MINIREVIEW

PRIMARY AND SECONDARY ENDOSYMBIOSIS AND THE ORIGIN OF PLASTIDS¹

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The theory of endosymbiosis describes the origin of plastids from cyanobacterial-like prokaryotes living within eukaryotic host cells. The endosymbionts are much reduced, but morphological, biochemical, and molecular studies provide clear evidence of a prokaryotic ancestry for plastids. There appears to have been a single (primary) endosymbiosis that produced plastids with two bounding membranes, such as those in green algae, plants, red algae, and glaucophytes. A subsequent round of endosymbioses, in which red or green algae were engulfed and retained by eukaryotic hosts, transferred photosynthesis into other eukaryotic lineages. These endosymbiotic plastid acquisitions from eukaryotic algae are referred to as secondary endosymbioses, and the resulting plastids classically have three or four bounding membranes. Secondary endosymbioses have been a potent factor in eukaryotic evolution, producing much of the modern diversity of life.

Key index words: plastids; endosymbiosis; chloroplast; gene transfer

If phycology is the study of algae, what indeed are algae? The Penguin Dictionary of Botany defines algae as an extremely diverse group consisting predominantly of aquatic plants showing relatively little differentiation of tissues and organs as compared with bryophytes and tracheophytes. More importantly, from the perspective of this minireview, the definition goes on to say "algae include both prokaryotic and eukaryotic organisms ranging from unicells through to filamentous forms to parenchymatous seaweeds over 50 metres long." The key here is that algae embrace both prokaryotic and eukaryotic forms. Although cladists may be moan algae as a paraphyletic concept, there is a vital link between those prokaryotic and eukaryotic algae; it is endosymbiosis. Endosymbiosis creates a nexus through all the algae back to the prokaryotic forms. The plastid, the photosynthetic organelle common to all algal groups, is ultimately derived through endosymbiosis of a cyanobacterial-like organism. The plastid thus links our discipline from seaweeds to phytoplankton and all the way back to the first photosynthetic prokaryotes. This minireview examines the more influential contributions to our understanding of endosymbiotic origin of plastids. I explore early light microscopic contributions to our understanding of plastid origins, the subsequent (albeit delayed) contributions of EM and molecular biology to endosymbiotic theory, and the emerging understanding of the special role for secondary endosymbiosis in creating algal diversity.

The first person to write about plastid origins was not, strictly speaking, a phycologist. A. F. W. Schimper was a physiological ecologist; indeed, he launched the discipline and is best remembered for coining the term "rain forest" (*der Regenwald*, in his native German) in his book classifying the globe's vegetation types (Schimper 1903). But Schimper was also interested in plastids, and in an article on the development of different plastid types in plants, he made a passing observation in what is arguably the most important footnote in phycology. Schimper noted that the plastids of plant cells, which divided by binary fission independently of the surrounding plant cell, bore an uncanny resemblance to free-living cyanobacteria (Schimper 1883).

Schimper's insight does not seem to have had much impact, but later articles by Mereschkowsky (1905, 1910) are extraordinary in that they laid out much of the endosymbiosis theory for us. Mereschkowsky (1905, 1910) likened plastids to "little green slaves" working for their host cells to produce food from sunshine. Mereschkowsky waxed lyrical about the secret of endosymbiosis (for a translation into English, see Martin and Kowallik 1999) and was much taken with the philosophical implications of heterotrophy versus autotrophy. Looking back at his contributions, one is awed by what he was able to infer with the limited tools on hand at the time. Although the details he proposed in his reticulated tree of endosymbiosis all may not be correct, he undoubtedly grasped concepts such as multiple endosymbioses to produce different plastid types and also recognized the relationship between oomycetes and heterokont algae-a concept we had to "rediscover" in the late 20th century using approaches such as DNA sequencing and EM.

A testament to the remarkable insight of Mereschkowsky is the paucity of new ideas that emerged in the 50 odd years after his articles. We pick up the story again in 1957 when we are nearing the end of what Taylor (ever an erudite commentator) termed the "eclipse period" of endosymbiosis (Taylor 1987); little intellectual light shone on the origin of plastids in this period prompted Klein and Cronquist (1967) to lament that "this bad penny has been circulating for a long time." Technology, however, came to the rescue

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of endosymbiosis when it was discovered that plastids contained DNA, thereby lending the theory some serious credibility. Stocking and Gifford (1959) made a shrewd choice of alga by labeling plastids of *Spirogyra* with tritiated thymidine. Autoradioraphs of the alga identified DNA in the helical spiral plastids of this green alga (Stocking and Gifford 1959). Subsequently, DNA was identified in various algal and plant plastids and, armed with their newfound genetic credentials, plastids (and mitochondria) assumed new importance on the evolutionary stage. From this point onward the idea took deeper and deeper root in the literature, propelled largely by the persuasive writings of Margulis (1970). For example, the microbiologist Woese (1977) asserted that "the case [for origin of plastids and mitochondria from endosymbiotic eubacteria] is a clear cut one, and it has now been proven." Pace et al. (1986) also stated that plastid origin by endosymbiosis was "beyond reasonable doubt." Gray (1991) went further by saying that "it seems pointless to consider seriously alternative explanations." But leave it to a phycologist to provide the pithiest assessment—"flogging a dead horse" was Taylor's summing up of arguments for the endosymbiotic origin of plastids (Taylor 1987).

Plastid DNA was the key to establishing the endosymbiotic theory, and the rest of this story retells an inexorable ascendancy from fringe hypothesis to a widely accepted theory. Pigott and Carr (1972) were the first to measure the relatedness of plastid and bacterial DNAs with a quantitative hybridization study between Euglena plastid DNA and cyanobacterial DNA. Bonen and Doolittle (1975) constructed T1 rRNA catalogues of rhodoplasts from the red alga Porphyra purpurea. T1 cataloguing was an early technique for comparing genes at the sequence level and preceded the development of nucleotide sequencing. Using this painstaking approach, Bonen and Doolittle (1975) showed that the genes of *Porphyra* plastids were most closely related to genes of cyanobacteria, strongly supporting endosymbiotic origin. Buetow (1976) soon followed up with a similar result using DNA from the plastids of Euglena. These early crude measures of sequence similarity were further substantiated by both protein and RNA or DNA sequencing that demonstrated (beyond reasonable doubt?) that plastids are reduced cyanobacteria (Schwartz and Dayhoff 1978). In time, nucleotide sequencing (either directly from rRNA or from the DNA encoding rRNAs) became increasingly more tractable and quickly overtook protein sequencing, to the point that we now have a tremendous database of plastid and bacterial gene sequences at our disposal. With only one major exception (rbcL; see Boczar et al. 1989, Palmer 1995), the sequence data reaffirm the endosymbiosis concept. Ribulose bisphosphate carboxylase sequences were initially perplexing in that different algal groups were shown to have noncyanobacterial enzymes, but these differences are now rationalized as lateral gene transfers or gene substitution events from mitochondrion to plastid (Palmer 1995, 1996)

The next major milestone in plastid sequencing was the completion of entire plastid genome sequences for algae, and again it was Euglena and Porphyra that pioneered the way (Hallick et al. 1993, Reith and Munholland 1993). The algal plastid genomes were found to contain many of the same genes as plant plastid genomes, although Porphyra plastids proved far more gene rich (Reith and Munholland 1993). Nevertheless, the overall architecture, particularly the arrangement of genes in operons, further supported the endosymbiotic origin of plastids (Reith and Munholland 1993). Complete plastid genomes are now also available for a diatom (Kowallik et al. 1995), a glaucophyte (Stirewalt et al. 1995), a green alga (Wakasugi et al. 1997), a prasinophyte (Lemieux et al. 2000), and a dinoflagellate (Zhang et al. 1999). The latter may not be a complete genome, because each gene is carried on a single DNA minicircle and the number of minicircles is uncertain (Zhang et al. 1999).

The sequencing of DNA gathered momentum, and eventually entire genomes became tractable goals. Although no eukaryotic alga has yet been completely sequenced, a cyanobacterial genome from Synechocystis was recently determined (Kaneko et al. 1996). The cyanobacterial genome contains 3229 genes and gives us a reference point for the gene content of the plastid endosymbiont at the outset of endosymbiosis (Kaneko et al. 1996). Because the plastid genome of Porphyra contains only 200 genes (Reith and Munholland 1993), it is clear that plastid genomes have undergone substantial reduction during endosymbiosis. Many of the missing genes were eventually found to have relocated to the nucleus, but numerous other genes were apparently lost-redundant in their new role as an endosymbiont (Martin and Herrmann 1998, Martin et al. 1998). *Chlamydomonas* is amenable to genetic and cell biological analysis, and much of what we know about gene transfer in algal plastid endosymbiosis comes from studies of Chlamydomonas. Transfer of genes from plastid to nucleus typically requires return passage of the gene product to its place of function. This mechanism is central to the establishment and refinement of endosymbiosis. The first vagrant gene to be studied was the RUBISCO small subunit (rbcS) of Chlamydomonas reinhardtii, and it was established early that a precursor form was produced in the cytosol and targeted. Subsequently, it was established that the precursor bears an N-terminal extension, the so-called transit peptide, which mediates transport. There were several key developments in the understanding of this process. Dobberstein et al. (1977) established that RbcS began as a precursor with a small (3.5 kDa) extension that is proteolytically removed within the plastid before assembly of the holoenzyme. Schmidt et al. (1979) determined the Chlamydomonas RbcS leader sequence and the site of processing. We now know that these transit peptide leaders mediate translocation into plastids using a complex apparatus embedded in the envelope membranes of the plastid. Plant cell biologists have stolen the march on phycologists in studying protein import (Vothknecht and Soll 2000), but the handful of studies on algae suggest that red algae, green algae, and even the glaucophytes use the same basic mechanism (reviewed in Schwartzbach et al. 1998).

The endosymbiotic origin of plastids from cyanobacterial ancestors thus seems confirmed. But a lingering question remains. Do all plastids have a common single origin (i.e. derive from a partnership between one host and one endosymbiont) or were there several endosymbiotic partnerships (perhaps between different hosts and/or different endosymbionts) that spawned the multifarious photosynthetic organelles encountered among the algae? Mereschkowsky (1910) postulated seven independent endosymbioses that gave rise to the dinoflagellates, heterokont algae, red algae, diatoms, brown algae, green algae and plants, and the phycomycetes. The endosymbionts are depicted as deriving from separate parts of the cyanobacterial radiation, and each entered into independent associations with a different lineage of nucleated cells producing a "polyphyletic plant world" (Mereschkowsky 1910). Although Mereschkowsky (1910) did not specify particular cyanobacterial lineages for each endosymbiosis, he proposed that at least three typesred, brown, and green-were involved. Mereschkowsky's inclusion of an endosymbiosis for phycomycetes is particularly insightful. Recognizing that this group (which includes the water molds and downy mildews or oomycetes and related organisms) was more closely related to algae than fungi, he invoked a symbiosis and subsequent loss of pigmentation to explain their origin.

Pigments have a central role in algal systematics, and it was established early that phycobilins in eukaryotic algae like *Porphyridium* were homologues of those in cyanobacteria (Gantt and Conti 1966a,b). By 1970, the underlying biochemical differences between the different colored algal groups was better understood, and this prompted Raven (1970) to streamline Mereschkowsky's scheme to a minimum of three separate endosymbioses. (Raven's scheme also allows for as many as nine independent plastid origins depending on interpretation.) Raven's (1970) proposed endosymbioses involved three types of photosynthetic prokaryotes: 1) regular cyanobacteria with chl a and phycobilins, which gave rise to red algae, cryptophytes, and Cyanidium (an anomalous red alga); 2) "green prokaryotes" possessed of chl b, from which derived the plastids of euglenoids, green algae, and their descendants, the plants; and 3) "yellow prokaryotes" with chl c, from which derived the plastids of brown algae, dinoflagellates, xanthophytes, and golden flagellates. The green prokaryotes and yellow prokaryotes were hypothetical organisms, presumed not to exist any longer as freeliving organisms.

Part of the satisfaction of science is the fulfillment of predictive aspects of a hypothesis. Hence, the discovery of a green prokaryote by Lewin and Withers (1975) generated considerable excitement because it provided a possible free-living relative of the plant plastid. Lewin created a new division, Prochlorophyta, for these organisms and orchestrated intensive biochemical, structural, and molecular analyses. Eventually, it emerged that chl *b* is widespread in the cyanobacteria, and phylogenetic trees suggest that the socalled prochlorophytes are not direct predecessors of plastids (Turner et al. 1999). Current pigment cohorts may not be indicative of immediate ancestry. The spectrum of pigments in extant algae may simply derive from differential losses (as first argued by Bryant 1992) because cyanobacteria/prochlorophytes have now been demonstrated to possess chl *a*, *b*, and perhaps *c*, as well as phycobilins (Larkum et al. 1994, Hess et al. 1996, Tomitani et al. 1999).

Exactly which prokaryotic lineage gave rise to plastids remains unclear. Considerable effort has been devoted to sequencing genes from extant cyanobacteria. Phylogenetic trees constructed from these genes clearly demonstrate that cyanobacteria and plastids share a common ancestor and the plastids, of every kind, unite as a monophyletic assemblage, but no particular extant cyanobacterial lineage can be clearly allied with the plastids (Turner et al. 1999). In the face of this uncertainty, an alternate approach to divining the number of plastid origins has been pursued. Plastid genes, like bacterial genes, are organized in units known as operons. By comparing the operons of plastid genomes with those of bacteria, including cyanobacteria, it is possible to determine ancestral arrangements of particular gene sets (Reith and Munholland 1995, Douglas and Penny 1999, Stoebe and Kowallik 1999). These comparisons reinforce the concept that plastids derive from cyanobacteria because many operon structures are conserved. More importantly, all plastids examined to date possess a number of unique gene arrangements not observed in extant cyanobacteria. They are believed to have been assembled after the establishment of endosymbiosis, thereby suggesting that all plastids with these arrangements derive from a common primary endosymbiosis (Reith and Munholland 1995, Douglas and Penny 1999, Stoebe and Kowallik 1999). It remains possible that these unique operon structures are not derived but primitive. Similar arrangements may occur in extant (or now extinct) cyanobacteria, and multiple endosymbioses from this stock could have produced the current structure of plastid genomes, but this scenario seems unlikely in my opinion.

A second line of evidence also suggests a single origin for all plastids. As mentioned above, when plastid protein genes are relocated to the nucleus, a mechanism to return the gene product to the organelle from the cytoplasm is required. We know very little about this process in algae, but several studies have demonstrated that the N-terminal leader components (transit peptides) of targeted algal proteins are recognized by the plant import apparatus and imported (Apt et al. 1993, Jakowitsch et al. 1996, Lang et al. 1998, Wastl and Maier 2000). This interchangeability of targeting motifs suggests a common evolutionary origin for the import machinery and hence a common origin for the plastids. Moreover, the residual genes in modern plastid genomes are consistent with a common primary origin (Martin et al. 1998). Finally, gene phylogenies (either from nuclear, mitochondrial, or plastid genes) now suggest that red algae and green algae/plants are sister taxa, which is consistent with a shared acquisition of the plastid (Gray et al. 1999, Moreira et al. 2000). Thus, the molecular data favor the early arguments of Cavalier-Smith (1982) and Bryant (1992) for a single primary endosymbiosis ultimately producing all plastids.

Although much of our understanding of endosymbiosis has come from biochemistry and molecular biology, important insights have come from studies of fossils, both living and dead. The connection tracing plastids back to cyanobacteria was revealed to be an extremely long one. Two papers in 1980 identified cyanobacterial-like fossils that were as much as 3.8 billion years old (Lowe 1980, Walter et al. 1980). Indeed, these are the oldest known evidence of life on our planet. Another key "fossil" is an enigmatic little flagellate known as Cyanophora paradoxa. First described by Korshikov in 1924, C. paradoxa is now recognized as the "coelocanth" of endosymbiosis. Cyanophora paradoxa belongs to a group known as the glaucophytes, which are unusual in having plastids with a strong morphological resemblance to cyanobacteria (Hall and Klaus 1963). The glaucophyte plastids (referred to as cyanelles) have chl a and phycobilin and a sturdy peptidoglycan (murein) wall of similar composition to the walls of gram-negative bacteria like cyanobacteria (Pfanzagl et al. 1996). Nevertheless, molecular biology demonstrates that cyanelles are indeed a "missing link" between plastids and cyanobacteria. Cyanelle genomes are plastid-like (Stirewalt et al. 1995), but their pigments, their membranes, and their walls are very cyanobacterial.

Two other extant organisms, cryptomonads and chlorarachniophytes, have also proved to be missing links in the endosymbiotic story. These two algae are our best lines of evidence for the extraordinary second chapter of the endosymbiosis (Fig. 1). Plastids of red algae, green algae, and glaucophytes have two bounding membranes. These plastids originated by the kind of endosymbiosis outlined above, and we usually refer to this as the primary endosymbiosis. However, plastids of many algae have three or four membranes, and their origins are slightly more complex. Secondary endosymbiosis rationalizes these extra membranes by postulating engulfment of a primary alga (a red, green, or glaucophyte alga with a double plastid membrane) by a heterotrophic eukaryote. Tomas and Cox (1973) and Lee (1977) first mooted the idea that these multimembraned, or complex, chloroplasts were acquired indirectly and not by the classic (primary) mechanism of endosymbiosis of a cyanobacterial-like prokaryote. However, their models were probably inaccurate and focused on inappropriate examples. Gibbs (1978), however, seems to have hit the nail right on the head when she proposed that the Euglena chloroplast derived from a eukaryotic (green) alga that had been engulfed by an eukaryotic phagotroph. The engulfed cell underwent drastic reduction such that in most cases the only residues are the chloroplast and the extra membranes created by the engulfment (Fig. 1).

Acquisition of chloroplasts in this secondary manner occurred multiple times (the number of acquisitions is argued hotly) and thus explains the patchy distribution of chloroplasts across the eukaryotic tree and the fragmentary nature of algae as a concept. Secondary endosymbiosis was first verified in cryptomonads where a minute residue of the endosymbiont nucleus was identified. Greenwood (1974) dubbed this little structure the nucleomorph. Gibbs and colleagues (Ludwig and Gibbs 1985) studied its morphology and ultrastructure and, along with Sitte's group (Hansmann et al. 1987), demonstrated the presence of DNA within this miniature nucleus. The nucleomorph was interpreted as representing a vestige of the endosymbiont's nucleus, and indeed, sequencing of nucleomorph genes revealed that they share a common ancestry with the red algal nucleus (Douglas et al. 1991). Recently, the entire nucleomorph DNA sequence was completed, and the data demonstrate that cryptomonads represent an intermediate stage in the development of a secondary plastid acquisition (Douglas et al. 2001). The cryptomonad nucleomorph encodes a mere 511 genes, 30 of which are for proteins required by the plastid (Douglas et al. 2001). The remainder, which comprise so-called housekeeping or genetic apparatus components, are apparently necessary for the perpetuation and production of the 30 plastid proteins (Douglas et al. 2001). The nucleomorph then is

FIG. 1. Schematic showing the origin and distribution of plastids through primary and secondary endosymbiosis. There appears to have been a single primary endosymbiosis in which a photosynthetic cyanobacterial-like prokaryote was engulfed and retained by a eukaryotic phagotroph. This event spawned three extant lineages: red algae, green algae/plants, and glaucophytes. These primary plastids are bounded by two envelope membranes. At least three secondary endosymbioses, in which a eukaryotic alga is engulfed and retained by eukaryotic phagotroph producing plastids with either three or four membranes, are known. A green alga was engulfed to produce chlorarachniophytes. Vestiges of the green algal nucleus persist in the form of a nucleomorph. Another green alga was probably engulfed by a different host to produce euglenoid algae. At least one other secondary endosymbiosis, this time involving the engulfment of a red algal-like endosymbiont, occurred. This event led to the cryptophytes, which still retain a remnant of the red algal nucleus, namely the nucleomorph. Some interpretations (see text) suggest that this secondary endosymbiotic event was the origin of heterokonts, haptophytes, dinoflagellates, and apicomplexan plastids. Alternative scenarios propose several separate secondary endosymbiotic events, each involving a red algal-like endosymbiont.



a relic whose sole task appears to be the coding of a handful of essential proteins required for the ongoing maintenance of the plastid.

In other algae with secondary endosymbiotic plastids (sometimes referred to as complex plastids), the nucleomorph is thought to have disappeared after transfer of all the necessary plastid protein genes to the host nucleus (Fig. 1). Evidence for such nucleomorph-to-host-nucleus gene transfer exists (Deane et al. 2000). In addition to being a frozen snapshot of secondary endosymbiosis, nucleomorphs are also of interest as simple examples of the nucleocytoplasmic system. Because nucleomorphs harbor an almost complete set of machinery for the copying, distribution, and expression of genes, they are a model for the eukaryotic genome (Douglas et al. 2001).

The second missing link in secondary endosymbiosis is the chlorarachniophytes. First described by Geitler (1930), the significance of these organisms was not understood until ultrastructural examination by Hibberd and Norris (1984) revealed that they also possessed a nucleomorph. However, unlike the cryptomonads, which contain a red-algal like symbiont, chlorarachniophytes have a green algal symbiont (Hibberd and Norris 1984, Gilson and McFadden 1996, Ishida et al. 1997, McFadden et al. 1994, Van de Peer et al. 1996). The chlorarachniophyte nucleomorph is superficially similar to the cryptomonad nucleomorph (each contains three tiny linear chromosomes), but analysis of the genes clearly demonstrates a separate origin (Gilson and McFadden 1995, 1996, Douglas et al. 2001). These two extant intermediates demonstrate unequivocally that secondary endosymbiosis occurred, and at least twice. Secondary endosymbiosis is also proposed for haptophytes, heterokonts (stramenopiles), dinoflagellates, and apicomplexa (Delwiche 1999, Cavalier-Smith 2000), all of which are assumed to have lost the nucleomorph through transfer of essential genes into the host nucleus. This second set of endosymbiotic plastid acquisitions has thus been an immensely important evolutionary driver, creating a large component of algal diversity.

Exactly how many secondary endosymbioses occurred is debatable (Fig. 1). Initially, the lack of any obvious relationships (either molecular or morphological) led many to the conclusion that most of these algal groups derived from separate secondary endosymbioses (Bhattacharya and Medlin 1995, Delwiche and Palmer 1997). Conversely, Cavalier-Smith (1999) argued on the basis of similarities in the protein import mechanisms that cryptomonads, heterokonts, haptophytes, dinoflagellates (peridinin-containing plastids), and apicomplexa all share a common secondary origin of plastids from a red algal endosymbiont (Fig. 1). Indeed, recent evidence based on analysis of glyceraldehyde-3-phosphate dehydrogenase genes supports the notion that all these organisms share a common plastid origin (Fast et al. 2001). If this turns out to be true, it revolutionizes our understanding of algal evolution and poses some intriguing questions about organisms such as ciliates, which by this reasoning would be secondarily lacking in plastids—assuming they do indeed lack a plastid. It is also very unclear whether the nonphotosynthetic members of lineages such as the Euglenozoa, stramenopiles, and dinoflagellates are primitively or secondarily lacking in plastids. These are important questions, particularly because many of these organisms are pathogens or environmentally important. Indeed, the protist responsible for malaria, *Plasmodium* spp., is now known to harbor a relict plastid (McFadden et al. 1996, Wilson et al. 1996, Köhler et al. 1997).

The better known examples of primary and secondary endosymbiosis, which encompasses most of the widely recognized algal species, are increasingly well understood. There are, however, a number of plastid endosymbioses about which we know relatively little. These plastid acquisitions probably fall into the category of "works in progress." The first example is perhaps an independent primary endosymbiosis. Lauterborn (1895) described a filose amoeba with blue green (cyanobacterium-like) endosymbionts that he dubbed Paulinella chromatophora. The endosymbionts resemble cyanelles of glaucophytes. Sequencing of the host rRNA confirms that Paulinella is a filose amoeba but revealed that it is allied to the lineage in which chlorarachniophytes belong (Bhattacharya et al. 1995). Here we have a curious situation. The cyanelle-like endosymbionts of Paulinella may be an independent primary endosymbiosis, yet this occurs in a lineage that has also availed itself of plastids through secondary endosymbiosis (chlorarachniophytes). Details of the molecular biology and biochemistry of the Paulinella endosymbionts are eagerly awaited.

Other unusual plastid acquisitions occur in dinoflagellates. The standard dinoflagellate plastid, which contains peridinin pigment, apparently originates from a secondary red algal endosymbiont (Durnford et al. 1999, Zhang et al. 1999, Fast et al. 2001), but several dinoflagellates have anomalous plastids with different pigments. Molecular and biochemical analyses of these plastids suggest they derive from various endosymbionts, including green algae, diatoms, cryptophytes, and haptophytes (reviewed in Delwiche 1999, Tengs et al. 2000). These acquisitions appear to have replaced the peridinin plastid and are referred to as tertiary symbioses. However, in the case of Lepidodinium viride, which likely contains a green alga (Watanabe et al. 1990), we should probably refer to it as a serial secondary endosymbiosis because the endosymbiont likely harbors a primary plastid. We know little about these organisms. Some may retain two plastids, and their nuclei could potentially harbor a mixture of genes donated by different endosymbionts.

Another type of endosymbiosis in progress is the theft of plastids by animals. Many invertebrates harbor algae for their ability to synthesize food from sunshine, but some take only the parts they need—the plastid. Trench initiated studies of mollusks that he dubbed "crawling leaves." These sacoglossan slugs graze on algae and sequester the plastids into their cells (Trench 1969, Trench et al. 1969, Trench and Smith 1970). Astonishingly, these plastids remain photosynthetically active, at rates similar to those observed in the intact alga, for up to 9 months (Green et al. 2000) The slugs are clearly well adapted to this lifestyle and select particular species of algae from which to attain plastids. Moreover, the slugs appear to synthesize proteins for the plastids (Mujer et al. 1996, Pierce et al. 1996, Green et al. 2000, Hanten and Pierce 2001). These interactions hint at a very specific relationship between slug and plastid, but as yet there is no direct evidence of the slug nucleus having acquired algal genes for plastid proteins (Mujer et al. 1996, Green et al. 2000). Hence, it is an open question as to whether these borrowed plastids are true endosymbiotic organelles-an accepted definition of endosymbiotic organelle is the transfer of genetic material from symbiont to host (Cavalier-Smith and Lee 1985).

Whither endosymbiosis research? On balance it appears that all plastids probably derive from a single primary endosymbiosis. So why was this one interaction so spectacularly successful when evolution has left no trace (with the possible exception of Paulinella) of any other primary endosymbioses? Clearly, a better understanding of secondary endosymbioses is also a major frontier. Exactly how many secondary endosymbioses occurred? What symbionts were involved? How did the integration of symbiont proceed? Other important goals are to unravel further the mechanisms of plastid integration, both in primary and secondary endosymbiosis. How did intracellular gene transfer occur and why? Why are some genes still resident in the plastid? Why do some organisms retain colorless plastids when they abandon autotrophy?

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