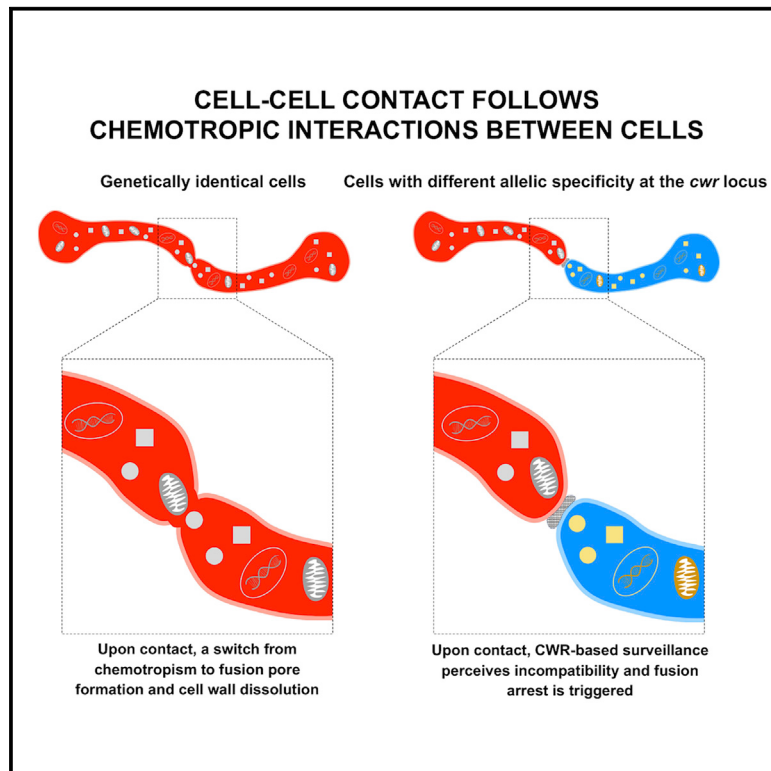


Current Biology

Allorecognition upon Fungal Cell-Cell Contact Determines Social Cooperation and Impacts the Acquisition of Multicellularity

Graphical Abstract



Authors

A. Pedro Gonçalves, Jens Heller, Elise A. Span, ..., Natalia Requena, Michael A. Marletta, N. Louise Glass

Correspondence

lglass@berkeley.edu

In Brief

Somatic cell fusion with conspecific partners entails risks of parasitism. Gonçalves et al. present an allorecognition system, triggered upon cell-cell contact and leading to a blockage in cell wall dissolution, that prevents the formation of chimeras between genetically non-identical spores and hyphae of a model fungus.

Highlights

- Conspecific cooperation and cell fusion are crucial for microbial development
- Allorecognition upon contact blocks cell fusion of genetically non-identical cells
- Incompatible partners cannot switch from communication to cell wall dissolution mode
- The independent appearance of polymorphic *cwr* alleles suggests convergent evolution

Allorecognition upon Fungal Cell-Cell Contact Determines Social Cooperation and Impacts the Acquisition of Multicellularity

A. Pedro Gonçalves,^{1,8,9} Jens Heller,^{1,2,8} Elise A. Span,^{3,10} Gabriel Rosenfield,¹ Hung P. Do,¹ Javier Palma-Guerrero,^{1,11} Natalia Requena,⁴ Michael A. Marletta,^{5,6,7} and N. Louise Glass^{1,2,12,*}

¹Department of Plant and Microbial Biology, University of California, Berkeley, Berkeley, CA 94720, USA

²Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

³Biophysics Graduate Group, University of California, Berkeley, Berkeley, CA 94720, USA

⁴Molecular Phytopathology, Botanical Institute, Karlsruhe Institute of Technology, 76131 Karlsruhe, Germany

⁵Department of Chemistry, University of California, Berkeley, Berkeley, CA 94720, USA

⁶California Institute for Quantitative Biosciences, University of California, Berkeley, Berkeley, CA 94720, USA

⁷Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, USA

⁸These authors contributed equally

⁹Present address: Institute of Molecular Biology, Academia Sinica, Taipei 11529, Taiwan

¹⁰Present address: Zymergen, Emeryville, CA 94608, USA

¹¹Present address: Plant Pathology Group, Institute of Integrative Biology, ETH Zürich, 8092 Zürich, Switzerland

¹²Lead Contact

*Correspondence: lglass@berkeley.edu

<https://doi.org/10.1016/j.cub.2019.07.060>

SUMMARY

Somatic cell fusion and conspecific cooperation are crucial social traits for microbial unicellular-to-multicellular transitions, colony expansion, and substrate foraging but are also associated with risks of parasitism. We identified a cell wall remodeling (*cwr*) checkpoint that acts upon cell contact to assess genetic compatibility and regulate cell wall dissolution during somatic cell fusion in a wild population of the filamentous fungus *Neurospora crassa*. Non-allelic interactions between two linked loci, *cwr-1* and *cwr-2*, were necessary and sufficient to block cell fusion: *cwr-1* encodes a polysaccharide mono-oxygenase (PMO), a class of enzymes associated with extracellular degradative capacities, and *cwr-2* encodes a predicted transmembrane protein. Mutations of sites in CWR-1 essential for PMO catalytic activity abolished the block in cell fusion between formerly incompatible strains. In *Neurospora*, alleles *cwr-1* and *cwr-2* were highly polymorphic, fell into distinct haplogroups, and showed trans-species polymorphisms. Distinct haplogroups and trans-species polymorphisms at *cwr-1* and *cwr-2* were also identified in the distantly related genus *Fusarium*, suggesting convergent evolution. Proteins involved in chemotropic processes showed extended localization at contact sites, suggesting that *cwr* regulates the transition between chemotropic growth and cell wall dissolution. Our work revealed an allorecognition surveillance system based on kind discrimination that inhibits cooperative

behavior in fungi by blocking cell fusion upon contact, contributing to fungal immunity by preventing formation of chimeras between genetically non-identical colonies.

INTRODUCTION

Complex multicellularity results from a developmental program that leads to differentiation of specialized structures and requires intercellular social cooperation [1]. In multicellular organisms, conspecific cooperation enhances adaptation to environmental variations due to the communal nature of produced goods (e.g., nutrients in a fungal colony). However, cooperation can cause conflict due to transmission of infectious elements and genotypes that negatively impact cellular fitness [2–6]. In this regard, high genetic relatedness and cooperation correlate positively to prevent exploitation of communal goods by cheaters [7–10].

The interconnected fungal mycelium is a prototype of a complex multicellular body. It operates as a polarized syncytium that expands via tip elongation and somatic cell-cell fusion [11, 12]. In *Neurospora crassa*, cell fusion between genetically identical cells (either germinated asexual spores [germlings] or between hyphae within a single colony) is a fitness character, as fusion mutants show a lag in colony development [3, 13]. Fusion between genetically non-identical cells results in a heterokaryotic syncytia that contains organelles of dissimilar genetic backgrounds. Although heterokaryon formation has been postulated to increase fitness in fungal populations [3], it is often precluded by allorecognition systems that either reduce somatic cell fusion between genetically distinct cells or cause cell death of fusion compartments [8, 14–16]. These allorecognition systems reduce transmission of mycoviruses, senescence plasmids, crippled

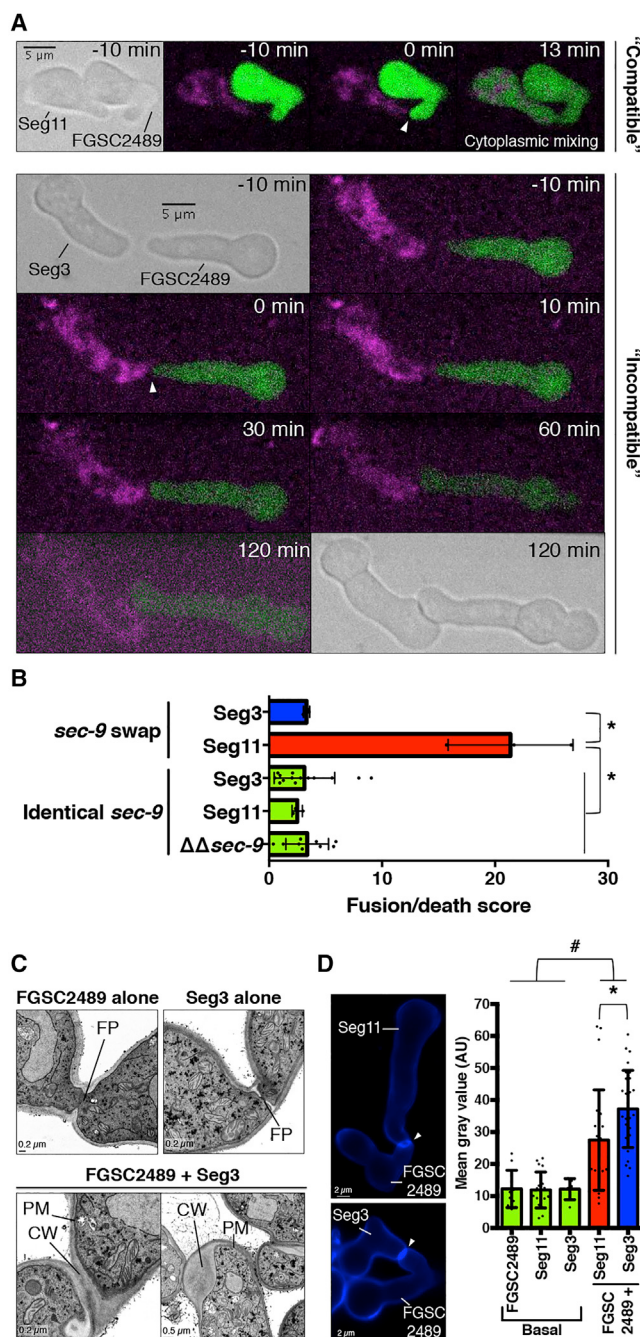


Figure 1. Cell-Wall-Associated Arrest Is Triggered to Prevent Cell Fusion of Nonself Cells

(A) GFP-expressing FGSC2489 germlings were paired with FM4-64-stained Seg11 (fusion-compatible, top) or Seg3 (fusion-incompatible, bottom) germlings, and cytoplasmic mixing (GFP exchange) was evaluated. Time point 0 was defined as the moment of cell-cell contact. Arrowheads indicate the zone of interaction between cells. See also Figure S1A and Videos S1 and S2. (B) Cell fusion as determined by propidium iodide (PI) uptake and flow cytometry. Indicated strains were paired with FGSC2489. Strains with identical *sec-9* alleles: mixed cells show no cell death upon fusion (green). Strains with *sec-9* swap alleles: mixed cells show cell death upon cell fusion (50% is theoretical maximum of cell death in this assay) [15]. * $p < 0.0001$ (one-way ANOVA followed by the Tukey post hoc test); $n \geq 3$. See also Figure S2. (C) Transmission electron microscopy of FGSC2489, Seg3, or FGSC2489 + Seg3 germling pairs. CW, cell wall; FP, fusion pore; PM, plasma membrane. (D) Mixtures of FGSC2489 cells with either Seg11 or Seg3 cells stained with calcofluor white. The mean gray value at the contact spot between cells (arrowheads) of ≥ 24 germlings pairs was quantified. "Basal" level of calcofluor white staining was obtained by quantifying cell wall segments in germlings not undergoing cell fusion ($n \geq 6$). * $p = 0.0226$; # $p < 0.003$ (one-way ANOVA followed by Tukey post hoc test). See also Table S1.

mitochondria, and defective nuclei between fungal colonies [2–4, 6, 17].

To examine cooperation during the acquisition of multicellularity, we assessed fusion dynamics between wild *Neurospora* isolates that showed chemotropic interactions and identified a cell wall dissolution arrest phenotype following contact [11, 12]. A population genomics analysis led to identification of cell wall remodeling checkpoint loci (*cwr-1* and *cwr-2*), whose allelic specificity regulates whether strains can transit from communication to cell wall dissolution and cell fusion. The *cwr* allelic recognition checkpoint displays signs of balancing selection and convergent evolution in distinct fungal genera and allows cells to undergo kind recognition, presumably to avoid cooperation with disadvantageous partners during development of syncytial, multinucleate colonies.

RESULTS

A Cell Fusion Checkpoint Is Triggered upon Cell-Cell Contact

Previously, we reported that allelic specificity at determinant of communication (*doc*) loci determines pre-contact kind recognition in *N. crassa* by regulating chemotropic behavior prior to somatic cell fusion [16]. The wild-type strains, FGSC2489 and JW258, are unable to establish chemotropic interactions prior to somatic cell fusion because they belong to different *doc* haplogroups (CGH1 and CGH2, respectively). However, these two strains can mate and produce progeny able to communicate with only one of their parents [16]. We evaluated whether post-chemotropic interactions were affected in progeny of the FGSC2489 $\times$ JW258 cross by staining germlings with FM4-64 and assessing cell fusion frequency when paired with an FGSC2489 strain expressing cytoplasmic GFP. Of these progeny, 62% underwent chemotropic interactions, cell fusion, and cytoplasmic mixing with FGSC2489 (Figure 1A, top; Video S1), and 38% did not show cell fusion and cytoplasmic mixing after chemotropic interactions and cell contact with FGSC2489 cells (Figure 1A, bottom; Video S2). Of progeny that underwent chemotropic interactions with JW258 cells, 52% showed cytoplasmic mixing with JW258, and 48% did not (Figure 1A). Over time, arrested cells redirected their growth, indicating that fusion was irreversibly blocked (Figure 1A, bottom).

To quantify cell fusion frequencies, we utilized a flow cytometry method based on a robust post-fusion death response mediated by genetic differences at *sec-9* [15]. Isogenic germlings (identical *sec-9* alleles) undergo cell fusion at high frequency and display low basal cell death levels (Figure 1B; Table S1), and germlings containing alternate *sec-9* alleles (but otherwise isogenic) show similar fusion frequencies but high post-fusion

(C) Transmission electron microscopy of FGSC2489, Seg3, or FGSC2489 + Seg3 germling pairs. CW, cell wall; FP, fusion pore; PM, plasma membrane. (D) Mixtures of FGSC2489 cells with either Seg11 or Seg3 cells stained with calcofluor white. The mean gray value at the contact spot between cells (arrowheads) of ≥ 24 germlings pairs was quantified. "Basal" level of calcofluor white staining was obtained by quantifying cell wall segments in germlings not undergoing cell fusion ($n \geq 6$). * $p = 0.0226$; # $p < 0.003$ (one-way ANOVA followed by Tukey post hoc test). See also Table S1.

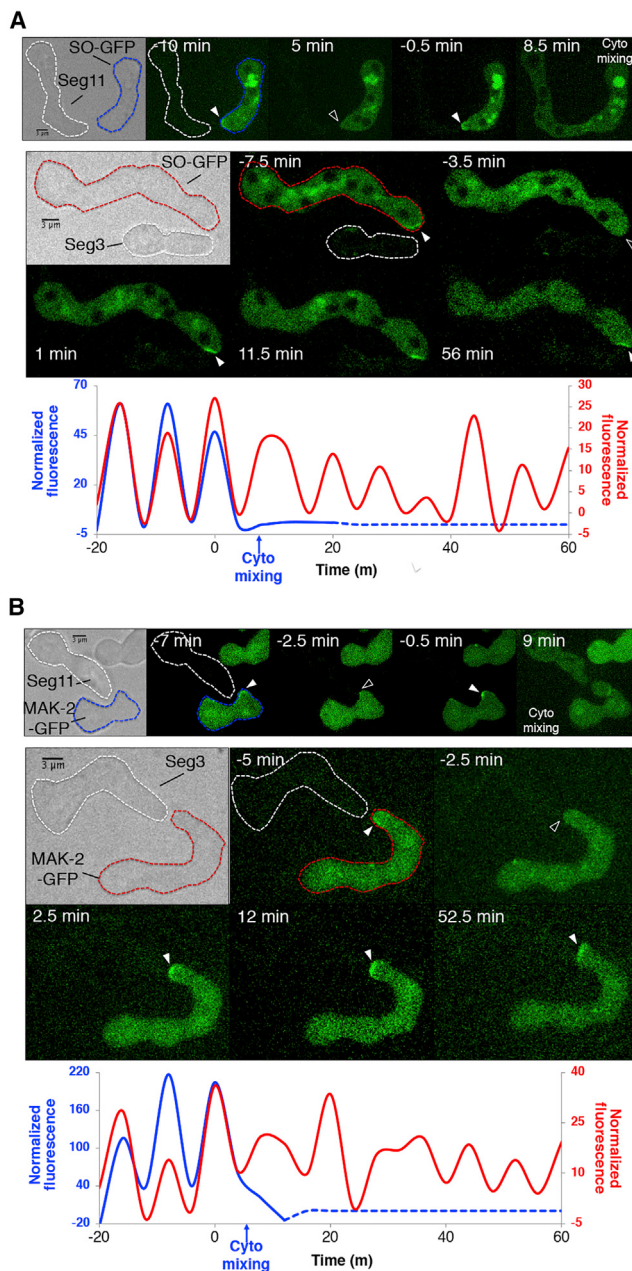


Figure 2. SOFT and MAK-2 Oscillations Continue at CAT Tips of Incompatible Fusion Partners after Contact

(A) FGSC2489 SOFT-GFP (SO-GFP) strain paired with Seg11 (compatible, top panels; blue line in plot) or Seg3 (incompatible, bottom panel; red line in plots); the mean fluorescence intensity at CAT tips was measured.

(B) FGSC2489 MAK-2-GFP strain paired with Seg11 (compatible, top panel; blue line in plot) or Seg3 (incompatible, bottom panel; red line in plots); the mean fluorescence intensity at CAT tips was measured.

Arrowheads indicate SOFT (A) or MAK-2 (B) localization at the zone of interaction; empty arrowheads indicate absence of SOFT (A) or MAK-2 (B) at CAT tips. Contact is defined as 0 min (time). Plots shown in (A) and (B) show oscillation of SOFT (A) or MAK-2 (B) to CAT tips over time with data from representative experiments. Arrows indicating “cyto mixing” in graphs indicates cytoplasmic mixing following cell fusion. Panels show representative experiments; $n \geq 4$.

See also Table S1.

death rates [15]. Thus, introducing alternate *sec-9* alleles in otherwise isogenic strains allowed us to use cell death as a proxy for fusion frequency. A program that allowed for automatic and unbiased gating and analysis of flow cytometry samples was generated (Figure S2). To assess cell fusion frequencies in progeny from the FGSC2489 $\times$ JW258 cross, we mixed FGSC2489 cells with Seg11 (progeny that undergoes chemotropic interactions and cell fusion with FGSC2489) or with Seg3 (progeny that undergoes chemotropic interactions but is blocked in cell fusion with FGSC2489). The relatively high death frequencies in FGSC2489 + Seg11 pairings and low death rates in FGSC2489 + Seg3 pairings indicated successful and blocked cell fusion, respectively (Figure 1B).

Transmission electron microscopy was used to assess whether cell fusion arrest observed for FGSC2489 + Seg3 pairings was due to failure in cell wall dissolution or in membrane merger. In samples of FGSC2489 cells alone or Seg3 cells alone, cell fusion was easily observed as indicated by dissolution of cell walls and plasma membrane at contact points (Figure 1C). In contrast, in mixtures of FGSC2489 + Seg3 cells, a high frequency of cell-cell contact sites showed an increase in cell wall material, consistent with a block in cell fusion during cell wall dissolution. To examine this phenotype further, we stained mixtures of FGSC2489 + Seg11 (compatible) versus FGSC2489 + Seg3 (incompatible) cells with the cell wall dye calcofluor white. Incompatible pairs of FGSC2489 + Seg3 germlings displayed significantly higher accumulation of dye at contact sites as compared to FGSC2489 + Seg11 pairs (Figure 1D). These data suggested that a cellular checkpoint is triggered upon cell-cell contact between genetically different cells that aborts fusion before cell wall dissolution is initiated.

Cell Fusion Arrest of Incompatible Cells Is Associated with Failure to Cease Communication

Members of a mitogen-activated protein kinase (MAPK) signaling complex (HAM-5/NRC-1/MEK-2/MAK-2) are recruited to fusion tips in germlings (termed conidial anastomosis tubes or CATs) [18] and to tips of fusion hyphae [12]. The MAK-2 complex assembles and disassembles at CAT tips every 8–10 min. A second protein complex bearing SOFT also assembles and disassembles at CAT tips but perfectly out of phase with the MAK-2 complex [19]. When FGSC2489 cells expressing either MAK-2-GFP or SOFT-GFP were paired with compatible Seg11 cells, MAK-2 and SOFT oscillated to CATs during chemotropic interactions and disappeared during cell wall dissolution and membrane merger (Figures 2A and 2B). In contrast, in arrested cell pairs (FGSC2489 + Seg3), MAK-2 and SOFT continued to oscillate at CAT tips long after cell contact (Figures 2A and 2B). These data indicated that germlings with a cell fusion block were unable to switch from communication to cell wall dissolution and membrane merger mode.

Cell Wall Remodeling Checkpoint Is Composed of Highly Polymorphic Linked Loci

To identify the causative locus of cell fusion arrest, we performed bulk segregant analysis (BSA) of progeny from the FGSC2489 $\times$ JW258 cross. After whole-genome resequencing, a region spanning approximately 1 Mb on chromosome V was identified that showed 100% SNP segregation between

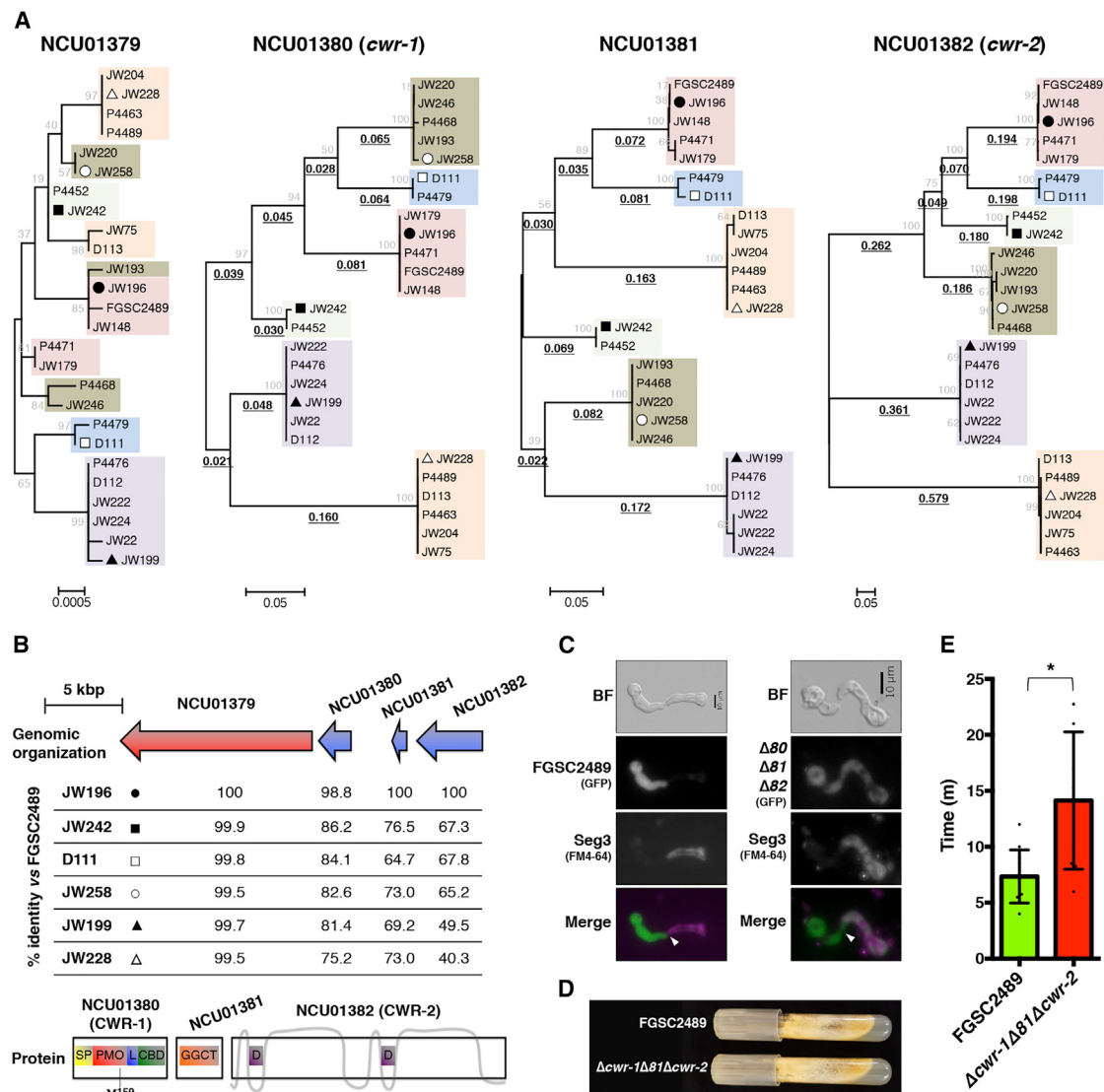


Figure 3. Identification of Genes Mediating Cell Fusion Arrest

(A) Amino acid sequences of NCU01379, NCU01380, NCU01381, and NCU01382 from 26 *N. crassa* isolates were used to build maximum-likelihood phylogenetic trees. Black underlined values below branches indicate length. Branch length values lower than 0.02 were omitted. Results from 100 bootstrap replicates are indicated at the nodes in gray. The six *cwr* haplogroups are indicated by rectangles of different colors. Selected isolates with correspondence to (B) are indicated with symbols. See also Figures S1B and S3.

(B) Graphical depiction of genomic organizations at the *cwr* region. The percentage of identity of the predicted protein sequences from wild isolates (JW196, JW242, D111, JW258, JW199, and JW228) was calculated using FGSC2489 as the reference. Conserved domains of NCU01380 (CWR-1), NCU01381, and NCU01382 (CWR-2) are shown. CBD, putative carbohydrate-binding domain; D, DUF3433 domain; GGCT, gamma-glutamylamine cyclotransferase domain; L, glycine- and serine-rich linker region; PMO, polysaccharide monooxygenase catalytic domain; SP, signal peptide. The predicted topology of CWR-2 transmembrane domains is shown in gray. See also Figure S4.

(C) FM4-64-stained Seg3 cells were paired with FGSC2489 GFP strain (left, no fusion) or the triple mutant (Δ NCU01380 Δ NCU01381 Δ NCU01382) GFP strain (right, fusion). Arrowheads indicate the zone of interaction between cells.

(D) Asexual development in slant tubes (7 days) for FGSC2489 and Δ NCU01380 Δ NCU01381 Δ NCU01382 strains.

(E) Time to cytoplasmic mixing after cell contact was quantified in pairs of FGSC2489 germings or Δ NCU01380 Δ NCU01381 Δ NCU01382 germings (self-fusion pairings). **p* = 0.0037 (Student's *t* test); *n* ≥ 8.

See also Tables S1 and S2.

FGSC2489 fusion-compatible and FGSC2489 fusion-incompatible pools of progeny DNA (Figure S1B). Using genomic sequences from 26 *N. crassa* isolates [15, 16, 20], we identified three linked genes (NCU01380, NCU01381, and NCU01382),

whose alleles showed high sequence diversity among individuals in this population and fell into six discrete haplogroups (Figure 3A). For example, the amino acid identity between FGSC2489 and JW228 for NCU01380, NCU01381, and

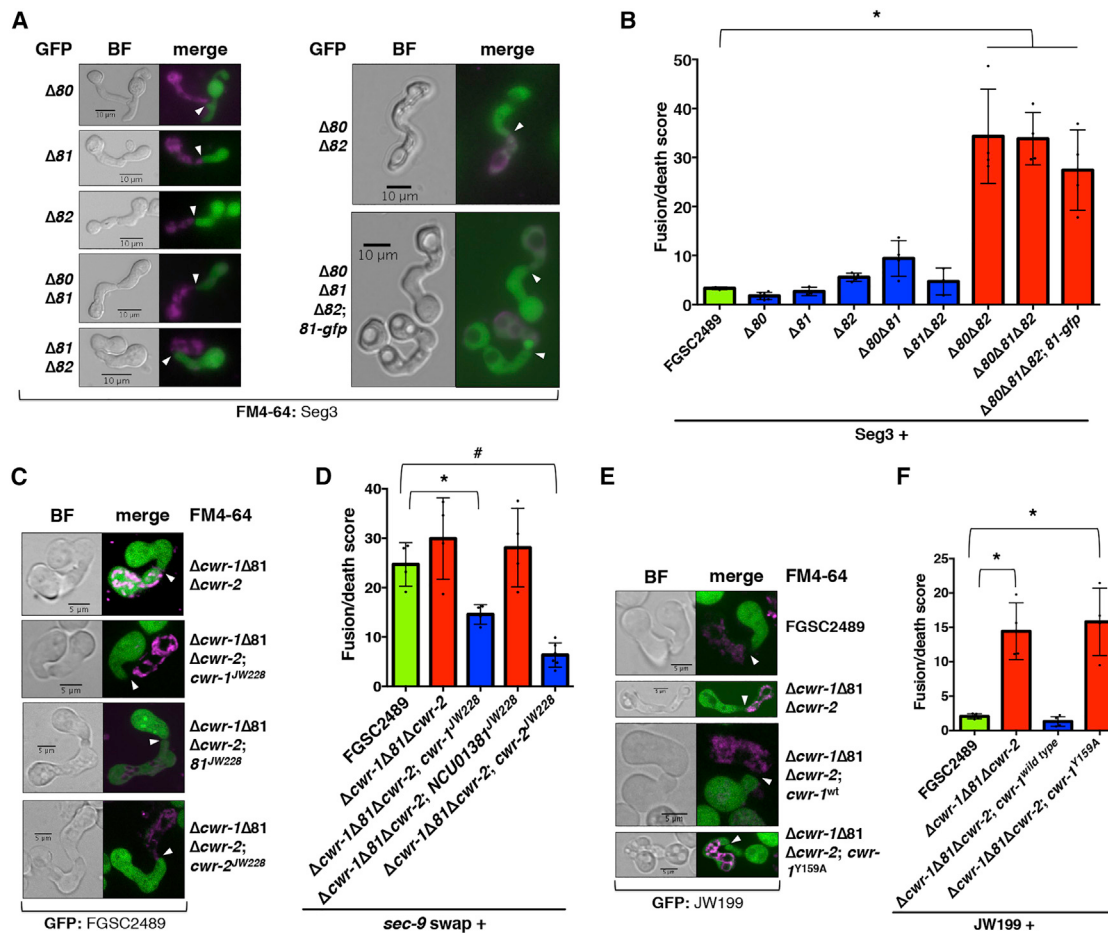


Figure 4. CWR-1 and CWR-2 Are Necessary and Sufficient to Induce Cell-Wall-Associated Arrest

(A) GFP-expressing cells (indicated on the left) were paired with FM4-64-stained Seg3 cells, and cytoplasmic mixing after cell contact (arrowhead) was evaluated. (B) Cell fusion as assayed by cell death score using *sec-9* swap assay, as determined by PI uptake and flow cytometry, was measured for Seg3 cells paired with cells from indicated strains. * $p < 0.0001$ (one-way ANOVA followed by Tukey post hoc test); $n \geq 4$. Seg3 and indicated strains contained incompatible *sec-9* alleles; cell death is a consequence of fusion of incompatible cells, with 50% the theoretical maximum. See also Figure S2. (C) FGSC2489-GFP cells were paired with indicated FM4-64-stained cells, and cytoplasmic mixing was evaluated upon contact (arrowheads). (D) Quantification of cell fusion as assayed by cell death was measured for pairings shown in (C). The FGSC2489 tester strain contained a *sec-9* swap allele. * $p < 0.01$; # $p < 0.0001$ (one-way ANOVA followed by Tukey post hoc test); $n \geq 4$. (E) JW199-GFP cells were paired with indicated FM4-64-stained cells, and cytoplasmic mixing upon contact (arrowheads) was evaluated. (F) Cell fusion, as assayed by cell death, was measured for pairings assessed microscopically in (E). * $p < 0.01$ (one-way ANOVA followed by Tukey post hoc test); $n \geq 6$. Green, FGSC2489 control strain; blue, strains blocked in cell fusion; red, strains that undergo cell fusion.

See also Tables S1 and S2.

NCU01382 was 75.2%, 73.0%, and 40.3%, respectively (Figure 3B). In contrast, NCU01379 showed 99.5% identity between FGSC2489 and JW228 (Figure 3B). The six haplogroups were completely conserved between alleles of NCU01380, NCU01381, and NCU01382 but only partially conserved for NCU01379 (Figure 3A), indicating recombination between NCU01379 and NCU01380-NCU01382.

The high sequence diversity observed for NCU01380, NCU01381, and NCU01382 is a property of genes involved in allorecognition, such as self-incompatibility loci in plants [21] and heterokaryon incompatibility loci in fungi [20, 22–24]. To test whether NCU01380, NCU01381, and/or NCU01382 were required for cell fusion arrest, we assessed the phenotype of mutants bearing deletions of NCU01380, NCU01381,

or NCU01382. Individual deletion strains Δ NCU01380, Δ NCU01381, and Δ NCU01382 were blocked in cell fusion with Seg3 cells (Figures 4A and 4B), a phenotype identical to parental strain FGSC2489. However, a strain bearing a deletion of all three genes (Δ NCU01380 Δ NCU01381 Δ NCU01382) underwent cell fusion with both FGSC2489 and Seg3 cells (Figures 3C and 4B).

To further test the hypothesis that allelic differences at NCU01380, NCU01381, and NCU01382 are causal for cell fusion arrest, we determined whether the block in cell fusion between wild isolates and FGSC2489 was dependent on NCU01380–NCU01382. First, we used strain JW196 (same haplogroup as FGSC2489; Figure 3A), and as predicted, JW196 fused with FGSC2489. However, cell fusion was blocked between

FGSC2489 and wild isolates from the other haplogroups (JW228, JW199, JW258, D111, and JW242; [Figure S3](#)). But when JW228, JW199, JW258, D111, or JW242 were paired with the triple-deletion strain Δ NCU01380 Δ NCU01381 Δ NCU01382, cell fusion was restored ([Figure S3](#)). These data confirmed that genetic differences at NCU01380, NCU01381, and NCU01382 were responsible for cell fusion arrest.

To determine whether all three genes were required for cell fusion arrest, we constructed double mutant strains Δ NCU01380 Δ NCU01381, Δ NCU01381 Δ NCU01382, and Δ NCU01380 Δ NCU01382. The double mutants Δ NCU01380 Δ NCU01381 and Δ NCU01381 Δ NCU01382 arrested during fusion in pairings with Seg3 cells. However, the Δ NCU01380 Δ NCU01382 double mutant underwent cell fusion with both FGSC2489 and Seg3 cells ([Figures 4A and 4B](#)). The triple mutant transformed with a GFP-tagged NCU01381 allele also underwent cell fusion with both FGSC2489 and Seg3 germlings ([Figures 4A and 4B](#)). These data indicated that allelic differences at NCU01380 and NCU01382, but not NCU01381, were required for the block in cell fusion. We named NCU01380 *cell wall remodeling checkpoint-1* (*cwr-1*) and NCU01382 *cell wall remodeling checkpoint-2* (*cwr-2*).

The morphological or growth phenotypes of any of the mutant combinations (including triple-deletion strain Δ cwr-1 Δ NCU01381 Δ cwr-2) were not significantly different from the parental FGSC2489 strain ([Figure 3D](#)). However, although triple mutant germlings underwent chemotropic interactions and self-fusion, they required significantly more time to fuse after contact, in comparison to FGSC2489 germlings ([Figure 3E](#)). These data indicated that *cwr-1*, NCU01381, and *cwr-2* contributed to the efficiency of self-fusion events during cell wall dissolution.

To determine whether CWR-1 or CWR-2 were sufficient to induce cell fusion arrest, we cloned *cwr-1*, *cwr-2*, and NCU01381 alleles from JW228 (incompatible with FGSC2489; [Figure S3](#)). The *cwr-1*^{JW228}, *cwr-2*^{JW228}, and NCU01381^{JW228} alleles were transformed individually into the Δ cwr-1 Δ NCU01381 Δ cwr-2 mutant. Homokaryotic strains bearing *cwr-1*^{JW228}, *cwr-2*^{JW228}, or NCU01381^{JW228} alleles were paired with FGSC2489, and fusion frequency was assessed microscopically and by flow cytometry. Consistent with *cwr-1* and *cwr-2* playing an essential role in a cell wall remodeling checkpoint, expression of either *cwr-1*^{JW228} or *cwr-2*^{JW228} alleles in the triple deletion strain was sufficient to induce cell fusion arrest with an otherwise isogenic parental strain FGSC2489 ([Figures 4C and 4D](#)). These data also indicated that incompatibility functioned in *trans* (between cells), as a strain carrying only one allele at *cwr-1* or *cwr-2* was blocked in cell fusion with an incompatible strain.

***cwr-1* Encodes a Polysaccharide Monooxygenase, and *cwr-2* Encodes a Protein with Predicted Transmembrane Domains**

cwr-1 encodes a predicted polysaccharide monooxygenase (PMO), with a signal peptide, a linker region rich in glycine and serine residues, and a carbohydrate-binding domain ([Figure 3B](#)). PMOs are an auxiliary activity (AA) within the carbohydrate-active enZymes database [25]. CWR-1 shows substantial homology to a chitin-active copper-dependent AA11 PMO from *Aspergillus*

oryzae [26] ([Figure S4A](#)). Of the 22 PMOs in *N. crassa*, four fell into the AA11 subtype, including NCU00822, NCU05932, NCU05404, and CWR-1 ([Figure S4B](#)). *cwr-2* encodes a protein containing two DUF3433 (domain of unknown function) and eight predicted transmembrane regions ([Figure 3B](#)).

The PMO portion of CWR-1 contained conserved amino acid residues associated with catalytic activity, including the histidine brace and a hydrogen-bonding network ([Figure S4A](#)) [25]. To test whether PMO catalytic activity was essential for cell fusion arrest, an allele with a substitution of Y159A (*cwr-1*^{Y159A}) was constructed ([Figures 4E and 4F](#)). This tyrosine is strictly conserved among AA11 PMOs and, when mutated in other AA families, leads to loss in catalytic activity [27]. Although introduction of *cwr-1*^{FGSC2489} into the triple-deletion mutant restored cell fusion arrest in pairings with incompatible JW199 cells, a triple-deletion mutant carrying *cwr-1*^{Y159A} underwent cell fusion. These data indicated that PMO catalytic activity is required for triggering the cell wall remodeling checkpoint.

Co-expression of Incompatible *cwr-1* and *cwr-2* Alleles within Single Cells Results in Asexual Developmental Defects

Our data indicated that *cwr-1* or *cwr-2* were sufficient for triggering cell fusion arrest in cells with incompatible *cwr* alleles. To investigate whether allelic versus non-allelic interactions were important for conferring this block, we expressed incompatible *cwr-1/cwr-2* alleles in a single strain. The *cwr-1*^{JW228} and *cwr-2*^{JW228} alleles were introduced into FGSC2489 (i.e., containing *cwr-1*^{FGSC2489} *cwr-2*^{FGSC2489}); strains co-expressing incompatible *cwr-1* and *cwr-2* alleles produced shorter aerial hyphae and asexual spores that were paler than parental FGSC2489 spores ([Figures 5A, 5B, and S5A](#)). This phenotype was not observed when *cwr-1*^{JW228} and *cwr-2*^{JW228} alleles were co-expressed in the triple deletion mutant (Δ cwr-1 Δ NCU01381 Δ cwr-2; [Figure S5A](#)). Introduction of a *cwr-1* allele from a different haplogroup (*cwr-1*^{D111}) into FGSC2489 also resulted in abnormal growth ([Figures 5A and 5B](#)). We used single-deletion strains of *cwr-1* or *cwr-2* (FGSC2489 background) expressing *cwr-1*^{JW228} or *cwr-1*^{D111} alleles to determine whether *cwr-1* allelic interactions or *cwr-1/cwr-2* non-allelic interactions were causative for the abnormal growth phenotype. Reversion from abnormal to wild-type morphology was observed when strains carried *cwr-1*^{JW228} or *cwr-1*^{D111} in a Δ cwr-2 mutant background and in strains carrying *cwr-2*^{JW228} in a Δ cwr-1 background ([Figures 5A and 5B](#)). These data indicated that non-allelic interactions between *cwr-1* and *cwr-2* were causal for the abnormal growth phenotype. FGSC2489; *cwr-1*^{JW228} asexual spores also displayed reduced germination, which was restored to wild-type levels when *cwr-2* was deleted ([Figure 5C](#)).

From the phenotype of strains carrying incompatible *cwr-1/cwr-2* alleles, we predicted that these strains would also show a block in self-fusion. Indeed, despite undergoing chemotropic interactions, cytoplasmic mixing in self-pairings was not observed ([Figures 5D and 5E](#)). Consistent with *cwr-1/cwr-2* non-allelic interactions being essential for this phenotype, the blocked self-fusion phenotype was reversed in *cwr-1*^{JW228} germlings carrying a Δ cwr-2 deletion ([Figures 5D and 5E](#)).

Mutants that show defects in germling fusion almost always show a block in hyphal fusion [12, 19, 28]. To test whether hyphal

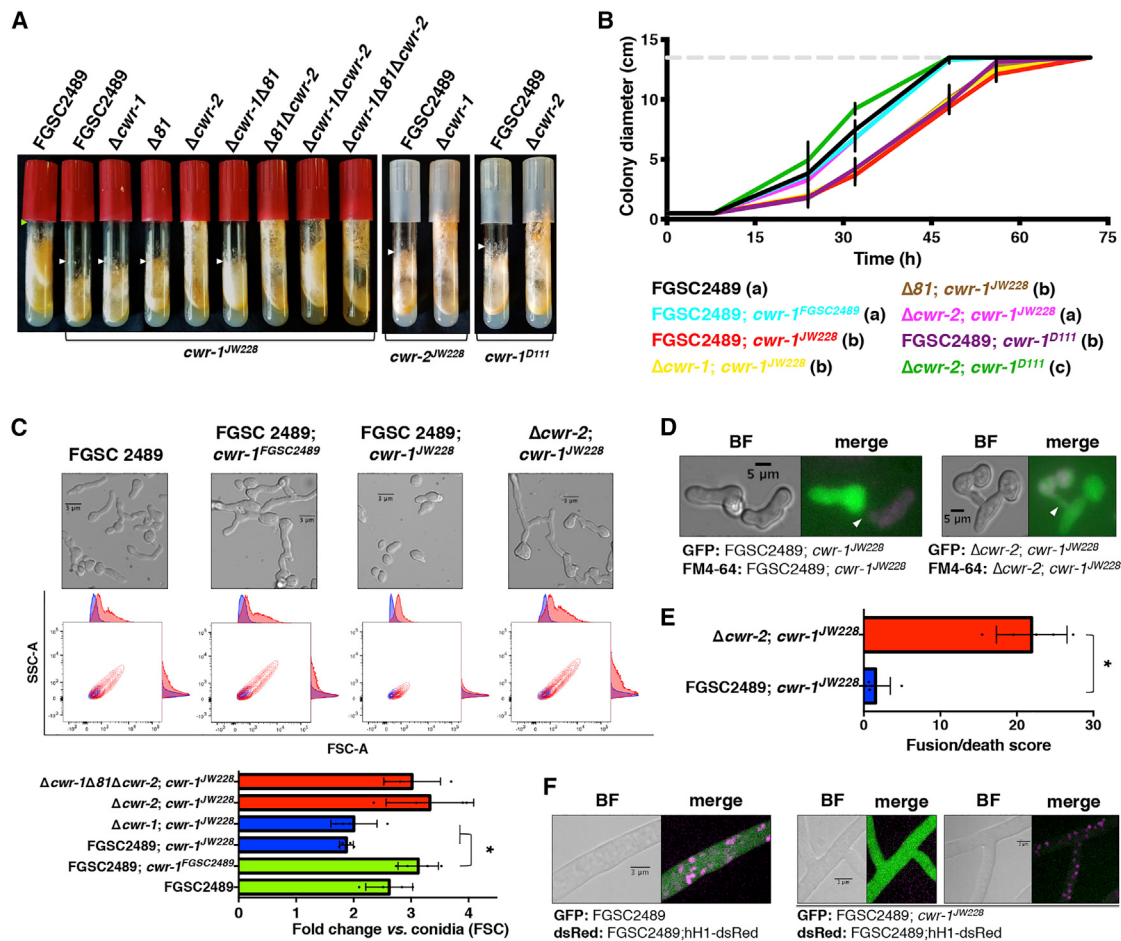


Figure 5. Genetic Interactions of Incompatible *cwr-1* and *cwr-2* Alleles Result in Reduced Germination, Aerial Hyphae Extension, Colony Establishment, and a Block in Cell Fusion

(A) Asexual development in slant tubes (7 days). Genes indicated below were introduced into the strain background indicated above. White arrowheads show aerial hyphae extension in comparison to FGSC2489 (green arrowhead). See also Figure S5.

(B) Colony diameter after 72 h of growth with color key shown below. Permutation tests (5,000) were used to identify statistically significant differences ($p < 0.05$); samples within statistical groups are represented by lower case letters; $n \geq 4$. See also Figure S5B.

(C) Bright-field micrographs (top panel) of 3.5-h-old germlings: strains co-expressing incompatible *cwr-1* and *cwr-2* alleles (third image) exhibit reduced germination. Middle panel shows flow cytometry contour plots of FSC (size) $\times$ SSC (granularity) profiles of 4-h cultures (red) versus conidial samples (blue). Bottom panel shows fold change of the mean FSC of 4-h cultures relative to conidial samples. * $p < 0.0314$ (one-way ANOVA followed by Tukey post hoc test); $n \geq 3$.

(D) Self-fusion in FGSC2489 $cwr-1^{JW228}$ (left) or $\Delta cwr-2$ $cwr-1^{JW228}$ (right) cells (isogenic cells expressing cytoplasmic GFP or FM4-64). Arrowheads indicate contact zone.

(E) Cell fusion, as assayed by cell death, was determined by PI uptake and flow cytometry, of FGSC2489 $cwr-1^{JW228}$ or $\Delta cwr-2$ $cwr-1^{JW228}$ cells measured by pairing isogenic strains except for incompatible *sec-9* alleles. * $p < 0.0001$ (Student's *t* test); $n \geq 5$. See also Figure S2.

(F) Images of contact area between FGSC2489 hyphae expressing hH1-dsRed (FGSC2489 H1-dsRED) and either FGSC2489 ($cwr-1^{FGSC2489}$)-GFP hyphae (left) or FGSC2489 ($cwr-1^{JW228}$)-GFP (right) hyphae, which was visually inspected for hyphae co-expressing GFP and dsRED, indicating hyphal fusion has occurred. See also Tables S1 and S2.

fusion is blocked in *cwr*-incompatible cells, FGSC2489; $cwr-1^{JW228}$ cells expressing cytoplasmic GFP were co-inoculated 5 mm apart onto a plate with FGSC2489 cells expressing dsRED-H1 (a nuclear marker). Hyphae containing both cytoplasmic GFP and dsRED nuclei were not observed (Figure 5F), in contrast to fusion-compatible pairings between FGSC2489-GFP + FGSC2489-dsRED-H1 (Figure 5F). These data indicated that hyphal allelic differences at the *cwr* loci.

CWR-1 and CWR-2 Show Trans-species Polymorphisms and Convergent Evolution

Orthologs of CWR-1/CWR-2 were only found in the Pezizomycotina subphylum. In some species, *cwr-1* and *cwr-2* were linked, although outside of Sordariomycetes and Leotiomyces, NCU01379 and NCU01381 were not linked to *cwr-1/cwr-2* (Figure S6A). To test whether allelic polymorphisms at CWR-1 and CWR-2 were retained in species where these loci are linked, we built phylogenetic trees that included multiple isolates of

N. tetrasperma, *N. discreta*, and various species of *Fusarium* (Table S3). In *N. crassa* and *N. discreta*, CWR-1 (Figure S6B) and CWR-2 (Figure 6) alleles clustered by haplogroup rather than by species, indicating that the age of allelic lines exceeds the age of speciation (7–10 mya) [29]. Six of the ten *N. discreta* isolates grouped into three *N. crassa* haplogroups, and the remaining four *N. discreta* isolates formed a haplogroup that was close to *N. tetrasperma* isolates (*N. tetrasperma* and *N. crassa* diverged 2.6–2.8 mya) [22]. In contrast, alleles of the highly conserved adjacent locus NCU01379 were reciprocally monophyletic (Figure 6). *N. tetrasperma* isolates did not show allelic differences at CWR-1 (Figure S6B) or CWR-2 (Figure 6), although they are polymorphic for other allorecognition loci [22, 30].

Alleles at the unlinked NCU01381 locus grouped by *Fusarium* species (Figure S6B), and, similar to *N. crassa*, linked *cwr-1* (Figure S6B) and *cwr-2* (Figure 6) loci showed allelic lineages. For example, ten orthologs of *cwr-1/cwr-2* from *F. fujikuroi* fell into four haplogroups, and four *F. verticillioide*s sequences fell into three haplogroups, with two of three haplogroups in *F. verticillioide*s clustering with three haplogroups of *F. fujikuroi*. Thus, in both *Neurospora* and *Fusarium*, *cwr-1/cwr-2* alleles showed trans-species polymorphisms, which is typical of allorecognition genes [20, 22, 23]. However, *cwr-1/cwr-2* haplogroups did not extend beyond genera (Figures 6 and S6B), indicating convergent evolution. This conclusion was supported by the lack of *cwr-1/cwr-2* allelic haplogroups in *N. tetrasperma*, suggesting that polymorphisms at these loci can be repeatedly lost and gained.

We calculated the average evolutionary diversity of *cwr-1/cwr-2* alleles in *Neurospora* and *Fusarium*, in isolates of *Trichoderma harzianum*, and in the plant pathogen *Zymoseptoria tritici*, with NCU01379 orthologs as an example of a non-diverse locus. *cwr-1/cwr-2* alleles showed increased diversity in all species except *N. tetrasperma* (Figure S6C), and diversity of *cwr*-linked NCU01381 alleles was only increased in *N. crassa* and *N. discreta* (Figure S6C).

DISCUSSION

In this study, we showed that cooperative cell fusion is under surveillance of an allorecognition checkpoint triggered by cell-cell contact. This checkpoint is controlled by two linked loci (*cwr-1* and *cwr-2*) with highly polymorphic alleles that segregated into discrete haplogroups. These two loci showed strict linkage disequilibrium and trans-species polymorphisms, consistent with balancing selection acting at these loci. *N. crassa* cells that harbored incompatible *cwr* alleles failed to initiate cell wall dissolution at the zone of contact and displayed extended MAK-2 and SOFT signaling, indicating an inability to transit between chemotropism and cell wall dissolution. CWR allorecognition acts in a negative manner, as cells containing alternate *cwr* alleles were blocked in cell fusion, and a strain carrying deletions of *cwr-1* and *cwr-2* underwent fusion with formerly incompatible cells. The *cwr-1* and *cwr-2* loci were not essential for self-fusion, although the $\Delta cwr-1 \Delta NCU01381 \Delta cwr-2$ mutant showed a slight delay in cell fusion, indicating that the CWR system is not necessary for fusion between compatible cells but rather blocks fusion between incompatible partners. The identification of CWR checkpoint in *N. crassa* will enable investigations into how the

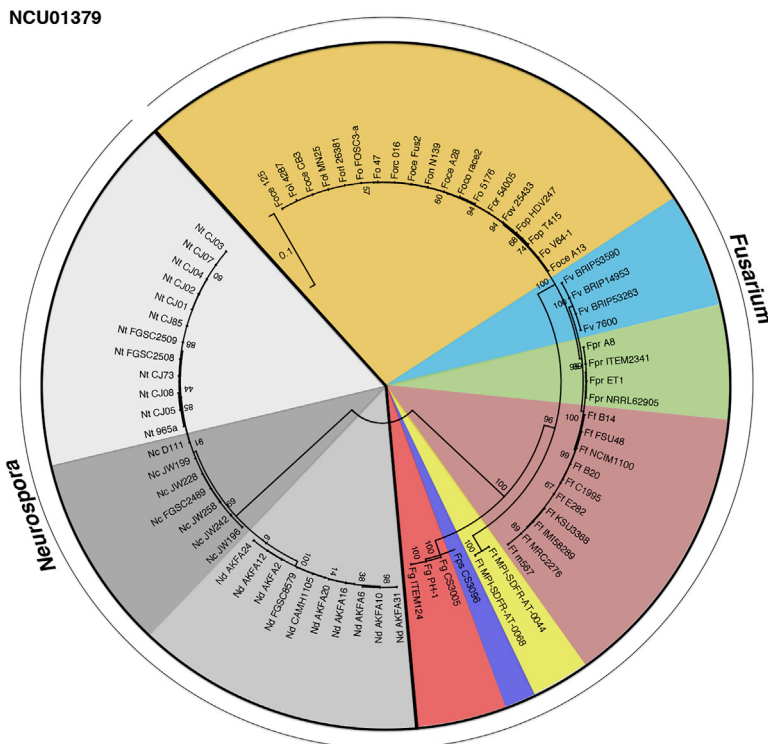
transition from adhesion to cell wall dissolution occurs, a process that is not well understood in fungi.

In the *N. crassa* genome, but not in other fungi, *cwr-1* and *cwr-2* are separated by NCU01381, which was dispensable for cell fusion arrest in *N. crassa*. Allelic diversity at NCU01381 is likely due to genetic hitchhiking, as NCU01381 was not identified in the *cwr-1/cwr-2* cluster in other species. Syntenic *cwr-1/cwr-2* pairs were only found in species in the Pezizomycotina, supporting the role of CWR proteins in cooperation and acquisition of complex multicellularity, which arose in this lineage 443–695 mya [31]. The linkage of *cwr-1/cwr-2* and TSP in *Fusarium* suggests that these loci could also regulate allorecognition in other fungi; *Fusarium* species are able to undergo germling CAT and hyphal fusion [32]. However, the fact that *cwr-1/cwr-2* haplotypes have not been maintained between *Neurospora* and *Fusarium* suggests repeated recruitment of these loci for allorecognition. This hypothesis is consistent with observations for *sec-9/plp-1*-mediated allorecognition; these two polymorphic loci regulate allorecognition in *N. crassa*, *Podospira anserine*, and *Cryphonectria parasitica* [15, 33], but polymorphisms segregate by genus rather than haplogroup [15].

Non-allelic interactions between *cwr-1* and *cwr-2* were necessary and sufficient to activate allorecognition and block cell fusion. CWR-1 is predicted to be a PMO11, and a mutation predicted to ablate catalytic activity abolished allorecognition. PMOs are typically active on recalcitrant polysaccharides [25], and the only characterized PMO11 acts on chitin [26], a fungal cell wall component. In one model for allorecognition, a specific cell wall modification by CWR-1 from a particular haplogroup would generate a signaling product, perhaps chitin-derived, that interacts with CWR-2; CWR-2 is a predicted transmembrane protein and could function as a receptor. However, this hypothesis would require CWR-1 from each haplogroup to form an allele-specific cell wall modification, which is not consistent with current models of PMO function [25]. An alternative model predicts interactions between non-cognate CWR-1 and CWR-2 proteins, where a conformational change in CWR-1 resulting from its catalytic activity leads to recognition by a non-cognate CWR-2. In this model, predicted catalytically inactive versions of CWR-1 (CWR-1^{Y159A}) would be incapable of polysaccharide degradation and presentation to its non-cognate CWR-2, allowing fusion to proceed. Further experiments to define regions of allelic specificity of CWR-1 and CWR-2, protein-protein interactions, biochemical analyses of CWR-1 from different haplogroups, and genetic suppression analyses will expand our knowledge on the molecular basis of allorecognition and cell fusion regulation by the CWR system.

By harnessing the power of population genomics, we identified three allorecognition checkpoints that act at distinct levels during early stages of cooperation and acquisition of multicellularity in filamentous fungi. The first checkpoint is regulated by allelic specificity at *doc* loci, where non-identity negatively regulates chemotropic interactions by preventing reinforcement of MAPK signaling [16]. The second checkpoint assesses identity at *cwr* loci and negative regulates the transition from cell adhesion to cell wall dissolution. Cell fusion can occur if cells have identical *doc* and *cwr* loci, but a third post-fusion checkpoint can trigger germling-regulated death. Allorecognition in this case is mediated by allelic differences at two linked loci, *sec-9*

Amino acid sequences of CWR-2 and NCU01379 from indicated isolates were used to build maximum-likelihood phylogenetic trees. Results from 100 bootstrap replicates are indicated in gray. Strains of the same species are shaded with identical colors; light gray, *N. tetrasperma*; medium gray, *N. discreta*; dark gray, *N. crassa*; light yellow, *F. tricinutum*; yellow, *F. oxysporum*; pink, *F. fujikuroi*; red, *F. graminearum*; green, *F. proliferatum*; blue, *F. verticillioides*; purple, *F. pseudograminearum*. See also [Figure S6](#). Phylogenetic trees of CWR-1 and NCU01381 are shown in [Figure S6B](#). Abbreviations and accession numbers are described in [Table S3](#).



and *plp-1*, which encode a SNARE and a fungal nucleotide oligomerization domain (NOD)-like receptor, respectively [15]. Unlike the *doc*, *cwr*, and *sec-9/plp-1* loci, which function in allorecognition in germlings and hyphae, genetic differences at *het* loci regulate heterokaryon formation following hyphal fusion; if hyphae differ in allelic specificity at a *het* locus, fusion compartments are walled off and rapidly killed [14, 34]. The existence of multiple allorecognition checkpoints prior and post cell fusion in *N. crassa* suggests an evolutionary pressure to avoid somatic nonself cooperation at all costs. Indeed, considering haplogroups identified for *doc* [16], *cwr*, *sec-9/plp-1* [15], and 12 *het* loci in *N. crassa* [20, 22], the likelihood of productive cell fusion events between non-identical cells is extremely small (~1,105,920 possible incompatible genotypes). Importantly, mechanisms regulating somatic allorecognition are suppressed during sexual reproduction, as wild isolates with allelic specificity differences at *doc*, *cwr*, *sec-9/plp-1*, and *het* loci are able to productively mate and produce meiotic progeny.

Examples of allorecognition upon cell-cell contact have been documented in the protochordate *Botryllus schlosseri*, where an inflammatory response resulting in allograft rejection is triggered upon contact between two colonies that lack identity at *fhc* [35]. In the social amoeba *Dictyostelium discoideum*, kin discrimination is mediated by direct binding of adhesion proteins TgrB1 and TgrC1 [10, 36]. In fungi, social cooperation and cell fusion enables interconnectedness of the mycelium, a network where public goods like organelles or nutrients are shared and transported over long distances [11, 12, 37, 38]. The mycelial soma is vulnerable to mycoparasites and/or cheaters that can gain access to the community via cell fusion, potentially exploiting shared resources and disturbing multicellular development [7, 8]. Hence, syncytial organisms, such as filamentous fungi, rely on allorecognition to ensure clonality, reducing the risk of transfer of infectious agents and cheaters and permitting formation of a fit cellular network consisting of near isogenic individuals with optimized competency to expand, forage, and mate.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - *Neurospora crassa*
- METHOD DETAILS
 - PCR
 - Transformation
 - Epifluorescence and confocal microscopy
 - Flow cytometry
 - Transmission electron microscopy
 - Radial growth
 - Bulk segregant analysis (BSA)
 - Bioinformatics
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.cub.2019.07.060>.

ACKNOWLEDGMENTS

We thank Marcus Roper (UC-Los Angeles) for his assistance with flow cytometry analyses. This work was supported in part by a National Science Foundation grant (MCB1412411) and a Laboratory Directed Research and Development Program of Lawrence Berkeley National Laboratory under US Department of Energy contract no. DE-AC02-05CH11231 to N.L.G. We thank the Berkeley Flow Cytometry Facility, the Robert D. Ogg Electron Microscope Laboratory, and the CNR Biological Imaging Facility for their technical support. This work used the Vincent J. Coates Genomics Sequencing Laboratory (UC Berkeley), supported by NIH S10 Instrumentation Grants S10RR029668 and S10RR027303.

AUTHOR CONTRIBUTIONS

A.P.G., J.H., M.A.M., and N.L.G. designed the study and experiments. A.P.G. and N.L.G. wrote the article. E.A.S. performed strain construction and analyzed the PMO family distribution. G.R. generated the flow cytometry analysis software. H.P.D. performed the calcofluor white-based cell wall measurements. J.P.-G. assisted in the bulk segregant analysis. N.R. contributed to the phylogenetic analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: June 2, 2019

Revised: July 16, 2019

Accepted: July 19, 2019

Published: August 29, 2019

REFERENCES

1. Knoll, A.H. (2011). The multiple origins of complex multicellularity. *Annu. Rev. Earth Planet. Sci.* 39, 217–239.
2. Bastiaans, E., Aanen, D.K., Debets, A.J., Hoekstra, R.F., Lestrade, B., and Maas, M.F. (2014). Regular bottlenecks and restrictions to somatic fusion prevent the accumulation of mitochondrial defects in *Neurospora*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369, 20130448.
3. Bastiaans, E., Debets, A.J., and Aanen, D.K. (2015). Experimental demonstration of the benefits of somatic fusion and the consequences for allorecognition. *Evolution* 69, 1091–1099.
4. Biella, S., Smith, M.L., Aist, J.R., Cortesi, P., and Milgroom, M.G. (2002). Programmed cell death correlates with virus transmission in a filamentous fungus. *Proc. Biol. Sci.* 269, 2269–2276.
5. Fernández-Busquets, X., Körnig, A., Bucior, I., Burger, M.M., and Anselmetti, D. (2009). Self-recognition and Ca²⁺-dependent carbohydrate-carbohydrate cell adhesion provide clues to the cambrian explosion. *Mol. Biol. Evol.* 26, 2551–2561.
6. Zhang, D.X., Spiering, M.J., Dawe, A.L., and Nuss, D.L. (2014). Vegetative incompatibility loci with dedicated roles in allorecognition restrict mycovirus transmission in chestnut blight fungus. *Genetics* 197, 701–714.
7. Aanen, D.K., Debets, A.J., de Visser, J.A., and Hoekstra, R.F. (2008). The social evolution of somatic fusion. *BioEssays* 30, 1193–1203.
8. Bastiaans, E., Debets, A.J., and Aanen, D.K. (2016). Experimental evolution reveals that high relatedness protects multicellular cooperation from cheaters. *Nat. Commun.* 7, 11435.
9. Diggle, S.P., Griffin, A.S., Campbell, G.S., and West, S.A. (2007). Cooperation and conflict in quorum-sensing bacterial populations. *Nature* 450, 411–414.

10. Kuzdzal-Fick, J.J., Fox, S.A., Strassmann, J.E., and Queller, D.C. (2011). High relatedness is necessary and sufficient to maintain multicellularity in *Dictyostelium*. *Science* **334**, 1548–1551.
11. Glass, N.L., Rasmussen, C., Roca, M.G., and Read, N.D. (2004). Hyphal homing, fusion and mycelial interconnectedness. *Trends Microbiol.* **12**, 135–141.
12. Fischer, M.S., and Glass, N.L. (2019). Communicate and fuse: how filamentous fungi establish and maintain an interconnected mycelial network. *Front. Microbiol.* **10**, 619.
13. Richard, F., Glass, N.L., and Pringle, A. (2012). Cooperation among germinating spores facilitates the growth of the fungus, *Neurospora crassa*. *Biol. Lett.* **8**, 419–422.
14. Gonçalves, A.P., Heller, J., Daskalov, A., Videira, A., and Glass, N.L. (2017). Regulated forms of cell death in fungi. *Front. Microbiol.* **8**, 1837.
15. Heller, J., Clavé, C., Gladieux, P., Saupe, S.J., and Glass, N.L. (2018). NLR surveillance of essential SEC-9 SNARE proteins induces programmed cell death upon allorecognition in filamentous fungi. *Proc. Natl. Acad. Sci. USA* **115**, E2292–E2301.
16. Heller, J., Zhao, J., Rosenfield, G., Kowbel, D.J., Gladieux, P., and Glass, N.L. (2016). Characterization of greenbeard genes involved in long-distance kind discrimination in a microbial eukaryote. *PLoS Biol.* **14**, e1002431.
17. Debets, F., Yang, X., and Griffiths, A.J. (1994). Vegetative incompatibility in *Neurospora*: its effect on horizontal transfer of mitochondrial plasmids and senescence in natural populations. *Curr. Genet.* **26**, 113–119.
18. Roca, M.G., Artl, J., Jeffree, C.E., and Read, N.D. (2005). Cell biology of conidial anastomosis tubes in *Neurospora crassa*. *Eukaryot. Cell* **4**, 911–919.
19. Fleissner, A., Leeder, A.C., Roca, M.G., Read, N.D., and Glass, N.L. (2009). Oscillatory recruitment of signaling proteins to cell tips promotes coordinated behavior during cell fusion. *Proc. Natl. Acad. Sci. USA* **106**, 19387–19392.
20. Zhao, J., Gladieux, P., Hutchison, E., Bueche, J., Hall, C., Perraudeau, F., and Glass, N.L. (2015). Identification of allorecognition loci in *Neurospora crassa* by genomics and evolutionary approaches. *Mol. Biol. Evol.* **32**, 2417–2432.
21. Fujii, S., Kubo, K., and Takayama, S. (2016). Non-self- and self-recognition models in plant self-incompatibility. *Nat. Plants* **2**, 16130.
22. Hall, C., Welch, J., Kowbel, D.J., and Glass, N.L. (2010). Evolution and diversity of a fungal self/nonself recognition locus. *PLoS ONE* **5**, e14055.
23. Muirhead, C.A., Glass, N.L., and Slatkin, M. (2002). Multilocus self-recognition systems in fungi as a cause of trans-species polymorphism. *Genetics* **161**, 633–641.
24. Paoletti, M. (2016). Vegetative incompatibility in fungi: from recognition to cell death, whatever does the trick. *Fungal Biol. Rev.* **30**, 152–162.
25. Span, E.A., and Marletta, M.A. (2015). The framework of polysaccharide monooxygenase structure and chemistry. *Curr. Opin. Struct. Biol.* **35**, 93–99.
26. Hemsworth, G.R., Henrissat, B., Davies, G.J., and Walton, P.H. (2014). Discovery and characterization of a new family of lytic polysaccharide monooxygenases. *Nat. Chem. Biol.* **10**, 122–126.
27. Harris, P.V., Welner, D., McFarland, K.C., Re, E., Navarro Poulsen, J.C., Brown, K., Salbo, R., Ding, H., Vlasenko, E., Merino, S., et al. (2010). Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. *Biochemistry* **49**, 3305–3316.
28. Fu, C., Iyer, P., Herkal, A., Abdullah, J., Stout, A., and Free, S.J. (2011). Identification and characterization of genes required for cell-to-cell fusion in *Neurospora crassa*. *Eukaryot. Cell* **10**, 1100–1109.
29. Corcoran, P., Dettman, J.R., Sun, Y., Luque, E.M., Corrochano, L.M., Taylor, J.W., Lascoux, M., and Johannesson, H. (2014). A global multilocus analysis of the model fungus *Neurospora* reveals a single recent origin of a novel genetic system. *Mol. Phylogenet. Evol.* **78**, 136–147.
30. Saenz, G.S., Stam, J.G., Jacobson, D.J., and Natvig, D.O. (2001). Heteroallelism at the *het-c* locus contributes to sexual dysfunction in out-crossed strains of *Neurospora tetrasperma*. *Fungal Genet. Biol.* **34**, 123–129.
31. Nagy, L.G., Kovács, G.M., and Krizsán, K. (2018). Complex multicellularity in fungi: evolutionary convergence, single origin, or both? *Biol. Rev. Camb. Philos. Soc.* **93**, 1778–1794.
32. Kurian, S.M., Di Pietro, A., and Read, N.D. (2018). Live-cell imaging of conidial anastomosis tube fusion during colony initiation in *Fusarium oxysporum*. *PLoS ONE* **13**, e0195634.
33. Choi, G.H., Dawe, A.L., Churbanov, A., Smith, M.L., Milgroom, M.G., and Nuss, D.L. (2012). Molecular characterization of vegetative incompatibility genes that restrict hypovirus transmission in the chestnut blight fungus *Cryphonectria parasitica*. *Genetics* **190**, 113–127.
34. Glass, N.L., and Dementhon, K. (2006). Non-self recognition and programmed cell death in filamentous fungi. *Curr. Opin. Microbiol.* **9**, 553–558.
35. Voskoboynik, A., Newman, A.M., Corey, D.M., Sahoo, D., Pushkarev, D., Neff, N.F., Passarelli, B., Koh, W., Ishizuka, K.J., Palmeri, K.J., et al. (2013). Identification of a colonial chordate histocompatibility gene. *Science* **341**, 384–387.
36. Hirose, S., Chen, G., Kuspa, A., and Shaulsky, G. (2017). The polymorphic proteins TgrB1 and TgrC1 function as a ligand-receptor pair in *Dictyostelium* allorecognition. *J. Cell Sci.* **130**, 4002–4012.
37. Roper, M., Simonin, A., Hickey, P.C., Leeder, A., and Glass, N.L. (2013). Nuclear dynamics in a fungal chimera. *Proc. Natl. Acad. Sci. USA* **110**, 12875–12880.
38. Simonin, A., Palma-Guerrero, J., Fricker, M., and Glass, N.L. (2012). Physiological significance of network organization in fungi. *Eukaryot. Cell* **11**, 1345–1352.
39. Vogel, H.J. (1956). A convenient growth medium for *Neurospora crassa*. *Microb. Genet. Bull.* **13**, 42–43.
40. Westergaard, M., and Mitchell, H.K. (1947). *Neurospora V*. A synthetic medium favoring sexual reproduction. *Am. J. Bot.* **34**, 573–577.
41. Colot, H.V., Park, G., Turner, G.E., Ringelberg, C., Crew, C.M., Litvinkova, L., Weiss, R.L., Borkovich, K.A., and Dunlap, J.C. (2006). A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. *Proc. Natl. Acad. Sci. USA* **103**, 10352–10357.
42. Gonçalves, A.P., Hall, C., Kowbel, D.J., Glass, N.L., and Videira, A. (2014). CZT-1 is a novel transcription factor controlling cell death and natural drug resistance in *Neurospora crassa*. *G3 (Bethesda)* **4**, 1091–1102.
43. Palma-Guerrero, J., Hall, C.R., Kowbel, D., Welch, J., Taylor, J.W., Brem, R.B., and Glass, N.L. (2013). Genome wide association identifies novel loci involved in fungal communication. *PLoS Genet.* **9**, e1003669.
44. Ellison, C.E., Hall, C., Kowbel, D., Welch, J., Brem, R.B., Glass, N.L., and Taylor, J.W. (2011). Population genomics and local adaptation in wild isolates of a model microbial eukaryote. *Proc. Natl. Acad. Sci. USA* **108**, 2831–2836.
45. Ellison, C.E., Kowbel, D., Glass, N.L., Taylor, J.W., and Brem, R.B. (2014). Discovering functions of unannotated genes from a transcriptome survey of wild fungal isolates. *MBio* **5**, e01046-13.
46. Szewczyk, E., Nayak, T., Oakley, C.E., Edgerton, H., Xiong, Y., Taheri-Talesh, N., Osmani, S.A., and Oakley, B.R. (2006). Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nat. Protoc.* **1**, 3111–3120.
47. Bardiya, N., and Shiu, P.K. (2007). Cyclosporin A-resistance based gene placement system for *Neurospora crassa*. *Fungal Genet. Biol.* **44**, 307–314.
48. Freitag, M., Hickey, P.C., Raju, N.B., Selker, E.U., and Read, N.D. (2004). GFP as a tool to analyze the organization, dynamics and function of nuclei and microtubules in *Neurospora crassa*. *Fungal Genet. Biol.* **41**, 897–910.
49. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675.

50. Gonçalves, A.P., Monteiro, J., Lucchi, C., Kowbel, D.J., Cordeiro, J.M., Correia-de-Sá, P., Rigden, D.J., Glass, N.L., and Videira, A. (2014). Extracellular calcium triggers unique transcriptional programs and modulates staurosporine-induced cell death in *Neurospora crassa*. *Microb. Cell* **1**, 289–302.
51. Katoh, K., and Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* **30**, 772–780.
52. Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **59**, 307–321.
53. Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**, 1870–1874.
54. Quevillon, E., Silventoinen, V., Pillai, S., Harte, N., Mulder, N., Apweiler, R., and Lopez, R. (2005). InterProScan: protein domains identifier. *Nucleic Acids Res.* **33**, W116–W120.
55. McGuffin, L.J., Bryson, K., and Jones, D.T. (2000). The PSIPRED protein structure prediction server. *Bioinformatics* **16**, 404–405.
56. Elso, C.M., Roberts, L.J., Smyth, G.K., Thomson, R.J., Baldwin, T.M., Foote, S.J., and Handman, E. (2004). *Leishmaniasis* host response loci (*lmr1-3*) modify disease severity through a Th1/Th2-independent pathway. *Genes Immun.* **5**, 93–100.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Hygromycin B	Thermo Fisher Scientific	10687010
Nourseothricin sulfate	Gold Biotechnology	N-500-100
FM4-64	Thermo Fisher Scientific	T3166
Calcofluor white M2R / Fluorescent Brightener 28	Sigma-Aldrich	F3543
Pluronic F-127	Sigma-Aldrich	P2443
Critical Commercial Assays		
Phire Plant Direct PCR Kit	Thermo Fisher Scientific	F130WH
Deposited Data		
Mapped reads for BSA / whole genome resequencing	This paper	https://www.ncbi.nlm.nih.gov/sra (SRA: PRJNA504906)
Computational code for flow cytometry analyses	This paper	https://github.com/gaberosenfield/Glass-Lab-Flow-Cytometry-Analysis
Experimental Models: Organisms/Strains		
<i>Neurospora crassa</i> strains (please see Table S1)	N/A	N/A
Oligonucleotides		
Primers: please see Table S2	N/A	N/A
Recombinant DNA		
pCSR-1	Fungal Genetics Stock Center	pCSR1
pMF272	Fungal Genetics Stock Center	pMF272
Software and Algorithms		
ImageJ	ImageJ	https://imagej.nih.gov/ij/
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
FlowJo	FlowJo, LLC	https://www.flowjo.com/
MAFFT	MAFFT	https://mafft.cbrc.jp/alignment/server/index.html
PhyML	ATGC	http://www.atgc-montpellier.fr/phyml/
Mega7	MEGA Software	https://www.megasoftware.net/
InterProScan	EBI	https://www.ebi.ac.uk/interpro/search/sequence-search
MEMSAT3/PSIPRED	UCL	http://bioinf.cs.ucl.ac.uk/psipred/

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, N. Louise Glass (Lglass@berkeley.edu). Strains and plasmids generated in this study are available from the Fungal Genetics Stock Center (<http://www.fgsc.net/>) and/or the Lead Contact.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Neurospora crassa

Vogel's minimal medium (VMM; with 2% sucrose and 1.5% agar) was employed for routine cultivation of *N. crassa* [39]. Asexual spores were obtained by growing strains in glass tubes with slanted VMM for 2 days in the dark, 30°C, followed by 4-6 more days at room temperature. For matings, synthetic cross medium (SC; with 1% sucrose and 1.5% agar) was used [40]. Briefly, a female parent strain that had been grown on SC until protoperithecia were observed – usually 8-10 days after inoculation – was fertilized with a conidial suspension of a male parent strain and grown for an additional 8-10 days until ascospores had been shot to the lid of

the Petri dish. Strains used in this study are listed in [Table S1](#). Deletion strains were constructed in a FGSC2489 genetic background [41]. Isolates from a Louisiana population have been previously described [16, 20, 42–45]. Genotypes were confirmed by PCR (Phire Plant Direct PCR Kit; Thermo Fisher Scientific) and/or Sanger sequencing. All experiments were performed at least in quadruplicates.

METHOD DETAILS

PCR

Fusion PCR [46] was employed to generate double and triple deletion mutants. For the Δ NCU01380 Δ NCU01382 double mutant, a Δ NCU01382 deletion was introduced in a Δ NCU01380 strain. A Pfu-based PCR strategy was used for site-directed mutagenesis of NCU01380. Key primers are described in [Table S2](#).

Transformation

Transformations were performed via electroporation based on pCSR-1 [47] or pMF272 [48] vectors. Conidia from one-week cultures of the recipient strain were harvested with ice-cold 1M sorbitol. After two washes, cells were resuspended in 90 μ l 1M sorbitol and mixed with 10 μ l containing of 1–2 μ g of DNA. The mixture was transferred to 1 mm gap cuvettes and electroporated using a Gene Pulser II (Bio-Rad) with the following settings: 1.5 kV, 25 μ F, 600 ohms. Ice-cold sorbitol (900 μ l) was quickly added to the sample, mixed with top agar medium (VMM with 1% agar, 1M sorbitol and a mixture of 2% sorbose, 0.05% glucose, 0.05% fructose as the carbon source; the three last components added after autoclaving) that was kept at $\sim$ 55°C and overlaid on previously prepared plates containing solidified bottom agar medium (identical to top agar but without sorbitol and with agar at a concentration of 1.5%) (containing 200 μ g/ml hygromycin B (Thermo Fisher Scientific) or 80 μ g/ml nourseothricin sulfate (Gold Biotechnology), when necessary for selection of transformants).

Epifluorescence and confocal microscopy

Conidia were diluted to 1.5×10^7 cells/ml. Strains bearing superfolder GFP (sGFP, hereafter referred as GFP) under the control of the *cgc-1* promoter or strains expressing SOFT-GFP or MAK-2-GFP were used to assess cell fusion [19]. Other strains were stained with 2 μ M FM4-64 (Thermo Fisher Scientific) for 15 mins in the dark. The FM4-64-stained cells were washed twice with sterile $\text{d}_2\text{H}_2\text{O}$ and mixed in a 1:1 proportion with the GFP-expressing cells. Eighty μ l of the strain mixture were spread onto 5 cm VMM plates and incubated at 30°C in the dark for 3.5–4 hr. In some experiments, 7 μ l of 5 mg/ml calcofluor white M2R (Sigma-Aldrich) was added to the sample. Fluorescence microscopy was performed on a Zeiss Axioskop 2 or on a Leica SD6000 confocal microscope equipped with a Yokogawa CSU-X1 spinning disk head or on a Zeiss LSM710. Images were analyzed using ImageJ [49]. For SOFT and MAK-2 oscillation experiments, mean pixel intensity was measured at the fusion spot and normalized with a region not involved in contact [19]. For hyphal fusion experiments, 3 μ l of 10^8 cells/ml were inoculated 5 mm apart on the center of a VMM plate.

Flow cytometry

Cultures were prepared as for microscopy, but 20% Pluronic F-127 (Sigma-Aldrich) [15] was used instead of agar. After 4 hr of incubation, samples were placed at -20°C for 10 mins, allowing for liquefaction of the Pluronic, followed by two washes with 1x PBS and final resuspension in 1x PBS containing 0.1 μ g/ μ l propidium iodide (PI) for cell death measurements. Cell death frequencies for at least 10,000 germlings were recorded on a BD LSR Fortessa X-20. Ungerminated conidia were run in parallel to allow gating of ungerminated from germinated spores. PI quantification was obtained using MATLAB (MathWorks). A computational code that automatically gates out ungerminated spores, recognizes fluorescence peaks and employs an exponential decay curve to fit and correct the data was used ([Figure S2](#)). For germination measurements, 20,000 total events were recorded and FlowJo (FlowJo, LLC) was used for analyses.

Transmission electron microscopy

Liquid VMM was inoculated with conidia from the indicated strains at a concentration of 10^6 cells/ml and incubated for 5 hr at 30°C (shaking at 200 rpm for 2.5 hr and without standing for 2.5 hr). After pelleted by centrifugation, cells were fixed with 2% glutaraldehyde, 4% paraformaldehyde, 0.04 M phosphate buffer (pH 7.0), followed by 2% KMnO_4 treatment. Samples were then dehydrated using a graded ethanol series before embedding the samples in resin.

Radial growth

For evaluation of radial growth [50], a 20 μ l inoculum containing 5×10^4 conidia was spotted on the center of 14.2 cm diameter Petri dishes and grown at 30°C, in constant dark. The colony diameter was recorded twice a day.

Bulk segregant analysis (BSA)

BSA followed by whole genome resequencing was performed as previously described [16]. Equal amounts of genomic DNA from 50 segregants in each pool were combined and used for library preparation.

Bioinformatics

Phylogenetic trees were constructed from MAFFT alignments [51] using PhyML [52] or Mega7 [53]. The mean evolutionary diversity was calculated from MAFFT alignments using Mega7 [53]. Conserved domains were identified manually or by using InterProScan [54]. The topography of NCU01382 was predicted using MEMSAT3/PSIPRED [55].

QUANTIFICATION AND STATISTICAL ANALYSIS

Prism (Graphpad Software) was used for the statistical analyses indicated in each figure, except for Figure 5B, in which the CGGC permutation test was used [56]. Data are presented as mean and standard deviation of multiple independent replicates and individual data points are shown.

DATA AND CODE AVAILABILITY

Mapped reads for each pool of DNA used for whole genome resequencing and bulk segregant analysis are available at <https://www.ncbi.nlm.nih.gov/sra> (SRA: PRJNA504906). The computational code used in this study for flow cytometry analyses (see also Figure S2) is available at <https://github.com/gaberosenfield/Glass-Lab-Flow-Cytometry-Analysis>.

Current Biology, Volume 29

Supplemental Information

Allorecognition upon Fungal Cell-Cell

Contact Determines Social Cooperation

and Impacts the Acquisition of Multicellularity

A. Pedro Gonçalves, Jens Heller, Elise A. Span, Gabriel Rosenfield, Hung P. Do, Javier Palma-Guerrero, Natalia Requena, Michael A. Marletta, and N. Louise Glass

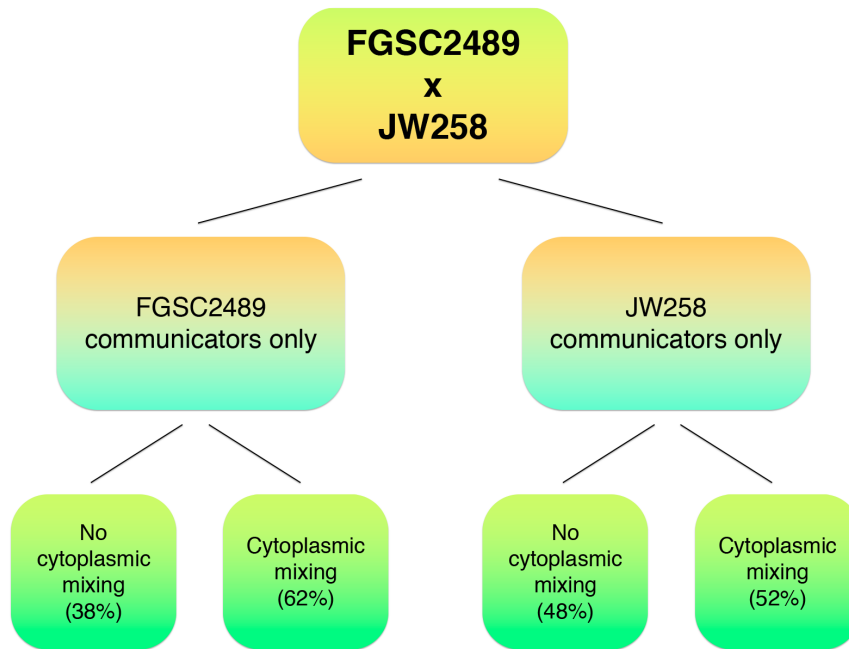
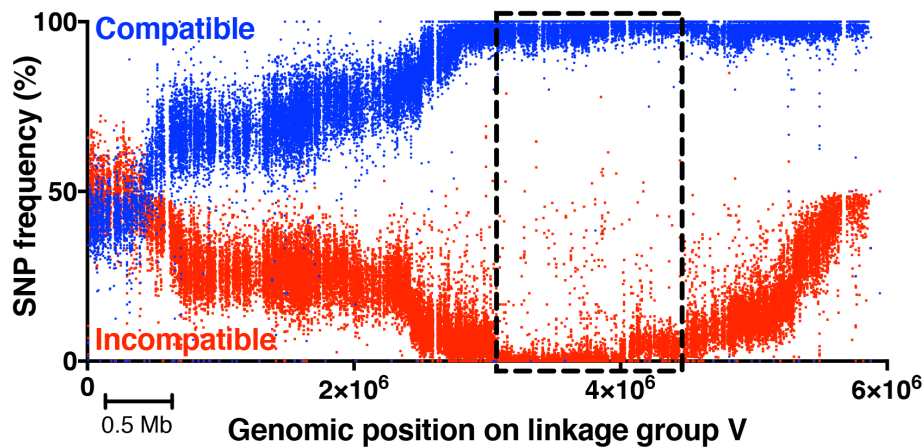
A**B**

Figure S1. Summary of the bulk segregant analysis and re-sequencing employed to identify the genetic basis of the cell wall arrest phenotype. Related to Figures 1 and 3. (A) FGSC2489 was crossed with the wild isolate JW258 and the progeny was initially screened for their ability to communicate with either parental strains [S1]. Cytoplasmic mixing was subsequently evaluated within each of the communication groups and the resulting percentages of progeny that showed cytoplasmic mixing or not are shown. **(B)** Whole genome resequencing of two pools of DNA obtained from progeny strains in the incompatible (38%; red) and compatible (62%; blue) groups of FGSC2489 communicators revealed a 1Mb region on chromosome V that showed 100% SNP segregation (highlighted by the dashed rectangle).

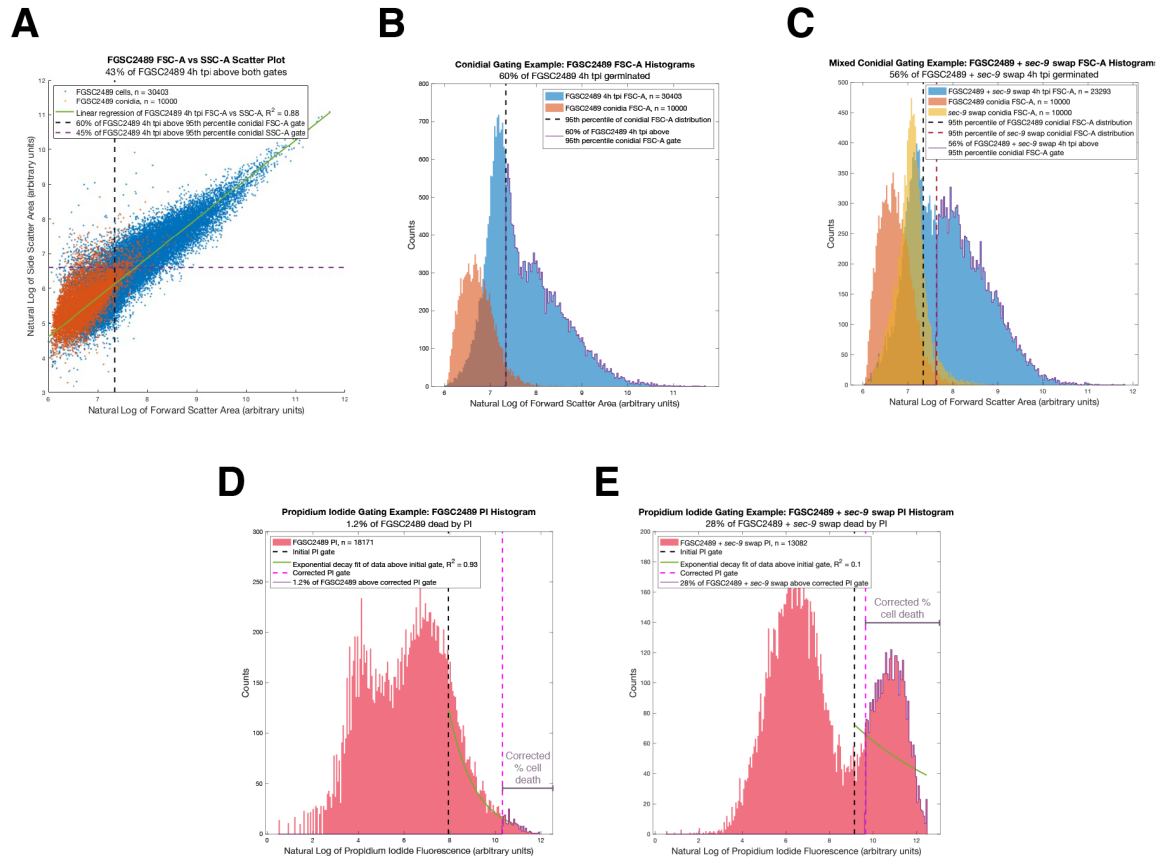
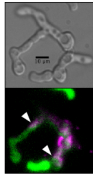
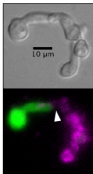
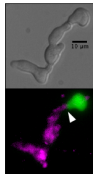
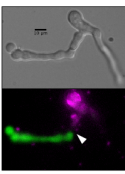
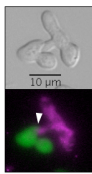
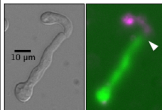


Figure S2. Automatic gating of germinated/ungerminated spores and quantification of cell death by flow cytometry. Related to Figures 1, 4 and 5. (A) Natural log of forward scatter area (FSC-A) versus natural log of side scatter area (SSC-A) of a representative experiment showing FGSC2489 ungerminated conidia (red) and the same conidia grown for 4 hrs as described in the Methods section (blue) with linear regression and germination gates. Gates were drawn at the 95th percentile of conidial FSC-A & SSC-A distributions (dashed lines). FSC-A was used for gating out conidia in subsequent analyses because forward scatter area is proportional to cell size, which increases as conidia germinate. SSC-A measures cell refractivity, which has a less obvious relationship with germination. FSC-A and SSC-A are correlated ($R^2 = 0.88$), such that using a two-dimensional gate (FSC-A and SSC-A, in this example, resulted in 43% of germination) is only slightly different than the germination rate obtained with using the more restrictive one-dimensional gate (SSC-A, in this example, resulted in 45% germination). Using the FSC-A one-dimensional gate resulted in 60% germination. (B) Histogram of the natural log of FSC-A of FGSC2489 ungerminated conidia (red) and the same conidia grown for 4 hours as described in the Methods section (blue), with germination gate. Gate drawn at the 95th percentile of conidial FSC-A distribution, yielded a 60% germination rate in this example. Data from cells considered germinated are outlined in purple. (C) Histogram of the natural log of FSC-A of FGSC2489 ungerminated conidia (red), 'sec-9 swap' ungerminated conidia (yellow) and mixed FGSC2489+'sec-9 swap' conidia grown for 4 hrs as described in the Methods section (blue), with germination gates. Gates drawn at the 95th percentiles of conidial FSC-A distributions. For mixed samples, the more restrictive 95th percentile conidial gate was used. In this example, the 'sec-9 swap' conidial gate was used and yielded a 56% germination rate. Data from cells

considered germinated are outlined in purple. (D) Histogram of the natural log of propidium iodide (PI) fluorescence area of germinated FGSC2489 cells with fluorescence gates and exponential decay fit. The initial gate (black dotted line) is defined using two-level Otsu's thresholding method [S2], with the higher threshold becoming the initial gate. Data above the initial gate is fit with an exponential decay curve, and the correlation coefficient is used to correct the percentage of the data above the initial gate according to the following formula: (% above initial gate) x (1 - correlation coefficient) = (corrected % cell death). In this example, 16.8% of germinated FGSC2489 cells are above the initial gate and $R^2 = 0.93$ for the exponential fit; this resulted in 1.2% corrected cell death. Data from events above the corrected gate are outlined in purple. (E) Histogram of the natural log of PI fluorescence area of germinated mixed FGSC2489+'*sec-9* swap' cells with fluorescence gates & exponential decay fit. In this example, 31.8% of germinated mixed FGSC2489+'*sec-9* swap' cells were above the initial gate and $R^2 = 0.1$ for the exponential fit; this resulted in 28% corrected cell death. Data from events above the corrected gate are outlined in purple. The percentage of corrected cell death is indicated as 'Fusion/death score' in various panels of Figures 1, 4 and 5. tpi = time post-inoculation.

Repository for analyses: <https://github.com/gaberosenfield/Glass-Lab-Flow-Cytometry-Analysis>.

	JW196	JW242	JW199	JW228	JW258	D111
FGSC2489						
$\Delta 80\Delta 81\Delta 82$	ND					



Cell wall dissolution and subsequent cell fusion



Arrest after cell-cell contact

Figure S3. Cell fusion analyses of selected wild isolates. Related to Figure 3. The ability to disassemble the cell wall at the zone of interaction and subsequently to fuse was tested for combinations of FGSC2489-background strains with selected wild isolates. Wild isolates were stained with FM4-64. FGSC2489-background strains expressed cytoplasmic GFP. For JW199 and JW228 pairings, FGSC2489 was employed; for the JW196, JW258 and JW242 pairings, the FGSC2489-background strain harbored a $\Delta doc-1\Delta doc-2$ double deletion; for the D111 pairing, the FGSC2489-background strain harbored a $\Delta doc-1\Delta doc-2$ double deletion and expressed *doc-1* and *doc-2* alleles from P4471. In the bottom panel, all FGSC2489-background strains harbored a $\Delta cwr-1\Delta NCU01381\Delta cwr-2$ triple deletion. ND, not determined. Arrowheads indicate the zone of interaction between cells. Colored rectangles on the strain name have correspondence to Figure 3A.

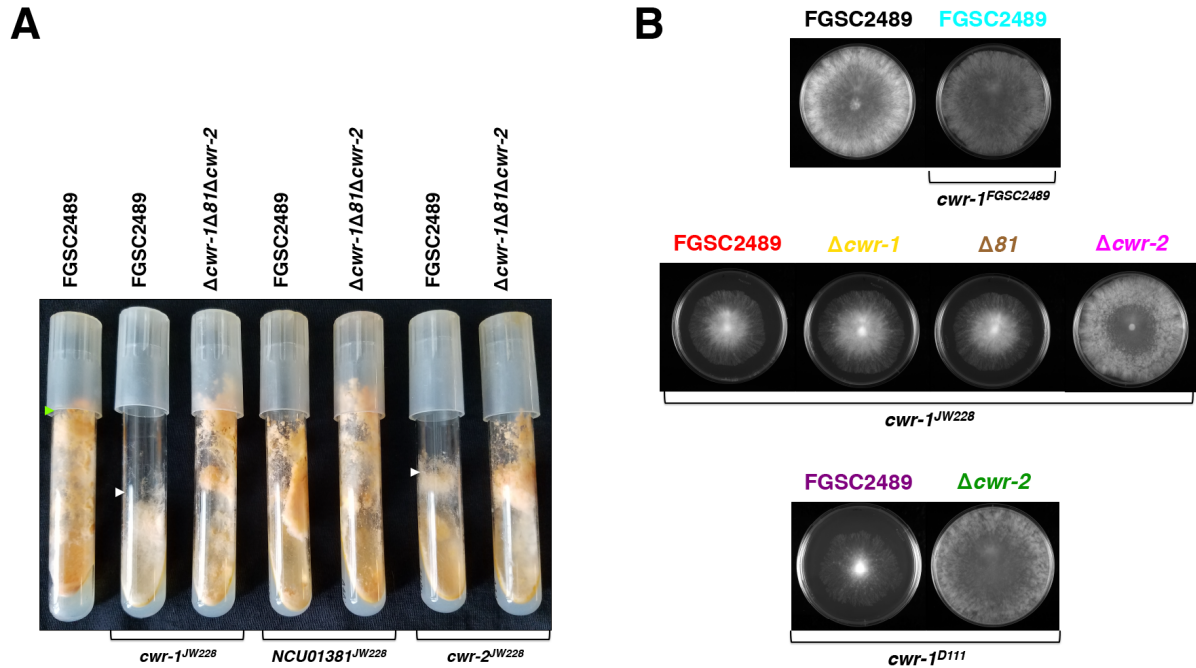
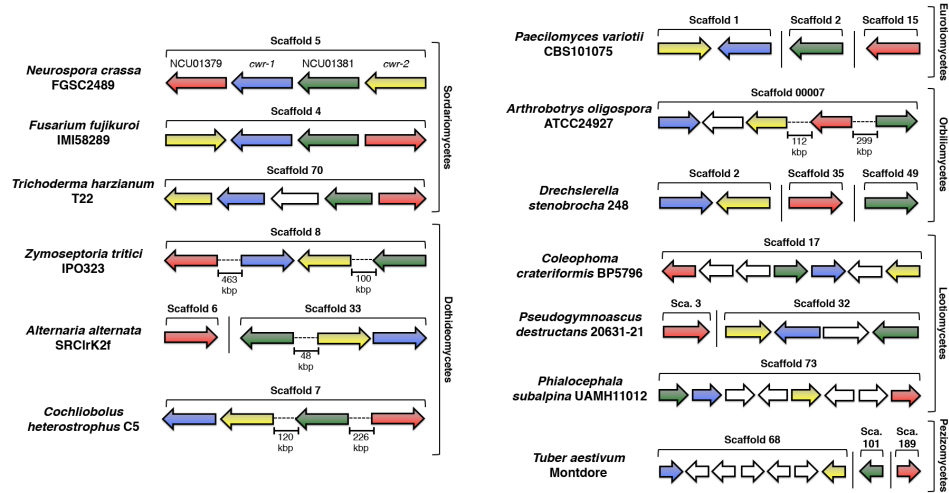
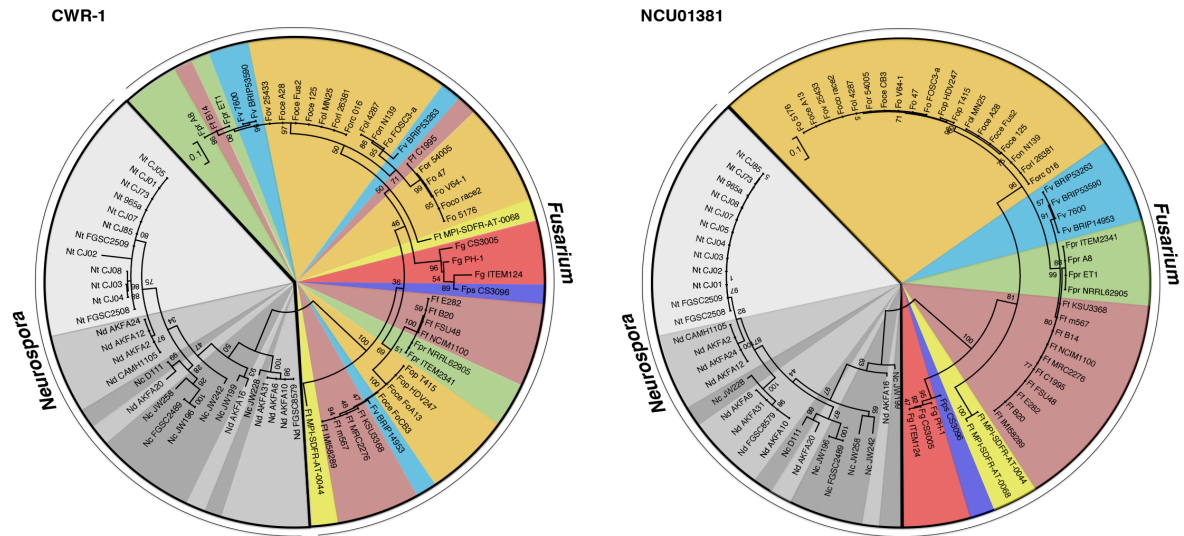


Figure S5. Expression of $cwr-1^{JW228}$ or $cwr-2^{JW228}$ in FGSC2489 results in morphological and fusion defects. Related to Figure 5. (A) Asexual development in slant tubes was evaluated after 7 days. Genes indicated below the tubes were introduced into the strain background indicated above the tubes. White arrowheads indicate reduced aerial hyphae *versus* the parental FGSC2489 strain (green arrowhead). In the $\Delta cwr-1 \Delta NCU01381 \Delta cwr-2$ strain, the expression of $cwr-1^{JW228}$ or $cwr-2^{JW228}$ does not result in reduced aerial hyphae. (B) Spores from indicated strains expressing $cwr-1$ alleles specified on the bottom were inoculated on the center of a Petri dish and photographs of the colonies were taken after 48 hrs of growth.

A



B



C

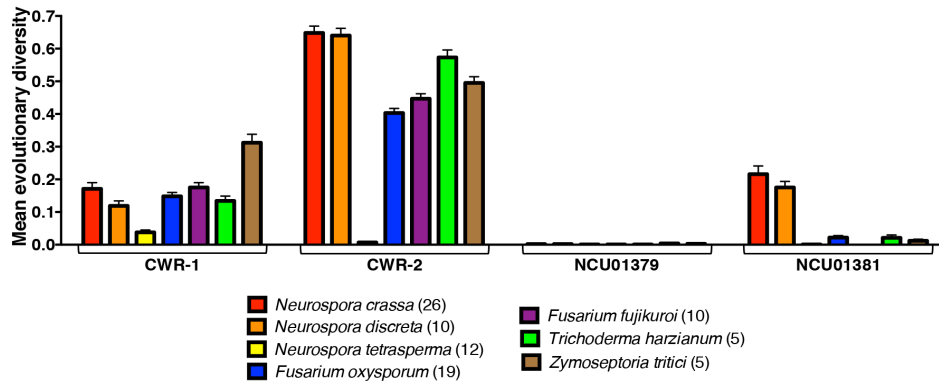


Figure S6. Distant CWR orthologs present features of balancing selection and convergent evolution. Related to Figure 6. (A) Genomic situation of *cwr-1* (blue), *cwr-2* (yellow), NCU01379 (red) and NCU01381 (green) orthologs in various fungal strains of the

Pezizomycotina subphylum. (B) The amino acid sequence of orthologs of CWR-1 and NCU01381 from the indicated isolates were used to build maximum likelihood phylogenetic trees. Bootstrap results (from 100 bootstrap replicates) are indicated at the nodes in grey. Strains of the same species are shaded with identical colors: light grey, *N. tetrasperma*; medium grey, *N. discreta*; dark grey, *N. crassa*; light yellow, *F. tricinctum*; yellow, *F. oxysporum*; pink, *F. fujikuroi*; red, *F. graminearum*; green, *F. proliferatum*; blue, *F. verticillioides*; purple, *F. pseudograminearum*. Note the mixed colors on several branches for CWR-1, indicating trans-species polymorphisms. TSP were also observed for NCU01381, but was unique to *Neurospora*. (C) The mean evolutionary diversity (MED) for each gene $\pm$ standard error (100 bootstrap replicates; JTT matrix-based model) was calculated for each of the indicated species. The number of alleles available for each species is indicated between parentheses.

Name	Genotype/Notes	Source	Fusion/ death score #
FGSC2489 (74-OR23-IV)	-	FGSC	2.05 ± 0.38
FGSC2489/GFP	<i>his-3::Pccg-1-gfp</i>	FGSC	ND
Seg11	Progeny of FGSC2489 x JW258	*	2.49 ± 0.46
Seg3	Progeny of FGSC2489 x JW258	*	3.54 ± 2.68
$\Delta\Delta sec-9$ (<i>sec-9</i> swap)	$\Delta plp-1(NCU09244)\Delta plp-2(NCU09244); sec-9(NCU09243)^{JW199}$	[S3]	3.37 ± 1.89
SO-GFP	<i>his-3::Pccg-1-NCU02794-gfp</i>	[S4]	ND
MAK-2-GFP	<i>his-3::Pccg-1-NCU02393-gfp</i>	[S4]	ND
$\Delta cwr-1\Delta 81\Delta cwr-2$	$\Delta NCU01380\Delta NCU01381\Delta NCU01382$	*	2.74 ± 2.20
$\Delta cwr-1\Delta 81\Delta cwr-2$ /GFP	$\Delta NCU01380\Delta NCU01381\Delta NCU01382; his-3::Pccg-1-gfp$	*	ND
$\Delta cwr-1$ /GFP	$\Delta NCU01380; his-3::Pccg-1-gfp$	*	ND
$\Delta cwr-1; \Delta\Delta sec-9$	$\Delta NCU01380; \Delta plp-1(NCU09244)\Delta plp-2(NCU09244); sec-9(NCU09243)^{JW199}$	*	2.74 ± 2.69
$\Delta 81$ /GFP	$\Delta NCU01381; his-3::Pccg-1-gfp$	*	ND
$\Delta 81; \Delta\Delta sec-9$	$\Delta NCU01381; \Delta plp-1(NCU09244)\Delta plp-2(NCU09244); sec-9(NCU09243)^{JW199}$	*	1.74 ± 0.91
$\Delta cwr-2$ /GFP	$\Delta NCU01382; his-3::Pccg-1-gfp$	*	ND
$\Delta cwr-2; \Delta\Delta sec-9$	$\Delta NCU01382; \Delta plp-1(NCU09244)\Delta plp-2(NCU09244); sec-9(NCU09243)^{JW199}$	*	7.69 ± 5.67
$\Delta cwr-1\Delta 81$ /GFP	$\Delta NCU01380\Delta NCU01381; his-3::Pccg-1-gfp$	*	ND
$\Delta cwr-1\Delta 81; \Delta\Delta sec-9$	$\Delta NCU01380\Delta NCU01381; \Delta plp-1(NCU09244)\Delta plp-2(NCU09244); sec-9(NCU09243)^{JW199}$	*	4.47 ± 5.38
$\Delta 81\Delta cwr-2$ /GFP	$\Delta NCU01381\Delta NCU01382; his-3::Pccg-1-gfp$	*	ND
$\Delta 81\Delta cwr-2; \Delta\Delta sec-9$	$\Delta NCU01381\Delta NCU01382; \Delta plp-1(NCU09244)\Delta plp-2(NCU09244); sec-9(NCU09243)^{JW199}$	*	4.35 ± 3.47
$\Delta cwr-1\Delta cwr-2$ /GFP	$\Delta NCU01380\Delta NCU01382; his-3::Pccg-1-gfp$	*	ND
$\Delta cwr-1\Delta cwr-2; \Delta\Delta sec-9$	$\Delta NCU01380\Delta NCU01382; \Delta plp-1(NCU09244)\Delta plp-2(NCU09244); sec-9(NCU09243)^{JW199}$	*	1.91 ± 0.96
$\Delta cwr-1\Delta 81\Delta cwr-2; \Delta\Delta sec-9$	$\Delta NCU01380\Delta NCU01381\Delta NCU01382; \Delta plp-1(NCU09244)\Delta plp-2(NCU09244); sec-9(NCU09243)^{JW199}$	*	3.28 ± 1.26
$\Delta cwr-1\Delta 81\Delta cwr-2; 81$ -GFP	$\Delta NCU01380\Delta NCU01381\Delta NCU01382; his-3::Pccg-1-NCU01381-gfp$	*	ND
$\Delta cwr-1\Delta 81\Delta cwr-2; 81$ -GFP; $\Delta\Delta sec-9$	$\Delta NCU01380\Delta NCU01381\Delta NCU01382; his-3::Pccg-1-NCU01381-gfp; \Delta plp-$	*	1.99 ± 0.73

	<i>1(NCU09244)Δplp-2(NCU09244); sec-9(NCU09243)^{JW199}</i>		
<i>Δcwr-1Δ81Δcwr-2; cwr-1^{JW228}</i>	<i>ΔNCU01380ΔNCU01381ΔNCU01382; his-3::Ptef-1-NCU01380^{JW228}</i>	*	2.73 ± 1.75
<i>Δcwr-1Δ81Δcwr-2; 81^{JW228}</i>	<i>ΔNCU01380ΔNCU01381ΔNCU01382; his-3::Ptef-1-NCU01381^{JW228}</i>	*	2.13 ± 1.22
<i>Δcwr-1Δ81Δcwr-2; cwr-2^{JW228}</i>	<i>ΔNCU01380ΔNCU01381ΔNCU01382; his-3::Ptef-1-NCU01382^{JW228}</i>	*	1.55 ± 1.26
<i>Δcwr-1Δ81Δcwr-2; cwr-1^{wt}</i>	<i>ΔNCU01380ΔNCU01381ΔNCU01382; his-3::PNCU01380-NCU01380</i>	*	1.83 ± 0.69
<i>Δcwr-1Δ81Δcwr-2; cwr-1^{Y159A}</i>	<i>ΔNCU01380ΔNCU01381ΔNCU01382; his-3::PNCU01380-NCU01380(475TAC>GCT)</i>	*	1.84 ± 0.93
<i>FGSC2489; cwr-1^{FGSC2489}</i>	<i>csr-1::Pgpd-1-NCU01380^{FGSC2489}</i>	*	ND
<i>FGSC2489; csr-1::cwr-1^{JW228}</i>	<i>csr-1::Pgpd-1-NCU01380^{JW228}</i>	*	0.76 ± 0.50
<i>Δcwr-1; csr-1::cwr-1^{JW228}</i>	<i>ΔNCU01380; csr-1::Pgpd-1-NCU01380^{JW228}</i>	*	ND
<i>ΔNCU01381; csr-1::cwr-1^{JW228}</i>	<i>ΔNCU01381; csr-1::Pgpd-1-NCU01380^{JW228}</i>	*	ND
<i>Δcwr-2; csr-1::cwr-1^{JW228}</i>	<i>ΔNCU01382; csr-1::Pgpd-1-NCU01380^{JW228}</i>	*	5.57 ± 5.26
<i>Δcwr-1Δ81; csr-1::cwr-1^{JW228}</i>	<i>ΔNCU01380ΔNCU01381; csr-1::Pgpd-1-NCU01380^{JW228}</i>	*	ND
<i>Δ81Δcwr-2; csr-1::cwr-1^{JW228}</i>	<i>ΔNCU01381ΔNCU01382; csr-1::Pgpd-1-NCU01380^{JW228}</i>	*	ND
<i>Δcwr-1Δcwr-2; csr-1::cwr-1^{JW228}</i>	<i>ΔNCU01380ΔNCU01382; csr-1::Pgpd-1-NCU01380^{JW228}</i>	*	ND
<i>Δcwr-1Δ81Δcwr-2; csr-1::cwr-1^{JW228}</i>	<i>ΔNCU01380ΔNCU01381ΔNCU01382; csr-1::Pgpd-1-NCU01380^{JW228}</i>	*	ND
<i>Δcwr-1; cwr-2^{JW228}</i>	<i>ΔNCU01380; his-3::Ptef-1-NCU01382^{JW228}</i>	*	ND
<i>FGSC2489; cwr-1^{D111}</i>	<i>csr-1::Pgpd-1-NCU01380^{D111}</i>	*	ND
<i>Δcwr-2; cwr-1^{D111}</i>	<i>ΔNCU01382; csr-1::Pgpd-1-NCU01380^{D111}</i>	*	ND
<i>FGSC2489; csr-1::cwr-1^{JW228}/GFP</i>	<i>csr-1::Pgpd-1-NCU01380^{FGSC2489}; his-3::Pccg-1-gfp</i>	*	ND
<i>Δcwr-2; csr-1::cwr-1^{JW228}/GFP</i>	<i>ΔNCU01382; csr-1::Pgpd-1-NCU01380^{FGSC2489}; his-3::Pccg-1-gfp</i>	*	ND
<i>FGSC2489; csr-1::cwr-1^{JW228}; ΔΔsec-9</i>	<i>csr-1::Pgpd-1-NCU01380^{FGSC2489}; Δplp-1(NCU09244)Δplp-2(NCU09244); sec-9(NCU09243)^{JW199}</i>	*	2.05 ± 1.67
<i>Δcwr-2; csr-1::cwr-1^{JW228}; ΔΔsec-9</i>	<i>ΔNCU01382; csr-1::Pgpd-1-NCU01380^{FGSC2489}; Δplp-1(NCU09244)Δplp-2(NCU09244); sec-9(NCU09243)^{JW199}</i>	*	6.28 ± 1.53
hH1-dsRed	<i>his-3::Pccg-1-NCU06863-dsRed; rid-1</i>	[S5]	ND
JW258	Wild isolate	[S6]	nd
JW199	Wild isolate	[S6]	2.11 ± 0.65

JW228	Wild isolate	[S6]	ND
JW242	Wild isolate	[S6]	ND
JW196	Wild isolate	[S6]	ND
D111	Wild isolate	[S6]	ND
$\Delta doc-1 \Delta doc-2$ /GFP	$\Delta NCU07191 \Delta NCU07192$; <i>his-3::Pccg-1-gfp</i>	[S1]	ND
$\Delta doc-1 \Delta doc-2$; <i>doc-1 doc-2^{CG3}</i> /GFP	$\Delta NCU07191 \Delta NCU07192$; <i>his-3::doc-1^{P4471} doc-2^{P4471}</i> ; <i>Pccg-1-gfp</i>	[S1]	ND
$\Delta cwr-1 \Delta 81 \Delta cwr-2 \Delta doc-1 \Delta doc-2$ /GFP	$\Delta NCU01380 \Delta NCU01381 \Delta NCU01382 \Delta NCU07191 \Delta NCU07192$; <i>his-3::Pccg-1-gfp</i>	*	ND
$\Delta cwr-1 \Delta 81 \Delta cwr-2 \Delta doc-1 \Delta doc-2$; <i>doc-1 doc-2^{CG3}</i> /GFP	$\Delta NCU01380 \Delta NCU01381 \Delta NCU01382 \Delta NCU07191 \Delta NCU07192$; <i>his-3::doc-1^{P4471} doc-2^{P4471}</i> ; <i>Pccg-1-gfp</i>	*	ND
FGSC2489; <i>his-3::cwr-1^{JW228}</i>	<i>his-3::Ptef-1-NCU01380^{JW228}</i>	*	ND
FGSC2489; 81 ^{JW228}	<i>his-3::Ptef-1-NCU01381^{JW228}</i>	*	ND
FGSC2489; 81 ^{JW228} /GFP	<i>his-3::Ptef-1-NCU01381^{JW228}</i> ; <i>csr-1::Pccg-1-gfp</i>	*	ND
FGSC2489; <i>cwr-2^{JW228}</i>	<i>his-3::Ptef-1-NCU01382^{JW228}</i>	*	ND

FGSC: Fungal Genetics Stock Center [S7]; *: This study. # Fusion/death score, as determined by flow cytometry, of the respective strains grown alone is shown as the mean $\pm$ standard deviation. ND: Not determined.

Table S1. *Neurospora crassa* strains. Related to Figures 1-5.

Name	Sequence	Purpose
hph F	CGGAGACAGAAGATGATATTGAAGGAGC	Hygromycin B resistance cassette
hph R	GTTGGAGATTTTCAGTAACGTTAAGTGGAT	Hygromycin B resistance cassette
Nat F	ctagctgattctggagtgacc	Nourseothricin sulfate resistance cassette
Nat R	agcttgcaaattaaagccttcgagc	Nourseothricin sulfate resistance cassette
NCU01382_5'_flank_f	ggccagactaagttatgtcggagg	Generation of Δ NCU01380 Δ NCU01381 Δ NCU01382 and Δ NCU01381 Δ NCU01382; flanking fragment
NCU01382_5'_fusion_r	GCTCCTTCAATATCATCTTCTGTCTCCGgccatcttgccgtggatcctg	Generation of Δ NCU01380 Δ NCU01381 Δ NCU01382 and Δ NCU01381 Δ NCU01382; flanking fragment
NCU01380_3'_fusion_f	ATCCACTTAACGTTACTGAAATCTCCAACaccctgagctttatgtgacc	Generation of Δ NCU01380 Δ NCU01381 Δ NCU01382 and Δ NCU01380 Δ NCU01381; flanking fragment
NCU01380_3'_flank_r	aagctctattggcatggagg	Generation of Δ NCU01380 Δ NCU01381 Δ NCU01382 and Δ NCU01380 Δ NCU01381; flanking fragment
NCU01382_5'_f_nested	ggatcgcgtaaacctggtgggtg	Generation of Δ NCU01380 Δ NCU01381 Δ NCU01382 and Δ NCU01380 Δ NCU01382 *; fusion PCR
NCU01380_3'_r_nested	gcaagttagttacgatgagcggcag	Generation of Δ NCU01380 Δ NCU01381 Δ NCU01382; fusion PCR
NCU01381_5'_flank_f	gaacgacagcgaagtcgtg	Generation of Δ NCU01380 Δ NCU01381; flanking fragment
NCU01381_5'_fusion_r	GCTCCTTCAATATCATCTTCTGTCTCCGgagtgaagccgaggagtg	Generation of Δ NCU01380 Δ NCU01381; flanking fragment
NCU01381_5'_f_nested	ggatatgtccaataagcgattg	Generation of Δ NCU01380 Δ NCU01381; fusion PCR
NCU01381_3'_fusion_f	ATCCACTTAACGTTACTGAAATCTCCAACgtctaaactttcttcgccgag	Generation of Δ NCU01381 Δ NCU01382; flanking fragment
NCU01381_3'_flank_r	gataattcggatcccagcg	Generation of Δ NCU01381 Δ NCU01382; flanking fragment
NCU01381_3'_r_nested	gttgactgggtgctgaacg	Generation of Δ NCU01381 Δ NCU01382; fusion PCR
NCU01382_aR(Nat)	gtcactccagaatcagctagGAAGGACAGCTTCAAACT	Generation of Δ NCU01380 Δ NCU01382; flanking fragment *
NCU01382_cF(Nat)	aaggctttaattgcaagctAATAAGCGATTGACTTGAC	Generation of Δ NCU01380 Δ NCU01382; flanking fragment *
NCU01382_cR	TGAAGCCGAGGAGTGTTAGCA	Generation of Δ NCU01380 Δ NCU01382; flanking fragment *
NCU01382_cnested	GGTGATATCAATAAATGACTCGGAC	Generation of Δ NCU01380 Δ NCU01382; fusion PCR *
80JW228_FXbaI	ACAAtctagaATGCACTTCACCAACTTG	Cloning of NCU01380 ^{W228} in pMF272
80JW228_REcoRI	ACAgaaattcGTAGCTTTGGAGATCCTA	Cloning of NCU01380 ^{W228} in pMF272

81JW228_FXbaI	ATAtctagaATGGCTGCCACAAGCAA	Cloning of NCU01381 ^{JW228} in pMF272
81JW228_REcoRI	ATAgaattcCATTCCCTTTGCAAGGC	Cloning of NCU01381 ^{JW228} in pMF272
82JW228_FXbaI	ATAtctagaATGGGCGTGGGAAAGTTCT	Cloning of NCU01382 ^{JW228} in pMF272
82JW228_RPspOMI	AAAgggcccTTTTATACCGAGAAAGTACA	Cloning of NCU01382 ^{JW228} in pMF272
cwr-1_wt_F	ATAgcgccgcACTGATTCATGCGGTAAA	Cloning of wild type <i>cwr-1</i> in pMF272
cwr-1_wt_R	CGCgaattcCTTTTTCAGGGCCACCATC	Cloning of wild type NCU01380 in pMF272
cwr-1_Y159A_F	CCGAGAGTTCGCTATGAACTGTG	Site-directed mutagenesis of NCU01380
cwr-1_Y159A_R	CACAGTTCATAGCGAACTCTCGG	Site-directed mutagenesis of NCU01380
cwr-1_JW228_insertFW	gggtgtacagcatgcgctagcATGCACTTCACCAACTTGAT	Cloning of NCU01380 ^{JW228} in pCSR-1
cwr-1_JW228_vectorRV	ATCAAGTTGGTGAAGTGCATgctagcgcagctgtacacc	Cloning of NCU01380 ^{JW228} in pCSR-1
cwr-1_D111_insertFW	gggtgtacagcatgcgctagcATGTTCTTCACACAAGCTTT	Cloning of NCU01380 ^{D111} in pCSR-1
cwr-1_D111_vectorRV	AAAGCTTGTGTGAAGAACATgctagcgcagctgtacacc	Cloning of NCU01380 ^{D111} in pCSR-1
cwr-1_JW228_D111_vectorFW	cggctcgttcagggttaattaattaatttaataagctccatgtcaacagg	Cloning of NCU01380 ^{JW228} and NCU01380 ^{D111} in pCSR-1
cwr-1_JW228_D111_insertRV	ggagctattaaattaattaattaaccctgaaacgaacgg	Cloning of NCU01380 ^{JW228} and NCU01380 ^{D111} in pCSR-1

*, for the generation of the Δ NCU01380 Δ NCU01382 double mutant, a PCR construct to delete NCU01382 was obtained (containing a nourseothricin resistance cassette) by PCR and transformed into a Δ NCU01380 strain (expressing a hygromycin B resistance cassette).

Table S2. Primers used in this study. Related to Figures 3-5

Abbreviation	Species	f. sp.	Strain name	NCU01379 ortholog	NCU01380 ortholog	NCU01381 ortholog	NCU01382 ortholog
Nc	<i>Neurospora crassa</i>	-	FGSC2489	XP_960936	XP_960937	XP_960938	XP_960939
Nc	<i>Neurospora crassa</i>	-	JW258	[S1, S3]			
Nc	<i>Neurospora crassa</i>	-	JW196				
Nc	<i>Neurospora crassa</i>	-	JW242				
Nc	<i>Neurospora crassa</i>	-	JW199				
Nc	<i>Neurospora crassa</i>	-	JW228				
Nc	<i>Neurospora crassa</i>	-	D111				
Nd	<i>Neurospora discreta</i>	-	AKFA16				
Nd	<i>Neurospora discreta</i>	-	AKFA10				
Nd	<i>Neurospora discreta</i>	-	AKFA31				
Nd	<i>Neurospora discreta</i>	-	AKFA6				
Nd	<i>Neurospora discreta</i>	-	AKFA24				
Nd	<i>Neurospora discreta</i>	-	AKFA12				
Nd	<i>Neurospora discreta</i>	-	AKFA2				
Nd	<i>Neurospora discreta</i>	-	CAMH1105				
Nd	<i>Neurospora discreta</i>	-	AKFA20				
Nd	<i>Neurospora discreta</i>	-	FGSC8579	Neudi1 71353	Neudi1 164991	Neudi1 164992	Neudi1 123145
Nt	<i>Neurospora tetrasperma</i>	-	FGSC2508	XP_009847286	XP_009847928	XP_009847927	XP_009847926
Nt	<i>Neurospora tetrasperma</i>	-	FGSC2509	EGZ75311	EGZ75312	EGZ75313	EGZ75314
Nt	<i>Neurospora tetrasperma</i>	-	CJ01	[S8, S9]			
Nt	<i>Neurospora tetrasperma</i>	-	CJ02				
Nt	<i>Neurospora tetrasperma</i>	-	CJ03				
Nt	<i>Neurospora tetrasperma</i>	-	CJ04				
Nt	<i>Neurospora tetrasperma</i>	-	CJ05				
Nt	<i>Neurospora tetrasperma</i>	-	CJ07				

Nt	<i>Neurospora tetrasperma</i>	-	CJ08				
Nt	<i>Neurospora tetrasperma</i>	-	CJ73				
Nt	<i>Neurospora tetrasperma</i>	-	CJ85				
Nt	<i>Neurospora tetrasperma</i>	-	965a				
Fg	<i>Fusarium graminearum</i>	-	PH-1	XP_011324713	XP_011324711	XP_011324712	XP_011324710
Fg	<i>Fusarium graminearum</i>	-	CS3005	EYB33121	EYB33119	EYB33120	EYB33470
Fg	<i>Fusarium graminearum</i>	-	ITEM124	PCD19322	PCD19324	PCD19323	PCD19325
Fps	<i>Fusarium pseudo-graminearum</i>	-	CS3096	XP_009255638	XP_009255636	XP_009255636	XP_009255635
Fo	<i>Fusarium oxysporum</i>	-	FOSC3-a	EWZ02246	EWZ02249	EWZ02247, EWZ02248	EWZ02250
Fo	<i>Fusarium oxysporum</i>	-	Fo47	EWZ48329	EWZ48332	EWZ48330, EWZ48331	EWZ48333
Foce	<i>Fusarium oxysporum</i>	<i>f. sp. cepae</i>	A28	RKL04166	RKL04196	RKL04185	RKL04184
Foce	<i>Fusarium oxysporum</i>	<i>f. sp. cepae</i>	CB3	RKL09064	RKL09062	RKL09063	RKL09061
Foce	<i>Fusarium oxysporum</i>	<i>f. sp. cepae</i>	A13	RKK69029	RKK69027	RKK69028	RKK69046
Fo	<i>Fusarium oxysporum</i>	-	V64-1	SCO82169	SCO82171	SCO82170	SCO82172
Foce	<i>Fusarium oxysporum</i>	<i>f. sp. cepae</i>	Fus2	RKK24812	RKK24810	RKK24813	RKK24811
Foce	<i>Fusarium oxysporum</i>	<i>f. sp. cepae</i>	125	RKK62586	RKK62600	RKK62634	RKK62599
For	<i>Fusarium oxysporum</i>	<i>f. sp. raphani</i>	54005	EXK97096	EXK97091	EXK97094	EXK97090
Fol	<i>Fusarium oxysporum</i>	<i>f. sp. lycopersici</i>	4287	XP_018243601	XP_018243596	XP_018243599	XP_018243595
Fol	<i>Fusarium oxysporum</i>	<i>f. sp. lycopersici</i>	MN25	EWZ93401	EWZ93405	EWZ93403	EWZ93406
Fop	<i>Fusarium oxysporum</i>	<i>f. sp. pisi</i>	HDV247	EXA42116	EXA42111	EXA42114	EXA42110
Fop	<i>Fusarium oxysporum</i>	<i>f. sp. pisi</i>	T415	FusoxT415 643914	FusoxT415 213834	FusoxT415 213802	FusoxT415 213849
Fon	<i>Fusarium oxysporum</i>	<i>f. sp. narcissi</i>	N139	RYC94909	RYC94911	RYC94964	RYC94910
Foco	<i>Fusarium oxysporum</i>	<i>f. sp. conglutinans</i>	race2	EXL80329	EXL80334	EXL80332	EXL80335
Fov	<i>Fusarium oxysporum</i>	<i>f. sp. vasinfectum</i>	25433	EXM33843	EXM33847	EXM33845	EXM33848
Fo	<i>Fusarium oxysporum</i>	-	5176	EGU85467	EGU85465	EGU85466	EGU85464
Forl	<i>Fusarium oxysporum</i>	<i>f. sp. radicis-lycopersici</i>	26381	EXL53658	EXL53662	EXL53661	EXL53663
Forc	<i>Fusarium oxysporum</i>	<i>f. sp. radicis-cucumerinum</i>	016	PCD41561	PCD41559	PCD41560	PCD41558

Ft	<i>Fusarium tricinctum</i>	-	MPI-SDFR-AT-0044	Fustr1 488323	Fustr1 340505	Fustr1 647008	Fustr1 489256
Ft	<i>Fusarium tricinctum</i>	-	MPI-SDFR-AT-0068	Fustr1 191053	Fustr1 191034	Fustr1 545313	Fustr1 528039
Ff	<i>Fusarium fujikuroi</i>	-	IMI58289	XP_023429181	XP_023429180	XP_023429601	XP_023429179
Ff	<i>Fusarium fujikuroi</i>	-	C1995	SCN91243	SCN91247	SCN91245	SCN91248
Ff	<i>Fusarium fujikuroi</i>	-	B14	SCV54708	SCV54704	SCV54705	SCV54703
Ff	<i>Fusarium fujikuroi</i>	-	FSU48	SCV34075	SCV34077	SCV34076	SCV34078
Ff	<i>Fusarium fujikuroi</i>	-	NCIM1100	SCO37804	SCO37801	SCO37803	SCO37800
Ff	<i>Fusarium fujikuroi</i>	-	E282	SCO01987	SCO01995	SCO01993	SCO01997
Ff	<i>Fusarium fujikuroi</i>	-	B20	SCN74057	SCN74067	SCN74061	SCN74070
Ff	<i>Fusarium fujikuroi</i>	-	m567	SCN95983	SCN95976	SCN95978	SCN95973
Ff	<i>Fusarium fujikuroi</i>	-	MRC2276	SCO40913	SCO40907	SCO40908	SCO40905
Ff	<i>Fusarium fujikuroi</i>	-	KSU3368	KLO91625	KLO91627	KLO91626	KLO91628
Fpr	<i>Fusarium proliferatum</i>	-	NRRL62905	CVK88004	CVK88002	CVK88003	CVK88001
Fpr	<i>Fusarium proliferatum</i>	-	ITEM 2341	RBA08679	RBA08681	RBA08680	RBA08682
Fpr	<i>Fusarium proliferatum</i>	-	A8	RKL41765	RKL41763	RKL41766	RKL41764
Fpr	<i>Fusarium proliferatum</i>	-	ET1	CZR38825	CZR38827	CZR38826	CZR38828
Fv	<i>Fusarium verticillioides</i>	-	BRIP53590	RBR10139	RBR10137	RBR10138	RBR10163
Fv	<i>Fusarium verticillioides</i>	-	BRIP53263	RBQ90282	RBQ90280	RBQ90281	RBQ90288
Fv	<i>Fusarium verticillioides</i>	-	BRIP14953	RBQ75871	RBQ75869	RBQ75870	RBQ75879
Fv	<i>Fusarium verticillioides</i>	-	7600	XP_018749309	XP_018749305	XP_018749306	XP_018749304
-	<i>Trichoderma harzianum</i>	-	T6776	KKP04788	KKP04790	KKP04789	KKP04791
-	<i>Trichoderma harzianum</i>	-	TR274	PKK52483	PKK52487	PKK52502	PKK52488
-	<i>Trichoderma harzianum</i>	-	CBS226.95	XP_024777899	XP_024777903	XP_024777900	XP_024777904
-	<i>Trichoderma harzianum</i>	-	M10	TriharM10_1 479745	TriharM10_1 256810	TriharM10_1 422315	TriharM10_1 371291
-	<i>Trichoderma harzianum</i>	-	T22	TriharT22_1 313179	TriharT22_1 313041	TriharT22_1 504702	TriharT22_1 461556
-	<i>Zymoseptoria tritici</i>	-	IPO323	XP_003850475	XP_003850030	XP_003850369	XP_003850392
-	<i>Zymoseptoria tritici</i>	-	ST99CH_3D7	SMQ53153	SMQ53351	SMQ53397	SMQ53352

-	<i>Zymoseptoria tritici</i>	-	ST99CH_1 A5	SMY26785	SMY26981	SMY27029	SMY26982
-	<i>Zymoseptoria tritici</i>	-	ST99CH_3 D1	SMR59590	SMR59792	SMR59840	SMR59793
-	<i>Zymoseptoria tritici</i>	-	ST99CH_1 E4	SMR56737	SMR56932	SMR56979	SMR56933

Table S3. Abbreviations and accession numbers. Related to Figures 6 and S6.

Supplemental References

- S1. Heller, J., Zhao, J., Rosenfield, G., Kowbel, D.J., Gladieux, P., and Glass, N.L. (2016). Characterization of greenbeard genes involved in long-distance kind discrimination in a microbial eukaryote. *PLoS Biol.* *14*, e1002431.
- S2. Otsu, N. (1979). Threshold selection method from gray-level histograms. *IEEE T. Syst. Man. Cyb.* *9*, 62-66.
- S3. Heller, J., Clave, C., Gladieux, P., Saupe, S.J., and Glass, N.L. (2018). NLR surveillance of essential SEC-9 SNARE proteins induces programmed cell death upon allorecognition in filamentous fungi. *Proc. Natl. Acad. Sci. U. S. A.* *115*, E2292-E2301.
- S4. Fleissner, A., Leeder, A.C., Roca, M.G., Read, N.D., and Glass, N.L. (2009). Oscillatory recruitment of signaling proteins to cell tips promotes coordinated behavior during cell fusion. *Proc. Natl. Acad. Sci. U. S. A.* *106*, 19387-19392.
- S5. Freitag, M., and Selker, E.U. (2006). Expression and visualization of red fluorescent protein (RFP) in *Neurospora crassa*. *Fungal Genet. Newsl.* *52*, 14-17.
- S6. Ellison, C.E., Hall, C., Kowbel, D., Welch, J., Brem, R.B., Glass, N.L., and Taylor, J.W. (2011). Population genomics and local adaptation in wild isolates of a model microbial eukaryote. *Proc. Natl. Acad. Sci. U. S. A.* *108*, 2831-2836.
- S7. McCluskey, K., Wiest, A., and Plamann, M. (2010). The Fungal Genetics Stock Center: a repository for 50 years of fungal genetics research. *J. Biosci.* *35*, 119-126.
- S8. Corcoran, P., Anderson, J.L., Jacobson, D.J., Sun, Y., Ni, P., Lascoux, M., and Johannesson, H. (2016). Introgression maintains the genetic integrity of the mating-type determining chromosome of the fungus *Neurospora tetrasperma*. *Genome Res.* *26*, 486-498.
- S9. Sun, Y., Svedberg, J., Hiltunen, M., Corcoran, P., and Johannesson, H. (2017). Large-scale suppression of recombination predates genomic rearrangements in *Neurospora tetrasperma*. *Nat. Commun.* *8*, 1140.