Is *ftsH* the Key to Plastid Longevity in Sacoglossan Slugs?

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Accepted: December 6, 2013

Data deposition: This project has been deposited at ENA under the accession numbers HG518425-74, HG794360, and SRR1038494.

Abstract

Plastids sequestered by sacoglossan sea slugs have long been a puzzle. Some sacoglossans feed on siphonaceous algae and can retain the plastids in the cytosol of their digestive gland cells. There, the stolen plastids (kleptoplasts) can remain photosynthetically active in some cases for months. Kleptoplast longevity itself challenges current paradigms concerning photosystem turnover, because kleptoplast photosystems remain active in the absence of nuclear algal genes. In higher plants, nuclear genes are essential for plastid maintenance, in particular, for the constant repair of the D1 protein of photosystem II. Lateral gene transfer was long suspected to underpin slug kleptoplast longevity, but recent transcriptomic and genomic analyses show that no algal nuclear genes are expressed from the slug nucleus. Kleptoplast genomes themselves, however, appear expressed in the sequestered state. Here we present sequence data for the chloroplast genome of *Acetabularia acetabulum*, the food source of the sacoglossan *Elysia timida*, which can maintain *Acetabularia* kleptoplasts in an active state for months. The data reveal what might be the key to sacoglossan kleptoplast longevity: plastids that remain photosynthetically active within slugs for periods of months share the property of encoding *ftsH*, a D1 quality control protease that is essential for photosystem II repair. In land plants, *ftsH* is always nuclear encoded, it was transferred to the nucleus from the plastid genome when Charophyta and Embryophyta split. A replenishable supply of ftsH could, in principle, rescue kleptoplasts from D1 photodamage, thereby influencing plastid longevity in sacoglossan slugs.

Key words: sacoglossa, plastid genomes, photosystem II, D1, ftsH, light stress.

Introduction

Several groups of animals enter into symbiotic relationships with algae, the zoochlorellae of *Hydra* being a well-known example (Habetha et al. 2003; Kawaida et al. 2013). Sacoglossan slugs are unique, however, in that they perform photosynthesis and fix carbon in a light-dependent manner using plastids that they sequester from the algae upon which they feed (Greene 1970; Marín and Ros 1989; Händeler et al. 2009). Five species among the sacoglossan slugs—*Elysia chlorotica*, *E. timida*, *E. crispata*, *E. clarki,* and *Plakobranchus ocellatus*—perform what is called long-term retention (LtR) of sequestered plastids (kleptoplasts). That is, when adult animals are given the opportunity to graze upon their preferred algal food source, they can survive subsequent starvation for up to several months, during which time they maintain active plastids with functional photosystems (Mujer et al. 1996; Pierce et al. 2006; Händeler et al. 2009), giving the slugs their characteristic green color (fig. 1). Though often described as "solar-powered slugs," it is not yet clear how, exactly, the slugs benefit from the kleptoplasts, as recent findings show that plastid-bearing *E. timida* and *P. ocellatus* survive starvation for months in the dark just as well as they do in the light (Christa et al. 2014). LtR species are distinguished from short-term retention (StR) species, the ingested plastids of which lose their photosynthetic ability rapidly over the first 2 weeks of starvation and are more rapidly digested than in LtR species (Händeler et al. 2009; Klochkova et al. 2013). Both LtR and StR sacoglossans feed by tapping the plastid-rich cytosol of siphonaceous algae, which have large cells, centimeters or more in length.

Because photosystems are known to have a relatively high rate of protein turnover in higher plants and algae studied so far (Aro et al. 1993; Lindahl et al. 2000; Komenda et al. 2012),

it was long speculated that the nuclear genomes of LtR species acquired genes of algal origin via lateral gene transfer (LGT): genes that encode products such as light harvesting complex proteins or psbO (Pierce et al. 2007; Rumpho et al. 2008) might help to maintain plastids in an active state by servicing the photosystems. A problem with the LGT hypothesis was that ability to perform LtR arose in multiple sacoglossan lineages independently, complicating the number and nature of putative transfers (Wägele et al. 2011). Moreover, direct tests of the LGT hypothesis using deep sequencing on *E. timida* and *P. ocellatus* (Wägele et al. 2011) and later on *E. chlorotica* (Rumpho et al. 2011) showed that plastid-bearing LtR sacoglossans do not express any genes of algal origin. Genome sequence data for *E. chlorotica* eggs furthermore showed that the slugs do not harbor algal DNA (Bhattacharya et al. 2013). Accordingly, LGT cannot be the mechanism underlying kleptoplast survival. In search of an explanation for kleptoplast longevity in LtR sacoglossans, we revisit square one.

LtR Slugs Feed on Specific Algae

There is a distinct trend among LtR slugs to specialize and often feed on a single algal species. The preferred algal species, however, are very different for different slug species and come from very distant corners of plastid diversity. *E. chlorotica* ingests plastids from *Vaucheria litorea*, a xanthophyte alga housing a plastid of secondary endosymbiotic (red algal) origin (Rumpho et al. 2001; Archibald 2009; Gould 2012), while *E. timida* ingests plastids from the ulvophyte green alga *Acetabularia acetabulum* (fig. 1; Marín and Ros 1989). These two slugs—together with *P. ocellatus* the slugs with greatest kleptoplast longevity—feed and survive from just the one species of alga upon which they have specialized. The closely related *E. crispata* and *E. clarki* sequester plastids from ulvophytes, namely *Halimeda, Bryopsis, Batophora, Caulerpa, Penicillus*, and *Codium* (Clark and Busacca 1978; Curtis et al. 2006). *Plakobranchus ocellatus* steals plastids from various algae, too, but during starvation, then strikingly retains only those of *Halimeda* (Christa et al. 2013). We have observed the same to occur in starvation experiments on *E. clarki*, during which plastids of *Halimeda* were detectable two weeks after the onset of starvation, while plastids of *Bryposis* were not, suggesting that the latter had been digested while the former had been retained. This was determined using a barcoding approach that, for *P. ocellatus,* recently provided similar results (Christa et al. 2013). Yet, if not all ingested plastids in LtR slugs are retained, could kleptoplast longevity in slugs be partly attributable to properties of the plastids themselves? We examined the issue from the perspective of plastid genomes.

Plastid Genomes of *Vaucheria* and *Acetabularia* are United by Encoding *ftsH* and *tufA*

The plastids sequestered by the different LtR species belong to algae from quite distantly related lineages, but could they have something in common that has been so far overlooked? The plastid genome of *V. litorea*, whose plastids remain photosynthetically active for up to 10 months in the slugs (Green et al. 2000), was already fully sequenced in the course of studies on *E. chlorotica* (Rumpho et al. 2008). No chloroplast genome sequence, however, was available for *A. acetabulum*, the sole food source of *E. timida*. The *A. acetabulum* plastid genome has been found to contain large repetitive elements (Tymms and Schweiger 1985) and estimated to reach a size of around 2,000 kb (Manhart et al. 1989). Through centrifugation, we generated a DNA fraction that was enriched for *A. acetabulum* plastid DNA and by shotgun sequencing of this chloroplast-enriched fraction, we obtained 138,285 kb of vector-trimmed raw data, from which we identified and assembled 63 contigs encoding proteins homologous to known plastid proteins of the UTC clade (Ulvophyceae, Trebouxiophyceae, and Chlorophyceae) of green algae. Of these contigs, 39 encoded full-length genes (table 1). These contigs had an average coverage of 56-fold (see supplementary fig. S1, Supplementary Material online) and an AT content of 69%, which is comparable to that of the ulvophyceans *Pseudendoclonium akinetum* (68.5%) and *Bryopsis hypnoides* (66.9%; Pombert et al. 2005; Lü et al. 2011). All contigs together represent a total length of approximately 350 kb, but we estimate the complete genome to be substantially larger, possibly as big as the 2,000 kb estimate of Manhart et al. (1989). Intron and intergenic region lengths, for example—often many kilobases long—by far exceed those identified from plastid genomes of related ulvophycean algae, whose genomes are less than 200 kb long (Pombert et al. 2005, 2006; Lü et al. 2011). The same is true for open reading

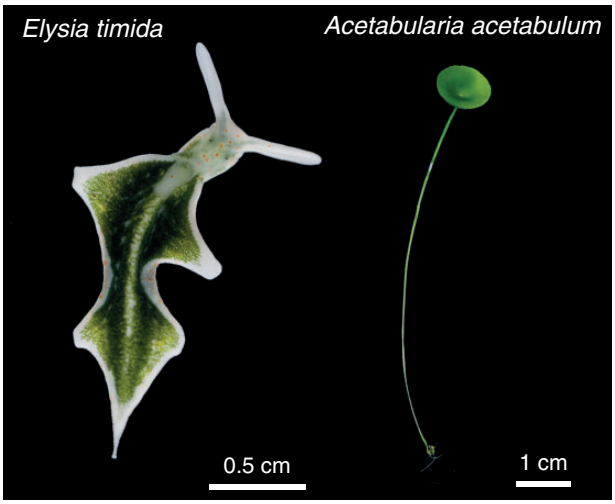

Fig. 1.—The sacoglossan sea slug *Elysia timida* feeds on the ulvophyte alga *Acetabularia acetabulum*. The sequestered kleptoplasts continue to perform photosynthesis for up to several months within specialized cells of the digestive gland of the slug.

Table 1

List of the 51 Full-Length Plastid Encoded Genes of *Acetabularia acetabulum* Identified

Gene	ORF Length (bp)	Contig Length (bp)	AT Content	Presence/Absence			Accession
				P. a.	*O. v.*	*B. h.*	
accD	885	13,807	69.4	•	•	•	HG18425
atpA*	1,509	8,868	65.9	•	•	•	HG18426
atpB*	1,440	2,571	65.9	•	•	•	HG18427
atpE_1*	399	5,694	66.2	•	•	•	HG18428
atpE_2	399	5,972	57.1				na
atpF	518	4,705	73.3	•	•	•	HG18429
atpH*	249		59.8	•	•	•	HG18430
chlB	1,575	2,407	67.3		•	•	HG18431
chlI	1,107	3,272	66.3	•	•		HG18432
chlL	864	16,526	70.1		•	•	HG18433
chlN	1,461		68.8		•	•	HG18434
psbM	105		76.2	•	•	•	HG18450
clpP	591	4,065	66.3	•	•	•	HG18435
cysA	693	2,504	72.0			•	HG18436
cysT	804	6,564	73.5			•	HG18437
ftsH	13,488	19,263	62.3	•	•		HG18438
infA	207	11,475	72.0	•	•	•	HG18439
rpl5	540		73.5	•	•	•	HG18462
rpl14*	369		69.1	•	•	•	HG18456
rps8*	414		74.2	•	•	•	HG18469
petA	924	3,120	71.5	•	•	•	HG18440
petB	648	4,808	67.1	•	•	•	HG18441
petD	462		65.6	•	•	•	HG18442
petG*	102	3,226	66.7	•	•	•	HG18443
psaB	2,082	3,305	59.1	•	•	•	HG18444
psaC	246	7,361	62.6	•	•	•	HG18445
psaJ	126	29,133	75.4	•	•	•	HG18446
psbJ	129		61.2	•	•	•	HG18448
ycf4	354		74.6	•	•	•	HG18474
psbA	1,035	9,700	59.5	•	•	•	HG794360
psbB	1,419	5,607	62.4	•	•	•	HG18447
psbK	132	7,445	70.5	•	•	•	HG18449
ycf12	102		75.5	•	•	•	HG18472
psbN*	135	6,264	72.6	•	•	•	HG18451
psbT*	96	5,851	71.9	•	•	•	HG18452
psbZ	189	4,962	72.5	•	•	•	HG18453
rbcL_1*	1,458	3,336	61.5	•	•	•	HG18454
rbcL_2	1,089	2,658	61.5				na
rpl12	528	7,371	68.4	•	•	•	HG18455
rps9	477		69.6	•	•	•	HG18470
rpl16*	429	4,236	64.6	•	•	•	HG18457
rpl19	339	4,654	76.7	•	•	•	HG18458
rpl2*	768	18,092	66.0	•	•	•	HG18459
rpl23	288		76.7	•	•	•	HG18461
rps19*	279		69.2	•	•	•	HG18466
rpl20*	351	5,356	78.4	•	•	•	HG18460
rps11*	381	9,845	63.5	•	•	•	HG18463
rps14*	303	8,626	72.6	•	•	•	HG18464
rps18	240	3,236	74.2	•	•	•	HG18465
rps4	609	5,619	73.7	•	•	•	HG18467
rps7*	471	16,827	67.5	•	•	•	HG18468
tufA	1,230	4,583	65.9	•	•	•	HG18471
ycf3	516	2,750	67.4	•	•	•	HG18473

NOTE.—The second column shows the gene length, the third column the contig length. For contigs encoding more than one gene, the length is given once. Final columns indicate presence/absence of the genes from the plastid genomes of the related *Pseudendoclonium akinetum, Oltmannsiellopsis viridis,* and *Bryopsis hypnoides.* Genes marked with an asterisk were used for the phylogeny shown in figure 3.

frames with no homology to known genes. Next to the *rpoC2* locus for example sits a 7,785 bp long open reading frame, potentially encoding a protein of 303 kDa with no significant similarity (e-value cutoff 10^{-10}) to any known proteins (fig. 2). Among the 51 protein coding genes with homology to common plastid genes, and for which we have full-length sequences (table 1), were *ftsH* and *tufA*, two proteins that we suggest in the following to be of particular interest with regard to understanding plastid longevity. These two genes are also encoded by the plastid genome of *V. litorea* (fig. 3; Rumpho et al. 2008), sole food source of *E. chlorotica*.

When we compared plastid genome data of algae and plants, it became apparent that in particular land plant plastids lack several genes commonly encoded by plastid genomes of a large variety of different algae, from the red, as well as the green lineage (rhodophytes and chlorophytes, respectively; fig. 3). Among those genes was the protease encoding *ftsH*—a protein essential for photosystem II maintenance—and *tufA* encoding the translation elongation factor Tu (Watson and Surzycki 1982). Early studies showed that sequestered plastids of *V. litorea* actively continue to transcribe and translate plastid-encoded *psbA* (Mujer et al. 1996), encoding the D1 protein of photosystem II, and transcripts of the *V. litorea* plastid-encoded *ftsH* and *tufA* were further found among RNA of *E. chlorotica* that had been starved for 2 months (Pierce et al. 2012). We found evidence for the presence of all three transcripts in *E. timida* slugs that had been starved for 1 month (fig. 4). Moreover, translation of *ftsH* transcript would be impaired in the absence of the crucial elongation factor Tu encoded by *tufA*. A replenishable supply of these gene products might be key to long-term plastid activity in slugs. How so?

ftsH Might Protect Kleptoplasts from Photodamage

Photosystem turnover in sequestered plastids of *A. acetabulum* and *V. litorea*, two preferred plastid sources for LtR slugs, has not been directly studied so far. Inferences for our hypothesis come from studies of model systems. Constant photodamage in land plant plastids demands a high level of protein import from the cytosol to replace affected components of the photosystems (Aro et al. 1993; Sakamoto et al. 2003; Nixon et al. 2010). Photosystem II (PSII), in particular its D1 protein, is affected the most by photodamage, and the latter is also a major culprit regarding subsequent damage, as a degenerate D1 leads to an accumulation of reactive oxygen species (ROS) (Nishiyama et al. 2006; Kato et al. 2009). Downstream, ROS not only affect a large variety of other biochemical pathways (Girotti 2001; Apel and Hirt 2004) but further inhibit the repair of the photosystem itself (Nishiyama et al. 2006). Essential for the repair of a damaged PSII is the removal of the faulty D1 protein, a process that is mainly mediated by the ftsH protease complex in plants and cyanobacteria (Nixon et al. 2010; Komenda et al. 2012). That observation is central to our arguments.

In organelles, ftsH proteins act as quality control proteases, as either hetero- or homo-oligomers (Janska et al. 2013). Twelve *ftsH* genes are encoded in the *Arabidopsis thaliana* nuclear genome, nine of which are targeted to the plastid (Sakamoto et al. 2003). Although all chloroplast-targeted ftsH proteases in the land plant can apparently assemble into functional hetero-oligomers—always consisting of A-type and B-type subunits—in mitochondria, the functional ftsH complex can consist of only a homo-oligomer (Janska et al. 2013). Furthermore, only one subunit needs to contain the functional proteolytic M41 domain (Zhang et al. 2010). In variegated *Arabidopsis* mutants, the loss of only a single *ftsH* gene results in high levels of accumulated ROS in the plastids, causing severe damage to the entire plant (Kato et al. 2009). As the plastid-specific *ftsH* gene is nuclear encoded in all land plants (fig. 3), the protease needs to be imported from the cytosol. In plastids sequestered by the slugs feeding only on one algal species, it is encoded by the plastid itself. This could explain why LtR species that feed on more than one algal species do not retain the ulvophyte *Bryopsis*—it does not encode *ftsH* on its plastid genome (fig. 3)—during prolonged starvation periods. The correlation between plastid-encoded *ftsH* and LtR deserves further study in algal grazing experiments.

Robust Plastids

That the plastids from some algal lineages, including ulvophytes, are more robust than land plant plastids was noted 40 years ago (Giles and Sarafis 1972). It was suggested that such robustness, sometimes bordering on apparent plastid autonomy, might be linked to the prolonged survival of plastids in what are now called LtR slugs (Trench et al. 1973; Rumpho et al. 2001). Although the reason(s) underlying the robustness of plastids that sacoglossans sequester remained obscure, the role of light stress and photodamage to PSII, in particular, has always figured prominently in the issue of sacoglossan plastid longevity (reviewed in Rumpho et al. [2011] and Cruz et al. [2013]). In that tradition, Jesus et al. (2010) recently showed, for *Acetabularia* plastids sequestered within in *E. timida*, that PSII recovers remarkably well subsequent to bleaching; they furthermore noted that "...*E. timida* kleptoplasts retain *A. acetabulum* photo-damage repair mechanisms...."

Here we are suggesting that kleptoplast robustness and photodamage repair at PSII are causally related, and that this conceivably could be attributable to only one or a few factors, with *ftsH* playing a pivotal role. Our reasoning here is guided by the observation that protein synthesis in land plant plastids has two temporally distinct roles: 1) biogenesis of the photosynthetic apparatus followed by 2) the maintenance phase during which the repair of photodamaged PSII in

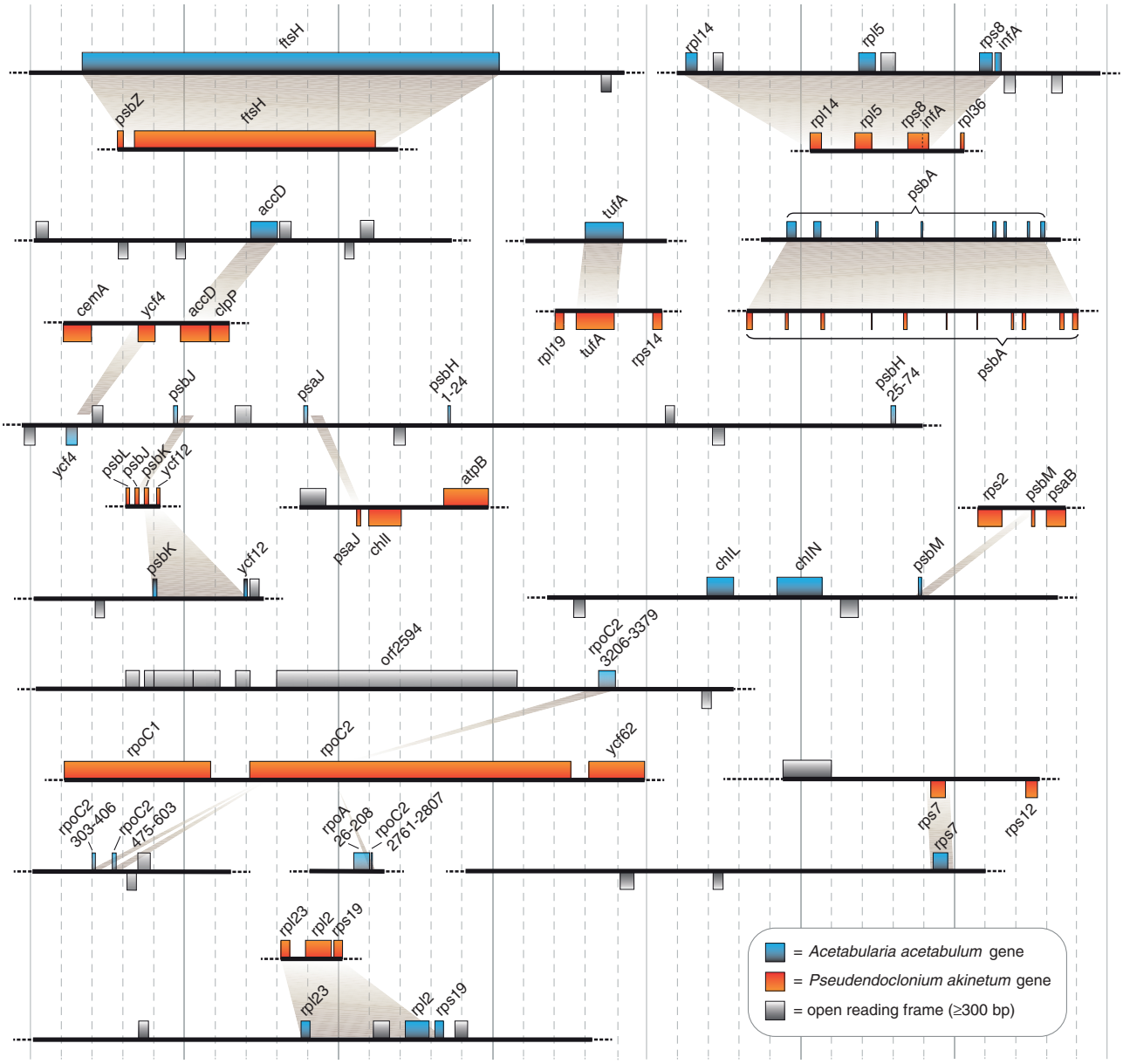

Fig. 2.—Sequencing contigs of the *A. acetabulum* plastid genome. Shown are contigs in comparison to the corresponding parts of the fully sequenced plastid genome of the phylogenetically related *Pseudendoclonium akinetum* encoding identical genes. The comparison illustrates the expansion of most intergenic and intron regions, and the increase in introns and open reading frames (ORFs ≥300 bp shown in gray) in *Acetabularia*. *Orf2594* encodes a hypothetical protein of 303 kDa within an apparent intron of *rpoC2*. Although the RNA polymerase *rpoC2* in *P. akinetum* is encoded by a single reading frame, in *A. acetabulum* it is highly fragmented across many dozen kilobase pairs. Many contigs assembled encode only a single gene (e.g., *accD* or *rps7*). Note that *psbA* is highly fragmented in both *P. akinetum* and *A. acetabulum*. Numbers beneath gene names represent the amino acid positions in the homolog of *P. akinetum*. Distance between two vertical gray lines in the background is 1 kbp.

particular plays the most prominent role (Nixon et al. 2010; Yao et al. 2012; Nickelsen and Rengstl 2013). Sacoglossans have no need for thylakoid biogenesis, as they acquire mature plastids, leaving the maintenance role as a possible factor.

If the kleptoplasts are robust, which they are, then either the slugs actively render them robust, or their robustness is an intrinsic property, or a combination of the two. The observation that most sacoglossans simply digest all ingested plastids, regardless of their source (Händeler et al. 2009; Christa et al. 2013), indicates that LtR species specifically provide an environment where plastids can persist. The observation that some LtR species such as *Plakobranchus* ingest plastids from several sources but retain only those from *Halimeda* long-term as kleptoplasts

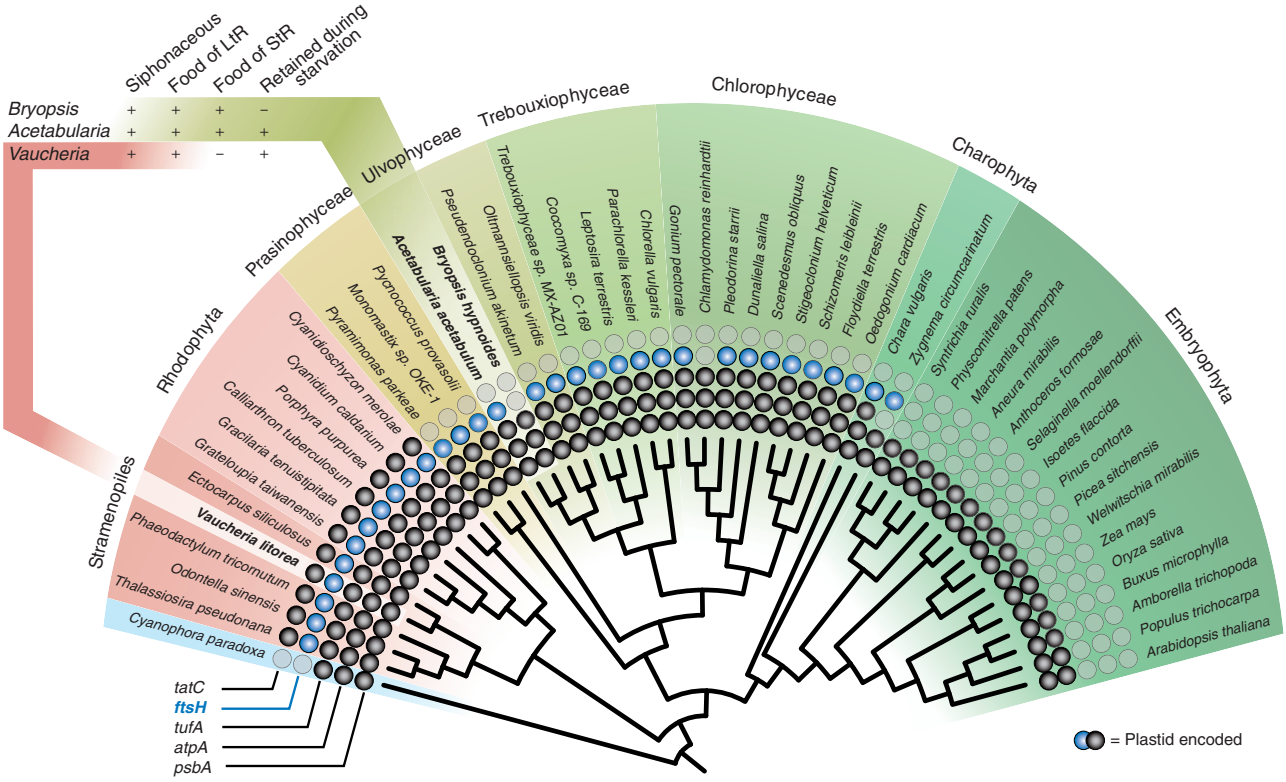

Fig. 3.—*ftsH* and *tufA* are encoded by the majority of algal plastid genomes. Different genes were lost from plastid genomes at different time points throughout evolution. *TatC,* for example, is only retained in plastid genomes of the red lineage, while *psbA* and *atpA,* for instance, are encoded by the plastid genomes of all 51 organisms analyzed. The majority of algae and water, but not land-dwelling streptophytes (embryophyta), encode *ftsH* and *tufA* on their plastid genomes. The cladogram is based on a multigene phylogeny of 17 genes (table 1) that are shared by all plastid genomes shown. Top left corner shows details on the three algae (in bold), whose plastids are being sequestered by slugs. Note the absence of *ftsH* in *Bryopsis hypoides*.

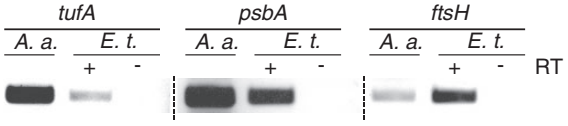

Fig. 4.—Transcripts of *tufA*, *psbA* and *ftsH* are present in starving *Elysia timida*. We isolated total RNA from slugs that had been starved for 1 month (31 days) and performed nonquantitative reverse-transcriptase PCRs to screen for the presence of mRNA of the three genes *tufA*, *psbA,* and *ftsH* in starving *E. timida* slugs (*E. t.*). RNA of *A. acetabulum* (*A. a.*) served as an additional positive control. +RT/−RT indicates the presence or absence of the reverse-transcriptase enzyme in the reaction.

(Christa et al. 2013) suggests that the ability to persist is a property intrinsic to the plastids. *Plakobranchus* could also selectively digest some plastids faster than others, implying the existence of digestive recognition mechanisms for individual food particles. But selective digestion, even if it exists, would still not explain why some kleptoplasts can survive for so long within slugs, again pointing to plastid intrinsic properties. Because *ftsH* connects PSII repair to a plastid intrinsic property—being plastid-encoded—in plastids that undergo LtR, it emerges as a prime candidate for a causal factor behind plastid longevity. A clear prediction of this hypothesis is that *Acetabularia* plastids should be particularly robust to high light intensities and recover faster from light stress, both in algae but especially in slugs, in comparison to plastids that lack *ftsH* genes, such as those of *Bryopsis*. A further implication of these findings is that they might open new avenues of pursuit for the engineering of higher plant plastids with increased tolerance to light stress.

Conclusion

Genes for *ftsH* and *tufA* are absent from the plastid genomes of higher plants (Martin et al. 1998), but present in the genomes of most algal plastids, including those that are currently known for being sequestered by LtR sacoglossan slugs (fig. 3). Thus, we posit—and it remains to be tested—that the gene content of *A. acetabulum* and *V. litorea* plastid genomes is directly involved in LtR of kleptoplasts. By bringing along their own replenishable supply of ftsH, these plastids might be better able to service photosystem II by removing the

damaged D1 protein in kleptoplasts. Hereby, they are better equipped for an extended "life" in a foreign cytosol than plastids that are dependent upon ftsH that is nuclear encoded and must be imported. By similar reasoning, a replenishable supply of tufA might aid sustained plastid translation, though our current focus is on ftsH. It is possible that the slugs do not directly provide any supporting functions at all in terms of proteins targeted to the organelle to help the kleptoplasts stay photosynthetically active for months in the cytosol of digestive gland cells. Even if D1 replacement does not occur in kleptoplasts, its removal by ftsH would prevent ROS damage and thus enhance longevity. Kleptoplast-encoded *ftsH* warrants further investigation regarding the nature of plastid longevity, not only in kleptoplasts of sacoglossan slugs.

Materials and Methods

The *A. acetabulum* DI1 strain we use to maintain lab cultures of *E. timida* was originally obtained from Prof. Menzel (Bonn, Germany) and grown at a 12 h/12 h light/dark rhythm, illuminated with 25 µm quanta $m^{-2}s^{-1}$ in 3.7% sea water (Tropic Marin). The plastids of *A. acetabulum* were isolated as previously described (Tymms and Schweiger 1985) but with penicillin–streptomycin (10 ml/l) and chloramphenicol (200 mg/l) added 48 h prior to the plastid isolation to reduce bacterial contamination. After disruption and filtration of the algal homogenate, the flowthrough was incubated for 30 min with lysozyme (2 mg/ml) and subsequently treated with 1 mg/ml DNase (Roche) for 1.5 h. As a final step, plastid DNA was extracted using Plant DNAzol (Invitrogen). DNA was sequenced using Roche GS FLX+system (GATC Biotech).

The 138,285 kb of vector-trimmed raw data (289,644 reads in total with a mean average read length of 477 bp) were manually assembled in packages using Sequencher V5.1 (gene codes). Assembly parameters for the first run were a minimum match percentage of 98 and a minimum overlap of 50, followed by a second assembly with a minimum match percentage of 90 and identical minimum overlap. There are 1,768 contigs (300 bp long with ≥10 reads/contig), and for 91 of them (average coverage of 56×), the best blast hits are plastid-encoded genes (e-value better than 10^{-10}). Only three contigs with coverage greater than 10-fold (average coverage of 14×) hit genes of potentially eukaryotic nuclear origin by the criterion of sequence similarity, but those three hits are sequences of low complexity. Although there were several bacterial and mitochondrial sequences among our >10× contigs, there is very little, if any, demonstrably algal nuclear contamination within our sequenced >10× contigs (supplementary fig. S1, Supplementary Material online), for which reason it seems likely that our plastid-related sequences represent bona fide *Acetabularia* plastid DNA, not nuclear pseudogenes thereof ("nupts"). Contigs of ≥1,000 bp length were screened for genes with homology to the plastid genomes of the UTC clade, and the coding sequences of identified genes (table 1) deposited at European Nucleotide Archive (ENA) (HG518425-74; HG794360) and reads submitted to the sequence read archive (SRR1038494). To generate the cladogram (fig. 3), protein sequences of plastid genomes were first downloaded from NCBI (September 2013). Orthologous gene clusters were then generated using BlastP (Altschul et al. 1997, Tatusov et al. 2001), then Needle (Rice et al. 2000) and Markov Cluster Algorithm (MCL) (Enright et al. 2002). Based on the 50 genes assembled for *A. acetabulum* (table 1), 17 universal clusters (genes) were identified that were encoded by all plastid genomes screened. These clusters were aligned by Multiple Alignment using Fast Fourier Transform (MAFFT) (Katoh et al. 2002) and concatenated, followed by tree construction using PhyML (Guindon et al. 2010).

RNA was extracted from four animals that had starved for 31 days using TRIzol (Life Technologies) according to the manufacturer's instructions, but with an additional DNase treatment (ThermoScientific). Reverse transcriptase PCRs were carried out using iScript Select cDNA Synthesis Kit from BioRad and the Phusion High-Fidelity DNA Polymerase (New England Biolabs). Primers used were: ftsHf 5′-CTGCAGAAAAGGTTTGGAGGC-3′, ftsHr 5′-GTCCGAGGGGAGTTGACTTG-3′; psbAf 5′-TGCATGGCCTGTAATCGGAA-3′, psbAr 5′-CGGTTGATAACGTCAGCCCA-3′; tufAf 5′-GCAAAACAAGTTGGCGTTCC-3′, tufAr 5′-GGCTAATAAAGCAGACCCGGA-3′.

Supplementary Material

Supplementary figure S1 is available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

Acknowledgments

This work was funded by a German Research Foundation (DFG) grant to S.B.G. and an European Research Council (ERC) grant to W.F.M. We thank Steffen Köhler (CAi, HHU Düsseldorf) for help with the images and Margarete Stracke for alga and slug culturing.

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Associate editor: John Archibald