Endosymbioses in Sacoglossan Seaslugs: Plastid-Bearing Animals that Keep Photosynthetic Organelles Without Borrowing Genes

Heike Wägele and William F. Martin

Abstract In this chapter, we summarize our knowledge on photosynthesis properties in the enigmatic gastropod group Sacoglossa. Members of this group are able to sequester chloroplasts from their food algae and store them for weeks and months in order to use them in a similar way as plants do.

Only four to five sacoglossan species are able to perform photosynthesis for months, others are less effective or are not able at all. The processes involved are not clear, but we show by this chapter that many factors contribute to the developing of a photosynthetic seaslug. These include extrinsic (environment, origin and properties of the nutrition and the plastids) and intrinsic factors (behaviour, physiological and anatomical properties). Maintenance of plastids is not enhanced by a horizontal gene transfer (HGT) from the algal genome into the slug genome, as was hypothesized for many years. We outline here the questions that now have to be asked and the research that has to be done to understand the factors that actually contribute to this unique metazoan phenomenon, which is not understood at all.

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Introduction

"Leaves that crawl" is a term that Robert Trench (1975) aptly used to characterize the species of sacoglossan seaslugs that sequester plastids from their algal food source and maintain them in an active photosynthetic state, effectively allowing the slugs to grow on CO_2 and light. The slugs steal the plastids from the alga upon which they feed; hence, the plastids in the slugs are called kleptoplasts. When the plastids become dysfunctional they have to be replaced, the symbiosis has to be reestablished. But the kleptoplasts can remain photosynthetically active for several months in some species, a circumstance that has captured the imagination of many researchers (and journalists) over the last 50 years and that has time and time again fuelled discussion of the possibility of horizontal gene transfer from the genome of the algal food source into this fascinating metazoan life form (Rumpho et al. 2000, 2001, 2006, 2011; Wägele et al. 2010a; Pelletreau et al. 2011). The incorporation of functional kleptoplasts, referred to for simplicity as chloroplasts or plastids here, is not unique to sacoglossans, because many protists incorporate "previously owned" plastids into their metabolism (e.g. Rumpho et al. 2006; Lee 2006; Stoecker et al. 2009; Johnson 2011). The fascination concerning molluscan kleptoplasty from higher chlorophytes (usually Ulvophyceae) or a heterokontophyte (*Vaucheria litorea*, Agardh 1823) stems, however, from the circumstance that these fully fledged metazoans become photosynthetic for a good portion of their life cycle, such that associations with "little green men" of science fiction, and global nutrition questions of the "what if" type are never far. This chapter serves to first introduce the discovery of the famous four sacoglossan species that—instead of just eating their algal food like most of the about 300 known sacoglossan species—have independently evolved so as to undergo long-term retention of their plastids and then to summarize the literature dealing with the different research perspectives towards unravelling the mystery behind functional plastid retention. Finally, recent findings have opened up new insights into the genetic basis behind the functional relationship between the photosynthesizing algal organelle and its gastropod digestive gland system.

History of the Famous Four

Detection of chlorophyll in Sacoglossa goes back to the late nineteenth century (De Negri and De Negri 1876), but the surprising property of using an organelle, the "enslaved" chloroplast, for the slug's metabolic purposes was described by Kawaguti and Yamasu only in 1965, based on the electron microscopical studies of *Elysia atroviridis* Baba, 1955. These authors showed that the green structures housed in the digestive gland cells and surrounded by a double membrane were structurally identical to the chloroplasts in the ulvophycean *Codium fragile* (Hariot, 1889) upon which *E. atroviridis* feeds. They assumed that these chloroplasts perform photosynthesis, produce oxygen and, while within the digestive gland cells, "*. . .will give their products of photosynthesis to the host cell and will receive, in return, waste substances of the animal. . .*" (Kawaguti and Yamasu 1965, p. 60). Since then, many studies have been performed on sacoglossans to investigate this unique feature from various perspectives. This led to the discovery of sacoglossans that were much more effective in this symbiotic relationship now known as long-term retention (LTR) forms: *Elysia crispata* (Mørch, 1863) (Fig. 1d; Caribbean Sea), *Elysia timida* (Risso 1818) [Fig. 1b; Mediterranean Sea], *Elysia chlorotica* Gould, 1870 (Fig. 1e; North Atlantic) and *Plakobranchus ocellatus* van Hasselt 1824 (Fig. 1a; Indopacific). Trench et al. (1969) detected, and Clark and Bussacca (1978) described, the long-term retention of chloroplasts in *E.* (designated there as *Tridachia*) *crispata*. Greene (1970) described retention in *P. ocellatus* (described there as *P. ianthobapsus*), characterized in more depth recently by Evertsen et al. (2007). Rahat and Monselise (1979) and especially Marín and Ros (1989) described long-term retention in *E. timida*, and finally Graves et al. (1979) did so for *E. chlorotica*. Since these discoveries, many other sacoglossan species have been investigated and the plastid retention time (ranging from several days to several weeks) has been recorded (Händeler et al. 2009).

Of all species investigated so far, only *E. chlorotica* survives for more than 1 year of starvation (that is, survives from its plastids) in culture (Rumpho et al. 2000). Händeler et al. (2009) reported retention for nearly 3 months in *P. ocellatus*; however, unpublished data (H.W.) suggests retention possibly for 4 months and more. *E. timida* retains chloroplasts for 50 days (Wägele et al. 2010a) and *E. crispata* for more than 40 days (Händeler et al. 2009). A recently described *Elysia* species—*E. asbecki* (Wägele et al. 2010b) (Fig. 1e)—exhibits photosynthetic activity in the first 10 days, similar to the performance seen in *E. timida* and *E. crispata* (Wägele et al. 2010b). Therefore, it is possible that this species might be the second long-term retention form known from the Pacific. If so, that would increase the circle of sacoglossans with long-term plastid retention to five species.

Händeler et al. (2009) and Wägele et al. (2011) clearly showed that evolution of long-term retention occurred in separate lineages (Fig. 2), but the ability to incorporate plastids without direct digestion goes back to a common ancestor in the lineage of the Plakobranchoidea. This taxon is characterized by parapodia that can

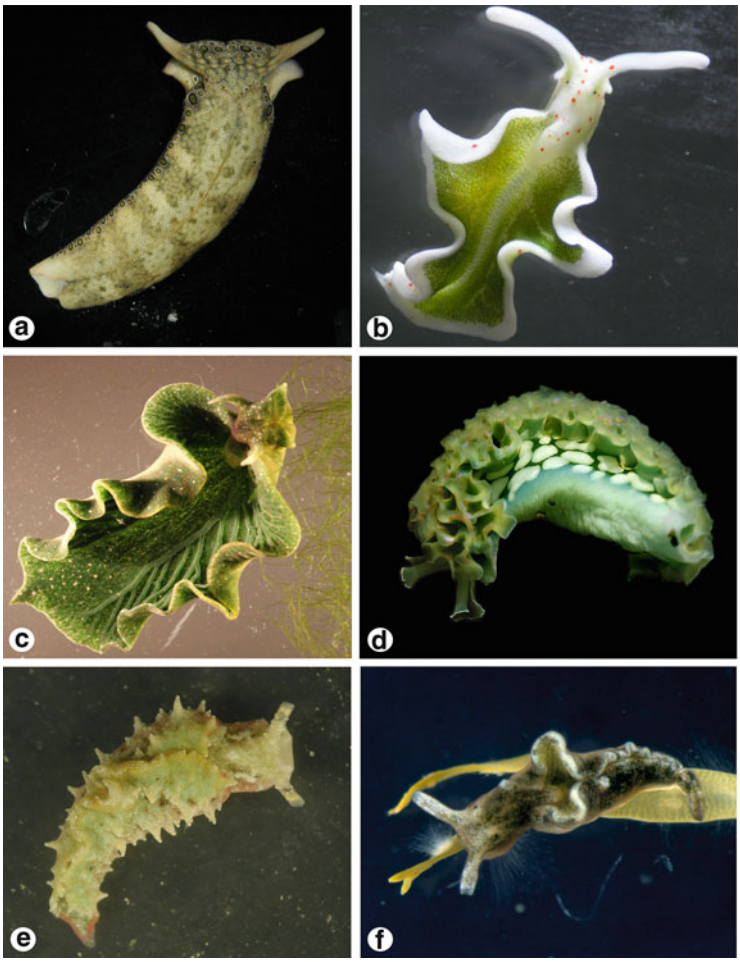

Fig. 1 The most intriguing sacoglossan species known for chloroplast retention. All species belong to the Plakobranchoidea, which typically have parapodia. (**a**) *Plakobranchus ocellatus*, (**b**) *Elysia timida*, (**c**) *Elysia chlorotica*, (**d**) *Elysia crispata*, (**e**) *Elysia asbecki* and (**f**) *Elysia viridis*. Photo of *E. chlorotica* provided with permission of M. Rumpho and K. Pelletreau

cover the whole dorsal body (Figs. 1a–f and 3d)—probably a key adaptation utilized by all long-term retaining species. Two other groups are described within Sacoglossa: the Limapontioidea that lack parapodia but can possess many flap-like dorsal appendages (Fig. 3a–c), and the shelled Oxynoacea (Fig. 3e, f). Maeda et al. (2010) contested the results of Händeler et al. (2009) by presenting an alternative phylogenetic hypothesis and claiming that kleptoplasty evolved at the base of the Sacoglossa, represented by the shelled *Cylindrobulla*. However, they used the term kleptoplasty to designate the act of merely engulfing and subsequently digesting chloroplasts. Immediate digestion is typical for nearly all

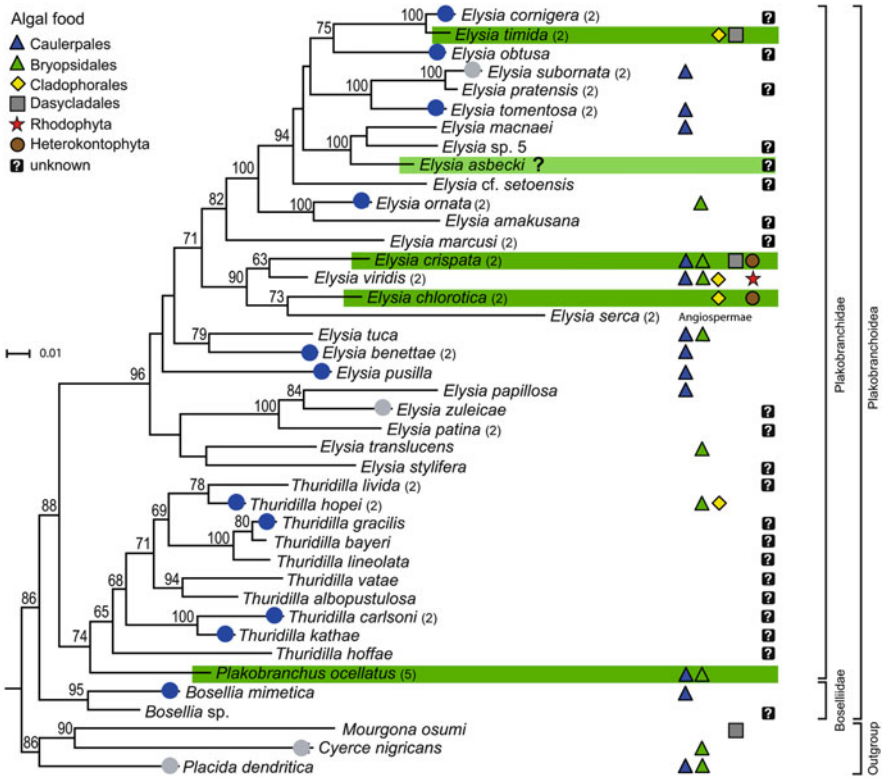

Fig. 2 Phylogeny of the Plakobranchoidea with information on food organisms [after Wägele et al. (2011)]. Maximum likelihood analysis of partial gene sequences (28S rDNA, 16S rDNA and *cox1*—first and second codon positions only). *Numbers* in *brackets* indicate the number of specimens included. *Numbers* above *branches* indicate bootstrap values. *Gray dots* and *blue dots* on the *branches* indicate non-retention or short-term retention of plastids, respectively. Long-term retention is pointed out by *green bars*, the fifth potential long-term form, *Elysia asbecki*, by a *light green bar* and a *question mark*

Limapontioidea and certainly all shelled Oxynoacea, it is clearly a trait ancestral within the Sacoglossa and should not be confused with kleptoplasty.

Clark et al. (1990) proposed six levels of chloroplast retention starting from no retention and direct digestion (level 1) to long-term function retention (level 6) with photosynthesis persisting for more than 1 week. Evertsen et al. (2007) extended this classification scheme by two additional levels in order to meet the capability of the long-term retention forms with a range of 1 week functional photosynthesis up to several months. With many species now known to exhibit a wide variety of chloroplast retention, and given the ambiguities of some reported photosynthetic measurements (due to environmental and individual factors, including the variety of ways to measure photosynthesis), these fine-grained levels of distinction are not used in the latest literature.

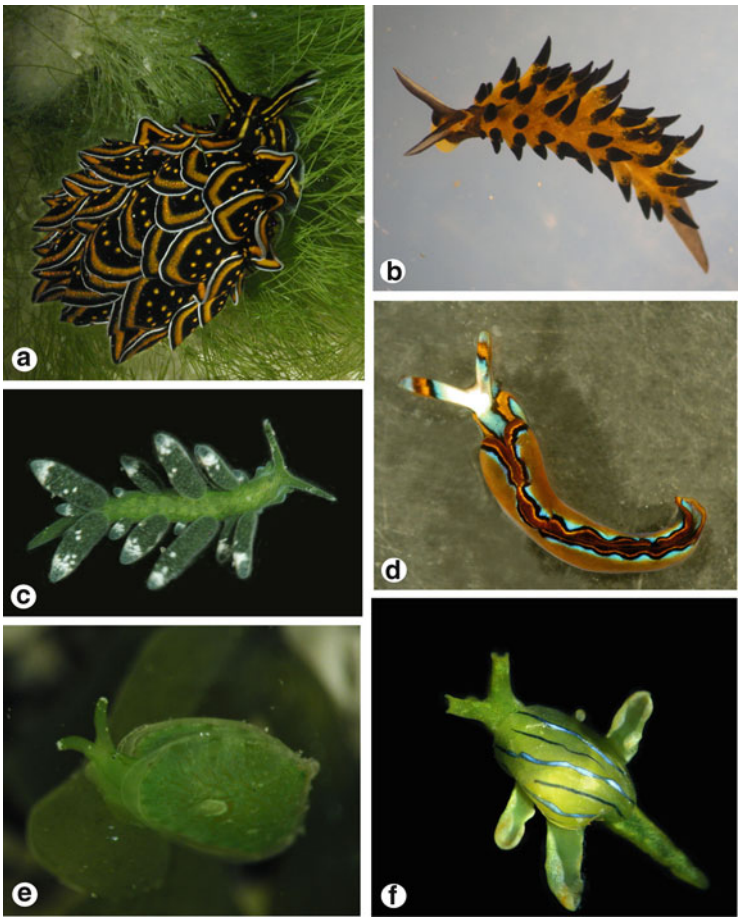

Fig. 3 Several sacoglossan members exhibiting only short-term or non-retention of plastids. (**a**–**c**) show members of the Limapontioidea, (**d**) of a Plakobranchoidea and (**e**, **f**) of shelled Oxynoacea. (**a**) *Cyerce nigricans*, (**b**) *Placida cremoniana*, (**c**) *Ercolania kencolesi*, (**d**) *Thuridilla neona*, (**e**) *Tamanovalva limax* and (**f**) *Lobiger viridis*

Investigation of Functionality of Plastids in Slugs from Different Perspectives

In the nearly 50 years following after the detection of functional plastids in the digestive gland cells of sacoglossans, the properties of the peculiar relationship between a plant organelle and a metazoan life form have been investigated from many perspectives. The following chapters review the various approaches.

Ultrastructure

The ultrastructure of sequestered plastids has been studied in several systems, usually in comparison with plastids in the natural algal source. Studies began with the investigation by Kawaguti and Yamasu (1965) on *E. atroviridis* and its food alga *Codium fragile*, followed by Taylor (1971) on *Hermaea bifida* (Montagu 1815) and its food *Griffithsia flosculosa* [now assigned to *Halurus flosculosa*, see Maggs and Hommersand (1993)]. Taylor (1968), Trench et al. (1973a, b) and Hawes (1979) studied *E. viridis* (Montagu, 1804) (Fig. 1f) feeding on C. *fragile*. Later Curtis et al. (2005, 2006) reported results for *E. crispata* and *E. clarki* (Pierce et al. 2006) and some of their food sources as *Bryopsis plumosa* (Hudson) (Agardh 1823), *Halimeda incrassata* (J. Ellis) Lamouroux, 1816 and *Penicillus capitatus* Lamarck, 1813. The ultrastructure of plastid retention was sometimes compared between long-term retention forms (LTR) and short-term (STR) or non-retention forms (NR): Graves et al. (1979) compared *E. chlorotica* (LTR) with a limapontioidean species, *Alderia modesta* (Loven, 1844) (NR); Evertsen and Johnsen (2009) compared *E. viridis* (STR) with *Placida dendritica* (Alder and Hancock, 1843) (NR).

These studies revealed a variety of plastid degradation stages within the digestive gland cells, depending on the species, its ability to incorporate plastids, as well as the duration of the starvation period. The occurrence of large starch grains in kleptoplasts and the higher frequency of plastoglobuli after several weeks of starvation—not observed in freshly fed species—is noteworthy [described, for example, for starved *Elysia viridis*, see Evertsen and Johnsen (2009)].

Of particular interest is the location of the plastid within the cytosol of the slug. Since plastids are incorporated via phagocytosis, one might expect three membranes to encircle an intact kleptoplast, one from the host phagosome as well as the inner and outer chloroplast membranes in the case of food plastids surrounded by two membranes; the (secondary) food plastids of *Vaucheria* are surrounded by four membranes in the alga. However, reports from the literature on this aspect from the various species remain incongruent (summarized in Table 1). *E. viridis* (STR), after 4 weeks starvation, has been reported to harbour a host membrane in addition to the intact plastid membranes. However, it was not observed in all sequestered plastids [Trench et al. (1973b), but see Hawes (1979) for different results] or the phagosome membrane appeared ruptured (Hawes and Cobb 1980; Evertsen and Johnsen 2009). Hirose (2005) investigated the ultrastructure of plastids in *P. ocellatus* (LTR), but came to no conclusion whether plastids were surrounded by host membranes or not. Muscatine et al. (1975) mentioned plastids with and without a phagosome membrane in *E. viridis* after feeding. Similar results were obtained for the limapontioidean species *Costasiella ocellifera* Simroth, 1895 (described as *C. lilianae*), which survived a 65-day starvation period after feeding on *Avrainvillea* (Clark et al. 1981). In this species, kleptoplasts were observed in the digestive gland cells with intact cp membranes, but not all were enclosed in a phagosome membrane. This was irrespective of starving condition

Table 1 Published results obtained from ultrastructural investigation of incorporated plastids with regard to presence of phagosome and chloroplast membranes

Species	PR	TEM results as outlined in the text	Number of membranes as specifically indicated in literature	Food and feeding	Authors
Alderia modesta	NR	Plastids surrounded by an "extrinsic" membrane, showing various degrees of degradation	3	*Vaucheria spec*	Graves et al. (1979)
Bosellia mimetica	STR	Plastids with a host membrane from a phagocytic vacuole	Probably 3	*Halimeda tuna*	Marín and Ros (1989)
Costasiella ocellifera (*as lilianae*)	?	Irrespective of plastid condition, a host membrane can be present or not. cp membrane is always intact		*Avrainvillea nigricans*	Clark et al. (1981)
Elysia australis	?	Plastids without host membrane			Marín and Ros (1993) [after Hinde (1983)]
Elysia chlorotica	LTR	Plastids bounded by an intrinsic double membrane and which contain parallel lamellae	2	*Vaucheria spec*	Graves et al. (1979)
Elysia chlorotica	LTR	Plastids with double membrane of cp and no host membranes	2		Rumpho et al. (2001)
Elysia clarki	LTR	Two membranes, the outer more loose, therefore probably from the host	2	*Penicillus spec.*	Curtis et al. (2006)
Elysia clarki	LTR	Plastids were separated from cytoplasm by a membrane		Various algae	Curtis et al. (2006)
Elysia maoria	?	Plastids without host membrane	2		Brandley (1981)
Elysia furvacauda	?	Plastids without host membrane	2		Marín and Ros (1993) Brandley (1984)
Elysia timida	LTR	Plastids with double chloroplast envelope; no host membrane	2		Marín and Ros (1989)

Elysia timida adults	LTR	Defunct plastids are surrounded by a host membrane. They are then digested	3		Marín and Ros (1993)
Elysia timida juveniles	LTR	Plastids with double chloroplast envelope and always a host membrane	3	*probably Cladophora*	Marín and Ros (1993)
Elysia translucens		Plastids with double chloroplast envelope; no host membrane!	2	*Udotea petiolata*	Marín and Ros (1989)
Elysia viridis	STR	Double membrane around plastids	2		Taylor (1968)
Elysia viridis	STR	Plastids envelope always intact, but external membrane outside the envelope sometimes present, sometimes not	2–3	*Codium fragile*	Trench et al. (1973b)
Elysia viridis	STR	Plastids surrounded by host membrane, but a similar number of plastids lack the animal membrane	2–3	After feeding	Muscatine et al. (1975)
Elysia viridis	STR	Plastids with host membrane and sometimes algal cytoplasm attached	3	*Codium fragile*	Hawes (1979)
Elysia viridis	STR	Plastids with host membrane after 28 days starvation. But membrane can be ruptured	3		Hawes and Cobb (1980)
Elysia viridis	STR	Feeding experiment: most plastids are intact with a phagosome membrane—these are the functional ones. Only few show a burst phagosome and double membrane with exposed thylakoid staples—they disintegrate	3 cp double membrane remains, phagosome and thylakoid membranes disintegrate	Fed with *Codium fragile*	Evertsen and Johnsen (2009)
Ercolania funerea	NR	Plastids with host membrane (Marín 1988)			Marín and Ros (1993) [after Marín (1988)]
Hermaea bifida	NR	Double membrane	2	*Griffithsia flosculosa*	Taylor (1971)

(continued)

Table 1 (continued)

Species	PR	TEM results as outlined in the text	Number of membranes as specifically indicated in literature	Food and feeding	Authors
Placida dendritica	NR	Feeding experiment: plastids intact with phagosome membrane and probably double membrane of cp. All degrade very quickly and simultaneously	3	*Fed with Codium fragile*	Evertsen and Johnsen (2009)
Placida dendritica	NR	10 min after feeding: Algal material in the lumen of digestive gland and in the vacuole containing the cp. Engulfing by microvilli Vacuole consisting of inner membrane of phagocytic vesicle	Probably 3	Starved for 4 days, then fed with *Codium fragile*	McLean (1976)
Thuridilla hopei	STR	Plastids with a host membrane from a phagocytic vacuole	3	*Cladophora vagabunda*	Marín and Ros (1989)

PR: period of retention as indicated in Händeler et al. (2009). *NR* non-retention, *STR* short-term retention, *LTR* long-term retention

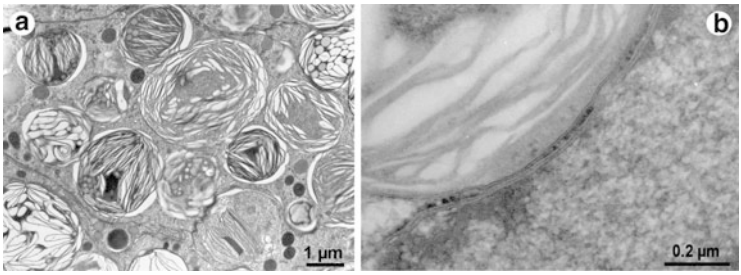

Fig. 4 Ultrastructural studies of incorporated chloroplasts in *Elysia timida* [after Wägele et al. (2011), originals with permission of R. Martin]. (**a**) *Acetabularia* chloroplasts in the cytosol of 2 months starved slug. (**b**) Chloroplast of fed slug in close contact to nuclear pore. Note the two membranes surrounding the chloroplast

and plastid condition. Marín and Ros (1989, p. 433) stated that the "*presence of a host membrane surrounding the chloroplasts in Thuridilla hopei and Bosellia mimetica seems to be closely related to fast plastid turnover*". That conclusion was supported by their results on two *Elysia* species, *E. timida* and *E. translucens* (Pruvot-Fol, 1957), which exhibited a much lower plastid turnover rate and showed no phagosome membrane.

The lack of a host membrane in *E. timida* but the presence of the two chloroplast membranes is confirmed in Wägele et al. (2011; Fig. 4). Interestingly, phagosome membranes exist in juvenile *E. timida* (Marín and Ros 1989). Similar to juvenile *E. chlorotica* [see Rumpho et al. (2000)], *E. timida* must first feed for a certain period of time before plastid retention with functional photosynthesis can develop. Brandley (1984) investigated *E. furvacauda* Burn, 1958, which not only feeds on *Codium* but also switches to other food organisms during the life cycle—including rhodophytes. They could not find a phagosome membrane in any of the incorporated plastids, although retention of the various plastid types was very different. *E. chlorotica* (LTR) show no phagosome membranes, but the plastids exhibit a double membrane, have direct contact to the cytosol and maintain their integrity even after 8 months of starvation (Mujer et al. 1996; Rumpho et al. 2001). Rumpho et al. (2001) emphasized the peculiar and unique situation in the relationship of *E. chlorotica* and its food *V. litorea*. Plastids in heterokontophyte alga *Vaucheria* exhibit four membranes: the double membrane of the plastid, followed by a third and fourth outer membrane, the latter of which is sometimes called the chloroplast endoplasmic reticulum. Ultrastructural investigations by Rumpho et al. (2001) confirmed the absence of the outer two membranes when the plastids are sequestered as kleptoplasts in the sacoglossan digestive gland cells. Rumpho et al. (2001) suggested that the outer two of the plastid's four membranes are probably also absent when plastids are transported in the lumen of the digestive gland.

In cases where a phagosome membrane is missing, plastids have direct contact to the cytosol of the slug's cell. Although the last word has not been spoken in regarding the presence or absence of a phagosome membrane in all these species,

the results to date suggest that functional kleptoplasts in long-term retention sacoglossans have a direct contact between the outer chloroplast membrane and the cytosol. Conversely, the presence of a phagosome membrane tends to indicate digestion. More investigations are needed, especially on STR forms such as *E. viridis*, regarding the number and nature of membranes surrounding the plastid at different stages of starvation. Clearly, a host phagosome membrane would serve to direct digestive enzymes towards the plastid, hardly a situation conducive to long-term retention. The situation in sacoglossans is perhaps similar to that observed for haptophyte kleptoplasts that are sequestered by the phagocytosing dinoflagellate *Dinophysis mitra*. Koike et al. (2005) reported various food particles and ingested cells within phagosomes, but never the ingested haptophyte kleptoplasts, leading them to conclude that the dinoflagellates "*. . .probably differentiate haptophytes from other prey, so that the haptophyte plastids are specifically treated not merely as food but as* "*special guests*" *in the D. mitra cytoplasm*" (Koike et al. 2005, p. 223).

The actual process of plastid engulfment has hardly been investigated so far but seems to occur in digestive gland cells quickly after feeding, according to McLean (1976), who studied the non-retention form *P. dendritica*. Evertsen and Johnsen (2009) described various stages of plastid degradation within *P. dendritica* (NR) and *E. viridis* (STR) after certain starvation periods. The latter species also show starch grains and nearly no recognizable remnants of plastids within phagosomes after several days of starvation.

Importantly, none of these ultrastructural investigations gave evidence of algal nuclei or nucleocytoplasm (Mujer et al. 1996) in the slugs' cells in any sacoglossan investigated yet. This is in contrast to some protists (Stoecker et al. 2009), where nuclei can be retained. Dividing chloroplasts were also never found in sacoglossans and plastids are not transmitted vertically via eggs (e.g. Trench et al. 1969; Marín and Ros 1993; Rumpho et al. 2001).

Photosynthetic Measurements

In early studies, various criteria for photosynthetic activity during starving periods were measured. Oxygen production was used by Graves et al. (1979) for *E. chlorotica* in comparison with the non-retaining form *A. modesta*. Taylor (1971) compared *H. bifida* with the food alga *G. flosculosa* and found a similar O_2 production during the first few days. This system is unusual so far in that *Hermaea* is not a member of the Plakobranchoidea, but of the Limapontioidea, which usually show no plastid retention at all, and the food alga is a rhodophyte. Chlorophyll contents were measured for several sacoglossans after various starvation periods (Clark and Bussacca 1978). Chlorophyll contents combined with the ability to fix labelled ^{14}C after various time periods were first used by Greene (1970) for *P. ocellatus* (LTR) and a short-term retention form, *E. hedgpethi* (Marcus 1961). Hinde and Smith (1972) used the same experimental design as

Greene (1970) and observed high photosynthetic activity in *E. viridis* over a period of 25 days. Marín and Ros (1989) and Clark et al. (1990) used similar methods for a survey of Mediterranean and Caribbean species. Carbon fixation, as a functional photosynthesis measurement, was further used by Hinde and Smith (1974) for some members of the Limapontioidea: *Limapontia capitata* (Müller 1774), *L. depressa* (Alder and Hancock 1862) and *A. modesta*. Their results on *Alderia*—exhibiting no photosynthesis—confirmed former studies [see also Graves et al. (1979)]. *L. capitata* (but not *L. depressa*) showed some carbon fixation during the first few days, but much less than *E. viridis*. The fixation rates reported in *C. ocellifera* by Clark et al. (1981) after 65 days of starvation corresponded to about 10 % of the algal rate, which appears very high since this species is a member of the Limapontioidea and not of the Plakobranchoidea. ^{14}C fixation rates measured in *E. chlorotica* (starved 4 months) were only slightly lower than in the food alga *Vaucheria* (Rumpho et al. 2001).

The incorporation of $^{14}CO_2$ as a proxy for photosynthetic activity in animals has a caveat, though. Animals can incorporate appreciable amounts of $^{14}CO_2$ into protein, lipid and carbohydrate (Agosin and Repetto 1963), but this can proceed through exchange reactions, without net CO_2 fixation (Louis Tielens and Manfred Grieshaber, personal communication). For example, many animals rely heavily upon propionate, a C_3 compound generated in the digestive tract, as the backbone of their carbon metabolism. The typical eukaryotic assimilation pathway for propionate involves carboxylation (incorporation of CO_2) to methylmalonyl-CoA, isomerization and thiolysis to succinate (Schöttler and Bennet 1991). In the presence of $^{14}CO_2$, this would lead to succinate (a C_4 compound) with label at either C-1 or C-4. En route to glucose, succinate is converted into triose phosphates (C_3 compounds) through decarboxylation, whereby the labelled or the unlabelled carboxyl group of succinate is removed with equal probability. This yields glucose with, on average, one labelled carbon atom for every two molecules of CO_2 incorporated, even though two carboxylations are balanced by two decarboxylations. The exchange reaction leads to incorporation of radioactive CO_2, but in a process that has nothing to do with photosynthesis.

In situ measurements of plastid function in Sacoglossa started with the use of a Pulse Amplitude Modulated (PAM) Fluorometer (Heinrich Walz, Germany) to detect in vivo fluorescence and to measure the quantum yield of charge separation in photosystem II in dark acclimated organisms to analyse the status of the photosynthetic activity (Wägele and Johnsen 2001). Further studies with PAM fluorometry on selected species followed (Evertsen 2006; Evertsen et al. 2007; Evertsen and Johnsen 2009; Jesus et al. 2010), including measurements of *P. ocellatus* showing nearly no decline of the quantum yield over a period of 2 months. Händeler et al. (2009) published measurements of nearly 30 species, including many genera never studied before. Yamamoto et al. (2009) focussed on Japanese sacoglossans, while Klochkova et al. (2010) focussed on sacoglossans in Korean waters.

Physiology

Physiological investigations were performed to understand the contribution of plastids towards energetic self-maintenance as well as supporting the slug. Trench and Smith (1970) detected synthesis of the photosynthetic pigments α- and β-carotene by tracing labelled ^{14}C in *E. viridis* and *E. crispata*; however, their results concerning xanthophyll pigments were negative and only small traces of chlorophyll *a* and *b* were detectable. Studies on *E. viridis* kept under starvation in light and dark conditions revealed a higher weight loss in darkness, implying that photosynthesis supports its metabolism (Hinde and Smith 1975).

Metabolites produced with the help of plastids were followed within slug tissues and traced to many precursor compounds, including lipids, amino acids, mono- and oligosaccharides (e.g. Hinde and Smith 1974). The role of different organs like neural tissues, the excretory system and the pedal gland have also been investigated (Trench 1969). Labelled ^{14}C was incorporated in the pedal gland of *P. ocellatus* (as *P. ianthobapsus*; Trench et al. 1973a) and *E. crispata* (as *Tridachia crispata* and *Tridachiella diomedea*; Trench 1973; Trench et al. 1974). Analysis of the mucus secretion after 3 weeks starvation revealed the presence of the photosynthetic products glucose and galactose, which are in turn precursors for the synthesis of mucus (Trench et al. 1969, 1970, 1973a, 1974). Subsequent studies confirmed these results for other species. Marín and Ros (1989) found about 7–8 % of net incorporated ^{14}C in the mucus of *E. timida*. Trench et al. (1973a, b) compared ^{14}C fixation in *E. viridis* and isolated plastids from their food (*C. fragile*) and showed that, while fixation rates are similar, the release of fixed carbon is much higher in the slugs than in isolated plastids. Also, the location of incorporated ^{14}C differs. When analysing *E. chlorotica*, Rumpho et al. (2001) mainly traced ^{14}C in water-soluble metabolites. In contrast, the food alga *Vaucheria* incorporated more ^{14}C in lipids and proteins.

Ireland and Scheuer (1979), analysing *P. ocellatus*, demonstrated a high incorporation of ^{14}C in secondary metabolites of the pyrone class (9,10-deoxytridachione). The in vivo photoconversion of this pyrone compound into photodeoxytridachione occurs when the animals are exposed to light. The same compounds have been found in the LTR forms *E. crispata* (Ireland and Scheuer 1979) and *E. timida* (Gavagnin et al. 1994). Ireland and Scheuer (1979) speculated that the photodeoxytridachiones function as a sunscreen, absorbing mainly ultraviolet light and therefore representing a biochemical adaptation to higher irradiances [but see Di Marzo et al. (1993), who assigned the function to defence]. Polypropionates seem to be more widespread within Sacoglossa, even in NR forms. The presence of these compounds within Plakobranchoidea, as well as Limapontioidea, was reported by Di Marzo et al. (1993) and Gavagnin et al. (2000).

Environmental Factors

Clark et al. (1981) showed the importance of environmental factors like temperature and irradiance on the survival of functional plastids within slugs. Net carbon fixation in *C. ocellifera* increased threefold from 20 to 30 °C, but declined again at 35 °C. The optimal temperature conditions for net carbon fixation in *E. timida*, measured in a range of 15–35 °C, were 25 °C (Marín and Ros 1989). Experiments with *E. tuca* Marcus and Marcus, 1967 in situ at its main locality (along the Key West, Florida) clearly showed seasonal and geographical variation in chlorophyll content (Waugh and Clark 1986, p. 485). These authors indicated that many factors, "*including feeding rate*, *metabolic rate of plastids*, *physiological support of kleptoplastids*, *and dietary selection may affect chlorophyll level*"—and they found the highest chlorophyll content during autumn period, when the animals also show highest reproductive activities.

High light intensity elicited few immediate effects on the direct carbon fixation in *C. ocellifera* (Clark et al. 1981), but observations over longer periods clearly indicate photodamage and subsequently lower carbon fixation compared to animals kept in lower irradiances (Clark et al. 1981). Marín and Ros (1989) found the highest carbon fixation in *E. timida* under light intensities of 200 μE m^{-2} s^{-1}. Giménez Casalduero and Muniain (2008) investigated chlorophyll content and oxygen production in starved *E. timida* kept in a normal day/night rhythm or kept in permanent darkness. Chlorophyll content decreased in similar amounts during the 28 days of starvation, irrespective of exposure to 12 h irradiance with 200 μE m^{-2} s^{-1} (for comparison, cloudless noontime irradiance in the tropics is about 1700 μmol photons/m^{-2} s^{-1}). However, the animals kept in light showed less shrinkage than those kept in the dark. This probably confirms the mentioned irradiance as an optimum for *E. timida* with low photodamage to the photosystems. Vieira et al. (2009) demonstrated that maximum quantum yield values decreased considerably quicker when specimens of *E. viridis* were exposed to medium light intensities (140 μE m^{-2} s^{-1}) during starvation experiments over a maximum of about 8 days, compared to lower light intensities (30 μE m^{-2} s^{-1}). Based on several analyses, including rapid light response curves (RLC), they suggested that photodamage of the D1 protein in the PSII reaction centre and the absence of repair mechanisms is the major factor of this decline. Evertsen and Johnsen (2009) reported similar results concerning the same species. Photoacclimation is a typical feature of plants adapting to changing light conditions, expressed by the angle α of the E_K curves when rapid light responses are measured. Vieira et al. (2009) could not detect any changes in α during low and high light experiments, inferring that no acclimation had occurred during the experiments. The chloroplasts showed the acclimation status of the light regime, when the slug consumed the alga. *E. chlorotica*, which incorporates plastids from the heterokontophyte *V. litorea*, was found to maintain functional levels of thylakoid membrane proteins over several months [see Mujer et al. (1996), Pierce et al. (1996), Green et al. (2000)]. *E. chlorotica* is the only species where evidence is given for active protein synthesis

in kleptoplasts (see below). However, studies on isolated *Acetabularia* plastids—the food of *E. timida*—indicate that the synthesis of chloroplast pigments (α- and β-carotene, chlorophyll *a*, chlorophyll *b* and xanthophylls) is still possible (Trench and Smith 1970) and therefore more studies on other long-term retention forms are needed.

Behaviour

Behavioural studies are few in number and were mainly performed on the Mediterranean species *E. timida*. Their major target was the importance of parapodia and their role in shading, therefore reducing photodamage in chloroplasts. Rahat and Monselise (1979) and Monselise and Rahat (1980) investigated the importance of parapodial movements in *E. timida*, first in the laboratory and subsequently in the sea, resulting in clear evidence of the parapodia closing over the body in darkness and very high irradiances. They showed that the eyes of the slugs are not important for measuring the light intensity, since opening degrees of the parapodia were similar in normal or eyeless slugs in various light regimes. Giménez Casalduero and Muniain (2008) interpreted the 65 % loss in chlorophyll content, but only a reduction of 25 % of photosynthetic production rate after 9 days starvation in *E. timida* as evidence for the ability of the slug to regulate its exposure to incident light by unfolding and refolding of its parapodia. Schmitt and Wägele (2011) reported results in support of that view by measuring fluorescence values in *E. timida* during various folding positions of the parapodia. This slug species clearly showed a strong, positive, phototactic behaviour, whereas reactions of *T. hopei* (Verany 1853), a close relative, were distinct, the latter species preferring dim light conditions. Since juvenile specimens of *E. timida* showed a strong phototactic reaction already before their first feeding, Schmitt and Wägele (2011) suggested that this behaviour might not be triggered by the chloroplasts.

Only one study addresses the recognition of light spectra. Weaver and Clark (1981) tested one long-term retention form (*E. crispata*), two short-term forms (*E. tuca* and *C. ocellifera*, as *C. lilianae*) and two non-retention forms [*Oxynoe antillarum* Mørch, 1863 and *Berthelinia caribbea* Edmunds, 1963]. Animals were allowed to choose between two different light spectra in two-trail experiments. Cellulose acetate filters were used with transmission of red, blue, yellow or green light, but wavelengths were not given. According to their interpretation, the three photosynthetic animals preferred green, yellow and blue light, thus shorter wavelengths, which at the same time correlated with higher light intensities. The two non-retention forms showed no light quality preferences but in general thrived under low light intensities or even darkness.

Ecophysiology

Jesus et al. (2010) addressed an ecophysiological problem not considered before in slugs: non-photochemical quenching (NPQ), typical for plants when quenching excessive light energy and removing excess energy from the photosynthetic electron transport chain by using accessory pigments. In *Acetabularia acetabulum* (Linnaeus) Silva, 1952 and *E. timida*, they clearly showed that pigments associated with the xanthophyll cycle behaved similarly in various light regimes and "were linearly related to NPQ capacity" (Jesus et al. 2010, p. 103). This was the first evidence that photo-regulation via the xanthophyll cycle, one of the most important photo-protection mechanisms in plants, can also operate in kleptoplasts isolated within slugs. They concluded that, in combination with behavioural responses, this mechanism could be important in retaining functional chloroplasts. Unusually however, a high xanthophyll ratio (higher zeaxanthin values) was observed in the dark experiments with *E. timida*. This was very different from *Acetabularia* kept under the same conditions. Since plastids in *E. timida* are not located within a phagosome membrane, resulting in a direct contact with the slug's cytosol, Jesus et al. (2010) suggested that there might exist some kind of interaction between the animal's metabolism and the electron transport chain of the kleptoplast. Another problem was addressed by Teugels et al. (2008), namely how the slugs overcome nitrogen deficiency during food shortages. By tracing ^{15}N-labelled ammonium, urea, nitrite and nitrate in dark and light experiments using *E. viridis*, they showed that N-uptake was higher during light exposure. However, nitrate was not used. They also measured ^{15}N uptake in dark green animals containing a high chloroplast density compared to light green animals with lower chloroplast density. When exposed to light, the uptake of ^{15}N molecules was much higher in the darker than in the lighter animals. Evidence of assimilation was observed by the presence of amino acids with ^{15}N signatures.

Chloroplast Origin

Early on, the species of food ingested, hence the type of chloroplasts sequestered, was considered as an important factor for functional photosynthesis in Sacoglossa. Accordingly, food algae were investigated by various authors with regard to performance in slugs' photosynthesis (Clark and Bussacca 1978). Food information was usually obtained by observing the slugs on specific substrates or during active feeding (e.g. Jensen 1980, 1993a, b; Thompson and Jarman 1989, for the most recent compilation see Händeler and Wägele 2007). Occasionally, pigment analyses were utilized to determine the origin of the incorporated plastids. For example, Greene (1970) rejected the former identification of cyanobacteria in *P. ocellatus* by Kawaguti and Yamasu (1965) based on the presence of chlorophyll *b*, which is not present in heterokontophytes but in chlorophytes. Additionally, the

combination of various pigments (especially siphonein and siphonaxanthin) indicated chlorophytes as the probable plastid source in *E. crispata* (Trench et al. 1969). Jesus et al. (2010) analysed pigment composition in *E. timida* and identified *Acetabularia* as the only probable food source. The conclusion that *H. filicina* is the food source for *E. timida* based on the observation that the slug was seen sitting on the alga [see Giménez Casalduero and Muniain (2008)] is tenuous.

Recent studies focus on barcoding the chloroplasts using single gene analyses (mainly *rbcL* and *tufA*) (Curtis et al. 2006; Händeler et al. 2010) to identify the source of ingested plastids. This is especially important when species are never observed to be feeding on algae (for example *P. ocellatus*), or when they feed on a variety of algae (for example *E. crispata* and *E. clarki*). Pierce et al. (2006) identified at least four different food items in *E. crispata*. A similarly diverse diet was confirmed for *E. clarki* (Curtis et al. 2006). Wägele et al. (2011) identified several food items belonging to different algal genera for the long-term retention form *P. ocellatus* by analysing *tufA*. From feeding and starvation experiments (Klochkova et al. 2010), it seems very unlikely that different kinds of sequestered plastids contribute in the same way and remain for the same time period. Therefore, the identification of retained plastids after weeks and months, especially in *E. crispata*, *E. clarki* and *P. ocellatus*, is essential to understanding the system. Interestingly, STR and NR forms can feed on the same algal species [*V. litorea*, see Pierce et al. (1996); *Caulerpa racemosa* (Forsskål) Agardh, 1873, see Händeler et al. (2010); *Chaetomorpha* spp., *Codium* spp., see Klochkova et al. (2010)] implying that long-term retention forms actively avoid direct chloroplast digestion.

A few studies deal with the impact of the alga on the feeding process. Waugh and Clark (1986) observed that the chlorophyll content varied in *Elysia tuca* according to the food organism on which the animals were collected. Animals collected from *H. incrassata* showed higher chlorophyll content than animals collected from *Halimeda discoidea* (Decaisne 1842). The authors considered the effort a slug must expend sucking the cytoplasm from the alga an important factor. Both *Halimeda* species calcify differently and therefore sucking might be more difficult in the more calcified *H. discoidea*. The relationship between *A. acetabulum* and *E. timida* has been well studied because of the close interaction during their annual life cycles: Juvenile slugs are able to accumulate chloroplasts, but grazing decreases when the alga increases calcification. The slug reaches its peak plastid accumulation when the alga is resistant to further grazing. Marín and Ros (1992, 1993) postulated the disappearance of food sources as a driving force for development of plastid retention in *E. timida*. Waugh and Clark (1986) hypothesised that algal wound-plug formation might retard the feeding process and therefore limit the uptake of chloroplasts. Jensen (1994) suggested algal cell wall structure, chemical composition and calcification as the most important factors governing the evolution of food preferences within Sacoglossa.

Händeler et al. (2009) mapped available food information on a phylogenetic tree of the Sacoglossa (Fig. 2, complemented with recent data). Diet data is however still unknown for many species. No pattern can currently be seen that would indicate a

preferred group of algae in the evolutionary process leading up to solar-powered sea slugs. We only know for sure that the algae have to be siphonaceous or siphonal (coenocytic), because sacoglossans suck out the contents of the filamentous algae and do not graze like members of the Anaspidea.

Properties of Plastids

The longevity and properties of isolated plastids as an indication of suitability for long-term retention was investigated in few cases. Giles and Sarafis (1972) isolated plastids from *Caulerpa sedoides* and maintained them in a functional state for more than 10 days within hens' eggs. CO_2 fixation was reduced to 48 % compared to the intact alga and decreased to 10 % after 27 days. Trench et al. (1973a) analysed carbon fixation rates in isolated *C. fragile* plastids. Fixation of ^{14}C still occurred after 5 days and is thus similar to results obtained from *A. acetabulum*, the exclusive food of *E. timida* [see Trench et al. (1973a, b)]. The authors discussed the putative robustness of siphonaceous algal plastids as an important factor in surviving engulfment into the slug's digestive cells. The stability of isolated plastids from *Codium* and *Caulerpa* was already emphasized by Grant and Borowitzka (1984a, b). Isolated *Vaucheria* plastids also exhibited a high structural and functional stability (Rumpho et al. 2001; Green et al. 2005).

Grant and Borowitzka (1984a) stressed the possibility that a contamination by extraplastidic material, enclosed in the so-called cytoplast, could enable the isolated plastids to produce many products (sugars and amino acids) for a long time. However, there are differences in the photosynthetic products when comparing isolated plastids from *Caulerpa*, *Acetabularia*, *Codium* and *Bryopsis*. Evidence for the release of photosynthetically produced substances from isolated plastids into the surrounding medium is also shown, but the uptake of exogenous material by isolated plastids seems to be very low [see Grant and Borowitzka (1984b)].

The autonomy of plastids as an important factor was also addressed by Rumpho et al. (2001). They assumed a higher autonomy of *Vaucheria* plastids due to the larger plastid genome, which contains many more genes necessary for photosynthesis, as compared to chlorophyte plastid genomes. But many LTR species retain chlorophyte plastids, so plastid gene content can hardly be a direct factor.

Functional Plastid Genomes and Lack of Horizontal Gene Transfer

How can a single organelle with a limited number of genes—between 60 and 200 protein coding genes for plastids (Timmis et al. 2004)—function without the complementary nuclear DNA that encodes for many proteins and enzymes necessary to uphold a functional photosynthesis over a period of several months? Less

than 10 % of the roughly 200–400 proteins required for photosynthesis in plastids are encoded by the plastid genome (Martin and Herrmann 1998) and thousands of genes that were acquired from cyanobacteria are present in the higher plant nuclear genome (Martin et al. 2002). That genes were transferred to the host nucleus during the origin of plastids from cyanobacteria has been known for some time (Martin and Cerff 1986; Martin et al. 1993, 1998); hence, the possibility that genes were also transferred during kleptoplast establishment has been considered as well.

The presence in sacoglossans of photosynthetically active sequestered plastids in the absence of sequestered algal nuclei led Pierce et al. (1996) to suggest that a horizontal gene transfer might have occurred from the algal nuclear genome into the slugs' nuclear genome. This would then enable the slug to encode the necessary proteins, subsequently targeted to the plastids they have sequestered. Their analyses with western blots on isolated plastids from *E. chlorotica* in comparison with plastids from the food alga *V. litorea* revealed a similar spectrum of proteins in both organisms. RuBisCo synthesis was experimentally interrupted in the slug by adding chloramphenicol, indicating plastid gene encoding. The authors concluded, however, that plastid proteins had to be synthesized on slugs' ribosomes within the slug cells, although, they did not mention the number of days the slugs were starved before performing the experiments. Pierce et al. (1996) discussed three possibilities as to where the genetic information could have come from. The first hypothesis suggests that the slug genome is genetically enriched via horizontal gene transfer (HGT) from the algal genome. The second proposes that the slug genome itself is already capable of encoding proteins targeted to the plastids. This is mentioned as dual targeting in Rumpho et al. (2000): The similarity of some proteins used in mitochondria and in plastids might simplify the process of redirecting. Rumpho et al. (2001, p. 310) considered that dual targeting "*. . .opens the possibility for animal proteins to be 'mis-directed' to the kleptoplasts*". The third hypothesis suggests that plastids release RNA to the slug cytosol, where the necessary proteins are synthesized and subsequently transported back to the plastids. Mujer et al. (1996) mentioned a fourth possibility, that kleptoplast proteins are very stable.

Mujer et al. (1996) stressed the necessity of molecular analyses for understanding the extraordinarily long plastid retention in the slugs. They reported the identification of several plastid-derived genes in isolated DNA from starved *E. chlorotica* using Southern blot analyses (*psaB*, *psbA*, *rrn16*, *rbcS* and *rbcL*). Protein products (D1, D2 and CP43) were still detected after several months of starvation. "*The ability of the symbiotic plastids to carry out transcriptional and translational functions helps explain their capacity for maintaining long-term photosynthetic activity*" (Mujer et al. 1996, p. 12336). The combination of functional proteins from PSI and PSII in the first few months of starvation, and the presence of certain larger proteins in an 8-month starved specimen, assigned to be products of *psaA* or *psaB*, led to their conclusion that PSI probably lasts longer in later starvation stages than PSII. Analyses by Green et al. (2000) suggested that kleptoplasts in *E. chlorotica* maintain photosynthetic oxygen production and electron transport activity through PSI and PSII for at least 5 and 6 months, respectively. Several proteins were identified by immunoblotting after even 7–9 months of

starvation, although some of them decreased considerably within this time. The identified proteins included some from PSII (D1, D2 and CP-43), PSI (PsaA, PsaB, PsaC and PsaD), and the cytochrome b_6/f complex (cytb_6 and cyt$f$), mobile electron carriers (cytc_6), RbcL, and the light-harvesting complex (FCP). Of these, only the last one is nuclear encoded. Translational ability was investigated by exposure to ^{35}S-labelled methionine in starved slugs and in the algae. Synthesis of D1, RbcL and several unidentified proteins was demonstrated even after 9 months, however not in the case of FCP. The authors discussed an extreme stability of this protein or a possible HGT for the specific gene. In general, the results confirmed former experiments (Mujer et al. 1996) that PSI remains functional longer than PSII. Due to negative results concerning presence of the algal ITS region in *E. chlorotica*, Green et al. (2000) ruled out the presence of an algal nucleus in the tissue of the slugs.

Rumpho et al. (2000) considered specifically the gene for phosphoribulokinase (PRK) as a possible candidate for HGT, since the encoded enzyme is needed for regenerating the CO_2 acceptor ribulose-1,5-bisphosphate to uphold photosynthetic CO_2 reduction (the Calvin cycle). In 2001, the authors reported PRK staying active for 9 months, but Southern blots and reverse transcriptase-PCR analyses were negative concerning the genes encoding PRK, RbcS and FCP. Rumpho et al. (2001, p. 310) did not entirely reject HGT as a consequence, but emphasized again the possibility that incorporated chloroplasts could be "*incredibly stable*". Hanten and Pierce (2001) published results on proteins belonging to the light-harvesting complex I (LhcI) in *E. chlorotica*. Since protein accumulation was blocked by cycloheximide, the authors inferred a nuclear-encoded synthesis from transferred genes in the slugs. The pigment protein FCP was again analysed by Pierce et al. in 2003, but they used another LTR species: *E. crispata*. Western blot analyses resulted in similar observations to those seen in *E. chlorotica*; cycloheximide appeared to block the synthesis of the protein. Pierce et al. (2003) tried to verify the identification of this protein through purification. The following determination of the N-terminal amino acid sequence (30 AA) and a subsequent BLAST search revealed 66 % sequence identity with FCP from the chromophyte *Cylindrotheca fusiformis*. The identification of three internal sequences lead to an 81 % overlap of one internal sequence (11 amino acids) with FCP protein sequence of the chromophyte *Macrocystis pyrifera*. Subsequently, the authors amplified a sequence (350 bp) of *Vaucheria* DNA after designing degenerate primers. Instead of applying the same primers to retrieve a sequence directly from the slugs, they indirectly identified part of the sequence by using Southern blot analyses against DNA taken from *E. crispata*. The resulting protein sequence showed a 51 % match with the FCP seen in *Laminaria saccharina*. These results, combined with western and Southern blot analyses, led the authors to infer that they had identified a gene that encodes for FCP within "*the genomic DNA of E. crispata, where it waits for the acquisition of new plastids in each generation of slugs*" (Pierce et al. 2003, p. 239). Pierce et al. (2007) reported evidence for gene sequencing of a transferred and targeted protein. The authors designed primers for FCP, lhc 1 and 2 (designated as lhcv 1 and 2 and as identified in former studies). The process of designing the

primers for the genes is not outlined, but subsequent sequencing of every amplicon of the three different genes revealed 100 % matches with respective genes in *Vaucheria* and *E. chlorotica*. They did not discuss however, whether these sequences match or mismatch respective GenBank sequences. They concluded that, due to the many unidentified proteins apparently blocked by cycloheximide and detected in former analyses, the number of genes transferred from the algal nucleus into the slug's nucleus must be very high.

Rumpho et al. (2008) then published results suggesting the presence of the algal gene *psbO* in *V. litorea* as well as in *E. chlorotica*. *PsbO* encodes MSP (the manganese stabilizing protein), a major protein associated with the oxygen evolving complex of photosystem II. They first designed a specific primer based on available *psbO* sequences in the GenBank database. Its product only showed a 48–68 % match to several MSP amino acid sequences from red algae. Nevertheless, by using the RACE method, they created a larger *psbO* product from *Vaucheria* nucleic acid extracts containing nearly 1,000 bp. Subsequently, new primers were designed from this long sequence. Amplification with these primers yielded PCR products with a 100 % similarity in comparisons of products obtained from *Vaucheria* and *E. chlorotica* nucleic acid extracts. A distressing aspect of that study is the description in detail of a bipartite targeting sequence in the putative *psbO* gene of the slug that would direct the product to the plastid. The plastids of *Vaucheria* are indeed surrounded by four membranes in the alga (Gould et al. 2008) and do require such complex targeting signals to traverse the four membranes, but the *Vaucheria* plastids sequestered in *Elysia* are only surrounded by two membranes, such that if those targeting signals were indeed present, the protein would exit the slug cell via the secretory pathway, and not be targeted to a plastid.

Rumpho et al. (2009) announced the presence of at least parts of the PRK gene (identical with *V. litorea*) in *E. chlorotica*. Activity of PRK protein was demonstrated for starved animals after 3 months, but redox regulation occurred only in PRK when studied in *Vaucheria* and not in the slug. Subsequent further identification of genes putatively subject to HGT was based on an EST analysis of *V. litorea* (Pierce et al. 2009; Schwartz et al. 2010). Knowing which genes are nuclear encoded in *Vaucheria* and necessary for upholding photosynthesis, they searched the *Vaucheria* EST library and subsequently designed primers. This resulted in algal sequences that exactly matched the slug's and are assigned to uroD, chlD, chlH, chlG, lhcv-3, lhcv-4 and PRK (Pierce et al. 2009; Schwartz et al. 2010). Although UroD (uroporphyrinogen decarboxylase) is synthesized in mitochondria and cytoplasm in animals, the authors rejected a possible dual targeting, at least for this gene. They suggested that the slug's UroD, which had only 27 % amino acid identity with UroD of the alga, is not responsible for the ongoing photosynthesis in *E. chlorotica*.

Notably, some of the identified genes (PRK, lhcv-3 and chlG) have an intron in *Vaucheria*, which is missing in the corresponding sequence obtained from *E. chlorotica*. Pierce et al. (2009) suggested that due to the high similarity of the genes in *Vaucheria* and *E. chlorotica*, the HGT must have been a very recent evolutionary event. They speculate that many more algal genes have been

transferred, "...—*perhaps even pieces of, or even entire, algal chromosomes are involved*" (Pierce et al. 2009, p. 127). Concerning the Sacoglossa in general, they also suggested that "...*gene movements have occurred many times across species and in different amounts*" (Pierce et al. 2009, p. 127).

In contrast, Rumpho et al. (2011, p. 307) emphasized that nearly all of the detected enzymes (except of two) are also encoded by the nuclear genome of animals. "...*it is possible that the animal could provide substitute proteins for the majority of the nuclear-encoded RPPP* (Calvin cycle) *enzymes if they were properly targeted to the foreign plastids*". This is possible, in principle at least, because the Calvin cycle and glycolysis/gluconeogenesis share a number of enzyme activities in common, and many Calvin cycle enzymes are indeed evolutionarily derived from duplicates of genes for cytosolic proteins that existed prior to endosymbiosis in the host cell that acquired the cyanobacterial ancestor of plastids (Martin and Schnarrenberger 1997).

Thus, in about 1996, the notion of HGT in sacoglossan kleptoplasty started to snowball, and it turned into a small avalanche with the 2008 report of the "transferred" psbO gene, the nuclear localization of which in the slug was not unequivocally shown, and which was obtained through PCR, not through a direct clone library (Rumpho et al. 2008). From the standpoint of classical endosymbiotic theory, one problem stood out in the *Elysia* gene transfer story that made us especially critical of the HGT claims. One of the crucial lines of reasoning behind the idea that plastids are derived from endosymbiotic cyanobacteria to begin with was the continuity of plastids through the egg cells of each generation, as Schimper (1883) and Mereschkowsky (1905) argued over 100 years ago. In other words, if the genes to support the plastids are present in sacoglossan slug nuclei, as some are claiming, why do the slugs reacquire the plastids every generation?

We reasoned that if there are transferred genes for photosynthetic functions in the nuclei of LTR slugs, we should be able to see those genes as expressed mRNA in a deep sequencing EST experiment using mRNA extracted from photosynthesizing slugs. We performed EST analyses of two LTR species, *P. ocellatus* and *E. timida* (Wägele et al. 2011) with 77,000 expressed sequence contigs for *P. ocellatus* and 25,000 contigs for *E. timida* (a total of 1.5 million reads and 64 Mb of nonredundant sequence data), made against mRNA that was extracted from animals that were demonstrably photosynthetic at the time of harvesting (PAM fluorescence) but removed from their food source for at least 3 weeks. We then compared the extensive *Arabidopsis* EST data, where a wealth of information on nuclear encoded genes for chloroplast biogenesis exists, to our slug ESTs and to the limited EST data then available for *Acetabularia*, the food alga of *E. timida*. The comparison to *Acetabularia* tells us whether, using *Arabidopsis* query sequences, we would be able to detect expressed algal genes in *Elysia* if they were there (a positive control for our computer analyses) while the comparison to the slugs tells us which homologues of nuclear encoded *Arabidopsis* genes for chloroplast proteins are expressed as mRNAs in the animals. The results (Fig. 5) clearly rule out the expression, by slugs, of horizontally transferred genes, from algae, as a component of plastid survival and functional photosynthesis in these two (out of the four

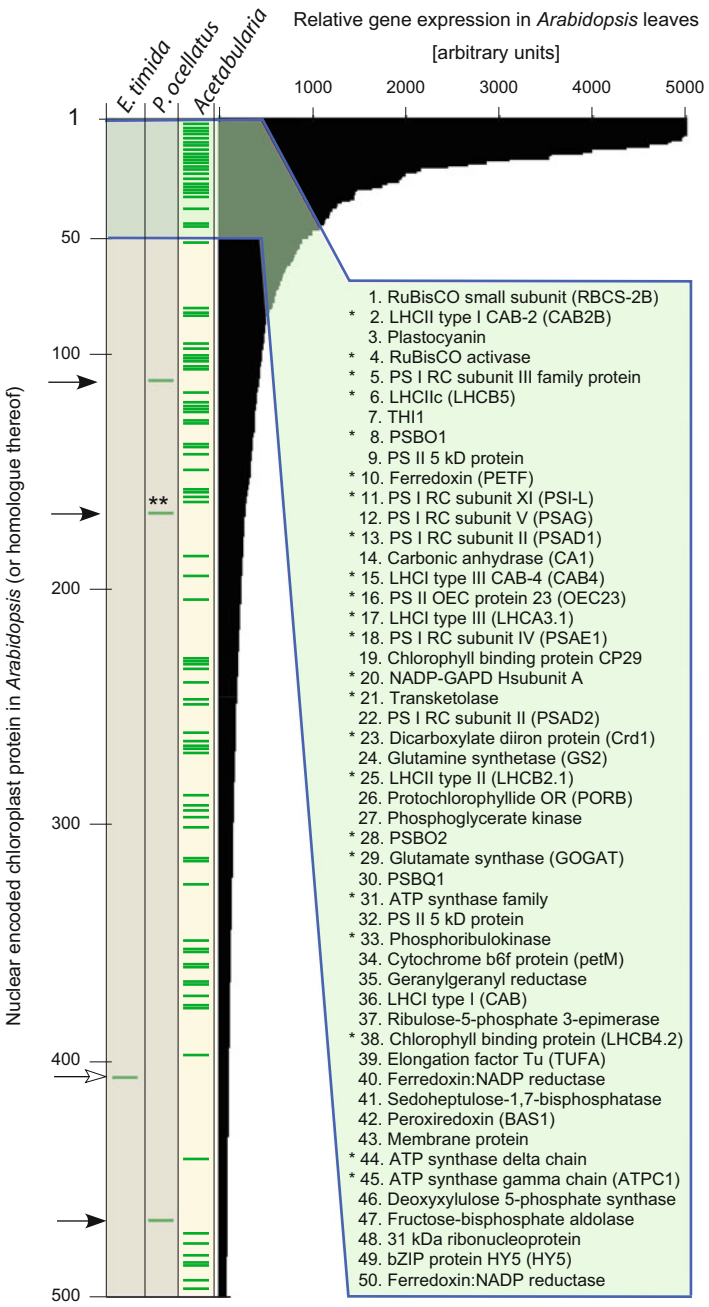

Fig. 5 Expressed genes in *Elysia timida* and *Plakobranchus ocellatus* compared to highly expressed homologous nuclear genes mainly encoding for photosynthesis proteins from the food alga of *E. timida*, *Acetabularia acetabulum*, and from *Arabidopsis* [after Wägele et al. (2011)]. For details see original publication. The most highly expressed 50 genes in *Arabidopsis* are listed. Matches with the *Acetabularia* contigs is indicated with an *asterisk*. Slugs' contigs showed no

well known) LTR sacoglossan species (Wägele et al. 2011). These results mean that the longevity of the sequestered plastids does not require transferred genes. The slugs have no need for HGT to support their photosynthetic symbiosis. We conclude that they just sequester long-lived plastids—which is possibly even more interesting.

But the HGT story is hard to stop, even with strong data for expressed genes of the type we presented. Pelletreau et al. (2011) repeated our EST experiment and analysed *E. chlorotica*, reporting 148 Mb of sequence data. They also found no evidence for expression of transferred genes, although they found a very low frequency of about 20 non-*Elysia* sequences (possibly contaminants) whereby they noted that "*None of these 20 ESTs, however, has a direct involvement in photosynthesis*". However, that did not stop them from concluding nonetheless that "*multiple lines of evidence that indicate that nuclear algal genes have been transferred*" (Pelletreau et al. 2011, pp. 1563, 1564). A problem with that conclusion is that the direct test of functionally relevant gene transfer (EST sequencing) comes up negative; all other data hinting at HGT in the slugs are indirect.

In the most recently published chapter of this story, Pierce et al. (2012) reported 98,000,000 reads and 8.8 billion base pairs of next-generation transcriptome sequencing data for *E. chlorotica*, a huge and valuable sequencing effort. They found many thousand reads that derive from the chloroplast genome, including 4,200 reads for the D1 protein of photosystem II alone. But among their 98 million reads, they only find 101 that match *Vaucheria* nuclear sequence data better than animal sequence data, corresponding to 52 transcripts. However, from that they conclude "*that a variety of functional algal genes have been transferred into the slug genome*" (Pierce et al. 2012, p. 3), even though evidence for expression of the collection of well over 500 nuclear-encoded genes that are required for functional plastids (Fig. 5) is altogether lacking. We disagree with their conclusion that their data represents evidence for HGT. In our view, the main finding of their study is that 98 million reads from *E. chlorotica* nucleic acid preparations produce 100 reads with undeniable similarity to *Vaucheria* nuclear genes; that finding suggests to us that their nucleic acid preparations used for sequencing were 99.9999 % free of contamination, because that is the frequency (one part per million) at which they find *Elysia* sequences. A purity of 99.9999 % is outstanding by any measure. But at the same time, the case for HGT to *Elysia* becomes a one in a million proposition.

Fig. 5 (continued) match with these first 50 genes or any *Acetabularia* gene. Three contigs of *P. ocellatus* (*black arrows*) had a match with one of the 500 most highly expressed *Arabidopsis* genes. One of these three (Rpl5, marked with *double asterisk*) is plastome encoded in chlorophytes and not nuclear encoded as in *Arabidopsis*. The other two are superoxide dismutase and a zinc finger protein. One match (*white arrowhead*) occurs in *E. timida*, it refers to ferritin. These three proteins/enzymes are ubiquitous

Future Research

If we accept the evidence from three laboratories showing that there is no horizontal gene transfer behind sacoglossan kleptoplasty (despite some interpretations of that evidence to the contrary), we have to ask again: what makes a slug photosynthetic? There are many unsolved problems concerning evolution, ecology, behaviour and genetics of these unique metazoan life forms. It starts with a founded hypothesis on the phylogenetic relationship of Sacoglossa and its sister taxon (Wägele et al. 2008; Händeler et al. 2009; Dinapoli and Klussmann-Kolb 2010; Jörger et al. 2011). This is a prerequisite to understanding when and why plastid incorporation has evolved, as well as the role of the food algae. At the moment, it seems that incorporation without digestion could have evolved once in the stemline of the Plakobranchoidea (Händeler et al. 2009), or alternatively multiple times independently. Available results, for example, on *C. ocellifera*, would support the latter hypothesis. This species, belonging to the Limapontioidea, shows a retention period of more than 60 days (Clark et al. 1981), in contrast to congeneric species [see Händeler et al. (2009)].

In the context of phylogeny, we can ask what the driving force behind the origin of long-term plastid retention was. Photosynthesis in slugs was certainly a by-product in evolution, fuelled by selective advantages (Ros and Marín 1990). Retaining or obtaining green coloration might have started as a means to becoming camouflaged (Clark et al. 1990; Rumpho et al. 2000; Wägele and Klussmann-Kolb 2005). Even shelled sacoglossans are green, although this colour is not derived from incorporated plastids. Additional input of energy by performing photosynthesis also provides an advantage for the food resources. These resources can be spared and food shortage (e.g. due to calcification) or even feeding on rare algal species becomes unproblematic (Marín and Ros 1989; Ros and Marín 1990; Teugels et al. 2008). Photosynthesis also helps the slug by supplementing the energetic demands of synthesizing defence compounds and possibly by facilitating nitrogen acquisition (Rumpho et al. 2006; Teugels et al. 2008). The ability to photosynthesize also helps relieve the energetic demands from reproductive behaviour, as Jensen (1987) demonstrated, when she observed reduced copulatory activity in starved *Ercolania nigra*, a non-retention form. It seems very likely that photosynthesis then also increases reproductive output.

We also have to ask whether the incorporation of plastids and subsequent ability to perform photosynthesis contributed to radiation within Sacoglossa. The number of sacoglossans is hardly known. Our own expeditions (H. Wägele) have revealed dozens of previously unknown sacoglossan species in need of formal description, and the same holds true for the collections of several colleagues. New molecular systematic analyses additionally revealed cryptic speciation (Carmona et al. 2011), and future analyses will show whether these new species are also characterized by their different and not yet investigated (photo-) biology. The few recently described species where photobiology was studied certainly raised the number of known photosynthetic slugs (Wägele et al. 2010b; Swennen 2011). However, there are

other factors to consider, for example chemical defence and protection against irradiance by uptake of secondary metabolites from food and/or their de novo synthesis (Gavagnin et al. 2000; Marín and Ros 2004).

Concerning the properties of the slug, we can ask whether they can regulate the uptake of chloroplasts that enable photosynthesis. Do they prefer certain plastids over others? Trench (1975) reported that *Elysia cauze* selected photosynthetic plastids for engulfment and rejected amylogenic ones. This would indicate a selective uptake of plastids from the same alga, depending on specific plastid features. Subsequently, we can ask about plastids from different algae and why they are not digested. Muscatine et al. (1975) and McLean (1976) have proposed that phagocytosis of plastids by symbiotic sacoglossans is followed by lysis of the enveloping host membrane, but the electron microscopic observations on enveloping membranes of sequestered plastids are still not completely clear, and even less so after plastid phagocytosis and incorporation for days up to months in STR and LTR slugs.

We also do not know the importance behind kleptoplast origin—a feature stressed by Evertsen and Johnsen (2009). Their studies showed that, in contrast to incorporated *Vaucheria* plastids in *E. chlorotica*, pigment proteins were not synthesized in *E. viridis* when they incorporate plastids from *C. fragile*. Therefore, the functionality of retained plastids could be investigated with regard to a slug's ability to synthesize pigments, proteins, lipids and starch. Recent success in molecular analyses of incorporated plastids by barcoding now provide the facility to clarify which food contributes to photosynthesis and which food does not (Händeler et al. 2010; Wägele et al. 2011).

Results taken from literature indicate that not only the plastid and its environment within the slug's cell counts but also the slug itself. Studies on ecology and adaptations in morphology and behaviour are also important. At least some species are able to reduce irradiance and therefore photodamage by phototactic behaviour and shading by parapodia (Schmitt and Wägele 2011). More studies on the influence of temperature and seasonality are needed, because their impact on photosynthetic performance seems to be very high [see Clark et al. (1981), Waugh and Clark (1986)].

We also know little concerning how much slug behaviour is influenced by the incorporation of plastids, or if their behaviour changes when enduring starvation. This seems very likely since reproductive efforts profit from photosynthesis (Jensen 1987; Middlebrooks et al. 2011); however, nothing is yet known about how this behaviour is triggered. Are there special photoreceptors as studies on eyeless *E. timida* specimens suggest (Rahat and Monselise 1979)?

Finally, what enables the slugs to perform photosynthesis for many months? Transcriptomic studies on three long-term retention forms—*Placobranchus ocellatus* (Wägele et al. 2011), *E. timida* (Wägele et al. 2011) and *E. chlorotica* (Pelletreau et al. 2011; Pierce et al. 2012) reject the hypothesis that HGT underlies plastid longevity [or should, see Pierce et al. (2012)]. Therefore, future analyses on plastid stability, protein stability and the slug's biochemical contribution should move to the fore in efforts to understand this beautiful, fascinating, and—among metazoans unique—symbiotic relationship.

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