CHAPTER EIGHT

Cell Biology of Chromerids: Autotrophic Relatives to Apicomplexan Parasites

Miroslav Oborník[*,†,‡,1], **Julius Lukeš**[*,†]

[*]Biology Centre, Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic
[†]Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic
[‡]Institute of Microbiology, Academy of Sciences of the Czech Republic, Třeboň, Czech Republic
[1]Corresponding author: e-mail address: obornik@paru.cas.cz

Contents

Abstract

Chromerida are algae possessing a complex plastid surrounded by four membranes. Although isolated originally from stony corals in Australia, they seem to be globally distributed. According to their molecular phylogeny, morphology, ultrastructure, structure of organellar genomes, and noncanonical pathway for tetrapyrrole synthesis, these algae are thought to be the closest known phototrophic relatives to apicomplexan parasites. Here, we summarize the current knowledge of cell biology and evolution of this novel group of algae, which contains only two formally described species, but is apparently highly diverse and virtually ubiquitous in marine environments.

International Review of Cell and Molecular Biology, Volume 306
ISSN 1937-6448
http://dx.doi.org/10.1016/B978-0-12-407694-5.00008-0

1. INTRODUCTION

The phylum Apicomplexa is a group of single-celled eukaryotes living as obligatory parasites of animals. These protists infect metazoan hosts ranging from invertebrates, such as polychaetes (Rueckert et al., 2010), sipunculids (Leander, 2006), cephalous molluscs (Kopečná et al., 2006), or various insects (Hecker et al., 2002), to reptiles, amphibians, and mammals, including humans (Duszynski et al., 1999; Smith, 1996). Apicomplexa also contains *Plasmodium*, the causative agents of malaria, the most devastating parasitic disease of humans, and the coccidium *Toxoplasma gondii*, likely the most prevalent parasite of humans with proposed influence on their behavior (Flegr, 2007). As one of the most speciose eukaryotic groups with an estimate of over a million species (Pawlowski et al., 2012), not surprisingly, the apicomplexans include many parasites of wild and domestic animals (Chartier and Paraud, 2012). Apicomplexan parasites taxonomically belong to alveolates, a group of protists, which also includes ciliates (Ciliophora), usually free-living heterotrophic protists possessing numerous cilia on their cell surface and a unique genetic system of macro- and micronuclei, and mostly phototrophic dinoflagellates (Dinophyta), algae with various complex plastids that are of high ecological importance in aquatic environments (Adl et al., 2012).

Most apicomplexans are known to contain a set of characteristic structures, in particular, the apical complex and the apicoplast. While the apical complex, a sophisticated apparatus usually composed of the conoid rink, rhoptries, and micronemes, is used to penetrate the host cell, the apicoplast represents a secondary nonphotosynthetic plastid derived from a putatively photosynthetic organelle. This remnant plastid is surrounded by four membranes reflecting its complex origin in secondary or tertiary endosymbiotic event (reviewed in Foth and McFadden, 2003; Lim and McFadden, 2010; Oborník et al., 2009; Roos et al., 1999). Its genome is highly reduced to the 35 kb-long DNA circle (Gardner et al., 1991; Kilejian, 1975) and lacks any traces of genes involved in photosynthesis. Apicoplast genome structure, gene content, and gene synteny are quite conserved among the apicomplexans, supporting a single origin of this formerly photosynthetic organelle (Denny et al., 1998; Lang-Unnasch et al., 1998). However, not all apicomplexans carry a plastid. It has been proved that members of the genus *Cryptosporidium*, parasitizing the intestine of vertebrates including humans, lack the apicoplast (Abrahamsen et al., 2004; Xu et al., 2004;

Zhu et al., 2000a). Moreover, all attempts to detect this organelle in eugregarines, early branching apicomplexans with huge cells associated with the invertebrate hosts, also failed (Toso and Omoto, 2007). According to molecular phylogeny (Carreno et al., 1999; Zhu et al., 2000b) and some morphological synapomorphies (Valigurová et al., 2007), both above-mentioned groups seem to be closely related. We suppose that these apicomplexans lost their plastid shortly after its acquisition, before the organelle became firmly established and indispensable for the parasite's survival (Oborník et al., 2009).

Still, the apicoplast has been found in the most species-rich and widespread groups of apicomplexan parasites, such as Coccidia, Piroplasmida, and Haemosporidia (Lim and McFadden, 2010; Oborník et al., 2009). In the best studied *Plasmodium falciparum*, this relic plastid was shown to be essential for the cell, its disruption leading to the so-called delayed death effect (Fichera et al., 1995; He et al., 2001; Pfefferkorn et al., 1992; Ramya et al., 2007). The apicoplast therefore represents a new promising target, even a proverbial Achilles' heel of these pathogens (Jomaa et al., 1999; McFadden and Roos, 1999; Soldati, 1999; Wiesner and Jomaa, 2007; Wiesner et al., 2008). The discovery of this organelle led to the groundbreaking suggestion that these heterotrophic parasites had evolved from a phototrophic ancestor, particularly, an alga hosting a complex plastid (McFadden et al., 1996). Although the apicoplast apparently lost the main plastid function and is thus not photosynthetic anymore, several likely essential metabolic pathways still take place in this organelle, such as the heme biosynthesis (Kořený et al., 2011, 2013; van Dooren et al., 2012; Wilson, 2002; Williams and Keeling, 2003), fatty acid synthesis (Goodman and McFadden, 2008), or nonmevalonate isoprenoid synthetic route (Jomaa et al., 1999; reviewed by Ralph et al., 2004). It has been proposed that particularly the heme (tetrapyrrole) biosynthetic pathway plays an important role in the plastid losses, which are known to occur frequently, especially during the evolution of alveolates and stramenopiles (Barbrook et al., 2006; Kořený et al., 2011, 2012; Kořený and Oborník, 2011). As a matter of fact, substantial attention has been paid to the heme pathway when searching for a suitable antimalarial drug target (Seeber and Soldati-Favre, 2010; van Dooren et al., 2012). However, it has been shown recently by an elegant chemical rescue of *Plasmodium* freed of the apicoplast that for its erythrocytic (=bloodstreams) stages, the only truly essential compound produced by the apicoplast is isopentenyl pyrophosphate, a product of the nonmevalonate isoprenoid pathway (Yeh and DeRisi, 2011).

Although thanks to the number of membranes surrounding the apicoplast it was evident that it is a complex plastid originating from an at least secondary endosymbiotic event (Köhler et al., 1997; McFadden et al., 1996), its particular origin from within the green or red plastid lineage had remained unknown for a long time. So far, only two groups of algae with secondary green plastid are known, namely, the photosynthetic Euglenophyta and Chlorarachniophyta, belonging to the excavates and rhizarians, respectively. Both protist groups are supposed to have acquired their plastids relatively recently (Archibald, 2012). All the other groups of algae known to harbor secondary plastids, such as Stramenopila, Alveolata, Cryptophyta, and Haptophyta, obtained them via the endosymbiotic relationship with a red alga. However, even within dinoflagellates, a species-rich group of alveolate algae mostly possessing a secondary red plastid, two species (*Lepidodinium viride* and *L. chlorophorum*) with the green secondary plastid have been described (Takishita et al., 2008; Watanabe et al., 1990). Before the discovery of chromerids (Moore et al., 2008), the dinoflagellates represented, due to their sister position to the Apicomplexa, the closest known phototrophic relatives of these obligatory parasites (Zhang et al., 2000). Unfortunately, since the apicoplast lost all its photosynthetic functions and since the genome of the dinoflagellate peridinin-pigmented plastid was reduced to an extremely narrow set of photosynthetic genes (Barbrook and Howe, 2000; Green, 2004; Zhang et al., 1999), the plastid genomes of these related alveolates virtually do not overlap (Keeling, 2008) and are, hence, beyond meaningful comparison. The only genes shared by both groups are those coding for rRNAs; however, their extreme AT richness and divergence make a trustable phylogenetic analysis highly questionable (Dacks et al., 2002; Howe, 1992; Oborník et al., 2002; Zhang et al., 2000). Consequently, numerous phylogenetic analyses of the apicoplast genes led to contradictory results. While the *tufA* gene-based analyses supported the origin of the apicoplast from within the green lineage (Egea and LangUnnasch, 1995; Köhler et al., 1997), other authors suggested its origin outside of the green lineage (Blanchard and Hicks, 1999) or inside the red plastid lineage (Williamson et al., 1994). The latter origin is further supported by the structure of the super plastid operon of the apicoplast genome, gene synteny, which is homologous to the red rather than the green plastid genomes (Blanchard and Hicks, 1999; McFadden and Waller, 1997; Stoebe and Kowallik, 1999; Zhang et al., 2000). In spite of that, the green scenario came once more into the game, when the uniquely split cox2 genes were found in the nuclear genomes of both apicomplexans and leguminous plants (Funes et al., 2002). However, other researchers have

shown that such an arrangement is present already in ciliates and had obviously evolved multiple times during evolution (Waller and Keeling, 2006; Waller et al., 2003). The ambiguous phylogeny of the apicoplast reflects the extreme divergence of its fast-evolving genes. Indeed, the AT content of the *P. falciparum* apicoplast genes can reach up to 97% and phylogenetic analyses of such biased sequences are heavily affected by various phylogenetic artifacts, including the long-branch attraction phenomenon (Dacks et al., 2002).

However, the discovery of a new group of photosynthetic alveolates called Chromerida represented a true breakthrough in this respect (Moore et al., 2008; Oborník et al., 2012). These algae contain relatively conserved plastid genomes with the gene repertoire overlapping with those of the apicomplexan and dinoflagellate plastids. Moreover, it was unambiguously shown that the chromerid plastid is the closest known phototrophic relative to the apicoplast. Accumulating evidence derived from nucleus-encoded genes further showed that chromerids share a common ancestry with the Apicomplexa (Janouškovec et al., 2010; Kořený et al., 2011; Moore et al., 2008; Oborník et al., 2009).

2. CHROMERIDA: A NEW GROUP OF ALGAE ISOLATED FROM AUSTRALIAN CORALS

Dinoflagellates are photosynthetic alveolates with complex plastids well known as important symbionts of corals (Freudental, 1962; Trench, 1993). During an investigation of the coral-associated flora in Australia, new algae were isolated from stony corals by a procedure usually used to isolate intracellular symbionts. When shape and morphology are considered, one of these algae to some extent resembled symbiotic dinoflagellates of the genus *Symbiodinium*, but molecular phylogeny complemented with ultrastructural and metabolic analysis revealed its unexpectedly close relationship with the apicomplexan parasites (Janouškovec et al., 2010; Kořený et al., 2011; Moore et al., 2008; Oborník et al., 2009, 2011).

Along with the formal taxonomic description of *Chromera velia*, the new phototrophic phylum Chromerida was established in the frame of the alveolates (Moore et al., 2008). Recently, the life cycle and morphology of *Vitrella brassicaformis* heralded the description of another chromerid lineage (Oborník et al., 2012). Topology of phylogenetic trees based on the SSU rRNA genes suggested the affiliation of chromerids to colpodellids, free-living heterotrophic predators of algae closely related to the apicomplexan parasites (Fig. 8.1; Janouškovec et al., 2012a,b; Moore et al., 2008).

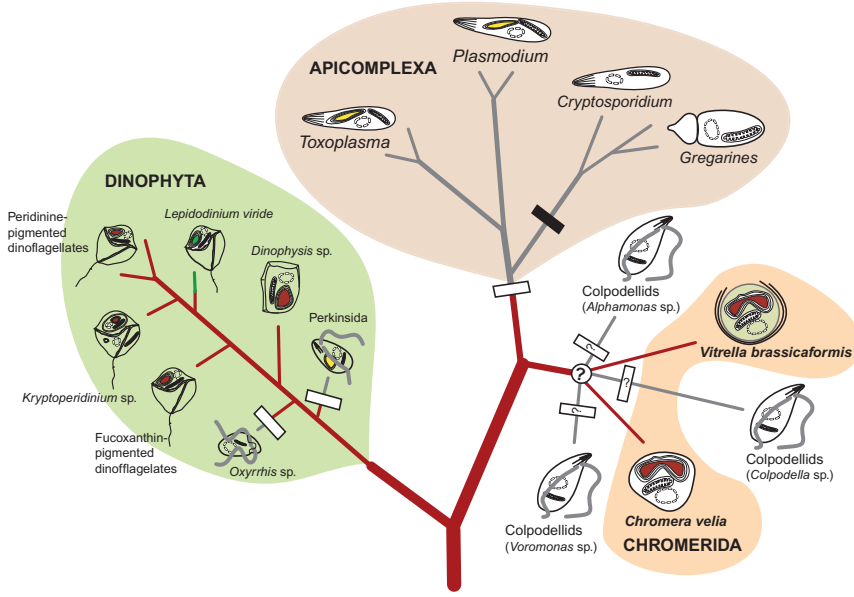

Figure 8.1 Phylogenetic position of Chromerida in frame of the Apicomplexa–Dinophyta tree. White rectangles indicate the loss of photosynthesis; black rectangle indicates the loss of plastid. Branching order in the Chromerida–Colpodellida clade remains unresolved. Red color depicts photosynthetic lineages, gray color marks secondary heterotrophs. Green color shows a rear case of replacement of the original red secondary plastid with the green secondary plastid.

However, colpodellids do not constitute a monophyletic group and, consequently, *Chromera* and *Vitrella* seem to be sitting within colpodellids in different positions on the root of the Apicomplexa (Fig. 8.1). This suggests possible multiple losses of plastids as well as unexpectedly large but so far mostly hidden diversity of the basal Apicomplexa and their rapid and massive early radiation.

2.1. *C. velia*: A new alga from Sydney Harbor

The first chromerid species, *C. velia*, was isolated from the coral *Plesiastrea versipora* growing in the relatively cold waters (~20 °C) of Sydney Harbor (Moore et al., 2008). Although *C. velia* was isolated directly from coral tissue by a procedure usually used to isolate intracellular symbionts (York, 1986), its symbiotic nature has so far not been confirmed and remains a subject of

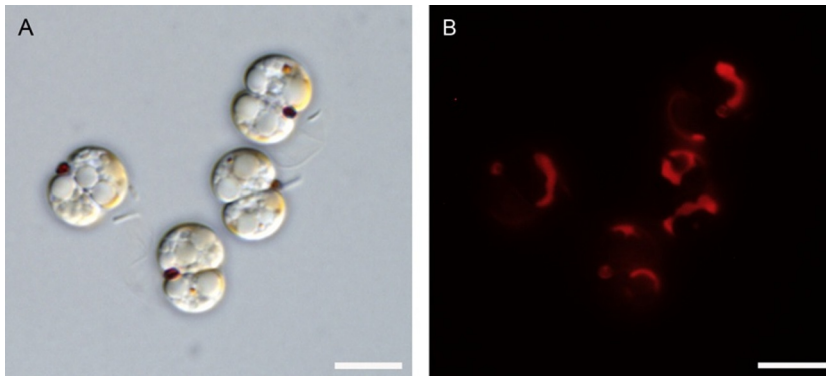

Figure 8.2 Light microscopy of cultured *Chromera velia*. Coccoid cells after the first round of cell division (left panel). Autofluorescence of the plastids (right panel) (scale bar = 5 μm).

discussion (see Chapter 7 for details). The culture, derived from the originally isolated strain, is being maintained in our lab for about 8 years as a free-living full phototroph in a simple algal F2 medium without any external source of organic carbon. Nonmotile brownish coccoid cells from 5 to 7 μm in diameter represent the originally isolated stage and also dominate the culture (Fig. 8.2). These vegetative coccoids form colonies where individual cells are kept together by some kind of a sticking gel produced by the alga. In the stationary culture, individual cells as well as colonies of *C. velia* sediment, forming a brownish layer at the bottom of the cultivation flask, with a tendency to adhere to its surface, suggesting possible endophytic character of chromerid algae. In addition to the predominant nonmotile coccoids, flagellated cells termed zoospores possessing two heterodynamic flagella were observed in the culture (Moore et al., 2008). As found later on, formation of zoospores is driven by exposure to light (Oborník et al., 2011; Weatherby et al., 2011). Since the rhythmicity of zoospore formation is preserved also under stable light condition, it is likely subject to circadian clock proteins that have indeed been found in the *C. velia* genome (Kručinská et al., unpublished results). Under culture conditions, the abundance of flagellates is also influenced by salt in the cultivation medium, as higher salinity of the medium resulted in a lower level of zoospore production (Guo et al., 2010). The function of zoospores in the life cycle of *C. velia* remains to be established. However, when the culture is exposed to spotlight of high intensity, coccoids massively transform into zoospores, which are

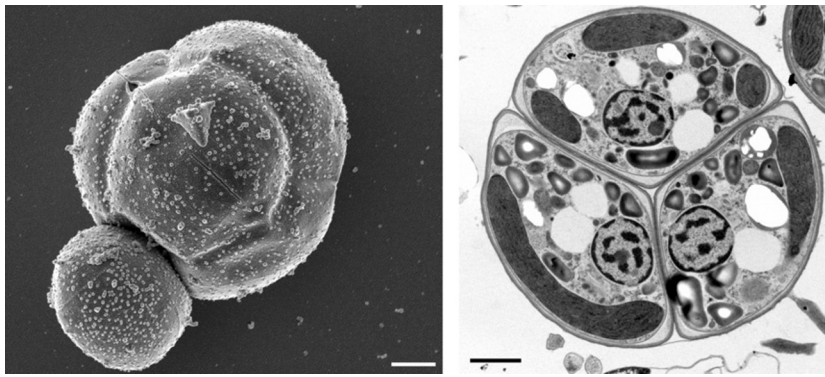

Figure 8.3 Ultrastructure of *C. velia*. Scanning electron microscopy of an autosporangium enclosing four tightly bound autospores, and a single free autospore (left panel). Transmission electron microscopy of a cross-sectioned autosporangium containing autospores (right panel). Note prominent sausage-shaped plastids (scale bar = 1 μm). *The right panel is reprinted from Oborník et al. (2011), Copyright (2011), with permission from Elsevier 2011.*

better equipped to escape from such unfavorable and harmful light conditions. We speculate that zoospores may also represent the stage invading the coral larvae.

Since various phylogenetic analyses supported a close relationship between the chromerids, dinoflagellates, and apicomplexans, we have investigated the ultrastructure of *C. velia*, seeking structural synapomorphies shared by these protists (Oborník et al., 2011). The vegetative stage composed of up to four coccoids (Fig. 8.2) contains sutures on the surface of its sporangium that resemble those seen on the coccidian cysts, such as *T. gondii* and related coccidian genera (Fig. 8.3; Kopečná et al., 2006). Further, vegetative cells and autosporangia are reminiscent of the apicomplexan cysts, particularly those belonging to the genus *Cryptosporidium*, a waterborne causative agent of human cryptosporidiosis (Hunter and Nichols, 2002). The alga divides asexually by binary division (Fig. 8.4), forming tetrads or triads in an autosporangium enclosed by an additional membrane (Fig. 8.5). At the ultrastructural level, *C. velia* displays other characters typical for alveolates, including cortical alveoli—flattened vesicles underlying the plasma membrane (Fig. 8.6) and supported by a single-layered sheet of microtubules forming a corset surrounding the entire algal cell. A single prominent cone-shaped plastid (Fig. 8.2B) is bound by four membranes (Fig. 8.7), a feature shared with the apicoplast, which reflects their complex evolutionary history. In contrast to *V. brassicaformis*, the plastid does not contain a conspicuous pyrenoid.

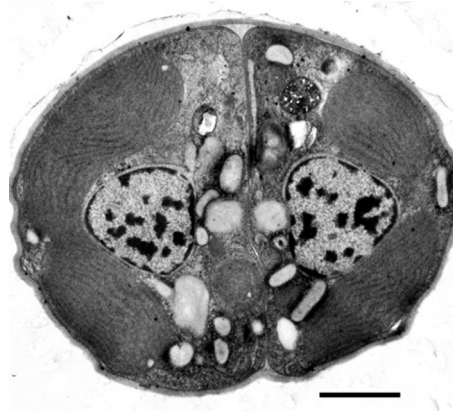

Figure 8.4 Ultrastructure of the asexual cell division of *C. velia*. The longitudinally sectioned plastids are filled with thylakoids arranged in stacks of three. The autospores also contain an oval nucleus with nucleoli, as well as numerous granules likely with amylopectin and lipid contents (scale bar = 2 μm).

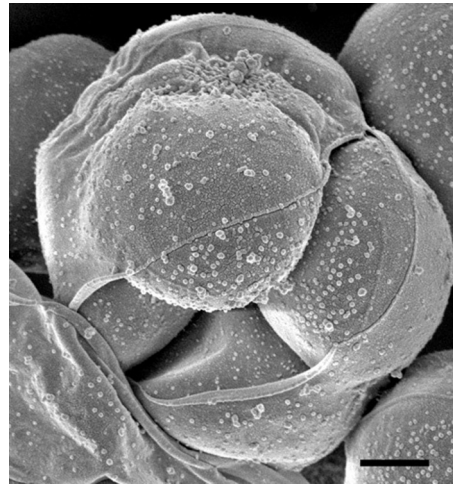

Figure 8.5 Detailed view of an autosporangium of *C. velia*. Ruptured wall reveals the presence of four tightly bound autospores (scale bar = 1 μm).

Along with the nonmotile cells, elongated zoospores possessing two prominent flagella (Fig. 8.8) and retaining a relatively large and evenly pigmented plastid (Fig. 8.9) were observed to move very fast in a characteristic zig-zag manner (Oborník et al., 2011; Weatherby et al., 2011). Zoospores of chromerids highly resemble colpodellids in morphology, overall cell shape, and the presence of two heterodynamic flagella, both thinly tapered at their terminus (Leander et al., 2003). The motile stages display

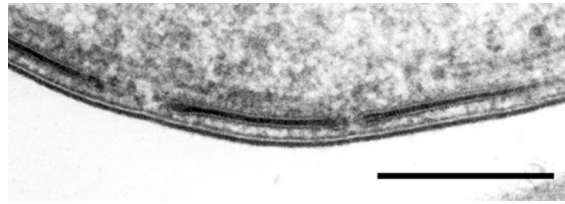

Figure 8.6 Cortical alveoli below the plasma membrane of *C. velia*. Section through the periphery of a vegetative cell reveals the presence of flat electron-dense alveoli and a single layer of underlying microtubules (scale bar = 200 nm).

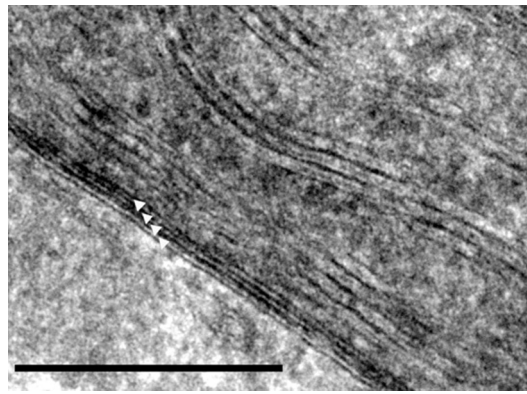

Figure 8.7 Membranes of the *C. velia* plastid. Arrowheads point to four membranes surrounding this secondary plastid. Note the arrangement of thylakoids in stacks of three (scale bar = 100 nm).

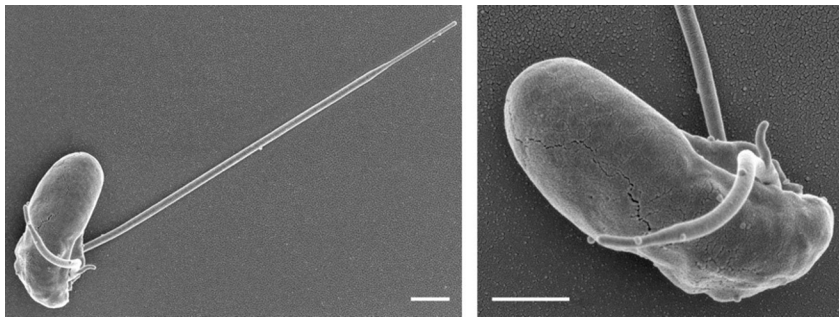

Figure 8.8 Scanning electron microscopy of *C. velia* flagellated zoospores. The elongated zoospores are equipped with two heterodynamic flagella. The shorter flagellum contains a typical finger-like projection (scale bar = 1 μm). *The left panel is reprinted from Oborník et al. (2011), Copyright (2011), with permission from Elsevier 2011.*

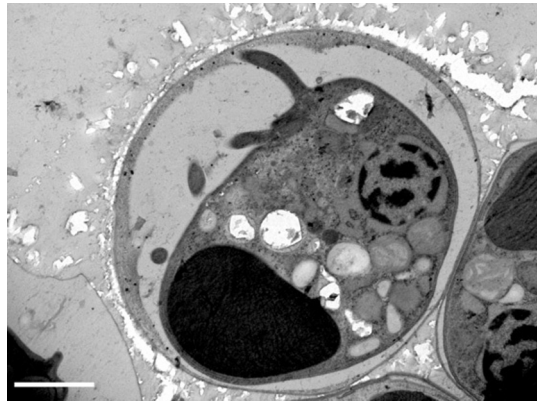

Figure 8.9 Ultrastructure of immature zoospore of *C. velia*. While both flagella of this zoospore have already been formed, it remains encysted. Note the large plastid and numerous cytosolic granules (scale bar = 1 μm). *Reprinted from Oborník et al. (2011), Copyright (2011), with permission from Elsevier 2011.*

a unique finger-like projection located close to the root of the shorter flagellum (Fig. 8.8), a feature absent from the other ultrastructurally studied chromerid *V. brassicaformis* (Oborník et al., 2012), or the closely related colpodellids (Leander et al., 2003). It appears that axonemes are formed inside the round aflagellar cell, with the flagella being at once ejected to its surface (Fig. 8.8) (Oborník et al., 2011). It is worth noting that such an unusual way of flagellar formation has been earlier described in *Plasmodium* (Briggs et al., 2004; Killick-Kendrick and Peters, 1978). Moreover, a structure resembling the pseudoconoid of some colpodellids and apicomplexans is also present in *C. velia* (Oborník et al., 2011).

2.2. *V. brassicaformis*: An alga from the Great Barrier Reef

The second chromerid species known so far was isolated by Robert Moore from the stony coral *Leptastrea purpurea* in the vicinity of One Tree Island, the Great Barrier Reef, and was formally described as *V. brassicaformis* (Oborník et al., 2012). Both chromerid species have very different morphology and life cycle, as well as the structure of their plastid genomes and evolutionary tempo of respective plastid-encoded genes (Janouškovec et al., 2010; Oborník et al., 2011, 2012). Recent results of a large-scale investigation of chromerid sequences demonstrated an unexpectedly high abundance of *Vitrella*-like sequences in the ocean, relatively rare occurrence of those affiliated with the genus *Chromera*, and the presence of several novel chromerid lineages, for which morphological data have yet to be obtained (Janouškovec et al., 2012a,b).

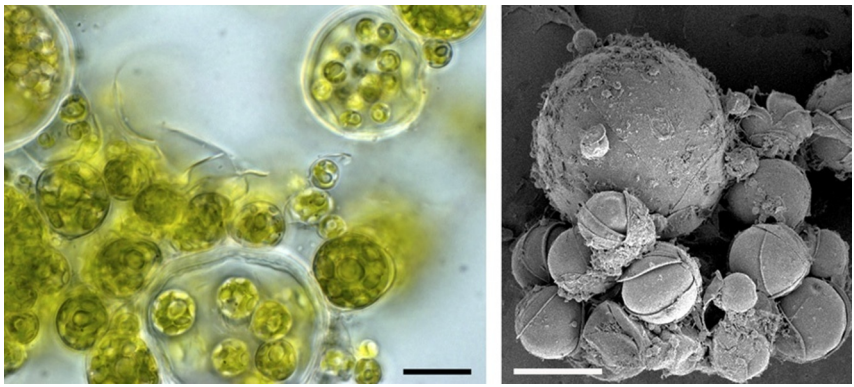

Figure 8.10 Light and scanning electron microscopy of cultured *Vitrella brassicaformis*. Vegetative cells are highly variable in size. Despite a thick and multilayered cyst wall, the cysts are fully transparent (left panel) (scale bar = 5 μm). Major size heterogeneity of vegetative cells in a stationary culture (right panel) (scale bar = 5 μm). *The right panel is reprinted from Oborník et al. (2012), Copyright (2012), with permission from Elsevier 2011.*

V. brassicaformis differs from *C. velia* on the first look, mainly because of the green color of its vegetative cells and autosporangia, which are of highly variable diameter (3–30 μm) and represent the predominant cultured stage of the alga (Fig. 8.10). Its development starts by a release of tiny vegetative autospores (3–4 μm in diameter), which will reach up to 30 μm in diameter and develop into either autosporangia full of autospores or zoosporangia filled with zoospores (Oborník et al., 2012). In contrast to *C. velia*, in which the autosporangia and zoosporangia contain up to four autospores and ten zoospores, respectively (Kručinská et al., unpublished results), the *V. brassicaformis* sporangia contain many dozens of spores. By forming additional layers, the laminated cell wall becomes thicker with the growing size of the vegetative cell. Indeed, stages surrounded by up to 12 unevenly thick cell wall layers were found. The layers frequently do not enclose the cell in its entirety, resulting in a cabbage-like structure (Fig. 8.11), which gave the alga its species name (Oborník et al., 2012). Interestingly, despite the thickness of its walls, the cell is fully transparent for light.

Flagellated zoospores (Fig. 8.12) formed inside of the zoosporangia (Fig. 8.13) were also present under culture conditions. However, in contrast to *C. velia*, the zoospores of *V. brassicaformis* contain a very tiny plastid, which in our opinion may even not be photosynthetically active. Another feature

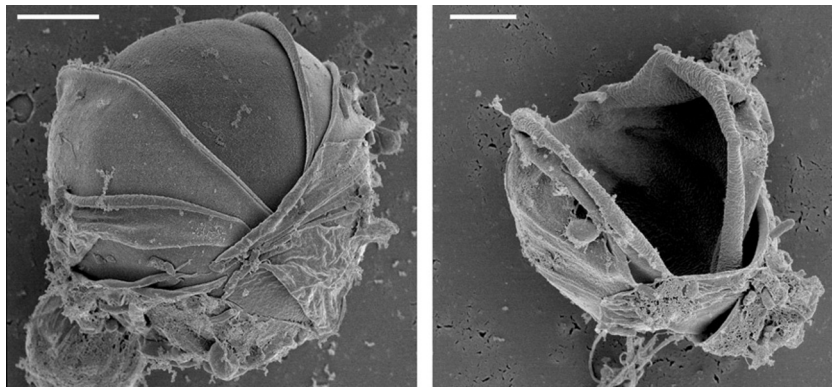

Figure 8.11 Scanning electron microscopy of *V. brassicaformis*. Vegetative cells and sporangia are enclosed by a wall composed of numerous superimposed layers resembling cabbage head (left panel) (scale bar = 5 μm). Empty multilayered wall of a ruptured sporangium (right panel) (scale bar = 5 μm). *Reprinted from Oborník et al. (2012), Copyright (2012), with permission from Elsevier 2011.*

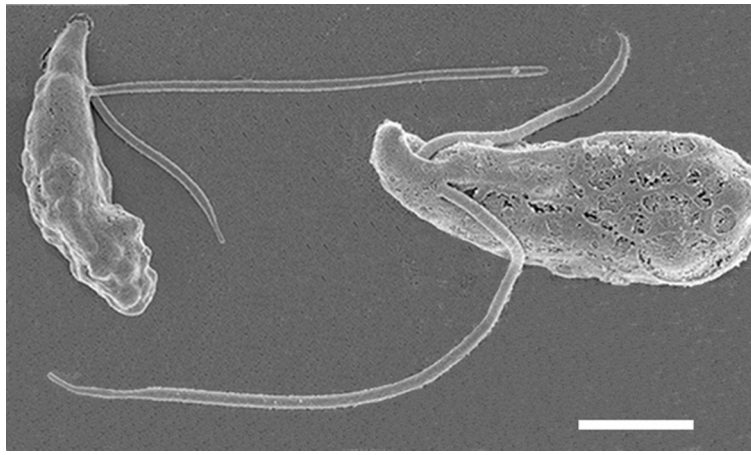

Figure 8.12 Scanning electron microscopy of *V. brassicaformis* flagellated zoospores. The elongated tapered zoospores are equipped with two heterodynamic flagella. Note the presence of a longitudinal ridge on the ventral side of the zoospore. The shorter flagellum lacks the finger-like projection present in zoospores of *C. velia* (scale bar = 2 μm). *Reprinted from Oborník et al. (2012), Copyright (2012), with permission from Elsevier 2011.*

characteristic of this chromerid species is a single round operculum in its cyst wall (Fig. 8.13; Oborník et al., 2012). Although both *C. velia* and *V. brassicaformis* contain very similar cortical alveoli and subalveolar sheet of microtubules, in the latter alga we have found no traces of pseudoconoid,

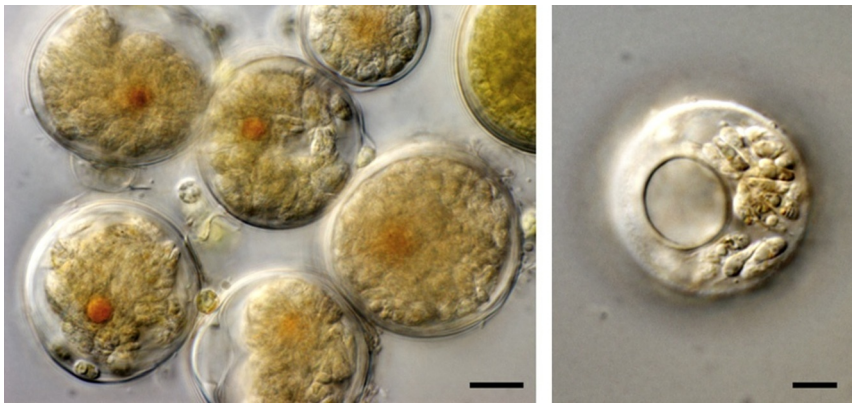

Figure 8.13 Light microscopy of *V. brassicaformis*. Stationary culture is composed of green autosporangia in various phases of sporulation, and orange-colored zoosporangia filled with tiny zoospores (left panel) (scale bar = 10 μm). Each sporangium is equipped with a prominent circular operculum (right panel) (scale bar = 10 μm). *The right panel is reprinted from Oborník et al. (2012), Copyright (2012), with permission from Elsevier 2011.*

with its plastid being equipped with a conspicuous pyrenoid. In addition to that, *V. brassicaformis* also substantially differs from *C. velia* in the composition of its pigments (Oborník et al., 2012; see below). All in all, both chromerids for which morphological and life cycle data are available differ substantially and likely represent only the tip of the iceberg where diversity of this emerging and widespread group of algae is concerned.

3. LIFE CYCLE

Since no signs of sexual reproduction have been found so far, only vegetative life cycles are considered for both chromerid algae (Oborník et al., 2011, 2012), which likely live in a similar environment, namely, on the surface of corals, or as symbionts inside of them (Cumbo et al., 2013; Janouškovec et al., 2012a,b). However, the frequent presence of *Vitrella*-like sequences in waters beyond the coral-inhabited territory strongly indicates their capacity to live independently of these ancient metazoans (Janouškovec et al., 2012a,b). The life cycle of *C. velia* (Fig. 8.14A) starts with the division of a vegetative coccoid, which will divide into two, three, or four daughter cells in one or two sequential binary divisions. Autosporangia containing more than four cells have never been observed in the culture. Autospores can be released from all types of

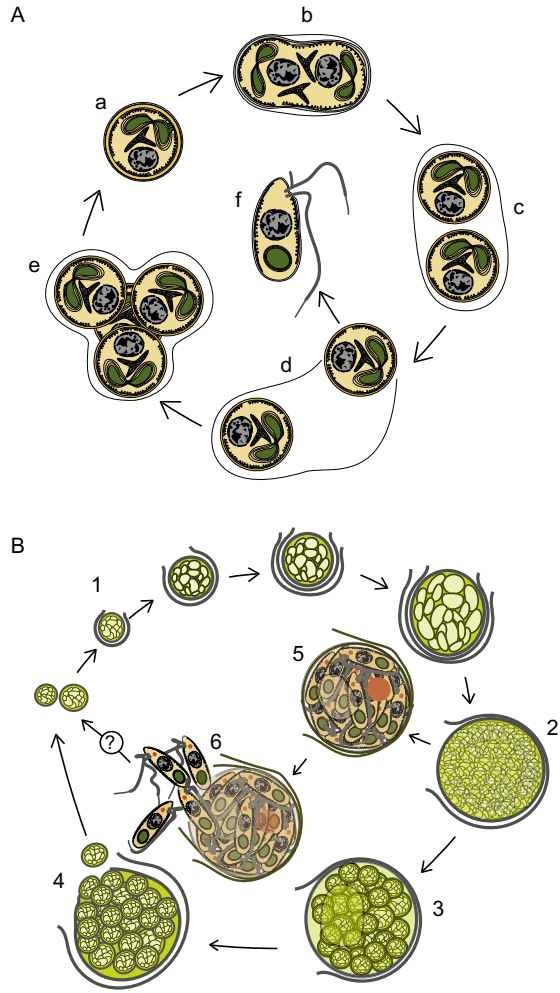

Figure 8.14 Life cycles of *C. velia* (A) and *V. brassicaformis* (B). Vegetative cell (a, 1) is a predominant stage in the cultures of both algae. It divides in *C. velia* (b) into two (c) to four (e) vegetative cells forming an autosporangium (c, e) bounded by additional membrane. Autospores are released from sporangia (d, e) and start a new life cycle. It appears that zoospores (f) can be formed after light exposure from released autospores but likely also from other stages (not shown). Vegetative cell of *V. brassicaformis* (1) grows up to 10–30 μm in diameter and forms cell with fine-grained content (2). It develops either into autosporangium (3) containing dozens of autospores (4) or zoosporangium with numerous zoospores (5). Both types of spores are released (4, 6), autospores start the new life cycle. Fate of zoospores is yet unknown. *(A) Reprinted from Oborník et al. (2011), Copyright (2011), with permission from Elsevier 2011. (B) Reprinted from Oborník et al. (2012), Copyright (2012), with permission from Elsevier 2011.*

sporangia, and flagellated zoospores are supposed to emerge from the released immotile autospores (Oborník et al., 2011). However, by light microscopy, we have recently observed extremely large cells (=zoosporangia) in the culture of *C. velia*, which contain four or eight flagellated zoospores. Excystation of zoospores from these zoosporangia occurs within about 2 min (Kručinská et al., unpublished results). Although the function of zoospores remains unknown, we have observed their very fast encystation, during which the cells under unfavorable conditions discarded their flagella, and within 10 min, the thin zoospores secreted a thick wall. Vegetative cell can be formed either from released autospore or encystated zoospore and commences a new life cycle (Oborník et al., 2011).

In contrast to *C. velia*, *V. brassicaformis* forms sporangia containing dozens to hundreds of autospores or zoospores (Figs. 8.2 and 8.13). After reaching about 10–30 m in diameter, the content of vegetative cells becomes fine grained and the forming sporangium becomes colored according to the type of spores it will produce. Inside of the green autosporangium, autospores formed gradually from the periphery of the cell are eventually released and start a new life cycle. An alternative pathway results in the production of lightly brown zoosporangia containing a conspicuous orange structure similar to the reddish-like globule found in the Eustigmatophyte algae. Interestingly, newly formed zoospores equipped with two heterodynamic flagella move very quickly within the still intact zoosporangium and swarm out of it after the rupture of its wall. The fate of the released zoospores is yet to be established. The high number of spores and the size of its sporangia may imply that *V. brassicaformis* should grow faster than *C. velia*, yet the opposite is the case. The number of spores in both types of sporangia represents the main difference between the life cycles of these chromerid algae (Oborník et al., 2011, 2012).

4. EVOLUTION OF EXOSYMBIONT

Origin of an alga with a secondary plastid is generally complex. Particularly in the case of the alveolates, which also include dinoflagellates known to host plastids of various origins in the frame of the same nuclear (=exosymbiont) lineage, phylogenetic positions of both secondary host (=exosymbiont) and complex plastid (=endosymbiont) should be carefully specified. In addition to that, the nuclear genomes of secondary algae (=algae harboring secondary plastid) are hybrids, as they bring together eukaryotic

genes of the exosymbiont and the algal endosymbiont (=the plastid remnant of an engulfed eukaryotic alga), possibly mitochondrial genes of both symbiotic participants, plastid (=cyanobacterial) genes (Oborník and Green, 2005), and quite often a high portion of other, usually bacterial genes, likely obtained by lateral gene transfer (Bowler et al., 2008). It was proposed that algae within the recently established SAR group (Adl et al., 2012), particularly diatoms, passed through an ancient endosymbiotic event involving a green alga, since they allegedly encode a high proportion of genes displaying green algal origin (Moustafa et al., 2009). However, one has to keep in mind that taxon sampling strongly influences such topologies, and so far, genome of a single rhodophyte, *Cyanidioschyzon merolae*, has been fully sequenced (Matsuzaki et al., 2004). Moreover, a reevaluation of these data questioned the above-mentioned conclusions (Deschamps and Moreira, 2012).

The origin of chromeran nuclear lineage (=exosymbiont) was proposed based on several phylogenetic analyses, initially the SSU and LSU rRNA genes (Moore et al., 2008), followed by trees computed from the combined dataset of eight nuclear genes (hsp90, hsp70, α-tubulin, β-tubulin, second paralogue of hsp70 [biP], elongation factor 2, and SSU and LSU rRNA genes) with a total of 7,137 characters (Janouškovec et al., 2010). Finally, a complex phylogenetic analysis of 3000 expressed sequence tags has been performed (Woehle et al., 2011). Unfortunately, the latter extensive analysis was damaged by the presence of a substantial contamination by land plant sequences, which in combination with low requirements for trees evaluated as valid (for instance, bootstraps were not computed) led to doubtful results showing a high fraction of green genes in the chromerid transcriptome. Indeed, a careful reevaluation of these data was compatible with a more conservative evolutionary scenario (Burki et al., 2012a).

It should be mentioned that all phylogenetic analyses invariably demonstrated the position of *C. velia* on the root of Apicomplexa (Burki et al., 2012a; Janouškovec et al., 2010; Moore et al., 2008; Oborník et al., 2009). Moreover, other characters also support the conclusion that chromerid algae are genuine relatives of the apicomplexan parasites rather than some unusual dinoflagellates. The *C. velia* genome contains typical histones H2A and H2B (Oborník et al., 2009), while the related dinoflagellates have permanently condensed chromosomes (Rizzo and Burghardt, 1982), which are packed by a recently described dinoflagellate-specific family of highly basic nuclear proteins (Gornik et al., 2012). Further, *C. velia* differs from these relatives by not having its chromosomes condensed during its entire cell cycle (Oborník et al., 2009, 2011). Last but not least, when the endosymbiont-derived genes are taken into

account, an ordinary plastid-encoded *tufA* gene was, in contrast to dinoflagellates, amplified from *C. velia* (Janouškovec et al., 2010; Oborník et al., 2009) and maximum-likelihood phylogenetic analysis placed it on the root of its apicomplexan homologues (Oborník et al., 2009). It should be mentioned that chromerids also share some molecular characters with dinoflagellates, such as the use of bacterial type II Rubisco and oligoU tails in their plastid transcripts. However, this is not surprising, since dinoflagellates represent the closest phototrophic relatives of the chromerid algae, with both phototrophic alveolates and their plastids sharing a common ancestry (Janouškovec et al., 2010).

5. EVOLUTION OF CHROMERID ORGANELLES

Like the related algae, chromerids contain two semiautonomous organelles: the mitochondrion and the plastid. The complex plastid is, according to the number of surrounding membranes, of secondary origin, presumably from an as yet unspecified engulfed rhodophyte. Unexpectedly, the plastids of both described chromerid species substantially differ, and this difference is based on available data, attributed to different speed of evolution (Janouškovec et al., 2010; Oborník et al., 2012). The level of mutual divergence between *C. velia* and *V. brassicaformis*, as well as between their plastids, is mainly reflected by the structure of plastid genomes. In addition to the mitochondrion and the plastid, a unique structure called chromerosome is found in almost each *C. velia* vegetative cell. The function of this large and conspicuous organelle-like structure remains unknown (Oborník et al., 2012).

5.1. Evolution of chromerid plastids

Tom Cavalier-Smith proposed that the entire eukaryotic supergroup Chromalveolata appeared, evolved, and expanded thanks to the secondary endosymbiotic event between a heterotrophic eukaryote (=exosymbiont) and a rhodophyte (=endosymbiont), giving rise to the complex plastids surrounded by more than two membranes (Cavalier-Smith, 1999). Although the taxon Chromalveolata has been already virtually replaced by the SAR (=Stramenopila, Alveolata, Rhizaria) group (Adl et al., 2012), secondary endosymbiosis remains the unquestionable major player in the evolution of these complex ubiquitous eukaryotes. However, the exact number of endosymbiotic events that occurred in the evolutionary history of algae with secondary red plastids remains controversial. Current extensive phylogenomic analyses are compatible with two rather than a single

acquisition of secondary plastid of a red lineage (Burki et al., 2012b). We tend to believe in a scenario involving two independent secondary endosymbioses with red algae, one having occurred on the root of the SAR group, with the second one being responsible for the emergence of an independent lineage of cryptophytes (Burki et al., 2012b). Such an evolutionary pattern is further supported by the presence of the nucleomorph, a remnant nucleus of the endosymbiotic alga, which was found in cryptophytes (Curtis et al., 2012).

The number of membranes surrounding the chromerid plastid betrays their origin via a secondary or even other complex endosymbiotic event (Janouškovec et al., 2010; Moore et al., 2008). A substantial evidence supports its common ancestry with the apicoplast, the nonphotosynthetic relic plastid of Apicomplexa, in particular, molecular phylogeny inferred from the plastid SSU rRNA genes (Moore et al., 2008), followed by a comprehensive analysis of 30 conserved plastid protein-coding genes (Janouškovec et al., 2010). However, it should be noted that in most constructed phylogenetic trees inferred from the plastid genes, chromerid, apicomplexan, and dinoflagellate plastids constitute very long branches. Unfortunately, phylogenetic analyses containing such genes tend to be affected by various artifacts (Mindell and Thacker, 1996; Oborník et al., 2002; Philippe and Germot, 2000; Stiller and Hall, 1999). Therefore, nonphylogenetic evidence supporting a relationship between chromerid plastid and the apicoplast is of particular importance, with the potential to counterbalance conclusions based on artifactual tree topology. Such is the case of the alternative genetic code for tryptophan in the plastid-encoded genes in *C. velia* (also see below) (Janouškovec et al., 2010; Moore et al., 2008), which is a conspicuous synapomorphy with the apicoplast of Coccidia, a group of advanced apicomplexan parasites, and the same applies for the noncanonical pattern of heme biosynthesis (also see Chapter 6.1.) which is homologous to the Apicomplexa (Kořený et al., 2011). Further, analysis of gene synteny in the plastid ribosomal operon suggests that plastids of these enigmatic coral-associated algae share a common origin with plastids from the apicompexans, the heterokont algae, and dinoflagellates (Janouškovec et al., 2010).

One of the obstacles preventing determination of the origin of the apicoplast has been the absence of any pigmentation of this nonphotosynthetic organelle. Since the chromerids apparently share origin with the apicomplexans, and their plastid is closely related to the apicoplast, we have investigated their pigments, the analysis of which showed surprising

results. First of all, both chromerids lack chlorophyll *c* in their plastids, the pigment hallmark of the red-derived secondary plastids (Moore et al., 2008; Oborník et al., 2012). So far, only a single stramenopile group named Eustigmatophyceae has been shown to lack this pigment (Sukenik et al., 1992). Moreover, in a single chromophyte species (*Xanthonema debile*) belonging to Xantophyceae, chlorophyll *c* is also absent (Gardian et al., 2011). An absence of this particular pigment from not only the chromerid algae but also from other independent lineages may suggest that in the evolution of the SAR group, chlorophyll *c* has been lost multiple times. However, since the pigment composition of *V. brassicaformis* is virtually identical to that found in the eustigmatophyte *Nannochloropsis lineata* (Oborník et al., 2012), the scenario postulating the origin of the chromerid plastid in tertiary endosymbiosis involving an eustigmatophyte alga cannot be fully rejected. Since the tertiary red plastids of dinoflagellates, the second group of photosynthetic alveolates, are bound by a four-membrane envelope, the number of membranes in chromerid and apicomplexan plastid supports their possible tertiary origin as well. Such a scenario can be preferred also by the unusual structure of the *C. velia* plastid genome (see below; Janouškovec et al., 2010), which resembles that of the tertiary plastid of the dinoflagellate *Karlodinium veneficum* (Gabrielsen et al., 2011). On the other hand, the presence of canonical highly conserved and compact plastid genome in *V. brassicaformis* makes this evolutionary pathway much less likely (Oborník et al., 2012). Although both chromerid species lack chlorophyll *c*, the composition of other pigments is again different. While in addition to chlorophyll *a*, violaxanthin, and β-carotene, the *C. velia* plastid also contains novel isoform of isofucoxanthin (Moore et al., 2008), *V. brassicaformis* is instead pigmented by the carotenoid vaucheriaxanthin (Oborník et al., 2012).

Together with unusual pigmentation of its plastid, *C. velia* has other special characters associated with its photosynthetic activity, which was measured either under continuous low light (15 mol m^{-2} s^{-1}) or high light (200 mol m^{-2} s^{-1}). The alga was able to acclimatize to the particular light conditions; under the low-light regime, it contains more C and N, shows higher chlorophyll *a* quotas and the connectivity of photosystem II is increasing. In contrast, when exposed to the high-light regime, the alga produced more photoprotective carotenoids, violaxanthin and isofucoxanthin. This adjustment was accompanied by a significant decrease of chlorophyll content and consequently led to the increase of the carotenoid:chlorophyll ratio (Quigg et al., 2012). The ability to adjust to different light conditions

makes the photosynthesis of *C. velia* very efficient. It was shown that violaxanthin and isofucoxanthin contribute significantly also to the light-harvesting capacity of *C. velia*. In addition to that, violaxanthin is able to quickly deepoxidize to zeaxanthin and thus effectively protect the cell against high light by nonphotochemical quenching (NPQ), similar, for instance, to diatoms (Coesel et al., 2008). Consequently, cells grown under high light show higher content of violaxanthin than those cultivated under low-light conditions, and NPQ is doubled when compared to less-illuminated cells (Kotabová et al., 2011). When the alga is grown under a light–dark cycle with a sinusoidal shape of light intensity, it is able to notably increase the photosynthetic rates. The changes in illumination in *C. velia* are reflected by stimulation of photorespiration and NPQ. It was also suggested that under sinusoidal illumination in this coral-associated alga, oxygen-consuming processes such as chlororespiration allow high CO_2 assimilation rates (Quigg et al., 2012).

Evolutionary history of light-harvesting complexes (LHC) composed of nuclear-encoded proteins posttranslationally targeted to the plastid where they function, which are involved in the capture of photons and transfer of energy in photosynthesis, was recently investigated in *C. velia*. Phylogenetic analyses by Pan et al. (2012) showed that out of 23 LHC homologues from *C. velia*, 17 formed one large and compact clade, while the rest appeared in four other clades dispersed throughout the tree, all groups containing homologues from algae with secondary plastids. Particularly, LHC from dinoflagellates and diatoms, and fucoxanthin chlorophyll-binding proteins formed a sister group to the major clade of LHC from *C. velia*. Only three LHC proteins from the chromerid alga appeared in close proximity to the red algal homologues (Pan et al., 2012).

Plastid genomes of both chromerids, assembled using 454 sequence data (Janouškovec et al., 2010), contain a truly astonishing number of differences. *C. velia* possesses a highly divergent plastid genome, which is large in size (120 kb), especially when its relatively low number of genes (80 protein-coding genes) is taken into account. These genes are generally highly divergent, AT-rich, and subject to the noncanonical UGA code (Janouškovec et al., 2010; Lang-Unnasch and Aiello, 1999; Moore et al., 2008; Wilson and Williamson, 1997). Chromeran plastid is also known to polyuridylylate its transcripts, as shown by the comparison of genomic sequences of *psbC*, *psbB*, and *psaA* genes with corresponding cDNA sequences. It has been demonstrated by pulse-field gel electrophoresis and hybridization with the *psbA* probe that a substantial fraction of the *C. velia* plastid genome exists

in linear form and contains inverted repeats of *AtpH*, *psbA*, and *ORF1* at both ends of the linear DNA molecule (Janouškovec et al., 2010; Janouškovec et al., submitted for publication). Moreover, it has been shown recently that *psaA* is uniquely split into two genes and consequently two independently formed transcripts and even two proteins (psaA1 and psaA2) are formed in *C. velia* (Janouškovec et al., submitted for publication). In contrast, the second chromerid alga, *V. brassicaformis*, possesses a highly conserved and compact 85 kb-long circular plastid genome, yet it still contains almost the same number of genes (81) as the *C. velia* plastid. Interestingly, many above-described molecular characters, including the non-canonical code, the split *psaA* gene, and the linear form of the plastid genome with inverted repeats at both ends likely resembling telomere-like sequences, are absent from the *Vitrella* organelle. Reassuringly, the sequences of both plastid genomes provide evidence for a common origin of the heterokont, chromerid, and apicomplexan plastids through the homologous structure of their plastid ribosomal operon (Janouškovec et al., 2010).

5.2. Reduced mitochondrial genomes of chromerids

Apicomplexan parasites as well as dinoflagellates are known to possess a tiny mitochondrial genome. If the highly reduced mitochondria-derived organelles such as the mitosomes and hydrogenosomes lacking any genome (Tovar et al., 1999) are not considered, the apicomplexan mitochondrion carries the smallest known genome, encoding only three protein-coding genes (*cox1*, *cox3*, and *cytb*), supplemented by extensively fragmented rRNA genes (Feagin, 1992; Vaidya et al., 1989; Waller and Jackson, 2009). However, based on available data, it appears that *C. velia* has an even smaller mitochondrial genome than *Plasmodium*, with just two protein-coding genes, namely, *cox1* and *cox3* being found so far (P. Flegontov, D.-H. Lai, J. Janouškovec, P. Keeling, A. Pain, M. Oborník, and J. Lukeš, unpublished results).

5.3. Chromerosome: *C. velia* as a possible mixotroph

C. velia also contains a rather mysterious structure in its cell (Fig. 8.15). Originally incorrectly identified as a mitochondrion (Moore et al., 2008), this prominent structure now labeled chromerosome (Oborník et al., 2011) contains solid reinforcement of unknown nature and is terminated by a kind of flexible extension resembling a flagellum. However, electron microscopy

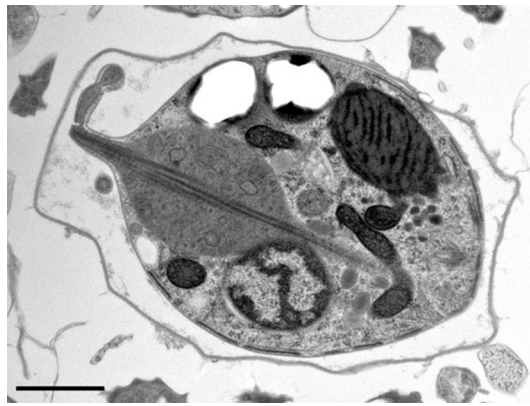

Figure 8.15 Transmission electron microscopy of chromerosome, the unique organelle-like structure in the cell of *C. velia*. Longitudinally sectioned chromerosome with a twisted proboscis and central bundle of fibers. Function of this organelle-like structure remains unknown (scale bar = 1 μm).

also showed that this flagellum-like extension does not contain any microtubular structures. Since it roughly resembles trichocysts of dinoflagellates, we have speculated that chromerosome could be used for hunting algae or other prey, if *Chromera* was a mixotroph (Oborník et al., 2011). On the other hand, no support for this alimentation has been found so far, since *C. velia* grows as a full phototroph on light without any external source of organic carbon.

6. METABOLISM OF CHROMERIDS

6.1. Unique pathway for tetrapyrrole biosynthesis

Tetrapyrroles are cyclic compounds that are, with a single known exception, essential for life (Kořený et al., 2012). Organisms synthesize tetrapyrroles either *de novo* (the most abundant being usually heme and chlorophyll) or have to scavenge them from their diet. The tetrapyrrole pathway is extremely conserved in both prokaryotes and eukaryotes, with the synthesis of the first common precursor, aminolevulinic acid (ALA), representing the only exception from this rule. In α-proteobacteria and all primary heterotrophic eukaryotes, this crucial compound is synthesized by condensation of succinyl-CoA and glycine in the so-called C4 or Shemin pathway in two steps catalyzed by ALA synthase (ALAS) in the mitochondrion. ALA is consequently exported into the cytosol where the next four steps take place usually. Yet in heterotrophic eukaryotes such as animals and fungi,

the last two steps catalyzed by coproporphyrinogen oxidase (CPOX) and ferrochelatase (FeCH) invariably happen back in the mitochondrion (Fig. 8.16; Oborník and Green, 2005). It was proposed that such an arrangement of the pathway reflects a major need of tetrapyrrole products in this organelle of primary heterotrophs (Kořený et al., 2011; Kořený et al., 2013). All other organisms, such as bacteria except α-proteobacteria and eukaryotic phototrophs, synthesize ALA by C5 pathway via NADPH-dependent reduction of glutamyl-$tRNA^{Glu}$ catalyzed by glutamyl-tRNA reductase. Resulting labile glutamate-1-semialdehyde is converted by glutamyl-1,2-semialdehyde aminomutase in a pyroxidal-5-phosphate-dependent reaction into ALA (Beale, 1999).

In eukaryotic phototrophs, the entire tetrapyrrole pathway is located within the plastid, which corresponds to the main requirement for the tetrapyrrole products in this compartment of phototrophic eukaryotes (Fig. 8.16; Kořený et al., 2011; Oborník and Green, 2005). It is well known that the apicomplexans synthesize heme via a noncanonical pathway, which is actually an unprecedented combination of both above-mentioned routes. Hence, ALA is synthesized by the C4 pathway in the mitochondrion and subsequently exported into the apicoplast where the next steps occur. The last two steps, which are localized in the mitochondrion and catalyzed by CPOX and FeCH, terminate the metabolic route (Nagaraj et al., 2009, 2010; Ralph et al., 2004; Sato and Wilson, 2003).

It has been supposed that mitochondrial location of the initial and terminal steps of the heme biosynthesis in the Apicomplexa is a consequence of their heterotrophic, particularly, parasitic lifestyle. However, the tetrapyrrole route in their closest phototrophic relative *C. velia* starts with the synthesis of ALA by the heterotrophic C4 pathway. ALA is then exported to the plastid, where the rest of the pathway takes place. Several of its enzymes are present in multiple versions of apparently different origins, likely the remnants of passed endosymbioses. Therefore, the *C. velia* cell contains three nuclear-encoded plastid-targeted copies of uroporphyrinogen decarboxylase (UROD), each of which originates either from a cyanobacterium, nucleus of the exosymbiont (=secondary host), or nucleus of the endosymbiont (=primary host). CPOX is also present in two copies, one coming from the exosymbiont nucleus while the origin of the other CPOX cannot be specified with certainty. Last but not least, one of the two ferrochelatases is of cyanobacterial origin, while the second homologue was probably acquired by lateral gene transfer from a proteobacterium (Kořený et al., 2011). Interestingly, while the cyanobacterial ferrochelatase was apparently

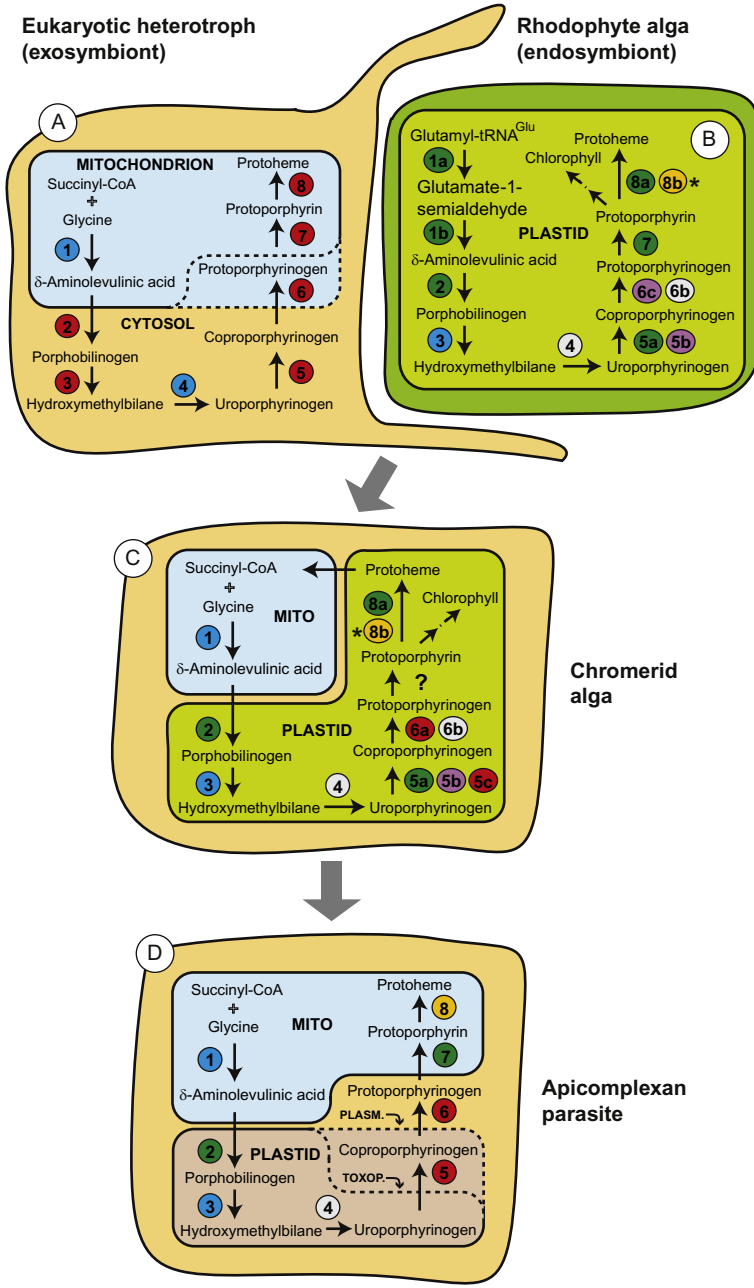

Figure 8.16 Evolution of tetrapyrrole (heme) pathway in chromerids and apicomplexan parasites. Chromerid algae evolved through the endosymbiotic relationship between primarily eukaryotic heterotroph (A) and phototrophic rhodophyte alga (B). Heme (C4) pathway of heterotrophic eukaryotes is localized in mitochondrion and cytosol, respectively (A) while the red algal (C5) pathway is entirely located to the plastid (B).

Continued

lost in the apicomplexan lineage, the proteobacterial homologue was retained and relocated to the apicomplexan mitochondrion (Sato and Wilson, 2003), terminating the pathway as in other primary heterotrophic eukaryotes. We should also note that both ferrochelatases are putatively located within the *C. velia* plastid (Fig. 8.16), as they contain the bipartite targeting presequence necessary to import the enzyme into this complex organelle. Since most phylogenies addressing the relationship between apicomplexans and chromerid algae are burdened by long branches and other possible artifacts, the shared presence of a noncanonical heme pathway by both protist groups represents, in our opinion, the so far strongest evidence for their common origin (Kořený et al., 2011).

Moreover, the fact that *C. velia* produces ALA by the heterotrophic-like C4 pathway makes it the only known photoautotroph synthesizing chlorophyll from glycine and not from glutamate. This fascinating metabolic curiosity was proved by feeding *Chromera* with radiolabeled glycine and glutamate with chlorophyll being made only from glycine (Kořený et al., 2011). How this unique heme pathway evolved is a subject of speculation. However, it was proposed by van Dooren et al. (2012) that the formation of ALA in the mitochondrion of a phototroph was made possible by the emergence of the ALA transporter in the ancestral apicomplexan plastid.

Figure 8.16 —Cont'd The noncanonical pathway of *C. velia* (C) starts, based on predictions, in mitochondrion by synthesis of aminolevulinic acid (ALA) catalyzed by ALAS, the enzyme of the C4 pathway. ALA is then transported to the plastid where the rest of the pathway takes place. In the noncanonical pathway of Apicomplexa (D), shown with depicted differences between *P. falciparum* and *T. gondii*, the last two steps of the pathway (PPOX, FeCH) were relocalized back to the mitochondrion, as it is in eukaryotic heterotrophs. Apicomplexa has also lost two copies of UROD, one copy of CPOX and FeCH, which were multiplied in *C. velia*. Numbers of the individual steps of the synthesis represent the following abbreviated enzymes: 1, ALA synthase (ALAS); 1a, glutamyl-tRNA reductase (GTR); 1b, glutamate-1-semialdehyde-aminomutase (GSA-AT); 2, ALA dehydratase (ALAD); 3, porphobilinogen deaminase (PBGD); 4, uroporphyrinogen synthase (UROS); 5, uroporphyrinogen decarboxylase (UROD); 6, coproporphyrinogen oxidase (CPOX); 7, protoporphyrinogen oxidase (PPOX); 8, ferrochelatase (FeCH). Although in both the rhodophyte and chromerid alga proteobacterial ferrochelatases were found (8*), they appear to be of independent origins. The colors of the enzymatic steps stand for the origin of the genes. Blue, mitochondrial origin; green, plastid origin; red, origin in exosymbiont nucleus; violet, origin in endosymbiont nucleus; yellow, origin in proteobacteria (likely by LGT); white, origin cannot be specified with certainty.

6.2. Other metabolic features of *C. velia*

Two main strategies are usually used to uptake iron by terrestrial microorganisms and plants. By way of reductive uptake mechanism, the extracellular ferric complexes are, via transplasma membrane electron transfer, dissociated by reduction. Afterward, the high-affinity permease system together with copper-dependent oxidase will import free iron. The alternative mechanism uses specific copper-independent receptors that take up siderophores without prior dissociation. In contrast to both canonical mechanisms, *C. velia* uptakes iron in a novel manner (Sutak et al., 2010). It was shown that its transplasma membrane electron transfer system is absent and therefore extracellular ferric chelates cannot be reduced. Hydroxymate siderophores also cannot be used by this alga as a source of iron. A ferrous chelator does not inhibit an uptake from ferric citrate, but a higher concentration of ferric ligand strongly reduces the iron uptake. Moreover, it was demonstrated that the cell wall of *C. velia* contains many receptors of iron, which is concentrated in close proximity of the transport sites. It seems that aqueous ferric ions are first concentrated in the cell wall and subsequently taken up without prior reduction (Sutak et al., 2010).

The most abundant category of lipids associated with the plant and algal plastidial membranes is represented by galactolipids. Their fraction can reach up to 85% of all lipids in these cells (Jouhet et al., 2007). Galactolipids such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are otherwise found exclusively in cyanobacteria, the predecessors of plastids. These compounds are in phototrophic eukaryotes synthesized by galactosyltransferases located in the plastidial membranes (Botté et al., 2011; Jouhet et al., 2007; Joyard et al., 2010). Although no enzyme involved in the synthesis of galactolipids has been found in the apicomplexan genomes (Botté et al., 2005), galactolipid-like compounds displaying similar mobility as the plant MGDG and DGDG were found in the lysates of *T. gondii* and *P. falciparum* (Marechal et al., 2002). Moreover, a DGDG-like lipid was detected in total extracts from these coccidians (Bisanz et al., 2006), and anti-DGDG antibodies were instrumental in the detection of digalactolipids in the pellicle membrane of *T. gondii* (Botté et al., 2008). When studied in *C. velia*, both MGDG-like and DGDG-like galactolipid compounds comigrating with spinach galactolipids were found (Botté et al., 2011). In contrast to *T. gondii*, where the DGDG-like molecules were detected outside the apicoplast (Botté et al., 2008), the same galactolipids were located at multiple membranes of the complex *C. velia* plastid, which is in good

correlation with the situation in plants and some algae. However, the localization of galactolipids was so far not pinpointed to a specific plastidial membrane. This striking difference between chromerids and apicomplexan can be explained by a hypothesis formulated by Botté et al. (2011); since plants are known to relocalize galactolipids during phosphate starvation from the plastid membranes to the extraplastidial ones, apicomplexans may have the capacity to transfer galactolipids from their plastid in a similar way. Galactolipids of *C. velia* are enriched in very long chains of unsaturated fatty acid, with MGDG and DGDG containing noncanonical fatty acid combination C20:5 and C16:0, while the standard combination of plant and algal galactolipids is C18:3 and C16:3. In addition to that, genes coding for the MGDG and DGDG synthases from *C. velia* were sequenced and phylogenetic analysis demonstrated their relation to the diatom counterparts in the frame of the red-derived complex plastids. In particular, the chromerid DGDG synthase branches off very early on the root of the red-derived plastids, while the MGDG synthase forms a sister group to one of the paralogues found in diatoms (Botté et al., 2011). Such a phylogenetic position is compatible with the origin of galactolipid synthesis from the red algal endosymbiont, the ancestor of the chromerid plastid.

Sterols are cyclic lipid molecules required to build cellular membranes and to maintain their integrity. Sterol composition is widely used in chemotaxonomy and in the case of dinoflagellates, sterols can be used even as a biomarker to seek particular species (Giner et al., 2003; Leblond and Chapman, 2002; Leblond and Lasiter, 2012; Mooney et al., 2007). Sterols can be synthesized *de novo*, as is the case in plants, algae, and many protists. However, in the apicomplexan parasites, these membrane compounds are obtained from the host (Coppens et al., 2000; Nishikawa et al., 2005; Sehgal et al., 2005). The sterol composition together with the genes encoding sterol-synthesizing enzymes was recently investigated in *C. velia*. It appears that chromerid sterol repertoire resemble that found in the apicomplexans (Leblond et al., 2012), although the latter group has been shown to rely on import of sterols from their hosts (Nishikawa et al., 2005). Surprisingly, the dinoflagellates synthesize different spectrum of sterols than *C. velia*, although these two groups are actually the closest known phototrophic alveolates. Still, sterols of *C. velia* appear to be more closely related to the sterol compounds of other phototrophs, such as glaucophytes and chlorarachniophytes, suggesting the sterol synthesis in *Chromera* is very ancient, probably ancestral to other groups of algae (Leblond et al., 2012).

7. CHROMERIDS AS POSSIBLE SYMBIONTS OF CORALS

It is well known that dinoflagellates of the genus *Symbiodinium* form symbiotic relationships with corals, and this relation appears to be essential for the survival of coral reefs (Bourne et al., 2009). Such symbiosis is well described and many species of *Symbiodinium* have been isolated so far. In fact, chromerids were initially obtained as a kind of by-product of a study on symbiotic dinoflagellates in Australian stony corals, in particular, *C. velia* from *Plesiastrea versipora* and *V. brassicaformis* from *Leptastrea purpurea* (Moore et al., 2008; Oborník et al., 2012). In order to visualize *C. velia* within corals, a highly specific fluorescence *in situ* hybridization protocol has been recently developed (Morin-Adeline et al., 2012). However, serious doubts have been expressed concerning the putative symbiotic nature of these algae, since a vast majority of chromerids have been detected as an epiphytic algae growing on the surface of corals (Janouškovec et al., 2012a,b).

Consequently, a new group of so far uncultivated alveolates possessing plastids has been found, based on the analysis of environmental samples from corals and their close surroundings (Janouškovec et al., 2012a). The fact that most of these alveolates were detected in depths not exceeding 5 m indicates their possible photosynthetic nature. However, recent histological investigations of larvae of the reef coral *Acropora digifera* and *A. tenuis* by use of *C. velia*-specific probe showed their ability to form a symbiotic relationship with coral larvae (Cumbo et al., 2013). This may suggest *C. velia* as a facultative symbiont of corals, because a substantial fraction of chromerid algae lives epiphytically on the coral surface. The latest analysis of chromerid-related sequences found in the genomic databases identified a novel clade of chromerids, termed ARL-V, which is likely associated with more than a dozen symbiotic corals (Janouškovec et al., 2012b). Indeed, these authors even observed an enrichment for these sequences in healthy coral tissues, a finding with potentially very important environmental consequences. It can be concluded that while some chromerid-related sequences were isolated from marine environments lacking corals, the association of at least some chromerids with coral reefs is stronger than ever.

8. CONCLUSIONS

Chromerid algae, *C. velia* and *V. brassicaformis*, isolated from corals in Australia, are the closest known phototrophic relatives of the apicomplexan

parasites, and their plastids share common origin with the apicoplast. This notion is based not only on accumulating phylogenetic evidence but is further supported by molecular and metabolic synapomorphies shared by the apicomplexans and chromerids, such as the use of alternative code for tryptophan in the plastid-encoded proteins, and the noncanonical arrangement of the heme biosynthetic pathway. Both chromerid species are mutually very different in morphology and life cycle, and also their plastid genomes are substantially distinct. Although chromerids are clearly related to the apicomplexans, they seem to form several independent evolutionary lineages rather than a single sister group, suggesting their fast radiation during the early evolution of the Apicomplexa.

ACKNOWLEDGMENTS

We thank Dr. Robert Moore for kindly sharing several chromerid isolates with us and Dr. Arnab Pain for discussions. This work was supported by Czech Science Foundation, grants no. P501/12/G055 and P506/12/1522, and the project Algatech (CZ.1.05/2.1.00/03.0110) to M. O., the Praemium Academiae Award to J. L., and the award FIC/2010/09 by the King Abdullah University of Science and Technology to M. O. and J. L., who is a fellow of the Canadian Institute for Advanced Research.

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