

Variable retention of kleptoplast membranes in cells of sacoglossan sea slugs: plastids with extended, shortened and non-retained durations

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Abstract Six species of sacoglossan sea slugs engulf and store chloroplasts from their algal food (kleptoplasts) in digestive gland cells for weeks. The question is unresolved as to why kleptoplasts are retained only in certain species, while in most others they are digested. We recently showed phagocytosis of algal chloroplasts by digestive cells in the long-term retention form *Elysia timida*. Chloroplasts with functional thylakoids and stroma, but devoid of inner and outer envelopes, had been taken up. The stored plastids in the slug cells had only a phagosome membrane. In the present study we report that in cells of another long-term retention species, *Plakobranthus ocellatus*, only one membrane is present. In contrast, chloroplasts in digestive cells of the non-retention form *Thuridilla hopei* were enveloped by inner and outer chloroplast membranes, as well as a phagosome membrane. On the other hand, *Elysia viridis*, which has been considered to be a short-term retention form, had some chloroplasts with and without chloroplast envelopes. The hypothesis is proposed that the absence of chloroplast envelopes in long-term retention forms help to avoid digestion.

Keywords High-pressure cryofixation · Sacoglossa · Long-term retention · *Elysia viridis* · *Plakobranthus ocellatus* · *Thuridilla hopei* · Kleptoplast membranes

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Introduction

Why are chloroplasts retained in cells of certain sacoglossan sea slugs (Gastropoda, Opisthobranchia) while they are not retained in others? This is an unresolved question in the story of kleptoplasty (“stolen” chloroplasts), pertaining to the survival of chloroplasts in slug cells. The vast majority of these slugs are either non-retention (NR), or short-term retention (StR) forms, in which the kleptoplasts lose their photosynthetic capacity, either immediately, or within the first weeks, respectively (Händeler et al. 2009). In contrast, long-term retention (LtR) forms retain functional chloroplasts for at least a month during starvation. Six species in the latter group have been identified: *Elysia chlorotica* Gould, 1870, *Elysia timida* (Risso 1818), *Elysia crispata* (Mörch 1863), *Elysia clarki* Pierce et al. 2006, *Plakobranthus ocellatus* Van Hasselt 1824, *Costasiella ocellifera* Simroth 1895 (de Vries et al. 2014). Functional kleptoplasty cannot be solely based on the alga species (Christa et al. 2013). Sequestered chloroplasts are able to photosynthesize in the light, but this light-dependent CO₂ fixation is not essential for the slugs to survive starvation, since LtR animals survived several months in complete darkness. Chloroplasts seem to be slowly digested as food reserves (Christa et al. 2014).

We found that cells of *E. timida* phagocytosed chloroplasts, which were devoid of inner and outer envelopes. In the digestive cells of the slug, chloroplasts with thylakoid membranes and stroma were surrounded by one membrane only, presumably, the phagosome membrane (Martin et al. 2013; see Fig. 1). In the present report, we compare the membranes of chloroplasts of *Thuridilla hopei*, a NR retention form, and *Elysia viridis*, possibly a StR form (Händeler et al. 2009; Fig. 2), with the LtR form *P. ocellatus*, which has led to the hypothesis that chloroplasts devoid of chloroplast envelopes survive in the slug cells.

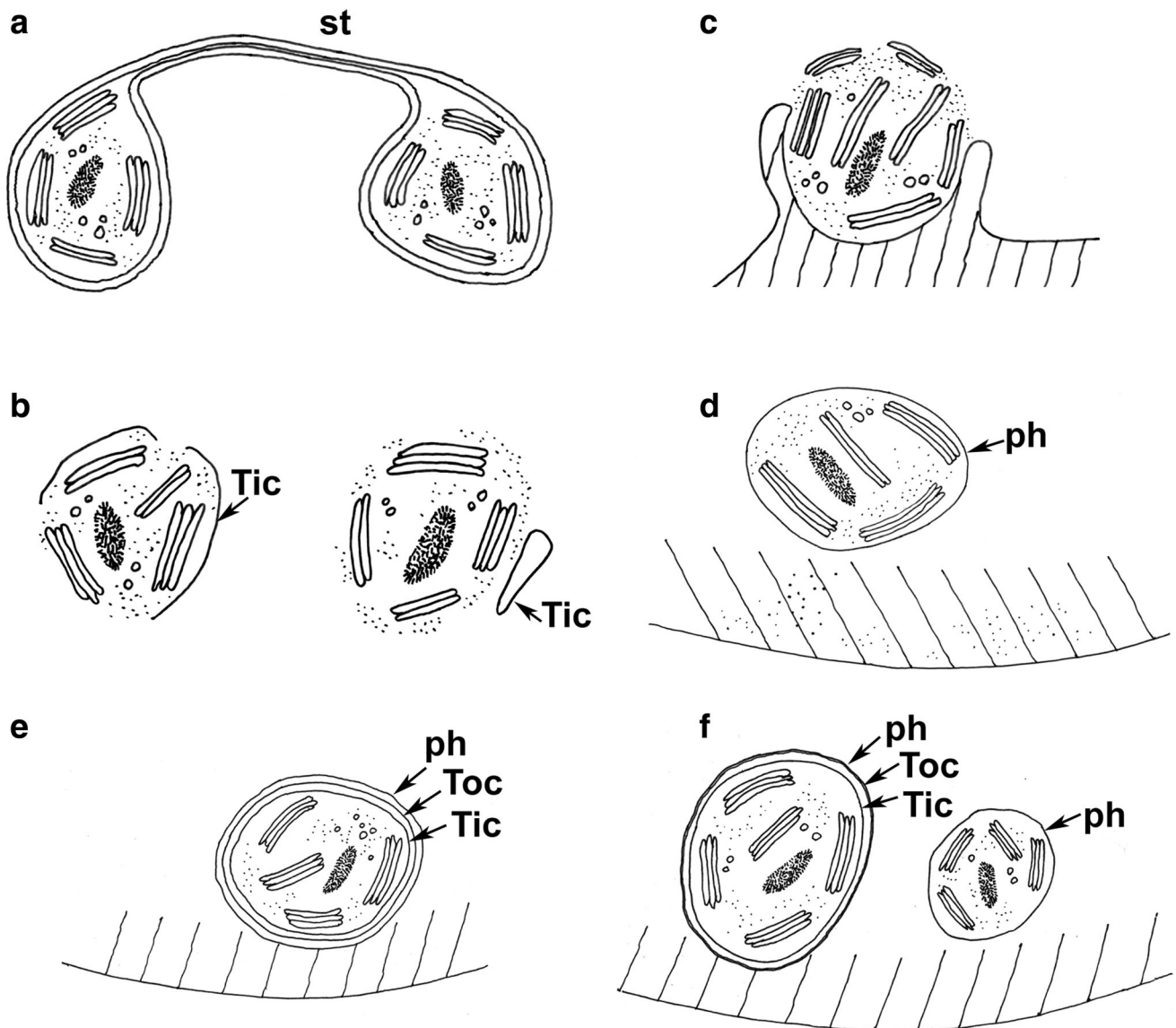


Fig. 1 Schematic drawings of the uptake of algal chloroplasts into digestive cells of the long-term retention slug *Elysia timida*. **a** Interconnected chloroplasts with stromules (*st*) in the alga *Acetabularia* species, **b** plastids in the gut of the sea slug, denuded or with detached Tic-membranes (*Tic*), **c** uptake of a denuded chloroplast by phagocytosis, **d** a kleptoplast with the phagosome membrane (*ph*) in a digestive gland cell of the slug. **e** A chloroplast

with three membranes (*ph*, *Toc*, *Tic*) in a digestive cell of the non-retention form *Thuridilla hopei*. **f** Two chloroplasts in a digestive cell of *Elysia viridis*. The larger plastid with three membranes (*ph*, *Toc*, *Tic*); this chloroplast has been found also in degeneration. The smaller plastid of unknown origin, with one membrane only, has never been found in degeneration

We applied high-pressure cryofixation, a method known to preserve the ultrastructure of membranes particularly well (Walther and Ziegler 2002).

Materials and methods

Specimens of *E. viridis* and of *T. hopei* were collected by scuba diving at the Isola del Giglio, Tyrrhenian coast, Italy. We examined one specimen of *P. ocellatus* from the

Republic of the Philippines, provided by Frank Richter (Meerwasseraquaristik Richter, near Chemnitz, Germany).

Conventional chemical fixation was conducted in the marine biological laboratory at sea. For high-pressure cryofixation, specimens were transported to the University of Ulm, Germany. We examined 15 specimens of *E. viridis*, of which eight were high-pressure cryofixed and seven were chemically fixed. Of *T. hopei*, four slugs were high-pressure cryofixed and four were chemically fixed. The specimen of *P. ocellatus* was high-pressure cryofixed.

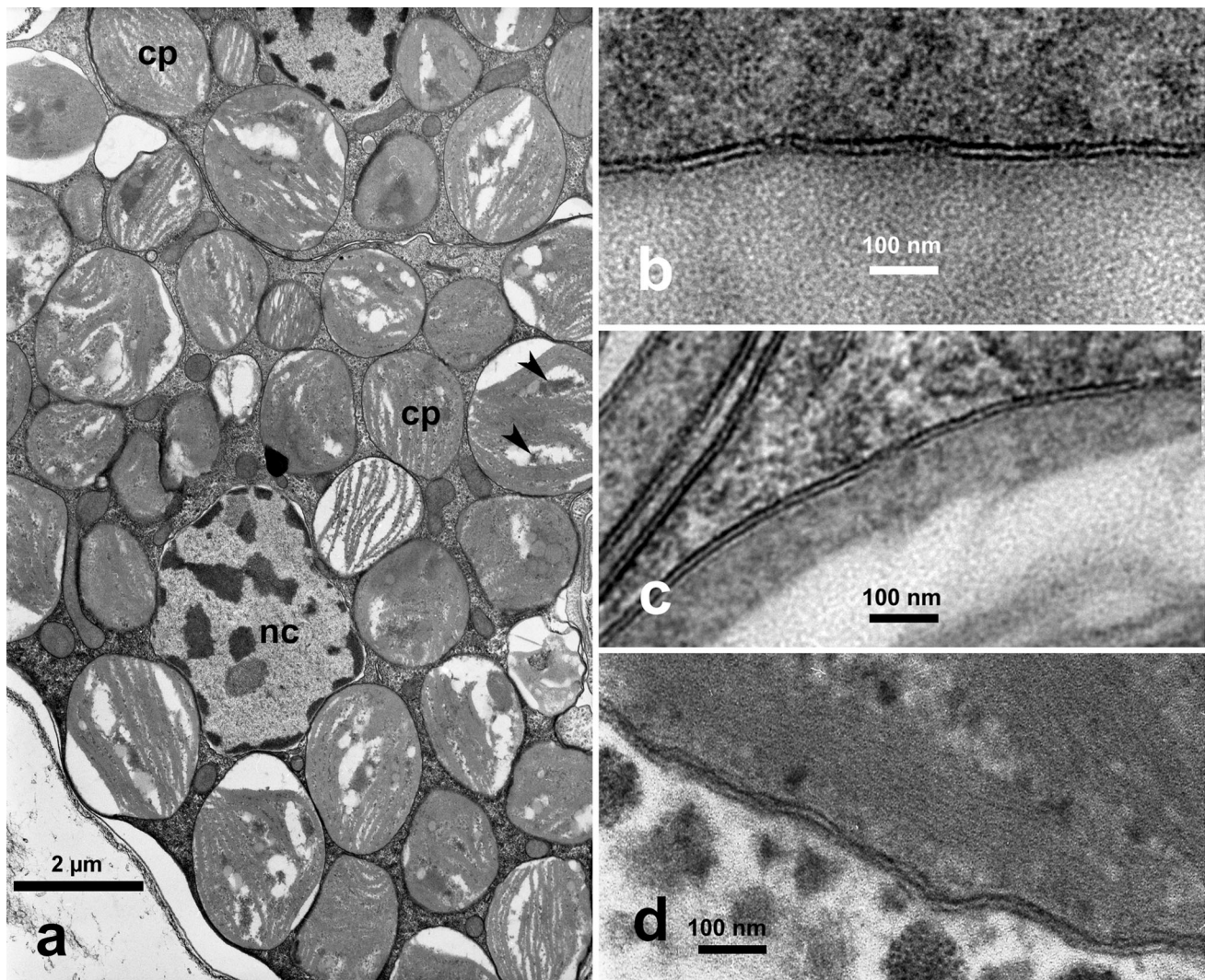


Fig. 2 **a** Kleptoplasts in digestive gland cells of the long-term retention form *Plakobranthus ocellatus*, high-pressure cryofixed, with nuclei (*nc*) and chloroplasts (*cp*) after unknown time of

starvation. The starch granules have been almost completely consumed (*arrowheads*). **b–d** Surfaces of kleptoplasts with the phagosome membrane, showing the membrane bilayer structure

For high-pressure cryofixation, slugs were killed by a cut through the head at the level of head ganglia and pieces of the parapodia were fixed. Parapodia were immersed in hexadecene-filled aluminum disks (bore holes 0.2 mm deep and 3 mm wide). These specimens were frozen under 2000 bar pressure of liquid nitrogen (Müller and Moor 1984) in a Compact 01 high-pressure freezing machine (Engineering Office M. Wohlwend GmbH, Sennwald, Switzerland). Frozen samples were kept in liquid nitrogen. They were then immersed in 2 % OsO₄ and 4 % H₂O in acetone (Walther and Ziegler 2002) at –90 °C in a freeze substitution machine assembled in the Ulm laboratory. The temperature was slowly raised to 0 °C over 19 h, and samples were transferred into a graded series of propylene

oxide/Epon mixtures at room temperature and embedded in Epon. Sections 70 nm thick prepared for transmission electron microscopy were contrasted with lead citrate (0.3 % for 15 s) and examined in a Zeiss EM10 or a Zeiss ‘912’ electron microscope.

For conventional chemical fixation, specimens were immersed for up to one week in fixative at room temperature, freshly prepared daily, consisting of 2 % paraformaldehyde and 2 % glutaraldehyde in filtered seawater, which had been mixed 1:1 with H₂O, containing 0.35 mol l⁻¹ sucrose, 0.17 mol l⁻¹ NaCl and 0.1 mol l⁻¹ Na-cacodylate, pH 7.6; measured osmolality without the aldehydes was 1.4 Osmol. They were dehydrated and embedded in Epon after osmification (2 % for 2 h, at room

temperature) and contrasted in 2 % uranyl acetate. Sections were further contrasted with lead citrate.

Results

The membrane of chloroplasts in digestive gland cells of the LtR form *P. ocellatus*

The cells of this slug were filled with chloroplasts, apparently of one type only (Fig. 2a). Surprisingly, intact starch granules and plastoglobuli were missing. There were small remnants of the granules (arrow heads). We do not know on which algae this specimen had fed and how long it had starved. The plastids, without exception, had one enveloping membrane often directly contacting the stacks of thylakoid membranes with stroma that was, presumably, the phagosome membrane (Fig. 2b–d). There were some whole cells with vacuoles (not shown). These observations indicated digestion of the chloroplasts in cells examined. This and the lack of starch granules support the conclusion that the slug had been starved for quite some time.

The membranes of kleptoplasts in digestive gland cells of the NR form *Thuridilla hopei*

The cells of this slug, when conventionally fixed shortly after collection, displayed many vacuoles containing dark remnants of chloroplasts (Fig. 3a). We infer that the plastids were degenerating by apoptosis, since lysosomes were not detectable (Mondy and Pierce 2003). Several apparently intact chloroplasts with starch granules and/or plastoglobuli, had distinctly more than one membrane (arrows); i.e., the two chloroplast envelopes and the phagosome membrane (Fig. 3b–e).

The plastids in digestive gland cells of *Elysia viridis*

Four of our specimens of this species included two types of chloroplasts: large chloroplasts with distinct thylakoid membranes (I), and small dark chloroplasts (II) (Fig. 4a). Other specimens only showed the small dark plastids. There were also vacuoles. The large plastids possibly stem from *Cladophora* species, since the slugs were feeding on this alga. Some of the large plastids were degenerating (Fig. 4a). The small dark plastids showed one membrane (Fig. 4c, d), while the large plastids usually had one membrane, sometimes there were three membranes (Fig. 4b). The small dark plastids, presumably, were retained long-term, the degenerating large plastids indicated short-term retention.

Discussion

This study is concerned with the question of why algal chloroplasts are stored for weeks in cells of certain sacoglossan slugs while they are not in others. Previously, we found that digestive gland cells of the slug *E. timida*, a LtR form, phagocytosed chloroplasts without chloroplast envelopes, wrapped in the phagosome membrane only, in large numbers without exception (Martin et al. 2013). Extending those observations, we also report the presence of only a phagosome membrane around the plastids in the long-term retention form *P. ocellatus* and in many cells of *E. viridis*.

The uptake of denuded thylakoid stacks with stroma containing prokaryotic ribosomes could be an important factor favoring retention. Whether or not the absence of chloroplast envelopes is a common feature in the long-term retention forms should and could be controlled. It suggests that the import signaling mechanism of enveloping membranes for nucleus-encoded proteins may be absent. Therefore, horizontally transferred algal gene products (Pierce and Curtis 2012), at least in *E. timida*, ‘would not be able to find their way to the inner membranes’ (Pierce et al. 2015). Other factors than absence or presence of chloroplast envelopes appear to be relevant too, since *Elysia cornigera*, having fed on *Acetabularia* species, such as *E. timida*, die when starved due to increased levels of reactive oxygen species (de Vries et al. 2015). Factors influencing the internalization and longevity of chloroplasts might include that plastids without the chloroplast envelope in cells of the LtR forms *E. timida*, and possibly *P. ocellatus*, are not recognized as foreign by the slug cells, and therefore are not digested. The phagosome membrane, which is still present, is an eukaryotic slug membrane. We assume that, on the other hand, the prokaryotic envelope membranes of the chloroplast with exposed recognition molecules, carrier proteins and porins, are recognized being foreign by the slug cell. These molecules on thylakoid membranes are lacking. The denuded chloroplasts with stacks of thylakoid membranes and stroma with ribosomes appeared compact. They were shown to be able to photosynthesize (Händeler et al. 2009; Christa et al. 2014) and therefore must have endogenous functionally active systems operating in autonomous plastids (Green et al. 2000).

To our knowledge in the literature, phagocytosis of chloroplasts has not been shown in any of the LtR forms, with the exception of *E. timida* (Martin et al. 2013). It would be interesting to see what happens to the membranes in *E. chlorotica*, a sacoglossan feeding on the alga *Vaucheria litorea*, with up to 9-month retention during starvation (Mujer et al. 1996). It is reported to take up

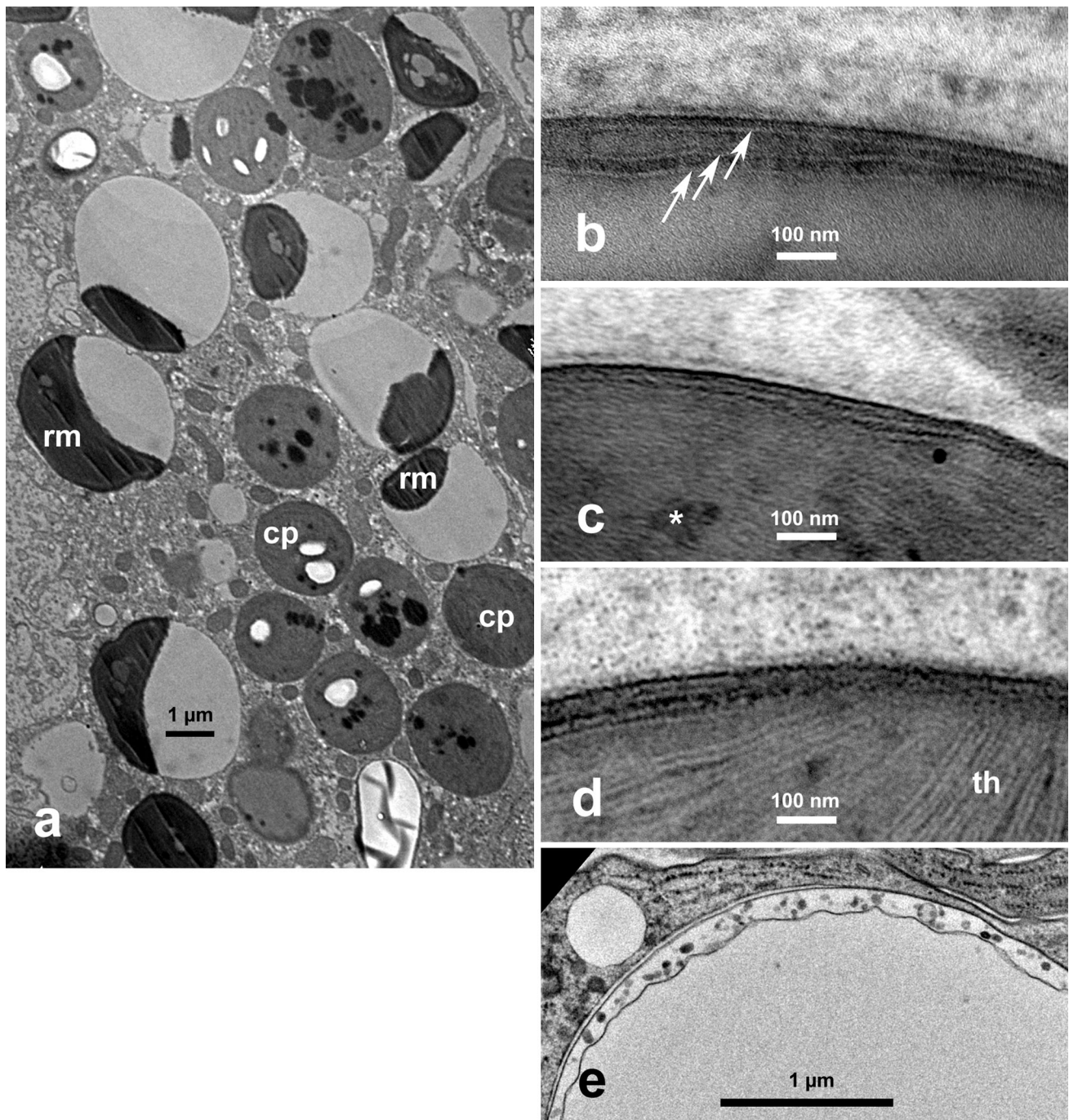


Fig. 3 **a** Kleptoplasts in digestive gland cells of the non-retention form *Thuridilla hopei*, fixed conventionally, shortly after collection. There are large vacuoles with apoptotic remnants of chloroplasts (*rm*) and, apparently, intact chloroplasts with plastoglobuli and starch

chloroplasts with two membranes (Rumpho et al. 2001). However, in a previous work a chloroplast in the slug cell is shown at large magnification with only one membrane (Rumpho et al. 2000; Fig. 2b).

As postulated by our hypothesis, the NR form *T. hopei*, in which the chloroplasts are digested within days, had two envelope membranes, as well as a phagosome membrane.

grains (*cp*). **b–d** Kleptoplasts show the external phagosome membrane and two internal chloroplast membranes (*arrows*), surrounding thylakoid stacks (*th*) and stroma (*asterisk*). **e** A vacuole with three remaining membranes. **b–e** High-pressure cryofixation

It is not clear whether *E. viridis* is a StR or a LtR form (Händeler et al. 2009). This may depend on which algae the slugs were feeding. Some kleptoplasts in these slugs were found to be degenerating, while functional chloroplasts were retained after 40 days of starvation, 73 days, and even up to 3 months (Hawes and Cobb 1980; Evertsen and Johnsen 2009; Hinde and Smith 1972; respectively). In our

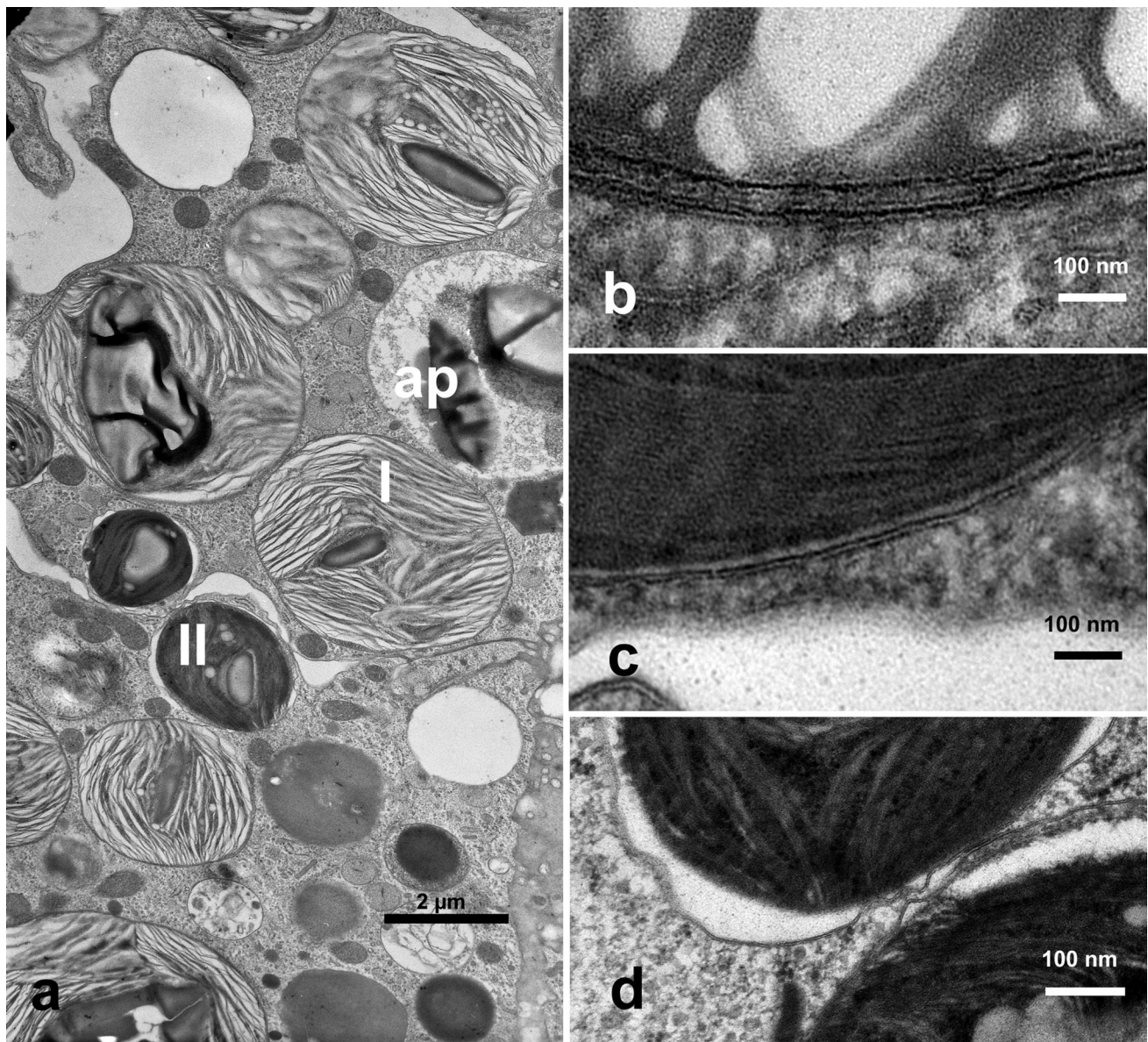


Fig. 4 **a** A digestive gland cell with two different types of chloroplasts (I, II) in *Elysia viridis*. Two of the large plastids, presumably of a *Cladophora* species, exhibited apoptotic

degeneration (*ap*). **b** High-power TEM of type I plastid with three membranes. **c**, **d** Type II plastids with the phagosome membrane only. High-pressure cryofixation

specimens, a majority of smaller chloroplasts had only one, while some of the larger plastids had three membranes. The latter, presumably derived from *Cladophora* species, were degenerating. *E. viridis*, when feeding on *Cladophora* species may be a StR form. Also Trench et al. (1973, Fig. 7) showed a chloroplast with two membranes in *E. viridis* fed with *Codium fragile*. However, another investigation reported retention not to be dependent of the sequestered algae species (Christa et al. 2013).

Nonetheless, algae with stromules might be a factor. A network of tubules formed by inner and outer chloroplast membranes in some algae, interconnected the plastids (Menzel 1994; Bourett et al. 1999; Gray et al. 2001;

Natesan et al. 2005) (Fig. 1a). This network at least is partially destroyed by mastication.

Are our results compatible with data in the literature? A comprehensive list of published ultrastructural investigations regarding membranes of incorporated plastids were reported in Table 1 of Wägele and Martin (2014). In this list, only plastids with two or three membranes were reported. This is in contrast to the plastids in the LTR forms *E. timida* (Martin et al. 2013) and *P. ocellatus*, where we always found one presumptive phagosome membrane only.

There may have been misinterpretations of published results. For example Hawes and Cobb (1980) in *E. viridis* (Plate 1, Fig. 2 and Plate 4, Fig. 2) and Hirose (2005) in

P. ocellatus (Fig. 27) showed plastids with one membrane only. Also, the high-power TEM image in Fig. 4b of Wägele and Martin (2014) with the bilayer structure of a single membrane around a chloroplast at a nuclear pore was wrongly interpreted as two membranes.

The single membrane—presumably the phagosome membrane—surrounding kleptoplasts in *E. timida*, in *P. ocellatus*, and, partially, in *E. viridis*, indicates that the outer (Toc) and inner (Tic) envelopes of chloroplasts containing the protein import mechanisms are absent. Moreover, “one can firmly conclude that no genes are transferred to [the nucleus of] those two species” (i.e., *E. timida* and *P. ocellatus*). Therefore, it can be concluded that “the slugs clearly have no need to support their photosynthetic plastids, that they just sequester long-lived plastids” (Martin et al. 2012). All considered, these observations signify that membrane-denuded algal chloroplasts may be more autonomous than chloroplasts of land plants. “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” (de Vries et al. 2014).

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