Of robust kleptoplasts and versatile embryoplasts

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Statement of authorship

I hereby declare that this dissertation is the result of my own work. No other person's work has been used without due acknowledgement. This dissertation has not been submitted in the same or similar form to other institutions. I have not previously failed a doctoral examination procedure.

Jan de Vries

Publications included in this thesis:

I **de Vries J**, Rauch C, Christa G, Gould SB. (2014a) A sea slug's guide to plastid symbiosis. Acta. Soc. Bot. Pol. 83:415-421

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IV **de Vries J**, Christa G, Gould SB. (2014b) Plastid survival in the cytosol of animal cells. Trends Plant Sci. 19:347-350

V Rauch C, **de Vries J**, Rommel S, Rose LE, Woehle C, Christa G, Laetz EM, Wägele H, Tielens AGM, Nickelsen J, Schumann T, Jahns P, Gould SB. (2015) Why it is time to look beyond algal genes in photosynthetic slugs. Genome Biol. Evol. 7: 2602-2607

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Additional publications:

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1 Summary

Photosynthesis in eukaryotes arose through endosymbiotic acquisition of a cyanobacterium. This endosymbiont turned into the heritable photosynthetic organelle of eukaryotes: the plastid. Various eukaryotes that lack heritable plastids engage in interactions with phototrophs to gain access to photosynthesis. Kleptoplasty is a very unique kind of symbiosis, in which a heterotrophic eukaryote steals plastids (kleptoplasts) from a photosynthetic one. This thesis focuses on the molecular mechanisms that mediate the only known case of functional kleptoplasty from the animal world, that occurring in some species of the sacoglossan sea slugs. Sacoglossans sequester plastids from their macroalgal food sources and store them in the cytosol of the digestive epithelial cells that envelop their digestive tract. Within the animal cytosol, kleptoplasts retain their photosynthetic activity, which in some slugs has been reported to last up to several months. Sacoglossan slugs that retain active plastids long-term display a remarkable starvation tolerance. It was long thought that photosynthates provided by the slugs' kleptoplasts were the basis of starvation tolerance. At the same time, kleptoplasts were thought to be chaperoned by products of genes that the slugs had acquired from their food alga through lateral gene transfer. Results of the present thesis challenge both of these concepts. Comparative analysis of two congeneric slugs with different modes of plastid retention revealed that, although carbon is fixed by the slugs' kleptoplasts, it has little influence on their starvation tolerance. Starvation tolerance is a property dependent on the animal. Global gene expression analyses showed that both slug species respond very little towards perturbations of the kleptoplasts' photosynthetic performance. The kleptoplasts, as a consequence, are not dependent on being fostered by their slug host. The absence of transcripts for algal nuclear genes (above noise level) indicates that the slug nuclear chromosomes have not been enriched with laterally acquired algal genes. In slugs that maintain their kleptoplasts for more than four weeks, the kleptoplasts' photochemistry perpetuates as measured through spectrofluorometry. Such kleptoplasts appear to have intrinsic properties that render them robust. To identify potential factors conferring plastid robustness, gene contents of algal and embryophytic plastid genomes were compared. These analyses uncovered the presence of specific genes, such as *ftsH*, which codes for an essential protein in the photosystem II repair cycle, in plastid genomes of kleptoplasts. It is conceivable that these genes equip the stolen algal plastids with a toolkit for greater *in situ* control over photosystem maintenance relative to plastids lacking genes such as *ftsH*. Comparisons of plastid genome content identified further genes unique to algal plastids, drawing a distinguishable line between algal and land plant plastids. This suggests the existence of a shift from *in situ* to nuclear control over plastid function during the evolutionary process of land plant origin. This postulated shift of control might explain why only the plastids of land plants can differentiate into such a broad range of plastid types.

2 Zusammenfassung

Die eukaryotische Photosynthese geht auf die endosymbiotische Akquisition eines Cyanobakteriums zurück. Diese Endosymbionten wandelten sich im Laufe der Evolution und wurden zu den vererbbaren photosynthetischen Organellen der Eukaryoten, den Plastiden. Viele plastidenlose Eukaryoten gehen Symbiosen mit phototrophen Organismen ein, um auf diese Weise Zugriff auf Photosynthese zu erhalten. Kleptoplastie ist eine besondere Form solcher Symbiosen, bei der ein heterotropher Eukaryot die Plastiden (Kleptoplasten) eines phototrophen Eukaryoten stiehlt. Fokus dieser Arbeit sind die einzigen bekannten plastidenstehlenden Tiere, die Schlundsackschnecken (Sacoglossa), und die molekularen Mechanismen die deren Kleptoplastie unterliegen. Sacoglossa grasen auf Makroalgen, stehlen deren Plastiden und lagern diese in den Epithelzellen ihres Verdauungstrakts ein. In dem tierischen Zytosol bleiben die Kleptoplasten photosynthetisch aktiv; einigen Berichten zufolge bis zu mehrere Monate. Zugleich weisen Sacoglossa, die aktive Plastiden über lange Zeit lagern können, eine bemerkenswerte Hungertoleranz auf, was der Bereitstellung von Photosyntheseprodukten der Kleptoplasten zugeschrieben wurde. Darüber hinaus galt die Ansicht, dass die Schnecken mittels lateralen Gentransfers mehrere Algenproteine zur Instandhaltung der Plastidenaktivität synthetisieren und nutzen können. Ergebnisse der hier vorliegenden Arbeit fechten beide Konzepte an. Vergleichende Analysen von zwei Schwestertaxa der Sacoglossa (mit jeweils unterschiedlichen Fähigkeiten der Plastidenlagerung) zeigten, dass der durch die Kleptoplasten fixierte Kohlenstoff die Hungertoleranz der Schnecken nur gering beeinflusst. Die Hungertoleranz der Sacoglossa scheint somit eine durch das Tier und nicht durch die Kleptoplasten determinierte Eigenschaft zu sein. Globale Genexpressionsanalysen zeigten, dass beide Schwesterarten transkriptionell nur gering auf Störungen der Photosyntheseleistung der Kleptoplasten reagierten. Darüber hinaus wurden in den Genexpressionsanalysen keine Signale von mRNA-Transkripten von Algengenen detektiert. Nichtsdestotrotz weisen einige Schneckenarten eine über Monate (durch Pulsamplitudenmodulations-Fluorometrie) messbare Photochemie auf. Die Kleptoplasten scheinen folglich von ihrem Schneckenwirt autonom, was eine auf intrinsischen Eigenschaften basierende Aufrechterhaltung der Plastidenaktivität nahe legt. Um die molekularen Mechanismen plastidärer Robustheit zu identifizieren, wurden die Gene, die in Plastidengenomen von Algen und Landpflanzen kodierten sind, verglichen. Hierbei stach die Anwesenheit von spezifischen Genen, wie *ftsH*, welches ein Schlüsselprotein im Photosystem II Reparaturzyklus kodiert, besonders heraus. Diese Gene befähigen Kleptoplasten möglicherweise zu einer direkteren *in situ* Kontrolle über die Instandhaltungsprozesse ihrer Photosysteme im Gegensatz zu solchen Plastiden, die Gene wie *ftsH* nicht in ihrem Plastidengenom kodieren. Weitere derartige Vergleiche von Plastidengenomen identifizierten zusätzliche algenspezifische Gene, wodurch sich eine klare Linie zwischen Plastiden von Algen und Landpflanzen abzeichnet. Diese Erkenntnisse legen eine Umverteilung der Kräfte von plastidärer *in situ* hin zu Zellkern-gesteuerter Kontrolle der Plastidenfunktion, nahe. Diese Kräfteumverteilung fand wahrscheinlich während des evolutionären Prozesses der frühen Landpflanzenevolution statt und könnte erklären, warum die Fähigkeit, sich in eine Vielzahl von spezialisierten Plastidentypen differenzieren zu können, auf Landpflanzenplastiden beschränkt ist.

3 Plastids: a brief account on photosynthetic eukaryotes[1]

Photosynthesis in eukaryotes was established through primary endosymbiosis. At least 1.2 Gya (Butterfield 2000; Douzery et al. 2004; Zimmer et al. 2007; Parfrey et al. 2011), an ancestral cyanobacterium, phylogenetically most closely related to section IV and V cyanobacteria (Dagan et al. 2013), was incorporated by a single-celled eukaryotic host (Cavalier-Smith 2000; Martin et al. 2015). This singular event gave rise to the first plastids (Cavalier-Smith 1982; Rodríguez-Ezpeleta et al. 2005; Gould et al. 2008; McFadden 2014). The lineage that can be traced back to this event forms a monophyletic group called Archaeplastida (Adl et al. 2005; Adl et al. 2012), which is comprised of the Glaucophyta, the Rhodophyta (red algae) and the Chloroplastida (green algae) (McFadden 2014; Zimorski et al. 2014; Archibald 2015a; Martin et al. 2015). But there are not only archaeplastidal plastids. There are secondary, or complex, plastids. Their origin encompassed the uptake of a rhodophyte or chlorophyte by a heterotrophic protist (Gould et al. 2008). In the latter case it is quite certain that this occurred twice, once leading to plastid-harbouring euglenids and once to the chlorarachniophytes (Gibbs 1978; Gilson et al. 2006; Archibald 2009). In case of the secondary red lineage, the number of events is still debated (Archibald 2015b; Stiller et al. 2014; Gould et al. 2015). Whatever the number of endosymbiotic events might have been, complex plastids can also be traced back to the archaeplastidal origin of plastids (Archibald 2015b). Next to this, there is also *Paulinella chromatophora* (Nowack 2014). This rhizarian amoeba has acquired a cyanobacterial endosymbiont that appears to be on its way of becoming a photosynthetic organelle (in that case coined chromatophore) independent of the archaeplastidal plastids (Nowack et al. 2008; Nowack 2014). That all major photosynthetic eukaryotes (i.e. those with heritable plastids and not *Paulinella*) share a common history is evident by the genes encoded by the plastids genome (Timmis et al. 2004) and also those of the mitochondrial genomes (Jackson et al. 2014).

The transition from an endosymbiont to a fully-fledged plastid encompassed a significant reduction in genome size and thus coding capacity (Timmis et al. 2004). The two major driving forces behind this genome erosion are outright gene loss and the transfer of genetic material to the nucleus (Timmis et al. 2004). The latter was termed endosymbiotic gene transfer (EGT; Martin et al. 1993). A consequence of EGT is that most of the genes required for on-going plastid function are encoded by the nuclear genome (i.e. the host's genetic material) (Martin and Cerff 1986; Martin et al. 1998; Timmis et al. 2004). Nuclear-encoded plastid genes are transcribed in the host nucleus, upon which translation in the cytosol and post-translational translocation to the plastid follows (Timmis et al. 2004; Soll and Schleiff 2004). The majority of these genetic changes occurred early on in the common ancestor of all Archaeplastida (cf. Timmis et al. 2004). This is the reason why components of the translocation machinery of plastids are highly conserved among all plastid-housing eukaryotes and can serve as molecular markers for studying plastid evolution (Gould et al. 2015). The same applies to all eukaryotes and the translocation machinery of mitochondria and mitochondria-related

[1] - *This section offers a brief introduction and an overview of the field of study. Specific introductions to the topics of the manuscripts associated with this thesis are found on pages 7-10 and 46-49.*

organelles (Garg et al. 2015). This is not surprising. Without a functioning protein translocation system that imports proteins from the cytosol, the organelles could not function at all (cf. Chen et al. 2002; Soll and Schleiff 2004). There is hence a strong selection pressure on maintaining a functional translocation system in the organelles. Plastids are, as a consequence, dependent on the host's nuclear material and can only be inherited with the respective nuclear context. This applies to both the simple vertical inheritance of plastids (i.e. from one generation to the next), but also to the special cases of lateral inheritance that occurred through secondary endosymbioses (Archibald 2009). The only eukaryotes that are truly photoautotrophic, that is the organism meets its carbon requirement solely through light-driven fixation of atmospheric carbon dioxide, are those that harbour heritable plastids (cf. Gould et al. 2008). But some eukaryotes that lack heritable plastids can also get hold of photosynthesis.

The most common way for a heterotrophic eukaryote to tap photosynthetic performance is through symbiosis. These photosymbioses are not limited to protists such as *Paramecium* (Esteban et al. 2010), but include wide-spread and palpable examples. Photosymbioses include the interaction between algae and/or cyanobacteria and fungi that in concert appear to form an entirely different organism, the lichens (Selosse and Le Tacon 1998; Rikkinen 2015). Also, various relationships between algae and animals have been documented, which are often fine-tuned and of high ecological importance for their respective environment (Trench 1993; Jeong et al. 2012; Frommlet et al. 2015). Especially in aquatic ecosystems there is a vast diversity of eumetazoan photosymbioses; many algae that can be associated with all kinds of marine invertebrates are known (Trench 1979; Stat et al. 2006; Rumpho et al. 2011). One of the best-studied models is *Symbiodinium*. This dinoflagellate enters a mutualistic relationship with a wide range of animals (ranging from sponges and corals to molluscs) but also protists (Stat et al. 2006; Stat et al. 2008; Venn et al. 2008). *Symbiodinium* provides its host with photosynthates, while it receives beneficial waste products in return (Muscatine and Porter 1977; Stat et al. 2008). Although in some systems vertical as well as horizontal symbiont transmissions occur (Stat et al. 2008), in most symbiotic relationships the photobionts are acquired anew by each generation (Rumpho et al. 2011). This is true for *Aiptasia* (that harbours *Symbiodinium*)*, Hydra* (harbouring *Chlorella*) or various ciliates and Foraminifera (Habetha et al. 2003; Stoecker et al. 2009; Johnson 2011; Grawunder et al. 2015). Main driving force behind the establishment of photosymbioses appears to be the supply of photosynthates during nutrient-poor conditions (Lowe et al. 2016).

Some eukaryotes engage in a genuinely different association to become photosynthetic: kleptoplasty. In that case not organisms, but just the photosynthetic organelles (termed kleptoplasts for stolen plastids), are incorporated by the host and into its cells (Clark et al. 1990; Rumpho et al. 2011). Kleptoplasty falls out of the classical concept of symbiosis, as the latter is usually defined by the interaction of an organism with a cellular compartment of another (cf. Raven et al. 2009). Kleptoplasty falls out of the concept of endosymbiotic acquisition of a novel or alien organelle, too. The problem is the lack of heritability (cf. de Vries et al. 2014a **I**). Because each generation of kleptoplastic organisms acquire their plastids anew (Rumpho et al. 2011), it is hard to imagine of how the stolen organelles could experience some kind of adaptation. At the same time, a wide range of

hosts is adapted to performing kleptoplasty. Diverse groups of protists engage in this process (Stoecker et al. 2009). Several genera of Foraminifera are known to acquire plastids (Bernhard and Bowser 1999), whose donors seem to be confined to specific groups of diatoms (Pillet et al. 2011). As for green sea slugs (see chapter 5; Wägele et al. 2011), the kleptoplasts of Foraminifera function without the support of genes that stem from the lateral transfer of algal DNA (Pillet and Pawlowski 2012). This is not the case for the ciliate *Myrionecta rubra* that acquires not only the plastids from its cryptophytic prey, but also the nuclei (Johnson et al. 2007). These stolen plastids, and then only the plastids, appear to be sometimes stolen again from the ciliate *Myrionecta* by the dinoflagellate *Dinophysis acuminate* (Wiscaver and Hackett 2010). The dinoflagellate retains the plastid surrounded by only two membranes, meaning that *Dinophysis* strips its kleptoplast from the outer two, eukaryotic, membranes (Lucas and Vesk 1990; Wiscaver and Hackett 2010). The above-mentioned examples illustrate that there are various strategies and mechanisms with independent origins for stealing and housing plastids across a diversity of distantly related protist lineages. This thesis focuses on the only case of kleptoplastic animals: the sacoglossan sea slugs.

4 Aims of the thesis

The major aim of this thesis was to gain a better understanding of kleptoplast longevity in sacoglossan sea slugs. Especially the molecular mechanisms that confer the obvious robustness of these plastids were of major interest. For a bipartite interaction, that is (i) the slugs and (ii) the alga (or rather their plastids), understanding of the contribution of each partner is crucial (Figure 1). Therefore, this thesis is structured in two parts, each dealing with investigations on the respective factors that confer compatibility of the two partners. Concomitantly, from these two interaction partners, two objectives were derived.

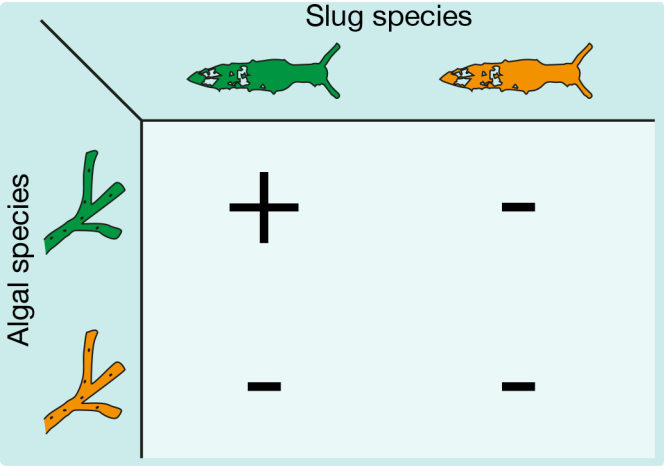

Figure 1: Sacoglossan kleptoplasty is a bipartite interaction.
Successful establishment of molluscan kleptoplasty requires compatibility (green) of both the host (slugs) and the source of plastids (algae). If either party is incompatible (orange), no long-term kleptoplasty can be established.
This figure is modified with permission from de Vries et al. 2014a **I**

Objective 1: **Investigations on the slug's compatibility**

Do the slugs sense their plastids and, if so, is that connected to the photosynthetic performance of their kleptoplasts?

What defines short-term and long-term retention of plastids on a molecular level in a comparable system?

Objective 2: **Investigations on the alga's compatibility**

What are the molecular factors that render a plastid long living, that is robust?

What is the evolutionary history of robust plastids?

5 Sacoglossan slugs steal semi-autonomous plastids

Photoautotrophy is limited to some prokaryotic and eukaryotic lineages. Animals are not among them. Yet, for a long time it seemed as if some slugs of the clade Sacoglossa (Figure 2) defied this textbook paradigm (cf. Rumpho et al. 2011; Cruz et al. 2013; Pierce et al. 2015): through the functional incorporation of plastids stemming from their food alga, these slugs were believed to transform into a system that depends on light energy alone. These stolen plastids (kleptoplasts) were considered to act as the photosynthetic factories the sacoglossans exploit to meet their carbon needs and that an animal had turned into a plant (cf. Rumpho et al. 2011; Cruz et al. 2013; Pierce et al. 2015). Here, an outline of how this view has changed is provided, emphasising on the contributions associated with this thesis.

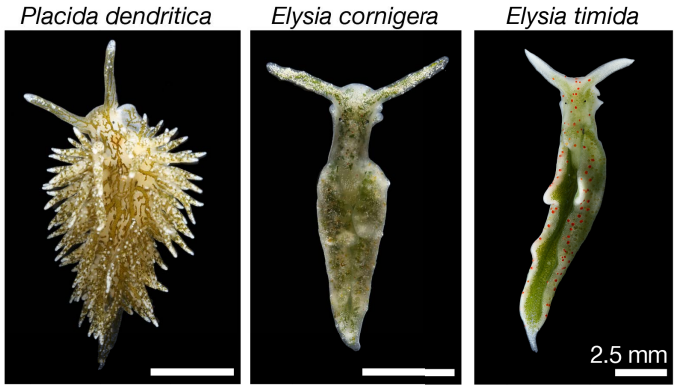

Figure 2: An appetizer of sacoglossan diversity. Three examples of sacoglossan slugs with different capabilities of plastid retention are shown. While *Placida* does not retain plastids at all, *Elysia cornigera* retains them for one to two weeks (short-term retention) and *E. timida* for several months (long-term retention). Note the morphological differences in the lateral appendices. *Placida dendritica* bears cerata while both *Elysia* species bear parapodia. The former is a defining feature of the Limapontioidea while the latter is a defining feature of the Plakobranchoidea (cf. de Vries et al. 2014b IV).

Pioneering work performed in the 1960s and 70s, spearheaded by Robert K. Trench, showed that carbon is fixed by the plastids residing within the cells of some sacoglossan slugs (Trench, 1969). Several experiments followed that investigated the relationship between the slugs and the allocation of fixed carbon (Greene and Muscatine 1972; Kremer and Schmitz 1976; Ireland and Scheuer 1979). This quickly resulted in work that analysed the dependency of the sacoglossans on light (Hinde and Smith 1972, West 1979, Marín and Ros 1989). In many studies it was observed that sacoglossans that were starved in the dark had a decreased survival rate compared to those that were starved in the light (Hinde and Smith 1972; Hinde and Smith 1975; Yamamoto et al. 2013). This was then attributed to the lack of photosynthetically fixed carbon (Hinde and Smith 1972; Hinde and Smith 1975; Marín and Ros 1989). But is it that simple? Light is crucial not only for plants, and many biochemical processes apart from photosynthesis are directly influenced by light, which ought to be considered when analysing the sacoglossans (de Vries et al. 2014a I). Thus, as discussed in de Vries et al. (2014a I), the experimental setup for analysing the interconnection between slug vigour and photosynthesis is crucial. Christa and colleagues (2014a) performed an experiment to address the importance of photosynthates for the slugs' survival during starvation using the photosynthesis inhibitor drug monolinuron (Arrhenius et al. 2004). In that study, slugs that were starved under conditions where photosynthesis was impaired (resulting in a close-to-zero carbon fixation rate) did not lose weight at a higher rate, let alone did they die earlier. Other experiments followed that used

the same approach (Christa et al. 2014b; de Vries et al. 2015a **III**), all leading to the same results: impairing photosynthesis did not result in an obvious decrease in slug vigour.

In retrospect, a survey of the literature showed that many experiments in the past added more weight to the importance of photosynthesis than supported by the data (Christa et al. 2014c **II**). How come? The reason likely rests with the methodology used in the work of Trench (cf. Trench 1969; de Vries et al. 2014a **I**). There, starvation was used as a method to evaluate the retention and longevity of plastids inside the cytosol of the animals. Starvation turned from a method into the key subject of research on photosynthetic slugs (cf. de Vries et al. 2014a **I**) and for a good reason: there are considerable differences in terms of the survival rates upon starvation in sacoglossan slugs (cf. Christa et al. 2014b). In order to have a handle on the factors that define a slug species as most compatible to the plastids the sacoglossans *Elysia cornigera* and *Elysia timida* were compared (de Vries et al. 2015a **III**). These slugs are sister species and can be fed the same alga, that is *Acetabularia acetabulum* (Marín and Ros 1992; Händeler et al. 2009; Krug et al. 2011; Christa et al. 2014d; Christa et al. 2015; de Vries et al. 2015a **III**). Thus, the 'factor' alga is the same in both in that setup. Yet, based on pulse amplitude modulation spectrofluorometry of the chlorophyll a fluorescence, *E. cornigera* was classified as a short-retaining species, meaning that the photosystem II (PSII) activity is only retained for one to two weeks which is accompanied with the death of the animals (Händeler et al. 2009; Krug et al. 2011; Christa et al. 2015). *E. timida* retains high PSII activity and survives starvation for several months (Händeler et al. 2009). In addition to the common evaluation of PSII activity, also the first comparative CO_2 fixation measurements for the two sister species over a period of starvation was performed (10 and 30 days for *E. cornigera* and *E. timida* respectively; de Vries et al. 2015a **III**). The results of de Vries et al. (2015a **III**) showed that carbon fixation declined at an equal rate in both species. This work demonstrated that (a) the performance of the kleptoplasts was the same (and likely dependent on their origin, i.e. the ulvophyte *A. acetabulum*) and (b) the different survival rates upon starvation do not depend on the carbon fixation by the plastids. But what defines the sacoglossans' differing abilities to endure starvation?

Comparative global gene expression analysis using RNA sequencing was chosen as an approach to highlight those factors that differed in starving *E. cornigera* and *E. timida* specimen (de Vries et al. 2015a **III**). To be able to connect starvation of the slugs to the biology of the kleptoplasts, treatments inducing a plastidal response were applied in that study; these treatments included chemical inhibition of photosynthesis through monolinuron and high-light treatment. Interestingly, either treatment had very little effect on the global gene expression response of both sister taxa (de Vries et al. 2015a **III**). Although there were slight indications that *E. timida* showed some response towards the plastid-impairing treatments, the main denominator in both species was whether they were starving or not (de Vries et al. 2015a **III**). A further observation made in de Vries et al. (2015a **III**) was that the higher starvation tolerance exhibited by *E. timida* was accompanied by a broad-scale downregulation of the metabolism. In that study we found that (a) upon progressing starvation, *E. cornigera*'s stress response signalling pathways showed a very early and definite induction while *E. timida*'s were less affected and (b) these trends were, again, little influenced by the treatments that

targeted the kleptoplasts. Another mechanism highlighted by the data of de Vries et al. (2015a **III**) includes an induced gene expression of reactive oxygen species (ROS) scavengers upon progressing starvation. In the same study we showed that only *E. timida*, but not *E. cornigera*, appears to accumulate ROS during starvation.

The ROS response is interesting for two reasons: First, ROS are a major hazard emitted by actively photosynthesizing organelles and, especially, damaged plastids (Aro et al. 1993; Kato et al. 2009). It is conceivable that effective scavenging of hazardous ROS underwent positive selection during the evolution of long-term kleptoplasty, as it directly influences longevity of both plastid and animal host. In another system, the *Paramecium-Chlorella* symbiosis, ROS tolerance was suggested to be a key factor for successfully housing a photosynthetic symbiont (Kawano et al. 2004) and we later hypothesised that the slugs' tolerance to photosynthesis-derived toxins might be a key factor for establishing kleptoplasty (de Vries et al. 2014b **IV**). Second, ROS are also known as signalling molecules especially during starvation (Scherz-Shouval et al. 2007; Li et al. 2013; Filomeni et al. 2015). Accordingly, the absence of an elevation of ROS-levels in *E. timida* (observed in de Vries et al. [2015a **IV**]) might simply reflect the weak starvation response that simultaneously results in reduced autophagous activity and thus less shrinkage of the animals and the interconnection of these factors. In summary, the different responses of the two sister taxa analysed in de Vries et al. (2015a **IV)** were almost completely uncoupled from the status of the kleptoplasts they housed. Starvation tolerance appears thus uncoupled from kleptoplasty and thus solely defined by the animal taxon.

Until today, one question that fascinates and intrigues researchers from many disciplines was summarised in a manuscript title by Rumpho and colleagues (2001): "How can isolated chloroplasts continue to function for months in the cytosol of a sea slug in the absence of an algal nucleus?" For more than a decade it appeared as if the answer was at hand: lateral gene transfer (LGT) from the algal to the slug nucleus (Pierce et al. 1996; Rumpho et al. 2008). By such means the lack of algal nuclei would be nullified as genes required for the maintenance of on-going photosynthesis would be encoded on the slugs' own chromosomes (Pierce et al. 2003; Schwartz et al. 2010; Pierce et al. 2012). The major reasoning behind this assumption, as reasoned for example in Rumpho et al. (2008), was founded upon the high turnover of photosynthesis-associated proteins in land plants (Aro et al. 1993). If one applies this reasoning to the situation in the slugs, one needs to expect that the laterally acquired factors are expressed on a high level to meet the equally high demands set by the turnover. This was the basis for testing the "LGT in slugs" hypothesis through global gene expression analysis (Wägele et al. 2011). All analyses of global mRNA abundance through sequencing did not detect an overrepresentation of transcripts coding for factors related to plastid maintenance or photosynthesis (Wägele et al. 2011; Rumpho et al. 2011; Pelletreau et al. 2011). On the contrary, they were below the levels of contamination (de Vries et al. 2015a **III**; Rauch et al. 2015 **V**). There has been some debate with regard to the interpretation of this data: it has been claimed that even the rare detection of transcripts associated with photosynthesis is important for understanding the mechanisms behind kleptoplasty (Pierce et al. 2012). But, as pointed out (Rauch et al. 2015 **V**), there is very little room for such interpretations. Stoichiometry is a pillar of all large-scale sequencing analysis, as discussed in Rauch et al. (2015 **V**). Although low abundant transcripts translating into low

abundant proteins can have a strong effects for a cell, the mode of action of the proteins these transcripts code for must be considered. Most of the factors necessary for on-going photosynthesis act directly in the biochemical processes of the photosynthetic apparatuses (Allen et al. 2011) and are expressed on such a high level that in photosynthetic tissues, such as leaves, they make up a major portion of the total transcripts (Bhalerao et al. 2003; cf. Wägele et al. 2011). And they need to be, but this is not the case in the slugs, as first shown by Wägele et al. (2011) and later by other studies, as summarised in Rauch et al. (2015 **V**). One cannot build a rationale for plastid maintenance in slugs upon canonical plastid biology without its full consequences. But what if canonical plastid biology does not apply here?

With LGT no longer a plausible explanation, another solution for the longevity of the plastids *in hospite* needed to be explored. Revisiting the performance of kleptoplasts from the same source (i.e. the ulvophyte *A. acetabulum*) residing within differently retaining species (the StR *E. cornigera* and the LtR *E. timida*), the answer to this question emerges. Stolen plastids performed equally in terms of carbon fixation in either slug species (de Vries *et al.* 2015 **III**). This implies that the *status quo* of the plastids perpetuates also outside of their natural, that is algal, cytosol. Importantly, this means that they are not rendered more robust by properties residing with the slug (cf. Rauch et al. 2015 **V**). The slugs simply provide the correct environment for housing the robust plastids (cf. de Vries et al. 2014a **I**; de Vries et al. 2014b **IV**; Rauch et al. 2015 **V**). It depends on the plastid to bring along the potential to be stored long-term. When these two factors (the right vessel, i.e. slug, and the right plastid) meet, long-term storage of kleptoplasts within the slug can occur (de Vries et al. 2014a **I**). Long-term kleptoplasty is currently solely analysed by starving the slugs (de Vries et al. 2014a **I**). As a consequence of this methodology, only for those slugs that can endure starvation concomitant with long-term retention of plastids can be detected. Future research should try to untangle these two factors.

This chapter outlined the kleptoplasts‘ contribution to the animals longevity and in turn the animals’ contribution to plastid longevity. Both appear rather minor. The plastids likely contribute to the slugs’ cell biology and metabolism through some means (de Vries et al. 2014b **IV**), but what is not known. The animals provide the correct environment for housing the plastids (i.e. the kleptoplasts) but the kleptoplasts seem to act indifferent of their animal host. This creates the necessity for two major attributes of the plastids: First, they must be intrinsically robust to bring along the potential to endure captivity long-term. Second, they must be able to regulate and maintain their function more autonomously than our current understanding of plastid biology would suggest.

Publication I: A sea slug's guide to plastid symbiosis

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A sea slug's guide to plastid symbiosis

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Abstract

Some 140 years ago sea slugs that contained chlorophyll-pigmented granules similar to those of plants were described. While we now understand that these "green granules" are plastids the slugs sequester from siphonaceous algae upon which they feed, surprisingly little is really known about the molecular details that underlie this one of a kind animal-plastid symbiosis. Kleptoplasts are stored in the cytosol of epithelial cells that form the slug's digestive tubules, and one would guess that the stolen organelles are acquired for their ability to fix carbon, but studies have never really been able to prove that. We also do not know how the organelles are distinguished from the remaining food particles the slugs incorporate with their meal and that include algal mitochondria and nuclei. We know that the ability to store kleptoplasts long-term has evolved only a few times independently among hundreds of sacoglossan species, but we have no idea on what basis. Here we take a closer look at the history of sacoglossan research and discuss recent developments. We argue that, in order to understand what makes this symbiosis work, we will need to focus on the animal's physiology just as much as we need to commence a detailed analysis of the plastids' photobiology. Understanding kleptoplasty in sacoglossan slugs requires an unbiased multidisciplinary approach.

Keywords: kleptoplasty; sacoglossan slugs; photosynthesis; plastid biology; photosynthetic slugs; evolution

Of leaves that crawl

Plastids are the main difference that distinguishes a plant or algal cell from animal cells. However, in 1876 de Negri and de Negri [1] described some green sea slugs to harbor granules that appeared to be stained green through chlorophyll pigments, similar to those of plant and algae plastids. It took almost another century before Kawaguti and Yamasu [2] could demonstrate that the globular chlorophyll bodies were identical to the plastids of the slug's algal food source (Fig. 1). Due to the nature by which the slugs acquire the plastids from their algal food source, these stolen organelles were termed kleptoplasts: stolen plastids. In his work on *Elysia crispata* (at that time known as *Tridachia crispata*), Trench [3] was one of the first to suggest that the slugs might specifically sequester the organelles for their ability to photosynthesize. Trench [3] not only demonstrated the incorporation of $^{14}CO_2$ through the plastids that are embedded within the epithelial cells that form the digestive glandular tubules, but also analyzed the perpetuation of the kleptoplast-slug relationship by separating the slugs from their algal prey. Ever since, starving the slugs has been a common approach to determine the slug's capacity to maintain functional kleptoplasts [4]. Along these lines the presence of photosynthesizing kleptoplasts was generally associated with the ability of some sacoglossans to survive starvation periods that can last many months [5]. This led researchers to coin the term of "leaves that crawl" [6].

In contrast to plants and algae, plastids of slugs are not vertically inherited; kleptoplasts have to be acquired by each new slug generation. Sacoglossan sea slugs have a highly specialized radula that consists of individual, serially organized teeth [7]. Only one tooth is used at a time and, when idle, stored in an autapomorphic structure called "saccus" [8], eponymous for the sacoglossan group. Some slug species can feed only on a single algal species and this might be associated with a specialized radula of mature animals [7]. In other cases, such as *Elysia viridis* that can feed for instance on *Codium* and *Bryopsis*, animals seem to have a more generally adapted radula allowing them to feed on a variety of different species [7]. However, this remains an observed correlation and it is difficult to imagine how one could provide empirical evidence at this point. Feeding experiments in the laboratory alone do not do the trick and radula mutation is far from feasible. Currently we do not know where food source selection actually begins. From what we know it could very well be that the "selective" animals can penetrate different siphonaceous algae and selection occurs downstream, not mechanically (hard and soft or small and large), but biochemically (sweet and sour or fresh and putrid).

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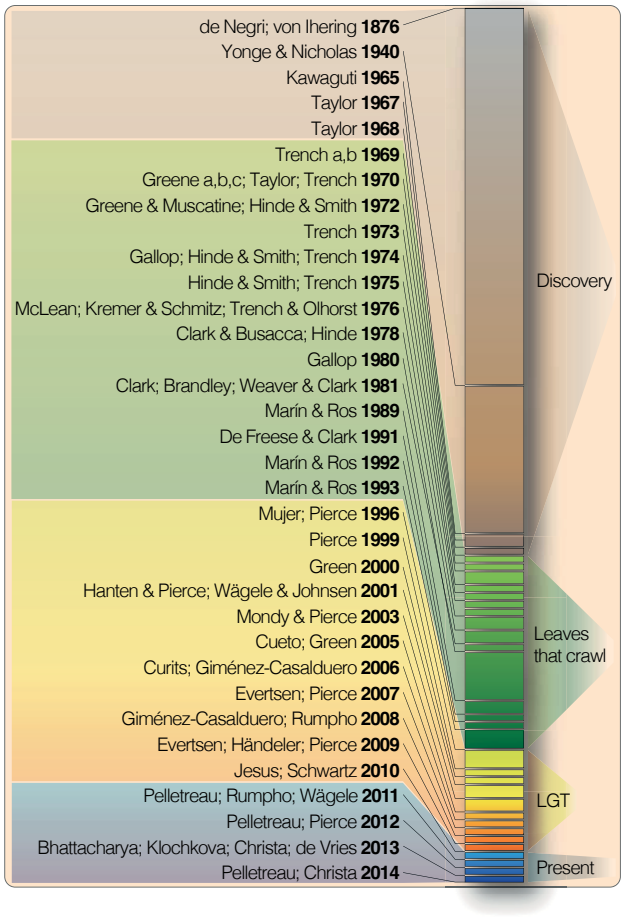

Fig. 1 Overview of about 140 years of research on green sacoglossan slugs. The timeline highlights key publications [1–6,8,10–17,20–23,27,33,40–43,46–49,52,53,56,61,63–94] on sacoglossan slugs since 1876. Four main periods (on the right) can be distinguished: the discovery phase, in which slugs and "chlorophyll-pigmented granules" were morphologically described (**a**), evidence for the incorporation of CO_2 suggested that slugs are "leaves that crawl" (**b**), and at a time where in general more and more gene transfers from one genome to another were identified, the concept was born that lateral gene transfer (LGT) from algae to slugs could support kleptoplasty in sacoglossans (**c**). The LGT concept dominated the field for 15 years until it was challenged for the first time in 2011 [12], which changed the way kleptoplasty in sacoglossan slugs is now viewed and studied. The listed manuscripts all refer to primary data manuscripts except the 1975 review by Trench, in which the term 'leaves that crawl' was coined. If an article has more than two authors, only the first author is listed.

The slugs do not feed on the entire alga, but rather use the radula's tooth to penetrate the cell wall of siphonaceous algae. They then suck out the entire cytosolic content of the algae including the organelles and all other compartments. This is not yet special, but in a few sacoglossan species only the plastids are selectively sequestered from the phagocytosed material. Individual food vacuoles can initially contain several kleptoplasts [9], but these are subsequently released into the cytosol after the vacuole is degraded. Considering that the vast majority of sacoglossans appear to treat incorporated plastids just like any other food particle, we can assume that kleptoplast retention happens on purpose. Still, the molecular mechanism of how plastids are initially recognized and subsequently released into the cytosol remains entirely unknown.

The ability of some kleptoplasts to remain functional inside the animal cells was long spearheaded by the idea that the slugs express genes they obtained through lateral gene transfer (LGT) from the algal nuclei, and which encode proteins that maintain plastid functionality [10,11]. When the first slug transcriptomes emerged that concept was challenged [12,13]. It has been discussed elsewhere in detail why LGT cannot support the stolen plastids [12,14], but, in brief, the reasons are (***i***) the meagre amount of photosynthesis-related transcript identified among slug messenger RNA – "one in a million reads" – and (***ii***) that algal genes have never been identified within in the genomic context of slug nuclear DNA. It now seems that intrinsic properties the stolen plastid bring along render some algal plastids more robust (that is "longer-living" in a foreign environment outside the algal/plant cytosol) than others [15–19]. How exactly is not known, but it is important to note that plastid transcription and translation can continue for months in some species and that their genomic coding capacity varies from that of land plastids [11,17].

The topsy-turvy of functional plastid retention among sacoglossan slugs

Based on pulse amplitude modulation (PAM)-fluorometry, and the determined maximum quantum yield (F_v/F_m) of photosystem II (which is commonly used to determine the photosynthetic capacity of slugs [20]), the majority of sacoglossans do not retain any functional plastids (non-retention species, NR) or for only a few days and up to two weeks (short-term retention species, StR). Seven non-monophyletic species are currently known to retain kleptoplasts with F_v/F_m values that remain on a level that is generally considered to account for the presence of a functional PSII for several months: *Elysia chlorotica*, *E. timida*, *E. crispata*, *E. clarki*, *E. viridis*, *Plakobranchus ocellatus* and *Costasiella ocellifera* [3,4,19,21–25]. All latter species are referred to as long-term retention (LtR) slugs.

But what really makes an LtR species? Genotyping incorporated algal plastids shows that the algal food sources of NR species sometimes matches to those of StR and LtR species [26]. The amount of plastids sequestered and stored in the digestive tubules also does not seemingly differ between long- and short-term retainers (Fig. 2; [19]). To make the matter even more complicated, some LtR species feed on several algae simultaneously [27–29]. What that means is that the food source alone cannot explain the long-term retention of functional kleptoplasts in LtR species. Interestingly though, plastid genome barcoding throughout starvation in the two polyphagous LtR species *E. clarki* and *P. ocellatus* suggest that the speed with which kleptoplasts are digested differs and depends on the plastid source [27,30,31]. It demonstrates that it takes two to tango: the right slug and the right plastid source.

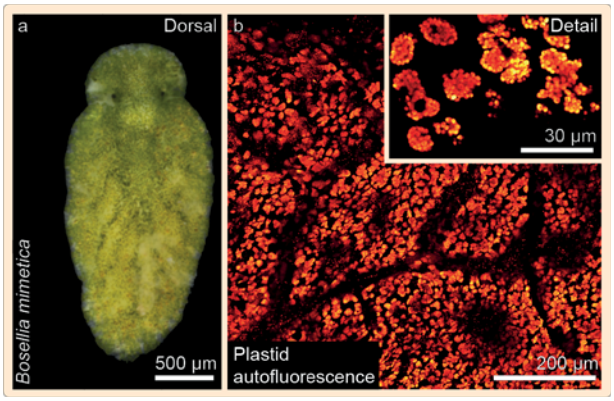

Fig. 2 Quantity does not equal quality. **a** The sacoglossan slug *Bosellia mimetica* harbours numerous kleptoplasts in its digestive tubular network, which (**b**) appear much denser in comparison to some LtR species (see [19,91]). Yet, *B. mimetica* is classified as a StR species; within a few days of starvation F_v/F_m values drop below those that are considered to represent functional photosynthesis [4].

Phylogenetic and photosynthetic analyses recently provided evidence that functional kleptoplasty evolved multiple times within the Plakobranchacea [25,32]. In turn, all basal shelled species are NR forms, but these species do not form a monophyletic group [4,19]. Virtually no molecular study has yet addressed the issue of uncovering the animals' mechanisms that determine the mode of retention. Comparative analyses of different species, in particular the molecular differences between NR, StR and LtR species, represent a promising tool to do so. For example, the trend that NR slugs engulf plastids in phagosomal membranes was noticed before [33,34]. NR species seem to keep the incorporated plastids inside phagosomes for immediate degradation all the time; they do not appear to ever release them into the cytosol [33,34]. StR and LtR forms on the contrary are known to retain kleptoplasts – that are released from phagosomes – in a similar fashion throughout starvation [19]. That is, StR do not appear to degrade kleptoplasts quicker than LtR species, but empirical evidence (for example based on $^{14}CO_2$ incorporation data) is currently lacking. Both, StR and LtR species, should have, in theory, the same "point of departure" when starvation commences and should have the same potential to make use of the kleptoplasts sequestered. Yet they do not. Notably, all LtR species have in common that cytosolic kleptoplast are left surrounded by two membranes only; they are those that constitute the canonical two membranes we are familiar with from land plant plastids [35–37]. This is even true for *E. chlorotica* that feeds on the stramenopile *Vaucheria*, but which houses complex plastids that in the alga are surrounded by four membranes [36,38,39]. That always these two membranes remain – the two that trace back to outer membrane and plasma membrane of the original cyanobacterial endosymbiont [37] – might hint at how, and in fact that, substrate and metabolites are actively exchanged between animal and plastid.

Darkness is more than the absence of photosynthesis

Although it is commonly accepted that the slugs benefit from photosynthesis, direct evidence is surprisingly scarce. Various studies on kleptoplastic slugs have analyzed survival rates or demonstrated that $^{14}CO_2$ is fixed by the acquired plastids [40–45]. To what degree the quantity of such carbon compounds is then physiologically relevant has, however, not yet been satisfactorily shown. Photosynthates sustaining the slugs might not be the sole necessity for sacoglossans to survive (long lasting) starvation periods. Note that for example the NR species *Costasiella nonatoi* survives starvation for about a month without showing any measurable PSII activity [25]. Similar observations have been made for *Elysia nigrocapitata* [46]. The question remains to what degree carbon fixation in sacoglossan slugs is needed to endure starvation (Fig. 3) and whether there is maybe another primary reason for starvation survival. If so, benefiting from functional kleptoplasts comes second.

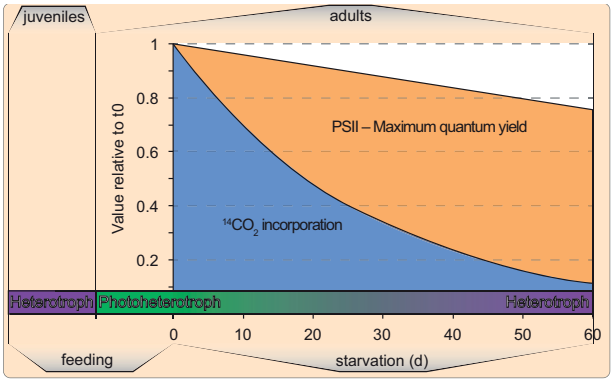

Fig. 3 Schematic overview of kleptoplast performance in LtR species. When the slugs hatch, they immediately need to feed on algal cytosol and begin to sequester their first plastids. During this phase, called transient kleptoplasty, juveniles are basically heterotrophic. Adult slugs are photoheterotrophic animals: they graze upon algae as long as these are available, but at the same time house CO_2-fixing kleptoplasts. When deprived of their food LtR species likely benefit from photosynthesis during the early phases of starvation, but the amount of incorporated carbon cannot sustain the animal. They switch back to heterotrophy through efficiently digesting their own tissue and degrading kleptoplasts. The latter is the reason why all starving LtR species are observed to shrink. Published CO_2 fixation rates for *C. ocellifera*, *E. viridis*, *P. ocellatus* and *E. timida* (blue curve; [14,22,40,43,69]) and photosystem II maximum quantum yield (F_v/F_m) values for *C. ocellifera*, *P. ocellatus* and *E. timida* (orange curve; [14,25]) of various studies on LtR species were pooled and plotted in relation to the values measured for freshly fed animals. The contrast between the two curves highlights that caution is warranted when kleptoplast productivity is evaluated on F_v/F_m values alone.

Previous studies reported the incorporation of photosynthetically fixed carbon (stemming from [^{14}C] bicarbonate) in a variety of slug metabolites [47,48]. Neglecting for now that we do not know enough about what photosynthate

supports the animal (and to what degree), it is important to ask: how do the slugs acquire products of photosynthesis? Kleptoplast-synthesized substrate will inevitably end up being metabolized, no matter whether that substrate was actively provided by an intact kleptoplast or whether it stems from an organelle degraded (e.g. through an autophagosome). It is important to determine which initial route the labeled CO_2 took to be incorporated into slug-specific metabolites. Microscopic analyses of *E. viridis* suggest that kleptoplasts that originate from *Codium fragile* accumulate substrate such as starch during starvation [49,50], which raises the question if any is actively secreted by the stolen organelles. Apart from any energy support the plastids might provide for the slugs, the animals might benefit from various other biochemical pathways the stolen plastids bring along [19,51]. It is also possible that photosynthates and kleptoplast-derived metabolites play a more crucial role for the proper development of juveniles than for the maintenance of mature adult slugs as recently suggested [14]. This is supported by recent observations on juveniles of *E. chlorotica* [52]. The data provided evidence with regard to the need of kleptoplast-derived lipid production for the proper development of the animals.

Animals are often kept in the dark, as a control for the slugs' dependency on photosynthates [52–54]. This is a good time to remember that the absence of light translates into more than just the absence of plastid photosynthesis. A few essential biochemical processes, such as the synthesis of vitamin D [55], occur in a light-dependent manner. Sacoglossans can synthesize a series of unique secondary metabolites including elysiapyrones [56] and tridachione [44] and it has been suggested that their synthesis is light dependent. Their synthesis could still be linked to the presence of kleptoplasts, but not photosynthesis. It has been hypothesized that an essential function of these polypropionates is linked to the quenching of ROS [56,57], hereby protecting the kleptoplasts' photosystems and/or the slugs' own tissue from oxidative stress.

Morphology does not equal function

The lateral foot expansions – the parapodia – are the morphological foundation from which the term "leaves that crawl" originates. In many plastid-bearing slugs the parapodia are intervened with numerous digestive tubules that harbor the green kleptoplasts; the wing-shaped structures are reminiscent of a leaf (Fig. 2). Some studies claim that the opening and closing of the parapodia is a controlled response to different light intensities, to either expose or shield the kleptoplasts to and from sunlight, respectively [58,59]. But these observations are to be interpreted cautiously [60]. There is no question that the position of the parapodia affects the amount of light reaching the kleptoplasts [61], but the response of some species is slow and it takes many minutes for the slugs to close the parapodia. Moreover, the LtR species *P. ocellatus* does not appear to alter the position of its parapodia at all. Even species that completely lack parapodia can retain functional plastids for a long time, as the case of the LtR species *C. ocellifera* demonstrates [25]. Parapodia are furthermore not limited to sacoglossan slugs. A wide range of heterobranchs have evolved parapodia that are used for various purposes including swimming and digging. In a nutshell, there is currently no evidence, or reason to assume, that sacoglossan parapodia have evolved as a consequence of housing kleptoplasts.

Two steps forward, one step back

Sacoglossan sea slugs puzzle researchers, as much as they fascinate. Ed Yong said it best when he commented on a recent analysis, which provided evidence that some adult slug species survive in the dark and do not loose weight faster than those kept in the light [14]: "Good science is about resisting the pull of easy conclusions. It's about testing stories that seem like they should be right to see if they actually are right. This is no easy task. Consider the case of the 'solar-powered' slugs" [62]. Several concepts have been put forward trying to explain how an organelle, adapted to plant cells, remains functional inside the cytosol of a eumetazoan cell. Two key concepts, slugs become photoautotrophs through kleptoplasty and kleptoplasts are supported by laterally transferred genes, are currently challenged [12,14,46], in particular the latter [12,13]. Research on the animal-plastid symbiosis in sacoglossan sea slugs is at a turning point. We know that some slugs sequester plastids through a sophisticated phagocytic mechanism whose details remain currently unknown. The kleptoplasts can continue to photosynthesize in the cytosol of the slugs' epithelial cells, but adults do not strictly depend on ongoing photosynthesis to survive starvation periods long-term. Laterally transferred algal genes do not support stolen plastids: the kleptoplasts' very own

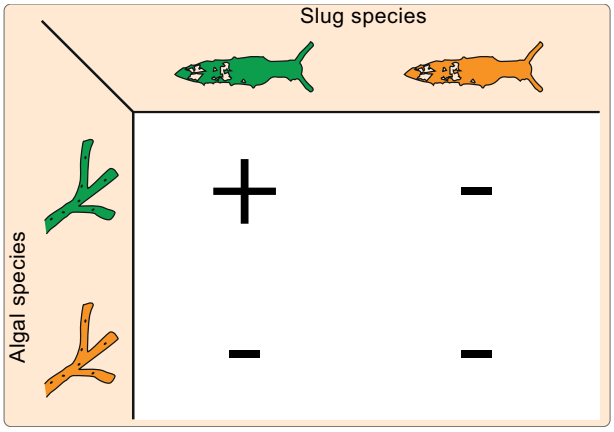

Fig. 4 Animal-plastid compatibility is determined by a two component system. In order to profit from functional kleptoplasts long-term (+), a slug species – component A – with an adapted physiology and digestive mechanism must acquire plastids from an algal species – component B – that naturally has robust plastids (both in green). The multifaceted factors that determine the compatibility of both partners in the plastid-slug system are yet to be discovered, but they could include dealing with plastid-derived toxins in case of the slugs and an adapted photobiology in case of the plastids. If any of the two partners lacks these requirements (orange), only short- or non-functional kleptoplast retentions are established (–).

biochemistry continues to function more independently than previously anticipated. From what we can tell, animal-plastid symbiosis depends on a combination of a robust plastid and a slug species whose physiology has evolved to tolerate (and likely service through substrate and metabolite exchange) an alien organelle (Fig. 4).

From the animals' perspective the following questions are hence key to better understand this unique symbiosis and how functional kleptoplasty has evolved multiple times in different slug species: (***i***) What are the benefits for the slugs next to carbon fixation? (***ii***) What underpins kleptoplast compatibility in the few species so far identified that house functional plastids for months? (***iii***) What are the molecular details of how plastids are recognized by the animals and released into the cytosol? A multidisciplinary approach will contribute to our understanding of how an organelle functions in a cytosol for which it did not evolve, and how some algae plastids have evolved to become as robust as they are. Through recent developments we now have the opportunity to make progress on this unique biological system of general evolutionary interest, for the purpose of better understanding plant animal symbioses.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: all authors have contributed equally to the review of literature. JV and SBG drafted the manuscript, whose final version was written and approved by all four authors.

Competing interests

No competing interests have been declared.

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Publication II: Switching off photosynthesis: the dark side of sacoglossan slugs

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Minor (drafted parts of the manuscript, helped in preparation of the figure, developed minor parts of the rationale)

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ARTICLE ADDENDUM

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Switching off photosynthesis

The dark side of sacoglossan slugs

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Sometimes the elementary experiment can lead to the most surprising result. This was recently the case when we had to learn that so-called "photosynthetic slugs" survive just fine in the dark and with chemically inhibited photosynthesis. Sacoglossan sea slugs feed on large siphonaceous, often single-celled algae by ingesting their cytosolic content including the organelles. A few species of the sacoglossan clade fascinate researcher from many disciplines, as they can survive starvation periods of many months through the plastids they sequestered, but not immediately digested – a process known as kleptoplasty. Ever since the term "leaves that crawl" was coined in the 1970s, the course was set in regard to how the subject was studied, but the topics of how slugs survive starvation and what for instance mediates kleptoplast longevity have often been conflated. It was generally assumed that slugs become photoautotrophic upon plastid sequestration, but most recent results challenge that view and the predominant role of the kleptoplasts in sacoglossan sea slugs.

Keywords: Sacoglossa, *Elysia*, kleptoplasty, photoautotrophy, photosynthesis, carbon fixation, photosynthetic slugs

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Our results[1] on the 2 slugs *Elysia timida* and *Plakobranchus ocellatus* came as a surprise to many and as such have generated quite some media attention: the article was featured in Nature, Scientific American, on a blog by Ed Yong at National Geographic, and many other sites such as science.org and phys.org. First of all, we need to stress that our findings do neither "*…disprove the idea that the slugs somehow derive energy from the photosynthesizing cells…*" nor that they are not "*solar-powered*" at all as some reports claim (phys.org and Ed Yong, respectively). Our results do, however, question the importance of photosynthesis for the so-called "crawling leaves"[2] and their survival during starvation. A critical examination of the topic generally highlights the importance to again think about statements such as "*…, after which plastids are able to support continued growth of the animal*" or "*…i.e. an animal (*E. chlorotica*) is able to sustain itself solely by photoautotrophic CO_2 fixation, as a plant.*" and "*…enabling their animal host to survive photo-autotrophically.*"[3-5, respectively] Juvenile slugs need to feed after hatching to establish stable kleptoplasty and adults are also observed to continuously feed as long as algae are available in the wild, demonstrating that, if anything these slugs are photoheterotrophic. Furthermore, we would argue that currently no single line of evidence for a single slug species exists that demonstrates the animals are phototrophic to a degree that allows the slugs to maintain, let alone gain, body mass through on-going photosynthesis and CO_2 fixation during prolonged starvation.

Costasiella ocellifera, Elysia viridis, *E. chlorotica*, *E. timida* and *P. ocellatus* have all been demonstrated to fix CO_2 in a light dependent manner.[1,6-8] But for how long during starvation, and is the amount of fixed CO_2 sufficient for them to really grow? While we currently have good evidence, based on pulse amplitude modulation (PAM) measurements, that the "light-dependent" reactions of the photosystem II continue to work inside the slugs[9-13] – in some cases for months – we lack the same kind of evidence for the "light-independent" reactions of the Calvin-Benson-Bassham (CBB-) cycle. In fact, the sparse

Figure 1. Sacoglossan slugs shrink during starvation. The image shows 2 individual *Plakobranchus ocellatus* specimens. The slug on the right was approximately of equal size, before it was starved for 6 months.

amount of data available suggests that the activity of the CBB-cycle declines rather rapidly. Experiments on *P. ocellatus*, *C. ocellifera*, *E. viridis* and *E. timida* show the CO_2 fixation rate decreases during the first 5–20 d to a rate comparable to that observed for slugs kept in the dark.[7,14-16] While for *P. ocellatus* the experiment was terminated after 27 d, *C. ocellifera* and *E. viridis* were kept alive for another 50 or 80 d, respectively, all while apparently not fixing CO_2. In *E. timida* the CO_2 fixation rate after 5–10 d of starvation had declined to the level measured for *Thuridilla hopei* for comparison, and whose kleptoplasts loose their photosynthetic ability over the first few days of starvation.[16] Should this be confirmed, through experiments dedicated to investigating CO_2 fixation rates over time, we will need to find an explanation of what happens with the ATP and $NADPH^+$ generated by the light-dependent reaction. During the early phases of starvation CO_2 fixation is probably advantageous for the slugs, but suggesting these animals to survive starvation because they are photoautotrophic is currently not supported by the data available. This would, at the very minimum, require evidence for on-going CO_2 fixation and the perpetuation of body mass during starvation.

Our results demonstrate that starving *E. timida* and *P. ocellatus* shrink and loose weight in the absence of fresh food (see also **Fig. 1**), whether being able to photosynthesize or not.[1] This was also observed by West,[17] who, for *E. chlorotica*, concluded that "*Statistical analysis of the growth experiment demonstrated that light intensity is not important to the size*." Klochkova and colleagues[18] questioned the importance of the kleptoplasts' remaining photosynthesis in *E. nigrocapitata*, since under natural light conditions they "*may not properly function in the natural habitat for a long time without recruit of new chloroplasts*". This not only contradicts the 'photoautotrophic concept', but also raises the question of the kleptoplasts true purpose.

Our current working hypothesis is that in adult slugs kleptoplasts are primarily stored as a rich nutritional source, but it is further noteworthy to mention that viewing plastids as organelles that fix only CO_2 to transform it into 'sugar cubes', whose energy is then available to the host, oversimplifies the biochemistry of the organelle. Their biochemical properties further include, but are not limited to, fatty acid, iron-sulfur cluster and amino acid synthesis.[19] Hence, the interaction of kleptoplasts and slug is more complex than it appears at first sight. Photosynthesis might still be beneficial for the animals, but exactly how and when remains to be determined. To distinguish the importance of light for the animals development from photosynthesis presents a further challenge, as the slugs likely require light, like most animals including us humans do, for their normal development and living.

The last few years have reminded us not to judge a book by its cover, nor a slug just by its color. New data forces us to become more critical about what we think we know, and reconsider the prime role of kleptoplasts in greenish sacoglossan slugs. In any case, we should not refer to these slugs as photoautotrophic animals, but determine for how long and to what degree they are phototrophic during starvation. Notwithstanding, the most previous results do not belittle the phenomenon of kleptoplast longevity and recent data suggests that the genomes of the plastids being sequestered might hold the key after all.[20] Their higher genetic autonomy in comparison to land plant plastids might translate into a better servicing of damaged photosystem II, hence less leakage of reactive oxygen species. In turn this might allow the slugs to store their kleptoplasts for longer periods of time, as their degradation through ROS-induced autophagy[21] is postponed. Sacoglossan slugs and their robust plastids remain a fascinating and rich field to study, presenting ever more avenues for future research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Publication III: Comparison of sister species identifies factors underpinning plastid compatibility in green sea slugs

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Contribution of Jan de Vries, first author:
Major (drafted the manuscript, prepared five figures and the majority of the supplemental content, developed parts of the rationale, performed the majority of the lab work and parts of the analysis)

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Comparison of sister species identifies factors underpinning plastid compatibility in green sea slugs

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The only animal cells known that can maintain functional plastids (kleptoplasts) in their cytosol occur in the digestive gland epithelia of sacoglossan slugs. Only a few species of the many hundred known can profit from kleptoplasty during starvation long-term, but why is not understood. The two sister taxa *Elysia cornigera* and *Elysia timida* sequester plastids from the same algal species, but with a very different outcome: while *E. cornigera* usually dies within the first two weeks when deprived of food, *E. timida* can survive for many months to come. Here we compare the responses of the two slugs to starvation, blocked photosynthesis and light stress. The two species respond differently, but in both starvation is the main denominator that alters global gene expression profiles. The kleptoplasts' ability to fix CO_2 decreases at a similar rate in both slugs during starvation, but only *E. cornigera* individuals die in the presence of functional kleptoplasts, concomitant with the accumulation of reactive oxygen species (ROS) in the digestive tract. We show that profiting from the acquisition of robust plastids, and key to *E. timida*'s longer survival, is determined by an increased starvation tolerance that keeps ROS levels at bay.

1. Introduction

Some sacoglossan slugs can house functional plastids for months in the cytosol of cells that line the animals' digestive tubules. The slugs steal the plastids (kleptoplasts) from siphonaceous algae upon which they feed. Theory has it that the presence of functional kleptoplasts [1,2] allows some of the sea slug species to survive starvation periods that can last almost a year [3,4]. Recent reports question the categorical importance of photosynthesis during starvation for several species [5,6] and while the animals fix CO_2 in a light-dependent manner [5,7–10], for how long and how much during starvation is not well documented. Animals induce autophagy to reallocate resources through the recycling of tissue when facing starvation [11] and mitochondria-generated reactive oxygen species (ROS) signalling has been found to be a key mediator of autophagy triggered by nutrient deprivation [12,13]. Over the past 20 years, research on 'photosynthetic (green) slugs' has focused on understanding how the stolen plastids can remain functional in a foreign cytosol [14], but the molecular response to starvation has not, to our knowledge, been assessed in sacoglossan slugs until now.

The kleptoplasts' stability outside of the algae disagrees with our knowledge about the sensitivity of land plant plastids, whose photosystem II is highly

susceptible to photosynthesis-associated damage [2,15,16]. Recent works [16,17] are reviving old observations that demonstrated some algal plastids are naturally more robust than land plant plastids [18]. Kleptoplast longevity therefore appears an intrinsic property the stolen organelles bring along, and depending on the slug species this results in different modes of kleptoplast retention and lengths of survival during starvation [2,16]. It is generally suspicious that among 300 sacoglossan species that feed on siphonaceous algae, only seven species are known to maintain functional kleptoplasts and survive long term [2,19,20].

The two congeneric species *Elysia cornigera* and *Elysia timida* both feed on the ulvophycean alga *Acetabularia*, but only *E. timida* tolerates prolonged starvation [2,21]. Why? We compared the two sister taxa by monitoring their kleptoplasts' photosynthetic capacity and characterizing the slugs' physiological response to starvation focusing on gene expression modulation and ROS development. Our results indicate that algal cytosol are the slug's main food source and furthermore demonstrate that the plastid-bearing slugs' capacity to endure prolonged periods of starvation is not determined by the photosynthetic activity of their kleptoplasts, but by how they have evolved to respond to starvation, in particular the ability to cope with ROS.

2. Results and discussion

(a) Performance of stolen plastids does not depend on the slug species

We fed *E. timida* and *E. cornigera* solely on *Acetabularia acetabulum* DI1 from the day they hatched in our laboratory. After eight and six weeks, respectively, and at which time the animals had reached maturity, the slugs were separated from the alga and routinely kept at 25 µmol quanta $m^{-2}\,s^{-1}$ for 12 h every day. All sacoglossans that starve are observed to shrink [10,22] and the same was true for our two species. Yet, *E. cornigera* decreased in size more rapidly than *E. timida* (figure 1*a*) and rarely survived more than two weeks without food, which is consistent with previous reports [19,21]. Loss in size was accompanied by a slow fading of the animals' greenish coloration owing to the loss of the pigments chlorophyll (Chl) *b* and, most prominently, Chl *a* (electronic supplementary material, table S1). In *E. cornigera*, pigment concentration declined from starvation day 2 to 4 by 32.9%, but then increased again for a few days in relation to the dry weight of the animals (figure 1*b*). At day 10, the Chl *a* and *b* concentration had dropped from 3109 to 1544 pmol mg^{-1} animal dry weight (49.7%). These values correlate with the rapid decrease observed in body size between day 4 and 7 of starvation (figure 1*a*). In *E. timida*, we observed a similar, yet less steep, decline in the concentration of pigments in relation to body mass over the 10 days of starvation measured (from 2193 to 1606 at day 4 (73.2%) and 1638 pmol Chl $a + b$/mg animal dry weight at day 10 (74.7%); figure 1*b*). The increase of photosynthesis-associated pigments around day 6 demonstrates that the animals we measured metabolize their own tissue at an apparently faster rate than the kleptoplasts degrade, which was also observed for starving juveniles of *E. chlorotica* [23].

Photosynthetic capacity was determined through measurements of photosystem II quantum efficiency (derived from Chl fluorescence analyses determined by pulse-amplitude modulation (PAM) fluorometry) and the amount of incorporated $^{14}CO_2$. Freshly fed slugs showed a similar initial maximum quantum yield (F_v/F_m of 0.83 ± 0.03 for *E. cornigera* and 0.85 ± 0.03 for *E. timida*). Measured F_v/F_m of *E. timida* declined slowly (-0.001 F_v/F_m per day starved) over the one-month period, reaching 0.77 ± 0.07 at day 30. In *E. cornigera*, the decline of F_v/F_m was more prominent (-0.008 F_v/F_m per day starved) over the 10 days measured, dropping to 0.75 ± 0.1, at which point the species began to die (figure 1*c*). At the same time, the amount of $^{14}CO_2$ incorporation had dropped to 34% ($\pm 15\%$) and 36% ($\pm 12\%$) in *E. cornigera* and *E. timida*, respectively, after 10 days of starvation (figure 1*c*). At that point, the F_v/F_m had dropped to 0.84 ± 0.03 and 0.75 ± 0.1 for *E. timida* and *E. cornigera*, respectively. After 30 days of starvation, the amount of $^{14}CO_2$ fixed in *E. timida* had dropped to $4 \pm 1\%$ in regard to the initial amount. In any case, after 10 days of food deprivation *E. cornigera* died in the presence of intact (electronic supplementary material, figure S1) and functional kleptoplast. The difference of profiting from kleptoplasty must depend on the animals' tolerance to starvation, not the stolen organelles' performance. This observation challenges the idea that kleptoplasts could act as food depot [5,10], and raised the question, why in particular *E. cornigera* dies in the presence of functional kleptoplasts.

(b) Global gene expression response is predominantly governed by starvation

To uncover the differences of the two species in the response to starvation, and test whether the slugs can to some respect sense the kleptoplasts' status, we analysed gene expression changes throughout starvation and under different environmental stimuli. We performed comparative transcriptomics of the slugs: (i) under starvation (S), (ii) under starvation and in the presence of the photosynthesis inhibitor drug monolinuron (S + M), and (iii) under starvation and including a daily bleaching pulse with 1000 µmol quanta $m^{-2}\,s^{-1}$ for 1 h d^{-1} (S + B). A total of 10 857 contigs for *E. cornigera* and 11 152 contigs for *E. timida* were assembled that were supported by at least 100 reads and homologous to eumetazoan sequences (electronic supplementary material, figure S2 and table S2). Global gene expression trends were confirmed for all conditions on six individual genes using quantitative reverse-transcription PCR (qRT-PCR) (electronic supplementary material, figure S3).

If the slugs have evolved to depend on the photosynthetic capacity of their kleptoplasts, we would expect to see a strong difference in the regulation of global gene expression profiles when kleptoplast electron transport is blocked (S + M) or when the kleptoplasts are under high light stress (S + B). Yet, when analysing the co-regulation of gene expression we found that starvation (S) in comparison to feeding (F) was the main denominator for the transcription response in *E. cornigera* (figure 2). The additionally applied treatments (S + B, S + M) only had marginal effects on the global expression profile of *E. cornigera* compared to starvation alone (S). In contrast to this, the response of *E. timida* throughout the first two weeks of starvation is more differentiated in regard to the individual stimuli and in comparison to *E. cornigera* (figure 2). After 4 d of S + M and 7 d S + B, the most differing expression was detected, indicating that *E. timida* indeed responded to the status of their kleptoplasts. Intriguingly, for *E. timida* a similar yet delayed transcriptional

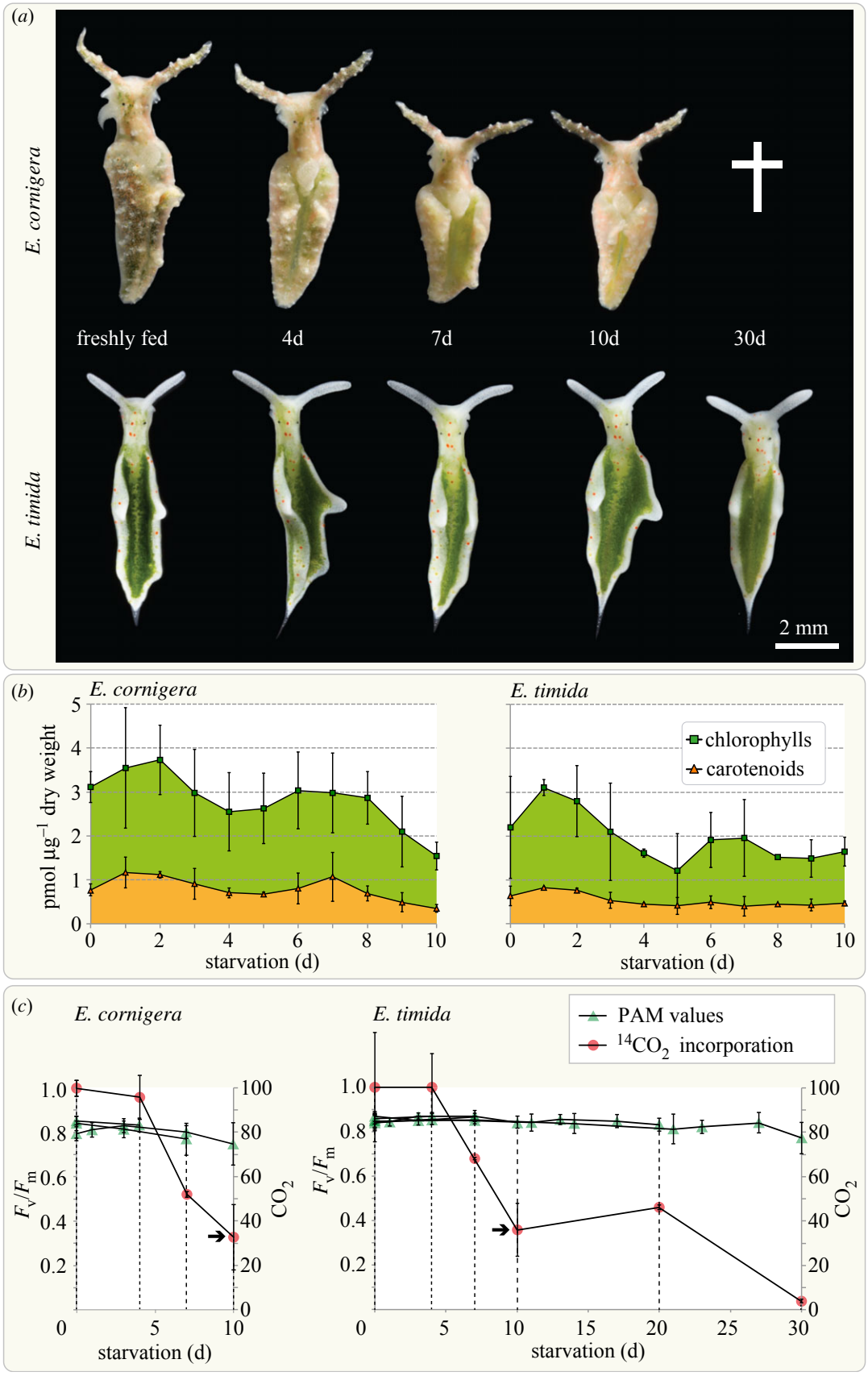

Figure 1. *Elysia cornigera* dies while showing comparable photosynthetic activity to *E. timida* during starvation. Adult slugs were fed on *Acetabularia acetabulum* and the effects of starvation at 25 µmol quanta m^{-2} s^{-1} on the photosynthetic capacity of both species evaluated. (*a*) Representative images of one single specimen of each species. *Elysia cornigera* shrinks more rapidly than *E. timida*, and while the *E. cornigera* specimens never survived starvation for more than two weeks, all *E. timida* specimens survived and retained an almost equal body length during the 30 d of starvation analysed. (*b*) Analysis of pigment concentration in relation to dry weight of the slugs during the first 10 days of starvation. Quantification was performed on three biological replicates per time point and normalized to an external standard; each pigment extract was derived from three to seven *E. cornigera* (in total 143 individuals) and two *E. timida* individuals (in total 66 individuals). (*c*) Assessment of the maximum quantum yield (F_v/F_m; measured using a PAM fluorometer) of photosystem II and net $^{14}CO_2$ fixation relative to freshly fed animals (t_0), during starvation within the same group of individuals. Each single F_v/F_m curve represents measurements on a group of at least 12 *E. cornigera* and nine *E. timida* specimens; from each group four *E. cornigera* (in total 48 individuals) and three *E. timida* (in total 54 individuals) were used for determining the $^{14}CO_2$ incorporation after 0, 4, 7, 10, 20 and 30 days of starvation (latter two only for *E. timida*).

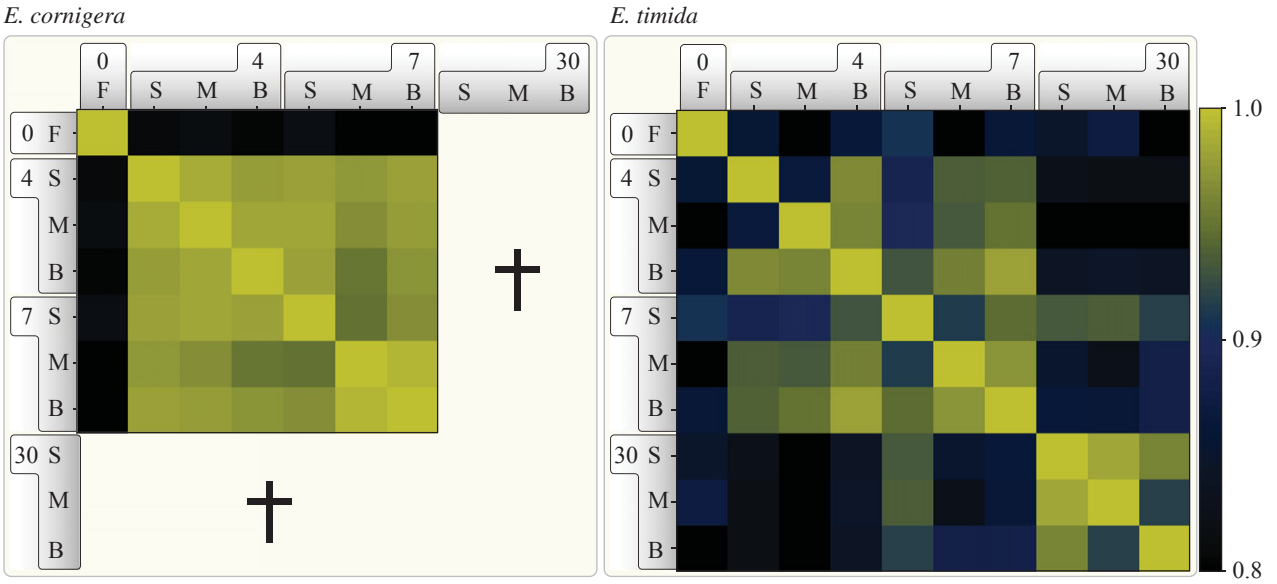

Figure 2. Global co-expression analysis of gene expression changes. Pearson correlations were calculated by comparing the FPKM (fragments per kilobase of exon per million reads mapped) values of filtered genes of the different samples. Note the clear separation of the control and the remaining conditions and for *E. timida* the shift between the time points of 4 and 7 days in comparison to 30 days. The colour key indicates the degree of co-regulation. The bold numbers represent time of treatment in days. No values for *E. cornigera* were obtained for 30 days, because all individuals had succumbed to starvation (indicated by a cross). F, freshly fed; S, starving; M, monolinuron; B, bleach.

response to starvation was observed, as transcriptional profiles at day 30 began to reflect those observed for *E. cornigera* earlier during starvation based on the correlation of co-regulated genes within each species (figure 2). The slugs predominantly respond to starvation and not to the photosynthetic capacity of the kleptoplasts.

(c) Host response underpins kleptoplast compatibility

When mapping the transcriptomes to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (figure 3), two main trends were observed: first, downregulation of carbohydrate, energy, and lipid metabolism associated pathways (mean $\log_2$FC of -0.32 versus an overall mean $\log_2$FC of -0.10). On average, 81 of the 89 metabolism pathways detected for *E. timida* were downregulated after 30 days of starvation. Monolinuron, blocking the quinon-binding site of the D1 protein [24], enforced the downregulation of core metabolic genes in both species (at 7 d S + M $\log_2$FC -0.44 in both). Second, in contrast to *E. timida* (mean $\log_2$FC of -0.11), starvation in *E. cornigera* triggered an elevated expression of environmental information processes and cellular and organismal systems including stress response and autophagy (mean $\log_2$FC 0.13). Light stress further promoted this reaction in *E. cornigera* (mean $\log_2$FC of 0.20 at 4 d/7 d S + B) compared to *E. timida* (mean $\log_2$FC of -0.03 at 4d/7d S + B). Starvation imposes a metabolic dormancy and gene expression profiles to a minor degree reflect kleptoplast manipulation. The activity of the kleptoplasts, however, does not explain the survival during starvation.

Ubiquitination is a key mechanism to tag organelles and misfolded proteins—accumulating under stress conditions such as starvation—for degradation [25,26]. Expression levels of polyubiquitin remained the same in both species, except for a single polyubiquitin homolog of *E. timida* (Eti000028, 4.5-fold upregulation, $p < 0.05$). For the ubiquitin binding autophagic receptor p62/SQSTM1 [27], an average of only 28 FPKMs (fragments per kilobase of exon per million reads mapped) were identified for *E. cornigera*, but 659 FPKMs for *E. timida*. And only for the latter species an upregulation of *p62/SQSTM1* was observed during starvation (electronic supplementary material, table S3). *Elysia timida* might be better equipped to detect damaged proteins and organelles than *E. cornigera*, allowing the former to better recognize and remove damaged organelles and misfolded hazardous proteins during starvation. The ability to recognize damaged kleptoplasts for their subsequent degradation could explain how the slugs profit from stored kleptoplasts. Only malfunctioning and ROS-leaking plastids would be detected by the autophagosomal machinery and removed; the profit of kleptoplasts would be an indirect one. This would also explain the more rapid decline of F_v/F_m values in *E. cornigera* in comparison to *E. timida*, while retaining the same CO_2 fixation rate (figure 1*c*). Accumulation of damaged plastids that fix no CO_2 would lead to a lower F_v/F_m ratio in *E. cornigera*. If *E. timida* specifically removes damaged plastids, then all malfunctioning photosystems are excluded from that equation.

We found that the upregulation of genes encoding ROS scavengers differed between the two sister taxa. With progressing starvation, *E. cornigera* elevated the expression of superoxide-dismutase homologs regardless of the treatment (e.g. Eco000152; on average 3.1-fold up at day 7, $p < 0.05$). Apparent changes in the expression of catalases were only detected for *E. timida* in the first days of starvation (Eti001444; up more than twofold on average). It was recently noted that a crucial part of the slug-plastid symbiosis is evolving mechanisms to cope with kleptoplast-associated toxins [2]. A difference in stress response and ROS evolution could explain the different survival rates of the two species observed during starvation.

(d) *Elysia cornigera* accumulates reactive oxygen species, not *Elysia timida*

ROS are known as signalling molecules [13] but also as deleterious cytotoxins [28]. To monitor hydrogen peroxide

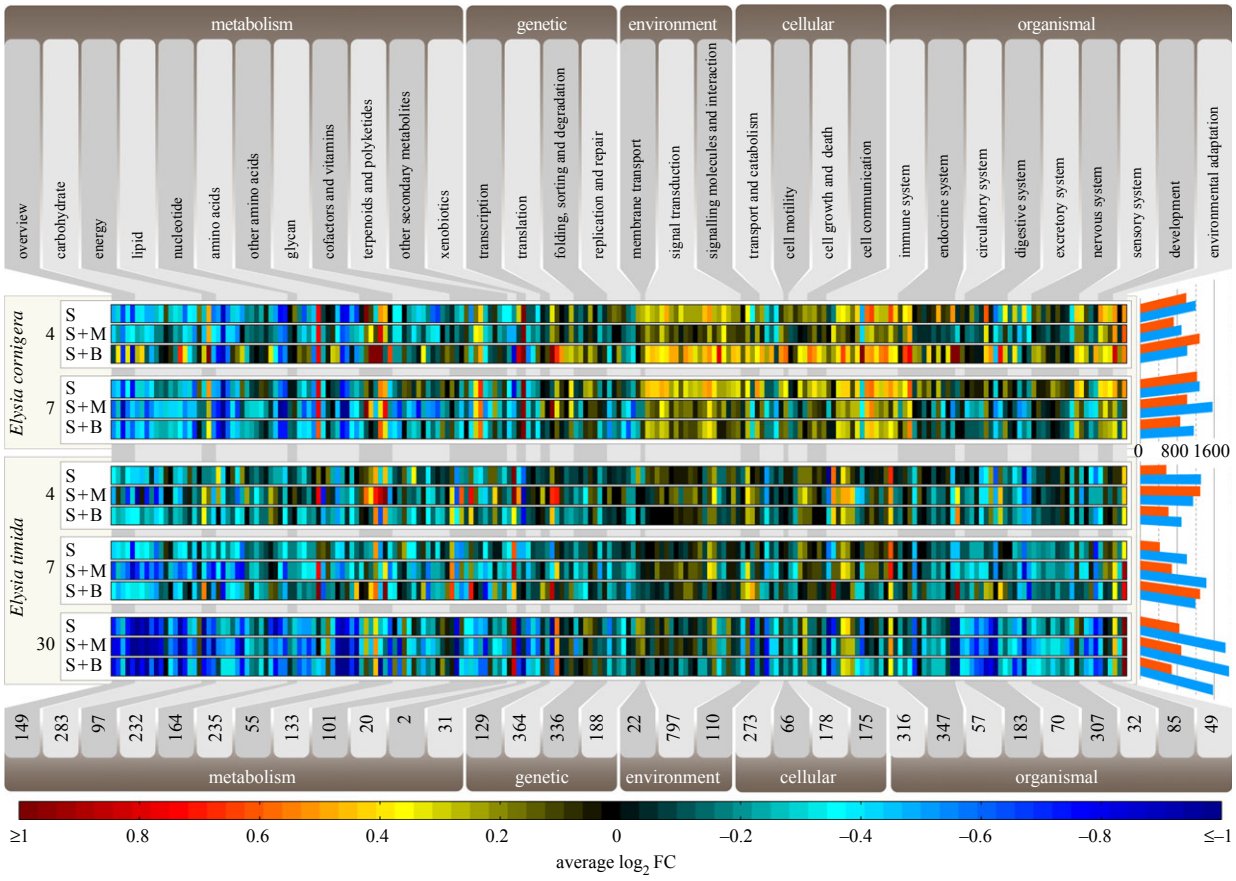

Figure 3. Gene expression changes of animal KEGG pathways. Transcriptomes were sequenced for both animals after 4 and 7 days of starvation (bold numbers), and additionally after 30 days for *E. timida*. *Elysia cornigera* and *E. timida* were subjected to three different conditions: starvation alone (S), starvation plus monolinuron (S + M; 2 µg ml^{-1}) and starvation plus bleaching (S + B; 1000 µmol quanta m^{-2} s^{-1} for 1 h each day). Upregulation is shown from red to black and downregulation from black to blue. The numbers at the bottom represents the amount of K-numbers (KEGG orthology group identifier) found within each reaction module (modules in alternating light and grey flags). The bars on the right show the total number of regulated genes (minimum of twofold) for the individual conditions analysed (blue bars, downregulation; red bars, upregulation).

(H_2O_2) production on tissue level we used 2′,7′-dichlorofluorescin (DCF). Freshly fed slugs showed no detectable amount of H_2O_2 (electronic supplementary material, figure S4), but at day 10 of starvation H_2O_2 was most prominently detected in *E. cornigera* within the lumen of the digestive tubules (figure 4*a*,*e*). Both the lumen and the epithelium of the digestive tubules—the localization of the kleptoplasts—showed intensive DCF staining (figure 5). By contrast, DCF staining in *E. timida* occurred only in some globular structures of 10–20 µm in diameter, but of otherwise unknown nature, within individual digestive tubules (figure 4*g*, arrowhead). After 30 d of starvation, the digestive glandular tubules of *E. timida* still showed no apparent increase in H_2O_2 levels (electronic supplementary material, figure S4), but DCF now additionally stained globular structures of approximately 100 µm in diameter that were situated between the epidermis and the digestive tubules, and independent from the latter. These structures could represent one of the secondary metabolite-harbouring vacuoles typical for these kind of slugs [29]. Stroma of the *A. acetabulum* kleptoplasts resting within the digestives tubule cells of both *E. cornigera* and *E. timida* showed equal intensity in regard to H_2O_2 levels (figure 4*c*, *f* insert). Mitochondria, a common source of ROS [13], were found to accumulate H_2O_2 as proved through the co-localization of the DCF signal with MitoTracker Red (figure 4*d*). Hydrogen peroxide, a critical ROS in terms of cytotoxicity [28], accumulates only in *E. cornigera*.

Animals respond to nutrient depletion by autophagy, and superoxide ($O_2^{\cdot-}$) is a key signal that triggers the autophagosomal machinery [12,13] and mammalian tissues cultures show strong increase in ROS after a few hours of starvation [12]. We conducted nine separate experiments, each time with different individuals, and monitored $O_2^{\cdot-}$ evolution using dihydroethidium (DHE) which stains the nuclei of cells that experience elevated levels of $O_2^{\cdot-}$ [30]. Chl autofluorescence was observed to decrease, but not completely fade, in both species throughout the 10 d monitored (figure 6*a*). This concurs with the pigment measurements and the ability of the remaining kleptoplasts to incorporate $^{14}CO_2$ (figure 1*c*). Feeding slugs showed only faint $O_2^{\cdot-}$ signals towards the edges of the parapodia, especially the epidermis (figure 6*a*). Staining did not predominantly dye nuclei of the plastid-bearing digestive gland cells, but instead those of surrounding and connective tissue of the parapodia. Levels of $O_2^{\cdot-}$, apparent through the number of nuclei stained and the intensity of the staining itself, steadily increased throughout starvation and predominantly in *E. cornigera* (figure 6*a*). While both slug species harboured functional kleptoplasts stemming from the same source, only *E. cornigera* exhibited increasing levels of $O_2^{\cdot-}$ that were significant, but not *E. timida* ($p < 0.001$). *Elysia cornigera* accumulates ROS to a much higher degree than its sister taxon (figure 6*b*), reflecting the different capacities of the two species with regard to managing starvation in the presence of functional kleptoplasts.

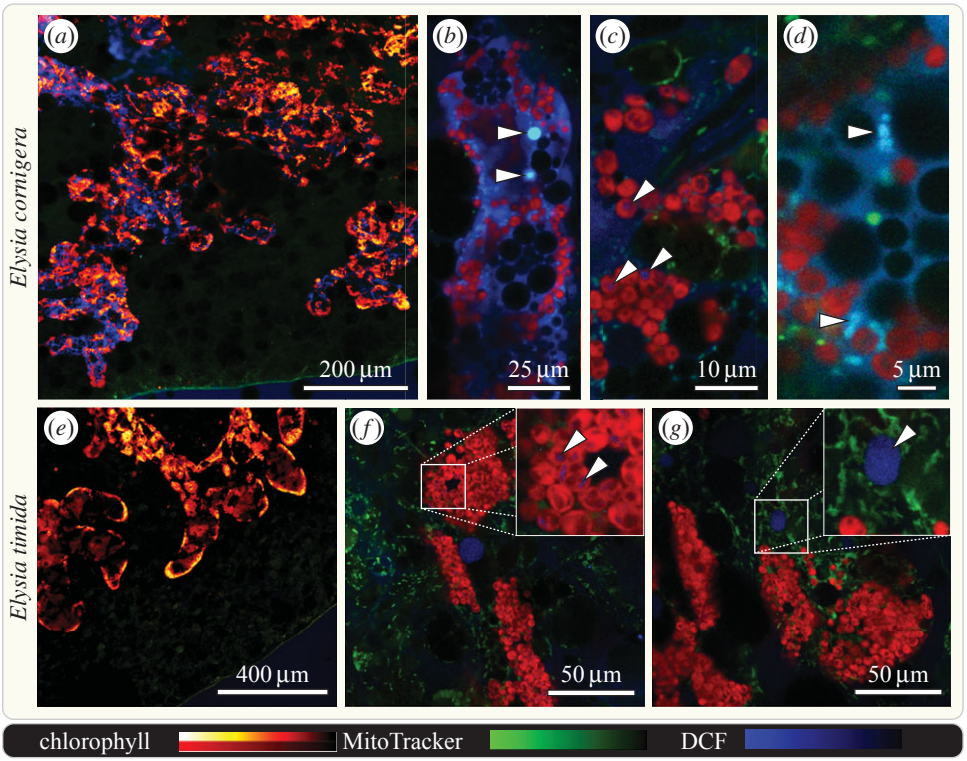

Figure 4. Hydrogen peroxide accumulates in the digestive tubules of starving *E. cornigera*, but not *E. timida*. Confocal laser scanning micrographs of the kleptoplast-bearing digestive tubules that pervade the parapodia of 10 days starved, whole mount *E. cornigera* and *E. timida* stained with 100 µM dichlorofluorescin (DCF; blue) and MitoTracker Red (green); chlorophyll autofluorescence in red to yellow (*a*,*e*) or red only (*b*–*d*, *f*, *g*). Digestive tubules of *E. cornigera* (lined with autofluorescent kleptoplasts) show well-defined DCF staining within the lumen and epithelium (*a*–*d*). Details of single digestive tubules revealing readily H_2O_2 accumulators of equal size as kleptoplasts (*b* arrowheads), kleptoplasts displaying weak DCF staining (*c*; see arrowheads) and MitoTracker Red co-localizing with DCF staining (*d*). Digestive tubules of *E. timida* displaying no distinguishable DCF signal in epithelium or lumen (*e*). Details of single digestive tubules revealing accumulating weak levels of H_2O_2 (*f*, arrowheads), comparable to those seen in *E. cornigera* (*c*, arrowheads) and showing globular structures of unknown nature accumulating H_2O_2 (*g*, arrowhead).

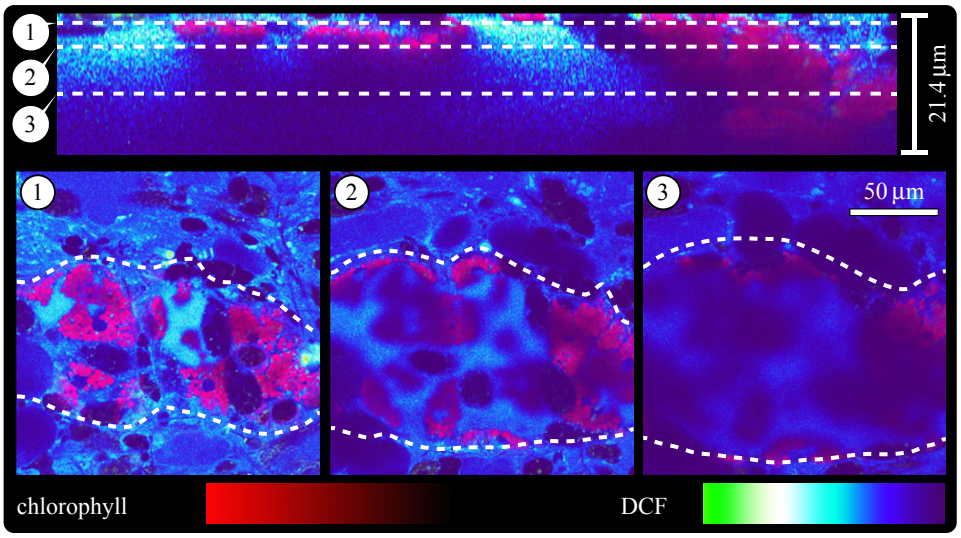

Figure 5. Hydrogen peroxide is present in the lumen and epithelium of digestive tubules of starving *E. cornigera*. Orthogonal view of a confocal laser scanning *z*-stack micrograph reconstructed from 58 individual optical sections (21.35 µm of depth in total) of a single digestive tubule (outlined by dashed lines in the bottom panel) of 10d starved *E. cornigera* stained with 100 µM dichlorofluorescin (DCF; purple-blue to green gradient); chlorophyll autofluorescence in red. At the top, a transverse section trough the stack is shown to illustrate at which level the three coronal sections (indicated by dashed lines) were captured.

3. Conclusion

Contrary to the current view our study demonstrates that the presence of functional kleptoplasts alone does not underpin the survival of green photosynthetic slugs. It is first and foremost the ability of some species to tolerate starvation. The best hints we currently have that underpin starvation tolerance include the upregulation of a set of canonical ROS quenchers such as catalases, and/or slug-specific polypropionate metabolites such as Elysiapyrone or Elysione [31], and/or the efficient and specific degradation of damaged kleptoplasts. Literature has divided slugs into non-, short- and long-term retention species, depending on for how

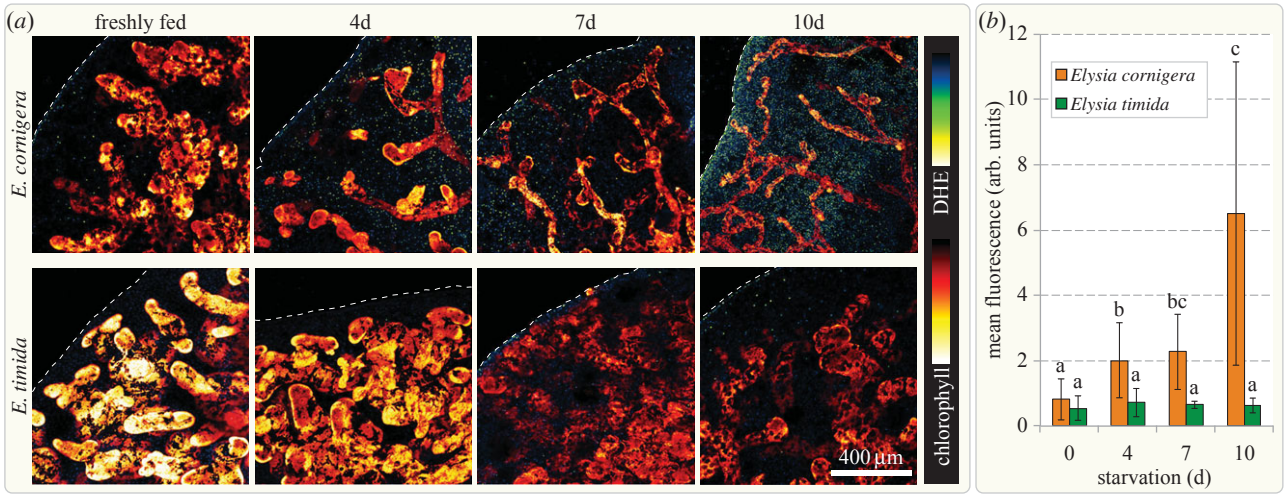

Figure 6. Superoxide accumulation in starving slugs. (*a*) Confocal laser scanning micrographs of the kleptoplast-bearing digestive tubules from similar positions on the parapodia of freshly fed and 4, 7 and 10 days starved, whole mount *E. cornigera* and *E. timida* (optical sections; pinhole set to 1 airy unit) stained with 100 µM DHE (staining $O_2^{\cdot-}$), shown in blue-yellow gradient; chlorophyll autofluorescence of the kleptoplasts is shown as red to yellow glow. Dashed lines mark the outer rim of the parapodia. DHE signal increases over time in *E. cornigera*, while the diameter of the digestive tubules decreases, visible due to their lining with kleptoplasts. Superoxide in the parapodia of *E. timida* does not significantly increase over time, while kleptoplasts lining the digestive tubules appear less densely packed. (*b*) Quantification of the mean DHE fluorescence intensity in the parapodial tissue; significance groups (for (*a*,*b*) $p < 0.01$; (*c*) $p < 0.05$) were determined using a Mann–Whitney *U*-test; measurement of fluorescence intensity was performed on greater than or equal to six individual specimens for each time point; error bars indicate standard deviation.

long kleptoplasts are functionally retained [19]. Our results demonstrate that it is equally important to divide the slugs into starvation-intolerant and starvation-tolerant species. That tolerance might be associated with the habitat and availability of the food source. *Elysia cornigera* was collected from the Caribbean Sea, but *E. timida* from the Mediterranean. Maybe only *E. timida* experiences food shortage due to seasonal differences and a difference in the calcification of individual algal species, and had to evolve accordingly. In any case, sacoglossan research has focused on how the animals chaperone their kleptoplasts, but it should just as much focus on how they manage starvation, because it will teach us on how they suppress ROS stress.

4. Experimental procedures

Elysia timida was collected on Giglio, Italy (42°22′ N, 10°52′ E and 42°21′ N, 10°52′ E) between 3 and 6 m depth. *Elysia cornigera* was collected at the Florida Keys (24°38′ N, 81°18′ W) at about 1 m depth. Both species were kept at a 12 L : 12 D rhythm at 25 µmol quanta $m^{-2} s^{-1}$ in 3.7% salt water (Tropic Marine) and at 21°C. *Acetabularia acetabulum* DI1 (isolate of Professor Diedrik Menzel, Bonn University, Germany) was grown at the same conditions as the animals, but in algal f/2 medium. For starvation experiments, animals were moved to Petri dishes (Ø200 mm) and 50% of the water was changed every other day. Monolinuron (JBL GmbH) was added from a stock solution of 4000 mg l^{-1} to a final concentration of 2 µg ml^{-1}. Bleaching was performed by illuminating the slugs with 1000 µmol quanta $m^{-2} s^{-1}$ for 1 h every day at the same time. For each pigment extraction, pooled slugs were transferred to 90% acetone for each time point, homogenized and kept at −20°C for 1 day. Extractions for every time point were carried out as biological triplicates, each replicate consisting of at least three *E. cornigera* and two *E. timida*. Crude extracts were filtered through a 200 nM polytetrafluoroethylene membrane and then analysed by reversed-phases high pressure liquid chromatography with ultraviolet/visible spectroscopy detection (Hitachi/Merk) as described earlier [32]. Pigment concentrations were determined using external pigment standards isolated from spinach thylakoids. Dry weight was determined on dried slug homogenate after extraction.

Photosystem II maximum quantum yield (F_v/F_m) was determined using a WALZ MINI-PAM as described previously [33], on the same group of individuals that were used for $^{14}CO_2$ incorporation measurements. For every time point for which $^{14}CO_2$ incorporation was determined, an individual group of slugs (at least 12 *E. cornigera* or nine *E. timida*) was kept in isolated dishes. For each of these groups, separate F_v/F_m measurements were performed, and each individual slug of a group was measured at least three times. For each data point, the mean of all individual triplicate measurements was calculated. The light-driven incorporation of $^{14}CO_2$ was determined as described earlier [5]. Briefly, for each triplicate measurement four individuals of *E. cornigera* and three of *E. timida* were used. Slugs were incubated in 1.2 ml artificial seawater supplemented with 0.40 mM [^{14}C]-$NaHCO_2$ (25 µCi per incubation, NEN-radiochemicals, MA, USA) for 2 h at room temperature and 72 µmol quanta $m^{-2} s^{-1}$ illumination. After rinsing, homogenization and acidification of the material with 150 µl 1 M HCl, all the substrate was expelled from the homogenate. Incorporation of ^{14}C by the slugs was determined in a scintillation counter after the addition of 12 ml LUMA-Gel scintillation cocktail (LUMAC, The Netherlands).

For ROS-imaging, slugs were incubated for 45 min with 100 µM DHE (DHE; excitation/emission HeNe 543/610 nm; Sigma) or 100 µM DCF (2′,7′-dichlorofluorescin; excitation/emission Argon 488/529 nm) plus 2 µM MitoTracker Red CMXRos (excitation/emission HeNe 543/599 nm; LifeTechnologies) in artificial seawater. Slugs were rinsed twice with seawater and decapitated before mounting. Confocal laser scanning microscopy was carried out using a Zeiss LSM 710. Imaging was always performed with the same settings and

at a similar position along the parapodial rim at the same depth relative to the epidermis. Images were processed using Fiji/ImageJ 1.48f [34] and statistics were performed using R [35]. Normality was tested via a Shapiro–Wilk test [36] and significance using a Mann–Whitney *U*-test [37].

Total RNA was extracted three times from seven *E. cornigera* (a total of 21) and three times from five *E. timida* (a total of 15) feeding on *A. acetabulum* (t_0, feeding control group), 28 individuals of *E. cornigera* that were starved for 4 days, of which nine individuals had only starved (S), nine had starved and were treated with monolinuron (S + M), and 10 of which had starved and were treated with 1000 µmol quanta m^{-2} s^{-1} for 1 h each day (S + B). The RNA of 20 *E. timida*'s (7 S, 7 S + M, 6 S + B) was isolated after four days of starvation (t_1), of 30 *E. cornigera* (10 S, 9 S + M, 11 S + B) and of 20 *E. timida* (6 S, 8 S + M, 6 S + B) after 7 days of starvation (t_2), and finally of 32 *E. timida* (11 S, 10 S + M, 11 S + B) after 30 days of starvation (t_3). RNA was isolated using TRIzol (Life Technologies) according to the manufacturer's protocol and an additional DNAse treatment (Thermo Fisher Scientific). Poly(A) mRNA enrichment, library preparation using the TruSeq kit (Illumina) and 100 bp paired end sequencing using the Illumina HiSeq2000 system was performed by the Beijing Genome Institute (Hong Kong). Reads with remaining adapter sequences, more than 5% of unknown nucleotides or more than 20% of nucleotides with quality scores less than 10 (Illumina 1.5) were removed as part of the raw data extraction.

A total of 1 186 405 486 reads (a minimum of 52 million reads/library) were obtained (electronic supplementary material, table S2). Reads were inspected by FastQC v. 0.10.1 [38] and filtered and trimmed using Trimmomatic 0.32 [39] (parameters: ILLUMINACLIP:TruSeq3-PE.fa:2 : 30 : 10; HEADCROP:10; TRAILING:3; SLIDINGWINDOW:4 : 20; MINLEN:36). Reads of all samples were assembled using Trinity r20131110 [40], which yielded 249 855 and 150 314 transcripts for *E. cornigera* and *E. timida*, respectively. Expression values for transcript clusters (or unigenes) were extracted and analysed using the Trinity pipeline [41] via RSEM 1.2.11 [42], Bowtie 1.0.1 [43] and edgeR 3.4.2 [44]. Only unigenes with raw read counts of greater than or equal to 100 over at least two samples were analysed, resulting in 36 380 and 32 897 unigenes, respectively. Differential expression was determined by $\log_2$fc of at least ± 1 compared to the control set of unigenes (with raw read counts of greater than or equal to 100). Expression change significance was assessed based upon the dispersion of available replicate information in edgeR. The longest isoform of each cluster was defined as the representative sequence for a unigene.

To plot the overall taxonomic distribution of the assembled transcripts, unigenes were subjected to a BLASTx-based [45] search (*e*-value cut-off 10^{-10}) against a database consisting of protein sequences of RefSeq version 64 [46] plus those of the genomes of *Crassostrea gigas* [47] and *Lottia gigantean* [48] (electronic supplementary material, figure S2). For all downstream analyses, only those genes were included for which top hits to the mentioned organisms or other metazoans were retrieved. Excluded were those with best blast hits to plants, protists, prokaryotes and viruses. Protein annotations for the 14 848 *E. cornigera* and 13 875 *E. timida* unigenes were extracted from mentioned BLAST hits and from a second BLASTx search to the UniProtKB/Swiss-Prot database [49]. KOG/NOG (EuKaryotic/Non-supervised Orthologous Groups [50–52]) categories were determined based on the best BLASTx hit to protein sequences of eggNOG v. 4 [53]. KEGG [54] accessions were obtained using the KAAS 1.6a webservice [55] against metazoa.

For qRT-PCR, 200 ng RNA was reverse transcribed using random hexamers and the iScript Select cDNA Synthesis Kit (Bio-Rad). Two-step qRT-PCR was performed on biological triplicates for each time point and treatment (each containing pooled RNA from greater than or equal to five slugs) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions on a StepOne Plus (Applied Biosystems) real-time PCR system. Primers were designed using Primer-BLAST [56] (for primer sequences, see the electronic supplementary material, table S4) and data were analysed according to Pfaffl [57], using the *Ec*RPL38 (Eco000149), *Et*RPL19 (Eti000121) and *Et*SETMAR (Eti000317) as reference genes.

Data accessibility. All sequencing data was deposited at the NCBI Single Read Archive (SRP047359) and the Transcriptome Shotgun Assembly Sequence Database (*E. cornigera*: GBRW00000000; *E. timida*: GBRM00000000).

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Author contributions. J.d.V., A.G.M.T., P.J. and S.B.G. carried out the main experiments. G.C., J.d.V. and H.W. additionally collected animals and C.W. processed the RNA-seq data. S.B.G. designed the experiments and wrote the manuscript together with J.d.V. All authors discussed the results, read and approved the final manuscript.

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Publication IV: Plastid survival in the cytosol of animal cells

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Contribution of Jan de Vries, first author:
Major (drafted the manuscript, prepared one figure, developed parts of the rationale)

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Forum

CellPress

Plastid survival in the cytosol of animal cells

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Some marine slugs sequester plastids from their algae food, which can remain photosynthetically functional in the animal's digestive gland cells in the absence of algal nuclei. The sequestered plastids (kleptoplasts) appear to maintain functional photosystems through a greater autonomy than land plant plastids. If so, kleptoplast robustness is a plastid-intrinsic property, and it depends on the animal to manage an alien organelle on the loose in order to maintain it long term.

Photoheterotroph through kleptoplasty

Heritable plastids that harness light energy to fix CO_2 and generate reduced carbon compounds occur only in algae and plants. Some marine slugs are special, because they appear to profit from photosynthesis not through symbiosis but by retaining only the source of photosynthesis – the plastids – from their algal food [1]. This process (kleptoplasty) has been described for ~75 sacoglossan species, who tap the cell walls of siphonaceous algae and feed by sucking out the algal protoplasm including organelles. Based on pulse amplitude modulation (PAM) measurements that record photosystem II activity, the vast majority of slugs are either non-retention (NR) or short-term retention (StR) forms, that is, the kleptoplasts lose their photosynthetic capacity either immediately or within the first 2 weeks, respectively [2]. Current research focuses on long-term retention (LtR) species, six of which have so far been identified: *Elysia chlorotica*, *Elysia timida*, *Elysia crispata*, *Elysia clarki*, *Plakobranchus ocellatus*, and *Costasiella ocellifera* (Figure 1). All six can retain functional kleptoplasts for at least a month during starvation. The morphology of animals, greenish colouring and ability to house photosynthetically active kleptoplasts is why the scientific and popular press often refer to these animals as 'leaves that crawl' or 'solar-powered slugs'. The system offers the unique chance to study photobiology from the perspective of an animal host, in plastids that 'live' in the cytosol of an animal cell. Recent results are changing the way we view kleptoplasty in sacoglossan slugs. New findings show that kleptoplast longevity occurs without the support of lateral gene transfer events, suggesting that observed plastid robustness is a trait the organelle itself brings along.

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'Crawling leaves' and photoautotrophy

Despite occasional claims to the contrary, sacoglossan slugs are not photoautotrophic; they are first and foremost heterotrophic. Because plastids are not transmitted vertically (i.e., from one slug generation to another via eggs), juveniles need to feed on algae for several days upon hatching before the first stable kleptoplasts are assimilated, a phase known as 'transient kleptoplasty' [3]. Adult slugs continue to feed as long as food is available too, but during starvation some species can survive for weeks or months whereas others cannot [1]. How, and in particular for how long, the plastids and slugs survive is less well understood than the literature might suggest. We recently pointed out that a few reports regarding the photosynthetic endurance of kleptoplasts during starvation are problematic [4]. In our hands, CO_2 fixation in slugs does not allow continued growth of the animals during starvation, and all species analysed so far have been shown to shrink and experience substantial weight loss – in some cases up to 93% – during starvation [5,6]. Undoubtedly, some slugs fix CO_2 in a light-dependent manner [6], but the question is for how long after the animals are deprived of their food, and is the amount of CO_2 fixation sufficient to support continued growth of the slugs?

The 'light-dependent reaction' measured through PAM fluorometry is currently the only method that has constantly provided evidence for a perpetuation of photosynthesis in several different slug species. Although F_v/F_m (maximum quantum yield) values provide a good indication about the general retention of functional photosystems in kleptoplasts [2], it is important to remember these values are relative and tell us nothing about what happens downstream of the photosystems. After weeks of starvation, a few remaining percent of plastids with functional photosystems might return high PAM values (which are ratios), but it is questionable whether the plastids can support the host with an amount of fixed carbon sufficient to be considered relevant. These details warrant attention, and the contribution of kleptoplasts in sacoglossan slugs is more complicated than one might think, especially when the behaviour of StR species is considered (Figure 2).

What mediates kleptoplast robustness?

Photosynthesis accompanies a constant high turnover of a subset of involved proteins, in particular the D1 protein of photosystem II [7]. Its maintenance requires accessory factors that are largely nuclear encoded. Algal nuclei, however, are rapidly digested after feeding [8]. In 1996, it was suggested that lateral gene transfer (LGT) from alga to animal might account for kleptoplast longevity [9]. This hypothesis became very popular, but has since been

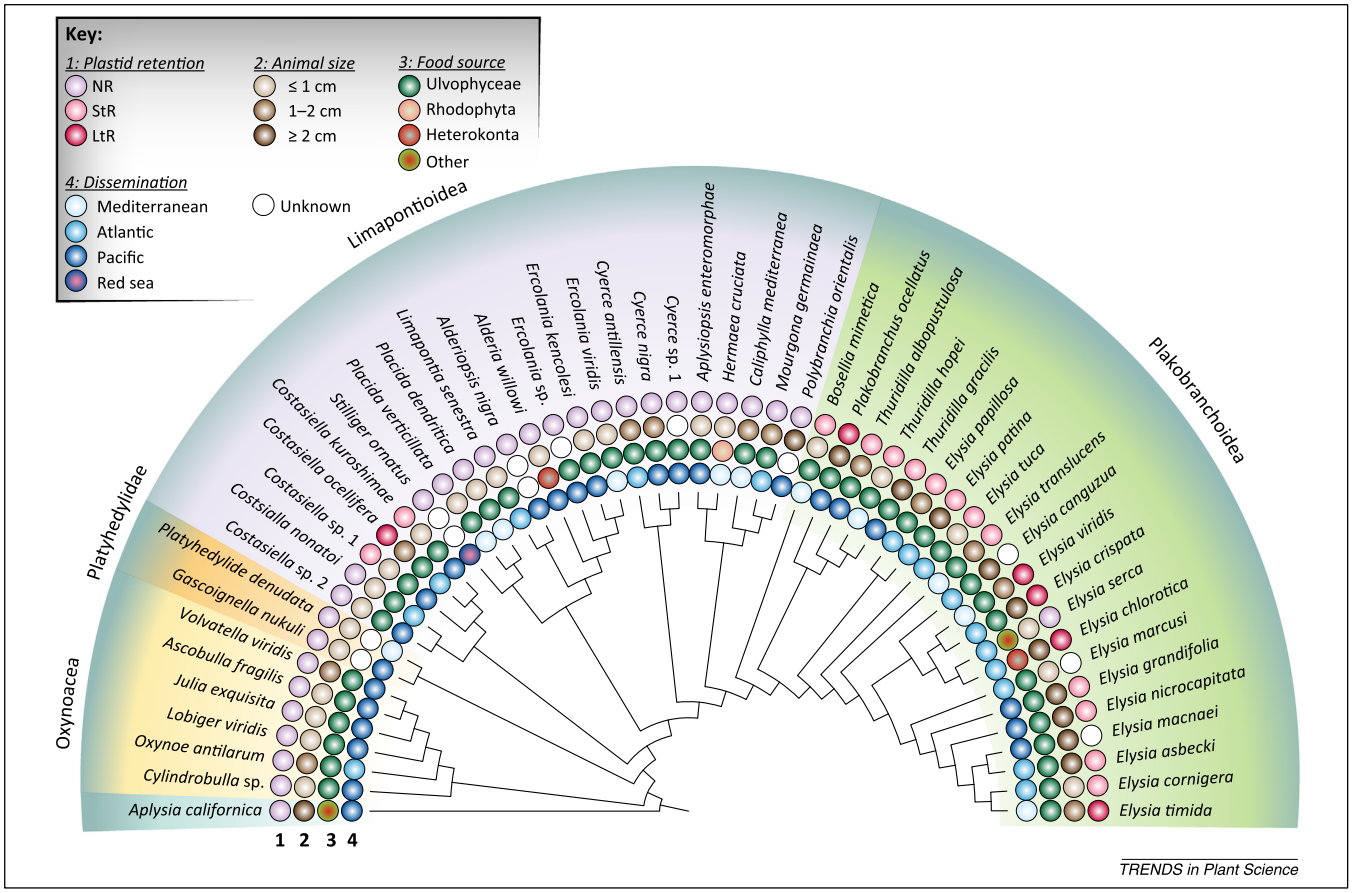

Figure 1. Phylogenetic relationship of the Sacoglossa. The shelled Oxynoacea are the most basal Sacoglossa. The sister taxon, the Plakobranchacea, unites the Limapontioidea and the Plakobranchoidea that are morphologically characterised by having either cerata (lateral appendices of the dorsal body) or parapodia (lateral appendices of the foot), respectively. The most basal Plakobranchacea are the worm-like Platyhedylidae that are, however, assigned to the Plakobranchoidea. Besides the genus *Costasiella*, neither the shelled Oxynoacea nor the Limapontioidea exhibit functional retention of kleptoplasts, which is common to nearly all Plakobranchoidea. Only six species (highlighted by dark red circles) are long-term retention (LtR) forms that are always larger than their corresponding sister taxa, and that are either non-retention (NR) or short-term retention (StR) forms. Besides some very specialised species, the majority of Sacoglossa feeds on ulvophycean algae, independent of whether being able to retain functional kleptoplasts or not. The cladogram is based on a four-gene Bayesian analysis using the anaspidean *Aplysia californica* (California sea hare) as the outgroup.

refuted [10,11] and the ability to service damaged photosystem II appears an intrinsic property of the plastids sequestered. Through sequencing the plastid genome of the ulvophycean alga *Acetabularia acetabulum* (food algae of the LtR species *E. timida*) and comparing it with other plastid genomes, we recently noticed that genomes of sequestered algae plastids encode, among other potentially crucial factors, FtsH [12]. This quality control protease specifically removes photodamaged D1, which is essential, because it stops the further generation of reactive oxygen species and allows the integration of *de novo* synthesised D1 into photosystem II [7]. Potentially, sequestered algal plastids bring along their own molecular toolkit for photosystem repair.

Could a single protein such as FtsH be that molecular toolkit and explain kleptoplast longevity? Apart from the contribution of slugs, it is likely that a combination of several mechanisms mediates kleptoplast robustness and allows ongoing photosynthesis in the cytosol of an animal's cell. These could include, for instance, non-photochemical quenching through a xanthophyll cycle and a generally slower turnover of plastid proteins. Interestingly, a first analysis suggests that the physiological photoregulation mechanism of kleptoplasts in slugs is surprisingly similar to that of the corresponding plastids in the algae [13]. The greater autonomy of some plastids sequestered by slugs, in comparison to land plant plastids, is a prerequisite for functional kleptoplasty, but it needs to be matched by the physiology of the slug in order to profit.

The origin of kleptoplasty and long-term retention in sacoglossans

The ability to survive prolonged starvation is rare among sacoglossans. Of the more than 300 described species, only six have been identified to retain functional kleptoplasts and withstand food deprivation for many months [2]. Five of the six species described belong to the Plakobranchoidea, one to the Limapontioidea [14], suggesting that this ability has evolved several times independently (Figure 1). The majority of sacoglossan sea slugs can feed on several algal species simultaneously, including the LtR forms *P. ocellatus*, *E. clarki*, and *E. crispata*, whereas some, including the LtR forms *E. timida*, *E. chlorotica*, and *C. ocellifera*, specialize on a single algal food source [2,14]. It is currently not known whether reliance on one source of kleptoplasts is more advantageous than a polyphagous life style. It is

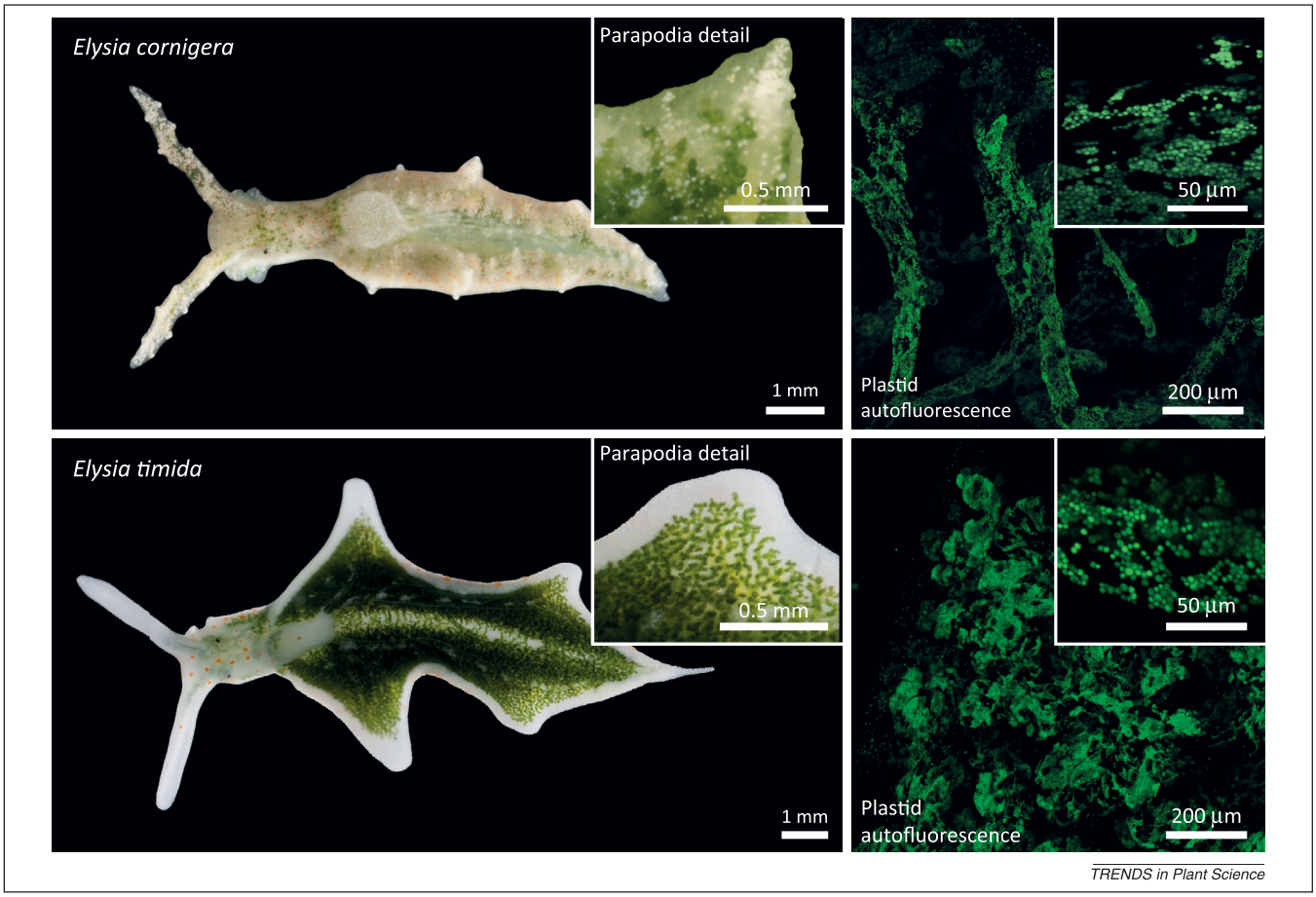

Figure 2. Kleptoplasts are biochemical lucky bags with a drawback. Pictured are the two phylogenetically close sacoglossans, *Elysia timida* and *Elysia cornigera*. Both slugs embed vast quantities of functional plastids they sequester from the ulvophycean alga *Acetabularia acetabulum* (plastid autofluorescence shown in green) and embed them within epithelial cells of digestive tubules that pervade the parapodia of slugs. Plastids are biochemical all-rounders and their abilities stretch beyond those of light-driven carbon fixation. Owing to their evolutionary legacy they can further synthesise, for example, certain amino acids, fatty acids, iron–sulphur clusters, haem, isoprenoid precursors, and certain cofactors. It has not been explored to what degree the slugs benefit from the biochemistry of kleptoplasts other than photosynthesis, but the vast amount of organelles stored reflects the rich nutritious depot from which the slugs could profit during starvation. Photosynthesis also accompanies the generation of toxic byproducts, such as reactive oxygen species or glycolates, for example. Their elimination is often complex and can require the interplay with other compartments. Intriguingly, when *E. cornigera* dies, the digestive tubules are still loaded with a quantity of kleptoplasts that visually appear similar to the quantity observed in *E. timida*, which however survives for many more weeks to come. LtR slugs might have developed strategies to better cope with self-contained, robust kleptoplast.

tempting to speculate that specializing on a single species as a food source is a prerequisite of evolution of stable kleptoplasty. Further work should address such issues.

Importantly, the ability of slugs to survive starvation is not strictly dependent upon the ability to retain functional plastids. Some NR species that have been analysed survive without food for many weeks to months too [5,14]. The LtR species *C. ocellifera*, *E. timida*, and *P. ocellatus* were shown to survive in the dark and with chemically blocked photosynthesis, all while not losing weight faster than the control group [4,14]. There is, furthermore, currently no evidence that the animals die when they are deprived of their food due to a lack of kleptoplasts and nutrition. In dying individuals of *E. chlorotica*, numerous kleptoplasts were still identified [15]. We observed that the StR species *Elysia cornigera* dies with digestive gland cells that are filled with a quantity of kleptoplasts that is similar to those of the phylogenetically closely related LtR species *E. timida*, whose starvation was commenced at the same time (Figure 2). Both species had been fed solely on the ulvophycean alga *A. acetabulum*, demonstrating that the food source alone cannot account for the differences observed in terms of how long an individual species survives food deprivation. Could there be another reason for slugs to die rather than a lack of nutrition? Research has largely focused on trying to understand how the slugs keep kleptoplasts functional in the absence of hundreds of algal nuclear genes, which are apparently not required. What if a more substantial part of slug–kleptoplast evolution is driven by the need to evolve mechanisms to cope with plastid-derived factors (possibly toxins generated through photosynthesis) rather than servicing the foreign organelle? In any case, some sacoglossan species can house functional plastids for months during starvation, but the exact nature of their contribution to slug biology is still not explored.

Future directions

An accumulating amount of data suggests that kleptoplast longevity is first and foremost an intrinsic property of the sequestered organelles, and a prerequisite for functional kleptoplasty. If these plastids are able to maintain

functional photosynthesis more autonomously than their relatives from higher land plants, this has two main implications for future research: (i) the slugs highlight those algae, whose photobiology is the most valuable to study; and (ii) we should begin comparing the biology of StR and LtR slugs to understand what it is that allows LtR animals to better match with kleptoplast robustness so as to profit from maintaining the organelles long term.

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Publication V: Why it is time to look beyond algal genes in photosynthetic slugs

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Contribution of Jan de Vries, second author:
Medium (wrote parts of the manuscript, helped preparing one figure, developed parts of the rationale)

Genome Biology and Evolution

SMBE

Why It Is Time to Look Beyond Algal Genes in Photosynthetic Slugs

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Abstract

Eukaryotic organelles depend on nuclear genes to perpetuate their biochemical integrity. This is true for mitochondria in all eukaryotes and plastids in plants and algae. Then how do kleptoplasts, plastids that are sequestered by some sacoglossan sea slugs, survive in the animals' digestive gland cells in the absence of the algal nucleus encoding the vast majority of organellar proteins? For almost two decades, lateral gene transfer (LGT) from algae to slugs appeared to offer a solution, but RNA-seq analysis, later supported by genome sequencing of slug DNA, failed to find any evidence for such LGT events. Yet, isolated reports continue to be published and are readily discussed by the popular press and social media, making the data on LGT and its support for kleptoplast longevity appear controversial. However, when we take a sober look at the methods used, we realize that caution is warranted in how the results are interpreted. There is no evidence that the evolution of kleptoplasty in sea slugs involves LGT events. Based on what we know about photosystem maintenance in embryophyte plastids, we assume kleptoplasts depend on nuclear genes. However, studies have shown that some isolated algal plastids are, by nature, more robust than those of land plants. The evolution of kleptoplasty in green sea slugs involves many promising and unexplored phenomena, but there is no evidence that any of these require the expression of slug genes of algal origin.

Key words: lateral gene transfer, kleptoplasty, photosynthesis, plastid biology, photosynthetic sea slugs.

Introduction

Sacoglossa are considered one of nature's curiosities. Inside some of these sea slugs, plastids sequestered from algae can continue to photosynthesize for weeks, or even months, in the absence of algal nuclei (Greene 1970; Rumpho et al. 2001; Händeler et al. 2009). That is conspicuous, because when land plant plastids are isolated and removed from their cellular context they rapidly degrade (Leegood and Walker 1983; Seftor and Jensen 1986; Polanská et al. 2004; Green et al. 2005). With the description of endosymbiotic gene transfer (EGT; Martin et al. 1993) and the concomitant genome reduction the organelles experienced (Timmis et al. 2004), the prime cause for the instability of isolated plastids quickly became apparent: the majority of proteins working in plastids are nuclear-encoded and posttranslationally imported from the cytosol (McFadden 2014). Hence, the duration with which kleptoplasts are kept functional in animal cells in the absence of algae nuclei encoding a 1,000 + plastid proteins presents an obvious contradiction. This required an explanation and in 1996 (Pierce et al. 1996) it was proposed that slugs had

acquired algal genes that encode proteins servicing the plastids through lateral gene transfer (LGT). Once the idea was presented, it was destined to be tested.

There Is No Evidence for Lateral Transfer of Algal Genes in Slugs

Let us first take a look at what we should expect if genes of algal origin were to play a role in kleptoplast maintenance. The slugs are sometimes referred to as "crawling leaves," because the entire appearance of the species in question (e.g., *Elysia chlorotica*, *Elysia timida*, and *Elysia viridis*) is greenish and in a few cases indeed leaf-like. Scientists noticed this already more than 150 years ago. They were particularly intrigued by the digestive tubules that pervade almost the entire body, sometimes including the head, and whose cells house the kleptoplasts (fig. 1*a*). Based on what we know about the biology of photosynthesizing cells (in plants, algae, and cyanobacteria), we must predict that transcripts of algal origin, which are supposed to maintain kleptoplast integrity in the slugs, are abundant. Yet, among all RNA sequencing data available for several species (Rumpho et al. 2011; Wägele et al. 2011; Pierce et al. 2012; de Vries et al. 2015), sequencing reads pointing toward transcripts from genes of LGT origin remain close to zero. In fact, they remain well below the counts representing obvious contamination (fig. 2*a* and *b*).

Single reads can easily be artifacts. In RNAseq analysis, it is common practice to filter for only those genes that are supported by a reliable number of independent reads. In the RNA-seq analysis by Pierce et al. (2012)—the last RNA-seq report published to claim expression of genes of LGT origin is relevant for kleptoplast performance—the highest read count for a single algal nuclear gene of potential LGT origin was two. Two among 98,238,204 reads. A favored argument to explain why only such few reads are detected is that "the symbiotic chloroplasts resides in only a few cells within the slugs" (Pierce et al. 2012) or that "only a relatively few cells in the slug contain plastids" (Pierce et al. 2015).

Both the appearance of the animal (fig. 1*a* and *b*) and factual numbers tell a different story. The chlorophyll content in a 6 mm long *Elysia cornigera* is around 3.1 nmol/mg dry weight and in a 10-day-old tomato seedling with fully developed green cotyledons it is about 22.4 nmol/mg dry weight (fig. 2*c*). A single chloroplast contains 2.5×10^8 chlorophyll (Chl) molecules (Stolz and Walz 1988). One nanomole of Chl thus corresponds to about 2 million chloroplasts, and hence about 6 million chloroplasts are found per mg dry weight in *E. cornigera*. Three nanomoles of Chl further translates into a mass of about 2.7 μg, and therefore accounts for about 3% of the total dry weight of slugs. Impressive, even if the estimations would be lower by a factor of 10. Also, if only a few cells of a photosynthetic sacoglossan slug would harbor kleptoplasts, then how would that match up with the concept that photosynthesis continues almost unabated for up to a year to support animal growth (Pierce et al. 2012)? Furthermore, among the RNA-seq data of 2012 from *E. chlorotica*, 4,234 reads for the plastid-encoded *psbA* were detected (Pierce et al. 2012). That is noteworthy, because the samples sequenced were enriched for poly(A)-tailed mRNA prior to sequencing and the plastid mRNA was copurified only as a contamination due to the high AT-content of the plastid transcripts. There is probably even more mRNA encoding *psbA* present than sequenced and even if not, the number of reads for this single *psbA* gene by far exceeds the total number of reads (111) found for the 52 genes of suggested LGT origin. Furthermore, all the reads interpreted to be of LGT origin are ≥99% identical in sequence to the algal transcripts and that would mean they are exempt from evolutionary codon adaptation in the slug's nucleus.

Slugs analyzed are mostly collected from the wild and then grown on their food alga in open aquaria in the lab. The cultures are not axenic; they cannot be and they do not have to be for the kind of experiments that are currently performed. Contamination of the isolated RNA is unavoidable, but not a problem as long it is monitored. In the most recent transcriptomic analysis on two slug species (de Vries et al. 2015), the number of reads obtained for genes of heterotrophic protists was much higher than those for any algal nuclear gene and they were hence omitted from downstream analysis. We predict the amount of contamination in the data set of *E. chlorotica* (Pierce et al. 2012) to be comparable. The entire sequence data have never been made publicly available, rather only that of the few dozen genes discussed and therefore it was not possible to assess this issue in *E. chlorotica*. But a thought experiment is possible: we 1) do not screen the slug RNA-seq data for potential contamination and we 2) accept the presence of a few mRNA reads as evidence for functional LGT that support kleptoplast maintenance. We could then conclude that hundreds of ciliate genes support kleptoplasty in *E. timida*, but fail to do the same for the short-retaining *E. cornigera* albeit present (fig. 2*a*). The most rational conclusion that remains is that RNA-seq offers no support for the expression of slug nuclear genes that originate from the food alga. These slugs are not what they eat, and they eat a lot (Christa et al. 2014).

Recently, evidence for algal LGT in Sacoglossa other than sequencing data emerged: a study by Schwartz et al. (2014) used fluorescence in situ hybridization (FISH) to localize genes of algal origin among slug chromosome spreads. That report was quickly picked up by the popular press and it currently scores among the top 5% of all articles so far evaluated by Altmetric.com. Evidently, the public cares a lot about LGT in slugs. The public, however, is likely less aware that FISH analysis can be quite deceptive, for example consider the case of *Chlamydomonas* "basal body DNA" (Hall et al. 1989). The recent FISH analysis on *E. chlorotica* (Schwartz et al. 2014) also provided no controls for the specificity of the probes used (*prk*, *actin*, and *rbcL*) in form of Southern blots. Apart

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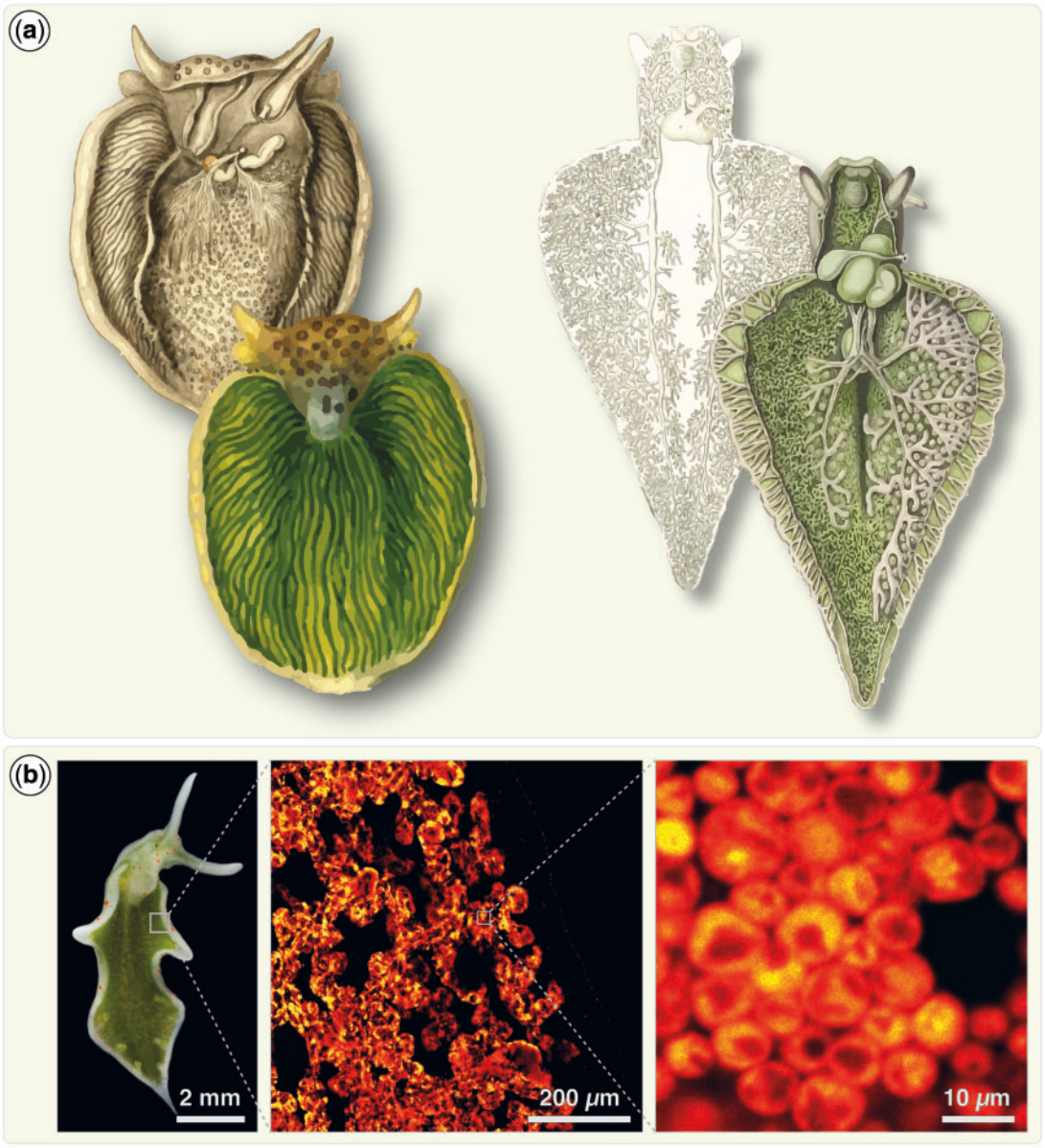

Fig. 1.—Sacoglossan slugs can house millions of kleptoplasts. (*a*) Shown are two of the earliest depictions of sacoglossan slugs and their "green" digestive tubules that can pervade the entire body. On the left a drawing by van Hasselt from 1824 showing *Plakobranchus ocellatus* and on the right a drawing of *Elysia viridis* by J. Thomas from 1852. Both demonstrate that an extensive digestive system, able to house millions of kleptoplasts, is not limited to *Elysia chlorotica*. Note how the digestive tubules of *E. viridis* pervade even the head of the animal. (*b*) The extent of stored plastids becomes apparent when viewing the slugs (here *Elysia timida*) under the microscope and filtering for the chlorophyll autofluorescence of the kleptoplasts (red-orange hue). In the middle, a detail of a region of the parapodia, with the individual digestive tubules being visible through the kleptoplasts' fluorescence. Zooming in further reveals the density with which the kleptoplasts are packed into the cytosol of the cells forming the digestive tubules.

from these technical issues, FISH analysis is not a suitable tool for providing evidence for LGT. The only reliable evidence for LGT would be to demonstrate the integration of algal DNA into the context of slug chromosomes (through DNA sequencing), from where it is expressed to support the stolen organelles by the product being specifically targeted to kleptoplasts. And although independent genome data of *E. chlorotica* is available (Pierce et al. 2012) to the authors of the FISH analysis, it has not been used to support their concept and also challenges published slug genome data that found no evidence for algal LGTs in *E. chlorotica* (Bhattacharya et al. 2013).

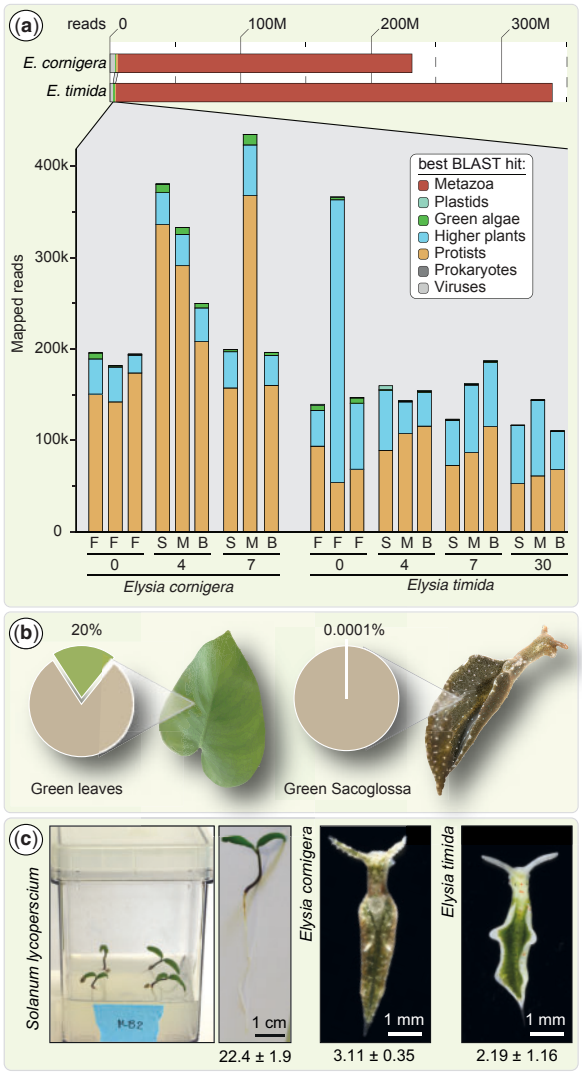

Fig. 2.—Among RNA-seq data, contaminating reads exceed reads of algal origin. (*a*) At the top the total number of reads (in million, M) recently sequenced for *Elysia cornigera* and *Elysia timida* (de Vries et al. 2015) are shown. Those reads were assembled into contigs and all contigs subjected to a BLAStx-based distribution analysis against RefSeq to determine their distribution among the taxonomic groups listed. Note that 1) the number of reads of protist origin in all cases exceeds those of green algal origin and that 2) in the LtR species *E. timida*, the amount of green algal reads declines with progressing starvation, while one would expect an elevated expression of genes supporting kleptoplasts. Slugs were freshly fed (F) and starved (S) for 4, 7, and 30 days under different conditions including monolinuron treatment blocking photosynthesis (M; 2 μg ml^{-1}) and high-light bleaching (B; 1 h of 1,000 μE $m^{-2}s^{-1}$ once per day). (*b*) While the percentage of nuclear mRNA transcripts associated with photosynthesis in a green leaf ranges around 20% (Bhalerao et al. 2003), slug transcriptomes return on average around 0.0001%. If the 52 genes described by Pierce et al. (2012) were truly transferred to the slug nuclear genome, they are expressed at a level that is 200,000 times too low to support photosynthesis. (*c*) The chlorophyll a+b concentrations of two slug species (from de Vries et al. 2015) versus those of entire 10-day-old tomato seedlings in nmol/mg dry weight.

As a last word on LGT, it should be mentioned that LGT to eukaryotes is manifest in two fundamentally different forms. First, there is gene transfer from organelles to the nucleus, or also called EGT. EGT is a continuous, ongoing process, and incontrovertibly documented in all sequenced genomes of eukaryotes (Timmis et al. 2004; Hazkani-Covo et al. 2010; Boto 2014). Second is outright LGT, where the donors are not chloroplasts or mitochondria. Newer findings show that latter, though it does occasionally occur, is extremely rare and does not manifest itself in the bigger picture of eukaryotic evolution (Ku et al. 2015). In this context, it is important to note that long-term retention of kleptoplasts evolved several times independently in sacoglossan sea slugs (Maeda et al. 2010; de Vries, Christa, et al. 2014; Christa et al. 2015). Hence, if the expression of nuclear genes of algal origin is the reason for robust kleptoplasts in one species, then the same should apply to other species as well. If all Sacoglossa retained functional kleptoplasts in a LGT-dependent manner, then they would have to be the record holders for LGT among animals, their LGT events outnumbering all other cases in animals thus far reported. Are Sacoglossa LGT magnets? Neither genome nor transcriptome data from these animals indicate that to be the case. Occam's razor dictates favoring a less assumptive scenario.

Stable Kleptoplasts in the Absence of LGT

Since the 1970s, it is known that some plastids sequestered by the sea slugs show a remarkable independent robustness (Giles and Sarafis 1972; Trench and Ohlhorst 1976; Green et al. 2005). The best explanations we have for robust plastids are effective photoprotection mechanisms (Serôdio et al. 2014; Cruz et al. 2015), a different coding capacity of the plastid genomes in question (Rumpho et al. 2000; de Vries et al. 2013) and maybe an overall difference in the stability (half-life) of essential proteins. That plastids sequestered by the slugs are intrinsically robust is, based on current information, the most parsimonious scenario. It explains how such a broad range of slug species can perform kleptoplasty (Christa et al. 2015) and why plastids of the same source can behave identically in slug species that differ in their ability to survive food deprivation (de Vries et al. 2015). Slugs acquiring robust plastids will not automatically retain them long-term and endure starvation as recently interpreted (Pierce et al. 2015). It is of equal importance that the slugs are physiologically adapted and require to retain them functionally (de Vries, Rauch, et al. 2014). This likely depends on whether they experience food deprivation in their habitat due to seasonal variation or not (Cruz et al. 2013; Wägele and Martin 2013; de Vries et al. 2015).

The ability to sequester and maintain an entire heterologous structure of foreign origin is not restricted to sacoglossa and their plastids. For the purpose of using them as a

defensive organ, some aeolidoidean sea slugs incorporate cnidocysts from their cnidarian prey to expose them on their surface (Obermann et al. 2012). Similar to the kleptoplasts, cnidocysts are first incorporated through oral feeding and as part of the regular diet. The peculiar thing is the release of the kleptoplasts from the phagosomes into the cytosol of the digestive epithelial cells. The latter appears more common when only organelles and not entire symbiotic organisms are retained by a host. The ciliate *Myrionectra rubra* releases transcriptionally active nuclei and plastids of its prey algae into the cytosol (Johnson et al. 2007), whereas symbiotic *Chlorella* algae of *Paramecium bursaria* or *Hydra viridis* remain inside a specialized digestive vacuole and isolated from the host's cytosol (Nowack and Melkonian 2010; Fujishima and Kodama 2012). It is not known how the plastids are specifically sorted from other food particles and then released into the cytosol or really why. Does it facilitate the easier exchange of substrate and metabolites? These observations, together with how Sacoglossa deal with kleptoplast-produced toxins such as reactive oxygen species and the general differences in starvation tolerance, remain promising research topics. All of these, however, are not associated with LGTs of algal origin.

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6 Plastid evolution – from autonomous robustness to controlled versatility

Plastids sequestered by the slugs are robust. Already in the 1970s studies were performed that investigated this robustness by isolating ulvophyte plastids and injecting them into egg albumen, where they stayed intact for several weeks (Giles and Sarafis 1972). Also plastids of the (secondary red) xanthophyte *Vaucheria litorea* were analysed for their *ex vivo* stability (Green et al. 2005). Green and colleagues (2005) analysed the plastids *in vitro*, meaning they were kept in an osmotically balanced buffer for two weeks, and revealed to be more stable than the chloroplasts of spinach to which they were compared. During this time the isolated plastids retained their transcriptional and translational activity (Green et al. 2005). The same applies to plastids residing within the slugs, as kleptoplast transcription and translation was found to remain active for up to several months (Trench and Gooday 1973; Mujer et al. 1996; Green et al. 2000; de Vries et al. 2013 **VI**). Unfortunately, molecular data on the machinery that underpins kleptoplast activity is scarce and limited to only a few species, mainly *E. chlorotica* (cf. Mujer et al. 1996; Green et al. 2000). Yet, photo-physiological methods have been applied to screen diverse sacoglossans (Händeler et al. 2009; Christa et al. 2015). For now, we can only assume (and occam's razor suggests to do so, cf. Rauch et al. [2015 **V**]) that in all those cases where kleptoplast photochemistry perpetuates for several weeks, it is due to a similar molecular chassis. What is more, the diversity of algal plastids retained by sacoglossans is considerable (de Vries et al. 2014b **IV**; Christa et al. 2014d). While most of the sacoglossans do not retain plastids long-term (Händeler et al. 2009; Christa et al. 2015), those that are retained do not form a distinct group: even though the majority of these plastids stems from ulvophytes, also plastids from secondary red origin, as in case of the famous *E. chlorotica*, can be retained (Christa et al. 2014d). Thus, not only are the slug species that perform kleptoplast long-term retention polyphyletic (Christa et al. 2015), but also are the algae (Christa et al. 2014d). This creates an intricate situation when it comes to soliciting specific factors and mechanisms that confer plastid longevity. As highlighted in the previous chapter, the animals' contribution to plastid longevity is rather marginal and we hence highlighted that it is the plastid biology that should be given attention (de Vries et al. 2014b **IV**). If one entertains the thought that not factors provided by the slugs but intrinsic plastid properties confer plastid robustness, certain expectations regarding the nature of these intrinsic factors arise. Factors conferring kleptoplast longevity should be (a) shared by all plastids that are being retained long term and (b) variation in these factors should, in turn, affect longevity of kleptoplasts. Here, the advances that were made with regard to understanding robust plastids are outlined.

It is fair to assume that, before being turned into kleptoplasts, plastids sequestered by the slugs are active and functioning in the algal cytosol. Hence, kleptoplasts freshly stolen from the algae should have all (and likely a reservoir of) the biomolecules they require for photosynthesis as well as other biochemical processes – which is their point of departure when they enter the slugs. Yet, over time, biomolecules are undoubtedly consumed and are being rendered useless through degradation. Robustness must accordingly either be conferred by stability of the biomolecules or a way to

replenish them (cf. Rumpho 2001). It is conceivable that universal biological currencies such as ATP or amino acids are imported by the kleptoplasts from the slugs' cytosol. The processing of those currencies should, however, then dependent on (a) the proteins that were imported from the algal cytosol before the plastids were sequestered or (b) proteins (and, for example, tRNAs) that are *de novo* synthesised from transcripts of genes encoded on the plastid genome. When discussing factors encoded on the plastid genome, a reasonable question is: why do canonical plastids retain genomes at all? In 1993, John Allen proposed that the reason why both chloroplasts and mitochondria retain genomes is to allow for *in situ* feedback between organellar redox chemistry occurring at the bioenergetics membranes and gene regulation (Allen 1993). Yet, some organelles of endosymbiotic origin exist without retaining any genetic material (Müller et al. 2012). Hydrogenosomes and mitosomes both evolved from mitochondria through reduction, but neither of the former two has retained a genome (Müller et al. 2012). Plastids can lose their genome, too: the chlorophyte *Polytomella,* a close relative of the model alga *Chlamydomonas*, has (Smith and Lee 2014) and so has the parasitic plant *Rafflesia lagascae* (Molina et al. 2014). Both, however, no longer perform photosynthesis and with it lost the electrochemical gradient across the thylakoid membrane (Figueroa-Martinez et al. 2015). The same applies to hydrogenosomes and mitosomes, which do not perform oxidative phosphorylation (Müller et al. 2012). Thus, there seems to be a connection between generating an ion gradient across a membrane and retaining specific genes on the organellar genome (Allen 2015). Yet, there are considerable differences when it comes to which genes are retained by plastid genomes when we take both land plants and algae into account (Allen et al. 2011). This warrants attention as some of these genes might impact the *in situ*, i.e. autonomous, regulation of essential components of the photosystem (de Vries et al. 2013 **VI**). Autonomous control, independent from any genetic information contained by the algal nuclei, over on-going photochemistry could help kleptoplasts to endure residing within the animal cytosol for a longer time (de Vries et al. 2014b **IV**).

One of the reasons why plastids that are sequestered by the slugs are able to synthesize proteins for such a long time might be that their genomes encode the elongation factor Tu (Baldauf et al. 1990; Martin et al. 1998; de Vries et al. 2013 **VI**). This protein should allow algal plastids in general to have a higher degree of control over their own protein biosynthesis, but plastid *tufA* was never intensively studied. Yet, one single additional protein associated with elongation is likely insufficient (as discussed in de Vries et al. [2014b **IV**]). Determining which components are required by the plastids to be important from their environment is one of the possible future avenues of research (cf. de Vries et al. 2014a **I**; de Vries et al. 2014b **IV**). But, as already mentioned in the previous chapter, many components that are associated with on-going photosynthesis, and in particular photosystem II, experience a high turnover (Aro et al. 1993; Schöttler et al. 2015). In that context it is important to remember that most of what is known about the turnover of photosystem components stems from a few model systems and some of the genes unique to certain algal plastid genomes could be relevant for photosynthesis and its adaptation to environmental cues (Simpson and Stern 2002). One of these is the *ftsH* gene (de Vries et al. 2013 **VI**). FtsH is a zinc-protease that is essential for the repair cycle of D1 (Lindahl et al. 2000) by recognizing and removing photo-damaged D1 protein (Nixon et al.

2005; Nixon et al. 2010; Janska et al. 2013). Once a damaged D1 is removed, a newly synthesised one can be inserted hereby restoring a functional PSII (Nixon et al. 2010; Chi et al. 2012; Komenda et al. 2012; Nickelsen and Rengstl 2013). If it fails to do so, as for example in *ftsh* knock-out mutants of *Arabidopsis*, deleterious ROS accumulate that can severely harm the organism (Kato et al. 2009). In land plants, *ftsH* is strictly nuclear encoded (Simpson and Stern 2002). Yet, various algal lineages encode *ftsH* on their plastid genomes (Wicke et al. 2011; de Vries et al. 2013 **VI**), while at the same time also having a nuclear copy of *ftsH* encoded (Itoh et al. 1999; Janska et al. 2013). How these are functionally linked awaits to be tested, as studies on plastid-encoded *ftsH*, although connections were repeatedly implied (Wolfe 1994; Takechi et al. 2000), are scarce (Itoh et al. 1999). We hypothesised in de Vries et al. (2013 **VI**) that this protease could represent a key factor that allows plastids to be more autonomous in the regulation of their photosystem and photosynthesis performance. At the same time it should equip isolated plastids with the tools to remove damaged D1, preventing the formation of ROS and hence result in higher longevity of plastids *in vitro* and *in hospite* (de Vries et al. 2013 **VI**). This would render the plastids robust due to ROS prevention.

The findings on FtsH (cf. de Vries et al. 2013 **VI**) not only shed new light on the special coding capacity of algal plastids but also, *vice versa*, the absence of specific genes from land plant plastid genomes. A recent study analysing the chloroplast genome of Charophytes has highlighted that something has happened to the coding region of FtsHs very early in streptophyte evolution (Civan et al. 2014). The putative *ftsH* homologs of the higher branching streptophyte algae (cf. Becker and Marin 2009; Wodniok et al. 2011) are more similar to the *ycf2* genes of land plant chloroplasts (Civan et al. 2014). Another protein with a similar and conspicuous evolution is Ycf1 (de Vries et al. 2015 **VII**). Ycf1 is one of the very few proteins that when knocked out generates an embryo-lethal mutant (Drescher et al. 2000). Moreover, it is exclusively found in the green, chloroplastidal lineage (de Vries et al. 2015 **VII**), although one report has suggested it might be homologous to the Tic20-like protein found in some red algae (Nakai 2015). Thus, the Ycf1 protein was gained through unknown means in the common ancestor of all Chloroplastida, but even within the Chloroplastida, detecting reliable homology of the protein sequences proved to be difficult (de Vries et al. 2015 **VII**). Our analyses (de Vries et al. 2015 **VII**) uncovered that this is due to differences between the Ycf1 encoded by the plastid genomes of chlorophytes and lower branching streptophyte algae and those of higher branching streptophyte algae and land plants. In that study, we hence termed these two Cte-Ycf1 and Ste-Ycf1. This clear split highlights that changes in plastid genomes occurred along the trajectory from streptophyte algae to land plants (Figure 3), tying the plastid genomes of the latter together with higher branching streptophyte algae (de Vries et al. 2016 **VIII**). What more can streptophyte plastid genomes tell us?

Various different types of algal plastids are distinguished in the literature (Steiner et al. 2001; McFadden et al. 2011), which correspond to the variety of algal lineages. Land plants also harbour a plethora of different plastid types, but in that case the plastid types differ with respect to the cell identity in which they reside (Figure 3; Jarvis and López-Juez 2013; Pfannschmidt et al. 2015; de Vries et al. 2016 **VIII**) and thus occur in one organism. Something must have happened with the

ability to control plastid differentiation within the streptophtyes, and we suggested that this occurred along the trajectory from freshwater-dwelling streptophyte algae to the first land-dwelling streptophytes (de Vries et al. 2016 **VIII**). Streptophyte algae are thought to have been pre-adapted for the transition from freshwater to land (Becker and Marin, 2009). Many exaptations have been described that are believed to have supported this transition (Delwiche and Cooper 2015; Holzinger and Becker 2015). As discussed (de Vries et al. 2016 **VIII**), various of these exaptations are, however, found among many streptophyte algal lineages and they do not help in pinpointing the singular common ancestor of all land plants. Plastid genome patterns do (de Vries et al. 2016 **VIII**) and, what is more, they uncover the same clade of streptophyte algae as the closest relative of land plants as recent phylogenomic analyses, the Zygnematophyceae (Wickett et al. 2014). The best indicator for this is the absence of *tufA* from the zygnematophyceaen plastid genomes (Baldauf et al. 1990; Turmel et al. 2005; de Vries et al. 2016 **VIII**). *TufA* is among the previously discussed factors that we propose to confer autonomy, which also means that land plants have never experienced this autonomy. There might be a reason for this. In de Vries et al. (2016 **VIII**), we posed that loss of *tufA* from plastid genomes can be considered as a first step of submission to nuclear control and that loss of plastid autonomy allowed the nucleus to exercise more control over plastid function, which was important to spread in an environment that posed many new challenges to plastids such as high irradiances (Maberly 2014; Delwiche and Cooper 2015). This control network was reinforced by the emergence of the proplastid, which can be converted into various plastid types (Figure 3; Jarvis and López-Juez 2013). This might explain why no robust land plant plastids were described: by losing their autonomy they became amendable through nuclear control and evolved into a plastid-type characteristic for higher land plants, which was termed embryoplast (de Vries et al. 2016 **VIII**).

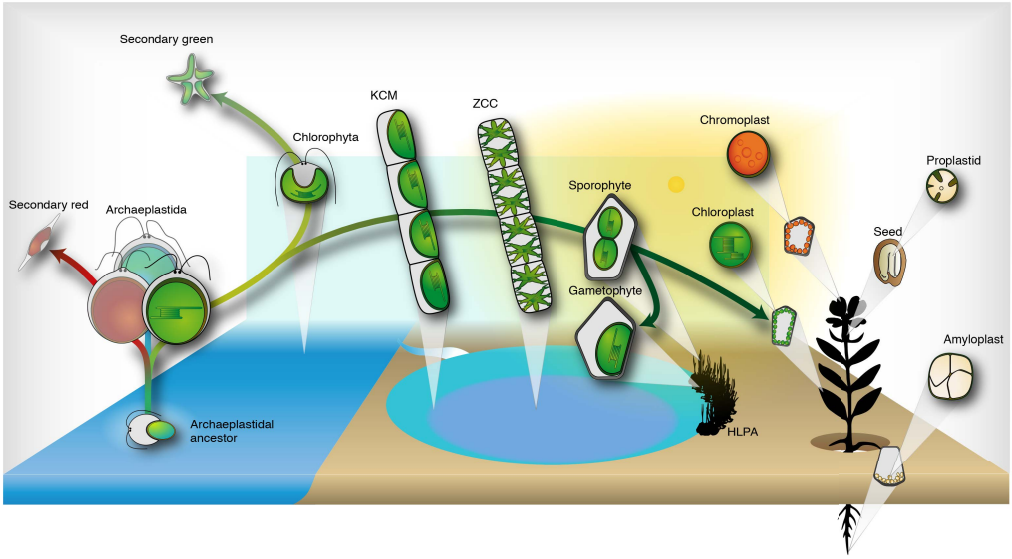

Figure 3: From the origin of plastids to the embryoplast.
The origin of plastids is marked by the uptake of an ancient cyanobacterium by a heterotrophic protist that is the ancestor to all Archaeplastida, from which three lineages arose, one of which are the Chloroplastida (the green lineage), encompassing the Chlorophyta and Streptophyta. Streptophytes include those freshwater algae (the KCM clade = Klebsormidiophyceae, Chlorokybophyceae, Mesostigmatophyceae and the ZCC clade = Zygnematophyceae, Coleochaetophyceae, Charophyceae) from which one lineage successfully gave rise to the hypothetical land plant ancestor (HLPA). From this HLPA, eventually, the complex higher vascular plant evolved. During streptophyte evolution, plastid numbers per cell have changed from monoplastidy in algae, to polyplastidal algae. The gametophytic stage-dominated HLPA likely housed only very few plastids per cell and, based on information on hornworts (Vaughn et al. 1992), cells of the HLPA might even have been monoplastidic during some stages of their life cycle. The sporophyte-dominated vascular plants have polyplastidic cells and an embryoplast that can differentiate into an unparalleled diversity of plastid types.

Publication VI: Is *ftsH* the key to plastid longevity in sacoglossan slugs?

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Contribution of Jan de Vries, shared first author:

Major (drafted the manuscript, prepared two figures, performed parts of the experimental work and computational analysis, developed parts of the rationale)

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Genome Biology and Evolution

SMBE

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

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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 *Bryopsis* 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

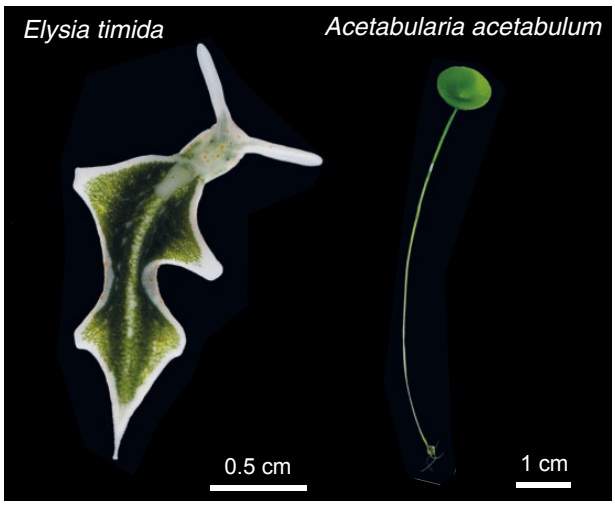

Fig. 1.—The sacoglossan sea slug *Elysia timida* feeds on the ulvophyte alga *Acetabularia acetabulum*. The sequestered kleptoplasts continue to perform photosynthesis for up to several months within specialized cells of the digestive gland of the slug.

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

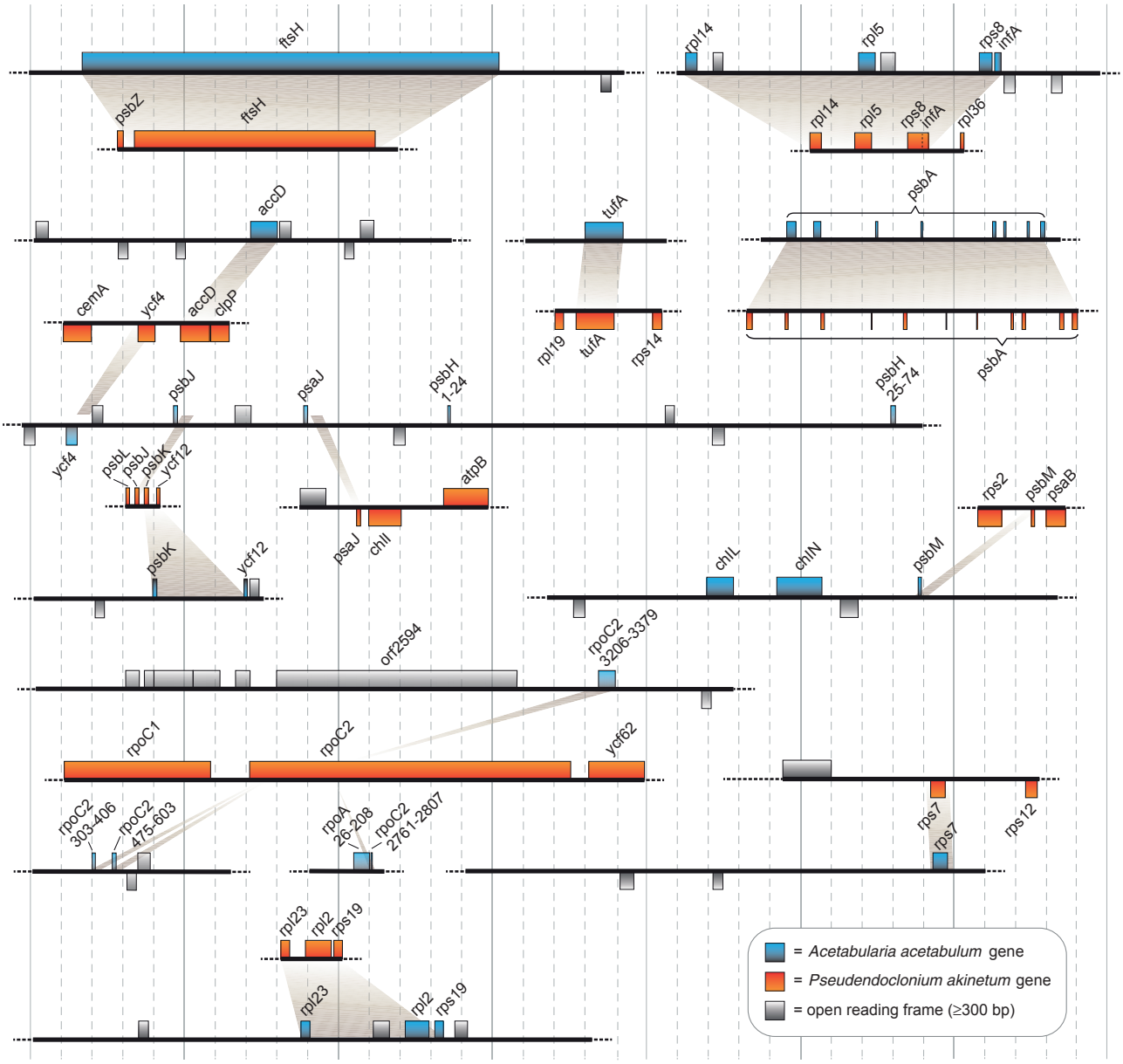

Fig. 2.—Sequencing contigs of the *A. acetabulum* plastid genome. Shown are contigs in comparison to the corresponding parts of the fully sequenced plastid genome of the phylogenetically related *Pseudendoclonium akinetum* encoding identical genes. The comparison illustrates the expansion of most intergenic 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

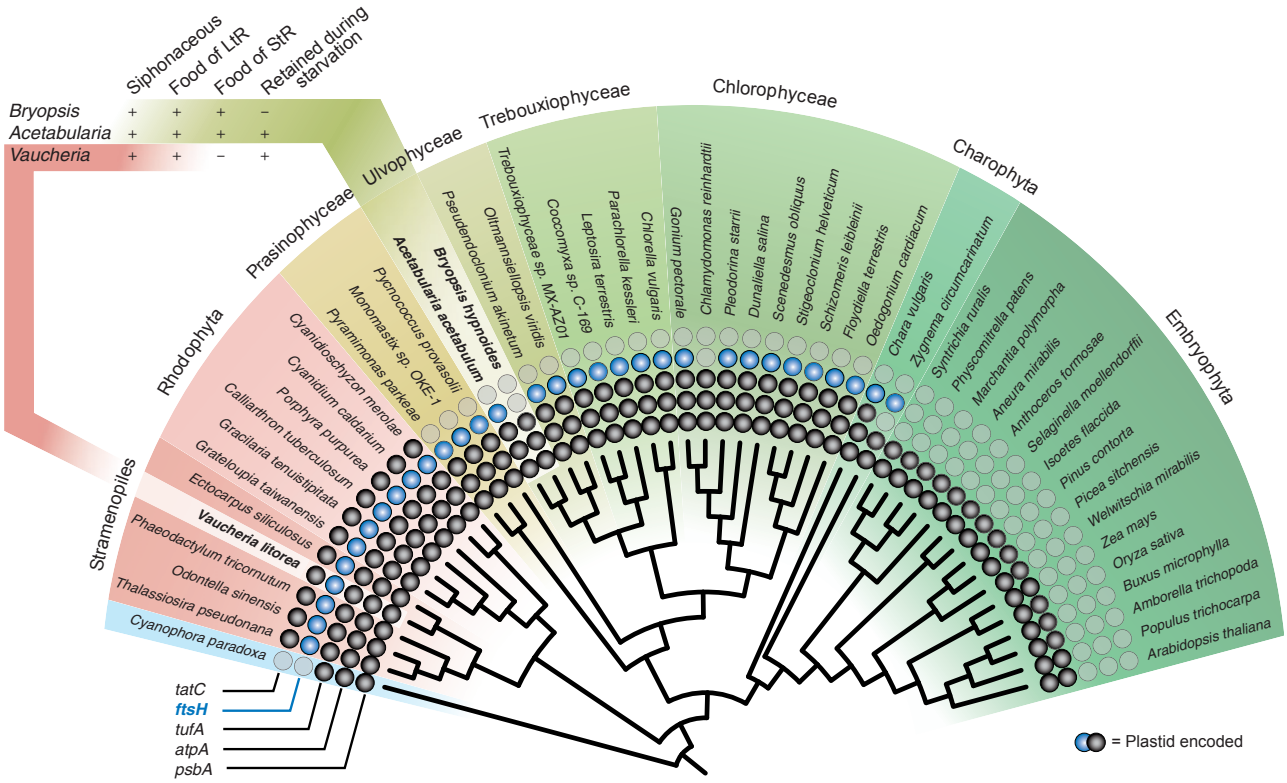

Fig. 3.—*ftsH* and *tufA* are encoded by the majority of algal plastid genomes. Different genes were lost from plastid genomes at different time points throughout evolution. *TatC,* for example, is only retained in plastid genomes of the red lineage, while *psbA* and *atpA,* for instance, are encoded 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*.

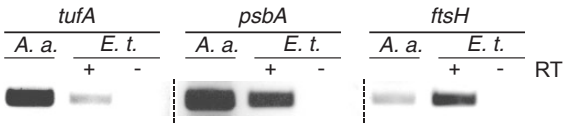

Fig. 4.—Transcripts of *tufA*, *psbA* and *ftsH* are present in starving *Elysia timida*. We isolated total RNA from slugs that had been starved for 1 month (31 days) and performed nonquantitative reverse-transcriptase PCRs to screen for the presence of mRNA of the three genes *tufA*, *psbA,* and *ftsH* 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′-CTGCAGAAAGGTTTGGAGGC-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/).

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Publication VII: YCF1: a green TIC?

Authors: Jan de Vries, Filipa L. Sousa, Bettina Bölter, Jürgen Soll, Sven B. Gould

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Major (drafted the manuscript, prepared three figures and supplemental material, developed parts of the rationale, performed parts of the analysis)

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YCF1: A Green TIC?

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A pivotal step in the transformation of an endosymbiotic cyanobacterium to a plastid some 1.5 billion years ago was the evolution of a protein import apparatus, the TOC/TIC machinery, in the common ancestor of Archaeplastida. Recently, a putative new TIC member was identified in *Arabidopsis thaliana*: TIC214. This finding is remarkable for a number of reasons: (1) TIC214 is encoded by *ycf1*, so it would be the first plastid-encoded protein of this apparatus; (2) *ycf1* is unique to the green lineage (Chloroplastida) but entirely lacking in glaucophytes (Glaucophyta) and the red lineage (Rhodophyta) of the Archaeplastida; (3) *ycf1* has been shown to be one of the few indispensable plastid genes (aside from the ribosomal machinery), yet it is missing in the grasses; and (4) 30 years of previous TOC/TIC research missed it. These observations prompted us to survey the evolution of *ycf1*. We found that *ycf1* is not only lacking in grasses and some parasitic plants, but also for instance in cranberry (Ericaceae). The encoded YCF proteins are highly variable, both in sequence length and in the predicted number of N-terminal transmembrane domains. The evolution of the TOC/TIC machinery in the green lineage experienced specific modifications, but our analysis does not support YCF1 to be a general green TIC. It remains to be explained how the apparent complete loss of YCF1 can be tolerated by some embryophytes and whether what is observed for YCF1 function in a member of the Brassicaceae is also true for, e.g., algal and noncanonical YCF1 homologs.

Some of the best evidence we have for the monophyly of plastids is how they import proteins (McFadden and van Dooren, 2004; Zimorski et al., 2014). Establishing a machinery that translocates proteins across the two membranes separating the organelle's stroma from the cytosol was crucial for the transition of the endosymbiotic diazotrophic-like cyanobacterium to a plastid in the heterotrophic host (Cavalier-Smith, 2000; McFadden and van Dooren, 2004; Dagan et al., 2013). It was also a prerequisite for the successful transfer of genetic material from the endosymbiont to the host nucleus through endosymbiotic gene transfer (EGT; Martin et al., 1993). Most of the EGT that stripped the plastid genome of its coding capacity occurred in the common ancestor from which the three archaeplastidal lineages—Glaucophyta, Rhodophyta (red lineage), and Chloroplastida (green lineage)—evolved (Martin et al., 1998; Zimorski et al., 2014).

Two main protein complexes mediate plastid protein import: the translocon on the outer envelope of chloroplasts (TOC) and the translocon on the inner envelope of chloroplasts (TIC). In accordance with the majority of EGT events, the main TOC/TIC components evolved in the common ancestor of Archaeplastida (Timmis et al., 2004; Kalanon and McFadden, 2008). Most proteins of the TIC complex are of cyanobacterial origin. However, all previously identified TOC and TIC components are encoded on nuclear DNA (Gould et al., 2008; Kalanon and McFadden, 2008; Shi and Theg, 2013), and, thus far, only TIC40 is unique to the Chloroplastida.

Recently, Kikuchi et al. (2013) found the plastid-encoded YCF1 to be part of a 1-MD membrane protein complex that included TIC20. This complex contained neither TIC40 nor TIC110 components that were previously identified (Kessler and Blobel, 1996; Chou et al., 2003), but three novel ones, including YCF1 (Kikuchi et al., 2013). YCF1 was the first plastid-encoded protein identified whose presence was shown to be essential for the survival of *Chlamydomonas reinhardtii* and tobacco (*Nicotiana tabacum*), but whose function remained elusive (Boudreau et al., 1997; Drescher et al., 2000). YCF1—or in accordance with its newly proposed function, TIC214—would represent the first plastid-encoded protein of the TIC complex. It would also add to the set of TIC components (including TIC40 and a full-length TIC62) that is unique to the green lineage. In the course of evolution, organelles have lost or transferred 1000s of genes to the host nucleus, but they hardly ever gained any. That alone is remarkable and the apparent lack of *ycf1* in Glaucophytes and Rhodophytes (whose import apparatus evolved from the same ancestral TOC/TIC as that of Chloroplastida) prompted us to inspect the distribution and sequence diversity of *ycf1* in more detail.

YCF1 AROSE VIA PLASTID GENE GAIN EARLY IN THE GREEN LINEAGE

The sequences of YCF1 proteins are highly divergent, with an average sequence identity well below 25%. This hinders the use of more classical BLAST searches and the generation of reliable phylogenetic trees. Hence, we chose to investigate the phylogenetic distribution of *ycf1* among the Archaeplastida using Hidden Markov Model (HMM) analyses on protein-coding genes of 558 archaeplastidal plastid genomes and a set of 55 cyanobacterial genomes from all five sections (NCBI; as of October 16, 2014). To test the sensitivity of our approach, we performed identical searches using protein alignments of 12 TIC20 and 16 TIC110 sequences from all major embryophyte (land plant) lineages (Figure 1; Supplemental Data Set 1). Through automatic genome annotations, protein-coding genes are sometimes incorrectly annotated as pseudogenes (that do not result in functional proteins) or are even missed entirely. To accommodate for this possibility, we performed a successive screen on those

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genomes for which no hits were detected in our first round of identifying YCF1 homologs. A tBLASTn search was performed (a search of translated nucleotide databases using a protein sequence query) and the output then added to an additional HMM search. By doing so, we identified several, yet with respect to sequence coverage and length, problematic YCF1 homologs. For instance, they might represent fragments of once full-length YCF1 proteins. These results are shown separately (Supplemental Figure 1 and Supplemental Data Set 2), as we do not wish to speculate on their possible functionality or correct annotation, and, more importantly, the difference is irrelevant for our conclusion.

A previous comprehensive analysis of the evolution of green plastid genomes suggested *ycf1* to represent one of the only few examples, together with *ycf2* and *matK*, of plastid gene gain in the green lineage (Wicke et al., 2011). However, there is also the often overlooked possibility of differential gene loss; the absence of homologs in available cyanobacterial genomes would support the former scenario. We found homologs encoded by cyanobacterial genomes only in the case of TIC20 that we used as a positive control (Figure 1B, vii), but not for YCF1 or TIC110 (Figure 1, i to vi and viii). The universal presence of TIC20 is in line with previous models (Kalanon and McFadden, 2008), and the lack of evidence for differential gene loss indeed supports the scenario that *ycf1* was introduced into the plastid genome of the common ancestor of Chloroplastida (Figure 2). However, there is a large degree of *ycf1* sequence variability in annotated genomes (particularly among the algae) that points to a more complicated evolutionary history.

YCF1 IS NOT RESTRICTED TO LAND PLANTS, BUT HMM ANALYSIS SUPPORTS A GENERAL SEPARATION BETWEEN STREPTOPHYTE AND CHLOROPHYTE YCF1 SEQUENCES

Using *Arabidopsis thaliana YCF1* (ATCG01130; Figure 3, i) as a seed sequence to search for a potentially conserved HMM characteristic for YCF1 returned 466 hits, 462 of which were restricted to embryophytes. Within the

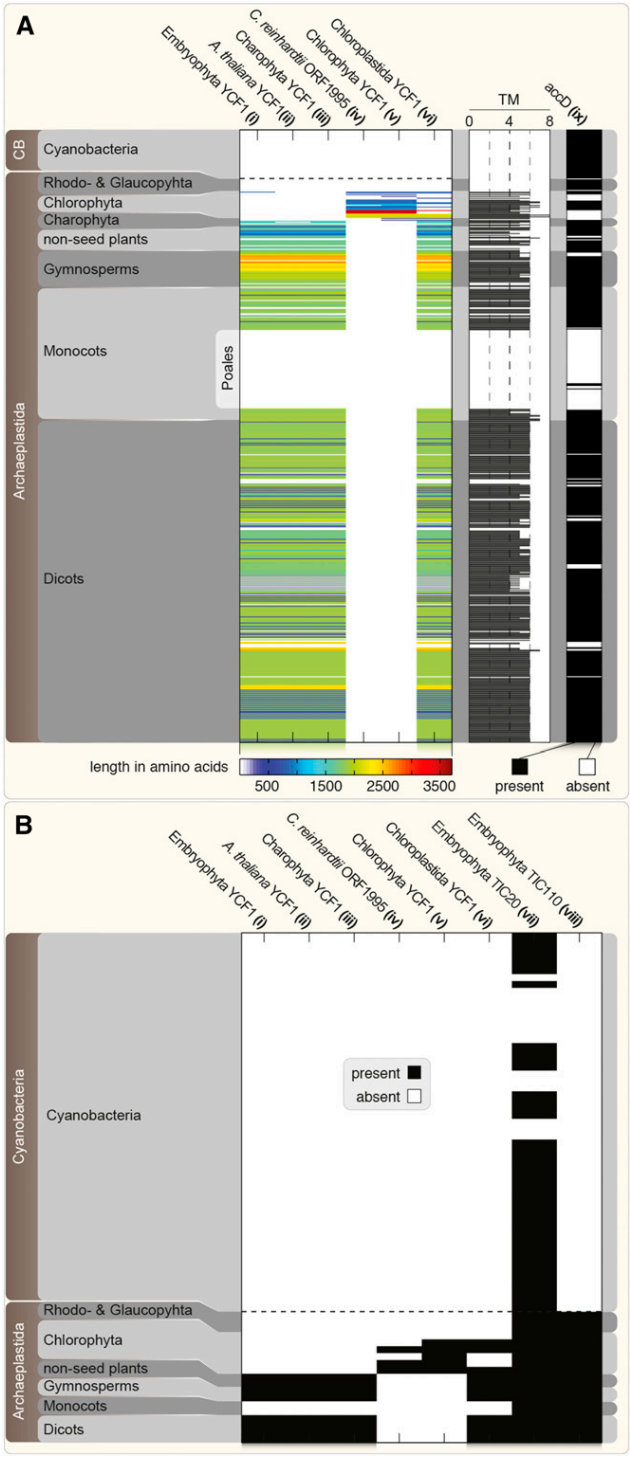

Figure 1. *ycf1* Distribution and Sequence Diversity.

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angiosperms, no YCF1 homolog was detected among Poales except *Typha latifolia* (Guisinger et al., 2010; Figure 1A, ii); this observation supports the early loss of *ycf1* in the grass lineage (Figure 2). A streptophyte-type YCF1 (Ste-YCF1) was detected only in genomes of charophytes that are currently thought to represent the streptophyte algae most closely related to land plants (these are Charophyceae, Zygnematophyceae, and Coleochaetophyceae; Civaň et al., 2014). A Ste-YCF1 was not detected in genomes of more distantly related taxa, including more distantly related charophytes (Chlorokybophyceae, Mesostigmatophyceae, and Klebsormidiophyceae), let alone rhodophytes, glaucophytes, and cyanobacteria (Figures 1A [ii] and 2; Supplemental Data Set 1). Thus, the approach detected the evolutionarily most distant land plant relative among charophytes using Arabidopsis YCF1 as a seed sequence to build the HMM. To exclude potential false negatives, we performed an HMM search based on an alignment of 25 YCF1 sequences from all major embryophytic lineages. This returned the same pattern, with hits limited to only streptophytes (HMM E-value $\leq 10^{-5}$) except for the chlorophyte alga *Pseudendoclonium akinetum* RF1 (YP_636263.1; Figure 1A, i) and differing in only two hits compared with the At-YCF1 results. Therefore, irrespective of whether we used one sequence or an alignment of 25 to commence our HMM search, the results were the same. This is in contrast to the results of Kikuchi et al. (2013), who found the *ycf1* gene present among all Chloroplastida, with the exception of the Poales, but including Chlamydomonas.

The potential ortholog of Ste-YCF1 sequences in Chlamydomonas (Chlorophyte lineage) is *Crorf1995* (GenBank X92726.1). Boudreau et al. (1997) were the first to notice structural similarities but were careful to draw a homology between *Crorf1995* and the land plant *YCF1* and the lack of the latter in the red and glaucophyte lineages. When we used Cr-ORF1995 as a seed sequence (in the same manner as At-YCF1; described above), the search for an HMM returned 23 hits, all limited to the chlorophyte lineage (Figure 1A, iv). Using an alignment of chlorophyte YCF1 sequences (a total of 17, all homologous to *Crorf1995* with an E-value $< 10^{-10}$) returned 28 hits among the chlorophyta and charophyta most closely related to Chlorophyta, but none among other streptophytes. Even when using the only nonstreptophyte protein retrieved above (the chlorophytic *P. akinetum* RF1; Figure 1A, i) as a seed sequence for an HMM search, we exclusively detected other chlorophyte hits (Supplemental Data Set 1). Only when we used a set of sequences from the alignment of Kikuchi et al. (2013), containing both streptophyte and chlorophyte YCF1 (henceforth named Chloroplastida YCF1), did we detect hits (481) among both Chlorophyta and Streptophyta (Figure 1A, vi). Importantly, the HMM search based on the Chloroplastida YCF1 alignment (Figure 1A, vi) returned hits that are, within the streptophyte lineage, fully consistent with the results obtained by building a streptophyte-only HMM (Figure 1A, i to iii). Yet, within the chlorophyte lineage, there is only a 50% match to the hits returned by using the chlorophyte alignment (Figure 1A, v). Although Cr-ORF1995 is present in the Chloroplastida YCF1 alignment, only 43.5% of the hits match those obtained by just using the Cr-ORF1995 sequence alone (Figure 1A, iv). This means that there is a general separation between streptophyte and chlorophyte YCF1 proteins (that is, Ste-YCF1 and Cte-YCF1; Figure 2). We might then ask how a connection between the algal Cte-YCF1 and the streptophyte Ste-YCF1 sequences was drawn in the first place.

The first chloroplast genome to be sequenced was that of the liverwort *Marchantia polymorpha* (Ohyama et al., 1986). Four additional embryophyte plastid genomes followed (Shinozaki et al., 1986; Hiratsuka et al., 1989; Wakasugi et al., 1994; Maier et al., 1995) before any algal plastid genome was sequenced. The first algal plastid genomes sequenced were not from green algae, but from the red algae *Porphyra purpurea* (Reith and Munholland, 1995), the glaucophyte *Cyanophora paradoxa* (Stirewalt et al., 1995), the heterokontophyte (secondary red) *Odontella sinensis* (Kowallik et al., 1995), and the euglenophyte *Euglena gracilis* (secondary green; Hallick et al., 1993). Finally, in 1997, the first plastid genome of a chlorophyte was reported, that of *Chlorella vulgaris* C-27 (Wakasugi et al., 1997). Wakasugi and colleagues detected a large open reading frame, Cv-ORF819 (NP_045891), for which no homolog was found among published algal plastid genomes, but it shared a few similarities (the N-terminal region encoding transmembrane domains) to the YCF1 found in the land plant plastid genomes available at that time. Wakasugi and colleagues raised

Figure 1. (continued).

(A) Phylogenetic distribution of YCF1 among 558 archaeplastidal plastid and 55 cyanobacterial genomes. HMM searches were performed based on different alignments or sequence seeds (i to viii). Hits (E-value $< 10^{-5}$) based on YCF1 alignments (i to vi) among all protein-encoding genes of 558 archaeplastidal plastid and cyanobacterial (CB) genomes are shown on the left, color-coded based on their amino acid length (blue to red; white means absence). Note the absence of YCF1 homologs among cyanobacteria, Rhodophyta, Glaucophyta, and Poales. For all detected YCF1 homologs, the number of TMHMM-predicted transmembrane helices (TM) is shown in the horizontal histogram. For comparison, the results (positive hit, black; no hit, white) of an HMM search against all plastid and cyanobacterial genomes using an alignment of 37 ACCD protein sequences is shown on the right (ix).

(B) The presence or absence (black and white, respectively) of genes encoding TIC20 and TIC110 among all protein-encoding genes of archaeplastidal nuclear and cyanobacterial genomes is shown. The screen is based on HMM searches using embryophyte sequence alignments (vii and viii). In comparison, the data from the YCF1 HMM searches in the archaeplastidal plastid and cyanobacterial genomes are shown in the same fashion. Dashed horizontal lines separate the cyanobacterial and archaeplastidal genome data. HMM and TMHMM results for every species analyzed are individually listed in Supplemental Data Set 1.

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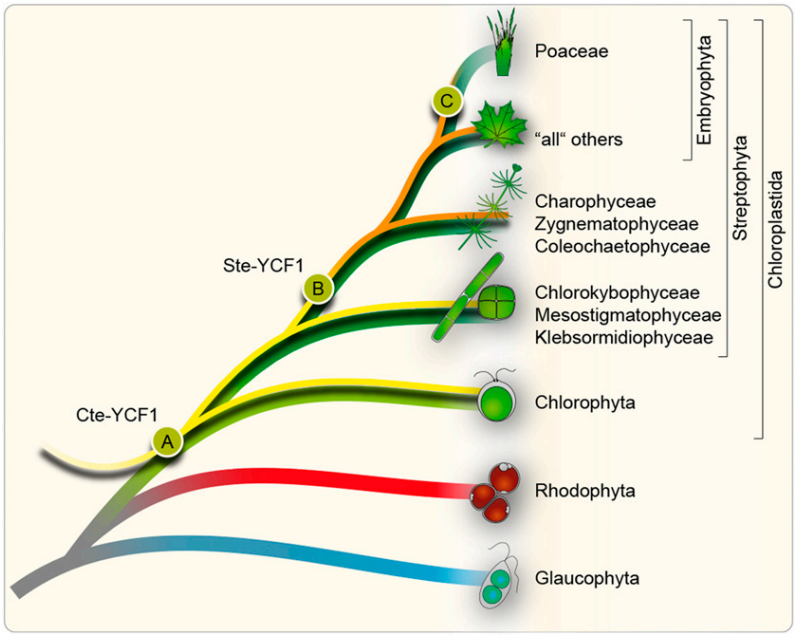

Figure 2. Cladogram of *ycf1* Evolution.

ycf1 was gained in the green lineage (event A), leading to the initial distribution of the ancestral chlorophyte-type (Cte)-YCF1 among all Chloroplastida (yellow trajectory). Within the charophytes, the ancestral "800 amino acid" Cte-YCF1 was transformed into the "1500 amino acid" Streptophyte-type (Ste)-YCF1 (event B), leading to its distribution among Embryophyta and their closest charophyte neighbors (orange trajectory). The vast majority of the embryophytes ("all" others) retained the Ste-YCF1, while it was lost in the Poaceae (event C) and a few other cases mentioned throughout the text. Note that in some chlorophyte algae, the ancestral Cte-YCF1 (initially 800 amino acids in length) also experienced some sequence duplication events that led to much longer versions of YCF1. However, this occurred independently from event B.

concerns with regard to the weak similarity but proposed Cv-ORF819 to represent a potential YCF1 ortholog (Wakasugi et al., 1997); thus, a tenuous bridge from algal chlorophytes to land plant streptophytes was established. With the release of the second chlorophyte plastid genome, that of *Nephroselmis olivacea* (Turmel et al., 1999), sequence discrepancies between streptophyte and chlorophyte YCF1s were no longer traceable. With the plastid genome data now available, our HMM-based analysis identified *Cvorf819* to be homologous to Cte-YCF1s, but its sequence similarity to Ste-YCF1s is somewhat obscure and warrants attention. Therefore, while there is an evolutionary connection between the Cte-YCF1 and Ste-YCF1 proteins, it becomes apparent that in streptophyte evolution, something special happened to YCF1 (Figure 2).

MASSIVE YCF1 SEQUENCE DIVERSITY AND HOW IT MIGHT HAVE EVOLVED

TIC214 is not always 214 kD. It is important to note that YCF1 sequences vary immensely in length. Those chlorophytes (prasinophytes) and charophytes (Mesostigmatophyceae and Chlorokybophyceae; Leliaert et al., 2012) most closely related to the common ancestor of all Chloroplastida possess a Cte-YCF1 of ~800 to 900 amino acids (Figure 3, vi). Yet in both the streptophyte and chlorophyte trajectories, some lineages encode YCF1 proteins that are >1500 amino acids in length, in some cases above 3000 amino acids. A rather conserved feature, and precisely the one that initially led to the original link of Cte- and Ste-YCF1s (Wakasugi et al., 1997), is the presence of N-terminally encoded transmembrane domains (TMs). For the vast majority of YCF1s, six TM domains are predicted based on TMHMM (Krogh et al., 2001), but the number ranges between four and eight (Figure 1A). In terms of being a TIC-associated membrane protein, the spruces *Picea morrisonicola* and *Picea abies* are interesting. Each spruce encodes a single YCF1 of ~1800 amino acids, but the N-terminal regions encoding the TM domains are missing. As discussed above regarding pseudogenization, we cannot exclude that this is due to a wrong annotation. A region encoding TMs is present upstream of the annotated genes (Figure 3, ii). However, the largest variation is found within the region downstream of the N-terminal TM domains.

Arabidopsis encodes a canonical YCF1 (ATCG01130), but also a very short version of only 343 amino acids (ATCG01000; Figure 3, i) largely lacking the sequence reaching into the stroma (according to Supplemental Figure 17 in Kikuchi et al., 2013). For ATCG01000, this truncation is likely due to the N terminus being encoded on the inverted repeat, but some seed plants, such as *Brassaiopsis hainla*, might encode only a short YCF1 (574 amino acids; Figure 3, iii). Some basal branching bryophytes even have a split version, maybe due to overlooked introns (Figure 3, iv). The domain of YCF1 reaching into the stroma harbors many highly variable charged motifs arranged in tandem (charge calculations are based on EMBOSS explorer charge prediction; Rice et al., 2000). Both the number and amino acid composition of these motifs vary, which explains why they are difficult to detect and align through classic BLAST searches, especially across distant taxa. This is reminiscent of the coiled regions of intermediate filament proteins, which function as "molecular Velcro" (Rose et al., 2005) and are rapidly evolving (Gould et al., 2011). Thus, what generally unites the streptophyte and chlorophyte YCF1 is not the primary sequence, but the presence of N-terminal TM helices that are followed by a sequence of varying length with repetitive charged motifs.

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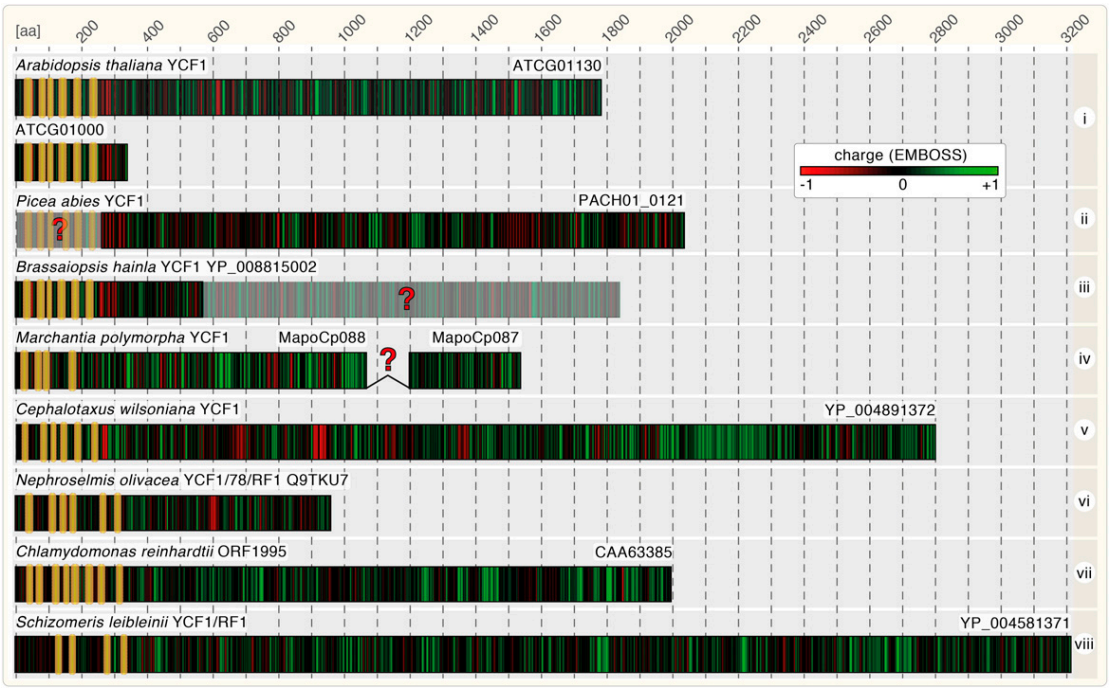

Figure 3. YCF1 Structural Features and Charge Distribution.

Structure and charge distribution of exemplary YCF1 proteins of eight species (i to viii). Proteins are schematically drawn, but to scale with regard to their amino acid length (scale at the top) based on current genome annotations. Potentially problematic annotations (red question marks) include a possibly incorrect open reading frame annotation (sequence ii), a frameshift (possible sequencing error; sequence iii), and an overlooked potential intron (sequence iv). ATCG01000 is a truncated copy of YCF1 present in the inverted repeat of the Arabidopsis plastid genome. Positive and negative charge distribution is shown in green and red, respectively, based on EMBOSS charge prediction using the standard settings. The positions of TMHMM-predicted TM helices are highlighted by yellow boxes. For each protein the gene ID or NCBI accession is shown at the C terminus of the respective sequence.

Whether streptophytic or chlorophytic, previous analyses indicate that *ycf1* emerged in the common ancestor of all Chloroplastida (Maul et al., 2002; reviewed in Wicke et al., 2011), apparently as an orphan gene because homologs outside of the green lineage are not known (Figure 2). Ste-YCF1 evolved within the Charophyta and was then vertically inherited by all Embryopyhta. This means that either—although it seems quite unlikely—Cte-YCF1 was lost in the later evolving lineages of charophytes and there was a gain of the Ste-YCF1 or, much more likely, the ancestral Cte-YCF1 (of around 800 amino acids) rapidly evolved into an ~1500-amino-acid-long protein (Figure 2); maybe through a recombination event of the sequence encoding the repetitive part of the protein as the amount of N-terminal TMs has more less remained unchanged (Figure 1A). For a better phylogenetic resolution, we performed an HMM search based on an alignment of five charophyte YCF1 sequences from charophytes most closely related to land plants (Charophyceae, Zygnematophyceae, and Coleochaetophyceae). This search returned 467 hits that matched the results of the HMM search based on the YCF1 Embryophyta alignment to 100% (Figure 1A, i and iii). Within the charophyte lineage, Ste-YCF1 is only found among these charophytes, but not in the more distantly related ones (Chlorokybophyceae, Mesostigmatophyceae, and Klebsormidiophyceae; Figure 2). This places the origin of Ste-YCF1 at the base of the Charophyceae, which, in accordance with the latest phylogenetic analysis (Civaň et al., 2014), explains their occurrence in the Zygnematophyceae, Coleochaetophyceae, and Embryophytes.

To complicate the matter, the Poales are not the only group within the angiosperms that lack YCF1; the parasitic plant *Orobanche purpurea* as well as *Vaccinium macrocarpon* and the genus *Erodium* have also lost the sequence. One might argue that the loss in parasites such as *Orobanche* is consistent with the fact that they are no longer phototrophic; however, apicomplexan parasites maintain all essential components of the protein import machinery for their highly streamlined and plastid-derived apicoplast (McFadden, 2011). Furthermore, this does not account for the loss in phototrophs such as *Vaccinium*, *Erodium*, and the grasses, and, most importantly, they all need to import proteins from the cytosol. For species for which no nuclear genome sequences are available, we cannot exclude the possibility that *ycf1* was transferred from the plastid to the nuclear genome, as it is a frequent event that can occur multiple times independently (Martin et al., 1998; Millen et al., 2001). But should this be the case, it would further

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complicate YCF1 evolution and diversity; moreover, grass nuclear genomes available do not encode an apparent *ycf1* ortholog.

Besides having lost *ycf1* from their plastid genome, grasses have replaced the plastid-encoded acetyl-CoA carboxylase D (accD) with a nuclear gene (Konishi et al., 1996). The corresponding protein is posttranslationally targeted to plastids and represents the eukaryotic large homomeric fatty acid (FA) synthase; in dicot plastids, the prokaryotic heteromeric form persists. The fact that grass plastids no longer assemble a heteromeric fatty acid-synthase complex, and at the same time have no need for YCF1, makes it tempting to speculate that YCF1 is involved in the assembly of the ACCase holoenzyme. Among embryophytes, in 80% of the cases where *ycf1* was lost from the plastid genome, it co-occurred together with the loss of *accD* (Delannoy et al., 2011), a trend that we were able to confirm by performing an HMM search using an alignment of 37 ACCD sequences from a broad range of embryophytes (Figure 1A, ix). In case of the above-mentioned *O. purpurea*, *accD* is still present in the chloroplast genome, although it does not encode a YCF1 homolog. *Erodium* and *V. macrocarpon*, on the other hand, lack ACCD. Whether or not this correlation translates into a functional causality is not known, but is worth investigating.

CONCLUDING REMARKS

Kikuchi et al. (2013) concluded that the complex containing YCF1 "constitutes a general TIC translocon." While we do not want to question the importance of YCF1 with regard to protein translocation in Arabidopsis, in the light of such sequence variation (some species appear to lack individual TM domains or even the entire gene), it is valid to ask how universal such a claim can be for all Chloroplastida. One could imagine that the emergence of YCF1 is associated with the substitution of phenylalanine at the +1 position of the ancestral N-terminal transit peptide: Based on this amino acid position, glaucophytes and rhodophytes are able to distinguish thousands of plastid proteins from all others translated in the cytosol (Patron and Waller, 2007; Gould, 2008; Bolte et al., 2009). The green lineage Chloroplastida lost this ability, and it has been speculated that the emergence of TOC159 and TOC64 was required to compensate for this remarkable evolutionary drift (Gould et al., 2008). However, these two proteins are needed for the recognition on the outside of the organelle; for TIC214, this scenario would only make sense if it is somehow associated with transit peptide recognition inside the stroma. Also, would not all Chloroplastida then require a functional YCF1? If this protein truly is "a general TIC translocon" (Kikuchi et al., 2013), its sequence diversity is unique in the light of the TOC/TIC machinery and its evolution. Future research needs to determine whether this finding applies to algal and noncanonical Ste- YCF1 homologs and how a complete YCF1 loss is tolerated by some embryophytes. Biochemical data from a broader range of species that also provide a functional explanation for the absence of YCF1 in Poales is direly needed.

Supplemental Data

Supplemental Figure 1. *ycf1* distribution based on HMM searches against a data set of all protein data from the plastid and cyanobacterial genomes plus additional tBLASTn hits.

Supplemental Data Set 1. Results for all YCF1 HMM searches against a data set containing all protein data from the plastid and cyanobacterial genomes as shown in Figure 1.

Supplemental Data Set 2. Results for all YCF1 HMM searches against a data set containing all protein data from the plastid and cyanobacterial genomes plus additional tBLASTn hits as shown in Supplemental Figure 1.

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AUTHOR CONTRIBUTIONS

J.d.V. and F.L.S. performed the computational analysis. S.B.G. and J.S. designed the study. All authors discussed the results and commented on the article that J.d.V., B.B., and S.B.G. wrote.

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Publication VIII: Streptophyte terrestrialization in light of plastid evolution

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Major (drafted the manuscript, prepared two figures, developed a major part of the rationale)

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Opinion

Streptophyte Terrestrialization in Light of Plastid Evolution

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Key steps in evolution are often singularities. The emergence of land plants is one such case and it is not immediately apparent why. A recent analysis found that the zygnematophycean algae represent the closest relative to embryophytes. Intriguingly, many exaptations thought essential to conquer land are common among various streptophytes, but zygnematophycean algae share with land plants the transfer of a few plastid genes to the nucleus. Considering the contribution of the chloroplast to terrestrialization highlights potentially novel exaptations that currently remain unexplored. We discuss how the streptophyte chloroplast evolved into what we refer to as the embryoplast, and argue this was as important for terrestrialization by freshwater algae as the host cell-associated exaptations that are usually focused upon.

Streptophyte Algae and Terrestrialization – A Simple Trajectory?

More than 1.2 billion years ago (Gya), a cyanobacterium entered an endosymbiotic relationship with a heterotrophic protist [1–3] (Figure 1, Key Figure). Their successful co-evolution gave rise to the first eukaryotic cell that carried a vertically inherited plastid. From the single origin of eukaryotic photosynthesis [4–6] three major lineages arose: the glaucophytes, the rhodophytes, and the **Chloroplastida** (see Glossary; Box 1). While there are many land-dwelling algae today, only a single **streptophyte algal** lineage successfully conquered land on a global scale and rose above its substrate.

Two aspects of the biology of streptophyte algae (also known as **charophytes**) are considered to have favored their transition to land: they are fresh-water algae and they possess various exaptations that allowed them to cope with the environmental stresses they then encountered [7,8]. Streptophyte algae underwent various specific biochemical adaptations that set them apart from chlorophytes [9,10]. Nevertheless, although they are all freshwater algae, the terrestrialization event by **Streptophyta** that marked the birth of land plants was a singularity. Why? The ability to tolerate desiccation cannot be the sole reason, because this is a feature of various streptophyte algal lineages [11]. The same is true for other key **embryophytic** characters important for the terrestrial lifestyle of the plants, such as gravitropism [12], parenchymatous tissues and meristems [13], cuticle-like substances [14], the possible shift from LHCSR (light-harvesting complex stress-related)- to PSBS (photosystem II subunit S)-dependent non-photochemical quenching [15], the presence of gene families involved in establishing symbioses with arbuscular mycorrhizal fungi [16,17], and the molecular chassis that underpins the physiology and development of streptophyte algae [18,19]. Plant terrestrialization is also thought to have benefited from a cell wall that was suggested to have specialized early in a streptophyte algal lineage already on land, owing to a lack of selection pressure on such a cell wall in aqueous environments [20]. However, different types of cell wall modifications are a universal feature of algae [21]; consider kelp, whose sturdy blades can reach many dozen meters in length, or *Calliathron* that synthesizes lignin [22]. Delwiche and Cooper recently provided a scholarly review

Trends

Interest in better understanding plant terrestrialization is growing, and there has been a longstanding debate regarding the nature of the single common algal ancestor of land plants. Emerging sequence data has now uncovered zygnematophycean algae as the closest relatives.

These data are also uncovering a growing list of exaptations among fresh-water (streptophyte) algae that are thought to be beneficial for conquering soil. Among them are factors often selected for in crop plants, such as the ability to cope with abiotic stress factors (e.g., high light stress) or the ability to engage in symbiotic relationships with fungi that aid in nutrient uptake.

None of these factors are, however, restricted to a single group of streptophyte algae, and the enigmatic question remains why terrestrialization was a single monophyletic event.

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Key Figure

Chronology and Key Steps in Plastid Evolution: From the Archaeplastidal Ancestor of Plastids to Plastid Diversification in Seed Plants

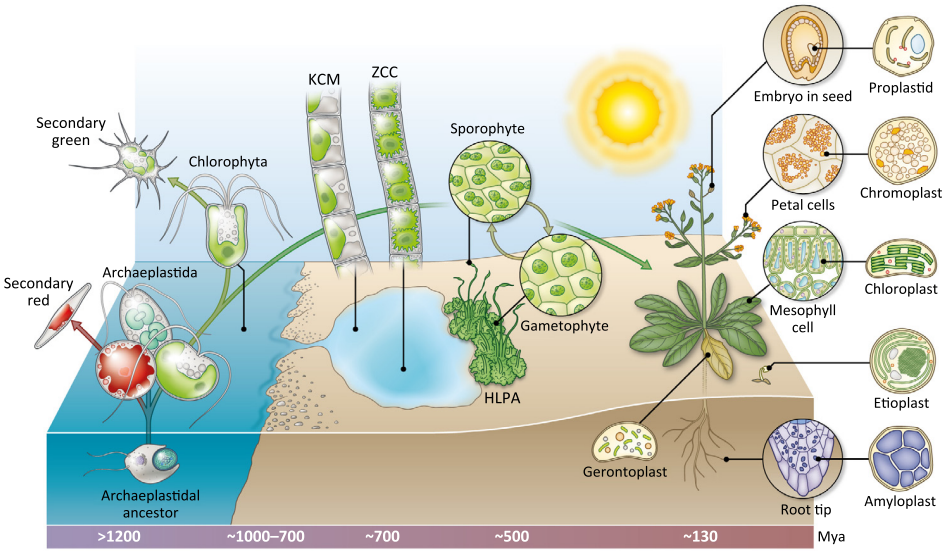

Figure 1. Through an endosymbiotic event that occurred at least 1200 Mya (million years ago), the heterotrophic common ancestor to all Archaeplastida acquired a cyanobacterium that was transformed into the first plastid. From this first phototroph, three major lineages arose. Members of the red and green lineage (Rhodophyta and Chlorophyta, respectively) engaged in secondary endosymbioses, giving rise to various kinds of meta-algae that harbor complex (secondary) plastids. The chloroplastidal lineage split some 1000–700 Mya into the Chlorophyta and Streptophyta. The first streptophytes were single-celled algae of the KCM clade (either Chlorokybophyceae or Mesostigmatophyceae). Within the KCM clade, the Klebsormidiophyceae evolved multicellularity and formed unbranched filaments. Algae of the ZCC clade, which arose from KCM streptophytes at approximately 700 Mya, are the first of the green lineage to house more than one plastid per cell. Although the ZCC clade bears algae of high morphological complexity, the unbranched filamentous Zygnematophyce are considered to be the closest algal relatives to the hypothetical land plant ancestor (HLPA). The HLPA likely evolved the alteration of generations early on, but was dominated by the gametophytic stage; polyplastidy might have occurred only in sporophytes of the HLPA (which is a trend, but not a rule, observed among extant hornworts, *cf.* [54]). With the rise of the angiosperms at approximately 130 Mya the plastid itself experienced tissue-specific specialization governed by the nucleus. All dates are based on [73–75].

of the relative contributions of these features to terrestrialization [8], but the 'fascinating, and enigmatic, question remains': what allowed only a single streptophyte algal lineage to conquer land? In fact, which lineage fits the bill was until recently still debated.

The evolution and diversification of land plants (embryophytes) has largely been inferred based on the analysis of plastid genes [23,24]. A crucial aspect that until recently has never been fully resolved is the basal branch of terrestrialization, that is, the identity of the group of algae that gave rise to plants. The evolution of streptophyte algae from single-celled green protists to complex multicellular 'thalli' was traditionally inferred based on morphological features, and was viewed as a clear trajectory from simple to complex. Recent phylogenetic analyses of hundreds of proteins conclude that the Zygnematophyceae constitute the streptophyte algal group that branches closest to embryophytes [25–28]. This might come as a surprise because gradual evolution from simple to complex is intuitive and seems logical, and Zygnematophyceae do not

Glossary

Amyloplast: a starch-storing plastid.
Archaeplastida: the monophyletic lineage of photosynthetic eukaryotes that arose at more than 1.2 Gya by the engulfment of a cyanobacterium – likely an ancestor of section IV or V cyanobacteria [66] – through a heterotrophic unicellular eukaryote. With the sole exception of *Paulinella chromatophora* [67], this event represents the only primary origin of photosynthesis in eukaryotes.
Charophytes: see streptophyte algae.
Chloroplastida: the monophyletic 'green' lineage of archaeplastida, uniting the chlorophytes and streptophytes.
Chromoplast: a type of plastid serving pigmentation.
Co-location of redox regulation (CoRR) hypothesis: a hypothesis that explains why bioenergetic organelles (plastids and mitochondria), that carry a membrane-bound electron transfer chain, retain genomes despite of gene loss and EGT.
Endosymbiotic gene transfer (EGT): the transfer of genetic material from the organellar to the nuclear genome and one of the major forces of organellar genome erosion [68].
Elaioplast: a lipid-storing plastid.
Embryophytes: the monophyletic, streptophytic lineage of land plants, ranging from basal hornworts and liverworts to flowering oaks.
Embryoplast: a type of plastid unique to the embryophyte lineage. It is characterized by a genome architecture that ties it to ZCC plastids and, most importantly, the ability to differentiate into subtypes (e.g., amyloplast or chromoplast; Figure 1) that in their diversity within a single species are restricted to vascular plants and likely orchestrated by the NEP.
Etioplast: a white plastid that differentiated in the dark (from a proplastid).
KCM clade: Klebsormidiophyceae, Chlorokybophyceae, and Mesostigmatophyceae. The clade of lower branching streptophyte algae.
Nucleus/plastid-encoded RNA polymerase (NEP/PEP): while the PEP is of cyanobacterial origin, the NEP is derived from the phage-type mitochondrial NEP; both service genes with their own specific promoters [63,65,69]. Green algae

Box 1. Plastids Are Special and Not All Plastids Are Alike

From the monophyletic origin of Archaeplastida, three major lineages arose (Figure 1). With only 15 described species, the glaucophytes represent the smallest group of Archaeplastida. Glaucophyte plastids have retained the cyanobacterial light-harvesting phycobilisomes and the ancestral peptidoglycan layer that is absent in all other plastids [76] except mosses [89]. The rhodophytes (~6000 described species) also retain phycobilisomes, and are further noteworthy in their production of floridean starch that resembles animal glycogen. Floridean starch is synthesized in the cytosol, with important implications regarding cytosolic versus stromal starch metabolism [77]. The most species-rich group is the chloroplastidal lineage with more than 350 000 species. It includes the algal Chlorophyta (~8000 species) and the Streptophyta that unite the streptophyte algae (~6000 species), and the land plants, the Embryophyta, that represent more than 95% of streptophyte diversity (*cf.* [78]; theplantlist.org). While the Chloroplastida lost the phycobilisomes, they are the only group to have retained the ancestral chlorophylls *a* and *b* [79]. Important features of the plastid were thus modified already early in the diversification of the archaeplastidal lineage.

The radiation of plastids in Archaeplastida did not end with the evolution of three primary lineages. Through secondary endosymbiotic events, primary plastids of the green and red algal lineages found residence in at least three other eukaryotic groups independently. These gave rise to several new lineages, one of which includes *Plasmodium*, the causative agent of Malaria [1–3]. While primary plastids are surrounded by two membranes, secondary plastids are surrounded by three or four, across all of which nucleus-encoded proteins need to be imported [80]. It was the presence of those additional membranes, separating the plastid stroma from the host cytosol, that prompted Gibbs to postulate that plastids had been passed from one eukaryote to another [81]. Plastids are not always retained for their ability to photosynthesize [82], but sometimes for their ability to synthesize lipids, FeS–clusters, heme, or isoprenoid precursors [83]. To a large extent this occurs in parasites, and it is noteworthy that whereas photosynthesis is lost early on during the emergence of a parasite of phototrophic origin, it is carbon fixation that is generally viewed as the most prominent attribute of plastid biochemistry. Plastids are also not always vertically inherited, and are sometimes 'stolen' (kleptoplasty) by each new generation [84,85]. Some plastids no longer have a genome [86] and sometimes the entire organelle can be lost [87]. Plastid diversity is immense and the success of photosynthetic eukaryotic lineages is undeniable.

feature the most complex morphology. However, Zygnematophyceae might have secondarily reduced their multicellular organization [27], and therefore the morphological features of algae, such as *Chara*, have misled us into placing Charophyceae as the closest algal relatives of plants.

There is evidence other than phylogenetic trees that Zygnematophyceae and embryophytes share the same ancestor. Most genes of the cyanobacterial progenitor of the plastid were either lost or transferred to the nucleus early in the evolution of **Archaeplastida** [29]. However, all known photosynthetic plastids retain a genome, which is still best explained by the **co-location of redox regulation (CoRR) hypothesis** [30]. Recent comparisons of plastid genomes have revealed curious distributions of particular genes among algae and land plants [26,31–34], and the coding capacity of plastid genomes offers support for the relationship between Zygnematophyceae and embryophytes (Box 2). It should be noted that the plastid-encoded proteins YCF1, YCF2, and FtsH highlight the changes that occurred in streptophyte plastids (Figure 2); these genes experienced a turbulent history during the evolution of Chloroplastida [26,32,34]. *Ycf1* and *Ycf2* are unique to the green lineage, and *Ycf2* is homologous to *ftsH*, from which it might have evolved by gene duplication. The YCF1 protein transformed from a chlorophyte version, Cte-YCF1, into a streptophyte version, the Ste-YCF1 protein, which is on average twice as large [34]. *FtsH* has undergone major changes too. Among the plastid genomes of Chlorophyta and the basal **KCM clade**, the FtsH protein is more similar to bacterial homologs than to the YCF2 of embryophytes or of the higher branching **ZCC clade** charophytes [26]. The evolutionary trajectory of streptophyte plastids is hence not only evident from the sequence features of particular genes, but also by simple gene content.

Another feature that unites the Zygnematophyceae with the land plants is the loss of the gene for elongation factor Tu (*tufA*) (Figure 2). The absence of *tufA* in the chloroplast genomes of both Zygnematophyceae and land plants was noticed in the 1990s [35]. At that time, however, phylogenetic evidence for the zygnematophyceaen origin of land plants was lacking and a sister relationship between the two appeared unlikely. We revisit this idea here and propose that, also through the right **endosymbiotic gene transfer** (EGT) at the right time, ceasing ever more

do not possess a plastid-targeted NEP. Their single RPOT (RNA polymerase T) gene encodes a polymerase targeted to mitochondria [70].

Paralog of accumulation and replication of chloroplast 6 (PARC6): cyanobacteria already encode ARC6, and its paralog PARC6 evolved in vascular plants and interacts with PDV proteins [71].

Phragmoplast: an accumulation of vesicles and cytoskeletal components at the division site that forms before cytokinesis.

Plastid division (PDV): PDV proteins are found only among land plants and localize to the outer midplastid ring during division.

Streptophyta: the monophyletic lineage of Chloroplastida that unites streptophyte algae and embryophytes. Characteristics include for example the hexameric organization of the cellulose synthase complex and the presence of a **phragmoplast** [72].

Streptophyte algae: freshwater algae with very diverse morphologies (unicellular, filamentous, thalli). Also known as charophytes or charophyte green algae.

ZCC clade: Zygnematophyceae, Coleochaetophyceae, and Charophyceae. The clade of higher branching streptophyte algae.

control over the plastid facilitated the initial steps towards terrestrialization and later allowed land plant plastid diversification. Plastid diversification was ultimately complemented by the emergence of a nucleus-encoded plastid RNA polymerase.

Exaptation Through Changes in Streptophyte Plastids

In 1972, Harman discussed the effects of mitochondrion-induced oxidative damage on cell survival and suggested that mitochondria work as a biological clock [36]. Mitochondria produce oxygen radicals that also affect their DNA [37]. The same problem exists for plastids, where photooxidative damage is an omnipresent challenge for the biochemistry and molecular biology of the organelle [38]. In an aquatic environment, algae can avoid high irradiances because light intensity is reduced by absorption by water, and many algae can adjust the depth in which they dwell [39]. Not so on land. When a streptophytic alga – an ancestor of *Zygnema* and all extant land plants – evolved to settle on land, a major obstacle to tackle was coping with light stress [7,8]. These stressors included higher photosynthetically active radiance and UV irradiances, both of which elevate the risk of accumulating deleterious mutations. Selection must have favored those streptophyte algae that bore chloroplasts less susceptible to such stress.

Several factors might have conferred irradiance resistance to streptophytic plastids. First, RNA editing was proposed to have evolved to compensate for non-synonymous changes that occurred on the DNA level and to revert the codon back to one that encodes the ancestral amino acid [40]. Compensation of higher UV-induced mutation rates through plastid RNA editing would have offered a significant selective advantage under terrestrial light conditions. Second, cyclic electron flow mediated by the *ndh* complex is thought to be essential for dealing with the reactive oxygen species (ROS)-associated stress response [41]. It was therefore proposed that retention of the '*ndh* complex' in the plastid genomes of streptophyte algae (and in light of the CoRR-hypothesis) was a key exaptation to terrestrial habitats [41], but the retention could also be connected to ATP synthesis under anaerobic conditions in roots [42]. A third potential factor involves the transfer of genetic information to the nucleus. This is beneficial for two reasons: in the nucleus (i) plastid protein-encoding genes are not exposed to photo-oxidative stress, and (ii) the impact of deleterious mutations can be minimized through sexual recombination, thus escaping Muller's ratchet [43]. EGT is a one-way street. More plastid-to-nucleus gene transfers have occurred in the green lineage than in the red [31], and in the latter a more autonomous regulation of photosynthesis can be expected. Whatever the extent of EGT, plastid genomes shrink, and

Box 2. Plastid Coding Capacity and Streptophyte Evolution

The coding capacity of plastid genomes can differ more than twofold, most notably when comparing the red and green lineages (Figure I). Major differences in the number of genes present mainly relate to carbon assimilation and the biosynthesis of pigments (*cf.* [31]). A large fraction of the genes found only in plastid genomes of the red (and secondary red) lineage are associated with transcription and translation (Figure I). This generally makes plastids in the green lineage more susceptible to nuclear control but, in and of themselves, these differences do little to help us to understand the split between streptophyte algae and land plants. The plastid NADH dehydrogenase complex, however, represents a more interesting case in which the majority of land plant plastid genomes retained genes that are otherwise absent. The 11 *Ndh* genes are found solely in streptophyte plastid genomes, with the exception of the basal branching chlorophyte *Nephroselmis* [24,32,41,88]. While coding capacity is largely conserved among chloroplastida [31], a few additional genes are nevertheless noteworthy in the context of terrestrialization. A step towards making chloroplasts more submissive to nuclear control was relinquishment of key genes involved in organelle division machinery. Only basal branching Chlorophyta and streptophyte algae possess the plastid division genes *ftsI, ftsW*, and *minD* on the plastid genomes (*cf.* [51]), while the latter is also present in other Chlorophyta (Figure I). ZCC clade algae and embryophytes either transferred these genes to the nucleus or lost them outright. Among streptophyte algal plastid genomes, those of the ZCC clade are already of embryophytic organization [23,24] (Figure I). The presence/absence pattern of *cysA* and *cysT* (encoding components of the sulfate ABS transporter system) ties together the liverworts, hornworts, and Zygnema. This pattern suggests at least two independent and early losses from the chloroplast genomes of the mosses and vascular plants (*cf.* [27,32]). Interestingly, considering all available ZCC clade plastid genomes, only Zygnema – and not any of the other zygnematophyceaen plastid genomes – possesses *cysA* and *cysT*. Of all extant streptophyte algae, *Zygnema* is most similar in plastid genome coding capacity to that of the hypothetical land plant ancestor.

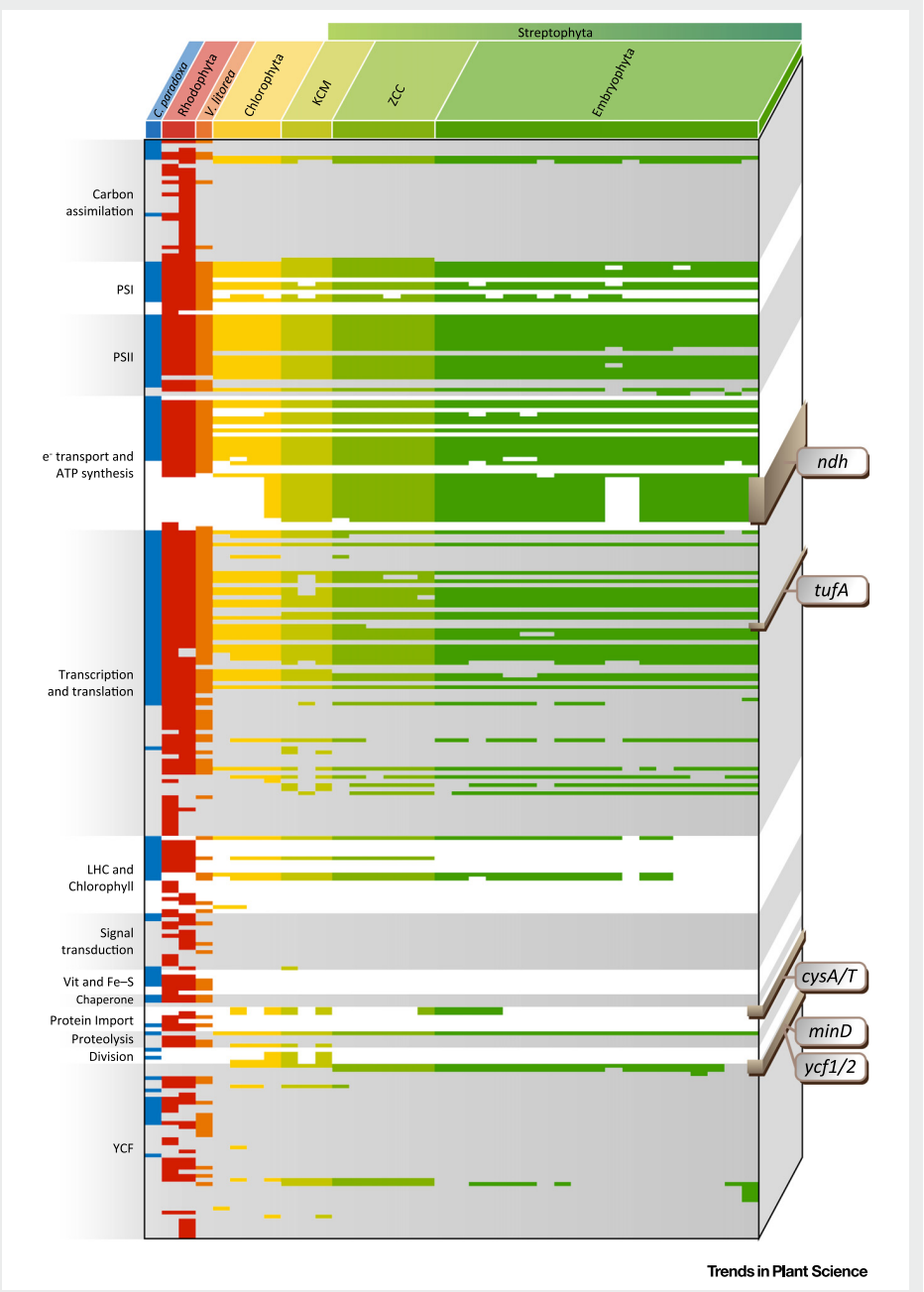

Figure I. Evolution of Plastid Coding Capacity. The presence/absence matrix of plastid-encoded proteins across 35 Archaeplastida and the heterokontophyte *Vaucheria litorea* as an example of an alga housing a red complex plastid; ticks are colored according to the algal groups and indicate the presence of a protein-coding gene. Proteins were sorted into functional categories according to Allen *et al.* 2011 [31]. Abbrevation: Vit, vitamins/cofactors.

thus the only place where additional regulatory systems could easily evolve was in the nucleus. Sometimes, however, the host still tapped into plastid biochemistry to do so.

In addition to light, drought is a major terrestrial stressor that the first plants would have had to deal with. Intriguingly, there is a 70% overlap in differential gene expression profiles between

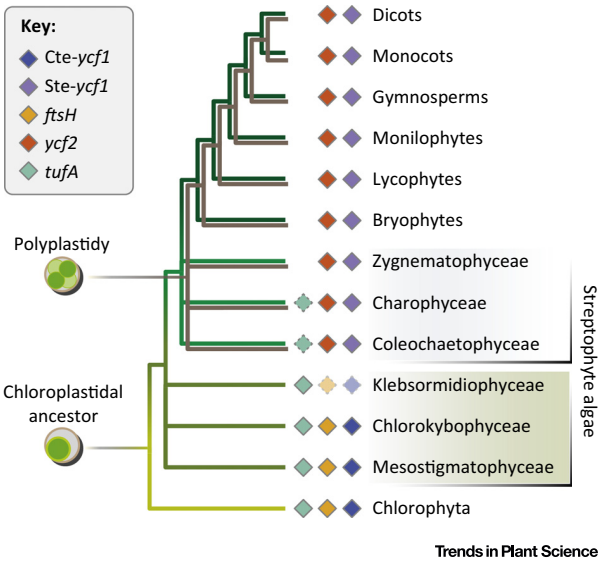

Figure 2. Changes in Plastid Gene Content Correlate with Phylogenetic Branching Patterns. Streptophytes and chlorophytes share a common, unicellular, and monoplastidic ancestor. Plastid genes such as *ycf1*, *ycf2/ftsH*, and *tufA* can act as indicators for the changes that occurred during streptophyte evolution. In the early-branching KCM clade of the charophytes, *ycf1* and *ftsH* have a higher similarity to their chlorophyte homologs and thus represent the ancestral state. In the higher branching ZCC clade (correlating with the emergence of streptophytic polyplastidy), these factors have higher similarity to their embryophyte homologs. In the case of *tufA* this is evident from the strong sequence divergence (dashed outlines) and the ultimate loss from the chloroplast genome in the Zygnematophyceae. Abbreviations: Cte-*ycf1*, chlorophyte type *ycf1*; Ste-ycf1, streptophyte type *ycf1*.

plants that have been challenged with drought and those exposed to high light stress [44]. One of the major components involved in the cellular response to both of these (terrestrial) stressors is abscisic acid (ABA), a compound that is to the largest degree synthesized in the plastid itself. Available data suggest that streptophyte algae bear all that is required for the ABA response [18,45], and ABA has repeatedly been suggested to be a major component of retrograde signaling [46]. ABA thus unites plastid–host stress signaling, and thus the scenario of streptophyte plastid exaptation comes full circle.

It is not enough for the host cell to be able to communicate with the plastid; it also needs to be able to control it. One way to gain control over the organelle is to control its protein synthesis. The transfer of *tufA* from the plastid genome to the nucleus might have been a crucial step, shifting control of a major switch for chloroplast activity from organelle to host. Plastid *tufA* has generally been associated with plastid translation, but it might in fact carry out two jobs. TufA is a determinant of bacterial cell shape by associating with the bacterial actin-like protein MreB [47]. MreB is essential for the coordination of cell division and polarity [48,49]. There is no actin cytoskeleton in plastids, and whether chloroplast TufA performs a similar function remains unknown. Nevertheless, given the progressive reduction of the plastid-encoded cell division machinery (Box 2) and the divergent nature of ZCC *tufA* sequences along the KCM–ZCC evolutionary trajectory [24,35], it would not be surprising if there were significant differences in how plastid division is coordinated in the different lineages. This is also important because the number of plastids residing in each cell can differ from lineage to lineage, including within the KCM and ZCC groups, and this can affect the rate of EGT: the presence of multiple organelles per cell, whether plastid or mitochondrion, is associated with an increased frequency of EGT [50].

There might be a connection between the loss of plastid division genes from plastid genomes and the emergence of polyplastidy. *Guillardia theta* and *Vaucheria litorea* both house complex plastids of secondary red algal ancestry [1–3]. *G. theta* is a single-celled alga with one plastid per cytosol, and here *MinD* and *MinE* are still plastid-encoded. *V. litorea* is a siphonaceous macroscopic stramenopile alga, with multiple red complex plastids per cytosol, and both genes are absent from the plastid genome. In the related stramenopile, *Thalassiosira pseudonana* (two plastids per cytosol [52]), *MinD* and *MinE* are also nucleus-encoded [51]. *MinD* is still plastid-encoded in some

KCM but not ZCC algae (Box 2). Among the streptophyte algae, only members of the ZCC group appear to house more than one chloroplast per cell [53]. One prerequisite for the evolution of polyplastidy could thus be the transfer of these genes to the host nucleus. Many algae, and especially basal-branching algae, house only one plastid per cytosol. A 'monoplastidic bottleneck' might be an evolutionary prerequisite for the transition from endosymbiont to organelle, and it still appears to be the default situation. The presence of only a single plastid might favor establishing synchronization with the host cell cycle. From what we can tell, true multiplastidy (three or more plastids per cytosol) evolved later and independently in the red and green lineages.

Regardless of the molecular basis of polyplastidy, only polyplastidic species of the ZCC clade appear to have had the capacity to explore plastid evolution to any great extent. A deleterious mutation or the transfer of the 'wrong gene' at the 'wrong time' can best be compensated for by the presence of one or more additional plastids – the more the better. There is a tendency for lower branching embryophytes such as hornworts to harbor only one plastid in the cells of their gametophytic tissues, and two in their sporophytes [54]. However, various hornwort species bear polyplastidic gametophyte cells [54]. We predict that whatever algal lineage managed to establish itself on land housed two or more chloroplasts per cell, at least during some stages of its life cycle (Figure 1). Polyplastidy was a trait that land plants have inherited from their streptophyte algal ancestors, and it was favored by an increased rate of EGT.

Land Plant Plastids: Control Governs Versatility

Plant and plastid development are tightly linked. They must be. This becomes evident when we consider the dormant seed of spermatophytes. It is a vessel that can endure desiccation and allow dissemination of progeny independent of water, and is kept dormant by plastid-synthesized ABA. The early evolution of sporopollenin in the charophytes paved the way [55], but the plastid also needs to be shut down, photosynthesis switched off, and the organelle transformed into some type of proplastid. The nucleus, and only the nucleus, needs to be able to determine when the photosynthetic machinery is to be degraded and later reactivated. Our understanding of the links between plastid differentiation and plant development is unfortunately still very limited [56]. Unique to vascular plants is (i) the ability to transform a proplastid to a **chromoplast**, **amyloplast**, **elaioplast**, and to the canonical chloroplast via the **etioplast** (Figure 1), and (ii) the interconversion between some of those forms [56]. Mosses lack such diverse plastid types [57], but ferns appear to form at least some type of proplastid [58]. The evolution of plastid differentiation in embryophytes appears to be associated with better control of plastid division (and being polyplastidic) through the emergence of associated gene families such as **paralog of accumulation and replication of chloroplast 6** (PARC6) and the **plastid division** (PDV) family [59,60].

An essential component that links plant development and chloroplast biogenesis is the division of labor in chloroplast transcription by the **nucleus/plastid-encoded RNA polymerase** (NEP/PEP) system. The nucleus exercises control over the existing PEP through the nucleus-encoded PEP-associated proteins (PAPs), which are crucial for transcriptional activity and, for example, for the transition from a proplastid to a chloroplast [61,62]. PAPs seem to be absent from chlorophytes and appear to have evolved in streptophytes, but why evolve a NEP and then keep the PEP? The PEP might be part of the CoRR machinery [30] and hence, for now, out of nuclear reach. Genes transcribed by the PEP are mostly those involved in ongoing photosynthesis, while biogenesis – and thus activation of the chloroplast – is carried out by the NEP [63,64]. NEP is therefore considered to be a main switch with the ability to kick-start chloroplast development [65]. While NEP and PEP seem to be present during all stages of plastid biogenesis [63], the PEP is kept silent by PAPs [61]. We speculate that the presence of an elaborate NEP/PEP/PAP system is what allows higher vascular plants to switch between an unparalleled variety of

different plastid types (Figure 1). However, that flexibility has its limits. Within each cell (and disregarding concentration gradients and the moment of transition) only one plastid type can be present at any given time. That is due to the inability of the nucleus to communicate with a subset of plastids in a single cell. One signal hits all.

Concluding Remarks: The Embryoplast

Increased taxon sampling and phylotranscriptomic analyses have demonstrated that extant land plants share a common ancestor with zygnematophycean algae. These findings prompt a revaluation of the catalog of exaptations that likely played a role in the conquering of land. Currently that catalog focuses on the host cell and it lists features that are ubiquitous among various streptophyte algae. The phylogenomic evidence that zygnematophycean algae branch at the base of embryophytes is supported by the pattern of some genes that remain plastid-encoded and some that have moved to the nucleus. To establish itself on dry land, the algal ancestor of land plants needed to evolve a plastid that it could control better and that was ready to cope with challenges posed, for example, by exposure to high light stress and desiccation. Steps towards such a plastid included the transfer of particular genes to the nucleus (e.g., *tufA* and *minD*) and the emergence of additional control mechanisms (e.g., NEP, PAP, PARC6, PDV). Bringing the plastid into the terrestrialization equation provides a new angle from which to ponder the question of land plant evolution (see Outstanding Questions). The combination of the factors we discussed is what transformed the streptophyte plastid into the **embryoplast**. The key character of the plant lineage – the plastid – played a decisive role in the transition of alga to land plant.

Outstanding Questions

Why is the vast majority of algae monoplastidic, and what was it that allowed some species of the red and green lineages to evolve polyplastidy independently?

Could the emergence of a sophisticated ABA signaling pathway, in addition to its importance for stress response, present another exaptation of streptophyte algae regarding terrestrialization?

Does the transfer of certain genes such as *tufA* and *minD* from the plastid to the nuclear genome provide the cell with increased control over the organelle that was essential for terrestrialization?

To what degree is the diversification into the various types of plastids, found only in vascular plants, associated with the emergence of the nucleus-encoded RNA polymerase (NEP) and PAP proteins?

We can also predict that the nuclear control over plastid function increases within the KCM to ZCC trajectory – a picture that should manifest itself in, for example, comparative studies on streptophyte algae.

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7 Summary of the obtained results and concluding remarks

Research on plastid-stealing sacoglossan slugs touches upon many different aspects of biology. The major interests in this field of research concern (i) what the slugs gain from the plastids, (ii) how the slugs handle their stolen plastids and (iii) what the basis for the diversity in food alga and modes of retention is. Publications **I** to **V** tackle these aspects. At the same time, and especially due to the latter aspect, the biology of the plastids is of major concern. Plastid function and photophysiology is to a large degree studied using a few model organisms and with a great bias towards the land plants. Therefore, we highlight in publication **VI** to **VIII** those factors that are, from our point of view, of major importance for understanding the difference between algal plastids (that are stolen by the slugs) and land plant plastids.

Publication I A sea slug's guide to plastid symbiosis

Research on the sacoglossan sea slugs was reviewed from a historical perspective, from the first species descriptions in the 19th century until the end of 2014. In this review, the methodology of how the photosynthetic sea slugs were studied was given great emphasis (the starvation in particular) and connected with its historical context. Also, instead of focusing on the retention of plastids, the animals' biology was highlighted. Here, three main aspects are in focus: i) behaviour of slugs during feeding and housing of the plastids, ii) the vast diversity of slug species with varying modes of plastid retention and iii) the pitfalls in analysing what slugs gain from photosynthesis. What is more, the concept of the two-component system was first formulated here, upon which a major portion of the rationale presented in this thesis rests. This creates a conceptual basis for future studies that should address the specification of the factors that define compatibility of the two-component system slug-alga.

Publication II Switching off photosynthesis: the dark side of sacoglossan slugs

Christa and colleagues (2014a) showed that impairing photosynthesis had no influence on the survival of plastid-harbouring sacoglossans during starvation. In light of that study, we here revisited the past literature on the sacoglossans and discussed it in context of the importance of photosynthetically fixed carbon for the slugs. This perspective article highlights that, although photosynthesis had been the focus of many studies performed in the past, few of these studies provided clear evidence for the carbon fixation rates remaining at a level that can support the animal during starvation. Indeed, all data suggests the contrary. We, here, encourage the scientific community to critically re-evaluate the (with regard to the plastid-housing sacoglossans) readily used term photoautotrophy. Sacoglossan slugs do not grow during starvation but shrink. Instead of only focussing on carbon fixation, other aspects of plastid biochemistry such as iron-sulfur cluster synthesis ought to be considered, too.

Publication III Comparison of sister species identifies factors underpinning plastid compatibility in green sea slugs

Sacoglossans have been categorized into three different forms of kleptoplast retention: long-term retention (LtR), short-term retention (StR) and non-retention (NR; Händeler et al. 2009). These classifications were based on the PS II maximum quantum yield determined through chlorophyll a fluorometry assessed during starvation of the animals. In many starving StR and NR species the fast decrease in PS II activity is accompanied by a fast death. The slugs' molecular chassis for these modes of retention and how that relates to the starvation tolerance of the animals were, however, not well defined and intermingled. In this research article, we compared two sister taxa with different modes of plastid retention: the StR *E. cornigera* and the LtR *E. timida*, both of which was raised on the same food alga, the ulvophyte *A. acetabulum*. Each slug species therefore had the same point of departure. While the analyses again showed that the PS II activity did indeed decline swifter in *E. cornigera* than in *E. timida*, the CO_2 fixation rates were the same in either species. Nevertheless, global expression analysis revealed that *E. cornigera* experienced pronounced starvation stress. Elevated ROS levels that were only observed in the StR but not LtR species underpinned this. Inhibiting CO_2 fixation of the plastids did not result in a pronounced response by either slugs species. Thus, the slugs' starvation tolerance is decoupled from the performance of their plastids. Systems chaperoning kleptoplasts also play a rather subordinate role during starvation. Main denominator of the slugs cellular response towards starvation was starvation itself. This manuscript highlights that the starvation response is a clear animal character that has little to do with photosynthates.

Publication IV Plastid survival in the cytosol of animal cells

By the time this opinion article was published, major concepts of the interaction between plastids and slugs had been overturned. The starvation tolerance of the sacoglossan slugs is not due to photosynthates provided by the plastids. The plastids, in turn, are not dependent on proteins that the slugs synthesise due to laterally acquired algal genes, because there are none. We highlighted the diversity of plastid retention and also the diversity of the plastids acquired. The slugs need to provide an environment in which they handle (i.e. scavenge) deleterious side products generated by the photosynthetic organelles. We pose that handling a foreign organelle might be one of the key characters the slugs need to be adapted for in order to house plastids long-term.

Publication V Why it is time to look beyond algal genes in photosynthetic slugs

A long standing concept that sought to explain the longevity of the plastids sequestered by the slugs was lateral gene transfer. It was believed that, through such transfer, the slug's chromosomes had been equipped with the molecular toolkit to express proteins that (i) chaperoned kleptoplasts and (ii) replenished degraded biomolecules. Here we explained that — although a conceivable concept — all sequencing data ever collected fails to offer support. On the contrary, it presents outright support for the absence of any laterally transferred algal genes to the nuclear genome of the slugs. Plastids must be rendered long-living by another, likely intrinsic, mechanism.

Publication VI Is *ftsH* the key to plastid longevity in sacoglossan slugs?

Plastids from many different algal "donors" are observed to stay photosynthetically active within the slugs' tissue for up to several months. The hypothesis that kleptoplasts residing in the slugs are supported through laterally acquired genes had been rejected. We, therefore, reconsidered the intrinsic factors of the plastids. To survey which intrinsic factors could render plastids robust, 51 available algal plastid genomes were screened, focusing on those sequestered by the slugs. Here, major interest was soliciting factors that (i) set algal plastid genomes apart from those of land plants, (ii) are shared by all plastid genomes of long-term retained kleptoplasts and (iii) help explaining stability of isolated plastids. In this dataset the draft plastid genome of the ulvophyte *A. acetabulum* (the sole food alga of *E. timida*) that was sequenced in course of this study was included. This screening approach unveiled two factors that potentially are of major importance for the longevity of plastids: the elongation factor Tu encoded by *tufA* and the D1 quality control protease encoded by *ftsH*. We reasoned that the former fosters on-going protein biosynthesis and the latter equips the plastids with the molecular toolkit to perform at least parts of the D1 repair cycle. Plants impaired in FtsH function have been shown to accumulate damaged D1 and experience elevated photooxidative stress. Plastids that bring along the ability to refill their supplies of FtsH protein, we hypothesised, would therefore experience a decreased risk of photooxidative stress emitting from the kleptoplasts during long-term storage in the slugs.

Publication VII YCF1: a green TIC?

Plastid genomes are limited in terms of coding capacity. Most factors that are retained in the plastid genome are linked to protein biosynthesis or photosynthesis (Allen et al. 2011; Allen 2015). Yet, in plastid genomes of the green lineage two proteins occur whose function remained elusive for decades: Ycf1 and Ycf2 (cf. Drescher et al. 2000). While the latter is most likely derived from (i.e. homologous to) the plastidal FtsH (Wolfe 1994; Wicke et al. 2011), their functional connection is unknown. A recent study proposed that Ycf1 acts as an essential and universal component of the translocon at the inner chloroplast envelope (Kikuchi et al. 2013). Representing an intriguing case of plastid genome coding capacity, the evolutionary history of Ycf1 was investigated in this article. Using a Hidden Markov Model approach protein data from all available plastid genomes (at that time 558) of Archaeplastida were screened for potential homologs of Ycf1. Our data show that Ycf1 is only found in the Chloroplastida and appears to have been lost multiple times independently from various chloroplastidal plastid genomes. Ycf1 homologs also showed a remarkable sequence diversity, ranging from ~400 to more than 3000 amino acids in length. This manuscript highlights that Ycf1 proteins of higher branching streptophytes reveals considerable differences to the Ycf1 proteins found in chlorophytes and lower branching streptophyte algae, even though they are homologous. We conclude that, due to this turbulent evolutionary history, Ycf1 would be a highly unusual key translocon component and this awaits further explanations.

Publication VIII Streptophyte terrestrialization in light of plastid evolution

Publications VI and VII highlight that three plastid genes experienced considerable changes during the course of chloroplastidal evolution: *tufA*, *ycf1* and *ycf2*. All of these factors have leaded us to conclude that, within the streptophyte lineage, the plastid experienced a pronounced evolutionary change. In this article, streptophyte evolution is discussed with an emphasis on the plastid, from its aquatic algal origin to the terrestrialization event and the following evolution on land. We highlight that simple presence/absence patterns of plastid factors alone help in carving out the closest algal relative to land plants. What is more, plastids factors offer a clear resolved guidance, which is in contrast to the often considered physiological and cellular complexity of streptophyte algae that is not confined to a single algal lineage (that gave rise to the land plants). The article discusses that, driven by loss and transfer of plastid genes, streptophyte plastids underwent subsequent loss of autonomy and higher submission to nuclear control along the trajectory from lower to higher branching streptophytes. We pose that a high degree of nuclear control over plastid function was one of the key characters that allowed for the successful terrestrialization of the streptophyte algal lineage that gave rise to the common ancestor of all land plants. On land, the plants' nuclear plastid control systems became more elaborate due to factors such as the nuclear encoded plastid RNA-polymerase, resulting in the diversity of plastid types (in one organism) only found in vascular plants.

Conclusion

Sacoglossan research was focused on two main aspects: (i) the plastid's photosynthesis is of major importance for the slugs and (ii) the slugs have a major contribution to the longevity of the plastids they steal. Photosynthetically fixed carbon appears a dispensable contribution to the slugs energy budget (discussed in publications **I**, **II** and **IV**). Photosynthates are not what allow the slugs to endure long-term starvation and influencing the plastids photosynthetic performance evokes little transcriptional response by the slugs during starvation (publication **III**). The latter goes hand in hand with the absence of laterally transferred algal genes that would maintain long-term kleptoplast function (publication **V**). This means that the two systems are disconnected from another and that the slugs' cytosol is merely an appropriate environment for housing stable plastids. The reason for the stability of the plastids rests with the plastids themselves, which results in an autonomy not observed among the plastids of land plants (publications **VI** and **VIII**). Intrinsic plastid robustness might be conferred by higher coding capacity resulting in *in situ* control over protein synthesis and photosystem maintenance (publication **VI**). Land plant plastids lack some of these components and, what is more, presence and constitution of plastid genes (such as *ycf1* and *ftsH*) draw a clear line between green (including basal streptophyte) algae and land plant plastids (publications **VII** and **VIII**). Loss of these factors might have been crucial for the transition to land, as it made plastids more amendable to nuclear control allowing for adaptation to terrestrial stressors (publication **VIII**). This suggests that the higher autonomy and the concomitant robustness of algal plastids (and kleptoplasts) was selected against and was, thus, lost from land plant plastids. What this autonomy implies for the molecular biology algal plastids (when resting within algal cytosol) is one of the outstanding questions for future research.

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