SYMBIOSIS (2005) 40, 31–40 ISSN 0334-5114

Stability of isolated algal chloroplasts that participate in a unique mollusc/kleptoplast association

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(Received June 24, 2005; Accepted August 19, 2005)

Abstract
The mollusc *Elysia chlorotica* Gould has acquired the ability to sustain itself photoautotrophically by forming a "symbiotic" or kleptoplastic association with chloroplasts of the heterokont alga *Vaucheria litorea* C. Agardh. The captured chloroplasts (kleptoplasts) remain intact and functional in direct contact with the animal cytosol for up to ten months with no detectable remnant of the algal nucleo-cytosol present. To determine if *V. litorea* chloroplasts display unusual stability or "robustness," isolated plastids were characterized over several days. Structural integrity was physically evaluated by phase-contrast microscopy using Image J software analysis and chemically, by measuring ferricyanide-dependent oxygen evolution. Light-dependent fixation of $^{14}CO_2$ and *in vitro* translation of chloroplast proteins were used to measure stroma-dependent functional stability. Spinach chloroplasts were used for comparison. It is concluded that *V. litorea* chloroplasts are more "robust" than typical land plant chloroplasts and this robustness may facilitate survival during incorporation of the plastids into the animal cytosol and contribute to their long-term functioning in the foreign cytosol.

Keywords: Chloroplast stability, *Elysia chlorotica*, heterokont algae, kleptoplasts, mollusc, symbiosis, *Vaucheria litorea*

1. Introduction

The possibility of maintaining isolated chloroplasts in a functional state for extended periods of time in an artificial medium or foreign cell type has intrigued scientists for many years, albeit with little long-term experimental success. Many of the earliest experiments were stimulated by the desire to characterize the autonomy of chloroplasts following some of the first reports of *in vitro* chloroplast activity (Arnon et al., 1954; Whatley et al., 1959; reviewed by Walker, 2003) and the observations that chloroplasts of some marine algae survive in the cytosol of certain marine gastropods (Kawaguti and Yamasu, 1965; Trench et al., 1969; Trench and Gooday, 1973; Trench, 1975). These observations stimulated attempts to sustain chloroplasts in artificial media (Ridley and Leech, 1970), introducing chloroplasts into phagocytic mouse fibroblasts (Nass, 1969) and hens' eggs (Giles and Sarafis, 1971), and fusing chloroplasts with land plant protoplasts (Potrykus, 1973; Bonnett and Eriksson, 1974; Bonnett, 1976; Fowke et al., 1979) and fungal protoplasts (Vasil and Giles, 1975).

One of the most remarkable displays of long-term isolated chloroplast activity occurs in a marine gastropod and algal chloroplast kleptoplastic association. In this case, the sea slug *Elysia chlorotica* feeds on coenocytic filaments of the heterokont alga, *Vaucheria litorea*, ingesting the chloroplasts and maintaining them in a functional state for up to ten months, the normal life-cycle of the mollusc (Pierce et al., 1996; Rumpho et al., 2000; 2001). This kleptoplastic association differs from other more commonly observed true symbioses in that the mollusc only captures part of *V. litorea*, the chloroplasts. The stolen organelles are maintained intracellularly in direct contact with the animal cytosol (Fig. 1; Mujer et al., 1996; Rumpho et al., 2000). Modern biochemical and molecular analysis has confirmed that chloroplasts are not genetically or biochemically autonomous (Givan and Leech, 1971; reviewed by

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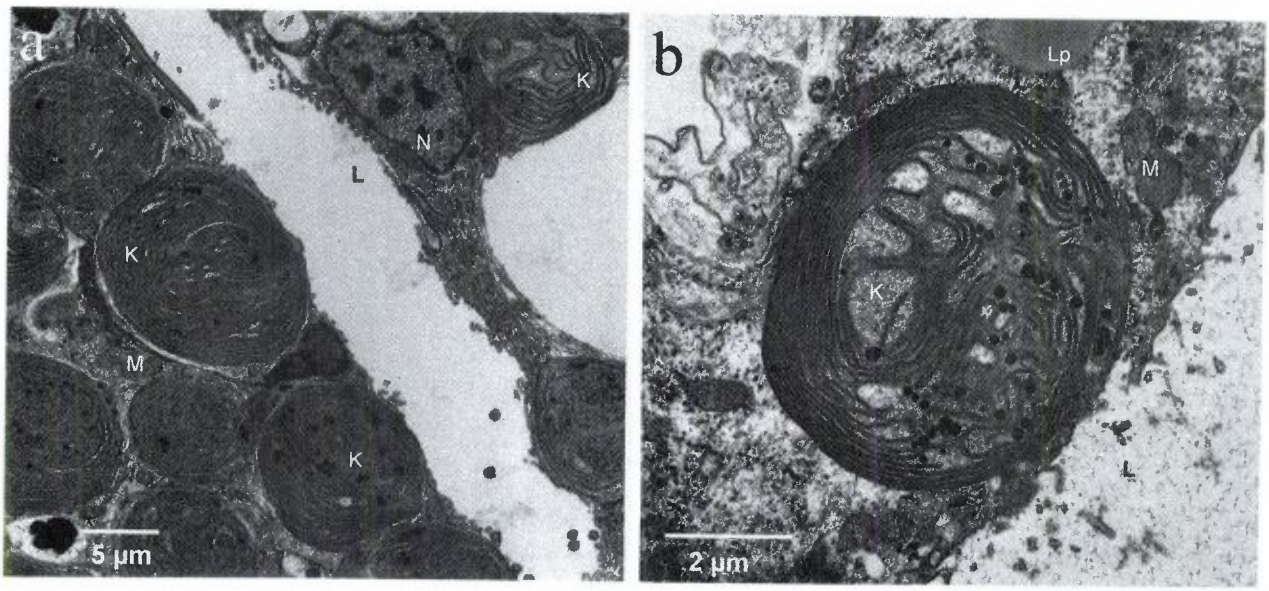

Figure 1. Electron micrographs of kleptoplasts in *E. chlorotica* immediately after uptake into cells lining the digestive tubules. Panels a and b illustrate low and high magnification, respectively, of kleptoplasts adjacent to the lumen of the tubules and apparently in the process of uptake. Numerous darkly-stained plastoglobuli are seen in the freshly acquired kleptoplasts, as well as several typical mollusc mitochondria, a mollusc nucleus, and lipid deposits. No chloroplast endoplasmic reticulum or encapsulating membranes are seen surrounding the kleptoplasts. K, kleptoplast; Lp, lipid deposit; L, lumen of digestive tubule; M, mitochondria; N, nucleus.

Rahgavendra, 1998; Martin et al., 2002; NCBI genome database http://www.ncbi.nlm.nih.gov/genomes/static/euk_o.html). Hence, the observation that certain chloroplasts remain structurally stable and functionally active for months in a foreign animal cell is that much more unusual and perplexing.

Vaucheria was selected in early chloroplast/protoplast fusion studies as a source of plastids because of the small size of the chloroplasts, the unique thylakoid ultrastructure which allowed the fused plastids to be distinguished from other chloroplast types (Bonnett, 1976), and the ability of the plastids to withstand manipulation (Bonnett and Eriksson, 1974). However, the authors did not elaborate or cite any experimental data to explain why they considered the plastids to be "hardy." Chloroplasts from other siphonaceous algae, e.g., the green algae *Codium* (Trench et al., 1973a,b) and *Caulerpa* (Giles and Sarafis, 1971), are also found in kleptoplastic associations with gastropods (see list in Williams and Walker, 1999). Trench et al. (1973a) hypothesized that plastids of siphonaceous algae possess unique properties that make them particularly amenable to uptake and survival by gastropods. Giles and Sarafis (1974) reported that isolated *Caulerpa* plastids appear intact by phase-contrast microscopy even after homogenizing in a blender, freeze/thaw treatment or sonication. It was necessary to use a French pressure cell to break them. This stability was attributed to the presence of a lysozyme-resistant "rigescent integument" surrounding the chloroplasts. It should be noted, however, that these observations were not supported by any biochemical (functional) data and, to our knowledge, no additional reports of such membranes have been reported in the literature.

It is well known that chloroplasts have some ability to adapt to changing cellular environmental conditions (Kaiser et al., 1981), but widely varying conditions will adversely and irreversibly affect their structure and function (Ridley and Leech, 1968). These variables may include: changes in osmotic pressure, light fluence, salt concentrations, metabolites, and pH, to name only a few (Mattoo et al., 1989; Mayfield et al., 1995). Plastids isolated from land plants, such as wheat, tobacco and spinach, remain structurally intact for only a few hours in a test-tube and photosynthetic activity is typically lost within 30 min to half a day of light exposure (Leegood and Walker, 1983; Seftor and Jensen, 1986; Polanska et al., 2004).

To determine if *V. litorea* chloroplasts display unusual stability, isolated algal plastids were characterized over a several day time-course. Phase-contrast microscopy (Walker, et al., 1987) and ferricyanide (FeCN)-dependent O_2 evolution (Lilley et al., 1975) were used to estimate structural intactness of the plastids. Incorporation of $^{14}CO_2$ and *in vitro* translation of chloroplast mRNA were used to assess functional stability (Huber et al., 1977). The data presented here contribute to a further understanding of not only this unique endosymbiosis, but to heterokont chloroplast biology in general.

2. Materials and Methods

Electron microscopy

Sea slugs were starved (deprived of any algal food source) for four months in culture and then allowed to feed on *V. litorea* filaments for 10 d, in an attempt to observe chloroplast uptake by electron microscopy. Prior to fixation, the sea slugs were relaxed in 3.4% $MgCl_2$, then immersed in fixative (2% glutaraldehyde in 0.15 M sodium cacodylate buffer pH 7.3 containing 0.58 M sucrose), sectioned, and incubated for 2 h on ice. Postfixation (2 h on ice) was in 1% osmium tetroxide in cacodylate buffer with sucrose, followed by in-block staining with 2% aqueous uranyl acetate, and dehydration in a series of acetone solutions. Samples were embedded in Epon/araldite and ultrathin sections stained with Reynolds lead citrate for 10 min and viewed with a Philips CM10 transmission electron microscope (Mujer et al., 1996).

Algal culture and chloroplast isolation

Vaucheria litorea C. Agardh was maintained in culture in enriched quarter-strength artificial sea water (ASW) (250 mosmol kg^{-1}) and a modified f/2 medium as described previously (Pierce et al., 1996), except that the cultures were illuminated by natural lighting and aeration was limited to daily manual swirling.

For algal chloroplast isolation, 1 to 4 g (wet mass) of algal filaments were diced with a razor blade in a minimal volume of isolation buffer (50 mM Hepes-KOH, 1 mM $MgCl_2$, 1 mM EDTA, 0.2% bovine serum albumin (BSA) (w/v), pH 7.6 and either 330 mM or 400 mM sorbitol), which had been chilled to a slush. Homogenization was carried out in 50 ml of the same isolation buffer using a Polytron (Brinkmann Instruments) and applying three short bursts of 2 to 4 s at speed 6. The homogenate volume was adjusted to 80 ml with isolation buffer, filtered through six layers of cheesecloth, and centrifuged at 1,930 g for 5 min at 4°C in a Beckman JA-17 fixed angle rotor with the brake off. The pellet was gently resuspended in 5 ml of isolation buffer and filtered twice through a 110 µm nylon net immediately prior to loading 1 to 2 ml of sample onto a Percoll step-gradient (30%/75%).

The samples were centrifuged at 8,000 g for 20 min at 4°C in a Beckman JA-13.1 swinging bucket rotor with the brake off. Intact plastids were collected from a band that formed near the 30%/75% interface and other bands were discarded after visually determining that they contained broken plastids and debris.

Purified chloroplasts were washed twice and resuspended to a concentration of about 5 µg chl μl^{-1} in isolation buffer minus BSA, aliquoted (25 µl $tube^{-1}$), and incubated at 4°C in the dark (unless indicated otherwise). An individual tube was gently agitated and an aliquot from a new tube analyzed for each time-point. Chlorophyll a and c concentrations were measured spectrophotometrically in 90% acetone extracts according to Sterman (1988).

Spinach chloroplasts were Percoll-purified according to Rumpho and Edwards (1984) and resuspended (5 µg chl μl^{-1}) in isolation medium containing: 330 mM sorbitol, 10 mM EDTA, 0.2% BSA, and 50 mM Hepes (pH 7.8). The concentration of chlorophylls a and b were measured spectrophotometrically in 96% ethanol extracts according to Wintermans and De Mots (1965).

Phase-contrast microscopy

Chloroplasts were viewed with phase-contrast optics and a 40X objective. Twenty images, containing between 25 and 130 plastids per image, were captured per slide and analyzed using the NIH imaging software program Image J (NIH freeware). Image J permitted chloroplast membrane 'halos' to be resolved from background based on the pixel intensities within the image. This allowed for comparison of lysed versus intact chloroplasts by establishing a range of intensities that could be used to differentiate a structurally intact chloroplast from those that were broken. Intact chloroplasts exhibited a bright halo and were highly refractive, whereas, broken chloroplasts exhibited a dull halo, were non-refractive, and had a much darker granal interior. Using the determined pixel range intensities, each of the twenty images per time-point were analyzed for percent intactness and averaged.

Ferricyanide-dependent O_2 evolution intactness assays

Chloroplast intactness was also estimated by measuring FeCN-dependent O_2 evolution with a Hansatech DW1 oxygen electrode in a final reaction volume of 1 ml at 18°C (Lilley et al., 1975; Rumpho and Edwards, 1984). Illumination was provided with a Hansatech LS2 light source providing 800 µmol photons m^{-2} s^{-1}. Each reaction was run in the appropriate assay buffer with the sorbitol concentration adjusted to 330 mM or 400 mM in 50 mM Hepes-KOH, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 3 mM $K_3Fe(CN)_6$ and 20 mM D,L-glyceraldehyde, at pH 7.6. NH_4Cl (20 mM) was added to uncouple the reaction, where indicated. Chlorophyll concentration used was 80 µg for algal plastids and 35 µg for spinach chloroplasts.

Photosynthetic incorporation of $^{14}CO_2$

Isolated algal chloroplasts (80 µg chl) were incubated in 1 ml isolation buffer containing 50 µCi $NaH^{14}CO_3$ (SR = 0.1 mCi/mmol) at 25°C under 500 µmol photons m^{-2} s^{-1} photosynthetic photon flux density (PPFD) for 10 min according to Huber et al. (1977). At the completion of labeling, the plastids were separated from the external medium by centrifugation at 1,000 g for 5 min. Pelleted plastids were washed with isolation buffer and centrifuged two times prior to quantification of incorporated acid-stable

^{14}C by liquid scintillation spectroscopy. Appropriate dark controls showed absence of incorporation of $^{14}CO_2$ in all cases.

In vitro *translation and gel analysis*

Chloroplast *in vitro* translation reactions were carried out in a reaction mix that contained 50 μCi [^{35}S]methionine, 0.5 mM amino acids minus methionine, and chloroplasts (80 μg chl) under 800 μmol photons m^{-2} s^{-1} or in the dark (control), at room temperature. After labeling for 20 min, chloroplasts were pelleted by centrifugation at 1,000 g for 5 min at 4°C and washed twice with the appropriate isolation buffer (described above). Chloroplast thylakoid- and stromal-enriched fractions were isolated by resuspending the pellet in hypotonic lysis buffer (10 mM Hepes-KOH, 10 mM $MgCl_2$ at pH 8) for 5 min on ice (Green et al., 2000). The lysed samples were centrifuged at 10,000 g for 10 min at 4°C. The resulting supernatant containing the stromal fraction was stored on ice prior to analysis. The remaining thylakoid-enriched pellet was washed twice with isolation buffer and resuspended in SDS-PAGE sample buffer (Laemmli, 1970). Polypeptides were separated by SDS-PAGE employing a 9 to 18% (w/v) linear polyacrylamide gradient. Samples were loaded on an equal radioactivity (100,000 dpm) or equal volume (volume corresponding to 100,000 dpm for the 0 h time sample) basis. Gels were incubated for at least 1 h in a solution of ethanol, methanol, glycerol and acetic acid (20:20:10:7), before transferring to a fluorography solution (0.4% 2,5-diphenyloxazole, 30% xylene, 9.5 N acetic acid and 15% ethanol) for 1 h. Gels were washed twice with deionized water, vacuum-dried on Whatman filter paper, and exposed to X-ray film (Fuji) at −80°C for 7 to 10 d (Mujer et al., 1996).

3. Results

Incubation conditions

Early experimentation on isolated *V. litorea* chloroplasts demonstrated that the organelles were fairly insensitive to a wide-range of media conditions. The following incubation conditions were found to be most favorable for sustaining intactness based on phase-contrast observations: final osmotic concentration between 250 and 400 mM, isolation in the presence of BSA (0.1 to 0.5% w/v), addition of $MgCl_2$ not to exceed 1 mM (reduces chloroplast clumping), a dense chlorophyll concentration (at least 5 μg chl μl^{-1}), and incubation in the dark at 4°C with no agitation. A representative experiment employing the above conditions (380 mM osmoticum) revealed that at least 40% of the isolated plastids appeared physically intact even after 14 d incubation in the dark vs. about 30%, if stored in the light (Fig. 2).

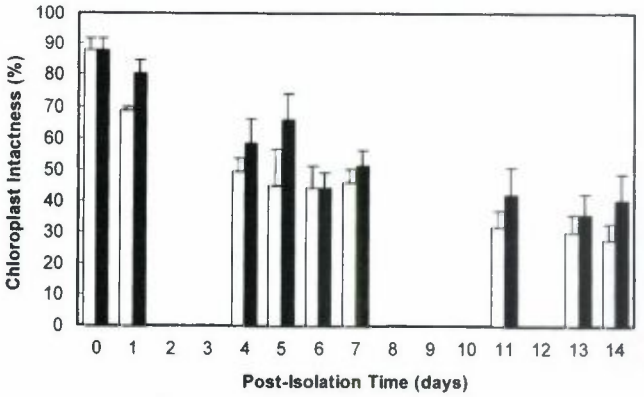

Figure 2. Intactness levels of isolated *V. litorea* chloroplasts estimated by phase-contrast microscopy over a 14 day time-course. Isolated chloroplasts were incubated in 380 mM total osmoticum in the light (open bars) or the dark (solid bars) and six independent samples counted. The averaged values for each time-point are shown +/-standard error of the mean.

Intactness estimates by phase-contrast microscopy

Isolated algal chloroplasts typically exhibited greater than 85% intactness at time zero as judged by phase-contrast microscopy (Fig. 3A and Fig. 4). Intact chloroplasts appeared to be surrounded by bright halos, whereas, broken chloroplasts exhibited halos which were less bright or absent altogether and their interior was much darker (Fig. 3B). At 24 h algal plastid intactness was about 75%, 48 h after isolation intactness exceeded 65%, and even after 72 h, algal plastids were still at least 52% intact (Fig. 4). No difference was noted for chloroplasts isolated and stored at 450 vs. 380 mM total osmoticum (Fig. 4). Spinach chloroplast intactness was compared at the same time and after an initial 90% intactness immediately after isolation, the value declined to only 25% intact after just 24 h (Figs. 3C and D, and Fig. 4).

Intactness estimates by FeCN-dependent O_2 evolution

Chloroplast intactness was also evaluated by measuring FeCN-dependent electron transport activity coupled to oxygen evolution in "intact" versus hypotonically lysed chloroplasts (Lilley et al., 1975). Algal chloroplasts isolated and incubated in isotonic media (380 mM) were 87% intact at 0 h and remained high at 73% and 62% intact at 24 and 48 h, respectively (Fig. 5). Intactness levels declined to 22% at 72 h. Likewise, algal chloroplasts isolated and maintained in hypertonic media (450 mM) exhibited an initial intactness of 90% and remained high at 86% and 66% intactness at 24 and 48 h, respectively, before declining to 25% intactness at 72 h. Spinach controls exhibited an initial chloroplast intactness of 97% before declining to 25% within 24 h (Fig. 5).

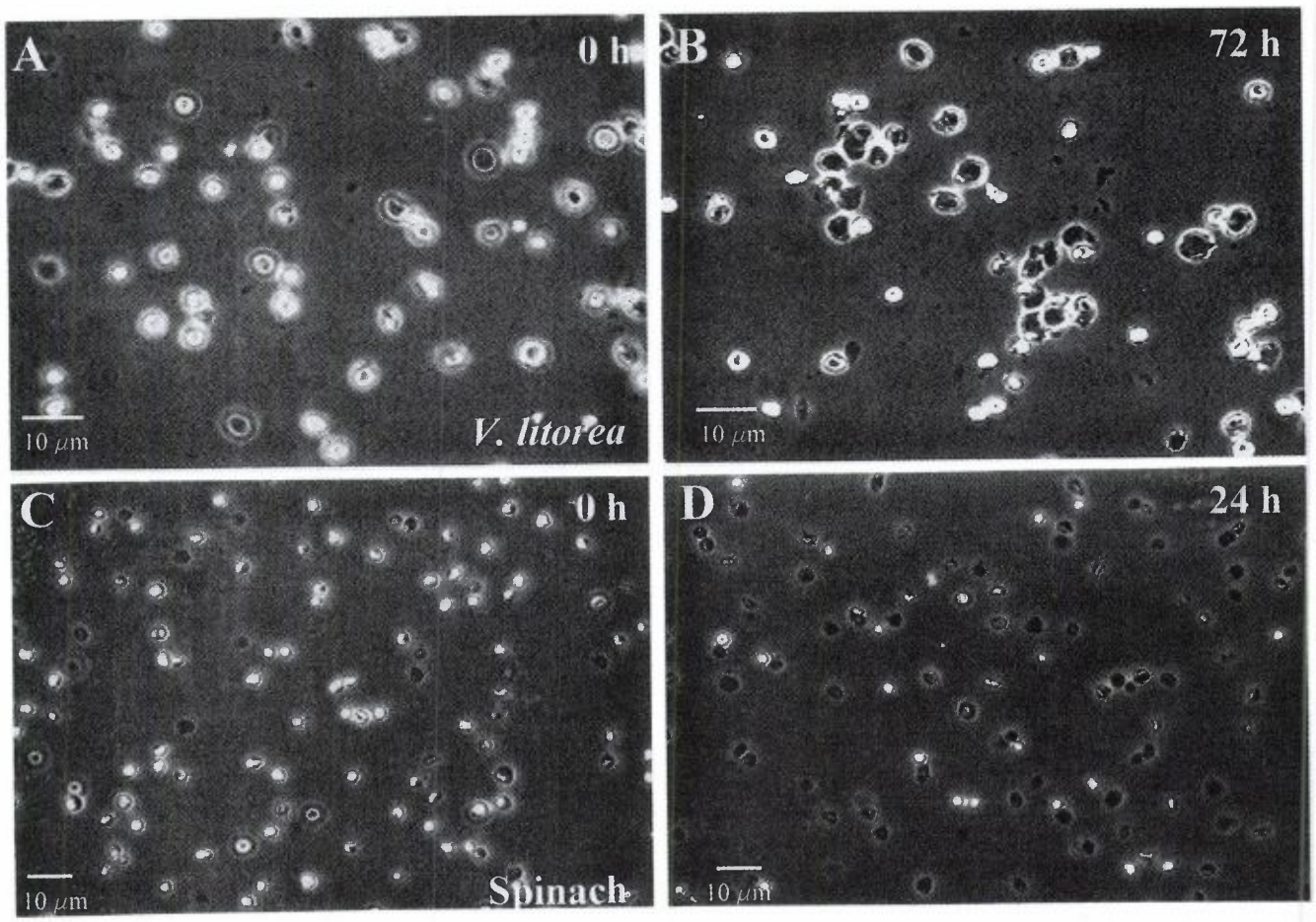

Figure 3. Phase-contrast microscopy images of *V. litorea* chloroplasts (isolated in 380 mM osmoticum) at 0 and 72 h post-isolation (panels A and B) and spinach chloroplasts at 0 and 24 h post-isolation (panels C and D). Structural integrity of the plastids was determined by measuring the pixel intensities of the refractive "halos" using NIH Image J software.

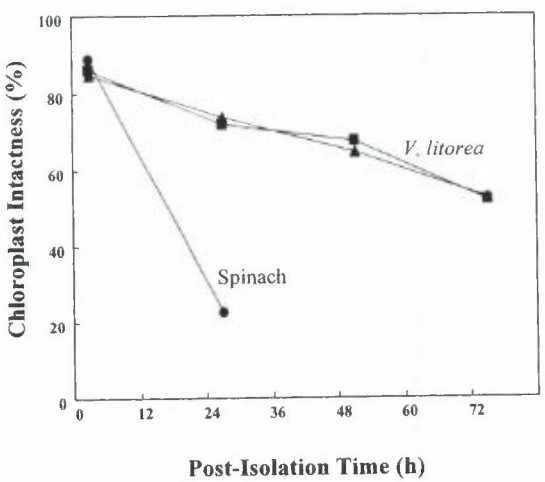

Figure 4. Percent plastid intactness during a 3-day post-isolation period based on phase-contrast microscopy and NIH Image J software analysis. For each time point, 20 images containing between 25 and 130 plastids per image were analyzed and an average percent intactness calculated. Values from a representative experiment are shown here; absolute values varied slightly between experiments, but the overall trends were the same. Plastids were isolated from spinach (●) and *V. litorea* in 450 mM (▲) or 380 mM (■) total osmoticum.

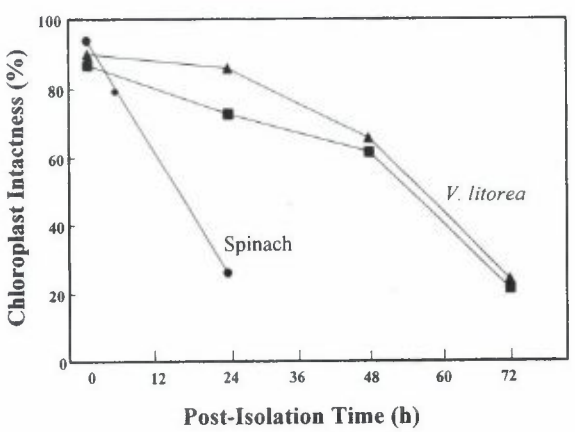

Figure 5. Plastid intactness based on FeCN-dependent O_2 evolution measurements. Percentage of *V. litorea* chloroplasts remaining intact over 72 h after isolation in hypertonic (450 mM) (▲) or isotonic (380 mM) (■) media, and control spinach chloroplasts (●). Each time point represents an average of at least three measurements from a representative experiment. Absolute values varied slightly between experiments, but the overall trends were the same.

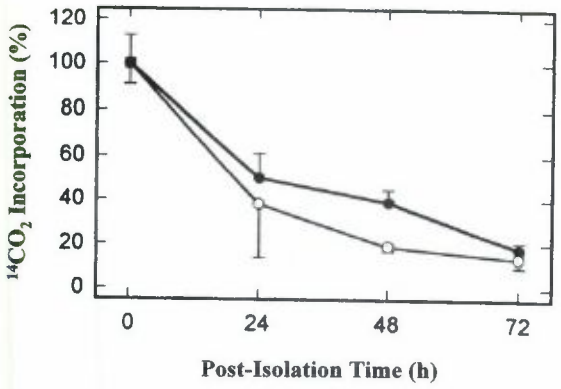

Figure 6. Incorporation of $^{14}CO_2$ by *V. litorea* chloroplasts isolated in 450 mM (●) or 380 mM (○) osmoticum and labeled at 0, 24, 48 and 72 h post-isolation time. Percentages (+/- standard error of the mean) are relative to the control (108 μmol mg^{-1} chl h^{-1}), assigned a value of 100% at time zero.

Incorporation of $^{14}CO_2$

It has been observed that chloroplast envelopes frequently break, reseal, and appear intact, but in fact, they have lost their stromal contents. Thus, a biochemical analysis of chloroplast functionality, dependent upon retention of soluble stromal contents was carried out. The ability of isolated algal plastids to incorporate $^{14}CO_2$ several hours post-isolation was evidenced by incorporation rates as high as 50, 40 and 20% of the control value (108 μmol CO_2 mg^{-1} chl h^{-1} at 0 h) at 24, 48 and 72 h post-isolation, respectively (Fig. 6). Again, the differing osmotic conditions did not significantly affect incorporation rates.

Chloroplast translational ability

The pattern and intensity of proteins translated by isolated algal chloroplasts incubated with [^{35}S]methionine were the same for the two osmotic concentrations (data not shown) and discussed together here. *De novo* synthesis of algal thylakoid polypeptides exhibited only minor changes in banding patterns and intensities over the 3-day time-course. This was true whether the extracts were analyzed on an equal radioactivity (Fig. 7A) or equal volume (Fig. 7C) basis. The photosystem II protein D1 (32 kD) was identified through comparisons of Western-blotting banding patterns (Green et al., 2000) and was found to be translated at all time points, but with less efficiency at 72 h post-isolation (see * in Figs. 7A and C). Similarly, the patterns of *de novo* synthesized stromal proteins (analyzed on either an equal dpm or equal volume basis by SDS-PAGE fluorography) exhibited no major qualitative changes over the 3-day time period (Figs. 7B and D). Both the large (55 kD) and small (12 kD) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), identified through previous Western-blot experiments (Green et al., 2000 and Green, 2001), continued to be translated at 72 h post-isolation (see *s in Figs. 7B and D). Dark controls were performed for all samples and no translation products were revealed (data not shown).

4. Discussion

The observation that *V. litorea* chloroplasts survive the uptake phase and remain functional for months in the foreign cytosol of the marine gastropod *E. chlorotica* suggests that the plastids may possess an unusual structural integrity as well as unusual biochemical and genetic autonomy. As shown here, chloroplasts isolated from the alga and maintained in the dark at 4°C in a simple medium containing sorbitol as the osmoticum, remain structurally intact and functionally capable of CO_2 incorporation and protein translation for at least three days. Incubation in the two different osmotic concentrations, 450 mM vs. 380 mM, did not affect algal plastid structure or function over the time-course. Control spinach chloroplasts were very unstable, both structurally and functionally, in comparison to the algal plastids.

The data obtained by phase-contrast microscopy agreed well with the FeCN-dependent O_2 evolution intactness measurements for isolated *V. litorea* chloroplasts through 48 h. As discussed above, incubation in isotonic versus hypertonic osmotic conditions did not affect overall intactness levels as estimated biochemically. Although a greater number of osmotic concentrations would need to be tested to determine the osmotic range that could be tolerated by isolated *V. litorea* chloroplasts, it is clear that they can withstand at least a 70 mM change in their external environment. This might seem surprising since changes in osmotic concentrations often have serious adverse effects on the ability of isolated plant plastids to maintain structural integrity and photosynthetic capacity (Kaiser et al., 1981). However, *V. litorea* is naturally found in brackish water of widely varying osmotic concentration. This alga can be successfully cultured at osmotic concentrations varying from quarter-strength to full-strength sea water and transferred between the two concentrations with little to no adaptation necessary (unpublished observations).

One difference in phase-contrast microscopy estimates of intactness versus FeCN-dependent O_2 evolution estimates was observed in algal plastids at 72 h. Phase-contrast revealed an algal chloroplast intactness of ~50%, exceeding the estimate of ~25% obtained by the functional FeCN assay. This difference may be due to "leaky" plastids. Within the algal filaments, chloroplast endoplasmic reticulum (ctER) is associated with the *V. litorea* chloroplasts (Mujer et al., 1996; Rumpho et al., 2000; 2001), but the ctER is lost upon uptake of the plastids by the mollusc (Fig. 1) and upon isolation of the plastids from the algal filaments (Rumpho et al., 2001). The association of ctER with chloroplasts is typical of the heterokont algae and reflects their secondary endosymbiotic origin (Ishida et

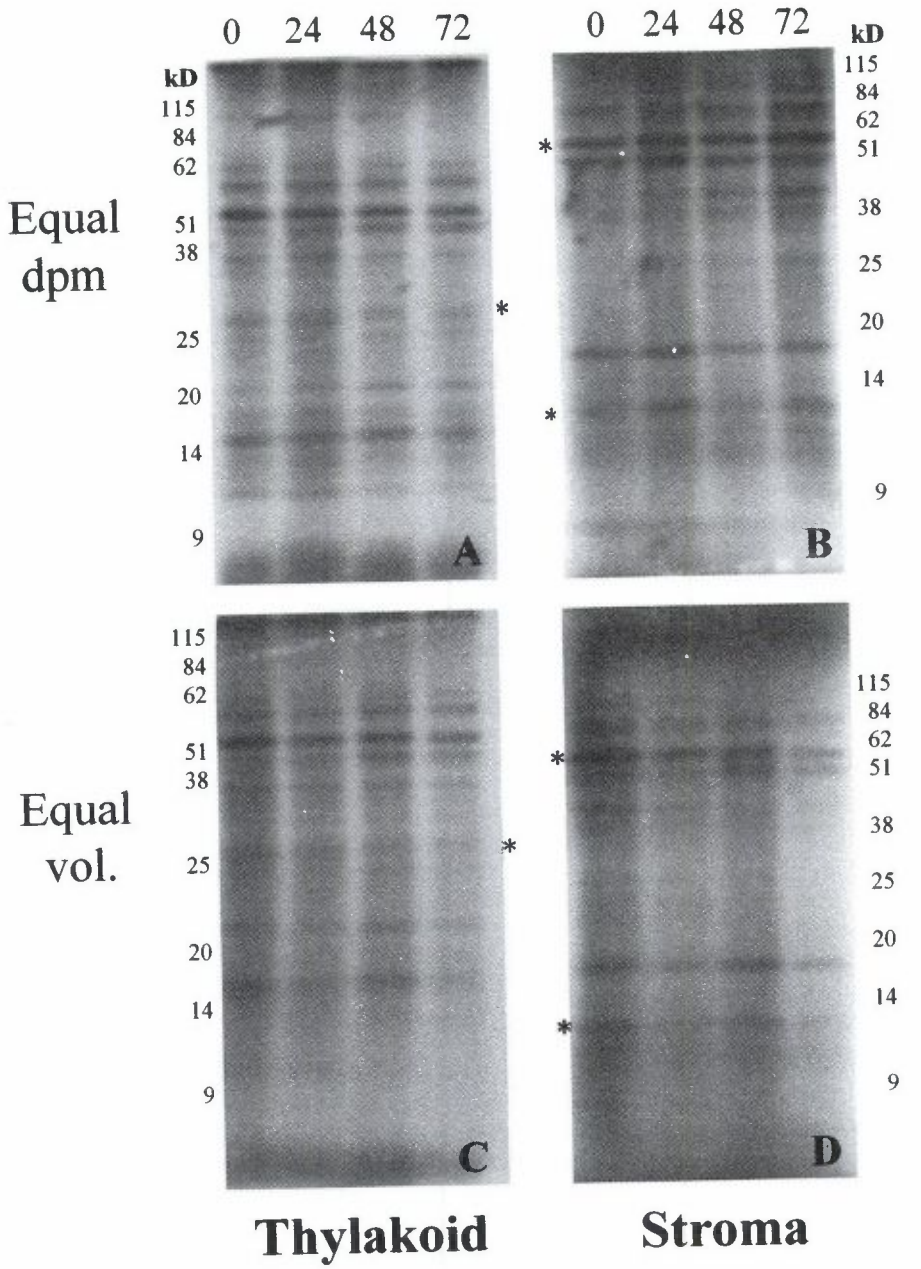

Figure 7. *In vitro* translation of thylakoid and stromal proteins by *V. litorea* chloroplasts labeled with 35S-methionine at 0, 24, 48 and 72 h post-isolation time. Chloroplasts were isolated and labeled in 380 mM osmoticum. Extracted proteins were loaded on an equal radioactivity basis (A and B) (100,000 dpm lane−1) or an equal volume basis (volumes equal to 100,000 dpm for the zero hour sample) (C and D). Sizes of molecular weight markers are shown alongside each fluorogram. The * in panels A and C indicates the 32 kD photosystem II D1 protein and in panels B and D the *s denote the large (55 kD) and small (12 kD) subunits of Rubisco.

al., 2000; Chaal et al., 2003). Depending on the degree of attachment of the ctER, it is conceivable that holes develop in the chloroplast envelopes during the isolation process. This would cause an initial loss of small metabolites and ions depending on the size of holes produced and the amount of time required for the plastids to reseal. Resealing of chloroplasts is very common and was first reported by Lilley et al. (1975). Ultimately, this disruption and resealing could cause potential functional problems for the chloroplasts *in vitro* without affecting the apparent structural integrity as judged by refractivity of the chloroplast envelope using phase-contrast microscopy (Leech, 1965; Spencer and Unt, 1965; Lilley et al., 1975; Schulz et al., 2004). The absence of a limiting membrane is frequently observed in electron micrograph images of otherwise normal looking kleptoplasts in *E. chlorotica* (Mujer et al., 1996; Rumpho et al., 2001). This same observation was made in earlier studies of *Vaucheria* chloroplasts fused with carrot protoplasts (Bonnett, 1976). However, this unusual structure is not universal and does not appear to seriously disrupt kleptoplast function as photosynthetic, translational, and transcriptional activity by the kleptoplasts was demonstrated for a minimum of six months association with the mollusc (Mujer et al., 1996;

Pierce et al., 1996; Green et al., 2000; Rumpho et al., 2001).

Chloroplasts isolated from *V. litorea* filaments and maintained in artificial media also exhibited translational activity that persisted for the entire 3-day time-course, with only a very small decrease in translational efficiency noted. Similar long-term *in vitro* translation ability has not been demonstrated in other algal and land plant chloroplasts; rather translational activity typically ceased within minutes to a few hours post-isolation (Kirk and Tilney-Bassett, 1967; Morgenthaler and Mendiola Morgenthaler, 1976; Mayfield et al., 1995). This lack of long-term translational activity is presumably due to the general instability of plastids and loss of essential ions, cofactors, metabolites, mRNA and/or the greater requirement for nuclear-encoded chloroplast-targeted polypeptides or "signals" to sustain the activity. Comparable studies on the requirement for nuclear encoded factors to regulate transcription and/or translation in higher plant chloroplasts, e.g., the photosystem II D1 protein (Mullet, 1988), have not been carried out in heterokont plastids. In *V. litorea* chloroplasts, synthesis of the D1 protein either does not require nuclear factors or the required factors are stable enough in the plastids to allow translation to persist since *de novo* synthesis was observed over the entire three days.

The large and small subunits of Rubisco were also major translation products by isolated *V. litorea* plastids, even at 72 h. Both subunits are plastid encoded in *V. litorea* (Rumpho, et al., 2001) and co-transcribed in all chromist plastids examined to date (Starnes et al., 1985; Fujiwara et al., 1993; Reith and Munholland, 1993). Here, as well, the possible requirements for nuclear encoded factors to assist in regulating the stoichiometric synthesis of the two subunits as well as assembly and activation of the holoenzyme are unknown for heterokont algae. Nonetheless, the sea slugs are able to fix CO_2 for at least six months (Green et al., 2000; Rumpho, 2001), retain high levels of Rubisco protein which can be activated by preincubation with CO_2 (unpublished data) and, as shown here, *V. litorea* chloroplasts exhibit the ability to fix CO_2 *in vitro* for at least three days post-isolation (Fig. 6).

The extended *in vitro* stability of the *V. litorea* chloroplasts correlates well with studies by Trench et al. (1973a,b) and Gallop et al. (1980) of isolated chloroplasts from another coenocytic (although green) alga, *Codium fragile*. These chloroplasts are typically found in kleptoplastic association with the sea slug, *E. viridis*. When stored in the dark at 5°C, isolated *C. fragile* plastids remained structurally intact for at least five days as judged by phase-contrast microscopy. Translational activity was not investigated, but isolated plastids stored in the light demonstrated the ability to fix carbon for up to 7 days. However, there was a progressive and significant decline in the rate of carbon fixation per milligram of chlorophyll. If the chloroplasts were stored at 5°C in the light instead of the dark, they lost the ability to fix carbon at a much faster rate. Direct exposure of isolated *C. fragile* chloroplasts to sea water significantly decreased photosynthetic activity, but the plastids remained active in mannitol solutions varying in osmotic concentration from 300 and 600 mM (Gallop et al., 1980). Thus, *Codium* plastids also withstand the physical pressure of uptake by the sea slug and probably never come in direct contact with sea water during this natural process.

All shelled sea slugs reported on to date feed only on the coenocytic alga *Caulerpa* and the majority of sacoglossans lacking a shell feeds on a variety of succulent siphonaceous algae. However, some sea slugs do feed on and acquire kleptoplasts from non-siphonaceous algae, especially filamentous green algae, e.g., *Cladophora*, or siphonaceous algae that can become highly calcified, e.g., *Halimeda* and *Acetabularia*. The broad feeding specificity of sacoglossans is nicely reviewed by Williams and Walker (1999), based in part on studies reported by Jensen (1980). It is apparent that unlike the majority of land plant plastids, the isolated coenocytic algal plastids are extremely resilient and this characteristic may contribute to their success in establishing endosymbiotic relationships with marine molluscs. The persistent structural integrity evidenced by phase-contrast microscopy and FeCN-dependent O_2 evolution most likely plays a role in survival of the algal chloroplasts in the mollusc as it makes the transition from a young juvenile to the adult sea slug form. The modified tooth (uniserate radula) of the mollusc allows it to puncture the algal filament and literally drain the cell contents (Jensen, 1980; 1993; Williams and Walker, 1999; Marín and Ros, 2004). At this stage the chloroplasts are under a great deal of osmotic as well as physical and digestive (chemical) stress which could only be tolerated by plastids with an unusual degree of inherent stability. What specifically contributes to this structural integrity at the level of the plastid membrane of siphonaceous algae, is not known. No "rigescent integuments" have been reported associated with *Codium* chloroplasts, none were observed by electron microscopy surrounding the chloroplasts *in vivo* in *V. litorea* filaments (Mujer et al., 1996) or the kleptoplasts in *E. chlorotica* (Fig. 1), and none were noted *in vitro* surrounding isolated *V. litorea* chloroplasts. Bonnett and Eriksson (1974) did report, however, that on occasion they noticed that isolated *Vaucheria* chloroplasts aggregated with two or three chloroplasts appearing to be surrounded by a membrane. The authors have also noticed this in intact *V. litorea* filaments observed with confocal microscopy (unpublished observations), but the significance is unknown at this time.

It is shown here that *V. litorea* chloroplasts remain functional for at least three days in a test tube when provided with a minimal environment of osmoticum and buffer. Further optimization of the experimental conditions to include other chemical components (e.g., Ficol, $NaHCO_3$, ATP, NADPH, inorganic phosphate, and anti-oxidants) and variations in light intensity, temperature, and chlorophyll concentration, may extend the length of *in vitro*

functionality. Characterization of these particular chloroplasts may provide some answers to plant biologists interested in further understanding chloroplast autonomy and the requirements for nuclear-cytosolic interactions to sustain chloroplast structure and function. Ultimately, this may add to the information necessary for maintaining isolated organelles in culture for long periods of time, enabling large scale production of chloroplast secondary products in isolation and/or alternative forms of photosynthetic energy production to be developed.

Acknowledgments

The authors thank Kelley Edwards, University of Maine, for assistance with the electron microscopy imaging; Larry Griffing, Texas A&M University, for the use of his microscopes and technical advice; and Michelle Benoit and Curran Kennedy, University of Maine, for their technical assistance. This research was supported by National Science Foundation Grant IBN-9808904 and University of Maine Hatch Project #ME08756-01. This is Manuscript #2822 of the Maine Agriculture and Forestry Experiment Station.

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