Kleptoplasts (kP) are distinct among photosynthetic organelles in eukaryotes (i.e., plastids) because they are routinely sequestered from prey algal cells and function only temporarily in the new host cell. Therefore, the hosts of kleptoplasts benefit from photosynthesis without constitutive photoendosymbiosis. Here, we report that the euglenozoan Rapaza viridis has only kleptoplasts derived from a specific strain of green alga, Tetraselmis sp., but no canonical plastids like those found in its sister group, the Euglenophyceae. R. viridis showed a dynamic change in the accumulation of cytosolic polysaccharides in response to light-dark cycles, and $^{13}$C isotopic labeling of ambient bicarbonate demonstrated that these polysaccharides originate in situ via photosynthesis; these data indicate that the kleptoplasts of R. viridis are functionally active. We also identified 276 sequences encoding putative plastid-targeting proteins and 35 sequences of presumed kleptoplast transporters in the transcriptome of R. viridis. These genes originated in a wide range of algae other than Tetraselmis sp., the source of the kleptoplasts, suggesting a long history of repeated horizontal gene transfer events from different algal prey cells. Many of the kleptoplast proteins, as well as the protein-targeting system, in R. viridis were shared with members of the Euglenophyceae, providing evidence that the early evolutionary stages in the green alga-derived secondary plastids of euglenophytes also involved kleptoplasty.

The endosymbiotic origin of mitochondria and plastids has been established with a wealth of evidence at different levels of biological organization. All of the plastids in eukaryotes, except those of the amoeboid Paulinella chromatophore, ultimately originated from a single endosymbiotic event with a cyanobacterium (1–4). However, the subsequent evolutionary history of plastids is much more complicated. Three lineages of photosynthetic eukaryotes, namely the Viridiplantae (green algae and land plants), the Rhodophyta (red algae) and the Glaucophyta, have retained the plastids acquired directly from the ancient cyanobacteria, called “primary endosymbiosis.” Several other eukaryotic lineages obtained plastids independently from one another by consuming either green algae or red algae, called “secondary endosymbiosis.” The eukaryotic groups with green secondary plastids are the Euglenophyta and the Chlorarachniophyta (i.e., collectively, the “green lineages”). Phototrophic cells in Stramenopiles, Alveolata, Haptophyta, and Cryptophyta acquired their secondary plastids from red algal prey cells (i.e., collectively, the “red lineages”) (3, 5, 6).

The number of different endosymbiotic events and the associated evolutionary processes that generated the diversity of plastids in eukaryotes remain contentious; however, the explanations for this history generally fall into one of two models that clarify the order of early stages that occurred before permanent plastids were established. The prevailing model for the endosymbiotic origin of secondary plastids infers a integration step, in which a heterotrophic eukaryotic cell ingested an algal cell and maintained it as an endosymbiont rather than digesting it. The establishment of mechanisms for metabolic exchanges must have then followed, which reduced the endosymbiont to an organelle through gene loss and gene transfer to the host nucleus (7, 8). An alternative “targeting-ratchet” model for the secondary plastids postulates that a protein-targeting system was established early to express proteins encoded in the host nuclear genome within the internalized plastid. This crucial evolutionary step drove the permanent integration of a plastid within its host cell (2). In this model, the gradual acquisition of critical genes by regular horizontal gene transfer events from prey cells, and the development of their targeting signals, was a prerequisite for the protein-targeting system that ultimately facilitated a permanent algal endosymbiont within the host cell (9).

Here, we investigated a potential model organism, Rapaza viridis, to better understand the evolutionary process of plastid acquisition. R. viridis was first described in 2012 as the only mixotrophic Euglenid known so far and is the closest sister taxon to the

**Significance**

The integration of fully established plastids involved horizontal transfer of chloroplast-associated genes to the host genome. Analyses of the euglenozoan Rapaza viridis, however, demonstrated that the proteins associated with its kleptoplasts (kP) are derived from diverse algae other than its kleptoplast donor. Some of them are shared with the plastid-targeted proteins of its sister lineage, Euglenophyceae, whose canonical plastid originated from a distinct green alga. The gradual accumulation of repeated horizontal gene transfer events constructed a genome in R. viridis that facilitates transient photosynthesis in the kleptoplasts. The mechanism for maintaining functionally active kleptoplasts in R. viridis provides context for understanding the early stages in the secondary endosymbiotic origin of plastids in euglenophytes and beyond.
Euglenophyceae, a predominantly photoautotrophic group possessing a Pyramimonas-derived plastid (10). Behavioral data, ultrastructural data, and molecular phylogenetic analyses of \textit{R. viridis} demonstrated several intermediate morphological traits between those found in euglenophytes and phagotrophic euglenids (10). \textit{R. viridis} actively feeds only on a specific strain of \textit{Tetraselmis} sp. using a reduced feeding apparatus and dies if deprived of either light or this strain of prey cells. In this study, we examined \textit{R. viridis} and \textit{Tetraselmis} sp. using transcriptomics, plastid genomics and time-course observations with transmission electron microscopy in order to evaluate the level of integration between the plastids and the host cell. We demonstrate that \textit{R. viridis} has no canonical plastids but has only transient kleptoplasts (kP) that have been previously reported also from other systems (11–18) (\textit{SI Appendix}, section 1.1), which are regularly acquired by consuming \textit{Tetraselmis} sp. We also show that \textit{R. viridis} expresses a series of nucleus-encoded proteins with apparent “plastid”-targeting signals that are homologous to those found in the Euglenophyceae and are probably transported into the kleptoplasts. Therefore, \textit{R. viridis} provides a unique opportunity to examine the tenets of the targeting-ratchet model and the intermediate stages that occurred during the evolution of plastid-targeting systems in much greater detail than in any other previously reported organisms.

Results and Discussion

The First Case of Kleptoplasty in Euglenozoans. The original description of \textit{R. viridis} by Yamaguchi et al. (10) noted intermediate traits between those of phototrophic and heterotrophic (algivorous) euglenids and, in particular, a strong dependence on both modes of nutrition. It was inferred that \textit{R. viridis} possesses canonical plastids and feeds actively only on specific prey, \textit{Tetraselmis} sp., so the lack of either resource (light for photosynthesis or prey) was fatal (\textit{SI Appendix}, section 1.2). However, when we compared phagocytic process of \textit{R. viridis} with that of other algivorous cells, such as the phagotrophic euglenid \textit{Peranema trichophorum}, we found that the cytoplasm of \textit{R. viridis} lacked any typical digestive phagosome containing algal material. Among typical algivores, the light green color of the chlorophyll inside the phagosome fades to brown and the chlorophyll fluorescence disappears, suggesting the progression of digestion. However, this does not occur in \textit{R. viridis}. This observation indicated that the prey, \textit{Tetraselmis} sp., together with its plastids, is temporarily retained by \textit{R. viridis} (10).

To verify this, we used the Sanger technique to sequence the 16S ribosomal RNA (rRNA) genes of the plastid genomes in a culture containing starved \textit{R. viridis} (i.e., no trace of \textit{Tetraselmis}) as well as from a unialgal culture of \textit{Tetraselmis}. Unexpectedly, the single copies identified in each culture were identical and clearly matched the \textit{Tetraselmis} sequence typical of Chlororodendra phyceae. Neither Euglenophyceae nor Pyramimonadale sequences were amplified from \textit{R. viridis} (\textit{SI Appendix}, Fig. S1 and section 1.3). To confirm that the absence of Euglenophyceae 16S rRNA is not due to the low specificity of PCR, we assembled the whole plastid genomes from Illumina reads generated from the starved \textit{R. viridis} culture and the prey \textit{Tetraselmis} sp. culture. \textit{R. viridis} data contained only one type of plastid genome which was identical to \textit{Tetraselmis} sp. (\textit{SI Appendix}, Fig. S2 and section 1.4). This result suggested that all of the cytoplasmic photosynthetic bodies of \textit{R. viridis} were in fact kleptoplasts, or transient plastids snatched from its prey, \textit{Tetraselmis} sp.

Our time-course observations of \textit{R. viridis} using transmission electron microscopy revealed that the Rapaza-type plastid reported by Yamaguchi et al. (10) were derived from sequential transformation from the ingested \textit{Tetraselmis}-type plastids (Figs. 1 and 2) (details of the original report in the \textit{SI Appendix}, section 1.2). Within 30 min after ingestion of a \textit{Tetraselmis} sp. cell by phagocytosis of \textit{R. viridis} (Figs. 1 A–C and 2 A and Movie S1), the plastid of \textit{Tetraselmis} sp. was sorted and segregated from the other cellular material, which was eventually excreted from the cell (Figs. 1 D–F and 2 B ). At this stage, the nucleus of \textit{Tetraselmis} sp. was eliminated (\textit{SI Appendix}, Fig. S3). In the following hours, the segregated kleptoplast was subdivided into smaller pieces (Figs. 1 G–L and 2 C), and constrictions formed an hourglass-like shape as observed with transmission electron microscopy (TEM) (\textit{SI Appendix}, Fig. S4). Fission of the newly acquired kleptoplast was active 12 to 18 h after the ingestion event and resulted in the presence of ≤10 small pieces of kleptoplast in a single \textit{R. viridis} cell. A large pyrenoid surrounded by polysaccharide grains was observed in the original plastid of \textit{Tetraselmis}, but disappeared in an early stage before kleptoplast fission, while multiple smaller pyrenoids were formed that contain pyrenoid-penetrated thylakoids (Fig. 1 M–O). At this stage, the kleptoplast displayed a three-membrane-bounded envelope, which is a typical feature of the Rapaza-type plastid (\textit{SI Appendix}, Fig. S5) (10). The early kleptoplast still contained the starch grains presumably formed inside the \textit{Tetraselmis} plastid, indicating the \textit{Tetraselmis} origin of the kleptoplast. However, in time, the starch grains gradually disappeared in the further subdivided pieces of the kleptoplast (Fig. 1 O).

\textit{R. viridis} then initiated cell division in parallel with the later stages of kleptoplast fission (Fig. 2 D), resulting in a threefold increase in cell number 11 d after the ingestion event (\textit{SI Appendix}, Fig. S6). Because the subdivided kleptoplasts were simply apportioned among the daughter cells of \textit{R. viridis}, the number of kleptoplasts per cell decreased as cell division was repeated. The growth continued until the total number of cells had increased 10-fold in 3 wk, suggesting that 3 to 4 rounds of cell division occurred in \textit{R. viridis} after ingesting a \textit{Tetraselmis} cell. The total amount of chlorophyll per unit culture did not increase, despite the increased number of kleptoplast in cells, indicating that the amount of chlorophyll per cell of \textit{R. viridis} decreased with increasing cell division (\textit{SI Appendix}, Fig. S6). This shows that the practical potential for photosynthesis of the kleptoplast in the cell of \textit{R. viridis} did not increase after the ingestion event.

We then investigated whether \textit{R. viridis} actually obtains photosynthetic products from these kleptoplasts. We examined the daily dynamics of polysaccharide grains in the cytoplasm of \textit{R. viridis} both microscopically and with chemical quantification. After kleptoplast acquisition was complete, polysaccharide grains accumulated in the cytoplasm of the daughter cells during the light period and reached their maximum just before the transition to the dark period. The quantity of accumulated polysaccharide grains decreased during the dark period and they were rarely observed just before the transition to the light period (Fig. 3 and \textit{SI Appendix}, Fig. S7). \textsuperscript{13}C isotope labeling experiments demonstrated that the polysaccharide grains in the cytoplasm were composed of photosynthetic products that were assumed to have been derived from the kleptoplasts. We added isotope-labeled bicarbonate (H\textsuperscript{13}CO\textsubscript{3}\textsuperscript{−}) to the medium during the light period and purified the polysaccharide grains from the cells just before the transition to the dark period for mass spectrometry analysis. The extensive incorporation of \textsuperscript{13}C into the polysaccharide grains was detected (\textit{SI Appendix}, Table S1). However, no accumulation of polysaccharide grains was observed in the cytoplasm of \textit{R. viridis} when it was continuously shaded from light (Fig. 3 C), suggesting that the polysaccharide granules did not derive from a heterotrophic carbon source but from the fixation of carbon dioxide.

Neither genetic data nor morphological observations supported the presence of any bona fide Pyramimonas-type plastid in \textit{R. viridis}. These data instead indicated the retention of plastids derived from
its prey, *Tetraselmis* sp., providing the first evidence of kleptoplasty among euglenozoans. The increase in the number of kleptoplasts in the cells of *R. viridis* results from kleptoplast fission rather than the conventional division seen in other algae. Consequently, the multiplied kleptoplasts are distributed to the daughter cells with no increase in size. *R. viridis* appears to then become metabolically dependent upon the photosynthesis of these kleptoplasts.

The kleptoplasts must be supplemented by regular phagocytosis of fresh *Tetraselmis* prey cells. We observed that *R. viridis* can survive no longer than 35 d without acquiring new kleptoplasts (10). We have also observed that vacuoles develop within the cells of *R. viridis* after approximately 20 d. The development of vacuoles is interpreted as a progression of the recycling metabolism of intracellular substances, implying gradual deterioration of the kleptoplasts function during this period. In addition, *R. viridis* continues to retain the kleptoplasts until death and does not digest or remove them to become a colorless cell. Therefore, the retention of functional kleptoplasts seems to be a prerequisite for sustaining cells of *R. viridis*.

**Evidence of Nucleus-Encoded Genes for Kleptoplasty.** In the observations described above, the kleptoplasts in the cytoplasm of *R. viridis* remained functional at least for about 3 weeks after the elimination of the *Tetraselmis* nucleus in the earliest stage of kleptoplasty. This implies that at the molecular level, the host controls the kleptoplast functions to some extent with its own, nucleus-encoded proteins, to exploit the photosynthetic products of the kleptoplasts and, at least temporarily, maintain their function. To verify this possibility, we examined the transcriptome of *R. viridis* for genes encoding proteins that may be transported into the kleptoplasts.

We first searched for possible signal peptide sequence features similar to those known to be responsible for the translocation of nuclear-encoded proteins to *bona fide* secondary plastids (19). As a result, we realized that the *R. viridis* sequence had features similar to the bipartite targeting system of Euglenophyceae, the closest relative of *R. viridis* (20, 21) *(SI Appendix, section 1.5)*. We predicted 2,241 putatively kleptoplast-targeted proteins based on the presence of the signal peptide (predicted with PrediSi or signalP) followed by the transit peptide (predicted with chlororP) and based on their homology to plastid-targeted proteins identified in *Euglena gracilis* (21). We then sifted through these candidate sequences using their predicted functions in plastids, based on their homology to either the predicted plastid proteome of *E. gracilis* (21) or the plastid-targeting proteins in the UniProt database (22). These methods identified 678 candidate genes *(SI Appendix, section 1.6)*. After the manual removal of potential...
contaminants, mitochondrial paralogues, and candidates with no transmembrane typical for the euglenid bipartite targeting signal. 274 putative kleptoplastic-targeted proteins were identified. We classified 166 of these as class I proteins with bipartite signal sequences and 73 as class II proteins with only a signal peptide (Dataset S1 and SI Appendix, section 1.5); we also annotated 35 proteins as transporters, constituting integral membrane proteins of the chloroplast envelope (Dataset S1). To validate the specificity of our approach on a negative control, we performed the same analysis on the predicted proteome of heterotrophic euglenid, Rhodomonas costata, previously suggested to ancestrally lack chloroplasts (23). Here we predicted 2,082 candidates with putative bipartite signals, which we then checked for contaminations, transmembrane domains and annotated their functions yielding 63 proteins (Dataset S2). Among those, 38 represent transporters (majority among them are ABC transporters and sugar transporters) with ambiguous localizations, and of the remaining 25, only one (RC0055040, ornithine carbamoyltransferase) has a hit to a plastid-targeted protein in E. gracilis. Yet, the predicted N-termini signal in R. costata is much shorter than the one in the homologue from R. viridis, and therefore, we conclude this protein is a false positive hit.

If these proteins of R. viridis are truly targeted to the kleptoplast and, for classes I and II, function inside the Tetraselmis-derived kleptoplasts, we should identify a translocation complex in the triple-membrane envelope of the kleptoplast. Indeed, we identified one orthologue of each Tic21 (RV36988), Tic20 (RV1840) and Tic110 (RV32406) proteins in the transcriptome of R. viridis (Dataset S1). Tic20 and Tic110 were identified based on targeted searches using profile hidden Markov models (HMMs). Only two proteins (Tic21 and Tic32 isoforms) have been identified in E. gracilis (24), and only Tic21 was confirmed in a proteomic study (21), suggesting highly reduced or derived translocation machinery in Euglenophyceae. Because Tic21 of R. viridis forms a well-supported clade with orthologues from Euglenophyceae (Fig. 4), it probably originated in their common ancestor, which likely possessed this unique translocation machinery and bipartite targeting signals on targeted proteins. However, other components (Tic20 and Tic110), which have together with Tic21 been proposed to form the inner envelope channel, are present specifically in R. viridis but not in Euglenophyceae. This suggests that these were either obtained later in the lineage of R. viridis or present in the common ancestor of Rapaza and Euglenophyceae and lost or diverged in Euglenophyceae. The independent origin of Tic20 is further supported by its relationship to “red-lineage” (Fig. 4B) while the Tic100 is relative to “green-lineage” (Fig. 4C). Overall, we conclude that R. viridis expresses nucleus-encoded proteins targeted to the kleptoplast and possess membrane translocons built from parts common with Euglenophyceae and other parts unique to R. viridis. Diverse Phylogenetic Origins of Kleptoplastic Proteins. To investigate the origins of proteins putatively targeted to kleptoplast, we calculated phylogenies for the candidate proteins and their orthologues from a custom protein sequence database (Datasets S3 and S4). This database includes the genomes and transcriptomes of 77 algal lineages covering the major eukaryotic and cyanobacterial groups, in addition to representatives of Discoba. We also included the transcriptome of the prey Tetraselmis sp. to evaluate possible contamination.

Some of these genes in R. viridis encoding suspected kleptoplast-targeted proteins were confidently shown to originate from multiple lineages of green algae other than Pyramimonas (Praunophyta), from which the plastids of Euglenophyceae originated (25–27). Although many plastid-targeted genes grouped together within the Tetraselmis lineage, their sequences were never identical to those of the prey strain Tetraselmis sp. used in the culture system in this study. This observation strongly suggests a long-lasting, close interaction between R. viridis and Tetraselmis, which is inferred to have driven gene transfer from Tetraselmis to R. viridis. A clear such example is the chaperonin 60 beta subunit (Fig. 5A) in R. viridis clustered robustly with the Tetraselmis lineage of core chlorophytes, while homologues of Euglenophyceae formed a robust cluster with that of Pyramimonas spp., as anticipated. However, in other cases, including the ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RCA) gene, the sequence of R. viridis together with those of the Euglenophyceae formed a sister group (Fig. 5B). Other trees suggested a green algal origin of the genes for plastid-targeted proteins in all euglenophytes, but their exact origin was not possible to pinpoint, such as the gene encoding fshH (SI Appendix, Fig. S8A), a D1 quality control protease that is essential for photosystem II repair. Most interestingly, almost 30% of the genes encoding kleptoplast-targeted proteins were phylogenetically related to algal clades with “red lineages,” rather than to any green algae. R. viridis homologues often clustered with Euglenophyceae (Euglena and Enteropriida) (Fig. 5B and SI Appendix, Fig. S8) in trees, suggesting gene transfer in a common ancestor of these euglenids (21, 28).

The observations described above support two inferences. First, these genes have been acquired by R. viridis in multiple horizontal gene transfer events involving a wide variety of algae, among which the core chlorophytes were predominant. This may reflect degrees of intimate interactions between R. viridis and these genetic sources, possibly as prey in kleptoplasty or algiwry. Second, the shared presence of HGT genes is most likely attributable to their ancestral origin and must have been introduced into the lineage before the divergence of R. viridis and the Euglenophyceae. Notable such case is Tic21, a component of the TIC translocation machinery, which, together with the existence of similar bipartite targeting signals, indicates that the targeting system was established before the divergence of Rapaza and Euglenophyceae. Thus,
it is also likely the pre-existing target system was involved in the establishment of the permanent plastid in Euglenophyceae. Our results agree with both the shopping bag hypothesis (29) and the red-carpet hypothesis (30), even though the origin of the plastids of Euglenophyceae is to be sought in the genus Pyramimonas (27).

Our data cannot fully resolve the relative timing of the symbiogenetic events, including the one that resulted in the Pyramimonas-derived plastid in Euglenophyceae. We infer that euglenids participated in more than a single symbiogenetic association at about the time when Rapaza and Euglenophyceae split. One scenario is that the last common ancestor of R. viridis and Euglenophyceae was a phagotrophic algivore without permanent plastids, which, however, might have already conducted kleptoplasty. Alternatively, one could assume the last common ancestor of R. viridis and Euglenophyceae had already established a permanent plastid derived from Pyramimonas together with a protein import system, which was subsequently eliminated in Rapaza that instead developed kleptoplasty with Tetraselmis. However, the former is a more plausible scenario considering 1) that Pyramimonas-derived genes are only minor in R. viridis and 2) that Tetraselmis-derived genes are rather abundant in Euglenophyceae; hence, the close association with Pyramimonas in the lineage of Euglenophyceae would have postdated the divergence.

**Metabolic Transporters for Exploiting the Kleptoplast.** We demonstrated that the products of photosynthesis from the kleptoplasts accumulate in the cytoplasm of R. viridis, which indicates the presence of a system for transporting organic molecules from the kleptoplast. We also observed that the starch grains inside the original Tetraselmis sp. plastid rapidly diminished in the earliest stage of kleptoplasty and did not form again in the later stages. R. viridis does not thrive in the absence of light, indicating its dependence on kleptoplasts for energy and the presence of transporters that deliver the necessary nutrients to the kleptoplasts. Therefore, we inferred that R. viridis expresses some metabolite transporters that pass through the envelope of the kleptoplast and transport the photosynthetic products. The importance of transporters for organellogenesis has been discussed previously in the endosymbiotic evolutionary model, where the targeting of transporters by the predator to its prey can facilitate the enslavement of the endosymbiont (2, 31).

We evaluated the metabolite transporters in the predicted proteome of R. viridis. Among the 274 predicted kleptoplast-targeting proteins, we identified 35 candidates that could function as metabolite transporters in the kleptoplast envelope (Dataset S1). The transporters seemed to be of exotic origin and transferred from prey cells representing either green algae (Fig. 6A and Dataset S1) or the red lineages (Fig. 6B). The phylogenetic trees indicated sister
relationships between the homologues of *R. viridis* and Euglenophyceae, suggesting that the acquisition of the transporters predated their common ancestor. **Targeting-Ratchet Model of Kleptoplasty in *R. viridis***. Kleptoplasty in *R. viridis* raises new insights into the order of events during symbiogenesis within the context of the targeting-ratchet model...
that differs from ideas proposed previously (32–34). Genes from both green algae and red lineages have been reported in the genome of Euglenophyceae, which are expressed in the plastids and confer essential functions (21). Many of these genes are also shared with *R. viridis* (Figs. 5 and 6), suggesting that these genes were already present in their last common ancestor. Furthermore, some of

Fig. 5. Diverse origins of the kleptoplast-targeted sequences in *R. viridis*. Maximum likelihood trees were estimated with 1,000 rapid bootstrap replicates in IQ-TREE. Photosynthetic Euglenids are highlighted in bold, and *R. viridis* is highlighted in purple. Support values above 50 are shown; black circles (∗) denote bootstrap support >95. (A) Phylogeny of chlorophenol 60 beta subunit (cpn60) alpha chain. (B) Phylogeny of ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RCA) showing sister relationship of clade of *R. viridis* and the Euglenophyceae. Full trees (RV20740. tree, RV30951.tree) are available at https://doi.org/10.5061/dryad.37pvcvpn.
the shared genes include essential transporters for exploiting the plastids/kleptoplasts. Therefore, if the plastid in Euglenophyceae postdated the split from *R. viridis*, then the acquisition of their shared exotic genes and the plastid-targeting mechanism must have predated the first association of the common ancestor of Euglenophyceae with *Pyramimonas*. Alternatively, if a permanent plastid had been already established in their common ancestor, still the shared genes and the "kleptoplast"-targeting mechanism...
must have predated the modern association of R. viridis with Tetraselmis; little evidence of a close relationship between the common ancestor and Tetraselmis can be found in the repertoire of the shared genes. In either case, the acquisition of the gene and the establishment of the target mechanism had to precede the new symbiogenetic association.

This stands in contrast to the orthodox hypothesis for symbiogenesis inferred from previously reported intermediate organism (e.g., Hatena arenicola), in which endosymbiosis is inferred to have occurred before the transfer of endosymbiotic genes and the loss of the endosymbiont's nucleus (34). Instead, plastid-targeting evolved early, probably during a long period of predation and kleptoplasty, and before the organelle became a permanent fixture of the cell. This has also been observed in a tertiary plastid origin (15), and these data suggest this may be a common route to endosymbiotic organelle origins. Additional comparisons of R. viridis, members of the Euglenophyceae, and potentially other yct-to-be-discovered lineages that diverged near R. viridis will continue to clarify the order of evolutionary steps involved in the establishment of kleptoplasts and permanent plastids.

Conclusions

Previous behavioral data, ultrastructural and molecular phylogenetic analyses demonstrated several intermediate traits in R. viridis that fall between those in Euglenophyceae, and phagotrophic euglenids. Members of the Euglenophyceae possess secondary plastids originating from Pyramimonas-like green algae; however, many details about the origin and early evolution of euglenophyte plastids remain unclear. R. viridis is the only mixotrophic euglenid known and branches as the sister lineage to the Euglenophyceae, so R. viridis provides a unique opportunity to gather insights into the order of events associated with the origin(s) of plastids. We performed time-course TEM observations of kleptoplast acquisition, biochemical analyses, plastid genomics, and transcriptomics from R. viridis and its green algal prey Tetraselmis sp. Our data showed that R. viridis 1) represents the first case of kleptoplasty (i.e., functional plastids stolen from prey cells) in euglenozoans; 2) lacks the canonical plastids present in Euglenophyceae; 3) has nucleus-encoded genes for plastid-targeted proteins with euglenid-type targeting signals to the kleptoplasts; 4) has nucleus-encoded plastid-targeted proteins that originated from many different lineages of algae in addition to Tetraselmis; and 5) has plastid transporters to facilitate targeting of the proteins into the kleptoplasts. These data advance our understanding of the origin and evolution of plastids not only in the Euglenophyceae but also eukaryotes as a whole.

Materials and Methods

Cultures. The strains of R. viridis (PRA-360) and Tetraselmis sp. (PRA-361) established by the authors of the original study (10) were also used in this study. These strains had been deposited in the American Type Culture Collection (Manassas, VA, USA) as PRA-360, but they subsequently died. They have now been deposited into the Microbial Culture Collection at the National Institute for Environmental Studies (Tsukuba, Japan) as NIES 4477 for R. viridis and NIES 4478 for Tetraselmis sp. Both strains were incubated in either I/2 medium (for genome and transcriptome sequencing) or Daigo IMK medium (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) for the remaining experiments, at 20 °C under illumination of 25 to 75 μmol photons m⁻² s⁻¹ with a 14-h light/10-h dark photoperiod (14L:10D). The R. viridis culture was maintained by regularly inoculating fresh medium with it, together with three times the number of Tetraselmis sp. cells (1:3) every 2 wk. After inoculation, the cells of Tetraselmis sp. were usually completely consumed by R. viridis within 12 h, resulting in a monospecific culture containing R. viridis exclusively. The monospecific culture of Tetraselmis sp. was maintained by inoculating fresh medium with an aliquot every 2 to 4 wk.

To set the zero point for the time course experiments involving serial light microscopy, TEM and chlorophyll analyses, fresh Daigo IMK medium was inoculated simultaneously with R. viridis cells and an equal number of Tetraselmis sp. cells (1:1) for rapid consumption, which was generally completed within 30 min. The cell numbers of both strains were counted with a particle counter/analyser (CDA-1000, Sysmex, Kobe, Japan). For microscopic observations, the culture was incubated at 20 °C under 14L:10D (50 μmol photons m⁻² s⁻¹), and the first dark period started 6 h after the inoculation. For the chlorophyll analysis, the culture was incubated at 20 °C under continuous light (50 μmol photons m⁻² s⁻¹). Quantification of polysaccharide accumulation was conducted with a culture of R. viridis 8 to 9 d after fresh Daigo IMK medium was inoculated simultaneously with R. viridis and three times the number of Tetraselmis sp. cells (1:3). The culture was then incubated at 20 °C under 14L:10D (50 μmol photons m⁻² s⁻¹).

Light Microscopy. Differential interference contrast and bright-field images were generated with an inverted microscope (IX71, Olympus, Tokyo, Japan) equipped with a color charge-coupled device camera (FX630, Olympus) and the Novol Image Filing System (Novol, Chofu, Japan). Fluorescent images and the associated monochromatic bright-field images were generated with an inverted microscope (IX73, Olympus) equipped with a complementary metal oxide semiconductor camera (ORCA-Flash 4.0, Hamamatsu Photonics, Hamamatsu, Japan) and cellSens Dimension v1.18 software (Olympus). In general, the sampled cells were held between two glass coverslips (0.13 to 0.17 mm) for observation. To stain the nuclei of live cells, SYBR Green I (Lonza, Basel, Switzerland) was applied to the cultured cells before observations.

Transmission Electron Microscopy. Chemical fixation was performed with a previously described method (35). Briefly, the cells were treated with glutaraldehyde and osmium tetroxide and then dehydrated through a graded series of ethanol before they were embedded in Spurr's resin (Polysciences Inc., Warrington, PA). Rapid freezing and the freeze substitution method were performed as previously described (36). The condensed cell cultures were cryofixed by impact-freezing onto a liquid-nitrogen-cooled copper block using a freezing device (VFZ-1, Japan Vacuum Device, Ltd., Mito, Japan) and were then freeze-substituted in acetone containing 1% OsO₄ for 3 d. The specimens were returned to room temperature with a stepwise temperature rise before they were embedded in Spurr's resin (Polysciences Inc.). Finally, ultrathin sections were stained with EM Stainer (Nisshin EM, Tokyo) and lead citrate before they were observed by TEM (Hitachi H7100, Hitachi Ltd., Tokyo, Japan).

Quantitation of Polysaccharide Grains. The polysaccharide grains were extracted from R. viridis cells and purified as previously described (37). The precipitated insoluble polysaccharide was fully dissolved in 1 M NaOH and quantified with the phenol–sulfuric acid assay (38), with reference to standard glucose solutions.

High Performance Liquid Chromatography (HPLC) Analysis. The pelleted cells frozen in liquid nitrogen were homogenized in acetone (1 mL) in an ice-cooled ultrasonication bath for pigment extraction. The acetone supernatant was immediately separated from the residue by centrifugation and directly injected into the HPLC apparatus for analysis. Analytical HPLC was performed with a liquid chromatograph system (Neexera X2, Shimadzu, Kyoto, Japan) coupled to a personal computer configured to run the Shimadzu LabSolution software (Shimadzu). Reversed-phase HPLC was performed according to the method of Kashiyama et al. (39).

Isotope Labeling Experiment. In the labeled culture experiment, a 2 mM solution of isotopically labeled sodium bicarbonate (NaH¹³CO₃; Cambridge Isotope Laboratories Inc., Tewksbury, MA) was added to a batch culture of R. viridis and to a culture blank medium (Daigo IMK) in a ratio of 1:10,000. In the control culture experiment, a 2 mM solution of unlabeled NaHCO₃ (Nacalai Tesque, Kyoto, Japan) was added to a batch culture of R. viridis in a ratio of 1:1,000. An aliquot of the labeled blank medium was immediately dried in vacuo, and the carbon isotopic composition of the solid residue obtained was analyzed. Subsamples (17 mL) of each of the labeled and control cultures were placed in a 50 mL vent-capped disposable culture flask and incubated at 20 °C for 9.5 h under continuous light (50 μmol–photons m⁻² s⁻¹). All the cells in each culture were then pelleted and frozen in liquid nitrogen. The purified polysaccharide grains were prepared from the frozen pellets using the method described above for polysaccharide quantification and their carbon isotopic compositions were analyzed by Shoko Science
(Yokohama, Japan) and are expressed in the conventional 8-notation against Vienna Peedee Belemnitile (613C (‰) = 10[(13)C/(12)C]_Sample/[(13)C/(12)C]_Standard - 1). Amplification and Sequencing of Partial Plastid 16S rDNA. The total DNA was extracted from cells harvested from 16-d-old cultures of *R. viridis* and *Tetraselmis* sp., with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI), according to the procedure provided by the manufacturer. To enhance the extraction efficiency, the *Tetraselmis* sp. cells were bead-treated before the extraction procedure. The partial 16S rDNA in the plastid was amplified with PCR using a universal primer set that recognizes the known sequences of the family Chlororophyceae, including *Tetraselmis*, and those of both Pyramimonadales and Euglenophyceae (TPE-16S_FWD: 5’-GGTGGCAAGGMCCGGTATAC3’; and TPE-16S_RV: 5’TGTGACGGCGCCTGGTKCAAR3’). The amplified products were gel-purified with the Wizard SV Gel and PCR Clean Up System (Promega) and then cloned into the pGEM-T Easy Vector (Promega). The inserted DNA fragments in the cloned plasmids were sequenced in both directions by Eurofins Genomics (Tokyo, Japan).

Sample Preparation for Genomic and Transcriptomic Sequencing. The total genomic DNA of *R. viridis* and *Tetraselmis* sp. was extracted with the QiAamp DNA Micro Kit (Qiagen, Hilden, Germany). The total RNA was isolated from *R. viridis* and *Tetraselmis* sp. with the Nucleospin RNA XS Kit (Macherey-Nagel, Düren, Germany). To avoid contamination with its food *Tetraselmis* sp., we extracted the DNA and RNA from 2-wk-old cultures of *R. viridis*. We observed no *Tetraselmis* sp. cells in these cultures. We compared the small subunit ribosomal RNA (SSU rRNA) gene sequences with the genome database of *R. viridis* and did not recover any *Tetraselmis* SSU rRNA sequences. This indicates that there was no contamination from *Tetraselmis* in the 2-wk-old cultures of starved *R. viridis*.

Sequencing libraries were prepared and sequenced was performed on the Illumina MiSeq platform using 150-bp paired-end reads for the *R. viridis* genome, and with the Illumina HiSeq platform using 100-bp paired-end reads for the *R. viridis* at the University of British Columbia Sequencing and Bioinformatics Consortium, University of British Columbia, Vancouver, Canada. The *Tetraselmis* sp. genome and transcriptome was sequenced on the Illumina MiSeq platform using 200-bp paired-end reads at the Biotechnology and Biomedicine Center in Vester, Charles University, Prague, Czech Republic. The raw reads are available in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) for *R. viridis* Bioproject number: PRJNA4901946, SRR22333116-SRR22333117 and for *Tetraselmis* sp. Bioproject number: PRJNA901983, SRR22356821-SRR22356822).

Plastid Genome and Transcriptome Assembly and Annotation. Quality control of the Illumina MiSeq reads from the genomes of *R. viridis* and *Tetraselmis* sp. was performed with the FastQC tool v.10.11.6 (40). Adapters, shortest reads (<36 bp), and poor-quality reads (mean Phred quality value of <15) were removed with the Trimmomatic tool v.0.38 (41). The plastid genomes were pre-assembled with SPAdes v3.10.1 (42), and the plastid genes were identified in the assembled contigs using the BLASTX algorithm (43) and extracted. Contigs that contained the *rbcL* gene (encoding the large subunit of ribulose bisphosphate carboxylase/oxygenase) were extracted and used as the seeds for the final assembly of the plastid genomes with NOVPlas v2.6.3 (44). The chloroplast genome sequence has been deposited to the Dryad data repository (http://datadryad.org) accession doi:10.5061/dryad.37pvmcpv. The plastid genome was annotated automatically with the cpGAVAS (45) and DOGMA tools (46), and manually corrected afterward. Plastid genome map was created with the OGDRAW tool (47).

Quality control of the Illumina HiSeq reads of the *R. viridis* and *Tetraselmis* sp. transcriptomes was performed with the FastQC tool v.10.11.6 (40). Adapters, shortest reads (<36 bp), and poor-quality reads (mean Phred quality value of <15) were removed with the Trimmomatic tool v.0.38 (41). The transcriptomes were assembled with Trinity v.2.0.6 (48) (https://github.com/trinityrnaseq/Trinityrnaseq/releases/tag/Trinityrnaseq-v5.0.2) and have been deposited to the Dryad data repository (http://datadryad.org) accession doi:10.5061/dryad.37pvmcpv. The completeness of the transcriptomes was estimated with Benchmarking Universal Single-Copy Orthologs (BUSCO) v.4.0.6 (49) with the eukaryote_odb10 dataset of 255 BUSCO groups. The predicted *R. viridis* proteome contained 87% complete BUSCOs and 3.5% fragmented ones, and 9.5% BUSCOs were missing. By comparison, the predicted proteome of *E. gracilis* (50) had 15.7% missing BUSCOs, suggesting that the *R. viridis* transcriptome was fairly complete. Similarly, the transcriptome of *Tetraselmis* sp. was estimated to be nearly complete, containing 83.9% complete BUSCOs and 7.8% fragmented BUSCOs, and 8.3% missing BUSCOs.

**Prediction of Kleptoplast-Targeting Proteins in *R. viridis* and *R. costata***. The possible plastid-targeting proteins in the translated transcriptome (predicted proteome) of *R. viridis* and *R. costata* were predicted by searching for the typical bipartite plastid-targeting signal of euoplastids (20) and by searching for orthologues of proteins identified in the plastid proteome of *E. gracilis* (21). The bipartite plastid-targeting sequences were identified as described by Novák Vančová et al. (21) using a combination of SignalP v. 4.1 (51) and PrediSi (52) to predict the signal peptides, and ChloroP v. 1.1 (53) to predict the chloroplast transit peptides after the in silico removal of the predicted signal peptides at their putative cleavage sites. The sequences were then truncated to a maximum length of 100 amino acid residues because the predictor searched for potential cleavage sites within the 100 most N-terminal residues. The preliminary dataset of *R. viridis* and *R. costata* plastid-targeting proteins (1,563/1,817 sequences) consisted of transcripts that had positive scores in SignalP + ChloroP, PrediSi + ChloroP, or SignalP + PrediSi + ChloroP analyses.

The translated transcriptome of *R. viridis* and *R. costata* was also compared with BLAST against the 1,402 plastid-targeting proteins of *E. gracilis* (21), and the 874/276 candidate proteins with bidirectional best hits (e-value of 1e−20) were identified as plastid-targeting proteins. We combined both datasets, which resulted in 2,241/2,082 nonredundant candidate proteins, and annotated them automatically with BLAST at the *E. gracilis* plastid proteome (21) and UniProt databases (22). We manually annotated the proteins and excluded the redundant isoforms with the same annotation, mitochondrion-targeting proteins, and hypothetical and predicted proteins. We obtained a dataset of 678/261 annotated predicted plastid-targeting proteins and used it in the subsequent steps of the analysis.

We searched for the components of translocon proteins using profile hidden Markov models (HMMs). The models of the translocon proteins were obtained from PFAM. Searches were performed using HMMER (http://hmmer.janelia.org/) with E-value threshold (E-value ≤ 10).

**N-Terminal Plastid-Targeting Sequences in *R. viridis* and *R. costata***. We evaluated the set of predicted *R. viridis* and *R. costata* chloroplast proteins (678/260 entries) for the characteristic *E. gracilis* plastid-targeting sequences. Potential membrane-spanning regions were identified with the hidden-Markov-model-based program TransMembrane prediction using Hidden Markov Models (TMHMM) (54). The prediction was performed with sequences truncated to a maximum of 200 amino acids and that started with methionine, suggesting that they had complete N-termini. After the removal of too-short sequences, we obtained 274/64 sequences with at least one transmembrane domain (Datasets S1 and S2). Sequences with one transmembrane domain (73 proteins) were annotated as class II plastid-targeting proteins, and sequences with two transmembrane domains were annotated as class I (166 proteins) (Dataset S1). Sequences with three transmembrane domains (12 sequences) may represent those targeted across the thylakoid membrane, and were also classified as class I, according to Durford and Gray (20). The remaining 35 proteins were annotated as transporters and constituted integral membrane proteins in the chloroplast envelope (Dataset S1).

**Determination of the Evolutionary Origins of Plastid-Targeting Proteins**. The sequences of *R. viridis* predicted to be plastid-targeting proteins with at least one transmembrane domain were used as queries for a Protein Basic Local Alignment Search Tool (BLASTP) search against a custom database. The custom database consisted of predicted proteins, clustered at 90% similarity using Cd-hit (55), from the genomes and transcriptomes of 77 organisms, consisting of various groups of algae (including the transcriptome of *Tetraselmis* sp. sequenced in this study), several cyanobacteria, and species from the Discoba (including *E. gracilis* and *Eutreptiella* sp., representing the photosynthetic euglenids). The e-value cut-off used when collecting the sequences was 10−5. Each protein dataset was aligned using the Multiple Alignment using Fast Fourier Transform (MAFFT) algorithm (with the default parameters) from the MAFFT package v7.271 (56). Regions of doubtful homology between sites were removed from the alignments with Block Mapping and Gathering with Entropy (BMGE) (57), with the
default parameters. At this step, we discarded alignments with fewer than 70 sites after trimming (92 alignments) and those with fewer than 20 sequences (three alignments). The reduced protein datasets were realigned with the MAFFT-L-INS-i method in the MAFFT package, and then trimmed with BMGE (settings as previously described). Maximum likelihood (ML) trees were constructed for the remaining 179 protein alignments using the IQ-TREE software v1.6.12 (58), with the evolution model automatically selected with the -m TEST parameter, and statistical support from 1,000 rapid bootstrapping replicates (59). The trees were inspected manually to identify the relationships of the sequences from R. viridis with those from other euphleojangles and other algae included in the database, and detected potential contamination (20 trees) and non-plastid paralogues (12 trees). The remaining 147 trees were deposited in (http://datadryad.org) accession doi: 10.5061/dryad.37pycmvn and summarized in Datasets S3 and S4. To improve presented trees, we added additional homologs based on the NCBI database searches and manually inspected alignments to remove long branches and sequences shorter than half of the alignment and calculate the trees as described above.


