Title: Kleptoplasty relying on a host-derived component in the Euglenid protist, *Rapaza viridis*

Running title

Molecular study of kleptoplasty in Rapaza viridis

Manuscript category

Commentary for Maruyama et al. (2023) doi: 10.1093/pcp/pcad044

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The diversity in current eukaryotic phototrophs was established via multiple endosymbiotic events (Sibbald & Archibald, 2020). In primary endosymbiosis, a eukaryotic host engulfed a cyanobacterial ancestor and evolved into Archaeplastida, which includes viridiplantae (plants and green algae), rhodophyte, and glaucophyte algae. Subsequent secondary (or higher) endosymbiosis spread plastids derived from red or green algae across different eukaryotic lineages, establishing extensive diversity. Secondary endosymbiosis has a significance in shaping the aquatic ecosystem of contemporary oceans because the major primary producers, including diatoms and haptophytes, have secondary plastids of red algal origin.

Upon establishment of endosymbiosis, the host and endosymbiont are integrated into a single photosynthesising cell. The key steps of this process include the installation of metabolite exchange systems, gene transfer from the symbiont to the host nucleus (i.e. endosymbiotic gene transfer, EGT), and establishment of protein-targeting systems to functionalize nuclear-encoded proteins in plastids (Sibbald & Archibald, 2020). Secondary plastids are surrounded by additional membranes (three or four, compared to two in primary plastids), and thus more complexity occur in the translocation of metabolites and proteins than in primary plastids.

Despite the evolutionary importance of secondary endosymbiosis, the mechanism underlying the integration of two eukaryotes remains elusive. Although we cannot observe past phenomena, observations of living organisms displaying intermediate stages of endosymbiogenesis are useful for the construction of rational models of evolution. Organisms that use ingested algae as temporal plastids, a phenomenon known as kleptoplasty, are excellent candidates for such research. Kleptoplasty is observed across diverse lineages including both multicellular and unicellular organisms, such as the sea slug *Elysia viridis*, which bears green algal kleptoplasts, and the ciliate *Mesodinium rubrum*, which bears cryptomonad-derived kleptoplasts. The euglenid *Rapaza viridis* (Rapaza hereafter) contains kleptoplasts derived from a green alga *Tetraselmis* sp. (Yamaguchi et al., 2012). Rapaza engulfs the *Tetraselmis* alga, but retains only its chloroplasts, discarding other parts including the cytoplasm and nucleus (Karnkowska et al., 2023). Rapaza appears to be an obligate photoautotrophic organism that depends on the photosynthesis of its kleptoplasts, because a regular supply of *Tetraselmis* and light illumination are essential for its survival.

Like algae and plants, Rapaza can grow with nitrate as its sole nitrogen source. In the general model of nitrate assimilation (Long et al., 2015), external nitrate is taken up by plasma membrane transporters, followed by nitrate reduction to ammonium ions through reactions catalysed by nitrate reductase (NR) and nitrite reductase (NiR) localised in the cytosol and plastids, respectively. The resulting ammonium ions are assimilated into carbon skeletons to yield amino acids. This process involves various transporters/enzymes located in different compartments, raising the fundamental question of how this complex pathway is constructed in Rapaza, which exhibits the intermediate traits of endosymbiogenesis.

In this issue, Maruyama et al. (2023) identified a putative *NR* (Rv*NaRL*) gene encoded in the Rapaza genome, which was unexpected, because heterotrophic euglenids generally do not have enzymes for nitrate assimilation. Therefore, they examined the function of Rv*NaRL* in nitrate assimilation by applying RNAi-mediated knockdown (KD) and CRISPR-Cas9-mediated knockout (KO) to Rv*NaRL*. The KD and KO lines were unable to grow when nitrate was supplied as the sole nitrogen source. Furthermore, mutant lines in nitrate medium exhibited polysaccharide granule hyperaccumulation, as observed in wild-type Rapaza deprived of nitrogen. These results unequivocally demonstrate the essentiality of the host-derived component in the photoautotrophic lifestyle conferred by the kleptoplast (Fig. 1). An obvious homolog of NiR in Rapaza has not yet been identified, which implies that further nitrite reduction is catalysed by NiR from *Tetraselmis* residing in the kleptoplast (Fig. 1). Such cooperative nitrate assimilation involving host-derived components have not been demonstrated in other organisms exhibiting kleptoplasty. For example, *E. viridis* cannot assimilate nitrate (Teugels et al., 2008), presumably due to the absence of NR in the host and the digestion of algal cytosol containing NR. By contrast, the ciliate *M. rubrum* can assimilate nitrate (Tong et al., 2015); however, a contribution of NR from ingested algal cytosol remains possible because *M. rubrum* retains whole algal cells as kleptoplasts. Compared with these organisms, Rapaza is likely at an advanced stage of endosymbiogenesis, in which the host and kleptoplast established a tight metabolic interplay.

Furthermore, Maruyama et al. (2023) suggested that Rv*NaRL* was acquired via horizontal gene transfer (HGT), rather than via EGT from ingested *Tetraselmis*. In addition to Rv*NaRL*, the parallelly conducted study of the Rapaza transcriptome detected

numerous HGT-derived sequences in putative kleptoplast-targeted proteins (Karnkowska et al., 2023). Phylogenetic analysis suggested that HGT gene donors are notably diverse, including green algae across different lineages and secondary algae with red plastids (Karnkowska et al., 2023). Intriguingly, genome mosaicism with HGT has also been reported in *Euglena gracilis* (Novák Vanclová et al., 2020), which belongs to a photosynthetic sister group of Rapaza, and in diatoms, a distant secondary lineage with a red plastid (Moustafa et al., 2009). Based on these studies, secondary endosymbiogenesis was suggested to be a complex process involving diverse gene sources, rather than a simple union of two eukaryotes. While the evolutionary process shaping the mosaic genome remains under debate, gene acquisition via hidden past endosymbiotic events (cryptic endosymbiosis) prior to establishing current kleptoplast/plastids has been proposed in Rapaza, *E. gracilis* and diatoms.

In summary, Maruyama et al. (2023) experimentally demonstrated the indispensable role of host-encoded RvNaRL in nitrate assimilation by molecular genetic approaches. The contribution of HGT-derived genes, including Rv*NaRL*, to the photoautotrophic metabolisms suggests that Rapaza has reassembled metabolic pathways using diverse gene sources rather than a simple utilization of parts from ingested alga (Fig. 1). The successful application of genome editing to Rapaza enables further identification of host-derived essential components, such as the plasma membrane nitrate transporter, for which a candidate exists (Maruyama et al., 2023). Conversely, examining the contribution of factors donated from ingested *Tetraselmis*, such as NiR, is also necessary to reveal host-kleptoplast interplay. As genome editing has recently succeeded in the genus *Tetraselmis* (Chang et al., 2020), functional validation of *Tetraselmis*-derived components could be possible. Although the disruption of essential genes is generally difficult due to lethality, the defective mutants of the nitrate assimilation pathway, from nitrate uptake to ammonium production, can readily be isolated by replacing the nitrogen source with ammonium. Taking this advantage, the research on nitrate assimilation in Rapaza will be an excellent model to obtain a snapshot of metabolic integration at the early stage of secondary endosymbiogenesis.

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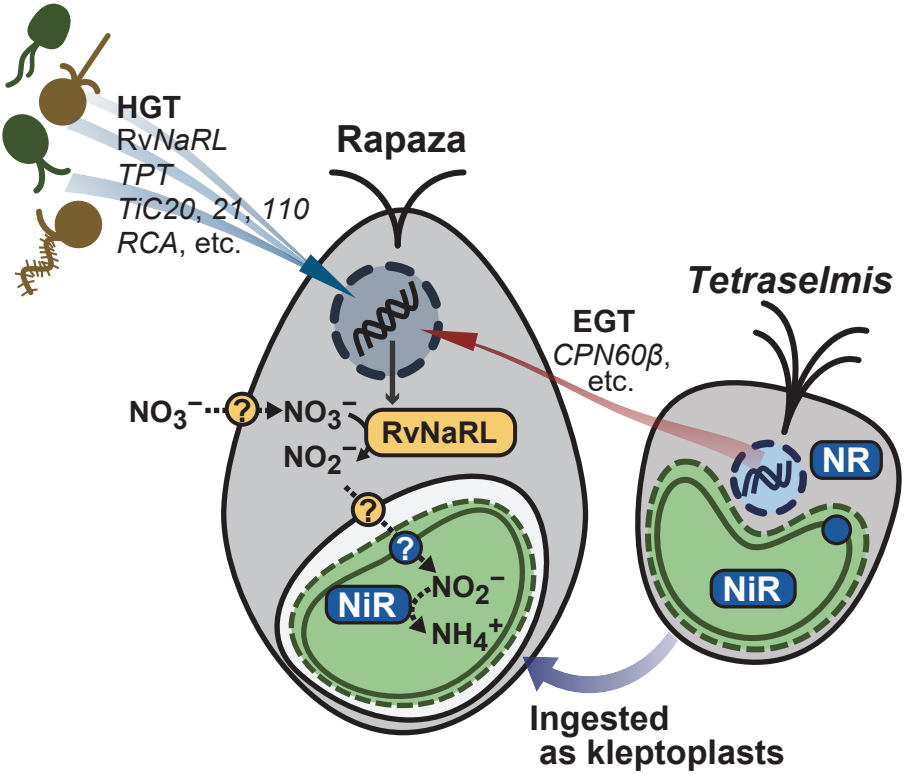
Figure legend

Fig. 1 A hypothetical model of endosymbiotic evolution shaping genome mosaicism and cooperative nitrate assimilation in Rapaza with kleptoplast (based on Maruyama et al., 2023; Karnkowska et al., 2023). Nitrate is reduced to nitrite by RvNaRL, whose gene was encoded in the nuclear genome of Rapaza. The absence of N-terminal targeting signals suggests that RvNaRL is localised in the cytosol. NiR in the kleptoplast may catalyse subsequent reduction to ammonium. Phylogenetic analysis suggested that RvNaRL was acquired via HGT, although its origin was not resolved (Maruyama et al., 2023). Karnkowska et al. (2023) identified many HGT-derived genes as well as genes from Tetraselmis (EGT). HGT-genes might presumably be acquired from diverse algae with which Rapaza was temporarily associated as prey or kleptoplast before establishing current kleptoplasty. The kleptoplast is surrounded by three membranes, of which the outermost membrane is the phagosomal membrane of Rapaza, while inner two are envelopes of *Tetraselmis* plastid (Karnkowska et al., 2023). Transporters on plasma- and phagosomal membranes (yellow) would be encoded in Rapaza genome, while the nitrite transporter on inner envelope (blue) is from Rapaza and/or *Tetraselmis*. TiC, Translocon at the inner envelope membrane of chloroplasts; TPT, Triose Phosphate Translocator; RCA, Rubisco activase. CPN, Chaperonin.


HGT
RvNaRL
TPT
TiC20, 21, 110
RCA, etc.
Rapaza
Tetraselmis
EGT
CPN60β,
etc.
NO3−
?
NO3−
NO2−
RvNaRL
?
?
NiR
NO2−
NH4+
NR
NiR
Ingested
as kleptoplasts