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ARIADNE'S THREAD: GUIDING A PROTEIN ACROSS FIVE MEMBRANES IN CRYPTOPHYTES[1]

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Cryptophytes are the most archetypal chromalveolates, with their complex plastid having retained many features of the red algal secondary endosymbiont. Most important of these is the remnant nucleus, the nucleomorph, that is kept between the inner and outer membrane pair of the endosymbiont in the highly reduced cytosol, the periplastidial compartment (PPC). Because the nucleomorph's coding capacity is very limited, proteins need to be imported from the host cytosol across the outer two membranes into the PPC and across all four membranes into the stroma. How this is accomplished has puzzled researchers for >20 years. Recent findings show that in both cases, a bipartite topogenic signal, a signal and subsequent transit peptide (TP), is responsible for targeting proteins correctly into these two compartments. An aromatic amino acid–based motif at the +1 position of the TP holds the information determining into which compartment the precursor protein is finally transported. Together with the identification of a novel endoplasmic reticulum associated degradation (ERAD)–derived translocon in the second-outermost membrane, these findings help us to understand the sophisticated targeting mechanisms across four membranes and clarify a key innovation during chromalveolate evolution.

Key index words: **chromalveolate; cryptophyte; endosymbiosis; nucleomorph; periplastidial compartment; stramenopile**

Abbreviations: **BIP, binding to immunoglobulin precursors; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum associated degradation; GFP, green fluorescent protein; PPC, periplastidial compartment; TP, transit peptide; UGGt, UDP-glucose-glycogen glucosyltransferase**

The enormous diversity exhibited by plastid-harboring protists has been driven, at least in part, by the dynamic process of secondary endosymbiosis, whereby phototrophic eukaryotes (green or red alga) are engulfed and integrated into heterotrophic eukaryotes. These events have left behind so-called complex plastids with additional membrane layer(s) surrounding the outer and inner membrane envelope of the organelle. For example, cryptophytes (e.g., *Guillardia theta* D. R. A. Hill et Wetherbee) harbor a complex plastid embedded within their endoplasmic reticulum (ER) that is surrounded by four distinct membranes (Fig. 1). Cryptophytes represent the most archetypal chromalveolate (a supergroup unifying organisms with complex plastids of red algal origin, Cavalier-Smith 1999), retaining many endosymbiont features, which have been reduced or lost in other phylogenetically related groups. A striking example is the vestigial endosymbiont nucleus, the nucleomorph, localized inside the PPC of the former red algal cytosol (Douglas et al. 2001). The retention of ancestral features and the fact that the nucleomorph genome has been fully sequenced make cryptophytes an excellent model for investigating the targeting and transport of nuclear-encoded complex plastid precursor proteins across membranes (Gould et al. 2006a). The combination of a detailed topogenic signal analysis via green fluorescent protein (GFP)–localization studies in the stramenopile *Phaeodactylum tricornutum* and the computer-based search for putative translocons has shifted our view of complex plastid protein-targeting in chromalveolates (Patron et al. 2005, Gould et al. 2006a, Gruber et al. 2007, Sommer et al. 2007). Here we summarize the basic pattern of protein-targeting in chromalveolates with an emphasis on the cryptophytes.

Nuclear-encoded plastid proteins. Nuclear-encoded plastid proteins of organisms that harbor a plastid surrounded by more than two membranes contain an N-terminal bipartite leader essential for organelle targeting. This leader comprises a signal peptide for ER targeting, followed by a TP for targeting across the subsequent membranes. In several chromalveolates, a conserved phenylalanine-based motif at the +1 position has been reported and determined to be essential for correct localization of the mature protein (Kilian and Kroth 2005, Patron et al. 2005, Gould et al. 2006a). Recent findings also suggest that the phenylalanine at this position is a crucial trigger for chromalveolates with four membranes, enabling them to distinguish between precursor proteins crossing the outer two membranes from those entering the PPC, or those crossing all four

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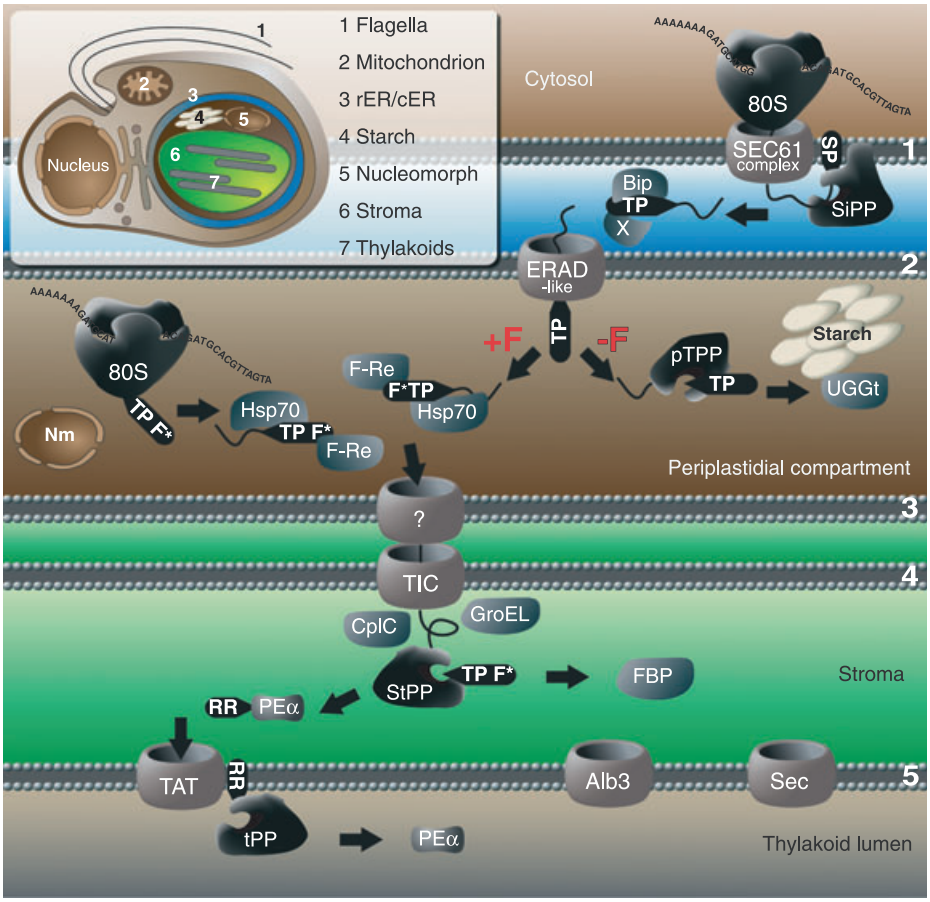

FIG. 1. Model for the preprotein import into the complex plastid of *Guillardia theta*. The complex plastid of the cryptophyte is located inside the host endoplasmic reticulum (ER). Hence, nuclear-encoded precursor proteins are first translocated cotranslationally across the ER membrane (1). Here, the signal peptide (SP) is cleaved by the signal peptide peptidase (SiPP), whereupon the remaining transit peptide (TP) is recognized by binding to immunoglobulin precursors (BIP) and an unknown receptor (X). Across the periplastidial membrane (2), precursors are translocated via a modified endoplasmic reticulum associated degradation (ERAD) system and inside the periplastidial compartment (PPC) where their fate depends upon the +1 position of the TP. Those without an aromatic amino acid lose their TP by a periplastidial transit peptide peptidase (pTPP) and get folded into a mature protein, like the UDP-glucose-glycogen glucosyltransferase (UGGt). Those with a phenylalanine (F) are kept in an unfolded state by Hsp70 and an unknown receptor (F-Re) and are subsequently translocated across the plastid envelope by an unidentified translocon in the third membrane (3) and the Tic machinery in the fourth membrane (4). Nucleomorph (Nm)-encoded and in the PPC translated plastid proteins are translocated accordingly. Inside the stroma, the precursors are received by the two chaperones ClpC and GroEL. The TP is cleaved by a stromal processing peptidase (StPP), and proteins like the fructose 1–6 bisphosphatase (FBP) are then released into the stroma. Thylakoid proteins can be translocated across the final membrane, the thylakoid membrane (5), by at least three different known pathways, namely, Albino3, the Sec, and the twin arginine translocon (TAT), the latter being responsible for the translocation of, for example, phycobiliproteins (PEα).

membranes to the plastid stroma (Gould et al. 2006b). For a few rare cases (cryptophytes and stramenopiles), other aromatic amino acids are present at the +1 position of the TP, also leading to correct stromal targeting (Gruber et al. 2007). This aromatic amino acid–based motif is also relevant for targeting in glaucophytes and rhodophytes (Steiner et al. 2005). Several plastid proteins, like some phycoerythrin subunits, need to be translocated across a fifth membrane within the plastid, the thylakoid membrane (Gould et al. 2007). The known thylakoid translocation pathways of land plant plastids (i.e., Tat, Albino3, and Sec-dependent) are also present in the cryptophyte thylakoid membranes.

Nuclear-encoded periplastidial proteins. The importance of phenylalanine for correct complex plastid targeting was demonstrated in experiments whereby this amino acid was substituted by alanine or methionine, resulting in imperfect import and location within the plastid envelope (Kilian and Kroth 2005). In the cryptophyte *G. theta,* the first nuclear-encoded periplastidial protein, UDP-glucose-glycogen glucosyltransferase (UGGt) was recently identified (Gould et al. 2006a). It was purified from starch grains isolated from the former red algal cytosol or PPC (Deschamps et al. 2006). Surprisingly, the bipartite leader sequence on UGGt is similar to nuclear-encoded stromal proteins, with the only detectable difference being serine at the +1

position of the TP instead of phenylalanine. Substitution of this single serine residue with phenylalanine results in mistargeting to the plastid stroma, where the TP is recognized by a peptidase and cleaved (Gould et al. 2006a). Again, this suggests that the aromatic motif is a crucial trigger for correct subtargeting in the complex multimembrane plastid. Furthermore, it suggests that in the course of evolution, a regular plastid TP was adapted to periplastidial targeting, together with a still unknown receptor on the ER-facing side of the periplastidial membrane, which recognizes the substituted non-aromatic amino acid motif at the +1 position of the TP (Fig. 1).

Many nuclear-encoded periplastidial proteins without a phenylalanine at the +1 position have since been identified, and they often encode regulatory factors. Interestingly, the principle of a bipartite leader with a nonaromatic residue at the +1 position for periplastidial localization also holds true for stramenopiles with some indication that this may also be the case for apicomplexa; however, further experiments are needed to substantiate this hypothesis (Gould et al. 2006b, Sommer et al. 2007). Nevertheless, the fact that TPs are responsible for the targeting across the second outermost membrane of four-membrane bounded plastids supports the theory of a translocator-dependent import mechanism for complex plastids.

An ERAD-derived translocon for the periplastidial membrane. In the nucleomorph of *G. theta*, proteins showing homology to ERAD components are encoded. This finding is unexpected, because of the absence of an ER inside the periplastidial space. One ERAD component (ORF201) was shown to localize to the periplastidial membrane of the cryptophyte's plastid and to rescue a yeast Der1p-deletion mutant (Sommer et al. 2007). The yeast mutant is normally unable to export incorrectly folded proteins from the ER (Knop et al. 1996, Hitt and Wolf 2004), but the expression of ORF201 in this yeast strain restored export of misfolded proteins to the cytosol for their degradation. Interestingly, in stramenopiles and also other chromalveolates, some of these proteins were observed to be encoded in two copies in the nucleus: one copy for the canonical host ERAD system and a second copy for the endosymbiont, bearing an N-terminal leader for periplastidial/periplastidial membrane localization. It was proposed that these components might serve as a translocon for nuclear-encoded plastid precursors inside the periplastidial (second outermost) membrane of chromalveolate plastids. This translocation machinery could pull precursor proteins with cleaved signal peptides out of the ER after the recognition of the remaining TP and into the PPC, the space between the second and third membranes (Fig. 1).

Concluding remarks. The chromalveolate supergroup hypothesis of Cavalier-Smith (1999) has been gaining support over the last few years as more and more comprehensive phylogenies have been published (e.g., Harper and Keeling 2003, Hackett et al. 2007). Much of these data at least tangentially support the idea of a monophyletic origin of a red algal endosymbiont engulfed by a predatory host (secondary endosymbiosis). New findings on protein-targeting pathways, like the ERAD-derived system with a leader for periplastidial localization, also provide compelling evidence for the chromalveolate supergroup hypothesis. It seems very unlikely that such a unique and complex protein-import system for translocating proteins across the membranes of the endosymbiont has evolved more than once in protist lineages. However, once the genome of the chlorarachniophyte *Bigelowiella natans* with its green algal endosymbiont is available, we should be able to resolve whether the possession of a second ERAD-based system for putative preprotein translocation is restricted to complex plastids of red algal origin.

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