Rapid Mass Movement of Chloroplasts during Segment Formation of the Calcifying Siphonalean Green Alga, *Halimeda macroloba*

Anthony W. D. Larkum\(^1,4\), Anya Salih\(^2\), Michael Küh\(\text{\textregistered}^3,4\)

1 School of Biological Sciences, University of Sydney, Camperdown, New South Wales, Australia, 2 Confocal Bio-Imaging Facility, School of Natural Sciences, University of Western Sydney, Richmond, New South Wales, Australia, 3 Marine Biological Section, Department of Biology, University of Copenhagen, Helsingør, Denmark, 4 Plant Functional Biology and Climate Change Cluster, University of Technology, Sydney, Broadway, New South Wales, Australia

Abstract

**Background:** The calcifying siphonalean green alga, *Halimeda macroloba* is abundant on coral reefs and is important in the production of carbonate sediments. The process by which new green segments are formed overnight is revealed here for the first time.

**Methodology/Principal Findings:** Growth of new segments was visualised by epifluorescence and confocal microscopy and by pulse amplitude modulation (PAM) fluorimetry. Apical colourless proto-segments were initiated on day 1, and formed a loose network of non-calciﬁed, non-septate filaments, containing no chloroplasts. Rapid greening was initiated at dusk by i) the mass movement of chloroplasts into these filaments from the parent segment and ii) the growth of new filaments containing chloroplasts. Greening was usually complete in 3–5 h and certainly before dawn on day 2 when the first signs of calcification were apparent. Mass chloroplast movement took place at a rate of \(~0.65 \, \mu m/s\). Photosynthetic yield and rate remained low for a period of 1 to several hours, indicating that the chloroplasts were made *de novo*. Use of the inhibitors colchicine and cytochalasin d indicated that the movement process is dependent on both microtubules and microfilaments.

**Significance:** This unusual process involves the mass movement of chloroplasts at a high rate into new segments during the night and rapid calcification on the following day and may be an adaptation to minimise the impact of herbivorous activity.


Editor: Ivan Baxter, United States Department of Agriculture, Agricultural Research Service, United States of America

Received November 9, 2010; Accepted May 13, 2011; Published July 5, 2011

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Funding: The study was financed by the Australian Research Council (AWDL, AS), the Danish Natural Science Research Council (MK) and the Carlsberg Foundation (MK). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: a.larkum@sydney.edu.au

Introduction

Calcifying siphonalean green algae in the genus *Halimeda* are abundant on coral reefs around the world [1] and are important in the sequestration of carbon from the atmosphere [1,2]. *Halimeda* species typically produce segmented plants that are attached by a holdfast, which can be attached to rock or coral or buried in sediment. Bundles of the aragonite crystals from dead *Halimeda* plants are an abundant source for the fine-grained sediments [1], and *Halimeda* species have been shown to produce massive carbonate deposits in deeper-water sites, both in the modern world [9,10,11] and in the geological past [12]. *Halimeda* is thus important both in geological sediment production [2,11,12] and in making a significant contribution to carbon sequestration [2].

A number of studies have been carried out on *Halimeda spp.*, with a view to clarifying processes of photosynthesis, growth, calcification and chloroplast movement. Borowitzka and Larkum studied calcification and chloroplast formation [3,13,14,15,16,17,18]. They concluded that photosynthesis during the day causes alkalisation of the inter-uterliclar space and that this is the trigger for calcification [3]. Subsequent microelectrode studies [19] supported this hypothesis, although it has not yet been possible to obtain *in situ* results for the inter-uterliclar space with pH microelectrodes because wound reactions lead to the release of acids around the microelectrode. While photosynthesis undoubt-
edly plays a key role, ion transport processes across the utricular filament membranes [3,15] and nucleation sites in the inter-utricular space [20] are also important for the calcification process. Borowitzka & Larkum [13,14] also studied chloroplast formation: they showed that new plastids formed from proplastids and that a ‘thylakoid organising body’ was an intermediate step in the maturation process [14].

Plastid movement in *Halimeda* species has been studied by Drew and coworkers. In the dark, chloroplasts are withdrawn inwards in the radial outer filaments [21], returning to the perimeter the following day just before dawn. The segments thus pale visibly in the dark, turning from a dark green colour to almost white. This is largely a response to light, since segments will also pale during the day if shaded, but there is also a regulatory component with an endogenous rhythm [22].

New segments of *Halimeda* plants are formed monthly [23]. In this process, a new colourless proto-segment is initiated during the course of the first day. This flaccid young segment is formed of a loose network of non-calcified, non-septate filaments, bathed in seawater and initially containing no chloroplasts. During the following night, chloroplasts move from the mature parent segment below into the developing segment. Apart from qualitative descriptions of this process, no detailed reports of the sequence of events or the possible mechanisms are available. Here we have used confocal microscopy and variable chlorophyll fluorescence imaging, with inhibitors and light manipulations, to study the process of the formation and greening of new segments in *Halimeda macroloba*.

**Materials and Methods**

**Sampling**

Plants of *Halimeda macroloba* Decaisne were collected on the reef flat adjacent to the Heron Island Research Station (152°06’E, 20°29’S) on the Great Barrier Reef, Australia. Plants were kept in running seawater tanks under shaded conditions at 23–25°C. For the confocal work, material was kept in a seawater aquarium at the University of Sydney over a period of 3 months at 25°C under white growth-light fluorescence tubes at an irradiance of ~250 μmol photons m⁻² s⁻¹ during a 12 h day.

**Microscopy**

Initial work was carried out using a compound microscope (BH2-RFL, Olympus, Japan) with a reflected light fluorescence attachment at 10X (DPLAPO10XUV, Olympus, Japan) and 40X (DAPO40UV/Rio, Olympus, Japan) magnification. The excitation light source was a 100W high-pressure mercury lamp (HBO 100W/2, Olympus, Japan). An excitation filter (UG-1, Olympus, Japan) was used in combination with a dichroic filter U-V (400–455 nm) for chlorophyll fluorescence (~680 nm), and an excitation filter BP-545 in combination with a dichroic filter B-G for green (cell wall) fluorescence (500–580 nm).

**Confocal Microscopy**

*H. macroloba* thallus segments were imaged in seawater mounted on a glass cover slip, which was fitted over a drilled glass slide. Typically, three regions of interest were imaged at different time intervals – the bottom, the middle and the growing edge of each sampled new segment. Confocal imaging was done on a Leica DMI80MBE inverted microscope fitted with a TCS SP2 confocal head (Leica Microsystems, Heidelberg, Germany) using 10X NA 0.35 dry, 40X PLAPO CS 0.75 oil or 63X HCX PLAPO CS water immersion objectives (Leica Microsystems, Heidelberg, Germany). We used the 488 nm excitation line of an Argon multi-line laser and the triple dichroic TD-488/543/633 nm beam splitter. Fluorescence emissions of cell walls and the meshwork of growing filaments were detected in photomultiplier (PMT) 1 at 500–530 nm and PMT 2 at 570–620 nm; chlorophyll in chloroplasts was imaged by fluorescence at 670–700 nm. Structural analysis was performed by 3-dimensional (3D) reconstruction of z-stacks containing serial sections (“xyz” scan mode) taken from each sample surface at 5 μm steps and to a depth of 40–120 μm. Complete 3D models of the specimen were rendered and examined at different orientations using Leica Microsystems software. Chloroplast streaming was imaged using the “xyt” time-scan mode. Spectral changes during segment growth and maturation were monitored at 488 nm or 514 nm laser line excitation and imaging was done by microspectral detection in “xyλ.” mode at 500–700 nm or 520–700 nm, respectively, using a 500 RSP dichroic filter to block excitation light.

**Variable chlorophyll fluorescence imaging**

The distribution and photosynthetic activity of chloroplasts in *Halimeda* plants was mapped simultaneously with an imaging pulse-amplitude-modulated chlorophyll fluorimeter (Imaging-PAM, Walz GmbH, Germany)[24]. The system uses weak modulated blue light to probe the status of PSII by measuring the chlorophyll fluorescence yield in the dark-adapted state (F₀) and during a strong saturation pulse (Fm), which drives photosystem II into a closed state. By this saturation pulse method [24,25] it is possible to determine the maximum quantum yield as:

$$\Phi_{max} = \frac{F_m - F_0}{F_m} = \frac{\Delta F}{F_m}$$

Similarly, the effective quantum yield of PSII (ΦPSII) can be determined under a known actinic irradiance, using the fluorescence yield under ambient irradiance (F) and the fluorescence yield from a saturating pulse (Fₘᵢₙ), as follows:

$$\Phi_{PSII} = \frac{F_m - F}{F_m} = \frac{\Delta F}{F_m}$$

These quantum yields can be transformed to a relative measure of photosynthetic electron transport rate (ETR) as follows:

$$rETR = A \cdot PAR \cdot \Phi_{PSII}$$

where the absorptivity, A, is either set to a constant or estimated from reflectance measurements. The imaging-PAM (i-PAM) system estimates A from imaging the reflectance of red (photosynthetically active) light (R) and near-infrared (photosynthetically inactive) light (RNIR) from a sample:

$$A = 1 - \frac{R}{RNIR}$$

Many other parameters of photochemical and non-photochemical
quenching can be determined by the saturation pulse method [24,25]. In this study we took advantage of the ability of the i-PAM system to map i) the distribution of chlorophyll/chloroplasts via fluorescence yield measurements, ii) the absorption of red light, and iii) chloroplast activity via the quantum yield of PSII over several time points at intervals of 5–15 minutes.

Oxygen microelectrode measurements

We recorded the O2 conditions in the proto-segment during its maturation with an O2 microelectrode with a tip diameter of 10 µm [26]. The microelectrode was connected to a pA-meter (PA2000, Unisense AS, Denmark) and measuring signals were acquired on a strip-chart recorder (SE110, ABB Goertz, Austria). The microelectrode was linearly calibrated from readings in aerated seawater and water deoxygenated with gaseous nitrogen. The microsensor was mounted in a manually operated micromanipulator (MM33, Martzhaeuser, Germany) and the measuring tip was carefully positioned into the center of the Halimeda proto-segment, which was kept in a small chamber with aerated seawater at a temperature of 25°C. We used a fiber-optic tungsten-halogen lamp with a collimating lens (KL2500, Schott GmbH, Germany) with the output fiber as a light source. The irradiance used in the morning after measuring the O2 depletion during darkness was ~365 µmol photon m⁻² s⁻¹.

Inhibitors and statistical analysis

Inhibitors were obtained from Sigma Chemical Co. A standard "R" statistics package was use for the chi-square analysis.

Results

General Observations and Microscopy

Observations in the field of >200 plants and over two summers indicated that white to yellowish proto-segments were formed during daylight hours (day 1) (Fig. 1A–B). Ten plants with proto-segments were collected in the late afternoon and were kept under ambient light in flowing seawater at 25°C. The proto-segment is formed of large-diameter (40–80 µm) poorly branched ramuli, called scaffold filaments in the following. There appears to be a general synchronicity in the process of proto-segment formation with most plants showing new growth at the same time, i.e. the same day of the month, once a month. However, on any given day, apart from the major event, about 10% of plants showed one or more new segments on one or more branches of the thallus.

Epifluorescence microscopy showed strong green fluorescence of the cell walls of filaments in newly formed proto-segments (Fig. 1C; 2A); after 1–2 hours, this fluorescence gradually increased and changed from green to bright yellow-orange emission (compare Fig 2A-D vs E-H). Confocal microscopy analysis of filament tips showed peak emissions at 540 and 583 nm, with 488 and 514 nm excitation, respectively. The yellow-orange fluorescence was especially prominent and was present in cell walls and the cytoplasm (Fig. 2J).

After dusk on the first day (ca 18.30 h in summer), greening of proto-segments occurred as a result of two processes: i) phase 1, movement of plastids in the cytoplasm inside the preformed scaffold filaments, originating from the parent segment, and then into smaller branches (Fig. 2A–H), and ii) phase 2, production of new green filaments by the parent segment, which grew into the proto-segments, intertwining the scaffold filaments (Fig. 2I, K).

Phase 1. In the first process, the rate of movement of chloroplasts was observed by confocal “xyt” imaging. Plastids moved by cytoplasmic streaming, with plastids being carried randomly; some individual chloroplasts moved in the reverse direction, but there was a net movement of chloroplasts in the distal direction (see Fig. 3). In 56 randomly selected plastids, in 14 separate filaments, we obtained a rate of 0.48 (±0.24) µm s⁻¹ (mean ± SD; n = 56). The greening process was observed visually many times on Heron Island with freshly collected material as well as in the laboratory with confocal microscopy, over 6 non-consecutive nights using 5 plants kept in the aquarium under normal (dark conditions) and 5 plants kept illuminated over night. Much binary fission of chloroplasts was observed under high power microscopy (x1200).

Phase 2. The initial rapid movement of chloroplasts into the scaffold filaments was followed by ramification of the initial ramuli, which formed many sub-branches, and by the growth of new ramuli from the parent segment. By dawn on day 2 (ca 05.30 h in summer), the secondary filaments had penetrated throughout the proto-segment and projected further in all directions, but particularly perpendicular to the surface, where many small branchlets were formed that became young utricles. These young utricles could be seen packed closely together from
dawn on day 2 (Fig. 2I) and thereafter began to fuse forming a closed, hexagonal surface identical to the surface of mature segments (Fig. 2L). During the morning of day 2, calcium carbonate (aragonite) crystals [3] were observed in the inter-utricular spaces, close to the cell walls of the young utricles (Fig. 4); aragonite crystals are highly light-scattering and their appearance within cells was visualized by using confocal microscopy in reflection mode (see Materials and Methods).

This developmental pattern was typical for most plants; however, in a few plants (<10%) it took most of the night to
complete the growth of green filaments. Such slow growth was particularly noted in young plants with thick mature segments and few branches; in one such case the new segments (8 in all) were still white the following morning and greening occurred only over the course of 5 h in daylight on day 2.

In all cases, growth continued on day 3 and by day 4 the new segments approached the size of mature segments. In the aquarium-grown material, segment formation did not appear to follow any periodicity and occurred randomly in plants from day to day, possibly as a result of disruption of their natural diurnal light cycles. Nevertheless, it was still possible to identify proto-segments on the afternoon of day 1 and to then observe greening during the night and calcification on the following day.

In an experiment with 10 plants under illumination (\( \sim 200 \, \mu\text{mol photon} \, \text{m}^{-2} \, \text{s}^{-1} \), white light) from 16.00 h on day 1 until 07.00 h on day 2 no green filaments were produced during the night. After transfer to natural light, green filaments began to grow in the early morning (ca 8.00 h, 2.5 h after dawn) of day 2, and by the afternoon of day 2 they were similar to filaments on plants darkened overnight. Plants \((n = 5)\) collected after 17.00 h on day 1, but illuminated overnight, as before, tended to have new segments that greened overnight, although the timing was delayed. Plants \((n = 5)\) irradiated with red light (\( \sim 100 \, \mu\text{mol photon} \, \text{m}^{-2} \, \text{s}^{-1} \), light >600 nm) overnight all had white proto-segments on day 2 and greened up (in daylight) on day 2.

**Imaging-PAM fluorometry**

On 4 nights using field material, a sub-sample of the normal (dark) treatment was taken for imaging PAM fluorometric analysis. This necessitated subjecting the plants to low modulated blue light pulses and short (<1 s) saturating blue light pulses every 5–15 min. A typical dataset is shown in Fig. 5 for one such treatment (and an animation is shown in Supporting Information: Movie S1). At time

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**Figure 3. Movement of chloroplasts into a scaffold filament of a proto-segment of Halimeda macroloba.** Confocal images were taken at intervals of 1.7 min (A–F) showing movement of chloroplasts near the base of a young proto-segment soon after chloroplasts had begun to flow into the filament.

**Figure 4. Surface view of maturing proto-segment in Halimeda macroloba showing the outer face of a utricular filament, in which the tips have fused and deposition of aragonite crystals is occurring.** The confocal images represent a 3D view of serial images taken at 0.2 mm increments under 458 nm excitation. (A) Reflection mode showing newly formed aragonite crystals in the intra-utricular spaces imaged at 450–470 nm. (B) In fluorescence mode showing cell wall emission at 510–590 nm, where it is fused to adjacent utricular filaments. (C) Chlorophyll emission from chloroplasts at 680–700 nm. (D) Composite of A), B) and C).

doi:10.1371/journal.pone.0020841.g003
doi:10.1371/journal.pone.0020841.g004
zero, the white proto-segment exhibited weak chlorophyll fluorescence and absorptivity and low quantum yield confined to the area closest to the old segment. Over the course of 3–6 hours, the new green segment was formed and chloroplasts migrated into its filaments. The greening of the segment was largely completed after ~3–5 h, while active photosynthesis was first detected after 5 hours. In the following 12 hours, the segment calcified and gained volume, while the maximum quantum yield was largely unchanged.

Usually, chloroplasts had reached the tips of the scaffold filaments after 180 min and the process of chloroplast movement was complete in ~210 min (Fig. 5). The chloroplasts moved at a fairly constant rate, as evidenced by the rate of increase and spread of $F_t$ throughout the new segment. Based on a mean base-to-tip length for the proto-segment of 7.0±0.1 mm (mean ± SD; n = 10) and a greening rate of 3 h, this would represent a chloroplast movement rate of 0.65 µm s$^{-1}$, which is similar to rates observed

![Figure 5. Sequence of new segment development and the onset of photosynthetic activity in Halimeda macroloba. The images were obtained by digital photography, absorptivity (Abs), fluorescence yield (F) and quantum yield of PSII ($\Phi_{PSII}$) over a 17 h period using microscopy and imaging-PAM fluorimetry. An animation of the full dataset, i.e., images taken every 5–10 minutes is available in Supporting Information: Movie S1. All colour images were normalised to the same relative colour code shown in the lower part of the figure.](image-url)
directly under confocal imaging (unpublished data), but somewhat slower than the more random movement observed above for individual chloroplasts. Initially the areas with chlorophyll fluorescence showed little or no quantum yield, i.e., they were unable to carry out photosynthesis. The capacity for photosynthesis lagged ~1 h behind completion of the chloroplast migration (see Fig. 5), as did the ability to carry out non-photochemical quenching (NPQ, not shown).

Oxygen concentration in the proto-segment

Microelectrode measurements showed O2 depletion (0–5% air saturation) in the center of the proto-segment throughout the dark incubation period where the maturation of the new segment occurred (data not shown). When actinic light was provided in the morning after the greening process had completed, the O2 quickly rose to levels exceeding those in the surrounding seawater kept at 100% atmospheric saturation. These measurements thus showed a pronounced O2 respiration in the proto-segment during maturation and fully competent photosynthesis only upon completion of the maturation process.

Inhibitor studies

Table 1 shows the results of the experiment with colchicine (0.5 mM) and cytochalasin d (2 μM), with 20 replicates. Alone, each inhibitor partially inhibited greening. However, with both inhibitors present together there was a total inhibition of new filament growth, where the proto-segments (n = 20) were approximately the same size on the morning of the second day, as at dusk the previous day, and without formation of utricle initials. Cytoplasmic streaming and filament growth were only fully inhibited in the presence of both inhibitors, while partial inhibition was induced when only one of the inhibitors was applied. Although the numbers are small, results are clearly significant and this was confirmed by using Pearson’s chi-square test on the complete set of data (p<2.2e−16).

Discussion

The siphonalean green alga Halimeda has been studied for many years because of its special features in terms of calcification [3,15,16,17,18] and circadian chloroplast movement [21,22]. The mechanisms involved in new segment formation have hitherto been unknown and our study shows for the first time that segment formation involves a complex series of events, whose further study could be a very valuable tool in plant cell research.

Once the proto-segment is formed, greening begins at dusk. Maturation is a three-part process, depending firstly on the import of green chloroplasts and proplastids into the scaffold filaments from the parent segment, together with replication of those plastids and secondly on the formation of new green filaments, which grow

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doi:10.1371/journal.pone.0020841.t001
development until the next morning when inhibition was alleviated and growth proceeded. Our finding that red light had a similar effect to white light in causing such a delay could indicate that a phytochrome system is involved.

The proto-segment is formed of scaffold filaments recruiting chloroplasts and becoming intertwined by secondary green filaments from the parent segment at night-time. We hypothesize that grazers attempting to feed on the newly arising structure during daylight of day 1 would be deterred by the tough nature of the proto-segment filaments, lack of nutrition and the possible presence of secondary metabolites. The walls of the proto-segment filaments are thickened and the fluorescent nature of the walls suggests that tannins or other phenolics may be present as a feeding deterrent. It is known that Halimeda spp. produce diterpenoid feeding deterrents [4,5,30] and such compounds may also be present in the proto-segment.

The end result of the greening process is a mature segment with protection from grazing by the presence of secondary metabolites and the production of unpalatable aragonite crystals (Fig 4). Borowitzka & Larkum [3] suggested that a major factor in the calcification process was the alkalinisation of the inter-utricular compartment, as a result of photosynthesis in adjacent chloroplast-packed utricular filaments in the light. This hypothesis was supported by the later work of Borowitzka [18] and De Beer & Larkum [19]. Unfortunately, it was not possible in the latter study to place a microsensor in the inter-utricular space owing to wound reactions. In mature segments, at night, chloroplasts are withdrawn deeper into the thallus and out of the inter-utricular reactions. In mature segments, the chloroplasts are imported into the proto-segments and by assessing the palatability of proto-segments and new green segments to herbivorous fish and other grazers, it should be noted, however, that Mantyka & Bellwood [31] have shown that many macroalgae on coral reefs, including several species of Halimeda, are susceptible to heavy grazing by reef fish, when more attractive algal species are not available.

In summary, the white proto-segment of Halimeda is a novel structure that has received little attention in the past. We present the first detailed study of the rapid structural changes and cellular transport processes involved in segment formation and maturation. The fast movement of H. macroloba chloroplasts, inactive in photosynthesis, into scaffold filaments seems to represent a new phenomenon of mass migration of plastids not previously reported. The whole system provides a fascinating field of research for future studies in plant cell biology.

**Supporting Information**

**Movie S1** An animation showing the greening of segments of Halimeda macroloba over 6 hours as imaged by an imaging-PAM (conditions were the same as for Fig. 5).

**WAV**

**Acknowledgments**

We wish to thank the staff of the Heron Island Research Station and the Australian Centre for Microscopy and Microanalysis, University of Sydney for support. We are grateful to Dr EA Drew and Professor R Overall, University of Sydney, who gave helpful general advice, and to Professor John Robinson, University of Sydney, who gave statistical advice. The field part of this study was carried out under Research Permit GB1000659, kindly supplied by the Great Barrier Reef Marine Park Authority, Townsville.

**Author Contributions**

Conceived and designed the experiments: AWDL AS MK. Performed the experiments: AWDL AS MK. Contributed reagents/materials/analysis tools: AWDL AS MK. Wrote the paper: AWDL AS MK.

**References**