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Protein Import and the Origin of Red Complex Plastids

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The number and nature of endosymbioses involving red algal endosymbionts are debated. Gene phylogenies have become the most popular tool to untangle this issue, but they deliver conflicting results. As gene and lineage sampling has increased, so have both the number of conflicting trees and the number of suggestions in the literature for multiple tertiary, and even quaternary, symbioses that might reconcile the tree conflicts. Independent lines of evidence that can address the issue are needed. Here we summarize the mechanism and machinery of protein import into complex red plastids. The process involves protein translocation machinery, known as SELMA, that arose once in evolution, that facilitates protein import across the second outermost of the four plastid membranes, and that is always targeted specifically to that membrane, regardless of where it is encoded today. It is widely accepted that the unity of protein import across the two membranes of primary plastids is strong evidence for their single cyanobacterial origin. Similarly, the unity of SELMA-dependent protein import across the second outermost plastid membrane constitutes strong evidence for the existence of a single red secondary endosymbiotic event at the common origin of all red complex plastids. We furthermore propose that the two outer membranes of red complex plastids are derived from host endoplasmic reticulum in the initial red secondary endosymbiotic event.

Introduction

Photosynthesis in eukaryotes stems from endosymbiosis. The single origin of plastids from a cyanobacterium gave rise to the glaucophytes, the red algae, the green algae and the plant lineages, whose primary plastids are surrounded by two membranes. All other photosynthetic eukaryotes that harbour plastids stemming from those lineages arose through secondary endosymbioses, or in rare cases, higher-order endosymbioses involving the uptake by a eukaryotic host of a eukaryote harbouring secondary plastids. Plastids of secondary symbiotic origin are usually surrounded by four membranes — three in some lineages — and are called complex plastids. The concept of secondary symbiosis was proposed in the 1970s [1–3]. Phycologists quickly embraced the idea of secondary endosymbiosis, because it helped explain the unusual combinations of characters displayed by protists with secondary plastids, especially the number of membranes that separate the host cytosol from the plastid stroma. Among specialists there is wide agreement that there were two independent secondary symbiotic events involving green algae — one leading to euglenoids, and one leading to chlorarachniophytes [4,5]. But that brings us to the question of how many independent endosymbiotic events led to complex plastids derived from red algae, and there the agreement stops.

In evolution, the hard problems are the good ones, and untangling the origin of complex red plastids is certainly a hard problem. For almost 30 years, evolutionary cell biologists have been debating the number and nature of symbiotic events underlying the origin of protists that harbour secondary plastids of red algal origin. There is currently no consensus in sight. No current proposal accounts comfortably for all of the data, and probably no proposal ever will, because there are simply too many conflicting characters, both cell biological and molecular. We make no attempt to comprehensively list all those characters or conflicts here — they have been discussed in much detail before (Table 1)[6–11]. Instead of aiming for another comprehensive overview, here we wish to focus on one specific aspect that we find particularly relevant. We aim to develop in more detail an idea sketched in a recent paper [12] that, upon closer inspection, deserves slightly more consideration — the origin(s) and homologies of the outermost two membranes of red complex plastids and how evolutionarily conserved protein targeting across them bears upon theories that address their origin.

The Standard Model of Membrane Homology in Secondary Symbiosis

Schemes depicting the symbiotic evolution of red complex plastids tend to share one property in common: they tend to depict the process of secondary plastid origin in a standard way, as we have in Figure 1A. That is, the plastid acquisition is drawn in such a way that the outermost membrane of the secondary plastid — called by convention, counting from the outside to the inside, membrane number 1 — appears to be homologous to a phagocytotic vacuole membrane of the host, while the second outermost membrane of the secondary red plastid (membrane 2, the periplastidal membrane, PPM) appears to be homologous to the plasma membrane of the endosymbiont. With notable exceptions [7,13,14], explicit statements about the actual homologies of the outer two membranes are generally rare in the literature, even though it was the number of membranes surrounding plastids that sparked the concept of secondary endosymbiosis in the first place.

Table 1. Key references that propose models on how red complex plastids have evolved, and/or that discuss many characters and conflicts associated with the different scenarios put forward.

Reference	Comment
[6]	Origin of the 'chromalveolate hypothesis'. Proposes all organisms harbouring a plastid of red algal origin trace back to one host and one red alga.
[7]	An early take on the likelihood of the chromalveolate hypothesis based on different characteristics, including phylogenetic data, and morphological and cellular characteristics.
[9]	A review on the pros and cons of the chromalveolate hypothesis. Offers testable models and raises the possibility of tertiary origin.
[8]	Discusses the origin of the CASH lineages in light of the eukaryotic tree of life and emphasises the impact of endosymbiosis on the host genome that today is of mosaic nature, uniting genes of at least four different origins (not taking LGT into account). Expands the chromalveolate hypothesis by adding multiple events of plastid loss.
[10]	Argues against [8]. Penalises especially plastid loss and favours multiple tertiary events.
[11]	Proposes the 'rhodoplex hypothesis'. It does not explicitly make a statement about the number of events, but that a single origin as proposed in the chromalveolate hypothesis is wrong. Also introduces the working term 'CASH lineages' (chromist, alveolate, stramenopile and haptophytes).

The standard approach to disentangling the number of symbioses involving red complex plastid lineages has been gene phylogenies. While gene trees are often valuable tools, it is fair to state that on this particular issue, gene phylogenies have delivered conflicting results. The application of gene trees and related molecular analyses to the question regarding the number of independent endosymbiotic events involving red algal endosymbionts has not narrowed down the number of competing hypotheses as everyone had hoped; rather it has spawned several new alternative theories. Very popular among the new variants are multiple tertiary symbioses [10,11,15–17], that is, the origin of complex plastids through the engulfment of algae having secondary plastids, followed by some very hefty — and often implicit — reduction of membranes and cell compartments to get back to the four membranes that surround the plastids in the modern algae, and even quaternary symbioses [11,17], which entails yet one more cycle of the same heavy lifting, accordingly (Figure 1B).

So what do the original, more traditional models say about where the two outer membranes come from? Melkonian [18] suggested that an autophagosomal membrane was involved to form the outer membrane (membrane 1). Cavalier-Smith [14] suggested that membrane 1 is a phagosomal membrane that ultimately fused with the nuclear envelope to become the plastid ER (Figure 1A). Let's focus briefly on how the membranes are organized in the different groups. In cryptophytes, haptophytes, and stramenopiles, membrane 1 is continuous with the host's rough ER and typically called the chloroplast ER [19]. In plastid-bearing alveolates (apicomplexans, chromerids, perkinsids and dinoflagellates), the outermost membrane is not continuous with the host's ER and does not carry ribosomes (Figure 2B). Moreover, as only three membranes surround the plastids of dinoflagellates, the question of outer membrane homology in that lineage is further complicated by the circumstance that at least one additional membrane has been lost.

Protein Import in Complex Red Plastids

Salient to the discussions surrounding homologies of the two (or, in the case of peridinin-containing dinoflagellates, one) 'extra' membranes that surround red secondary plastids is the issue of protein import. For primary plastids, the mechanisms of protein import are well known [20]. Most plastid proteins are nuclear encoded, synthesized as cytosolic precursors and imported into the plastid through the TIC and TOC complexes (for translocon of the inner and outer envelope of chloroplasts, respectively), which were present in the common ancestor of all Archaeplastida (organisms possessing primary plastids), and which thus represent very strong evidence for the single origin of primary plastids. Had primary plastids arisen on multiple occasions, it is unlikely they would have independently invented the same protein import machinery consisting of homologous proteins [21]. Fully fledged TOC/TIC systems do not exist in prokaryotes, many components of the TOC and TIC complexes are eukaryotic inventions [20]. Algae with red secondary plastids possess the components of the TIC and TOC machinery [22]. Exactly the same argument applies to mitochondria — the unity of TOM/TIM complexes across all eukaryotic lineages indicates a single origin of mitochondria [23].

How do nuclear-encoded proteins traverse the outer two membranes to get to membranes 3 and 4, where TOC and TIC reside? Here the plot thickens. In cryptophytes, stramenopiles and haptophytes, we have an excellent model for traffic of precursor proteins across the two outermost membranes. In these groups, complex plastid membrane topology is identical and in all cases the host's Sec61 complex is used to co-translationally target proteins across membrane 1 into the first intermembrane space, which thus corresponds, topologically, to the ER lumen (Figure 2A). For plastid-bearing alveolates it is a different, slightly more involved story. After co-translational targeting to the ER across the Sec61 complex, the precursors have yet to reach membrane 1 because the outermost membrane of the complex plastid is not contiguous with the host ER (Figure 2B). Some evidence for vesicle trafficking from the secretory system to the outer plastid membrane exists, and this is currently the most favoured mechanism [24–27]. In any case, having passed membrane 1 of red complex plastids the precursors then face one more membrane (number 2) before reaching the TOC/TIC machinery in membranes 3 and 4. How do proteins cross membrane number 2?

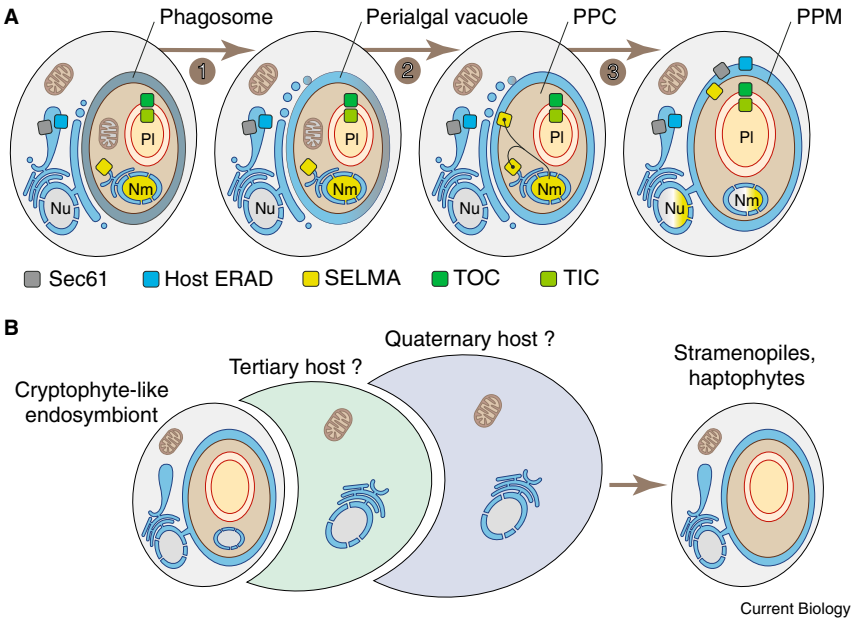

Figure 1. Phagosomal origin for the outermost membrane of red complex plastids.

(A) At the onset of endosymbiosis, the symbiont resided within a phagosomal vacuole. This vacuole was converted to a perialgal vacuole (1) to terminate symbiont degradation. Inside this vacuole the red alga began to reduce in complexity, losing, for example, its mitochondria (2). At the same time, the nucleomorph (Nm)-encoded SELMA was dual-targeted to (i) the reduced endoplasmic reticulum (ER) of the periplastidal compartment (PPC) of the endosymbiont and (ii) to its former plasma membrane (now the periplastidal membrane (PPM); membrane 2). Then the outer membrane of the perialgal vacuole (membrane 1) fused with the host ER, forming the 'chloroplast ER'. Once SELMA was functionally integrated into the PPM, import of proteins whose genes had been transferred from endosymbiont to host was ensured and the situation observed in cryptophytes today reached (3), with some SELMA genes having been transferred to the nucleus (Nu) and some still being Nm-encoded. In the chromalveolate hypothesis we must predict either a separation of the endosymbiont's compartment from the ER for alveolates or, alternatively, a bifurcation of alveolates prior to the fusion of the perialgal vacuole with the host ER. (B) Count the membranes. In 'rhodoplex hypothesis'-like scenarios that propose independent tertiary or quaternary endosymbiosis we must predict additional rounds of reduction that now also include the loss of the nucleomorph. We must also predict additional rounds of fusions of the perialgal vacuole (now containing a cryptophyte or stramenopile algae that are far more complex) with the host ER. Note that for stramenopiles and haptophytes we end up with a membrane topology identical to that found in the initial cryptophyte-like endosymbiont. It is as if the additional hosts never existed.

Crossing the Second Outermost of Four Plastid Membranes: SELMA

In the past few years it has become apparent that in all lineages bearing red secondary plastids that are surrounded by four membranes, a specific machinery resides in membrane 2 [28–30]. The exceptions are the dinoflagellates, whose plastids have lost one membrane (probably the membrane carrying this machinery). The first hints about this machinery came from the sequence of the nucleomorph genome of *Guillardia theta*, which was found to encode homologs of the ERAD system [30]. ERAD stands for ER-associated protein degradation and consists of about a dozen or so components. In a typical eukaryotic cell, ERAD serves to re-translocate (or extract) proteins from the ER lumen for cytosolic degradation [31], but the ERAD machinery in the cryptomonad endosymbiont has been adapted for another membrane trafficking role. Cryptophytes have their own, nuclear-encoded ERAD machinery, and the nucleomorph-encoded ERAD components were clearly distinct and were furthermore clearly derived from the red algal symbiont. Following a great deal of hard work to pursue the issue, the current understanding of protein import across membrane 2 is that the nucleomorph-encoded ERAD homologues — now called SELMA, for symbiont-specific ER-like machinery [32] — do the job of bringing proteins across membrane 2 and, in the case of plastid proteins, into contact with TOC and ultimately TIC. The molecular components of SELMA have been the subject of several recent reviews [23,33,34]. For *Guillardia*, where several components of SELMA are nucleomorph encoded and synthesized on ribosomes in the periplastidal compartment (PPC, the red algal symbiont cytosol; Figure 2A), SELMA is poised to be inserted, without evolutionary innovations, into a native ER membrane, as outlined below.

With the notable exception of dinoflagellates, all investigated secondary red plastid-containing lineages encode SELMA homologues in their nucleus, in addition to their genuine ERAD machineries. The conserved function of SELMA is to import plastid precursor proteins from the ER lumen into the PPC and towards membrane 3, the plastid. Importantly, the SELMA homologues in all secondary red lineages appear to trace to one and the same source [11,35]. The different red secondary plastid-bearing lineages thus all have the same SELMA, which is derived from one and the same symbiont's ERAD machinery [11,35]. Just as TOC and TIC indicate a single origin of primary plastids [20], SELMA indicates a single origin of secondary red plastids. Tom Cavalier-Smith will be quick to point out that that is exactly what the chromalveolate hypothesis said [6], albeit based on a different translocon, as SELMA had not been discovered then. But while the monophyly of SELMA provides strong evidence for the monophyletic origin of the secondary red algal plastid, it does not directly tell us how many hosts might have come to acquire it. Different tertiary (or quaternary) hosts can, in principle, make use of the same SELMA, just like different tertiary (or quaternary) hosts can, in principle, make use of the same TOC and TIC. But that does not mean that we can invoke tertiary (or quaternary) symbioses without some penalty for the number of events. And in comparison to TOC and TIC, which always reside in the same homologous membrane, the symbiont ER in which SELMA arose no longer exists, such that SELMA has been retargeted to a different membrane. How did that happen, how often, and to which membrane?

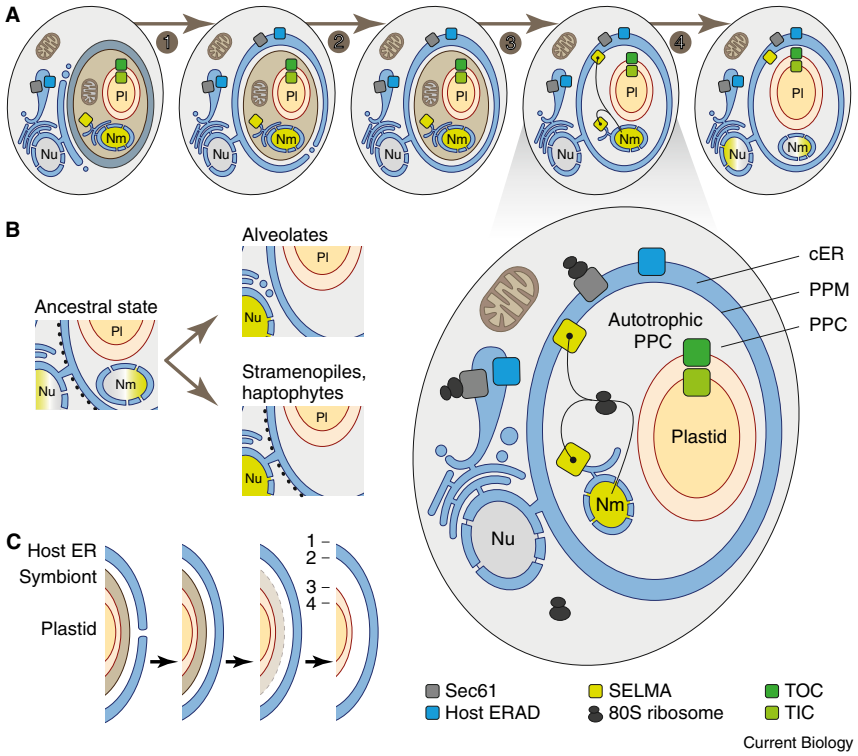

Figure 2. ER origin for the two outermost membranes of complex red algal plastids.
(A) In this chain of events, the phagosomal membrane is lost early, and the endosymbiont floats free in the cytosol and is subsequently surrounded by the host's ER (1), which ultimately closes (2) to form the 'chloroplast ER' (cER). Inside the cER the autotrophic endosymbiont lost its plasma membrane (3). The nucleomorph (Nm)-encoded SELMA, which is transcribed on 80S ribosomes inside the periplastidal compartment (PPC), was then dual-targeted to the reduced ER of the endosymbiont and the periplastidal membrane (PPM) that stems from the outer leaf of the host ER. Ongoing reduction further streamlined the endosymbiont and its PPC to the situation we observe today in cryptophytes, in which it also lacks an observable ER (4). Nu, nucleus; Pl, plastid. (B) The two different trajectories the complex plastid experienced to get from the ancestral state (similar to that of today's cryptophytes) to that observed in alveolates or haptophytes and stramenopiles. In both cases we lose the Nm, and all genes encoding SELMA have been transferred to the host nucleus. For other details, please refer to the text and Figure 1. (C) Overview to illustrate the key events of membrane fusion and loss. Numbers indicate the membrane nomenclature used throughout the manuscript.

SELMA Localization and the Origin of the Outer Two Membranes

Rather than invoking tertiary or higher order symbioses, we posit one single origin of the red secondary import machinery in membrane 2. That raises the question of where membrane number 2 comes from. Zimorski *et al.* [12] tersely suggested an ER-enclosure model for the origin of the outer two membranes; here we pursue that thought in more detail for the case of *Guillardia*. Crucial to our argument is that important components of the cryptophyte SELMA machinery are nucleomorph encoded. With regard to SELMA, *Guillardia* thus represents the ancestral state. In addition, recent phylogenetic analyses have it that cryptophytes branch deeply among photosynthetic eukaryotes [17,36], but that is not crucial to our argument.

Our model for the origin of the two outermost membranes posits an initial loss of the phagosomal membrane, and then a loss of the endosymbiont plasma membrane, but only after it became surrounded by host ER (Figure 2C). The autotrophic endosymbiont was maintained inside this cavity before the symbiont's plasma membrane was lost, leaving its remaining cytosol (today's periplastid compartment, or PPC) surrounded by host ER. In terms of homology, membranes 1 and 2 are thus 'leaves' of the host ER. SELMA either arose through duplication of the symbiont ERAD or, perhaps more simply, without duplication and simply by specialization of the symbiont's ERAD concomitant with the reduction and loss of the ER in the PPC. With loss of the symbiont ER, SELMA was no longer targeted to that membrane, but rather to the outer leaf of the host's ER, which is now membrane number 2 and is poised in the right orientation to have SELMA inserted (Figure 2C). This chain of events can transpire without the need for evolving any new targeting information for SELMA components. In membrane 2 SELMA retains its function, exporting proteins from the ER lumen, but now the host's ER lumen, which transports the proteins into the symbiont's cytosol. The substrates for SELMA translocation are inserted by the host's Sec61 machinery on the other ER face, which is now membrane number 1. This derives the situation exactly as it occurs in *Guillardia*. In this respect, our model does not require fusion of the ER with either a plasma membrane or its derivate, the phagotrophic vacuole.

Then come three alternatives for the origin of the remaining secondary plastids. In the chromalveolate hypothesis (i), one transfer (or, less likely, multiple independent transfers) of genes for SELMA from the nucleomorph to the host nucleus is needed, nothing more. Now synthesized by the host cytosolic ribosomes, targeting to the host's outer ER leaf puts SELMA in its natural location. The other two alternatives would either be (ii) multiple independent red secondary symbiotic events, which nobody is currently arguing, or (iii) multiple tertiary (or quaternary) symbioses involving a symbiont (of secondary symbiotic origin) that already has SELMA. Alternative (ii), already unpopular, would furthermore be incompatible with the single origin of SELMA, and will not be considered further. Alternative (iii) is quite popular at present but becomes a very complicated matter. Each tertiary or higher-order symbiosis entails multiple paths to derive the first and second membrane around the plastid (elaborated in the next section) and requires the repeated successful transfer of hundreds of nuclear genes for photosynthesis related functions, plus SELMA. Those transfers begin, in our view, to carry a fairly severe weight that adds at least as much burden to theories entailing multiple secondary or tertiary symbioses as does assuming gene losses for an unneeded (or even never

fully established) organelle in ciliates, cryptosporidia, oomycetes and *Goniomonas*, as a single origin would require. Gene losses are one thing, membrane losses are another.

Tertiary and Quaternary Symbioses: Not Simpler

When we put multiple independent tertiary (and especially quaternary [18]) endosymbioses into the equation, we add a whole new layer of membrane and cell-biological complexity. A quick sketch shows that the series of events leading from an engulfed cryptophyte bearing a nucleomorph to a chromerid, dinoflagellate or haptophyte complex plastid is quite involved (Figure 1B), and the sketch hardly does justice to the molecular details associated with each symbiotic event. Keeping track of four cryptophyte plastid membranes, cryptophyte ER, cryptophyte plasma membrane, host plasma membrane, and host ER en route to getting exactly the SELMA localisation as in the original cryptophyte, but with the SELMA and photosynthesis genes in the host nucleus, is not trivial (a good exercise for students and specialists alike).

Two points come to bear on the issue. The first concerns membrane losses. The higher order symbiotic models entail many multiple losses, in parallel and in multiple independent lineages, of the numerous membranes that necessarily surround the plastid at some point during these postulated symbiotic events. In the higher order models, those losses are invoked without penalty. Were membrane losses really that easy, why are the additional membranes only lost in the wake of independent tertiary and quaternary endosymbiotic events? Why have red complex plastids not lost their additional outer membranes together with SELMA-dependent targeting and why did they not revert to a simple TOC- and TIC-dependent import in a two-membrane bounded plastid? In dinoflagellates, one of the ancestral four membranes has been lost, yes, but only one, not both, and in the remainder of the lineages with red complex plastids, the two outer membranes have resisted further reduction since the secondary endosymbiont was established. The processes of membrane loss that gave rise to the four membranes of red complex plastids, and the additional loss that gave rise to three membranes surrounding dinoflagellate plastids, appear to be rare — and possibly lineage-defining — events.

The second point concerns great investment of cell biological activity for a conspicuous lack of evolutionary change. If the complex plastid of haptophytes is of tertiary or even quaternary origin as recently suggested [9,11,17], then it ended up with the exact same plastid membrane topology as observed today in cryptophytes — except for the additional loss of the nucleomorph (Figure 1B). That is remarkable and, if true, is cause for reflection. Why could the nucleomorph be lost in all secondary red lineages that engulfed a cryptophyte, but not the cryptophyte itself? Furthermore, all scenarios involving tertiary symbioses must not only predict the convergent and successful loss of the nucleomorph (and transfer of its genes, including the SELMA machinery, to the host), but also multiple independent fusion events of the outer complex plastid membrane with the host's ER and an additional round of translocon invention for the additional membranes initially present. Whatever model is correct, from the perspective of protein targeting and membrane complexity, Occam's razor would appear to demand fairly compelling arguments to depart from a single origin of red secondary plastids. The chromalveolate hypothesis was once quite popular. But popular does not mean correct.

Molecular Phylogenies are Equivocal on the Critical Issues

So why are there so many reports of evidence against the chromalveolate hypothesis? The nature of that evidence is mostly phylogenetic trees, but the trees are also massively conflicting at present on this matter — the red versus green debate to name one example [37–40] — so it is becoming increasingly difficult to say that trees harbour strong evidence one way or the other in the issue. It is true that alveolates have a very distinct cell biology, with their cortical alveoli and specific proteins underpinning that morphology [41]. That can be interpreted as evidence that their host lineage was distinct from that of the other three red secondary lineages, hence as indirect evidence for an independent plastid origin. But the observation is that all alveolates evolved their lineage-specific traits, and whether they evolved them in a lineage that had a red plastid or not does not change the number or nature of the inventions one iota. Thus, the cell biological distinctness of the alveolates indicates their undisputed common ancestry, but does not at all speak to the question of whether or not their common ancestor harboured a red secondary plastid.

A potential problem with the single-origin (the chromalveolate) hypothesis is that ciliates and oomycetes, and maybe also *Blastocystis* (both at the base of stramenopile algae) and *Goniomonas* (at the base of cryptophyte algae), would be secondarily non-photosynthetic. Because many genes are known to have been transferred from the primary plastid to the host nucleus [42], an expectation has arisen that these organisms should possess many genes in their nuclei that betray their photosynthetic past, relics of nucleus to nucleus gene transfers. Evidence for such photosynthetic relics is generally lacking in those genomes [43,44], which has been taken as evidence that ciliates and oomycetes were never plastid-bearing, hence as evidence against the chromalveolate hypothesis. But maybe the expectation is flawed. While it is true that hundreds of nucleus-to-nucleus gene transfers for photosynthetic genes have taken place in the evolution of the cryptophytes [39], the power of gene loss is great. *Cryptosporidium* is a relative of *Plasmodium*, and though *Plasmodium* has a plastid, neither genome has preserved a distinct photosynthesis-related gene repertoire. In flowering plants, the convergent and efficient loss of symbiont-specific genes was observed to have occurred several times independently when arbuscular mycorrhization was terminated [45]. 'Use it or lose it' might be the rule.

One might argue that plastid loss, not gene loss, is the problem. That argument, however, entails many unknowns about the degree to which the plastid had been integrated into the biochemistry of the host prior to loss. The red algal symbiont could have been lost early in some lineages before the mass migration of genes to the host nucleus had occurred, although that is not necessarily the case as evident by the loss of the plastid in a parasitic dinoflagellate [46]. Though there were opportunities to invent SELMA twice, for example in the chlorarachniophytes, it did not happen [47]. Why? Perhaps the ER did

not surround the endosymbiont, as in cryptophytes, and perhaps the chlorarchniophyte plastid is surrounded by a phagosomal membrane. And in *Euglena*, where there are three membranes as in dinoflagellates, the situation is different again. Something else appears to have happened in the green secondary lineages, underscoring the uniqueness of SELMA in the red lineage. Maybe the establishment of red secondary symbiosis is not so simple after all.

Alveolates and Chromists

The main difference between the morphology of the alveolate and chromist complex plastid is that membrane 1 is only continuous with the host ER in chromists. Hence, in the case of the phagosome model, the integration of SELMA into the red algal plasma membrane must have been preceded, or accompanied by establishing directional vesicle transport to the specialized vacuole that housed the endosymbiont. That machinery might still be used today in plastid-bearing alveolates, whose outer plastid membrane is not continuous with the host ER. From what we can tell, alveolate mitosis is closed and chromist mitosis is open. During chromist mitosis the nuclear membrane degrades, leaving a four-membrane bound complex plastid that had commenced division prior to the host nucleus [48]. Therefore, if the two lineages are monophyletic, alveolates might have switched to closed mitosis such that the outer membrane of the complex plastid no longer fused with the host rough ER once mitosis was completed. One can argue this is unlikely, and in fact speaks for the independent origin of the two lineages, but the difference between open and closed mitosis is not always clear cut [49] and the two can even coexist in a single species [50].

Conclusions

If the origin of red complex plastids had a simple solution, we would have found it by now. None of the models are easy. Because the endosymbiont was an autotroph, the problem is not how the endosymbiont was initially kept alive in the chain of events, whether it was inside a phagosome-derived compartment or a cavity of the ER. Rather, the major challenge in all models is how the bottleneck of establishing the transport of macromolecules was initially overcome. The ER origin of membrane 1 and 2 in secondary red lineages follows a natural series of events in a red algal cytosol (now the PPC of *Guillardia*), where SELMA once evolved. Some might complain that this concept 'doesn't prove anything', but it illuminates the problem from a new and potentially promising angle. SELMA evolved only once, and it allowed the diversification of lineages that possess red complex plastids. Gene trees are supposed to be tools to test hypotheses. In the face of many conflicting trees, our first reaction should be to ask whether the conflict stems from processes in biological history or whether it stems from processes in a sequence-analysing computer, not to ask how many additional symbioses with reduction to the same ground state would be needed to explain the trees. In the issue of how red complex plastids have come to reside in diverse lineages, it is helpful to have some benchmarks for orientation and reference. Protein import into red secondary plastids is, we offer, one such benchmark and it might be the best currently available.

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