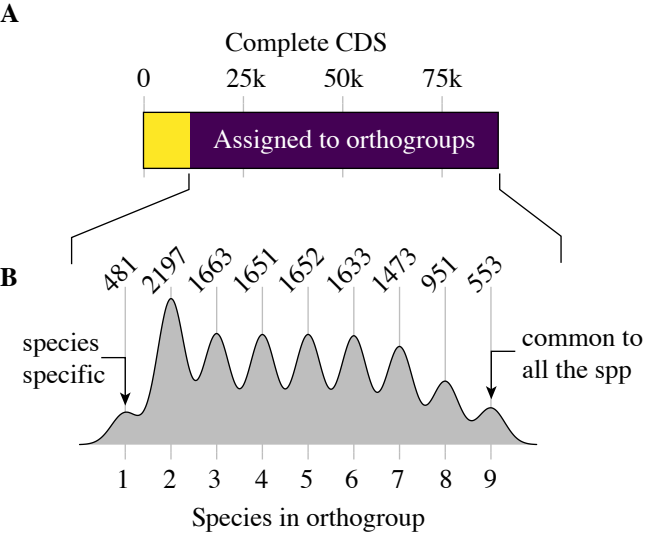


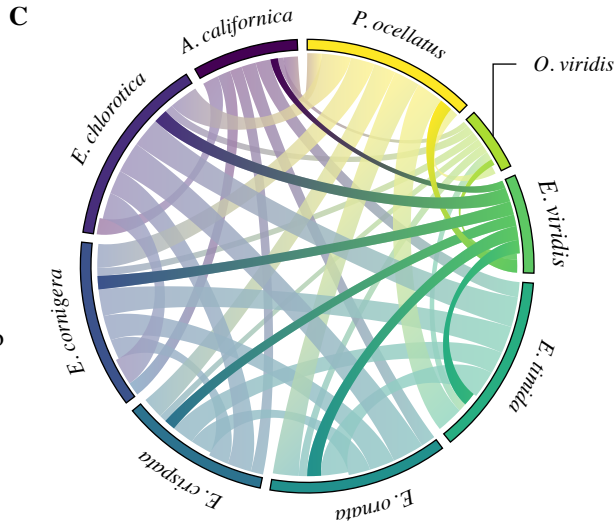


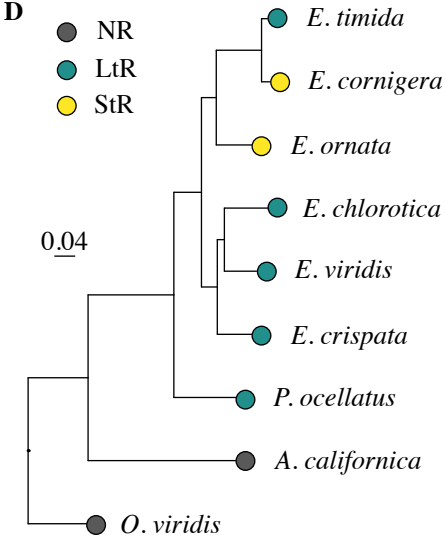


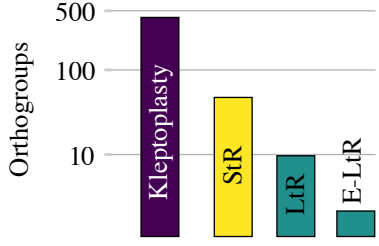


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| *P. ocellatus* | 744 | 0 | 10 | 0 |
| *O. viridis* | 0 | 0 | 0 | 0 |
| *E. viridis* | 623 | 0 | 9 | 2 |
| *E. timida* | 626 | 0 | 9 | 3 |
| *E. ornata* | 802 | 46 | 0 | 0 |
| *E. crispata* | 762 | 0 | 14 | 3 |
| *E. cornigera* | 731 | 51 | 0 | 0 |
| *E. chlorotica* | 834 | 0 | 15 | 2 |
| *A. californica* | 0 | 0 | 0 | 0 |