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Niche Engineering Demonstrates a Latent Capacity for Fungal-Algal Mutualism

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Abstract

Mutualistic symbioses shape the evolution of species and ecosystems and catalyze the emergence of biological complexity, yet how such symbioses first form is unclear. We show that an obligate mutualism between the yeast *Saccharomyces cerevisiae* and the alga *Chlamydomonas reinhardtii* —two model eukaryotes with very different life histories—can arise spontaneously in an environment requiring reciprocal carbon and nitrogen exchange. This capacity for mutualism is phylogenetically broad, extending to other Chlamydomonas and fungal species. Furthermore, we witnessed the spontaneous association of Chlamydomonas algal cells physically interacting with filamentous fungi. These observations demonstrate that under specific conditions, environmental change induces free-living species to become obligate mutualists and establishes a set of experimentally tractable, phylogenetically related, synthetic systems for studying the evolution of symbiosis.

Mutualistic symbioses—beneficial associations between different species involving persistent physical contact and physiological coupling—are central to many evolutionary and ecological innovations (1–3). These include the origin of eukaryotic cells, the colonization of land by plants, coral reefs, and the gut microbiota of insects and animals (4, 5). Despite their ubiquity and importance, we understand little about how mutualistic symbioses form between previously free-living organisms (5, 6). Like speciation, the birth of novel symbioses has rarely been witnessed, making it difficult to determine if co-evolution occurs before symbiosis begins or if chance ecological encounters initiate new symbioses (5, 7). Such “ecological fitting” (8, 9) occurs when both a particular environment and previously evolved traits allows a set of species to complement each other, giving rise to novel interactions without the need for prior coevolutionary adaptation.

We tested two genetically tractable organisms, the budding yeast *Saccharomyces cerevisiae* and the green alga *Chlamydomonas reinhardtii*, to determine if a reciprocal exchange of carbon and nitrogen would lead to obligate mutualism between algae and fungi such as those which occur naturally (10–13). In our scheme (Fig. 1A), *S. cerevisiae* metabolizes glucose to carbon dioxide (CO_2), a carbon source that *C. reinhardtii* fixes via photosynthesis, and *C.*

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reinhardtii reduces nitrite (NO_2^-) into ammonia (NH_3) (14), which yeast can use as a nitrogen source. Co-culturing experiments (15) indicate that by preventing access to atmospheric CO_2, *S. cerevisiae* and *C. reinhardtii* become obligate mutualists (Fig. 1B). This mutualism depends on the metabolic capabilities of the two organisms: *S. cerevisiae* cannot use nitrite as a nitrogen source and *C. reinhardtii* cannot use glucose as a carbon source. Cell proliferation did not require genetic engineering or fine-tuning of nutrient concentrations or starting ratios of the two species (Figs. 1B, S1, S2) and failed when either species (Fig. 1B, conditions 2–3), glucose, or nitrite was omitted from the experiment (Fig. 1B, conditions 4–5). Agitation attenuates this mutualism (Fig. 1B, condition 6), suggesting the importance of cell-cell proximity and spatial structure in establishing successful cooperation (16). Thus, a simple environmental change can induce free-living organisms to be mutualistic without requiring adaptive co-evolution.

In our scheme, mutualism can be obligate or facultative depending on the environment. Access to atmospheric CO_2 makes *C. reinhardtii* a facultative mutualist by removing its dependence on *S. cerevisiae* for carbon (Fig. 1B, condition 7), but the yeast remains dependent on the alga for nitrogen. In this environment, algal proliferation is improved by the presence of glucose-metabolizing, CO_2-generating budding yeast while yeast proliferation is reduced, although not extinguished (Fig. 1B, conditions 7 vs. 8). Conversely, adding ammonia (as ammonium chloride) to airtight co-cultures allows budding yeast to proliferate independently of the alga while the alga remains dependent on the yeast for carbon. Under these conditions, *S. cerevisiae* (~4 hrs doubling time in our conditions) out-proliferates *C. reinhardtii* (≥12 hrs doubling time) and drives the alga to near extinction (Fig. S1, condition 15). These results suggest that stable metabolic mutualisms require that the faster growing species be obligately dependent on nutrients produced by its slower growing partner.

The engineered obligate mutualism between *S. cerevisiae* and *C. reinhardtii* is not limited to our initial choice of input nutrient concentrations. Successful mutualisms were established over nearly two orders of magnitude in glucose and nitrite concentrations (Fig. 2). However, this resulted in complex population dynamics. We observed undulations and variations in stability across time similar to density-dependent population cycles predicted for mutualistic systems (17). Other carbon (e.g., galactose) or nitrogen (e.g., nitrate) sources, although less effective, also sustain mutualism between *S. cerevisiae* and *C. reinhardtii* (Fig. S3).

We also demonstrate that many different ascomycetous yeast and four Chlamydomonas species, spanning over 300 million years of evolutionary divergence in each clade, can form mutualisms (Fig. 3). Nearly all yeast species we examined form synthetic obligate mutualisms with *C. reinhardtii*, although with different degrees of productivity (Fig. 3A). Mutualistic productivity, as assessed by total cell counts, did not correlate with a yeast's preference for a fermentative or respiratory lifestyle (Fig. 3A), whether a yeast strain was isolated from soil (a potential habitat shared with *C. reinhardtii*), had an intrinsic growth rate, or nitrite-mediated inhibition of growth (Fig. S4, Table S1). Thus, we observe that mutualisms can be phylogenetically broad, but that the degree of success depends on species-specific traits.

Two yeast species and the alga *Chlorella vulgaris* did not form obligate mutualisms (Fig. 3). *C. vulgaris*, which can use glucose as a carbon source, out-proliferated *S. cerevisiae*, whereas *Hansenula polymorpha*, a yeast that can use nitrite as a sole nitrogen source, out-proliferated *C. reinhardtii*. The yeast *Kluveromyces polysporus* failed to form an obligate mutualism with *C. reinhardtii*. This yeast can grow in an ammonium-supplemented co-culture medium, suggesting that it fails to cooperate with *C. reinhardtii* likely because it either cannot grow at the low ammonia levels produced by *C. reinhardtii* or is more sensitive to nitrite inhibition at such low ammonia levels (Fig. S4). *Neurospora crassa* and *Aspergillus nidulans* are genetically tractable filamentous fungi that can use nitrite as a nitrogen source (18). The ability of these fungi to reduce nitrite keeps wild type strains from forming obligate mutualisms with *C. reinhardtii*. However, mutants that cannot reduce nitrite did form obligate mutualisms (Fig. S5), suggesting that a loss of gene function in one species could be complemented through mutualism (11, 19).

We observed that the filamentous fungi formed macroscopic structures such that the fungal hyphae were decorated with *C. reinhardtii* cells (Figs. S5, 4A–B, Movies S1–S6). However, physical associations between fungus and alga form even in the absence of any metabolic dependency (Figs. S6, S7, Movies S7–S16). Electron microscopy of interactions between *C. reinhardtii* and *A. nidulans*, which shares a most recent common ancestor with lichenous fungi within the class Eurotiomycetes (10), revealed a tight fungal-algal contact interface (Fig. 4C–D) reminiscent of wall-to-wall interfaces between fungal and algal cells in extant lichens (11). The walls of *C. reinhardtii* cells in contact with *A. nidulans* hyphae are less heavily stained and appear thinner than *C. reinhardtii* cells cultured separately (Fig. 4E) possibly due to locally secreted *A. nidulans* cell wall remodeling enzymes. We saw no evidence of any morphologically complex tissue structures, such as those seen in many lichens, nor of fungal hyphae penetrating algal cells (11, 20). Thus, these synthetic mutualisms may result in physical complexes but they do not form elaborate morphological structures at the cellular or organismal level.

The ease with which fungal-algal mutualisms were created suggests that ecological interactions may be relatively easy to establish (21). Furthermore, they do not require a prior facultative, commensal, or parasitic stage, or co-evolutionary adaptation (5–7, 22, 23). Our understanding of how “ecologically framed” pairs of species can be created due to environments that force them to depend on each other will be useful in the emerging field of synthetic ecology (24, 25) as well as for understanding the assembly of microbial communities in cases of disturbed or invaded habitats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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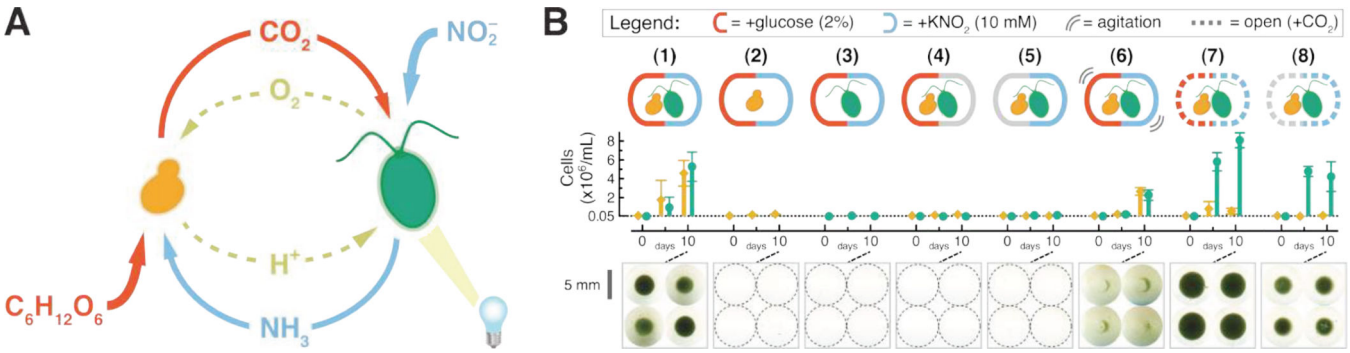

Figure 1. A synthetic mutualism between *S. cerevisiae* and *C. reinhardtii*

(A) A metabolic circuit for mutualism based on carbon and nitrogen exchange. *S. cerevisiae* (orange, left) metabolizes glucose ($C_6H_{12}O_6$) and releases carbon dioxide (CO_2), which is assimilated photosynthetically by *C. reinhardtii* (green, right) to release oxygen (O_2); *C. reinhardtii* metabolizes nitrite (NO_2^-) and releases ammonia (NH_3) as a nitrogen source for *S. cerevisiae*. An intrinsic, near-neutral pH balance between 6.8–7.4 is maintained by a metabolic exchange of protons between yeast and alga (15). (**B**) Proliferation of *S. cerevisiae* and *C. reinhardtii* under different co-culture conditions demonstrates that obligate mutualism can arise without any genetic engineering of metabolic pathways. Top: cartoons of the different conditions tested; middle: cell density of yeast and alga over the course of the experiment (mean ± 95% confidence interval; N=4); bottom: images of the cell populations from four representative examples of each culture condition (after 10 days). The dark green hue of the pellets is due to *C. reinhardtii* cells; *S. cerevisiae* cells are off-white and are interspersed throughout the pellet. See (15) for further details.

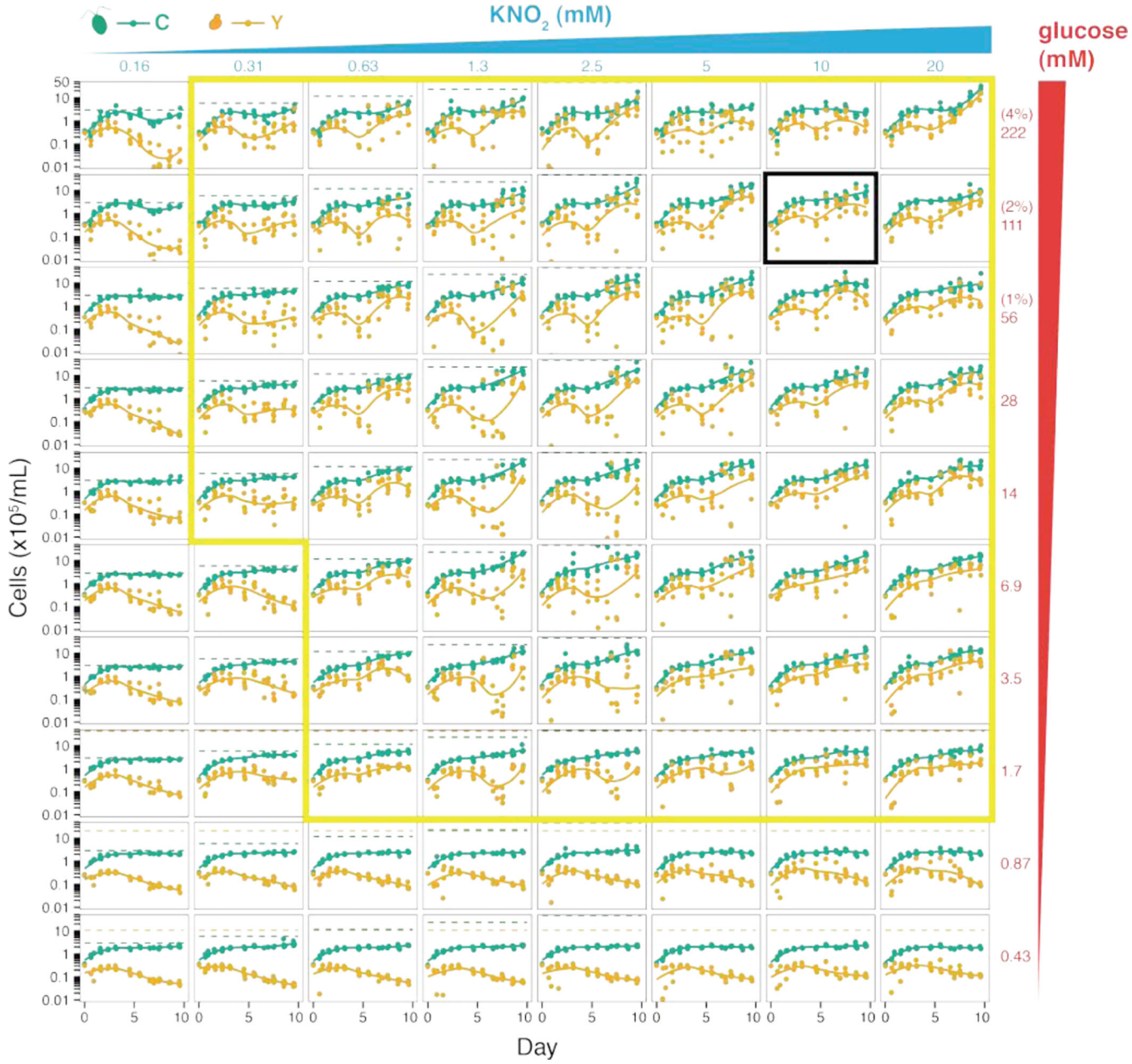

Figure 2. Landscape of mutualistic productivity

Cell densities over time for each species grown in co-culture (*C. reinhardtii* in green; *S. cerevisiae* in orange) grown from an initial inoculum of ~0.3×10^5 cells/mL for each species; irradiance=110 μmol/m^2/s. Each of 4 replicate point pairs (green and orange) are plotted. Local polynomial regression fits (by robust linear regression in R with y~x) for both cell types are plotted as a visual guide of cell proliferation. Co-culture conditions are denoted on the left in black and show increasing, respectively, left to right KNO_3 and bottom to top glucose concentrations. Dashed lines indicate the maximum predicted cell densities expected for *C. reinhardtii* (green) and *S. cerevisiae* (orange) for each co-culture (15). Net positive proliferation of both yeast and algae is supported within the region bounded by the yellow

outline. The limited proliferation of *C. reinhardtii* under conditions outside this region (in days 1–2) indicates residual atmospheric CO_2 in the wells of the sealed microtiter plate.

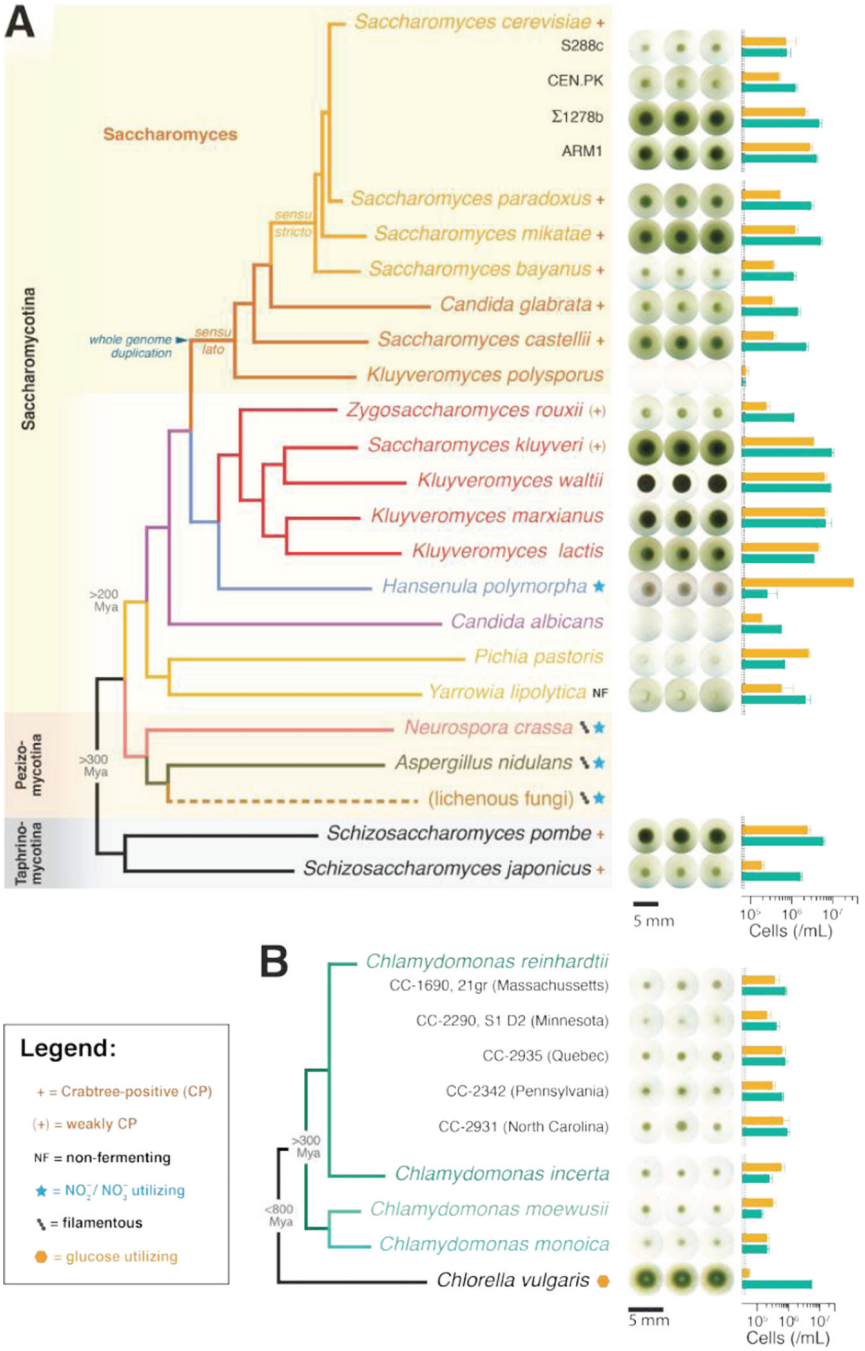

Figure 3. The capacity for mutualism is phylogenetically broad

(**A**) Annotated phylogenetic tree of select ascomycetous fungal species (adapted and modified from published work (15)) paired with *C. reinhardtii* (CC-1690, 21gr), in three representative 9 day-old cocultures (indicated by dashed lines in the histogram of cell densities) grown under 110 μmol/m^2/s of light. Measured cell counts (mean ± 95% confidence interval; N=4) for yeast (orange) and alga (green) are shown to the right of cell culture images. Subphyla of Ascomycota are indicated on the far left. Crabtree-positive yeasts (exhibiting a preference for fermentation over respiration even under aerobic

conditions) are indicated with a "+", and weakly Crabtree-positive yeasts with a "(+)". *Y. lipolytica* is a non-fermenting (NF) yeast. Nitrate/nitrite utilizing fungi are indicated by a light blue star and filamentous fungi by three connected dots (see Fig. S5). (**B**) Annotated phylogenetic tree (adapted and modified from published work (15)) of select algal species and Chlamydomonas cultivars (green bars at right) paired with *S. cerevisiae* (S288C) (orange bars, right) in representative 7 day-old co-cultures grown under 110 μmol/m^2/s of light (mean ± 95% confidence interval; N=12). *Chlorella vulgaris*, is an asexual alga, distantly related to *C. reinhardtii*, able to use glucose as a carbon source (orange hexagon). Descriptions of strains are provided in Table S1.

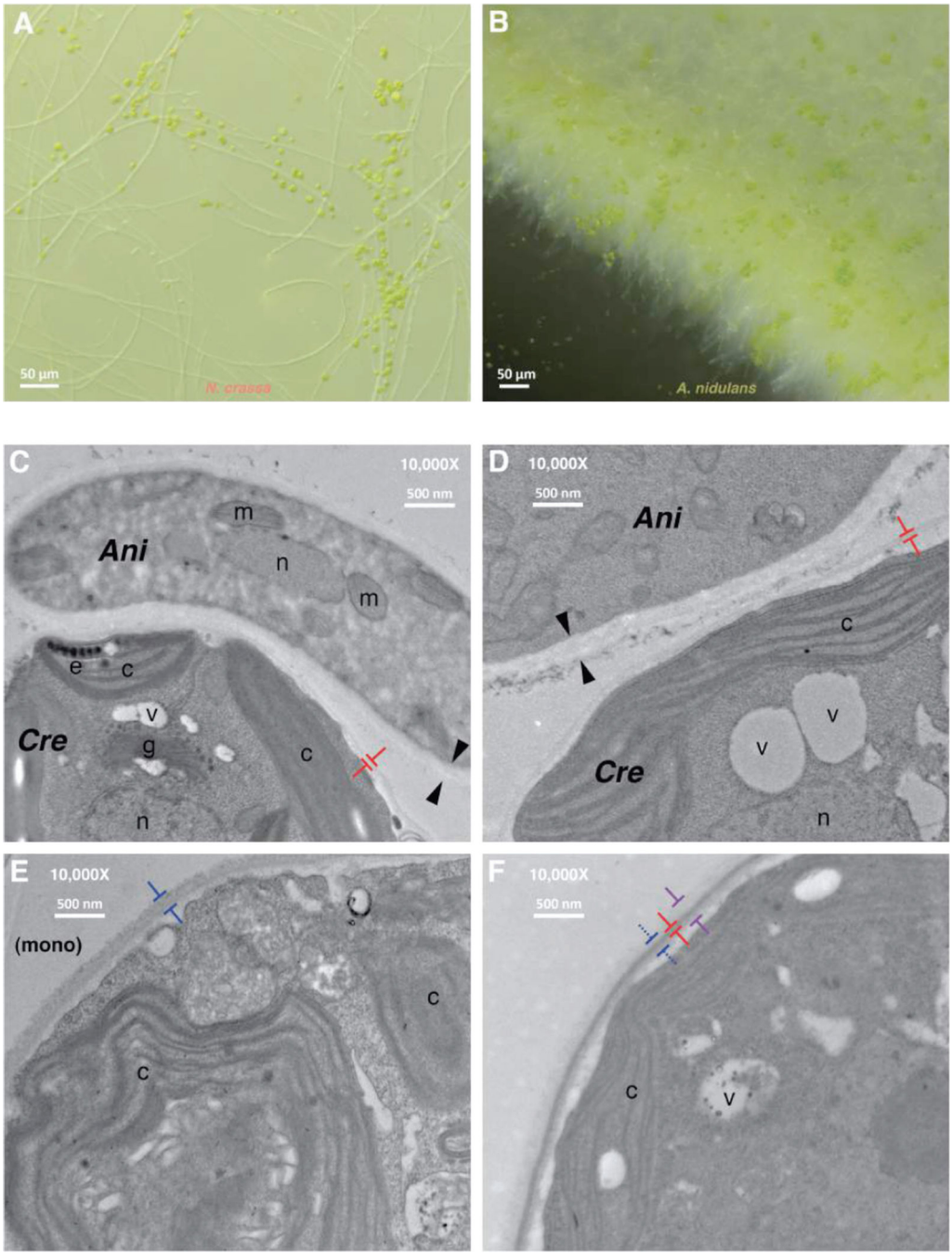

Figure 4. *C. reinhardtii* physically associates with *N. crassa* and *A. nidulans*

Representative light micrographs of the periphery of algal-fungal associations formed in obligate mutualistic co-culture. *C. reinhardtii* cells (green) stick to hyphae (white filaments) of (**A**) *N. crassa* (FGSC 11007 Δ*nit-4*) or (**B**) *A. nidulans* (TS003 *crnA*- *crnB*-). (**C–F**) Representative transmission electron micrographs reveal a simple wall-to-wall interface between *C. reinhardtii* (*Cre*) cells and *A. nidulans* (*Ani*) hyphae. Opposed arrows indicate the thickness of fungal cell walls and opposed colored T-bars indicate those of algal cells ((**C**): 51 ± 10 nm; (**D**): 60 ± 7 nm; mean ± SD). (**E**) *C. reinhardtii* grown in mono-culture

(160 ± 20 nm; blue T-bars) or (**F**) unattached *C. reinhardtii* isolated from the supernatant of the same co-culture [T demarcations: reference mono-culture cell wall thickness (red dashed; see E); (2) core (heavy) cell wall staining (blue): 50 ± 4 nm; (3) diffuse cell wall staining (purple): 260 ± 30 nm)] (15). Labeled intracellular components: m, mitochondria; c, chloroplast; e, eyespot; g, Golgi; n, nucleus; and v, vacuole.