From Endosymbiosis to Synthetic Photosynthetic Life[1]

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The chloroplasts of photosynthetic eukaryotes arose more than 1.6 billion years ago (Yoon et al., 2004) through the process of primary endosymbiosis, in which a cyanobacterium became permanently integrated into a heterotrophic mitochondriate eukaryote (Reyes-Prieto et al., 2007). Through subsequent secondary and tertiary endosymbioses (i.e. additional nested endosymbioses between plastid-bearing eukaryotes), plastids spread out into a vast array of photosynthetic and nonphotosynthetic organisms, the former contributing a large share of the global primary productivity and the latter encompassing a huge diversity of organisms ranging from the protists that cause malaria to parasitic plants. Thus, the plastids in nearly all present-day plastid-bearing organisms descended from a single primary endosymbiosis (Gould et al., 2008; Archibald, 2009; Keeling, 2010).

Considering that endosymbiotic relationships between bacteria and eukaryotes are common in nature (Nowack and Melkonian, 2010), it is remarkable that only one such primary association between cyanobacterium and host apparently took hold in ancient evolutionary history to produce a bona fide chloroplast and spawn the diversity of plastid-bearing organisms now extant on earth. Evidence for more recent endosymbioses leading toward the evolution of new primary plastids (Prechtl et al., 2004; Gould et al., 2008; Nowack et al., 2008; Archibald, 2009) indicate that the establishment of more such associations is possible, but the evolutionary transition from cyanobiont to organelle is extraordinarily rare. In contrast, secondary and tertiary endosymbioses yielding new types of plastid-bearing organisms have occurred multiple times (Gould et al., 2008; Keeling, 2010). This raises the intriguing question: What does it take to establish a permanent and functional plastid in a foreign host? As many aspects of plastid research are directly or indirectly rooted in the endosymbiotic origin of these organelles, continued pursuit of this question from various perspectives holds potential for illuminating a great deal about how plastid function is integrated with that of the host organism, which is still poorly understood. Here we highlight recent findings bearing on this question and consider novel approaches that could help provide answers.

The requirements for evolving a plastid in a host cell have been discussed in many reviews (e.g. Bhattacharya et al., 2007; Gould et al., 2008). Photosynthesis probably catalyzed evolution of the first plastids by providing reduced carbon to the host cell, thereby reducing or eliminating the need for feeding (Weber et al., 2006; Nowack and Melkonian, 2010). A stunning example of the significance of photosynthesis in this context is the kleptoplastic association between the sea slug *Elysia chlorotica* and the heterokont alga *Vaucheria litorea* (Rumpho et al., 2006). *E. chlorotica* sucks chloroplasts out of the algal cells, harbors them in the cytosol of cells lining the digestive track (Kawaguti and Yamasu, 1965; Taylor, 1967), and completes its life cycle photoautotrophically, with no additional ingestion of organic food sources. In fact, though this cannot be considered a permanent association because the plastids cannot divide or be transmitted to the next generation, the captured chloroplasts are maintained in a photosynthetically active state for up to 14 months. Since many components of the photosynthetic machinery have high turnover rates and must be frequently replaced with newly synthesized proteins, several potential explanations for kleptoplast longevity in the slug were considered, including the possibility that the algal plastid genome contains all the required genes (Green et al., 2000; Rumpho et al., 2000). However, while it was found that kleptoplast genes are expressed for at least 8 months in starved slugs (Mujer et al., 1996), sequence analysis showed that at least one gene required for photosynthetic electron transport was missing from the algal plastid genome and that the corresponding protein must therefore be provided by the slug (Rumpho et al., 2008). Indeed, a number of genes required for photosynthesis reside in the sea slug nucleus and were acquired from the alga by horizontal gene transfer (Pierce et al., 2007; Rumpho et al., 2009; Schwartz et al., 2010). Presumably the encoded proteins are targeted to the kleptoplast via the plastid import machinery, though this remains to be shown experimentally. With recent progress in genome sequencing technology, in the near future comparison of the *E. chlorotica* and *V. litorea* nuclear genomes should make it relatively straightforward to identify the full complement of algal genes transferred to the slug that may be critical

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for maintenance of kleptoplast activity. A recent transcriptome analysis of a dinoflagellate that also harbors kleptoplasts revealed five nuclear genes of algal origin that may contribute to kleptoplast longevity in that organism (Wisecaver and Hackett, 2010). In addition, comparison between the genomes of *E. chlorotica* and a related species incapable of hosting plastids for more than a few days (Rumpho et al., 2006) should reveal animal-specific candidate genes potentially important for kleptoplasty. This fascinating system thus promises to yield new insights into endosymbiont-organelle transitions.

The existence of nonphotosynthetic plastids in many organisms indicates that photosynthesis is not the only cyanobiont function contributing to the establishment or retention of organelles. Nitrogen fixation has been implicated as a driver of organelle evolution in the diatom *Rhopalodia gibba*, which contains nitrogen-fixing spheroid bodies derived from cyanobacteria that have lost the capacity for photosynthesis (Kneip et al., 2007; Gould et al., 2008; Nowack and Melkonian, 2010). The secondary plastids of the malaria parasite *Plasmodium falciparum* and other Apicomplexan protists retain just a remnant of the red algal plastid genome from which they probably descended (Janouskovec et al., 2010), yet these apicoplasts are essential for completion of the parasite life cycle. The reason for this is not yet clear, but may be because apicoplasts still possess predicted nuclear-encoded pathways for several critical plastid-associated metabolic processes, including fatty acid, isoprenoid, heme, and iron-sulfur cluster biosynthesis (Lim and McFadden, 2010).

Another prerequisite for the evolution and integration of plastids appears to be massive transfer to the nucleus and loss of cyanobiont genes. This process may be important for bringing the symbiont under control of the host, reducing the buildup of deleterious mutations in the symbiont genome, and reducing genetic redundancy associated with the coexistence of two genomes (Martin et al., 1998; Bock and Timmis, 2008). In fact, the extent to which gene transfer and loss from the cyanobiont have occurred may be an important factor in distinguishing symbiont from organelle, the lines between which are not always clear (Archibald, 2009). Symbiont integration further requires that critical genes transferred to the nucleus acquire eukaryotic promoters and terminators and targeting signals that enable translocation of the gene products back into the plastid via the protein import machinery (Martin, 2003; Bock and Timmis, 2008). However, although comparative genomics has clearly established the extent of gene transfer and loss in a variety of plastid-bearing organisms (Bock, 2010; Keeling, 2010), the processes by which gene transfer occurs are still poorly understood (Martin, 2003). In a series of elegant experiments, the Timmis and Bock laboratories monitored the transfer of an antibiotic resistance marker from transplastomic tobacco (*Nicotiana tabacum*) plastids to the tobacco nuclear genome to qualitatively and quantitatively examine the process of endosymbiotic gene transfer to the nucleus. It was shown that gene transfer is DNA mediated and that functional nuclear genes arise through rearrangements at the insertion site, leading to the capture of promoter sequences and 3′-untranslated regions stabilizing the transcripts (Huang et al., 2003; Stegemann et al., 2003; Stegemann and Bock, 2006). These studies constitute a foundation for understanding the mechanisms and biological consequences of endosymbiotic gene transfer.

Plastid origin through endosymbiosis did not only involve gene transfer from cyanobiont to host nuclear genome. In the case of secondary and higher-order endosymbioses in which plastids have been passed from one eukaryote to another, horizontal gene transfers between eukaryotic nuclei, including nuclear genes required for plastid functions, played a major role (Keeling, 2010). Such gene transfer events could also be detected between transgenic plant lines in tissue-grafting experiments (Stegemann and Bock, 2009), indicating that it is possible to study the mechanisms of horizontal gene transfer in laboratory experiments.

Merger between cyanobiont and host also requires the integration and coordination of the host and symbiont cell cycles. That is, host and cyanobiont must divide in a coordinate fashion, particularly in unicellular organisms in which the product of the merger is a single chloroplast—otherwise the photosynthetic organelle would be inherited by only one of the daughter cells and lost from the other, as occurs in the protist *Hatena arenicola* (Okamoto and Inouye, 2005, 2006). This coordination has apparently been achieved in part by combining components of the cell division apparatus present in the cyanobacterial ancestor of chloroplasts with components of host origin. The most widely conserved among these are the enodsymbiont-derived FtsZ and host-derived dynamin proteins (Osteryoung and Nunnari, 2003; Miyagishima et al., 2008; Yang et al., 2008). These nuclear-encoded, ring-forming contractile components of the plastid division apparatus function inside and outside the plastid, respectively, to constrict the organelle membranes. Both proteins appear to be present and are probably required for plastid division in all organisms with primary plastids. In contrast, the vestigial nonphotosynthetic plastids of apicomplexan parasites no longer use FtsZ for division, but still require a dynamin protein (though of distinct origin) for plastid fission (van Dooren et al., 2009). Thus dynamins may be universally required for division of plastids whereas FtsZ may be dispensable in some plastid types, perhaps in organisms in which photosynthetic capacity has been lost (Schmitz et al., 2009).

In addition to the cell-biological and genetic prerequisites, the metabolic networks and exchange of metabolites between host and cyanobiont have to become coordinated. Phylogenomic analyses indicate that the host tapped into cyanobiont photosynthates very early in the evolution of primary plastids (Weber et al., 2006;

Tyra et al., 2007). Presumably, the cyanobiont lost its ability to synthesize and store starch concomitantly with endosymbiosis, allowing reduced carbon in the form of ADP-Glc, the precursor for starch synthesis, to be withdrawn from the symbiont without interfering with its central carbon metabolism (Deschamps et al., 2008). Carbon export from the symbiont was apparently established by routing of a host-derived endomembrane sugar-nucleotide transporter to the cyanobiont membrane. The phosphate translocators of extant plastids evolved from these transporters shortly thereafter, enabling feedback control of metabolism through metabolite antiport (Weber et al., 2006; Bhattacharya et al., 2007). Additional third-party metabolite transporters may have come from intracellular parasites, such as *Chlamydia*, which might also have resided in the host cell during the original endosymbiotic association that gave rise to chloroplasts (Linka et al., 2003; Moustafa et al., 2008).

Since even animals are able to acquire and maintain plastids in a functional form, at least for several months, it is tantalizing to contemplate the possibility of recapitulating this process in the laboratory. Though this might at first glance seem an esoteric endeavor, the intellectual and scientific rewards of such an undertaking are potentially significant. Here we briefly consider two possible approaches for studying the requirements for plastid retention in a nonplant host—one based on the built-in biology of the *E. chlorotica*-*V. litorea* symbiosis and the other using synthetic biology to transfer plastids from a plant or alga and maintain them in a nonplant organism.

E. chlorotica already has the genetic capacity to maintain *V. litorea* chloroplasts in a photosynthetically active state for many months. If it were possible to culture and transform the plastid-bearing digestive tract cells, a plethora of novel experiments on the requirements for plastid maintenance in the slug would become possible. For example, RNAi might be employed to knock down expression of particular slug genes required for photosynthesis or other chloroplast functions (Pierce et al., 2007; Rumpho et al., 2008, 2009; Schwartz et al., 2010), or animal-specific genes implicated in plastid longevity. Other questions could be addressed as well; for example, it might be possible to introduce specific cDNAs or transgenes into the slug nuclear genome, such as those encoding FtsZ or dynamin, to understand what prevents plastid division in the slug and to engineer the process through step-by-step addition of selected genes. This system could thus constitute a tool for functional study of the organellar division machinery through gain-of-function approaches. This concept could be extended to the study of plastid-nucleus signaling, establishment of metabolic connections, interorganelle interactions, etc., thereby opening novel avenues for the testing of hypotheses derived from phylogenomics and systems approaches.

Plastids from other organisms might also be introduced into cultured *E. chlorotica* cells, either by phagocytosis, the normal route for uptake of *V. litorea* plastids into slug cells (Rumpho et al., 2006), or perhaps by microinjection. Since transplastomic tobacco plants can be readily generated, such as those used in the studies from the Bock and Timmis groups described above, the possibility would exist of introducing tobacco plastids expressing specific genes of interest into the slug cells. Similar experiments could be carried out in plastidless digestive tract slug cells if the latter could also be cultured. Efforts to develop such culture systems from *E. chlorotica* could thus pay dividends for studies of organelle integration and plastid-host coordination.

Perhaps more challenging is the idea of transferring plastids to a non-plastid-bearing host. Such a feat was attempted by several investigators 30 to 40 years ago (Rumpho et al., 2006), but developments in model systems and biotechnology make this potentially more feasible now. What does it take to begin charting this territory? First, a suitable host is needed. Yeast (*Saccharomyces cerevisiae*) would be an obvious first choice because of the availability of yeast artificial chromosomes that can carry the large amounts of genetic information that might be required for establishing a functional plastid in a foreign host. Since most proteins needed for plastid function are not encoded by the organelle, transferring large numbers of plant or algal genes to the synthetic host, many of which would encode plastid-targeted proteins, would no doubt be required to provide the plastid in its novel environment with the proteins it needs to survive and function. The recent cloning of several natural and synthetic *Mycoplasma* genomes in yeast demonstrates it is possible to insert and propagate very large pieces of foreign DNA in this organism (Gibson et al., 2008, 2010). Though the cloned bacterial genomes are small (up to 1.1 Mb), it will likely become possible in the near future to synthesize larger genetic units and transfer them into eukaryotic cells. The ability to perform targeted knockouts and gene replacements is also an advantage of yeast; for instance, it may be necessary to inactivate specific host genes to accommodate the plastid.

Selection of the right plastid type would also be a key requirement. Initial efforts might start with the transfer of nonphotosynthetic plastids, for example from a parasitic plant, because they would presumably be functionally less complex (Krause, 2008) and thus require the transfer of fewer plant genes to the synthetic host. Alternatively, one could develop a blueprint for plastid transplantation into yeast using *V. litorea* chloroplasts since they are predisposed for life in Opisthokonta, which include animals and fungi (Steenkamp et al., 2006). In the latter case, *E. chlorotica* genes potentially required for chloroplast viability and maintenance in yeast would be indicated by comparative genomic analyses described above. Starting out with solving basic issues, such as reliable import of host-encoded proteins into the transplanted plastid, effective organelle division in the new host to ensure

plastid propagation, and establishment of metabolic connections between plastid and host cells, by adding on additional plastid functions in a stepwise process it may eventually become possible to generate a fully functional photosynthetic plastid in yeast, though clearly many issues would need to be considered to accomplish this feat.

Beyond being a novel tool for addressing fundamental questions related to plastid origin and function, synthetic plastids have huge potential in biotechnological applications, such as the production of biofuels and high-value biomaterials. The advantage of a synthetic plastid-bearing organism is based on its construction principle: Ideally, the chassis used as a plastid acceptor permits easy genetic modification, such as multiple gene additions and knockouts. In this way, the synthetic host can be engineered to take full advantage of organic carbon backbones provided by photosynthesis through the plastid. Depending on the biotechnological goal, however, such systems don't have to be photosynthetic. Adding a nonphotosynthetic plastid, e.g. to yeast as described above, would provide an additional reaction compartment with unique properties, such as the capacity for fatty acid or terpene synthesis, that is not easily achieved in the host cell. In addition, since the synthetic plastid would harbor its own genome, if it were transformable it could be used as a reservoir for incorporation of additional genes of biotechnological relevance (Bock, 2007).

Clearly, transfer of the landmark organelle of photosynthetic organisms into plastid-free receptor cells presents major intellectual and technological challenges that will not be easily overcome. Conquering them will require a solid understanding of the primary and higher-order origins of plastids through endosymbiotic events. At the same time, synthetic approaches could drive novel discoveries and make it possible to test hypotheses derived from evolutionary studies, thereby providing deep insights into the evolution of organelles in particular and of eukaryotic life in general.

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