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Fungal and algal gene expression in early developmental stages of lichen-symbiosis

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Abstract: How plants and microbes recognize each other and interact to form long-lasting relationships remains one of the central questions in cellular communication. The symbiosis between the filamentous fungus *Cladonia grayi* and the single-celled green alga *Asterochloris* sp. was used to determine fungal and algal genes upregulated in vitro in early lichen development. cDNA libraries of upregulated genes were created with suppression subtractive hybridization in the first two stages of lichen development. Quantitative PCR subsequently was used to verify the expression level of 41 and 33 candidate fungal and algal genes respectively. Induced fungal genes showed significant matches to genes putatively encoding proteins involved in self and non-self recognition, lipid metabolism, and negative regulation of glucose repressible genes, as well as to a putative D-arabitol reductase and two dioxygenases. Upregulated algal genes included a chitinase-like protein, an amino acid metabolism protein, a dynein-related protein and a protein arginine methyltransferase. These results also provided the first evidence that extracellular communication without cellular contact can occur between lichen symbionts. Many genes showing slight variation in expression appear to direct the development of the lichen symbiosis. The results of this study highlight future avenues of investigation into the molecular biology of lichen symbiosis.

Key words: *Asterochloris* sp., *Cladonia grayi*, lichen, plant-fungal interactions, symbiosis

INTRODUCTION

Members of kingdom Chloroplastida are ubiquitous in terrestrial, aquatic and oceanic habitats and encompass the diversity of all green algal lineages and land plants (Adl et al. 2005, Lewis and McCourt 2004). Members of kingdom Fungi have developed a range of symbiotic interactions with Chloroplastida in which they have diversified into an array of terrestrial niches (Selosse and Le Tacon 1998, Taylor 1990). The most successful symbiosis, based on fungal species numbers alone, is the lichen. More than 13 500 species of lichen-forming fungi have been described (Kirk et al. 2008) based on the transformative properties of the symbiosis to the fungal phenotype, comprising nearly one-fifth of all known fungal species. Although the emergent properties of lichen thallus development first were recognized as the symbiotic growth of different organisms 140 y ago (Schwendener 1869), the molecular mechanisms of lichenization remain to be discovered. Fundamental questions need to be addressed on the evolution of symbiosis between Chloroplastida and Fungi (Arnold et al. 2009, James et al. 2006, Schoch et al. 2009) on the signaling pathways connecting lichen symbionts and on morphogenesis.

The initial stages in the development of lichen symbiosis can be examined in vitro through the axenic culturing of symbionts. In experimental resynthesis aposymbiotic cultures of mycobionts and photobionts are mixed and allowed to grow together on nitrocellulose filters over a reduced nutrient medium (Armaleo 1991, Backor and Fahselt 2003, Oliver et al. 1989). The development of a stratified lichen thallus follows four general developmental stages consisting of pre-contact, contact and growth together, growth into a transitional pre-thallus and growth into a stratified thallus (Armaleo 1991, Ahmadjian et al. 1978, Galun 1988). These stages are part of a dynamic continuum that originates anywhere novel fungal-algal contacts are made (Armaleo 1991). The synchronized growth of the latest stage in lichen development cannot be achieved regularly in the lab, and for this reason we focus on only the first two stages. For the lichen model system formed by the symbiotic growth of *Cladonia grayi* and *Asterochloris* sp. the first two stages of development (pre-contact, and contact) can be observed in vitro within 21 d, and the latter step is accompanied by a distinctive phenotypic change in the fungus (Joneson and Lutzoni 2009). The phenotypic change in *C. grayi* consists of a switch from apical hyphal growth with long internodes, to reduced apical growth and internode length with increased lateral branching (Armaleo 1991, Honegger 1997, Joneson and Lutzoni

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2009). Entry into lichen symbiosis is accompanied by changes in gene regulation and expression in the photobiont and mycobiont (Armaleo and Miao 1999, Trembley et al. 2002), although the genetic details remain to be determined.

The *Cladonia grayi-Asterochloris* sp. model system of symbiosis was used to explore the combined upregulated transcriptome of early lichen development. At this time published expressed sequence tags, genomic, and/or cDNA libraries are lacking for any closely related fungi or green algae of the lichen symbionts, and the annotation of any gene products relies on homology based searches. Suppressive subtractive hybridization (SSH) (Diatchenko et al. 1996) was used to determine symbiotically upregulated genes and quantitative PCR (qPCR) to verify the expression level of candidate genes. Extracellular communication between symbionts before contact generally has been disregarded because chemotropic signals do not appear to be exchanged (Ahmadjian 1959, Clayden 1998). We present here the first evidence that mycobiont and photobiont gene expression changes not only after symbiont cellular contact (stage 2) but is influenced by the proximity of physically separate symbiotic partners (stage 1). We present here novel fungal and algal genes differentially expressed in the early developmental stages of the lichen symbiosis.

MATERIALS AND METHODS

Experimental symbiosis.—Axenic cultures of *Cladonia grayi* and *Asterochloris* sp. as described in Armaleo and May (2009) were used. Cultures of *Cladonia* were maintained in 50–200 mL liquid cultures of malt yeast (MY, 20 g malt extract and 2 g yeast extract/L) in Ehrlemeyer flasks on a rotating shaker (100 rev/min) and regularly ground in a commercial Waring blender (every 6–8 wk). *Asterochloris* was grown on nitrocellulose membranes (47 mm diam, 0.22 μm pore, Millipore catalogue No. GSWP04700), over one-quarter strength *Trebouxia* Medium (Ahmadjian 1967), under approximately 40 μmol m^{-2} s^{-1} illumination for 12 h/d.

Day 1 denotes the beginning of the experiment, on which aposymbiotic and symbiotic cultures growing on nitrocellulose filters over 2% agarized plates made from a 99 : 1 mix of Bold's basal medium (Deason and Bold 1960) and MY (hereafter referred to as 99 : 1 medium) were created. *Cladonia* cultures were ground 1–2 d before the beginning of the experiment. On Day 1 liquid *Cladonia* cultures were concentrated at 7 K for 10 min. Decanted mycelia were resuspended in a minimum of liquid 99 : 1 medium, filtered through a 74 μm pore size nylon filter, and the filtrate concentrate determined with a spectrophotometer at 600 nm and a predetermined concentration of 1 OD = 0.75 mg, dry weight. On Day 1 cultures of *Asterochloris* were scraped and resuspended in a minimum of liquid 99 : 1 medium with a handheld glass homogenizer. The algae subsequently were filtered through a 74 μm nylon filter, and the concentration of the filtrate determined by spectrophotometer at 750 nm with a predetermined concentration of 1 OD = 0.8 mg, dry weight.

An ideal ratio of 7 : 3 fungus to alga dry weight determined the volume of fungus and/or alga to deposit onto filters by vacuum filtration, and liquid filtrates were adjusted appropriately with additional liquid 99 : 1 medium. Five milligrams dry weight of *Cladonia* and 2.14 mg dry weight of *Asterochloris* were used per replicate. Each symbiotic developmental stage was compared to aposymbiotic growth of the same age. For both stage 1 and stage 2 aposymbiotic growth *Cladonia* and *Asterochloris* cultures were individually vacuum filtered onto membranes and each filter culture grown in separate Petri dishes. For stage 1 symbiotic growth an *Asterochloris* filter was placed atop a *C. grayi* filter and grown in the same Petri dish. For stage 2 symbiotic growth both *Cladonia* and *Asterochloris* cultures vacuum filtered together onto a single membrane. All filter cultures were kept at room temperature (25 C) with 12 h 40 μmol m^{-2} s^{-1} illumination per day.

Stage 1 aposymbiotic and symbiotic filters were scraped on Day 9 and the materials lyophilized in a Freezemobile (Spectrofuge Corp.) at 0 C until dry. RNA extraction proceeded immediately, followed by first strand cDNA synthesis. Stage 2 materials were processed in the same manner on Day 21. The length of stage 2 was based on the minimal time needed to generate clearly symbiotic structures in culture (Joneson and Lutzoni 2009). The length of stage 1 was meant to be shorter than stage 2 but long enough to generate discernible responses.

Nucleic acid extraction.—RNA was extracted by first grinding lyophilized material on a Mini Bead Beater 8 (BioSpec Products Inc.) with 700 μm zirconia beads. Material and beads initially were frozen in liquid nitrogen, ground 10 s at full speed and placed back in liquid nitrogen. The samples were resuspended in guanidine-phenol buffer (Solution A, according to Xie and Rothblum [1991], with these molar substitutions: 25 mM sodium citrate and 4 M guanidinium thiocyanate) and ground 30 s at half speed. Samples were removed from beads, and RNA was extracted according to the DNA extraction in Armaleo and Miao (1999), with an additional DNase treatment (Promega USA, Madison, Wisconsin). Total genomic DNA was isolated for primer construction of housekeeping genes and *Lip3* with a modified protocol of Zolan and Pukkila (1986) using 2% sodium dodecyl sulphate as the extraction buffer.

DNA sequencing.—Sequencing was carried out on an Applied Biosystems 3730xl DNA automated sequencer (Duke IGSP sequencing facility) with Big-Dye Terminator Cycle Sequencing 3.1 (PE Applied Biosystems, Foster City, California). Sequences were analyzed with Sequencher 4.8 (Gene Code Corp., Ann Arbor, Michigan).

Single stranded cDNA (ss-cDNA) synthesis.—ss-cDNA for qPCR reactions was carried out with an oligo $d(T)_{16}$ and SuperScript II Reverse Transcriptase (Invitrogen USA, Carlsbad, California) according to the manufacturer's guidelines with the addition of an initial 80 C RNA denaturation step and a 2 h (total) elongation step. RNA

was degraded chemically with sodium hydroxide and heat. ss-cDNA was stored at −80 C.

SSH and cDNA library construction.—First and second strand cDNA was synthesized for SSH with the BD SMART™ PCR cDNA Synthesis Kit following the manufacturer's guidelines for cDNA subtraction, except for the following alterations (BD Biosciences Clontech, Palo Alto, California). *Rsa*I digested double-stranded cDNA (ds-cDNA) was cleaned with QIAquick PCR purification columns after reduction with n-butanol (QIAGEN USA, Valencia, California), eluted into a final 100 μL volume and precipitated on ice 30 min with a final wash of 80% ethanol.

SSH was carried out with the PCR-Select™ cDNA Subtraction Kit according to the manufacturer's directions and starting with adaptor ligation, (BD-Biosciences-Clontech, Palo Alto, California). A truncated (20 bp) version of Nested PCR 1 was used to later increase cloning efficiency, 5′CGAGCGGCCGCCCGGGCAGG3′. Fungal and algal stage 1 SSHs were performed independently with aposymbiotic cDNA as the driver and symbiotic cDNA as the tester. For stage 2 a mixture of algal and fungal aposymbiotic cDNA was used as the driver and symbiotic cDNA as the tester. The proportions of fungal and algal aposymbiotic cDNA used in the stage 2 driver were determined by qPCR. Algal and fungal specific LSU primers (SUPPLEMENTAL TABLE I) were run separately with ss-cDNA and compared to known mixtures of varying proportions of algal and fungal ss-cDNA. The ds-cDNA created through SSH was cloned into the pCR2.1-TOPO plasmid and transformed into TOP-10 *Escherichia coli* cells (Invitrogen USA, Carlsbad, California) with 7.5 ng cDNA/reaction.

Clone analysis.—Using Sequencher 4.5 default parameters (Gene Code Corp., Ann Arbor, Michigan), double-stranded sequences of clone inserts were cleaned of poor reads and vector sequence and assembled into contigs greater than 100 bp long. Local batch BLASTX algorithmic queries (Altschul et al. 1990) with default settings and a threshold e-value $< 10^{-5}$ were carried out with the set of non-redundant (nr) proteins from the National Center for Biotechnology and Information (www.ncbi.nlm.nih.gov, accessed 21 May 2008). Translated nucleotide sequences greater than or equal to 30 amino acids that also matched an nr database entry with equal or greater than 35% identity were considered significant. Protein coding sequences in proper reading frame were parsed and used to query the UniProt database (www.ebi.uniprot.org) of protein sequence and function with BLASTP. Datasets of BLASTX and UniProt results were managed with customized PERL and BioPERL scripts (Stajich et al. 2002).

Genome of origin was determined for genetic regions by manually classifying up to the top five BLASTX hits (if available). Sequences were labeled of fungal origin if the top hits were fungal sequences. Algal sequences were identified by their match to either all Chloroplastida or Chloroplastida and cyanobacteria. The remainder of sequences was classified as ''either''.

qPCR.—To confirm differential expression of select genes qPCR was performed on two experimental replicates per treatment. (Experimental replicates are different filters set up at the same time, and in each case a single culture was used to set up both the aposymbiotic or symbiotic growth of the fungus and the alga). (qPCR primers can be found in SUPPLEMENTAL TABLE I). qPCR in 20 μL reactions were run with these conditions: 0.4 units Apex Taq DNA Polymerase (Genesee Scientific, San Diego, California), 1× Apex PCR Buffer, 3 mM MgCl2, 0.16 mM dNTPs each, 2% (v/v) ROX passive reference (Eurogentec, San Diego, California), 2.5×10^{-5}% Sybr Green (Invitrogen USA, Carlsbad, California) and an amount of cDNA representative of a standard dilution curve of 1, 10E-01, 10E-02 and 10E-03. qPCR reactions were conducted in a Chromo4 thermocycler (Bio-Rad, Hercules, California) with these parameters: 95 C for 10 m, followed by 44 cycles of 95 C for 30 s, 65 C for 30 s and 72 C for 30 s. The fluorescence was measured at the end of the 72 C extension step. Three qPCR reactions were performed for each gene-dilution series (12 reactions per gene, per treatment), and no reverse-transcriptase (NRT) negative controls were run for each gene in each experimental replicate. Each sample was normalized against two controls, the efficiency of each primer pair determined with the standard curve method (Rasmussen 2001), and the final normalized relative expression levels of the symbiotic fold-change in expression was calculated according to Pfaffl (2001). Individual primer efficiencies were averaged within treatments between experimental replicates.

Control gene qPCR primers were determined as follows. Fungal *ef1*α primers were designed from the genomic sequence amplified and sequenced with *tef1*F and *tef1*R (Morehouse et al. 2003), *βtub* primers were designed from the genomic sequence amplified and sequenced with Bt1F (5′-CAGGTCCATCTCCAGACCGG-3′), and Bt10-LM (Myllys et al. 2001) and *act* primers were designed from an unpublished sequence from Tami McDonald (Duke University). Algal *act* primers were designed from the genomic sequence amplified and sequenced with ACT1T (modified as 5′-ACACCGTGCCCATCTAYG-3′) and ACT2T (Kroken and Taylor 2000) and *ef1*α, *GAPDH* and *RbcS* primers from SSH-cDNA-generated sequences.

For *Cladonia grayi*, an 838nt region of *ef1*α (GenBank accession number FJ56937) and a 1172nt region of *βtub* (GenBank accession number FJ56938) were amplified from genomic DNA. A 730nt region of *act* from *Asterochloris* sp. was amplified from genomic DNA (GenBank accession HM355478). All SSH-cDNA sequence trace files have been deposited in NCBI Trace Archives (www.ncbi.nlm.nih.gov/Traces/home/): stage 1 *Cladonia grayi* (TI 2274562157–2274562843), stage 1 *Asterochloris* sp. (TI 2274010227–2274011900) and stage 2 *C. grayi* and *Asterochloris* sp. library (TI 2274584348–2274585170).

Saccharomyces cerevisiae *cultivation and plasmid construction.*—As a proof of principle that SSH identified functional protein coding genes in *Cladonia*, a candidate gene was chosen for further characterization. *Lip3* was heterologously expressed in yeast because extracellular lipases play important roles in plant-fungal interactions, and *C. grayi* lacks a transformation system for testing gene function.

Saccharomyces cerevisiae strain W303-1A (MATa ade2-1 can1-100 his3-11,15 leu2-3112 trp1-1 ura3-1) and yeast expression vector p416GPD-2xHA were gifts from Dr Chandra Tucker (Duke University). W303–1A was grown in synthetic complete (SC) medium -ura with 2% glucose as the carbon source in transformant selection (Burke et al. 2000).

The partial 5′ and 3′ ends of the *Lip3* coding mRNA were sequenced with a 5′-and-3′-RACE library provided by Tami McDonald (Duke University). From this a coding region was determined and an amino acid sequence deduced with the standard eukaryotic codon system. The genomic sequence encoding *Lip3* subsequently was sequenced with primers outside the coding region, Clgr Lip3 0058F (5′-GTTTGTGACCGAGGCTCTTTCATC-3′) and Clgr Lip3 1377R (5′-CTGGGCGAGGTCTGGAAAGTCAAC-3′). The *Lip3* coding sequence was cloned by homologous recombination into the plasmid p416GPD (Mumberg et al. 1995). In this plasmid *Lip3* is under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter. Forward and reverse PCR primers were designed to complement the p416GPD vector at the insert site with Phusion (New England Biolabs). High efficiency transformation was carried out according to the Clontech user manual PT3529-1. All clones were verified by sequencing. The p416GPD-2xHA plasmid without *Lip3* was transformed in to W303–1A for use as a negative control.

Enzyme assay.—Olive oil plates were created with an SC-ura base made of 9 mL SC-ura, overlain with 3 mL SC-OO. SC-OO medium was prepared by adding 7.5 mL -OO/L to SC-ura medium and homogenizing in a Waring blender on high speed 1 min and placing on a heated stir plate at 60 C for 5 min to reduce foaming.

Before the enzyme assay yeast were grown in liquid SC-ura medium containing 2% glucose at 200 RPM and 37 C for 3 d. Culture density was measured with a Hausser Scientific-improved Neubauer haemocytometer and a light microscope. Spots of 50 000 cells in 10 μL were pipetted onto olive oil assay plates. Yeast cells were grown on SC-OO plates in a 37 C incubator 19 d. Plates were photographed, colonies were washed from the plates with 95% ethanol and the plates photographed again to observe lipase activity.

Protein characterization.—Protein molecular weight was determined with the protein molecular weight module available at Stothard's sequence manipulation suite (Stothard 2000). Signal peptide sequences and subcellular location prediction were determined with the N-terminal signal sequence prediction algorithms, TargetP 1.1 (Emanuelsson et al. 2000) and SignalP3.0 (Bendtsen et al. 2004). Predicted protein secondary and tertiary structure was determined with the SCRATCH protein predictor (Cheng et al. 2005). Multiple sequence protein alignment carried out at the MUSCLE Web service at EBI (Edgar 2004).

RESULTS

Filter culture observations.—All filter cultures visibly increased in size over the given developmental timeframes. *Asterochloris* sp. grew well regardless of treatment and appeared to grow the most in stage 1 symbiosis. *Cladonia grayi* grew best in stage 2 symbiosis, with markedly little growth in stage 2 aposymbiosis. Stage 2 symbiosis between *Asterochloris* sp. and *C. gray* was visible with light microscopy and lactophenol blue staining as an entanglement of hyphae and algae that were not separated by simple agitation or force.

Library construction and gene annotation.—Stage 1-specific SSH-cDNA libraries were created for *Cladonia grayi* and *Asterochloris* sp. Five hundred seventy-six randomly chosen fungal clones were sequenced. Five hundred twenty-two clones contained non-vector inserts 100–1510 bp, representing 420 unique gene regions. Based on a BLASTX comparison, 196 unique sequences (47%) significantly matched at least one entry in the GenBank nr database of protein sequences, e-value $< 10^{-5}$.

Four hundred eighty randomly chosen algal clones were sequenced. Four hundred thirty-five clones contained non-vector inserts 100–1173 bp, representing 369 unique sequences. Of these 121 (33%) significantly matched at least one entry in the GenBank nr protein database with a BLASTX comparison with an e-value $< 10^{-5}$.

A stage 2 specific SSH-cDNA library was constructed from 960 randomly chosen and sequenced clones. Eight hundred seventy-four clones contained non-vector inserts more than 100 bp and up to 1515 bp long, representing 674 unique gene regions. Three hundred seventy-seven unique sequences significantly matched at least one GenBank nr protein database entry (e-value $< 10^{-5}$). Of these 240 (64%) were of fungal origin, 118 (31%) were of algal origin and 19 (5%) were classified as ''either''.

The subset of SSH genes tested by qPCR gave a direct estimate of SSH efficiency. When looking at all fungal genes whose symbiotic/aposymbiotic expression ratio is higher than 1, 45% and 50% of fungal genes are upregulated in stages 1 and 2 (FIGS. 1, 2) respectively, and 28% and 47% of stage 1 and 2 algal genes (FIGS. 3, 4). However the discussion is limited to genes whose qPCR expression ratio is at least 2.

qPCR of select genes.—qPCR was performed to confirm differential expression of 21 and 20 fungal genes in the first and second stages of lichen resynthesis respectively. A list of these genes, their putative function and their top GenBank hits are included (TABLES I, II). Genes were chosen based on their putative function and a homologue's previous implication in plant and fungal interactions or general stress and defense responses, as well as genes of unknown function. In a similar manner qPCR was performed to confirm

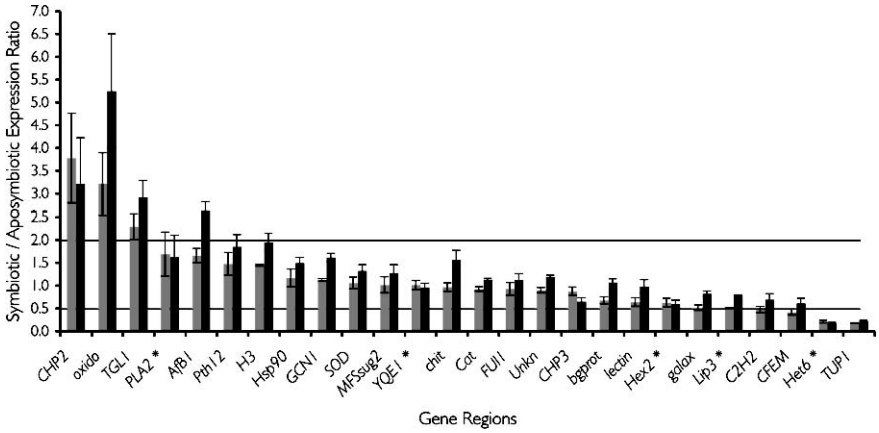

FIG. 1. Expression of 26 SSH-selected *Cladonia grayi* genes in stage 1 of lichen development (assayed by qPCR after 9 d symbiotic but segregated growth). Light gray bars correspond to expression levels normalized to *actin*, and dark gray bars to *beta-tubulin*. * denotes genes upregulated in stage 2 (and confirmed by qPCR) and tested by qPCR in stage 1. Standard error shown. Solid lines at 2 and 0.5 demark cutoff values above and below which expression levels were considered significantly different.

differential expression of 18 and 17 algal genes in the first and second stages of lichen synthesis respectively. A list of these genes, their putative function and top GenBank hit are included (TABLES III, IV). When the same gene is represented in more than one library, different GenBank accession numbers correspond to sequences from different libraries. The NRT controls of all fungal and algal genes showed at least a 32-fold lower background than in the same treatment using reverse transcriptase.

Relative expression levels of three fungal and four algal putative control genes were inspected because there was no prior knowledge of the expression of these genes. The fungal genes for actin (*act*), beta-tubulin (*βtub*) and translation elongation factor 1-alpha (*ef1α*) were normalized to each other to look for consistencies in expression level. In a similar manner the algal genes for actin (*act*), the rubisco small subunit (*RbcS*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were inspected. Fungal *act* and β*tub* behaved consistently relative to each other across replicates and treatments. On average the β*tub/act* expression ratio (± standard error) was 1.16 ± 0.37 in the aposymbiotic and 1.44 ± 0.45 in the symbiotic state. The expression level of *ef1α* varied within experimental replicates across multiple runs and therefore was dismissed as a control gene. Algal *act* and *GAPDH* behaved in a relatively similar manner across both experimental replicates in both treatments when compared to each other. On average

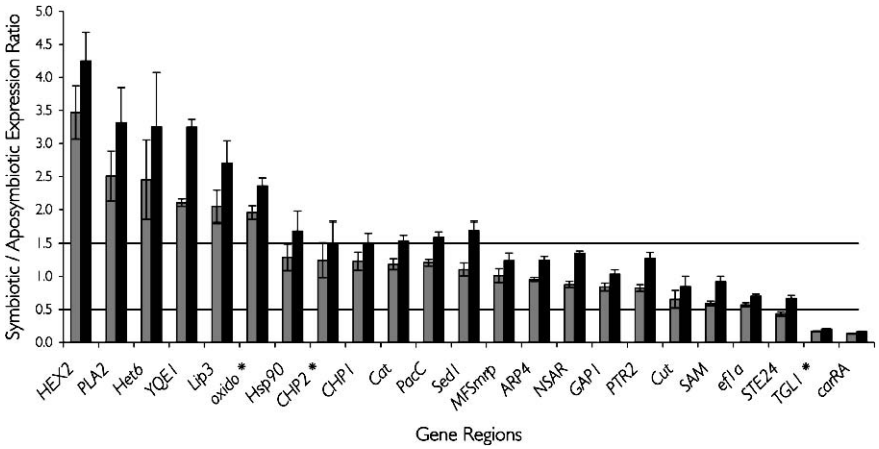

FIG. 2. Expression of 23 SSH-selected *Cladonia grayi* genes in stage 2 of lichen development (assayed by qPCR after 21 d symbiotic contact growth). Light gray bars correspond to expression levels normalized to *actin*, and dark gray bars to *beta-tubulin*. * denotes genes upregulated in stage 1 (and confirmed by qPCR) and tested by qPCR in stage 2. Standard error shown. Solid lines at 2 and 0.5 demark cutoff values above and below which expression levels were considered significantly different.

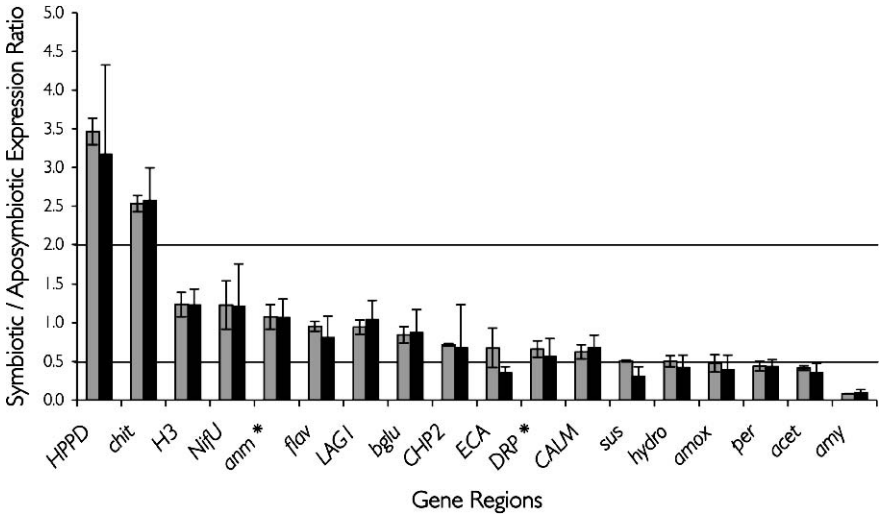

FIG. 3. Expression of 18 SSH-selected *Asterochloris* sp. genes in stage 1 of lichen development (assayed by qPCR after 9 d symbiotic but segregated growth). Light gray bars correspond to expression levels normalized to actin, and dark gray bars to GAPDH. * denotes genes upregulated in stage 2 (and confirmed by qPCR) and tested by qPCR in stage 1. Standard error shown. Solid lines at 2 and 0.5 demark cutoff values above and below which expression levels were considered significantly different.

GAPDH was expressed 1.14 ± 0.08 times the expression level of *act* in the aposymbiotic state and 1.09 ± 0.20 in the symbiotic state (standard error reported). As *RbcS* was expressed at least 20 times or greater than *actin*, which in itself is much higher than most of the genes being tested herein, *RbcS* was not considered further as a control gene.

Fungal qPCR.—Of 21 stage 1 fungal genes screened (FIG. 1), three genes (a conserved hypothetical protein 2 [*CHP2*], a D-arabitol dehydrogenase [*oxido*] and a triacylgyceride lipase [*TGL1*]) show at least a twofold increase in expression level in the symbiotic state. Of the remaining genes 18 (82%) did not change in overall expression (expression differential between 0.5 and 2), and 1 (4%) decreased. Five additional genes identified by SSH as induced in stage 2 were tested by qPCR in stage 1 (FIG. 1) but did not show more than a twofold increase. A gene of unknown function (*unkn*) has the highest expression in stage 1 relative to controls (26.6 to 37.8) and *oxido* the lowest (0.05 to 0.23) (SUPPLEMENTAL TABLE II).

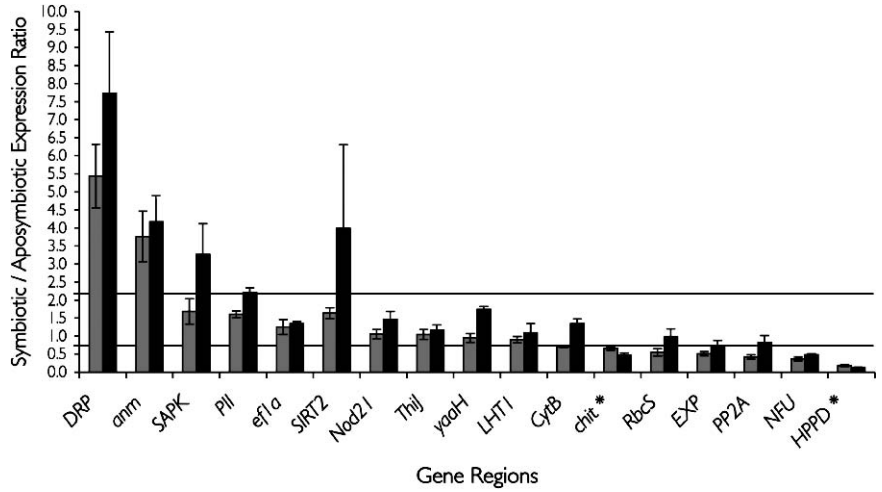

FIG. 4. Expression of 17 SSH-selected *Asterochloris* sp. genes in stage 2 of lichen development (assayed by qPCR after 21 d symbiotic contact growth). Light gray bars correspond to expression levels normalized to actin, and dark gray bars to GAPDH. * denotes genes upregulated in stage 1 (and confirmed by qPCR) and tested by qPCR in stage 2. Standard error shown. Solid lines at 2 and 0.5 demark cutoff values above and below which expression levels were considered significantly different.

TABLE I. Fungal gene regions selected from stage 1 of lichen resynthesis and analyzed by qPCR

Acronym	Putative function	GenBank accession number	Top GenBank BLASTX-hit accession and description
AfB1	Aflatoxin B1 aldehyde reductase	FJ756896	XP_001242002.1, hypothetical protein CIMG_05898 [*Coccidioides immitis* RS]
bgprot	g-protein, beta-subunit	FJ756898	EDN30908.1, guanine nucleotide-binding protein beta subunit [*Botryotinia fuckeliana* B05.10]
C2H2	C2H2 Transcription factor	FJ756899	ABH09883.1, MrgA [*Penicillium marneffei*]
Cat	Catalase	FJ756902	XP_385871.1, hypothetical protein FG05695.1 [*Gibberella zeae* PH-1]
CFEM	CFEM domain	GQ232742	EDN33600.1, predicted protein [*Botryotinia fuckeliana* B05.10]
Chit	Chitinase	FJ756903	No significant match NA
CHP2	Conserved hypothetical protein	FJ756905	XP_001224568.1, hypothetical protein CHGG_06912 [*Chaetomium globosum* CBS 148.51]
CHP3	Integral membrane protein	FJ756906	EDN27194.1, predicted protein [*Botryotinia fuckeliana* B05.10]
FUI1	Uracil Transporter	FJ756908	EAT84602.1, hypothetical protein SNOG_08326 [*Phaeosphaeria nodorum* SN15]
galox	Galactose oxidase	FJ756909	A38084, galactose oxidase (EC 1.1.3.9) precursor [validated] - fungus (*Cladobotryum dendroides*)
GCN1	GPI-anchored membrane protein	FJ756911	XP_660657.1, hypothetical protein AN3053.2 [*Aspergillus nidulans* FGSC A4]
H3	Histone 3	FJ756912	EDN09534.1, histone H3 [*Ajellomyces capsulatus* NAm1]
hsp90	Heat Shock Protein 90	FJ756916	EDN08176.1, ATP-dependent molecular chaperone HSC82 [*Ajellomyces capsulatus* NAm1]
lectin	Lectin	FJ756917	EAT77235.1, hypothetical protein SNOG_15302 [*Phaeosphaeria nodorum* SN15]
Lip3	Lipase 3	FJ756919	EAT86333.1, hypothetical protein SNOG_06502 [*Phaeosphaeria nodorum* SN15]
MFSsug2	Sugar transporter	FJ756921	EDN28217.1, hypothetical protein BC1G_08140 [*Botryotinia fuckeliana* B05.10]
oxido	D-arabinitol dehydrogenase	FJ756923	XP_503594.1, hypothetical protein [*Yarrowia lipolytica*] unnamed protein product [Yarrowia lipolytica CLIB122]
Pth12	Transcription factor	FJ756926	AAY62596.1, Pth12p [*Magnaporthe grisea*]
SOD	Superoxide Dismutase	FJ756931	Q8J0N3.2, Superoxide dismutase [Cu-Zn] copper-zinc superoxide dismutase [*Paecilomyces tenuipes*]
TGL1	Triacylglyceride lipase	FJ756932	XP_001242824.1, hypothetical protein CIMG_06720 [*Coccidioides immitis* RS]
Tup1	WD repeat transcription factor	FJ756933	XP_001257724.1, wd-repeat protein [*Neosartorya fischeri* NRRL 181]
unkn	Unknown	FJ756934	No significant match NA

Of 20 stage 2 fungal genes screened (FIG. 2), a negative regulator of glucose repressible genes (*HEX2*), a heterokaryon incompatibility locus (*Het6*), an extracellular triacylglyceride lipase (*Lip3*), a phospholipase (*PLA2*) and a dioxygnease (*YQE1*) all show at least a twofold increase in symbiotic expression. Of the remaining genes 14 (70%) did not change in expression, and one, a carotene synthesis subunit (*carRA*), decreased in expression. Three additional genes identified by SSH as induced in stage 1 were tested by qPCR in stage 2 (FIG. 2) but did not show more than an average twofold increase. In stage 2 (SUPPLEMENTAL TABLE III) a cutinase (*Cut*) has the highest expression (15.5 to 33.9), *Het6* the lowest (1.39E-02 to 5.04E-02). Based on N-terminal signal sequence prediction, the lipid metabolism proteins *Lip3* and *TGL1* are secreted and the secretion state of *PLA2* cannot be determined.

Algal qPCR.—Of 18 stage 1 algal genes tested (FIG. 3), two genes (*chit*, a chitinase-related gene, and *HPPD*, a putative 4-hydroxyphenylpyruvate dioxygenase) show at least a twofold increase in expression in the symbiotic state. Of the remaining genes 13 (71%) did not change in expression (expression

TABLE II. Fungal gene regions selected from stage 2 of lichen resynthesis and analyzed by qPCR

Acronym	Putative function	GenBank accession number	Top GenBank BLASTX-hit number and description
ARP4	Nuclear actin-related protein 4	FJ756897	XP_001819220.1, hypothetical protein [*Aspergillus oryzae* RIB40]
carRA	Carotene synthesis subunit	FJ756900	XP_383242.1, hypothetical protein FG03066.1 [*Gibberella zeae* PH–1]
Cat	Catalase	FJ756901	XP_385871.1, hypothetical protein FG05695.1 [*Gibberella zeae* PH–1]
CHP1	Conserved hypothetical protein	FJ756904	XP_001595791.1, hypothetical protein SS1G_03881 [*Sclerotinia sclerotiorum* 1980]
Cut	Cutinase	FJ756907	XP_001820550.1, hypothetical protein [*Aspergillus oryzae* RIB40] triacylglycerol lipase
ef1α	Elongation factor 1-alpha	FJ756937	ABG81871.1, translation elongation factor–1 alpha [*Canoparmelia caroliniana*]
GAP1	Amino acid permease	FJ756910	XP_001586021.1, hypothetical protein SS1G_13114 [*Sclerotinia sclerotiorum* 1980]
Het6	Heterokaryon incompatibility 6	FJ756913	XP_001909459.1, unnamed protein product [*Podospora anserina*]
HEX2	Negative regulator glucose repressible genes	FJ756914	XP_001553836.1, hypothetical protein BC1G_08029 [*Botryotinia fuckeliana* B05.10]
Hsp90	Heat shock protein 90	FJ756915	XP_001540846.1, ATP–dependent molecular chaperone HSC82 [*Ajellomyces capsulatus* NAm1]
Lip3	Lipase 3	FJ756918	XP_001796872.1, hypothetical protein SNOG_06502 [*Phaeosphaeria nodorum* SN15]
MFS1mrp	MFS1 transporter	FJ756920	XP_001549034.1, hypothetical protein BC1G_12442 [*Botryotinia fuckeliana* B05.10]
NSAR	Norsolorinic acid reductase	FJ756922	XP_961973.2, norsolorinic acid reductase [*Neurospora crassa* OR74A]
pacC	pacC, transcription factor	FJ756924	XP_001399922.1, transcription factor pacC–*Aspergillus niger*
PLA2	Phospholipase 2	FJ756925	EDU47685.1, platelet–activating factor acetylhydrolase precursor [Pyrenophora tritici–repentis Pt–1C–BFP]
PTR2	MFS Peptide transporter	FJ756927	XP_001247106.1, hypothetical protein CIMG_00877 [*Coccidioides immitis* RS]
SAM	Sterile alpha motif protein	FJ756928	XP_001591554.1, hypothetical protein SS1G_07000 [*Sclerotinia sclerotiorum* 1980]
Sed1	Cell wall glycoprotein	FJ756929	AAU07726.1, Sed1–1p [Saccharomyces pastorianus]
STE24	Sterile24	FJ756930	XP_001271650.1, CaaX prenyl protease Ste24 [*Aspergillus clavatus* NRRL 1]
YQE1	Dioxygenase	FJ756935	XP_001389856.1, hypothetical protein An01g14680 [*Aspergillus niger*]

differential 0.5–2), and three (18%; *acet, amy*, and *per*) decreased. Two genes from the stage 2 SSH library were tested as part of this set (*anm* and *DRP*) but did not show more than a twofold increase (FIG. 3). Relative expression levels of stage 1 genes are provided (SUPPLEMENTAL TABLE IV). The highest expressed gene, a putative cell wall-associated hydrolase (*hydro*), is expressed 29.47–83.28 times higher than both control genes. The lowest expressed gene, amylase (*amy*), is expressed 0.001–0.01 times the level of both controls.

Of 17 stage 2 algal genes tested (FIG. 4) a putative arginine methyltransferase (*anm*) and a putative dynamin-related protein (*DRP*) show at least a twofold increase in expression. Of the remaining genes 13 (71%) did not change in expression and two decreased in expression (*HPPD, NFU*). Two genes from the stage 1 SSH library were tested as part of this set (*chit* and *HPPD*) but did not show more than an average twofold increase in expression (FIG. 4). Relative expression levels of stage 2 genes are provided (SUPPLEMENTAL TABLE V). *RbcS* is expressed at levels 23.84–46.54 times that of the both controls. *HPPD* is the lowest tested expressed gene in stage 2 at 6.16E-04–2.62E-02 times the level of *actin* and *GAPDH*.

TABLE III. Algal gene regions selected from stage 1 of lichen resynthesis and analyzed by qPCR

Acronym	Putative function	GenBank accession number	Top GenBank BLASTX-hit number and description
amox	Amine oxidase	HM355448	YP_001866143.1, amine oxidase [*Nostoc punctiforme* PCC 73102]
amy	Alpha amylase	HM355449	CAL58037.1, alpha amylase 1 (IC) [*Ostreococcus tauri*]
bglu	Beta-glucosidase	HM355451	AAF03675.1, raucaffricine-O-beta-D-glucosidase [*Rauvolfia serpentina*]
CALM	Calmodulin	HM355452	CAL50295.1, calmodulin mutant SYNCAM9 (ISS) [*Ostreococcus tauri*]
chit	Chitinase-like protein	HM355453	NP_192013.1, glycosyl hydrolase family 18 protein [*Arabidopsis thaliana*]
CHP2	Conserved hypothetical protein	HM355454	AAG49030.1, ripening regulated protein DDTFR8 [*Lycopersicon esculentum*]
ECA	Calcium-transporting ATPase	HM355457	EAY96338.1, hypothetical protein OsI_18241 [*Oryza sativa* Indica Group]
flav	Flavoprotein	HM355460	XP_001756079.1, predicted protein [*Physcomitrella patens* ssp. *patens*]
H3	Histone 3	HM355461	CAL54140.1, Histones H3 and H4 (ISS) [*Ostreococcus tauri*]
HPPD	4-hydroxyphenylpyruvate dioxygenase	HM355462	CAR47957.1, p-hydroxyphenylpyruvate dioxygenase [*Chlamydomonas reinhardtii*]
hydro	Bacterial cell wall associated hydrolase	HM355463	ZP_01689674.1, cell wall-associated hydrolase [*Microscilla marina* ATCC 23134]
LAG1	Longevity assurance gene	HM355464	XP_001697632.1, hypothetical protein CHLREDRAFT_41962 [*Chlamydomonas reinhardtii*]
NifU	Nitrogen fixation protein nifU	HM355466	EEF47225.1, Nitrogen fixation protein nifU, putative [*Ricinus communis*]
per	Peroxisome biogenesis factor	HM355469	EEF44734.1, peroxisome biogenesis factor, putative [*Ricinus communis*]

Lip3 *nucleotide and protein sequences.*—The *Lip3* gene of *Cladonia grayi* consists of a 1066 bp open reading frame (GenBank accession number GQ232743) interrupted by two introns, 57 and 80 nucleotides long at positions 71 and 303 respectively. The encoded protein consists of 309 amino acids with a predicted molecular weight of 32.86 kDa (FIG. 5). The N-terminal region contains a deduced signal peptide, composed of the first 17 amino acid residues, and the putative cleavage site for the signal peptidase is predicted to be between residues 17 and 18. Cleavage of the signal peptide results in a peptide 292 amino acids long with a molecular weight of 31.05 kDa.

Lip3 contains conserved domains of the lipase 3 superfamily (FIG. 5). The catalytic triad Ser-Asp-His (Derewenda et al. 1992b) are found at the pre-peptide residues Ser170, Asp225, His284. *Lip3* contains an active site lid (Derewenda et al. 1992a) encoded in amino acids Val111-Asp117, which forms a short alpha helix that covers the active site in an aqueous environment. The conserved nucleophilic elbow motif GXSXG (Kohno et al. 1996) is encoded in amino acids Gly168-His169-Ser170-Leu171-Gly172. The *Cladonia grayi Lip3* pre-peptide amino acid sequence has been aligned with original peptide sequences from which conserved domains were defined (FIG. 5) (Derewenda et al. 1992a, b, 1994; Kohno et al. 1996). Forty-nine of the 292 amino acids in *C. grayi*'s post-translationally modified protein are identical to and shared between homologues in *Penicillium camembertii* (GI:999872), *Rhizomucor miehei* (GI:443485) and *Thermomyces lanuginosa* (GI:12084345).

The Saccharomyces Genome Database (SGD) was searched for extracellular triacylglyceride lipases that might interfere with the enzyme assay using BLASTP and the *Cladonia grayi Lip3* amino acid sequence, as well as with the words ''extracellular'' and ''lipase''. The best SGD match to *Lip3* is YJR107W, an uncharacterized protein with a class 3 lipase active site. This putative protein shares 27% identical residues on a 206 amino acid stretch with *Lip3* but is predicted to be intracellular. No genes for extracellular triacylglyceride lipases are present in the yeast genome.

Heterologous expression.—To determine whether *Lip3* from *Cladonia grayi* is secreted and lipolytically active in vitro the yeast heterologous expression system was

TABLE IV. Algal gene regions selected from stage 2 of lichen resynthesis and analyzed by qPCR

Acronym	Putative function	GenBank accession number	Top GenBank BLASTX-hit number and description
anm	Protein arginine methyltransferase	HM355450	XP_001702822.1, protein arginine N-methyltransferase [*Chlamydomonas reinhardtii*]
CytB	Cytochrome B	HM355455	NP_042262.1, cytochrome b [*Prototheca wickerhamii*]
DRP	Dynamin-related protein	HM355456	XP_001700931.1, dynamin-related GTPase [*Chlamydomonas reinhardtii*]
ef1α	Translation elongation factor 1- alpha	HM355458	ACL97363.1, elongation factor-1 alpha [*Ignatius tetrasporus*]
EXP	Expansin	HM355459	XP_001767753.1, predicted protein [*Physcomitrella patens* ssp. *patens*]
LHT1	Amino acid transporter (lysine-histidine)	HM355465	CAD89802.1, histidine amino acid transporter [*Oryza sativa* (indica cultivar-group)]
NFU	Iron-sulfur cluster assembly	HM355467	XP_001690805.1, iron-sulfur cluster assembly protein [*Chlamydomonas reinhardtii*]
Nod21	Nodulin 21	HM355468	XP_001700022.1, hypothetical protein CHLREDRAFT_112030 [*Chlamydomonas reinhardtii*]
PII	Nitrogen regulatory protein	HM355470	XP_001703658.1, nitrogen regulatory protein PII [*Chlamydomonas reinhardtii*]
PP2A	Protein phosphatase 2A	HM355471	XP_001752983.1, predicted protein [*Physcomitrella patens* ssp. *patens*]
RbcS	RuBisCO small subunit	HM355472	ACF16408.1, chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit [*Myrmecia incisa*]
SAPK	Serine/threonine protein kinase	HM355473	ACD44937.1, osmotic stress/ABA-activated protein kinase [*Zea mays*]
SIRT2	NAD-dependent deacetylase, sirtuin 2	HM355474	XP_001419772.1, predicted protein [*Ostreococcus lucimarinus* CCE9901]
ThiJ	ThiJ/PfpI domain protein	HM355476	XP_001759218.1, predicted protein [*Physcomitrella patens* ssp. *patens*]
yaaH	GPR1/FUN34/yaaH family of multi-pass cell membrane proteins	HM355477	XP_001835110.1, predicted protein [*Coprinopsis cinerea* okayama7#130]

used with an olive oil plate assay. Extracellular lipolytic activity is detectable by substrate hydrolysis, which causes a halo-like clearing in olive oil-supplemented agar. Yeast transformed with *Lip3* secreted a functional lipase as shown by a halo on assay plates, as compared to yeast transformed with an expression vector without *Lip3*, and lacked this halo when grown on olive-oil plates (FIG. 6). *Lip3* expression did not appear to inhibit yeast survival or growth because colony size remained constant regardless of treatment (data not shown).

DISCUSSION

The upregulation of novel fungal and algal genes in stages 1 and 2 of lichen development were identified with SSH and verified with qPCR. *Cladonia grayi* genes upregulated in symbiosis show significant matches to genes putatively encoding proteins involved in fungal self and non-self recognition, lipid metabolism and negative regulation of glucose repressible genes, as well as to a D-arabitol oxidoreductase, a dioxygenase, and a conserved hypothetical protein. *Asterochloris* sp. genes upregulated in symbiosis include putative homologues of a chitinase-related gene, an amino acid transporter, a protein arginine methyltransferase and a dynamin-related protein. These results include the first evidence that mycobionts and photobionts can communicate without direct cell-to-cell contact.

Most of the tested genes are never completely silent, and relatively limited changes in gene expression characterize both the transition from aposymbiotic to early symbiotic growth, as well as the developmental transition from stage 1 to stage 2. Although the expression of uniquely symbiosis-specific genes was not detected (such genes could be among the SSH-cDNAs that lacked GenBank matches), it appears that lichen symbiosis development relies on, at least in part, a set of genes that are common to many taxa and to varying metabolic pathways (TABLES I–IV). This is similar to other mutualistic symbiotic interac-

```
                                    v
Cladonia grayi                1 MFKIAAAITFFLSSTLAGPVQSRSIDSTTFANLQLFEQFAAAAYCPGNNNITKGGTKLSC  60
Penicillium camembertii       1 ------------------------DVSTSELDQFEFWVQYAAASYYEADYTAQVGDKLSC  36
Thermomyces lanuginosa        1 -------------------------EVSQDLFNQFNLFAQYSAAAYCGKNNDAPAGTNITC  36
Rhizomucor miehei             1 ------------------SINGGIRAATSQEINELTYYTTLSANSYCRT---VIPGATWDC  40
                                                                                  *   *

Cladonia grayi               61 PLSNNCPLVEADDVTTVYEFENSLLTDVTGYVAVDNTRSLTVLAFRGSESVRNFLADADF 120
Penicillium camembertii      37 S-KGNCPEVEATGATVSYDFSDSTITDTAGYIAVDHTNSAVVLAFRGSYSVRNWVADATF  95
Thermomyces lanuginosa       37 T-GNACPEVEKADATFLYSFEDSGVGDVTGFLALDNTNKLIVLSFRGSRSIENWIGNLNF  95
Rhizomucor miehei            41 I---HCDATEDLKIIKTWS---TLIYDTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTF  94
                                      *   *                *     *           **** *  *      *

Cladonia grayi              121 -PTVPTDICSGCEADQGFYNSWLEARTDVTSALHSAAAANPSYKVVVVGHSLGGAIAAIA 179
Penicillium camembertii      96 —VHTNPGLCDGCLAELGFWSSWKLVRDDIIKELKEVVAQNPNYELVVVGHSLGAAVATLA 154
Thermomyces lanuginosa       96 DLKEINDICSGCRGHDGFTSSWRSVADTLRQKVEDAVREHPDYRVVFTGHSLGGALATVA 155
Rhizomucor miehei            95 VPVSYPPV-SGTKVHKGFLDSYGEVQNELVATVLDQFKQYPSYKVAVTGHSLGGATVLLC 153
                                          *     **  *                    * *     ***** *

Cladonia grayi              180 AAEI--RDQGT-DADLYTY--GQPRIGGSTISSYITNQNKGGNFRVT-HYDDPVPRLPPL 233
Penicillium camembertii     155 ATDL--RGKGYPSAKLYAY--ASPRVGNAALAKYITAQ--GNNFRFT-HTNDPVPKLPLL 207
Thermomyces lanuginosa      156 GADL--RGNGY-DIDVFSY--GAPRVGNRAFAEFLTVQTGGTLYRIT-HTNDIVPRLPPR 209
Rhizomucor miehei           154 ALDLYQREEGLSSSNLFLYTQGQPRVGDPAFANYVVST--GIPYRRTVNERDIVPHLPPA 211
                                      *  *        *    ** *            *   * *    * ** **

Cladonia grayi              234 LLGFVHISPEYYID--TGNYVPVTAADIKELTGSINLLGNAGN-IGDGVDLDAHGWYFN- 289
Penicillium camembertii     208 SMGYVHVSPEYWIT--SPNNATVSTSDIKVIDGDVSFDGNTGTGLPLLTDFEAHIWYFV- 264
Thermomyces lanuginosa      210 EFGYSHSSPEYWIK--SGTLVPVTRNDIVKIEG---IDATGGNNQPNIPDIPAHLWYFG- 263
Rhizomucor miehei           212 AFGFLHAGEEYWITDNSPETVQVCTSDLET--------SDCSNSIVPFTSVLDHLSYFGI 263
                                    *  *   ** *         *   *                    *  **

Cladonia grayi              290 NISSCDTSDSLEFKRAVISG 309
Penicillium camembertii     265 QVDAGK-GPGLPFKRV---- 279
Thermomyces lanuginosa      264 LIGTCL-------------- 269
Rhizomucor miehei           264 NTGLCT-------------- 269
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FIG. 5. Alignment of *Cladonia grayi* Lip3 pre-peptide amino acid sequence with original post-translationally modified peptides from which conserved regions were defined: *Penicillium camembertii* (GI:999872), *Thermomyces lanuginose* (GI:12084345), and *Rhizomucor miehei* (GI:443485). Signal cut site of *C. grayi* marked with a v between residues 17 and 18. Catalytic triad (Ser, Asp and His) in underlined boldface. Active site flap/lid, as defined by an alpha helix in the protein's secondary structure, boxed in boldface. Nucleophilic elbow motif Gly-His-Ser-Leu-Gly highlighted in gray. Amino acids conserved among all taxa marked with asterisks.

tions between mycorrhizal fungi and their hosts (Duplessis et al. 2005, Johansson et al. 2004, Le Quéré et al. 2005, Martin et al. 2008, Martin and Nehls 2009, Peter et al. 2003, Polidori et al. 2002, Voiblet et al. 2001). In both mycorrhizal and lichen symbiosis, development appears to involve epistatic interactions between genes showing slight quantitative variation. Our methods cannot show the direct role of each gene in lichen symbiosis. Instead the results of this investigation are a resource of candidate genes for building hypotheses and future testing. The discussion therefore is restricted to those genes.

Fungal genes.—Three of the upregulated fungal genes appear to be involved in lipid metabolism, *TGL1, Lip3* (both are putative triacyglyceride lipases and predicted to be secreted) and *PLA2* (a putative intracellular phospholipase). Upregulation of *TGL1* is limited to stage 1, suggesting a role for this lipase when the fungus and alga are close enough to sense each other through diffusible signals yet are not in cell-to-cell contact. Conversely *Lip3* and *PLA2* are strongly induced in stage 2 (FIG. 2), where *PLA2*'s expression is 50% more than its level in stage 1 (FIG. 1). Unlike most other tested genes, the aposymbiotic expression of both *Lip3* and *PLA2* increases between days 9 and 21 (SUPPLEMENTAL TABLES II, III). However their symbiotic induction surpasses this aposymbiotic baseline increase.

Due to the important role of extracellular lipases in plant-fungal interactions, the candidate gene *Lip3* was chosen for further characterization. Lipases play a role in general fungal nutritional lipid digestion and development, in growing arbuscular mycorrhizae lipid metabolism is correlated with carbon allocation (Bago et al. 2002) and in pathogenic fungi extracellular lipases are involved in host cell and tissue

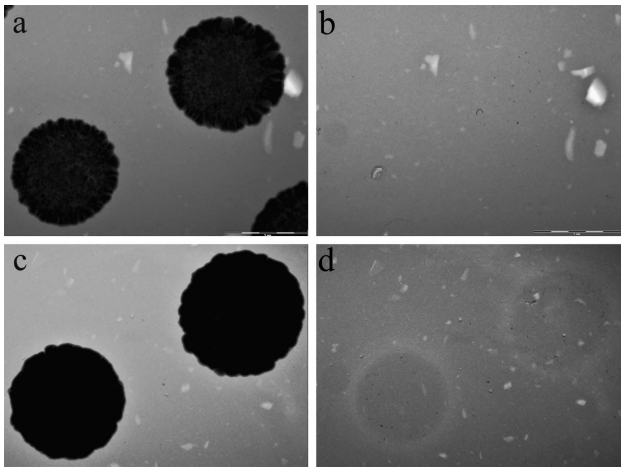

FIG. 6. Heterologous expression of Lip3 in yeast. a. Yeast transformed with expression vector without Lip3. b. Plate washed of yeast without Lip3. c. Yeast transformed with Lip3. d. Plate washed of yeast with Lip3, zone of hydrolysis visible as halo. In b and d compare abnormalities in agar with a and c to determine previous location of yeast. The original images of both b and d have had their sharpness adjusted + 40%.

adhesion and penetration (Berto et al. 1999, Chu et al. 2008, Comménil et al. 1998, Voigt et al. 2005). Extracellular lipases are thought to aid in host penetration through destruction of the secreted waxy outer layer of plants known as the cuticle (Berto et al. 1999, Voigt et al. 2005). Aquatic and terrestrial green algae are not known to produce a cuticle layer (Raven 1977), and the cell walls of closely related green algae are thought to be made of non-lipid derived cellulose, glycoproteins and polysaccharides (Domozych et al. 1980, Konig and Peveling 1984). Although lichen-forming green algae related to *Asterochloris* are known to produce large amounts of triacylglycerols, these are presumably storage products (Guschina et al. 2003) and not in their cell membrane. In lichen development secreted mycobiont lipase targets could be lipids released when *Asterochloris* sporangial mother cells lyse to generate auto- and zoospores or they could be of fungal origin. The algal cell wall becomes covered by a fungal secreted mixture of lipids and phenolic secondary metabolites (Honegger 1990). Changing morphogenetic needs could require the action of fungal lipases on this mixture. Due to its potential importance in lichen development, the functionality of one of the upregulated lichen lipases, *Lip3*, was tested by heterologous expression in yeast. This showed that the *C. grayi Lip3* gene produces a functional secreted lipase.

Self and non-self recognition in filamentous ascomycetes is mediated in part by proteins containing Het domains (Glass and Kaneko 2003). Het domain proteins also have been suggested to play a role in development and resistance to pathogens (Fedorova et al. 2005). The approximate 15-fold swing in expression (from fivefold repression in stage 1 to threefold induction in stage 2 (SUPPLEMENTAL TABLES II, III) suggests that the Het domain containing *Het-6* gene region in *Cladonia grayi* participates in the recognition process of lichen thallus formation between *C. grayi* and *Asterochloris* sp.

oxido, a putative D-arabitol dehydrogenase, was chosen based on its possible role in fungal carbohydrate metabolism. Symbiotic (and not aposymbiotic) lichen algae leak carbohydrates into the extracellular matrix shared with mycobionts (Green and Smith 1974, Richardson and Smith 1968, Richardson et al. 1968). In trebouxioid algae such as *Asterochloris* sp. this carbohydrate is mainly the sugar-alcohol ribitol. Once taken up by the mycobiont, ribitol is converted to arabitol and mannitol through the pentose phosphate pathway (Lines et al. 1989). This differs from the more often studied symbiotic mycorrhizal fungi that take up glucose and fructose from their symbiotic partners and use different carbon metabolic pathways in their symbiosis (Deveau et al. 2008, Nehls et al. 2010). The expression of *oxido* increases 1.95–5.25-fold in stages 1 and 2, suggesting that *Cladonia grayi* anticipates and/or senses algal secretion of ribitol even before cell-to-cell contact and that the effect persists after contact.

A significant increase in *Cladonia grayi* stage 2 expression of *HEX2* was observed. The *HEX2* homologue in *Saccharomyces cerevisiae*, *REG1*, is necessary to repress genes involved in alternative carbohydrate metabolism when glucose, the preferred carbohydrate of *S. cerevisiae*, is present (Niederacher and Entian 1987). The fact that *C. grayi* might use a similar pathway in turning off general sugar metabolism genes used in foraging when polyols from the alga become available is consistent with the upregulation of *HEX2*. Sugar metabolism genes downregulated by *HEX2* might be examples of the gene repression suggested to occur during symbiosis by Armaleo and Miao (1999) and observed in a number of unidentified genes by Trembley et al. (2002). Although *oxido* and *HEX2* both are connected to changes in carbohydrate metabolism, *HEX2* begins to respond only in stage 2 while *oxido* is already activated in stage 1.

In addition to the low number of true positives recovered through SSH and verified by qPCR, our ability to find candidate symbiosis genes for *Asterochloris* sp. was limited by an overall shortage of green algal gene information available in public databases and warrants further green algal based genetic symbiosis studies and genome sequencing. For

example only 31% of stage 1 *Asterochloris* sp. sequences had significant homology to the nonredundant set of proteins in GenBank, in contrast to 50% for *Cladonia grayi*.

Algal genes.—The *Asterochloris chit* gene sequence is upregulated in stage 1 of lichen development and belongs to the GH18 chitinase-like superfamily of glycosyl hydrolases. Plant chitinases (EC 3.2.1.14) hydrolyze the β-1,4-glycoside bond found in chitin, and chitin-like molecules found in fungal cell walls and bacterial Nod factors (Kasprzewska 2003). Although it is not known whether *chit* acts as a chitinase in *Asterochloris*, its activation raises the interesting possibility that chitin signaling might play a role in lichen development. In some symbiotic interactions with fungi, the hydrolyzed targets of secreted Chloroplastida chitinases can act as signals in a receptor-mediated pathway that a symbiont (either pathogenic or mutualistic) is present (Kasprzewska 2003). The putative chitinase in *Asterochloris* sp. is upregulated only in stage 1, suggesting that chitin signaling is important only in the very initial interaction between symbionts. This temporal expression is similar to that seen in *Lotus japonicus* after it has been grown with either of its mutualistic partners, the fungus *Glomus mossae* or the bacterium *Rhizobium* (Deguchi et al. 2007). The possibility that *Asterochloris* uses chitin signaling can be tested by surveying the transcriptional changes that occur on in vitro application of purified chitooligosaccharides.

The *anm* gene sequence in *Asterochloris* sp. is upregulated in stage 2 (FIG. 4) and shows high similarity with other protein arginine methyltransferases, such as with *Chlamydomonas reinhardtii* (XP 001702822.1) and *Arabidopsis thaliana* (NP 563720.1). The function of arginine methylation has been investigated primarily in histone modifications and epigenetics, although it also has been shown to be involved in RNA processing and export, translation, signal transduction, DNA repair and protein-protein interactions (Pahlich et al. 2006). Epigenetic changes during symbiotic development are likely and could be tested with chromatin immunoprecipitation-based technology.

We chose SSH to investigate differential gene expression in lichen development because we lack common tools available to model systems such as microarrays and genetic transformation. Studies have found 25–38% of SSH generated candidates show more than a twofold induction when tested with qPCR (Caturla et al. 2002, Herrero et al. 2007, Nowak et al. 2004). In the present study the number of SSH-cDNA generated candidate genes verified by qPCR were relatively low (11–28%), although the total number of upregulated fungal genes is higher than that in the alga (16% and 28% in stages 1 and 2 for *C. grayi* respectively, as opposed to 11% and 12% in *Asterochloris*). High throughput sequencing technology was not available at the time we started this project, and no lichen symbiont genome sequence has been published to date. Our understanding of the molecular basis of lichen symbiosis will be greatly advanced by the development of genomic tools.

In conclusion the present results show that in vitro lichen resynthesis is amenable to the molecular investigation of symbiosis. Differential gene expression suggests that mycobionts and photobionts communicate both before and including cellular contact. Up- and downregulated *Cladonia grayi* and *Asterochloris* sp. genes and their putative roles in additional Fungi and Chloroplastida provide compelling models on which to build testable hypotheses in lichen development.

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