Supplementary Materials for

Niche engineering demonstrates a latent capacity for fungal-algal mutualism

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Other Supplementary Material for this manuscript includes the following:
(available at www.sciencemag.org/345/6192/94/suppl/DC1)

Tables S1 to S8 (Excel)
Movies S1 to S16

Materials and Methods

Culture Medium and Pre-culturing

Cells were cultured in a defined basic medium designed to support the growth of various algal and fungal strains. A **C**hlamydomonas-**Y**east **B**asal (CYB) medium (25 mOsm) lacking carbon or nitrogen sources, consists of the following components (per liter): 0.125 g $MgSO_4 \cdot 7H_2O$, 0.074 g $CaCl_2 \cdot H_2O$, 0.4 g K_2HPO_4, 0.18 g NaH_2PO_4, 29.2 mg EDTA, 6.6 mg KOH, 1.2 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg $ZnCl_2$, 0.06 mg H_3BO_3, 0.2 mg $CoCl_2 \cdot 6H_2O$, 0.4 mg $CuCl_2 \cdot 2H_2O$, 0.05 mg $Na_2MoO_4 \cdot 2H_2O$, 0.018 mg KBr, 0.003 mg KI, 0.0018 mg Na_3VO_4, 0.0018 mg Na_2SeO_3, and the following vitamins: 4 mg myo-inositol, 0.4 mg 4-amino benzoic acid, 0.4 mg calcium D-pantothenate, 0.4 mg niacin, 0.4 mg pyridoxine hydrochloride, 0.4 mg thiamine hydrochloride, 0.002 mg biotin, 0.002 mg cyanocobolamin, and 0.002 mg folic acid. The pH of this medium is 6.8. The designation "CYM:2% glucose + 10 mM KNO_2" is used to indicate a **C**hlamydomonas-**Y**east **M**edium (CYM) with a CYB composition supplemented with 2% glucose and 10 mM potassium nitrite (final osmolarity of ~150 mOsm). All CYB, carbon, and nitrogen stock solutions were separately autoclaved (20 min at 120 °C), 0.2 µm-filter sterilized, and aged/equilibrated at room temperature for at least 24 hrs before use.

Prior to coculturing experiments, fungal strains are cultivated in log-phase for at least 12 hrs on CYM:2% glucose + 37.4 mM NH_4Cl. *C. reinhardtii* strains are cultivated for at least 24 hrs on either CYM:20 mM Tris•HCl (pH 7.0) + 10 mM KNO_3 or a modified Tris-acetate medium, TAP* (equivalent to CYM:20 mM Tris•Acetate (pH 7.0) + "N-stock" (see below), but *without* CYB vitamins). Other algal strains were cultivated in CYM:N-stock. "N-stock" is a mixture of different nitrogen sources with a 1X composition (per liter) as follows: 0.6 g urea, 0.32 g KNO_3, 0.12 g acetamide, and 0.04 g NH_4Cl. Prior to coculturing yeast and algae, cultures of individual organisms were centrifuged at 2000 x g (algae) or 4000 x g (yeast) for 3 min to pellet cells, and cells were gently resuspended in a volume of CYB equivalent to the pre-culture volume. The cells were washed two more times, with the final volume being half of the initial volume. Algal cells were allowed to recover for 30 min in CYB under light. Cell densities of these washed cultures were then measured using a Coulter counter (Z2 analyzer; Beckman Coulter, Inc., Danvers, MA) and a stock concentration of each cell type was prepared in fresh CYB medium for coculture inoculation.

Coculturing Experiments

Fluorescently labelled prototrophic *S. cerevisiae* (S288c expressing monomeric teal fluorescent protein (26) under the ENO2 promoter at the HO locus) and intrinsically fluorescent *C. reinhardtii* (strain CC-1690) cells were cocultured in a minimal salts CYB medium supplemented with glucose and/or potassium nitrite (see Culture Medium) in 1 ml volumes in 2 ml screw-cap tubes with silicone rubber gaskets (Neptune Scientific, San Diego, CA) [Figs. 1B, 3A, and fig. S1] or in 250 µL volumes in the wells of 96-well round-bottom, polystyrene microtiter plates (well capacity ~330 µL) (Corning, product #3788; Corning Inc., Corning NY) [Figs. 2, 3B, and figs. S2, and S3]. Unless otherwise indicated, cells were inoculated at a density of 0.05×10^6 cells/ml (dashed horizontal line in Fig. 1 and fig. S1) and grown at room temperature. To maintain an airtight environment, 2 ml tubes were tightly capped and 96-well microtiter plates were sealed with a 76.2 µm thick inert polyolefin film with pressure-sensitive-release silicone adhesive (ThermalSealRTS, Excel Scientific, Victorville, CA), double-sealed

with a 127 μm polyvinylidene fluoride film (CS Hyde Company, Lake Villa, IL), covered with a 1/8" thick custom glass plate, and vacuum sealed in a 76.2 μm thick nylon/polyethylene pouch (Doug Care Equipment, Springville, CA). Standing cocultures were exposed to 45 or 110 $\mu mol/m^2/s$ (400-700 nm, photosynthetic active radiation) of continuous light from a bank of six F40T12 Soft White/ALTO tubes (Philips, Arlington, MA), appropriately attenuated using neutral density filters (Roscolux #398 Neutral Grey, 40% transmission; Rosco Laboratories, Central Falls, RI). A rotary tube mixer (model 346; Fisher Scientific, Waltham, MA) operated at 25 rpm was used for agitated coculture experiments, (Fig. 1B, condition 6).

At appropriate experimental time points, cocultured cells were thoroughly mixed by vigorous vortexing and inversion and counted by flow cytometry (LSR Fortessa; BD Biosciences, San Jose, CA); 10 μL at 0.5 μL/sec) using custom gates to discriminate yeast and algal cells based largely on CFP (ex=440 nm; em=470/20 nm) and APC-Cy7 (ex=635 nm; em=780/60 nm) fluorescence, respectively. A coculture was sacrificed for each experimental measurement made. Cell counts were corrected for aggregate particle counts (typically less than 10%) by custom gating on particle side-scatter time-of-flight values (vs. side-scatter amplitude) and through multipeak-Gaussian fits of fluorescence amplitude histograms (27) assuming ≤4 aggregate populations using IgorPro (Wavemetrics, Lake Oswego, OR). Images of cocultures were generated by adding 250 μL of a resuspended culture in a well of a 96-well round-bottom microtiter plate, centrifuging at 4000 x g for 3 min, and imaging from below using an Epson Perfection V700 Photo transparency scanner (Epson, Long Beach, CA). Other fungi and Chlamydomonas strains (see table S1) were cocultured and analyzed by similar methods. Maximum predicted cell densities for *C. reinhardtii* and *S. cerevisiae* over the gradient landscape of Fig. 2 were calculated assuming $0.54x10^{-12}$ moles of nitrogen per *C. reinhardtii* cell grown under autotrophic conditions (28, 29) and $2.5x10^{-12}$ moles of carbon per *S. cerevisiae* cell (30).

Cocultures with filamentous fungi were started from small (≤1 mm^3) hyphal fragments (dissected with sterile forceps), washed in CYB, and grown with ~$1x10^5$ cells (total) of *C. reinhardtii* in 50 ml of CYB supplemented with 2% glucose and 10 mM KNO_2 medium in 150 ml polystyrene bottles (Corning, product #431153) under 45 $\mu mol/m^2/s$. For control experiments in which the dependence of fungi on *C. reinhardtii* for nitrogen is short-circuited, 20 mM NH_4Cl was added to the culture medium. For control experiments in which the dependence of *C. reinhardtii* on fungi for carbon is short-circuited, polystyrene bottles were only loosely capped, ensuring access to ambient CO_2. For complementary control experiments in which the dependence of fungus on *C. reinhardtii* for nitrogen is short-circuited, wild-type, nitrite-positive utilizing fungal strains were used and an additional 10 mM KNO_2 was added to the culture medium. All culture inoculation and assembly steps were conducted aseptically in a tissue culture hood. Images and movies of symbiotic composite associations were acquired using a Zeiss Axio Zoom.V16, and processed using Zen 2012 (Zeiss, blue edition; Zeiss, Thornwood, NY) software.

Transmission Electron Microscopy

Fragments of fungal-algal associations of <3 mm^3 were sterilely dissected and fixed at room temperature for 6-12 hrs in 2.5% glutaraldehye + 2% paraformaldehyde (made fresh) in 1X CYB medium supplemented with 10 mM KNO_3, 62.5 mM sodium cacodylate, and 16 mM sodium phosphate at pH ~7.2. Fixed fragments were transferred to 1X CYB supplemented with 10 mM KNO_3 and 63 mM HEPES, and stored at 4°C (< 72 hrs) until further processed. Fragments were subsequently osmium fixed, resin embedded, sectioned, and imaged by standard methods (31)

using a Hitachi H-7650 transmission electron microscope (Central Electron Microscope Facility, University of Connecticut Health Center, Farmington, CT). Cell wall thicknesses reported in Fig. 3 (mean ± SD) were determined by 25 independent measurements of randomly chosen cell wall cross-sections using the image processing suite Fiji (32).

Phylogenetic Trees

Evolutionary trees of Fig. 3 were adapted and modified from published analyses of ascomycetous fungi (33, 34) and algae (35). Dating of fungal branch points were obtained from (36-38) and for algal branch points from (39-41). References for annotated species characteristics (e.g., Crabtree-positive = ability to ferment in the presence of oxygen) can be found in table S1.

Fig. S1

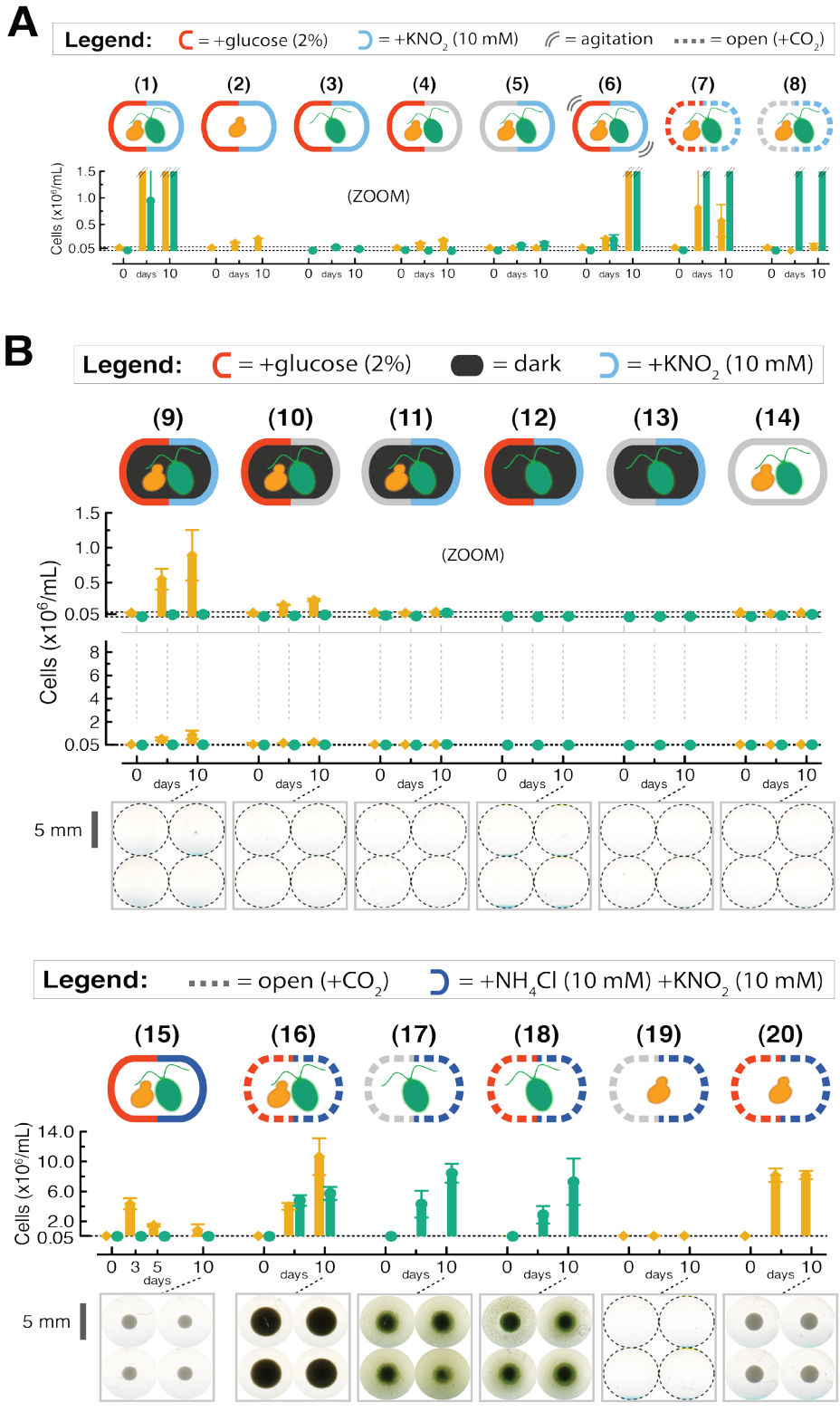
A
Legend: = +glucose (2%) = +KNO2 (10 mM) = agitation = open (+CO2)
(1) (2) (3) (4) (5) (6) (7) (8)
Cells (x10^6/mL)
1.5
1.0
0.5
0.05
(ZOOM)
0 days 10
B
Legend: = +glucose (2%) = dark = +KNO2 (10 mM)
(9) (10) (11) (12) (13) (14)
Cells (x10^6/mL)
1.5
1.0
0.5
0.05
(ZOOM)
8
6
4
2
0.05
0 days 10
5 mm
Legend: = open (+CO2) = +NH4Cl (10 mM) +KNO2 (10 mM)
(15) (16) (17) (18) (19) (20)
Cells (x10^6/mL)
14.0
10.0
6.0
2.0
0.05
0 3 5 10 days
0 days 10
5 mm

Fig. S1.

(**A**) Expanded view of Fig. 1B showing cell densities below $1.5x10^6$/mL. (**B**) Proliferation of *S. cerevisiae* and *C. reinhardtii* under additional coculture conditions. See Fig. 1B legend for details. Conditions 9 through13 were cocultured in the absence of light. Although *C. reinhardtii* does not assimilate CO_2 or proliferate (substantially) on nitrite without light (14, 42), the alga generates sufficient light-independent nitrite reductase activity and ammonia production exists to support residual growth of *S. cerevisiae* in the dark. Conditions 15 through 20 include 10 mM ammonium chloride as a nitrogen source, which would allow *S. cerevisiae* to grow independently of *C. reinhardtii*. Condition 15 leads to loss of the alga; budding yeast rapidly outproliferates the obligately dependent *C. reinhardtii* (although cell debris is accumulated; cf. image of pelleted cells for condition 15). Note different y-axis scales for different parts of the figure.

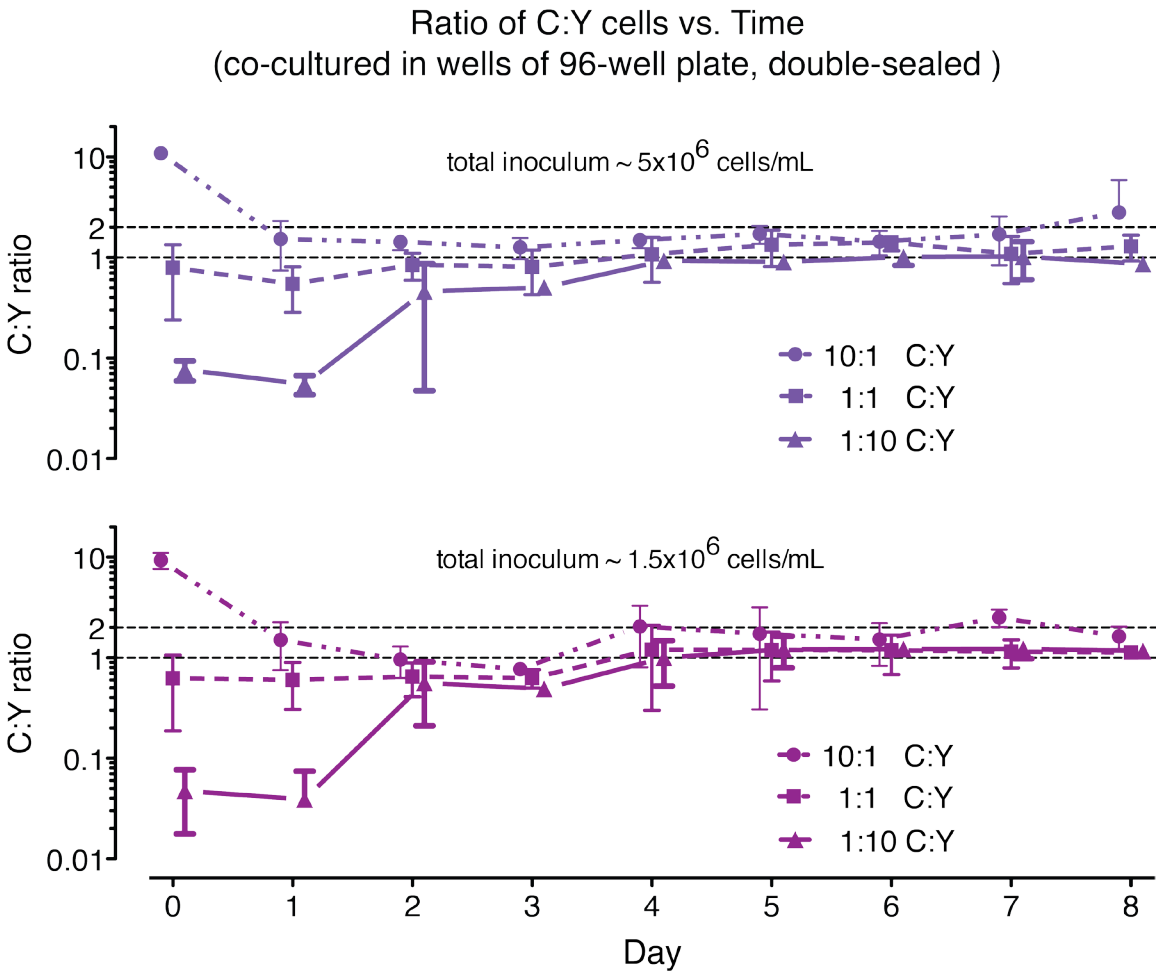

Fig. S2.

The ratio of *C. reinhardtii* to *S. cerevisiae* cells grown in sealed vessels (in CYM:2% glucose + 10 mM KNO_2 with 45 µmol/m^2/s light) converges towards a stable ratio range over time (95% confidence intervals shown), with dashed lines for ratios of 1 and 2 drawn as a visual guide. Co-cultivation was done in wells of a vacuum-sealed, 96-well microtiter plate with initial inoculation ratios (nominally) of 10:1, 1:1, and 1:10 *C. reinhardtii* to *S. cerevisiae* (and at two different total cell inoculums: ~5x10^6 (*top*), and ~1.5x10^6 (*bottom*) cells/ml in 250 µL; N=4). The cell ratio converges to 1-2 C:Y within five days, essentially independent of initial inoculation ratio.

Fig. S3

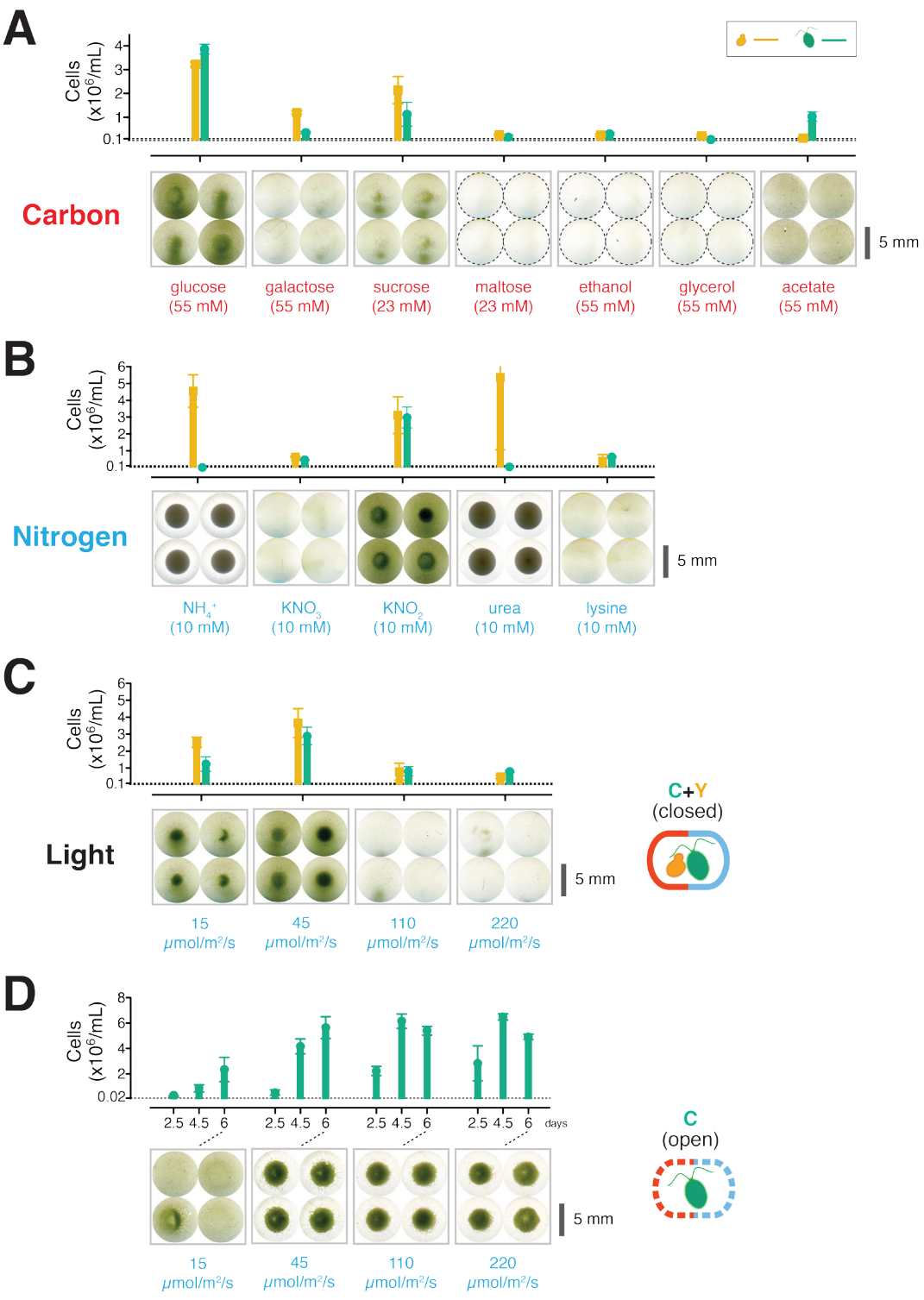
A
Cells (x10^6/mL)
4
3
2
1
0.1
Carbon
5 mm
glucose (55 mM)
galactose (55 mM)
sucrose (23 mM)
maltose (23 mM)
ethanol (55 mM)
glycerol (55 mM)
acetate (55 mM)
B
Cells (x10^6/mL)
6
5
4
3
2
1
0.1
Nitrogen
5 mm
NH4+ (10 mM)
KNO3 (10 mM)
KNO2 (10 mM)
urea (10 mM)
lysine (10 mM)
C
Cells (x10^6/mL)
6
5
4
3
2
1
0.1
Light
5 mm
15 µmol/m²/s
45 µmol/m²/s
110 µmol/m²/s
220 µmol/m²/s
C+Y (closed)
D
Cells (x10^6/mL)
8
6
4
2
0.02
2.5 4.5 6
days
5 mm
15 µmol/m²/s
45 µmol/m²/s
110 µmol/m²/s
220 µmol/m²/s
C (open)

Fig. S3.

Mutualistic growth after 6 days of coculture using different carbon and nitrogen resource nutrients, and light intensities. Unless otherwise indicated, the carbon source was 2% glucose (111 mM), the nitrogen source was 10 mM KNO_2, the irradiance was 45 μmol/m^2/s, and *S. cerevisiae* and *C. reinhardtii* were inoculated at ~0.1x10^6 cells/mL. (**A**) Carbon sources were used at the same concentration of 55 mM for comparison, with the exception of sucrose (disaccharide of glucose and fructose) and maltose (disaccharide of glucose), which were used at 23 mM. *C. reinhardtii* cannot use any of these carbon sources for growth, with the exception of acetate. Glucose is the preferred carbon source for *S. cerevisiae*, galactose is assimilated by respiro-fermentation, and ethanol, glycerol, and acetate are respired (30). Sucrose fermentation requires the induced expression of invertase, an extracellular enzyme that hydrolyzes sucrose. Although the *S. cerevisiae* strain used here has been corrected to enable maltose utilization (see table S1), the parental background may not be optimized to ferment it. (**B**) Nitrogen sources were used at the same concentration of 10 mM for comparison. *S. cerevisiae* can use NH_4^+ and urea as nitrogen sources, and by day 6, leads to the extinction of *C. reinhardtii*. Lysine is an extremely poor nitrogen source for *S. cerevisiae* (43). In the absence of preferred nitrogen sources, *C. reinhardtii* expresses an extracellular L-amino-oxidase that hydrolyzes L-lysine to α-keto-ε-aminocaproic acid, H_2O_2, and NH_3 (44-46); NH_3 becomes a public good that is subsequently imported as a nitrogen source or diffuses away. Nitrate requires an additional two-electron NAD(P)H-mediated reduction to nitrite (by nitrate reductase, which is highly regulated) before it can be further assimilated by *C. reinhardtii* (47, 48), and metabolized nitrate leads to a complex light- and CO_2-dependent release of both nitrite and ammonium into the medium (14). Unlike nitrite or ammonium, nitrate can be stored and sequestered within the vacuoles of *C. reinhardtii*. (**C**) Light intensity can modulate the productivity of mutualism, with an optimum at ~45 μmol/m^2/s, although all light levels examined lead to successful obligate mutualisms. (**D**) Proliferation of *C. reinhardtii* cells (alone) inoculated at ~0.02x10^6 cells/ml and grown under the same conditions as in (C) except with access to atmospheric CO_2. The reduced mutualistic productivity at higher light levels in (C) are not due to a decreased ability of *C. reinhardtii* cells to proliferate; in fact, higher light levels lead to faster proliferation of *C. reinhardtii* cells (see days 2.5-4.5).

Fig. S4

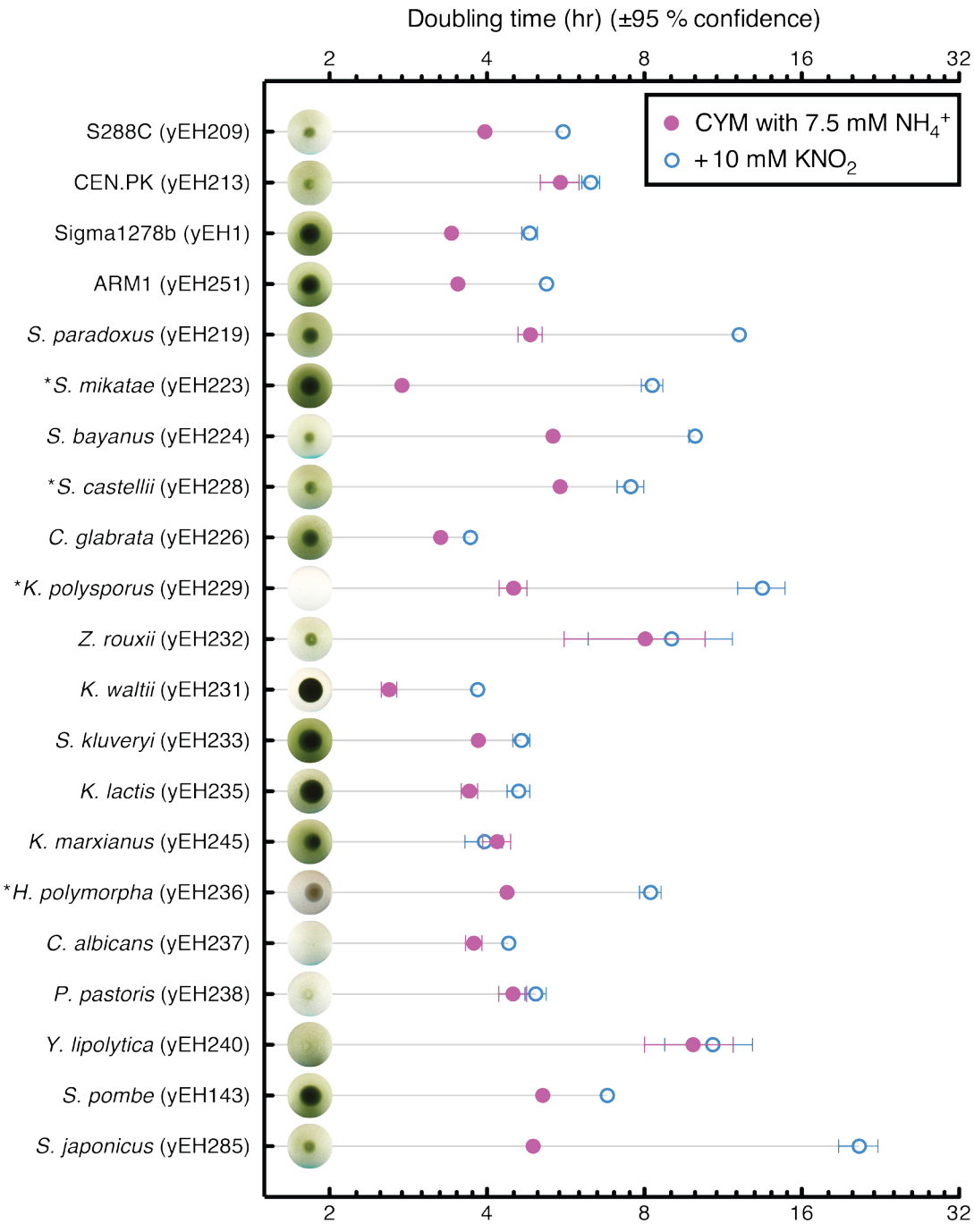
Doubling time (hr) (±95 % confidence)
2
4
8
16
32
CYM with 7.5 mM NH_4^+
+10 mM KNO_2
S288C (yEH209)
CEN.PK (yEH213)
Sigma1278b (yEH1)
ARM1 (yEH251)
S. paradoxus (yEH219)
**S. mikatae* (yEH223)
S. bayanus (yEH224)
**S. castellii* (yEH228)
C. glabrata (yEH226)
**K. polysporus* (yEH229)
Z. rouxii (yEH232)
K. waltii (yEH231)
S. kluveryi (yEH233)
K. lactis (yEH235)
K. marxianus (yEH245)
**H. polymorpha* (yEH236)
C. albicans (yEH237)
P. pastoris (yEH238)
Y. lipolytica (yEH240)
S. pombe (yEH143)
S. japonicus (yEH285)

Fig. S4.

Doubling times (with 95% confidence intervals) of different yeasts across the fungal tree of Fig. 3 with (blue) and without (purple) 10 mM nitrite present in the growth medium. Yeasts (see table S1 for full descriptions) were grown in CYM:2% glucose + 7.5 mM NH_4Cl with or without 10 mM KNO_2, and three replicate growth curves for each condition were generated using a Bioscreen C system (15 min interval measurements of 600 nm absorbance at 25 °C with shaking prior to measurement (Growth Curves USA, Piscataway, NJ) and an initial inoculum of $3x10^4$ cells/300 μL. Doubling times were determined by fitting phases of logarithmic growth by least-squares regression. Nitrite, an oxidant and common food preservative, generally inhibits growth, although to varying degrees depending on the yeast species and genetic background. Images of representative 9-day old cultures of yeast with *C. reinhardtii* (see Fig. 3A) have been superimposed for comparison with the success of mutualistic coculture in glucose and nitrite. Strains that were originally isolated from soil, a habitat in common with *C. reinhardtii*, are indicated by an asterisk.

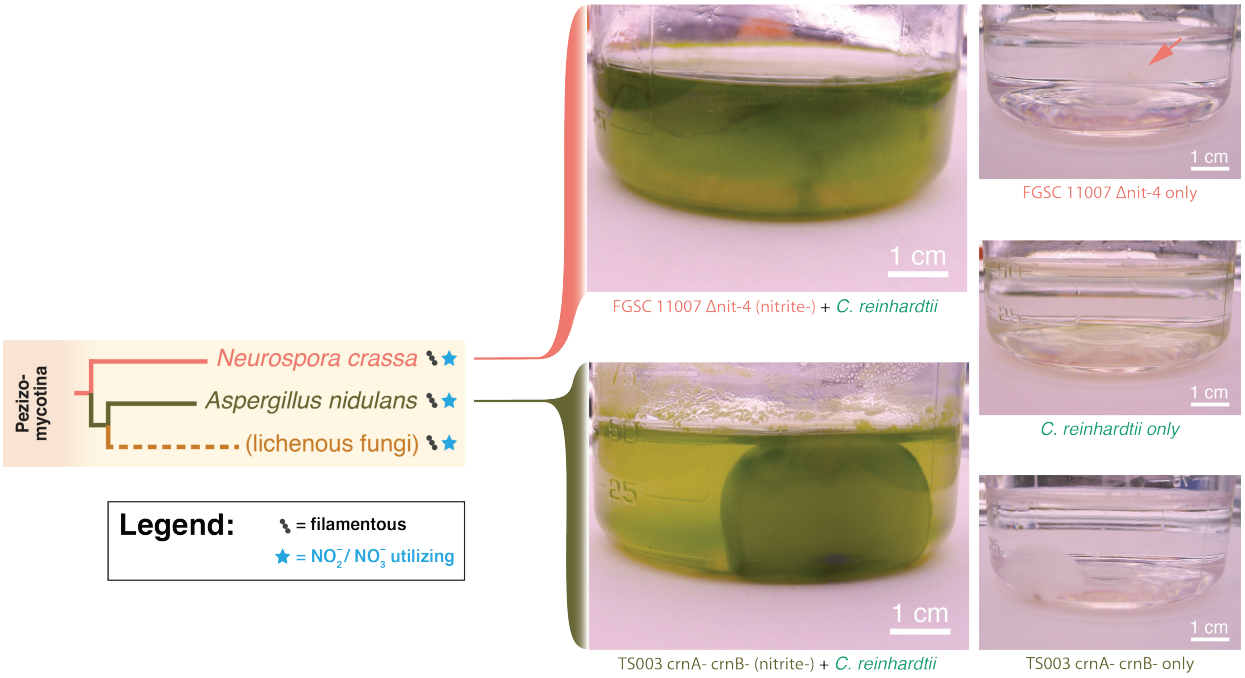

Fig. S5.

Mutualistic, symbiotic associations created between *C. reinhardtii* and model filamentous fungi carrying mutations that prevent nitrite utilization. *Left*: Annotated cladogram of showing the relationship between model filamentous fungi and lichenous fungi (cf. Fig. 3A). *Middle*: Representative 10 day-old cocultures of *C. reinhardtii* and nitrite-utilization deficient mutants of *N. crassa* (FGSC 11007 *Δnit-4*; top) or *A. nidulans* (TS003 *crnA- crnB-*; bottom). *Right*: Corresponding monocultures of *N. crassa* (top), *C. reinhardtii* (middle), and *A. nidulans* (bottom). These model filamentous fungi as well as the yeasts shown in Fig. 3 all lack a high-affinity ammonia transporter gene (MEPα) that characterizes many lichen-forming fungi (49, 50); thus, such a transporter is not essential for our synthetic fungal-algal mutualisms.

Fig. S6

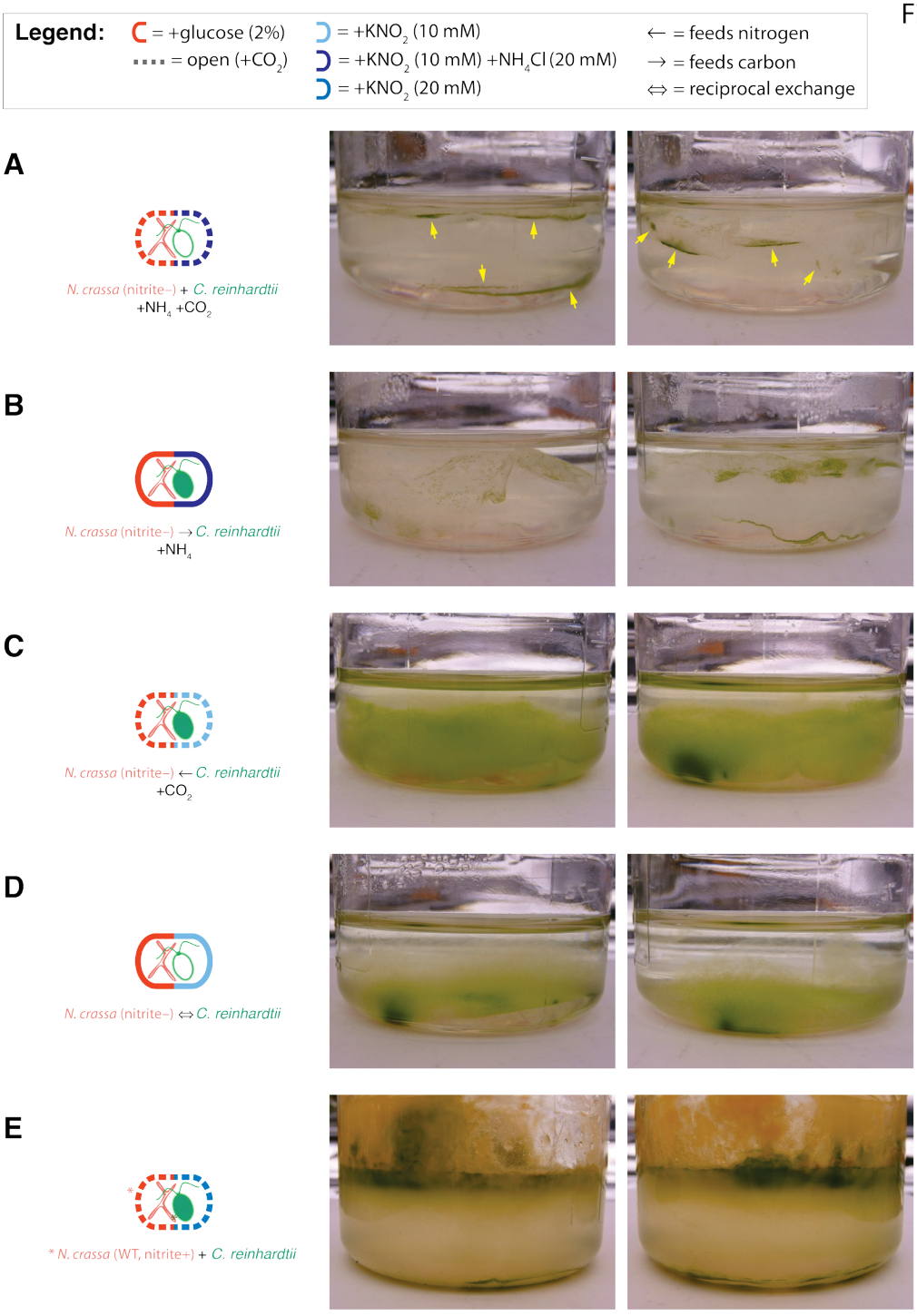
Legend:
= +glucose (2%)
= open (+CO2)
= +KNO2 (10 mM)
= +KNO2 (10 mM) +NH4Cl (20 mM)
= +KNO2 (20 mM)
← = feeds nitrogen
→ = feeds carbon
⇔ = reciprocal exchange
A
N. crassa (nitrite–) + C. reinhardtii
+NH4 +CO2
B
N. crassa (nitrite–) → C. reinhardtii
+NH4
C
N. crassa (nitrite–) ← C. reinhardtii
+CO2
D
N. crassa (nitrite–) ⇔ C. reinhardtii
E
* N. crassa (WT, nitrite+) + C. reinhardtii

Fig. S6.

Metabolic "short-circuit" experiments demonstrate that the physical association of *C. reinhardtii* to *N. crassa* occurs regardless of any obligate metabolic dependency (see corresponding movies S7 through S11). (**A**) *N. crassa* mutant (FGSC 11007 *Δnit-4*, a non-nitrite-utilizing mutant) grown in coculture for 8 days with *C. reinhardtii* (CC-1690) in CYM:2% glucose + 10 mM KNO_2 + 20 mM NH_4Cl and access to ambient CO_2; under these conditions, neither fungus nor alga requires the other for nitrogen or carbon. *N. crassa* grows faster than *C. reinhardtii*, leading to predominantly white fungal masses, some of whose edges are prominently green (yellow arrows) due to the attachment of *C. reinhardtii* (cf. movie S7). (**B**) *N. crassa nit-* mutant grown in coculture for 8 days with *C. reinhardtii* in CYM:2% glucose + 10 mM KNO_2 + 20 mM NH_4Cl and without access to ambient CO_2; under these conditions, the fungus does not require the alga for nitrogen but the alga is dependent on the fungus for CO_2. As in (A), *N. crassa* grows faster than and independent of *C. reinhardtii*, leading to a predominantly white material, with attached *C. reinhardtii* leading to green spots (left panel) and green peripheral edges (cf. movie S8). (**C**) *N. crassa nit-* mutant grown in coculture for 8 days with *C. reinhardtii* in CYM:2% glucose + 10 mM KNO_2 and access to ambient CO_2; under these conditions, the fungus requires the alga for nitrogen but the alga does not require the fungus for CO_2, producing green masses of the two organisms although the algal density is uniform (cf. movie S9). (**D**) *N. crassa nit-* mutant grown in coculture for 8 days with *C. reinhardtii* in CYM:2% glucose + 10 mM KNO_2 in a sealed environment; under these conditions, both fungus and alga are obligately dependent on one another for nitrogen and carbon (cf. fig. S5 and movies S1 through S3). This also leads to a balanced, green growth, although the overall yield is lower than (C), where *C. reinhardtii* has access to ambient CO_2 (cf. movie S10). (**E**) *N. crassa* (FGSC 2489) wild-type, nitrite-utilizing strain in coculture for 8 days with *C. reinhardtii* in CYM:2% glucose + 20 mM KNO_2 and access to ambient CO_2; under these conditions, as in (A), neither fungus nor alga requires the other for nitrogen or carbon. As in (A), *N. crassa* grows faster than *C. reinhardtii*, leading to a predominantly white fungal material below the gas-liquid interface with algal growth primarily at this interface, where there is likely greater access to ambient CO_2 (cf. movie S11). The deep orange "fuzz" along the sides of the vessel is due sporulating *N. crassa*, an indication that nitrogen has been significantly depleted in the culture medium (51).

Fig. S7

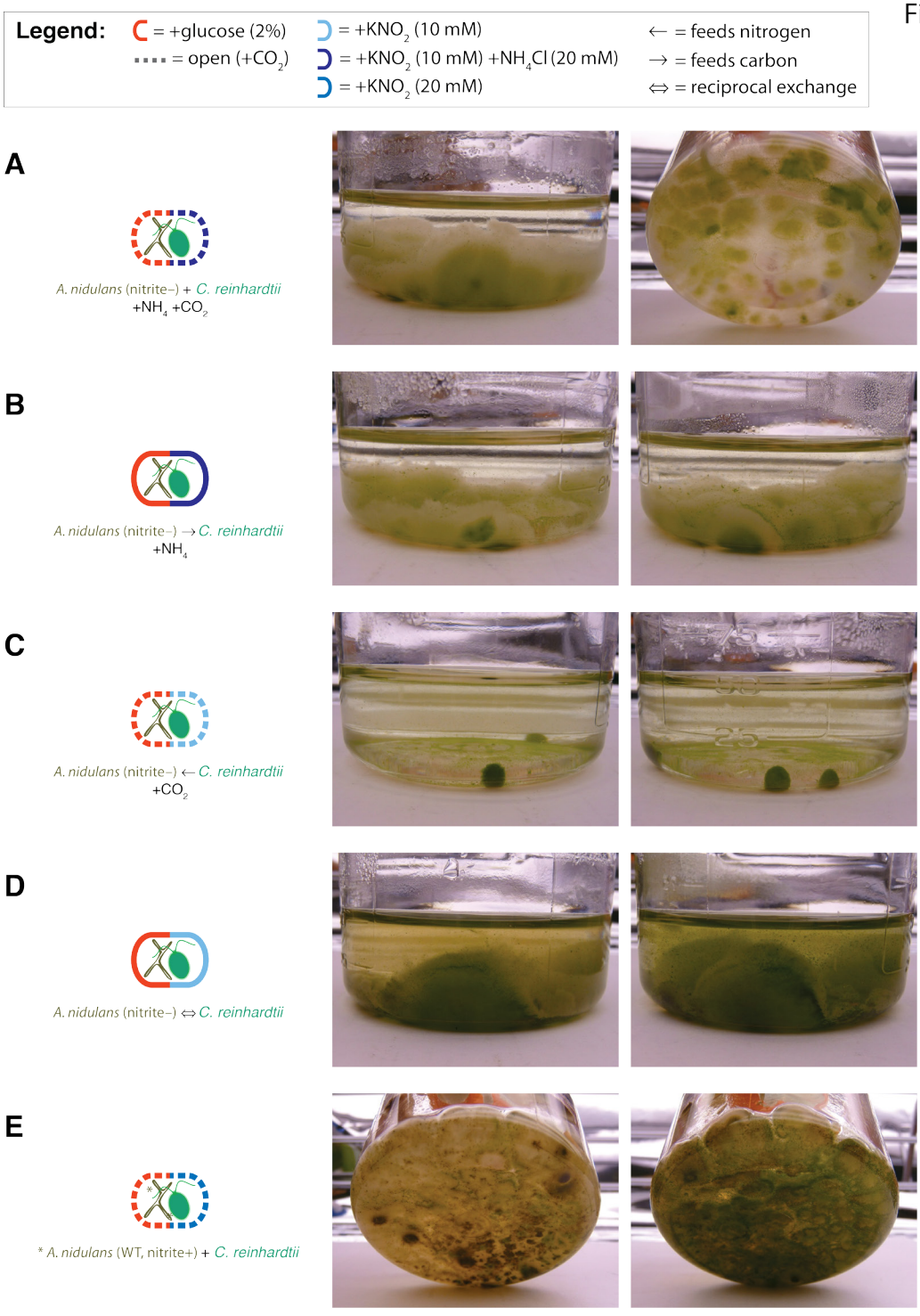
Legend:
= +glucose (2%)
= open (+CO2)
= +KNO2 (10 mM)
= +KNO2 (10 mM) +NH4Cl (20 mM)
= +KNO2 (20 mM)
← = feeds nitrogen
→ = feeds carbon
⇔ = reciprocal exchange
A
A. nidulans (nitrite–) + C. reinhardtii
+NH4 +CO2
B
A. nidulans (nitrite–) → C. reinhardtii
+NH4
C
A. nidulans (nitrite–) ← C. reinhardtii
+CO2
D
A. nidulans (nitrite–) ⇔ C. reinhardtii
E
* A. nidulans (WT, nitrite+) + C. reinhardtii

Fig. S7.

Metabolic "short-circuit" experiments demonstrate that the physical association of *C. reinhardtii* to *A. nidulans* occurs regardless of any obligate metabolic dependency (see corresponding movies S12 through S16, and Fig. 4). (**A**) *A. nidulans* (TS003 *crnA- crnB-*, a non-nitrite-utilizing mutant) grown in coculture for 8 days with *C. reinhardtii* (CC-1690) in CYM:2% glucose + 10 mM KNO_2 + 20 mM NH_4Cl and access to ambient CO_2. Under these conditions, neither fungus nor alga require the other for nitrogen or carbon, and yet composite "balls" of the two species form with a greener core and whiter periphery (cf. movie S12). (**B**) *A. nidulans nit-* mutant grown in coculture for 8 days with *C. reinhardtii* in CYM:2% glucose + 10 mM KNO_2 + 20 mM NH_4Cl and without access to ambient CO_2; under these conditions, the fungus does not require the alga for nitrogen but the alga is dependent on the fungus for CO_2. As in (A), composite balls form whose center is greener than their periphery; some *C. reinhardtii* accretion can be seen on top of the balls in the right panel (cf. movie S13). (**C**) *A. nidulans nit-* mutant grown in coculture for 8 days with *C. reinhardtii* in CYM:2% glucose + 10 mM KNO_2 and access to ambient CO_2. Under these conditions, the fungus requires the alga for nitrogen but the alga does not require the fungus for CO_2; this results in greener and more compact balls (cf. movie S14). (**D**) *N. crassa nit-* mutant grown in coculture for 8 days with *C. reinhardtii* in CYM:2% glucose + 10 mM KNO_2 in a sealed environment; under these conditions, both fungus and alga are obligately dependent on one another for nitrogen and carbon (cf. fig. S5 and movies S4 through S6). This leads to large green aggregates with concentrations of *C. reinhardtii* on their surface (cf. movie S15). (**E**) *A. nidulans* (FGSC A4) wild-type, nitrite-utilizing strain in coculture for 8 days with *C. reinhardtii* in CYM:2% glucose + 20 mM KNO_2 and access to ambient CO_2; under these conditions, as in (A), neither fungus nor alga requires the other for nitrogen or carbon. Green *C. reinhardtii* are embedded amongst *A. nidulans* hyphae (cf. movie S16), although the lack of *A. nidulans* dependence on *C. reinhardtii* leads to variable degrees of green coloration. Dark brown spots (left panel) are sites of *A. nidulans* spore formation, an indication that carbon or nitrogen has been significantly depleted in the culture medium (52).

Table S1.
Strains used in this study.

Table S2.
Data table for Fig. 1B graphs.

Table S3.
Data table for Fig. 2.

Table S4.
Data table for Fig. 3 graphs.

Table S5.
Data table for fig. S1B graphs.

Table S6.
Data table for fig. S2.

Table S7.
Data table for fig. S3 graphs.

Table S8.
Data table for fig. S4.

<u>Supplementary Movies S1 through S3</u>: Z-stack series of a representative symbiotic associations formed between *N. crassa* (*nit-*) and *C. reinhardtii* cultures under conditions that enforce mutualism. These movies serve to complement Fig. 4A in showing that the colocalization of *C. reinhardtii* on *N. crassa* hyphae is not merely due to a "hovering" of swimming algae in the vicinity; *C. reinhardtii* appear to be attached to hyphae, are resistant to gentle hydrodynamic displacement by liquid flow, and are predominantly immotile/de-flagellated.

Movie S1.
Low magnification (13X) z-stack movie series of a representative mutualistic symbiotic association formed by the interaction between *N. crassa* (FGSC 11007 *Δnit-4*, a non-nitrite-utilizing mutant) and *C. reinhardtii* (CC-1690) showing two types of organizational structure: (i) an even, dense distribution of *C. reinhardtii* cells (green) embedded within a dense meshwork of white, fine-haired fungal hyphae (top right), and (ii) less dense, peripheral regions where algal cells are concentrated into patches (left and middle). Each plane was imaged for 0.2 seconds and is 32 μm apart in the z-axis. Fungi and algae were cocultured in 50 ml of CYM:2% glucose + 10 mM KNO_2 medium in an airtight, 150 ml polystyrene container (Corning) under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). A segment of the resulting aggregate was sterilely

dissected, transferred to a plastic petri dish, bathed in CYB medium, and imaged from the top using a Zeiss Axio Zoom.V16 stereomicroscope.

Movie S2.

Z-stack image series at 50X magnification and 2X real time playback of a representative peripheral fragment of mutualistic symbiotic association formed by the interaction between *N. crassa* (FGSC 11007 *Δnit-4*) and *C. reinhardtii* (CC-1690). Each plane was imaged for 0.2 seconds and is 3µm apart in the z-axis. Numerous *C. reinhardtii* cells (green dots) can be seen coating strands of *N. crassa* hyphae (white filaments). The imaged sample was occasionally gently tapped to induce movement of the dissected fragment and liquid flow, and to demonstrate the attachment of *C. reinhardtii* cells to fungal hyphae. Fungi and algae were cocultured and imaged as in movie S1.

Movie S3.

Close-up of a representative mutualistic symbiotic association formed by the interaction between *N. crassa* (FGSC 11007 *Δnit-4*) and *C. reinhardtii* (CC-1690). The movie shows a z-stack, in which each plane was imaged for 0.2 seconds and is 1 µm apart in the z-axis. The images are shown at 100X magnification and 2X real time playback. They show *C. reinhardtii* cells (green) attached to *N. crassa* hyphae (white filaments) like beads on a string. Several *C. reinhardtii* cells can be seen freely swimming or jittering while attached to fungal hyphae (some via their flagella, e.g., see upper left, time = 10-12 sec). Fungi and algae were cocultured and imaged as in movie S1.

<u>Supplementary Movies S4 through S6</u>: Z-stack series of a representative symbiotic associations formed between *A. nidulans* (*nit-*) (a distant relative of lichenous fungi) and *C. reinhardtii* under conditions that enforce mutualism. These movies serve to complement Fig. 4B in showing that the colocalization of *C. reinhardtii* on *A. nidulans* hyphae is not merely due to a "hovering" of swimming algae in the vicinity; *C. reinhardtii* appear to be attached to hyphae in small clusters, are resistant to gentle hydrodynamic displacement by liquid flow, and are not visibly motile.

Movie S4.

Low magnification (20X) z-stack series of a representative mutualistic symbiotic association formed by the interaction between *A. nidulans* (TS003 *crnA- crnB-*, a non-nitrite-utilizing mutant) and *C. reinhardtii* (CC-1690) showing a patchy distribution of *C. reinhardtii* (green) cells on top of a homogenously green tissue center (top), a predominantly white hyphal periphery (lower right), and a greening edge (lower left). Each plane was imaged for 0.2 seconds and is 15 µm apart in the z-axis. Fungi and algae were cocultured and imaged as in movie S1.

Movie S5.

Z-stack image series at 50X magnification and 2X real time playback of a representative peripheral fragment of mutualistic symbiotic association formed by the interaction between *A. nidulans* (TS003 *crnA- crnB-*) and *C. reinhardtii* (CC-1690) (region in movie S2, expanded). Each plane was imaged for 0.2 seconds and is 3 µm apart in the z-axis. *C. reinhardtii* cells (green) have a patchy distribution and are associated with *A. nidulans* hyphae (which are more

densely packed than *N. crassa* hyphae) like clusters of grapes. Fungi and algae were cocultured and imaged as in movie S1.

Movie S6.

Close-up of a representative mutualistic symbiotic association formed by the interaction between *A. nidulans* (TS003 *crnA- crnB-*) and *C. reinhardtii* (CC-1690). The movie shows a z-stack, in which each plane was imaged for 0.2 seconds and is 2 μm apart in the z-axis. The images are shown at 100X magnification and 2X real time playback. They show *C. reinhardtii* cells (green) attached on top of and between densely packed *A. nidulans* hyphae (white filaments) like clusters of grapes on a vine. Several *C. reinhardtii* cells can be seen swimming within the fungal hyphal network and near the periphery of the algal-fungal association (e.g., time = 10-14 sec). Fungi and algae were cocultured and imaged as in movie S1.

<u>Supplementary Movies S7 through S11</u>: Representative associations formed between *N. crassa* and *C. reinhardtii* in series of combinatorial control experiments, demonstrating that physical association between fungus and alga is independent of any metabolic dependency (see fig. S6).

Movie S7.

Z-stack image series at 50X magnification and 2X real time playback of a representative peripheral fragment of mutualistic symbiotic association formed by the interaction between *N. crassa* (FGSC 11007 *Δnit-4*, a non-nitrite-utilizing mutant) and *C. reinhardtii* (CC-1690) grown together in a loosely capped 150 ml polystyrene container (Corning) containing 50 ml of CYM:2% glucose + 10 mM KNO_2 + 20 mM NH_4Cl medium, under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). Under these conditions, *N. crassa* (*nit-*) does not require *C. reinhardtii* for nitrogen nor does *C. reinhardtii* require *N. crassa* for carbon (cf. fig. S6A); despite the lack of metabolic dependency, both fungus and alga still physically associate. In addition to regions of densely packed green *C. reinhardtii* cells within a *N. crassa* hyphal network, several *C. reinhardtii* cells or cluster of cells can be seen attached to moving *N. crassa* hyphae (white filaments). The fragment was imaged as described in movie S1, although the illumination arrangement is different. Each plane was imaged for 0.2 seconds and is 4 μm apart in the z-axis.

Movie S8.

Z-stack image series at 112X magnification and 2X real time playback of a representative peripheral fragment of mutualistic symbiotic association formed by the interaction between *N. crassa* (FGSC 11007 *Δnit-4*) and *C. reinhardtii* (CC-1690) cocultured in 50 ml of CYM:2% glucose + 10 mM KNO_2 + 20 mM NH_4Cl medium in an airtight, 150 ml polystyrene container (Corning) under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). Under these conditions, *N. crassa* (*nit-*) does not require *C. reinhardtii* for nitrogen, although *C. reinhardtii* is obligately dependent on *N. crassa* for carbon (this feeding dependency is indicated in the movie as *N. crassa* → *C. reinhardtii*) (cf. fig. S6B). The fragment was imaged as described in movie S1, although the illumination arrangement is different. Each plane was imaged for 0.2 seconds and is 1 μm apart in the z-axis.

Movie S9.

Z-stack image series at 112X magnification and 2X real time playback of a representative fragment of mutualistic symbiotic association formed by the interaction between *N. crassa* (FGSC 11007 *Δnit-4*) and *C. reinhardtii* (CC-1690) cocultured in a loosely capped 150 ml polystyrene container (Corning) containing 50 ml of CYM:2% glucose + 10 mM KNO_2 medium, under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). Under these conditions, *N. crassa* (*nit*-) requires *C. reinhardtii* for nitrogen (this feeding dependency is indicated in the movie as *N. crassa* ← *C. reinhardtii*), while *C. reinhardtii* does not require *N. crassa* for carbon (cf. fig. S6C). Patchy clusters of *C. reinhardtii* cells are seen within a *N. crassa* hyphal network of white filaments. The fragment was imaged as described in movie S1, although the illumination arrangement is different. Each plane was imaged for 0.2 seconds and is 2 μm apart in the z-axis.

Movie S10.

Z-stack image series at 112X magnification and 2X real time playback of a representative peripheral fragment of mutualistic symbiotic association formed by the interaction between *N. crassa* (FGSC 11007 *Δnit-4*) and *C. reinhardtii* (CC-1690) cocultured in 50 ml of CYM:2% glucose + 10 mM KNO_2 medium in an airtight 150 ml polystyrene container (Corning), under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). Under these conditions, *N. crassa* (*nit*-) requires *C. reinhardtii* for nitrogen and *C. reinhardtii* requires *N. crassa* for carbon (this feeding dependency is indicated in the movie as *N. crassa* ⇔ *C. reinhardtii*) (cf. fig. S6D); these conditions are the same as in movies S1 through S3 and imaged as described in movie S1, although the illumination arrangement is different. Each plane was imaged for 0.2 seconds and is 1.4 μm apart in the z-axis.

Movie S11.

Z-stack image series at 112X magnification of a representative peripheral fragment of mutualistic symbiotic association formed by the interaction between a wild-type nitrite-utilizing *N. crassa* strain (FGSC 2489) and *C. reinhardtii* (CC-1690) cocultured in a loosely capped 150 ml polystyrene container (Corning) containing 50 ml of CYM:2% glucose + 20 mM KNO_2 medium, under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). Wild-type *N. crassa* does not require *C. reinhardtii* for nitrogen nor does *C. reinhardtii* require *N. crassa* for carbon (cf. fig. S5E); this is a similar metabolic arrangement as in movie S7, achieved using a nitrite-utilizing fungal strain. Despite the lack of metabolic dependency, both fungus and alga still physically associate. This fragment was imaged as described in movie S1, although the illumination arrangement is different. Each plane was imaged for 0.2 seconds and is 3 μm apart in the z-axis.

<u>Supplementary Movies S12 through S16</u>: Representative associations formed between *A. nidulans* (a distant relative of lichenous fungi) and *C. reinhardtii* in series of control experiments, demonstrating that physical association between fungus and alga is independent of any metabolic dependency (see fig. S7). Cf. images of algal attachments to fungal hyphae in lichens (71).

Movie S12.

Z-stack image series at 90X magnification and 2X real time playback of a representative peripheral fragment of mutualistic symbiotic association formed by the interaction between *A. nidulans* (TS003 *crnA- crnB-*, a non-nitrite-utilizing mutant) and *C. reinhardtii* (CC-1690) grown together in a loosely capped 150 ml polystyrene container (Corning) containing 50 ml of CYM:2% glucose + 10 mM KNO_2 + 20 mM NH_4Cl medium, under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). Under these conditions, *A. nidulans* (*nit-*) does not require *C. reinhardtii* for nitrogen nor does *C. reinhardtii* require *A. nidulans* for carbon (cf. fig. S7A); despite the lack of metabolic dependency, both fungus and alga still physically associate. *C. reinhardtii* cells (green) cluster onto *A. nidulans* hyphae (white filaments) like "grapes on a vine." Fungi and algae were imaged as described in movie S1, although the illumination arrangement is different. Each plane was imaged for 0.2 seconds and is 10 μm apart in the z-axis.

Movie S13.

Z-stack image series at 112X magnification and 2X real time playback of a representative peripheral fragment of mutualistic symbiotic association formed by the interaction between *A. nidulans* (TS003 *crnA- crnB-*, *nit-*) and *C. reinhardtii* (CC-1690) cocultured in 50 ml of CYM:2% glucose + 10 mM KNO_2 + 20 mM NH_4Cl medium in an airtight, 150 ml polystyrene container (Corning) under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). Under these conditions, *A. nidulans* (*nit-*) does not require *C. reinhardtii* for nitrogen, although *C. reinhardtii* is obligately dependent on *A. nidulans* for carbon (this feeding dependency is indicated in the movie as *A. nidulans* → *C. reinhardtii*) (cf. fig. S7B). As in movie S12, *C. reinhardtii* cells (green) cluster onto *A. nidulans* hyphae (white filaments). Fungi and algae were imaged as described in movie S1, although the illumination arrangement is different. Each plane was imaged for 0.2 seconds and is 10 μm apart in the z-axis.

Movie S14.

Z-stack image series at 112X magnification and 2X real time playback of a representative fragment of mutualistic symbiotic association formed by the interaction between *A. nidulans* (TS003 *crnA- crnB-*, *nit-*) and *C. reinhardtii* (CC-1690) cocultured in a loosely capped 150 ml polystyrene container (Corning) containing 50 ml of CYM:2% glucose + 10 mM KNO_2 medium, under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). Under these conditions, *A. nidulans* (*nit-*) requires *C. reinhardtii* for nitrogen (this feeding dependency is indicated in the movie as *A. nidulans* ← *C. reinhardtii*), while *C. reinhardtii* does not require *N. crassa* for carbon (cf. fig. S7C). In contrast to movies S12 and S13, under these conditions, *C. reinhardtii* cells are much more densely packed in association with *A. nidulans* hyphal network of white filaments (cf. smaller, compact tissue ball in fig. S7C), akin to the greener, interior sections of fragments in movies S4 through S6. Fungi and algae were imaged as described in movie S1,

although the illumination arrangement is different. Each plane was imaged for 0.2 seconds and is 10 μm apart in the z-axis.

Movie S15.

Z-stack image series at 112X magnification and 2X real time playback of a representative peripheral fragment of mutualistic symbiotic association formed by the interaction between *A. nidulans* (TS003 *crnA- crnB-*, *nit-*) and *C. reinhardtii* (CC-1690) cocultured in 50 ml of CYM:2% glucose + 10 mM KNO_2 medium in an airtight 150 ml polystyrene container (Corning) containing, under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). Under these conditions, *A. nidulans* (*nit-*) requires *C. reinhardtii* for nitrogen and *C. reinhardtii* requires *N. crassa* for carbon (this dependency is indicated in the movie as *A. nidulans* ⇔ *C. reinhardtii*) (cf. fig. S7D); these conditions are the same as in movies S4 through S6 and imaged as described in movie S1, although the illumination arrangement is different. The clustering of *C. reinhardtii* cells on *A. nidulans* white hyphal filaments is dense, but not as extreme as in movie S14 (*A. nidulans* ← *C. reinhardtii*; cf. movie S4). Each plane was imaged for 0.2 seconds and is 10 μm apart in the z-axis.

Movie S16.

Z-stack image series at 111X magnification of a representative peripheral fragment of mutualistic symbiotic association formed by the interaction between a wild-type nitrite-utilizing *A. nidulans* strain (FGSC A4) and *C. reinhardtii* (CC-1690) cocultured in a loosely capped 150 ml polystyrene container (Corning) containing 50 ml of CYM:2% glucose + 20 mM KNO_2 medium, under constant light (irradiance=45 μmol/m^2/s) for 7 days (15). Wild-type *A. nidulans* does not require *C. reinhardtii* for nitrogen nor does *C. reinhardtii* require *A. nidulans* for carbon (cf. fig S7E); this is a similar metabolic arrangement as in movie S12, achieved using a nitrite-utilizing fungal strain. Despite the lack of metabolic dependency, both fungus and alga still physically associate. However, *C. reinhardtii* cells are less densely clustered compared to movie S12, and substantially less so than in movie S14 (*A. nidulans* ← *C. reinhardtii*). Thus, the degree of dependency of *A. nidulans* on *C. reinhardtii* for nitrogen appears to dictate the density of *C. reinhardtii* cells embedded within the *A. nidulans* hyphal network. This fragment was imaged as described in movie S1, although the illumination arrangement is different. Each plane was imaged for 0.2 seconds and is 20 μm apart in the z-axis.

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