SYMBIOSIS (2009) 47, 109–115 ©2009 Balaban, Philadelphia/Rehovot ISSN 0334-5114

Compatibility and thigmotropism in the lichen symbiosis: A reappraisal

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(Received May, 29, 2008; Accepted September 19, 2008)

Abstract
The development of many complex stratified lichen thalli is made through stages of complex phenotypic interactions between a filamentous fungus (the mycobiont), and a trebouxioid alga (the photobiont). Typically, the second stage of this symbiotic development is marked by the envelopment of the photobiont by the mycobiont through increased lateral hyphal branching and the formation of appressoria. Previously, the mycobiont's envelopment of photobiont cells was considered thigmotropic (a growth response due to shape) as a mycobiont can envelop algal sized objects in its environment. However, after growing the mycobiont *Cladonia grayi* with various phototrophs and glass beads, we conclude that the mycobiont does not show this characteristic second stage morphological response when grown in non-compatible pairings. Instead, *C. grayi* displays a distinctive morphological growth response only in compatible symbiotic pairings, such as with its natural photobiont *Asterochloris* sp.

Keywords: Lichen, thallus-development, compatibility, selectivity, thigmotropism, *Cladonia, Asterochloris*

1. Introduction

A differentiated lichen thallus is formed through complex interactions between mycobiont and photobiont cells growing together to form a body unlike either of the two symbionts grown alone (Ahmadjian, 1966; Armaleo, 1991; Galun, 1988; Honegger, 1993). This paper focuses on the early development of stratified thalli that contain trebouxioid photobionts. The sequence of events leading to a stratified thallus can be summarized in four basic stages: stage one, pre-contact, which is prior to physical contact of symbionts but close enough that extracellular interactions are possible; stage two, early-contact, where fungal cells contact algal cells by appressoria and/or subsequently envelop them through an increase in short lateral branches; stage three, incorporation of growing fungal and algal units into an undifferentiated mass; and stage four, differentiation of layered tissues (Ahmadjian et al., 1978; Bubrick et al., 1985; Galun, 1988). The molecular mechanisms behind this sequence of events are unknown.

The initial stages of *in situ* and *in vitro* natural lichen development have been the focus of many studies investigating the compatibility and selectivity of symbionts towards their partners (Ahmadjian et al., 1980; Ahmadjian and Jacobs, 1981; Bubrick et al., 1985; Galun and Garty, 1988; Kon et al., 1993; Ott, 1987; Schaper and Ott, 2003). As compatibility and selectivity have been used differently by various authors (Galun, 1988), we here define them in the context of our experiments. Starting with stage one of development, if fungi and phototrophs do not interact to form any of the initial stages of lichen development they are considered non-compatible (which includes neutral and negative interactions). Mycobionts and photobionts are considered compatible when they enter into stage two of lichen development as shown by the envelopment of the alga through increased lateral branching. In some interactions, compatible mycobionts and photobionts form only the early stages of lichen development (stages one and two, sometimes three) but do not proceed to form a stratified thallus. Selection is the process through which appropriate symbionts are distinguished and enter into stage four of development, resulting in the formation of a stratified thallus. Only when symbionts have entered into stage four of development is selection completely successful. (Specificity, a term describing the relationship between mycobionts and photobionts, is excluded from the discussion as it describes the evolutionary ecology of the

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selectivity of the symbionts in stage four of development, and the degree to which they are specialists or generalists.)

Schwendener first recognized the dual nature of lichens as the symbiosis between mycobionts and photobionts in 1869. Investigations into the compatibility of the symbionts began shortly thereafter. From the detailed drawings of Bornet (1873) and Bonnier (1889a) it is clear that the scientists of the late 19th century were familiar with the hyphal branching of mycobionts and the envelopment of photobiont cells. Bonnier (1889a) was the first to microscopically inspect the growth of mycobionts with inanimate objects and heterologous phototrophs (i.e., all phototrophs other than the known photobiont(s) of a given mycobiont species). Bonnier grew mycobionts with sand, glass wool, and thinly cut straw, and concluded that because the fungi grew over these inanimate items in a manner similar to their photobionts, the mycobiont does not exhibit a distinct growth response to compatible photobionts. Further confirmation came after growing mycobionts with heterologous algae: the golden alga *Vaucheria sessilis*, and two species of the filamentous green alga *Trentepohlia*. However, according to his drawings it appears that the mycobionts failed to envelop animate and inanimate objects in the same manner as their natural photobionts.

Nearly 100 years later, similar experiments reinvestigated the thigmotropic response using either *Cladonia cristatella* or *Xanthoria parietina* and algal sized glass beads (Ahmadjian and Jacobs, 1981; Bubrick et al., 1985). Members of both *Cladonia* and *Xanthoria* form lichen thalli with trebouxioid algae and show a stage 2 specific response (Galun, 1988). In further experiments using *C. cristatella*, Ahmadjian and Jacobs (1981) reported that the mycobiont grew over glass beads in a manner similar to its compatible photobiont. However, in experiments with *X. parietina*, Bubrick et al. (1985) observed that the mycobiont did not interact with the glass beads when simultaneously grown with compatible photobionts. In neither case were illustrations provided. These results have been cited widely in the literature to suggest that a mycobiont does not change its growth habit in response to compatible trebouxioid photobionts; instead that mycobionts respond to thigmotropic stimuli (Galun, 1988; Honegger, 1993; Lawrey, 1984; Trembley et al., 2002).

As part of our on-going study of the molecular mechanisms underlying the development of mycobionts and photobionts into a stratified thallus, we revisited the thigmotropic growth response of mycobionts towards photobionts and inanimate objects. Here we compare the growth of the lichen-forming fungus *Cladonia grayi* in compatible and non-compatible pairings. We compare the growth of *C. grayi* with its known photobiont *Asterochloris* sp. (a trebouxioid alga) with the growth of this fungus with green algae never found associated with *C. grayi* in nature (*Chlorella vulgaris* and *Trentepohlia* sp.), with moss protonema (*Funaria hygrometrica*), and with glass beads.

2. Methods

Cultures and media

All cultures were maintained and used at room temperature, ca. 23–25ºC. Grinding, pipetting and plating took place in a Nuaire Class II, Type A/B3, Biological Safety Cabinet.

Axenic cultures of *Cladonia grayi* and *Asterochloris* sp. were provided by D. Armaleo (Department of Biology, Duke University). These cultures were originally created from the soredia of material deposited at DUKE (Accession No. 0038794). The *C. grayi* culture was maintained in 50–200 ml liquid cultures of Malt Yeast (MY, 20 g malt extract and 2 g yeast extract per liter), in Erlenmeyer flasks on a rotating shaker (100 rev/min), and regularly ground in a commercial Waring blender (every 6–8 weeks).

Asterochloris sp. was maintained on plates of one-quarter strength *Trebouxia* Medium (Ahmadjian, 1967). Cultures of *Chlorella vulgaris* (UTEX 30) and *Trentepohlia* sp. (UTEX 1227), two green algae never found with *Cladonia grayi* in stage four of lichen development, were maintained on plates of MY. All algae were grown under ca. 40 µmol m^{-2} s^{-1} illuminations for 12 hours a day and subsequently removed for experimental set up by pipetting sterile water onto the plates and pipetting up and down until algal cells were visibly resuspended in the solution.

Spores of *Funaria hygrometrica* were provided by J. Shaw (Department of Biology, Duke University). Spores were washed in bleach for 30 s, next in distilled water for 30 s, and then allowed to germinate on cellophane discs over a reduced nutrient medium made from a 99:1 mix of Bold's Basal Medium (Deason and Bold, 1960), MY medium, and solidified with 2% agar (hereafter referred to as 99:1 medium). Spores were germinated and protonema grown for 20 days under the same growth conditions as the green algae, before placing freshly ground *Cladonia grayi* onto the growing filaments. Although mosses never form lichen thalli with mycobionts, the protonemal stage was chosen based on previous observations that lichen-forming fungi can grow symbiotically with protonemal filaments (Bonnier, 1888, 1889b).

Glass bead preparation

Glass beads 3–10 µm and 10–30 µm in diameter (PolySciences, Inc., Warrington, PA) were washed with acid and thoroughly washed in water before mixing them individually or together with the mycobiont. In brief, enough 5.8 M HCl was used to cover the beads in an

Eppendorf tube, and incubated at room temperature for 1 hour. The solution was spun down at maximum speed in a tabletop centrifuge for 2 min. The acid was poured off, and the beads were flooded with autoclaved Ultrapure water (Hydro Picotech® 2, Durham, NC) before being vortexed and spun down at maximum speed for 2 min, removing the supernate by pipetting. Beads were washed in this manner until the pH of the supernate was increased to 6.3. The beads were subsequently dried in a 37˚C incubator overnight, and stored at room temperature.

Each experiment was conducted one to several times from 2004–2007. *Cladonia grayi* cultures were ground one to two days prior to the beginning of each set of experiments and maintained as above, until the experiments began. Experimental plates of *C. grayi*, *C. grayi* with green algae, and *C. grayi* with glass beads were set up by first pipetting a small amount of freshly ground *C. grayi* such that the mycelia could spread out and individual clumps were visible by eye. *Cladonia grayi* was pipetted on to autoclaved hand-cut circular cellophane discs approximately 47 mm in diameter over 2% agarized 99:1 medium, and allowed to dry until the fungus and cellophane disc were secured upon the agar surface (0–2 hours). Next, 100–200 µl of water mixed with either green algae or glass beads were pipetted on to the fungal material and allowed to dry until all supplemental liquid had evaporated. A minimum of algal cells and glass beads were placed on the plates to allow for even spacing of the cultures or glass beads. Experimental plates of *C. grayi* with moss protonema started with plates of germinated *Funaria hygrometrica* spores (above) onto which the liquid culture of *C. grayi* was pipetted. Plates were parafilmed and cultures allowed to grow under the same environmental conditions as the original algal and moss cultures.

Environmental Scanning Electron Microscopy (ESEM)

For each experimental setup, small sections of the cultures on cellophane were sampled under sterile conditions for up to seven weeks. Pieces of cellophane were attached to SEM stubs and visualized using a Philips XL30 ESEM microscope (FEI Company). Resulting micrographs were adjusted for improved contrast when needed. Days after initiation of symbiosis were noted on micrographs. This number represents the maximum length of time for possible interaction between symbionts. Micrographs may actually represent younger symbiotic interactions, as these interactions are initiated as the mycobiont grows to established green algae, moss protonema, or glass beads.

3. Results

Cladonia grayi grew in all conditions when grown on cellophane over the reduced nutrient medium 99:1 (alone, with green algae, with moss protonema, or with glass beads), as indicated by an increase in colony size after three weeks (data not shown). Growth of *C. grayi* alone is marked by strong apical growth and reduced lateral branching at the edge of a colony (Figs. 1a and 1b). This growth pattern did not change over the course of 43 days. Aerial hyphae regularly grew from marginal hyphae, as well as from mycelial clumps.

When grown with *Asterochloris* sp., *Cladonia grayi* grew around algal cells individually (Fig. 2a), and around algal cells in clumps (Fig. 2b). Around individual cells of *Asterochloris* sp. the fungus exhibited increased lateral branching (as shown by highly branched hyphae surrounding round algal cells), when compared to growth alone (Fig. 2a, and hyphae not in contact with algae in Fig. 2b). This pattern was regularly observed in the mixed cultures, although *C. grayi* did not grow around every algal cell (lower right corner of Fig. 2a). The envelopment of algal cells through increased lateral hyphal branching was visible by ESEM as early as 21 days after resynthesis, although this response probably occurs earlier. Within 10 days of resynthesis, symbiotic interactions were visible under a dissecting microscope at 16x as aerial hyphae of *C. grayi*, can be observed growing out of *Asterochloris* colonies (data not shown). Cells of *Asterochloris* sp. engulfed by *C. grayi* cannot be dislodged by a gentle stream of water, or mechanical force (squashing material between a cover slip and glass slide).

When grown with glass beads, *Cladonia grayi* never developed the second stage growth response found with compatible algae (Figs. 3a and 3b). *Cladonia grayi* grew over all sizes of glass beads in a non-compatible manner, including those of comparable size to *Asterochloris* sp. cells (15–20 µm in diameter). Glass beads at the edge of *C. grayi* colonies are in general not overgrown with hyphae (Fig. 3b). Many glass beads remained attached to *C. grayi* hyphae after gentle washing in water, and attempts to dislodge with mechanical force. Aerial hyphae were formed regularly on older clumps of mycelia, as well as from hyphae growing from amongst glass beads.

Cladonia grayi grew amongst proliferating cells of *Chlorella vulgaris*, but was never observed to grow around individual cells, and virtually never formed aerial hyphae within colonies of this green alga (Figs. 3c and 3d). *Chlorella vulgaris* grew quickly on the reduced nutrient medium 99:1, regardless of whether *C. grayi* was present or not. Colonies of *C. vulgaris* rapidly increased and covered both kinds of plates over a four week period. Aerial hyphae were visible on clumps of *C. grayi* mycelia growing alone.

Cladonia grayi grew over individual filaments and throughout clumps of *Trentepohlia sp.* (Figs. 3e and 3f). *Cladonia grayi* also formed aerial hyphae readily on mycelial mats, as well as on clumps of *Trentepohlia* sp. Although *C. grayi* grew throughout clumps of *Trentepohlia* sp. forming aerial hyphae, the mycobiont never exhibited

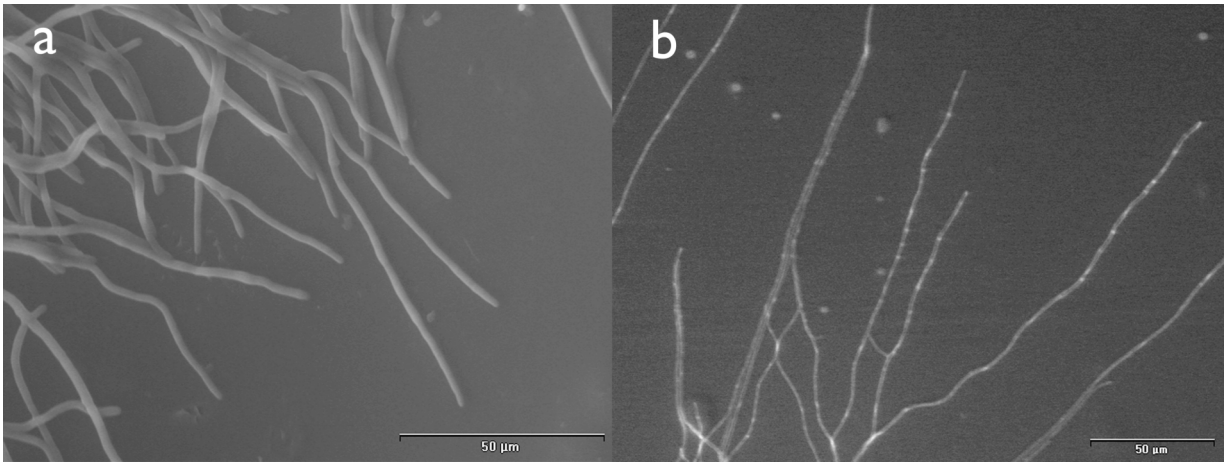

Figure 1. *Cladonia grayi* growing alone on cellophane over 99:1 medium. a) Edge of colony, day 5. Scale bar = 50 µm. b) Edge of colony, day 43. Bumps between hyphae are abnormalities in the cellophane. Scale bar = 50 µm.

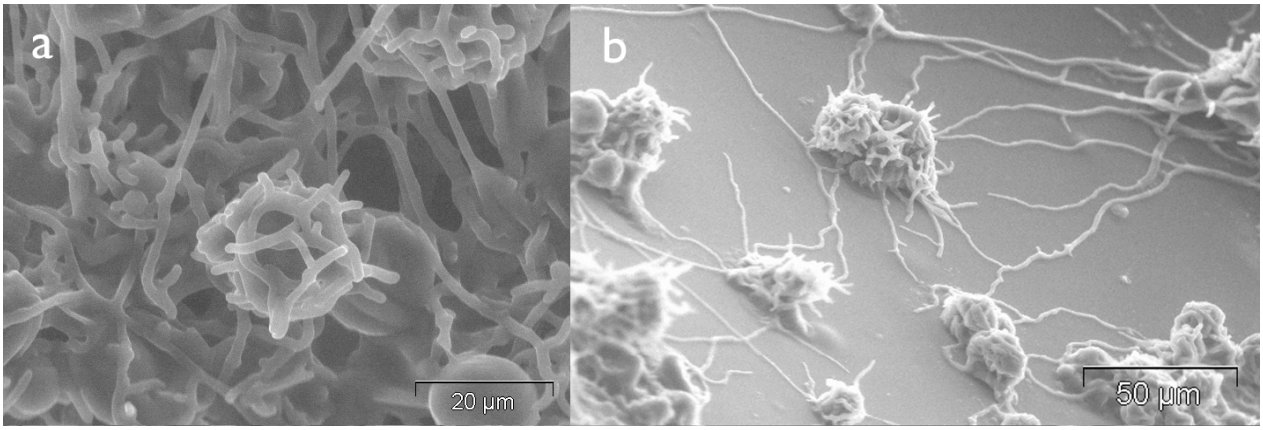

Figure 2. *Cladonia grayi* growing with *Asterochloris* sp., and surrounding algal cells through increased fungal branching. a) A single algal cell enveloped by fungal hyphae can be seen in the middle of the micrograph. At least one alga, lower right hand corner, is not engulfed by fungal hyphae, day 49. Scale bar = 20 µm. b) Clumps of algal cells and highly branched hyphae can be seen scattered throughout, connected by exploring hyphae, day 49. Note increased branching of *Cladonia* in clumps of algae, when compared to hyphae between clumps and not in contact with algae. Scale bar = 50 µm.

the symbiotic phenotypic response of the second stage of lichen resynthesis as found with *Asterochloris* sp.

When grown with the protonema of *Funaria hygrometrica*, C. *grayi* grew over the moss filaments, but never entered into stage 2 of thallus development (Figs. 3g and 3h). *Cladonia grayi* never grew on lateral aerial protonemal filaments, instead only over horizontal filaments (Fig. 3h). Aerial hyphae were present on individual hyphae growing away from mycelial clumps, on mycelial clumps, and within moss protonemal mats.

4. Discussion and Conclusions

The results of this study show that *Cladonia grayi* has a distinctive phenotypic response to its compatible photobiont, *Asterochloris* sp., during stage two of lichen development. Consistent with previous studies, the mycobiont *C. grayi* can grow over green algae, moss protonema, and glass beads. However we conclude that *C. grayi* expresses the stage two distinctive envelopment, through increased lateral hyphal branching, exclusively towards its compatible photobiont. This symbiotically induced change in branching pattern of *C. grayi* in response to *Asterochloris* sp. is most apparent when comparing the fungus grown with glass beads and heterologous phototrophs with that of the fungus on its own.

We have not ruled out the possibility that *Cladonia grayi* is interacting or communicating in some way with the heterologous phototrophic organisms presented, or even with the glass beads. The photosynthetic organisms that *C. grayi* was grown with did not appear on a macroscopic level to be inhibited by the growth of the fungus. Colonies of algae and protonema growing amongst the fungi

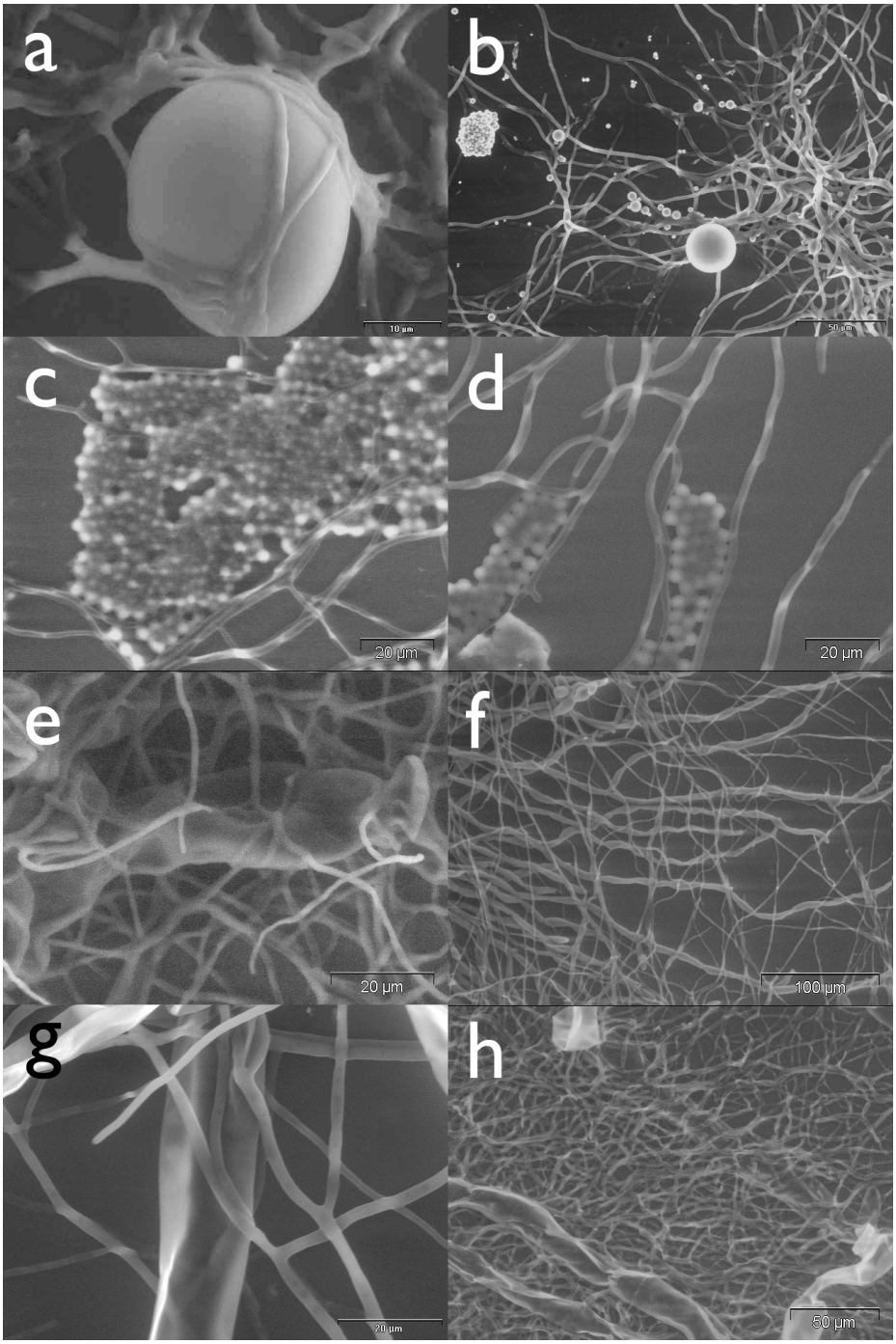

Figure 3. *Cladonia grayi* growing with glass beads and heterologous phototrophs. a) *Cladonia grayi* growing over a glass bead, towards the center of a mycelial mat. Increased hyphal branching with short lateral hyphae absent, day 29. Scale bar = 10 µm. b) *Cladonia grayi* growing with glass beads at the edge of a mycelial colony, day 8. Scale bar = 50 µm. c) *Cladonia grayi* growing with spherical cells of *Chlorella vulgaris*, at the edge of a mycelial colony, day 9. Scale bar = 20 µm. d) *Cladonia grayi* growing with *C. vulgaris*, day 38. Scale bar = 20 µm. e) *Cladonia grayi* growing with *Trentepohlia* sp., day 9. At least one filament of *Trentepohlia* sp. can be seen growing horizontally across the center of the micrograph. Scale bar = 20 µm. f) *Cladonia grayi* growing over the edge of a *Trentepohlia* sp. colony, day 38. Scale bar = 100 µm. Thick filaments of *Trentepohlia* are growing horizontally (mostly) across the micrograph. g) *Cladonia grayi* growing with protonema of *Funaria hygrometrica*, day 5. One filament of *F. hygrometrica* is shown growing vertically through the center of the micrograph. Scale bar = 20 µm. h) *Cladonia grayi* growing with several protonemal filaments of *F. hygrometrica*, day 24. *Cladonia grayi* has grown over part of one protonemal filament in the upper right hand third of the micrograph. Portions of the same filament remain free both above and below where *C. grayi* has overgrown it. Growth contacts between fungus and moss lack the phenotypic lichen-symbiotic response of *C. grayi*. Scale bar = 50 µm.

remained green and proliferated in a manner similar to the photosynthesizers grown on their own. No obviously dead colonies of photosynthesizers were found amongst fungi on the resynthesis plates. Therefore, it is assumed that *C. grayi* cannot kill these photosynthesizers under the given conditions (i.e., on cellophane discs over a reduced nutrient medium). If, however, *C. grayi* is recognizing or communicating with any of the above photosynthesizers other than *Asterochloris* sp., it does so without forming the diagnostic morphological response of the second stage of thallus development.

Previous authors have noted that in *in vitro* resynthesis, mycobiont growth is not directed toward compatible photobionts, instead it is a random growth pattern that brings the symbionts together (Ahmadjian, 1959; Clayden, 1998). This is consistent with the nutrition search strategy found in non-lichen-forming, filamentous ascomycetes (Gassmann and Ott, 2000), in which these fungi grow over

substrates and away from regions in which they have exhausted the resources. This hypothesis is consistent with our results showing that *Cladonia grayi* will grow over, and adhere to, glass beads in its vicinity. This hypothesis can also explain why more glass beads are grown over by *C. grayi* at the center of mycelial mats, than at the edge of fungal colonies. As the density of *C. grayi* hyphae increases, there is a higher chance that a glass bead will be contacted by a roaming hypha. It is interesting that *C. grayi* never adheres to *Chlorella vulgaris, Trentepohlia* sp., or *Funaria hygrometrica*, suggesting that a mycobiont will not adhere to everything with which it comes into contact. Perhaps the heterologous phototrophs resist attachment through a defense mechanism. Whether or not a roaming mycobiont will adhere to substrates it encounters cannot to date be predicted and remains to be investigated.

Although *Cladonia grayi* responds to cells of *Asterochloris* sp. in a stage 2 specific manner, the mycobiont does not respond in the same manner towards every photobiont cell (Fig. 2a), suggesting that the early stages of thallus development are more complicated than just compatible symbionts finding one another. The inability of every cell within a compatible photobiont species to be enveloped by a compatible mycobiont has also been reported from a similar stratified lichen thallus system formed by the mycobiont *Usnea strigosa* and the photobiont *Trebouxia usneae* (Ahmadjian and Jacobs, 1982). Newly divided algal cells appear to be the most suitable for the primary stage of thallus development between mycobionts and photobionts (Honegger, 1986). In the present experimental design we were unable to determine the age of the algae entering stage two of thallus development. Furthermore, it is still not clear at which point *C. grayi* recognizes compatible or selective photobionts. Mycobionts that form stratified lichen thalli *in situ* have been shown to proceed through stage three of development in *in vitro* resynthesis with compatible heterologous photobionts (Ahmadjian et al., 1980; Ahmadjian and Jacobs, 1981; Bubrick et al., 1985; Kon et al., 1993; Schaper and Ott, 2003), suggesting that there are shared molecular mechanisms in the early stages of lichen development between selective and non-selective (yet compatible) symbionts. The recognition of lichen symbionts towards one another has been suggested to be a multi-step process (Bubrick et al., 1985), and warrants further investigation.

The mycobiont *Cladonia grayi* displays a compatible symbiotic phenotype in response to *Asterochloris* sp. in the early contact stage between the two symbionts. This is in contrast to the mycobiont's non-compatible response to glass beads and heterologous green symbionts. Using this earliest stage exclusive to lichen symbiosis, we can now begin to study the genetic and molecular mechanisms underlying the development of a stratified lichen thallus.

Acknowledgements

We would like to acknowledge the assistance of Daniele Armaleo for supplying materials and support, Leslie Eibest for assistance with ESEM, and Jonathon Shaw and Matt Johnson for their help in using *Funaria*.

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