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The Predicted Mannosyltransferase GT69-2 Antagonizes RFW-1 To Regulate Cell Fusion in *Neurospora crassa*

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**ABSTRACT** Filamentous fungi undergo somatic cell fusion to create a syncytial, interconnected hyphal network which confers a fitness benefit during colony establishment. However, barriers to somatic cell fusion between genetically different cells have evolved that reduce invasion by parasites or exploitation by maladapted genetic entities (cheaters). Here, we identified a predicted mannosyltransferase, glycosyltransferase family 69 protein (GT69-2) that was required for somatic cell fusion in *Neurospora crassa*. Cells lacking GT69-2 prematurely ceased chemotropic signaling and failed to complete cell wall dissolution and membrane merger in pairings with wild-type cells or between *Δgt69-2* cells (self fusion). However, loss-of-function mutations in the linked regulator of cell fusion and cell wall remodeling-1 (*rfw-1*) locus suppressed the self-cell-fusion defects of *Δgt69-2* cells, although *Δgt69-2 Δrfw-1* double mutants still failed to undergo fusion with wild-type cells. Both GT69-2 and RFW-1 localized to the Golgi apparatus. Genetic analyses indicated that RFW-1 negatively regulates cell wall remodeling-dependent processes, including cell wall dissolution during cell fusion, separation of conidia during asexual sporulation, and conidial germination. GT69-2 acts as an antagonist to relieve or prevent negative functions on cell fusion by RFW-1. In *Neurospora* species and *N. crassa* populations, alleles of *gt69-2* were highly polymorphic and fell into two discrete haplogroups. In all isolates within haplogroup I, *rfw-1* was conserved and linked to *gt69-2*. All isolates within haplogroup II lacked *rfw-1*. These data indicated that *gt69-2/rfw-1* are under balancing selection and provide new mechanisms regulating cell wall remodeling during cell fusion and conidial separation.

**IMPORTANCE** Cell wall remodeling is a dynamic process that balances cell wall integrity versus cell wall dissolution. In filamentous fungi, cell wall dissolution is required for somatic cell fusion and conidial separation during asexual sporulation. In the filamentous fungus *Neurospora crassa*, allorecognition checkpoints regulate the cell fusion process between genetically different cells. Our study revealed two linked loci with transspecies polymorphisms and under coevolution, *rfw-1* and *gt69-2*, which form a coordinated system to regulate cell wall remodeling during somatic cell fusion, conidial separation, and asexual spore germination. RFW-1 acts as a negative regulator of these three processes, while GT69-2 functions antagonistically to RFW-1. Our findings provide new insight into the mechanisms involved in regulation of fungal cell wall remodeling during growth and development.

**KEYWORDS** *Neurospora*, cell fusion, allorecognition, mannosyltransferase, cell wall, chemotropism, glycosyltransferase, CAP59

In filamentous fungi, the interconnected mycelial network formed as a result of somatic cell fusion within an individual colony allows cytoplasm, nuclei, organelles, and nutrients to be shared, enhancing hyphal growth and rapid spatial expansion (1–5). Somatic cell fusion can occur between genetically identical and genetically dissimilar
fungal cells and colonies. Fusion between genetically dissimilar cells/colonies can facilitate the introduction and maintenance of genetic variation in populations for adaptive processes (6, 7). In some pathogenic fungi, intra- or interfungal species cell fusion events are important for virulence and host colonization (8, 9), or are required to broaden host specificity (10). However, cell fusion between genetically nonidentical colonies or cells can result in the transfer of infectious elements, such as mycoviruses or selfish genetic elements, or colonization by debilitated genotypes, such as dysfunctional mitochondria (11–13). To avoid such exploitation, filamentous fungi have evolved a variety of mechanisms to govern non-self-recognition processes (allorecognition) during both pre- and post-cell-fusion events (13–17).

In the filamentous ascomycete species *Neurospora crassa*, cell-to-cell communication and chemotropic interactions have been extensively studied and are important aspects that occur prior to cell fusion (18). In genetically identical germlings, intercellular communication promotes the formation of specialized structures in germinated asexual spores (germlings) termed conidial anastomosis tubes (CATs) that undergo chemotropic growth (19). An essential part of chemotropic growth between germlings and hyphae is the oscillation of a MAK-2 MAP kinase signaling complex and the SOFT protein to opposing CAT tips (20–22). So far, approximately 80 genes have been identified that are involved in the process of communication and/or fusion, ranging in function from intracellular signaling, calcium modulation, membrane merger, production of reactive oxygen species, actin regulation, vesicle trafficking, and transcriptional control (18, 23, 24).

Recently, allorecognition between genetically different germlings was investigated using a population sample of *N. crassa* (25–29). Three key checkpoints were characterized that regulate allorecognition in germlings/hyphae during the cell fusion process (17). The first checkpoint is controlled by allelic specificity at the determinant of communication (*doc*) loci, where nonidentity negatively regulates chemotropic interactions (25). The second checkpoint blocks the transition from cell adhesion to cell wall dissolution when adhered cells have nonidentity of *cwr-1* and *cwr-2* (cell wall remodeling) loci (29). The third checkpoint occurs postfusion and triggers a rapid cell death response in the fusion cells, which is determined by allelic differences at *plp-1/sec-9* (30) or *rcd-1* (27–30).

In this study, we identified the *gt69-2* gene from a cross between distantly related *N. crassa* isolates that segregated for a cell fusion phenotype. The *gt69-2* gene encodes a predicted alpha-1,3-mannosyltransferase that regulates cell wall dissolution during cell fusion and has similarity to the cryptococcal mannosyltransferase 1 (*CMT1*) gene from *Cryptococcus neoformans* (31). In *C. neoformans*, Cmt1p catalyzes the transfer of mannose from GDP-mannose to α-1,3-linked mannose disaccharides associated with capsule synthesis. Here, we show that loss-of-function mutations in *gt69-2* resulted in cells that were blocked in cell wall dissolution during cell fusion in *N. crassa*, a phenotype that was suppressed by loss-of-function mutations in *rfw-1*. Overexpression of *rfw-1* blocked cell fusion and also resulted in a conidial separation phenotype. Population analyses revealed two polymorphic haplotypes at *gt69-2*, with one haplogroup containing a linked *rfw-1* locus, which was absent in members of the second haplotype. These data indicate that the *gt69-2/rfw-1* loci are under balancing selection and provide new mechanisms regulating cell wall remodeling during cell fusion and conidial development in *N. crassa*.

**RESULTS**

Identification of highly polymorphic loci that segregate with a cell fusion arrest phenotype. Previously, we identified the cell wall remodeling (CWR) loci *cwr-1* and *cwr-2* that regulate cell wall dissolution during somatic cell fusion in *N. crassa* (29). During somatic cell fusion, hyphae and germlings (germinated asexual spores) that undergo chemotropic interactions, but carry incompatible alleles at *cwr-1* and *cwr-2* loci, fail to degrade the cell wall at the point of contact, thus preventing cytoplasmic mixing (29). Simultaneous deletion of *cwr-1* and *cwr-2* abolishes the block in cell fusion...
between some strains carrying alternative \(\text{cwr}\) alleles and cells complete the fusion process (29). However, in screening germinated conidia (germings) from a \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) mutant (\(\Delta\text{cwr-1} \Delta\text{NCU01381} \Delta\text{cwr-2}\)) (Table S1 in the supplemental material) against other wild-type \(N.\ crassa\) isolates, we observed that the \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) mutant failed to undergo cell fusion when paired with wild-type strain JW224 (Fig. 1A), suggesting the existence of a second locus that regulated cell wall dissolution during somatic cell fusion. To identify this second locus, we performed bulk segregant analysis (BSA) of progeny from a cross between FGSC2489 (the parental laboratory strain of the \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) mutant) and JW224. Progeny segregated into three classes: (i) progeny that underwent chemotropic interactions with FGSC2489 and JW224, but only completed cell fusion with FGSC2489; (ii) progeny that underwent chemotropic interactions with FGSC2489 and JW224, but only completed cell fusion with JW224; and (iii) progeny that failed to fuse with either parent. This third class of progeny was paired with the \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) mutant; approximately half of these progeny fused with the \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) strain, while the other approximately half did not. Genomic DNA from these two progeny pools of the third class, one pool of progeny that fused with the \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) mutant and the second progeny pool that failed to fuse with the \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) mutant, was isolated and subjected to whole-genome resequencing. From a comparison of single nucleotide polymorphisms (SNPs) between these two pools, a region spanning approximately 3 Mb on chromosome VI was identified that showed SNP segregation between the \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) fusion-compatible and the \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) fusion-incompatible pools of progeny (Fig. 1B). Upon further inspection of this 3 Mb region, mapped reads coverage to NCU05915 were significantly lower in \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) fusion-incompatible progeny pools compared to \(\Delta\text{cwr-1} \Delta\text{cwr-2}\) fusion-compatible progeny pools (Fig. S1A).

Using assembled genome sequences of 23 \(N.\ crassa\) isolates (26), we analyzed polymorphisms at NCU05915 and linked loci (NCU05914, NCU05916, and NCU05917) (Fig. S2). Among the 23 strains, alleles at NCU05914 and NCU05917 were highly conserved (>90 amino acid identity) (Fig. 1C, Fig. S1B and S2). In contrast, alleles of NCU05916 showed high sequence diversity and alleles fell into two haplogroups among the 23 wild isolates (Fig. 1C, Fig. S1B and S2). We defined the alleles of NCU05916 with high conservation to FGSC2489 (the laboratory strain; amino acid identity > 96%) as haplogroup I, and alleles that were highly similar to each other but different from haplogroup I alleles, and which included JW224, as haplogroup II (Fig. 1C, Fig. S1 and S2). Interestingly, all the strains within haplogroup II lacked the linked locus NCU05915, while within haplogroup I strains, NCU05915 alleles were highly conserved with above 98% amino acid identity (Fig. 1C, Fig. S1 and S2).

NCU05916 encodes a predicted 457-amino acid (aa) alpha-1,3-mannosyltransferase with a conserved “CAP59_mtransfer” protein domain (Fig. 2A), which showed 36% identity to \(C\).\ neoformans\ Cmt1p (\(C\).\ neoformans\ mannosyltransferase 1), an enzyme with alpha-1,3-mannosyltransferase activity (31). NCU05916 has been designated \(gt69-2\) to reflect its predicted biochemical activity as a glycosyl transferase member in family 69 (http://www.cazy.org/GT69.html). NCU05915 encodes a predicted 367-amino acid protein lacking identifiable functional domains except a transmembrane domain (Fig. 2A); we named NCU05915 as \(\text{regulator of cell fusion and cell wall remodeling 1}\) (\(\text{rfw-1}\)) for its phenotype (see below). Both NCU05915 and NCU05916 contained a predicted N-terminal signal peptide (SP) (Fig. 2A). Alignment of GT69-2 from 23 \(N.\ crassa\) isolates showed a region in the N terminus that was highly divergent (HD) between the two different haplogroups (Fig. 2A).

**Cell fusion deficient phenotype of \(\Delta\text{gt69-2}\) is suppressed by mutations in \(\text{rfw-1}\).**

To determine whether \(\text{gt69-2}\) and/or \(\text{rfw-1}\) was responsible for cell fusion arrest, we generated \(\Delta\text{gt69-2}\) and \(\Delta\text{rfw-1}\) single deletion mutants, and a \(\Delta\text{rfw-1}\Delta\text{gt69-2}\) double deletion mutant by replacing \(\text{gt69-2}, \text{rfw-1}\), or the whole region containing both \(\text{rfw-1}\) and \(\text{gt69-2}\) with a hygromycin B-resistance cassette in an FGSC2489 background (see the Materials and Methods) (Fig. S3A and B). Cell fusion assays were performed by
pairing FM4-64-stained mutant germlings with FGSC2489 germlings expressing cytoplasmic green fluorescent protein (GFP). The $\Delta gt69-2$ and $\Delta rfw-1\Delta gt69-2$ germlings underwent chemotropic interactions, but failed to complete cell fusion and cytoplasmic mixing with FGSC2489 germlings (Fig. 2B). In contrast, the $\Delta rfw-1$ mutant showed a wild-type cell fusion phenotype when paired with FGSC2489. These data indicated that $gt69-2$ was required for successful cell fusion with its wild-type parental strain.

To assess self-fusion defects, we crossed cytoplasmic GFP into the $\Delta gt69-2$, $\Delta rfw-1$, and $\Delta rfw-1\Delta gt69-2$ mutants. Similar to results obtained in pairings with the parental strain (FGSC2489), self fusion was observed in $\Delta rfw-1$ cells but was blocked in $\Delta gt69-2$ cells (Fig. 2C). However, to our surprise, the $\Delta rfw-1\Delta gt69-2$ double mutant cells underwent self fusion (Fig. 2C). These data indicated that the cell fusion arrest observed when the $\Delta rfw-1\Delta gt69-2$ double mutant was paired with its isogenic parent FGSC2489 (with functional alleles of $gt69-2$ and $rfw-1$) was alleviated in $\Delta rfw-1\Delta gt69-2$ self pairings.

To confirm that a deletion of $rfw-1$ suppresses the cell fusion defect of $\Delta gt69-2$, we generated a second double mutant by introducing a $\Delta rfw-1$ deletion into a $\Delta gt69-2$ mutant by replacing $rfw-1$ with a nourseothricin-resistance cassette (see the Materials and Methods) (Fig. S3A and B). This independently derived double mutant (ANCU05915 $\Delta gt69-2$) (Table S1) showed an identical slant phenotype to the $\Delta rfw-1\Delta gt69-2$ mutant (Fig. S3C) and, identical to the $\Delta rfw-1\Delta gt69-2$ mutant, underwent fusion in self pairings but not when paired with FGSC2489 (Fig. S3D). These data supported the original observation that deletion of $rfw-1$ suppressed the cell fusion defect of the $\Delta gt69-2$ mutant.

To quantify cell fusion frequencies in the mutants relative to wild-type cells, we utilized a flow cytometry method based on a robust postfusion death response in germinated spores that is mediated by genetic differences at sec-9 (29, 30). In brief,
Cell fusion is blocked in the Δgt69-2 cells, but is restored in Δrfw-1Δgt69-2 germlings. (A) Schematic drawing of the NCU05915 (RFW-1) and NCU05916 (GT69-2) proteins and amino acid alignment of the highly divergent region with gt69-2 orthologs from N. crassa and N. discreta wild isolates. SP, signal peptide; TM, transmembrane domain; CAP59_mtransferase superfamily, alpha-1,3-mannosyltransferase catalytic domain. (B) Assays of cell fusion with FGSC2489 by epifluorescence microscopy. FM4-64-stained FGSC2489, Δrfw-1, Δgt69-2, or Δrfw-1Δgt69-2 germlings paired with FGSC2489 expressing cytoplasmic GFP. (C) Assay of self-fusion phenotype in the indicated strains by epifluorescence microscopy. Self pairings of Δrfw-1, Δgt69-2, or Δrfw-1Δgt69-2 where half of the germling were stained with FM4-64 and the other half expressed cytoplasmic GFP. (D) Quantification of cell fusion via flow cytometry using a cell death assay activated upon cell fusion (29); sec9swap indicates that strains contain an incompatible allele of sec-9. FGSC2489 × FGSC2489 (WT × WT) pairing is the positive control for cell fusion and shows a high cell death score. FGSC2489 (WT) × cwr JW228 is a negative control (blocked in cell fusion) showing a low cell death score (29). *, P value < 0.0001 versus negative control (WT × cwr); #, P value < 0.001 versus positive control (WT × WT).
FGSC2489 and mutant strains were engineered to carry sec-9^GRD2 at the native sec-9 locus. When germlings carrying incompatible sec-9 alleles undergo cell fusion, cell death is induced within 20 min, which can be used as a proxy for cell fusion frequency using vital dyes and flow cytometry (29, 30). FGSC2489 + FGSC2489sec-9swap pairings were used as a positive control and showed a high death rate (~22%), while a negative-control pairing between cells unable to complete cell fusion (FGSC2489 with cwr-1JW228 + FGSC2489sec-9swap) showed a low death frequency (~5%) (Fig. 2D), a value consistent with that previously reported (29). As predicted by microscopic analyses, the Δgt69-2 + FGSC2489sec-9swap pairings, the Δgt69-2 + Δgt69-2sec-9swap pairings, and the Δrfw-1Δgt69-2 + FGSC2489sec-9swap pairings all showed a low death frequency (2 to 5%) (Fig. 2D), consistent with a block in cell fusion. In line with the microscopy results, the Δrfw-1 + FGSC2489sec-9swap pairings and the Δrfw-1 + Δrfw-1sec-9swap pairings both showed a high level of death frequency, showing that cells lacking rfw-1 are not affected in cell fusion (Fig. 2D). The Δrfw-1Δgt69-2 + Δrfw-1Δgt69-2sec-9swap self-pairings also showed a high death frequency (Fig. 2D), confirming that the lack of rfw-1 suppressed the cell fusion defect of the Δgt69-2 mutant. Additionally, these data also showed that neither GT69-2 nor RFW-1 was essential for cell fusion, as Δrfw-1Δgt69-2 germings showed self-fusion frequencies that were slightly higher than parental WT germings (Fig. 2D).

Genetic interactions between gt69-2 and rfw-1. The Δgt69-2 mutant showed a lower height of aerial hyphae compared to FGSC2489 (Fig. 3A), a phenotype that has been observed in other cell fusion mutants (21, 32, 33). However, this phenotype was not observed in the Δrfw-1 or Δrfw-1Δgt69-2 mutant strains, indicating that, analogously to the cell fusion process, the short aerial hyphae phenotype of Δgt69-2 was suppressed by deletion of rfw-1. To test whether the Δgt69-2 mutant showed a lower growth rate, we inoculated hyphal plugs or conidial suspensions of each strain on Vogel’s minimal medium (VMM) agar plates and measured the diameters of colonies up to 2 days postinoculation. When a conidial suspension was inoculated onto plates, the Δgt69-2 mutant showed a smaller colony diameter and fewer aerial hyphae compared to FGSC2489 (Fig. 3B and C). By plotting colony diameter over time, the Δgt69-2 showed a lower growth rate for 24 h, consistent with a lag in colony establishment, a phenotype that has also been observed in other cell fusion mutants (21) (Fig. 3C). In contrast, with hyphal plug inoculations—that is, after the colony was already established—the Δgt69-2 mutant and FGSC2489 showed a similar growth rate (Fig. 3C). These data indicated that gt69-2 was dispensable for growth rate of a mycelial colony, but important for colony establishment via germling fusion.

The cell fusion defect of the Δgt69-2 mutant was suppressed in the Δrfw-1Δgt69-2 double mutant (Fig. 2C). To further explore the genetic interactions between rfw-1 and gt69-2, we assayed the cell fusion phenotype of strains carrying different combinations of rfw-1 and gt69-2 deletions (wild-type alleles present in the respective strains are shown with a superscript plus [+]), sign) by microscopy and by flow cytometry (Fig. 4A and B). As shown in Fig. 2C, Δrfw-1Δgt69-2 + Δrfw-1Δgt69-2 germings undergo cell fusion, as did pairings between Δrfw-1gt69-2 + Δrfw-1Δgt69-2 germings (Fig. 4A), which was confirmed using flow cytometry (Fig. 4B). However, Δrfw-1gt69-2 and Δrfw-1gt69-2 germings showed a mixed cell fusion phenotype (Fig. 4A and B), where some pairs underwent cell fusion while others were blocked. Similarly, pairings between Δrfw-1Δgt69-2 + Δrfw-1Δgt69-2 pairs also showed a mixed cell fusion phenotype and reduced fusion frequency (Fig. 4A and B). These data indicated that in cells that lacked gt69-2 but contained rfw-1, cell fusion was fully or partially blocked. For successful fusion, gt69-2 was required in both cells if rfw-1 was present in either one or both cells. A summary of the cell fusion phenotypes of different combinations of rfw-1 and gt69-2 mutants is shown in Fig. 4C.

Cells lacking gt69-2 affect oscillation of MAK-2 and are blocked in cell wall dissolution. To assess when the cell fusion defect occurred in Δgt69-2 cells, we first used transmission electron microscopy to determine whether the fusion defect in Δgt69-2 cells was due to a failure in cell wall dissolution or in membrane merger.
FGSC2489 samples, cell wall and plasma membrane dissolution at the point of contact between germling fusion pairs was easily observed (Fig. 5A). In contrast, in Δgt69-2 pairings, we failed to find cell wall dissolution at contact points (Fig. 5A), and accumulation of cell wall material at cell-cell contact sites was not observed, in contrast to cell pairings between incompatible cwr strains (29). These data indicated that the block of cell fusion in Δgt69-2 mutant was caused by failure of cell wall breakdown upon contact between Δgt69-2 cells.

During chemotropic interactions between compatible cells, the mitogen-activated protein kinase (MAPK) signal transduction protein complex (NRC-1, MEK-2, MAK-2, and the scaffold protein HAM-5) are recruited to conidial anastomosis tubes (CATs) (19). The MAK-2 complex assembles and disassembles at CAT tips every 8 to 10 min; chemical inhibition of the phosphorylation activity of MAK-2 results in immediate cessation of chemotropic growth (20). A second protein complex bearing SOFT (SO) also assembles and disassembles at CAT tips, but perfectly out of phase with the MAK-2 complex (20). FGSC2489 (MAK-2-GFP) + FGSC2489 (SOFT-dsRED) cells display oscillation of MAK-2 and SOFT to CATs during chemotropic interactions until physical contact. Previously, we showed that in cell pairings between incompatible cwr strains, MAK-2 and SO continued to oscillate at the contact point, consistent with an inability of cwr incompatible cells to transit from chemotropic growth to cell wall dissolution (29).

To further explore the block in self cell fusion in the Δgt69-2 cells, we analyzed MAK-2-GFP localization in Δrfw-1Δgt69-2 germlings, in Δgt69-2 (mak-2-gfp) germlings, and in Δrfw-1Δgt69-2(mak-2-gfp) germlings. In wild-type pairings, MAK-2-GFP shows dynamic localization to CATs during chemotropic interactions, localizing to one CAT tip while disappearing from its partner cell every ~4.5 min (Fig. 5B). Consistent with microscopic observations showing wild-type levels of cell fusion, the Δrfw-1 cells showed normal dynamics of MAK-2 oscillation during chemotropic interactions.
In pairings between Δgt69-2 cells, oscillation of MAK-2 was observed during chemotropic interactions, but when Δgt69-2 germlings were in close proximity, MAK-2 localization to CATs was no longer observed (Fig. 5D). Additionally, MAK-2 localization at the contact point between Δgt69-2 germlings was not observed, which is apparent in wild-type pairings. These data indicated that Δgt69-2 germlings were affected during interactions when cells were in close proximity and in subsequent cell wall dissolution. Importantly, normal MAK-2-GFP dynamics during chemotropic interactions were restored in self pairings of Δrfw-1 Δgt69-2 germlings, consistent with the suppression of the cell fusion defect of the Δgt69-2 cells by deletion of rfw-1 (Fig. 5E).

GT69-2 and RFW-1 localization, overexpression phenotypes, and sensitivity to cell wall stress. Both GT69-2 and RFW-1 have predicted signal peptides. To characterize the subcellular localization of GT69-2 and RFW-1, we fused GFP to the N-terminal region of the predicted proteins immediately after the predicted signal peptides. The GFP-fused gt69-2 and rfw-1 were driven by the ccg-1 promoter and expressed in Δgt69-2 and Δrfw-1 cells, respectively; GFP fluorescence was not observed in constructs (Fig. 5C). In pairings between Δgt69-2 cells, oscillation of MAK-2 was observed during chemotropic interactions, but when Δgt69-2 germlings were in close proximity, MAK-2 localization to CATs was no longer observed (Fig. 5D). Additionally, MAK-2 localization at the contact point between Δgt69-2 germlings was not observed, which is apparent in wild-type pairings. These data indicated that Δgt69-2 germlings were affected during interactions when cells were in close proximity and in subsequent cell wall dissolution. Importantly, normal MAK-2-GFP dynamics during chemotropic interactions were restored in self pairings of Δrfw-1 Δgt69-2 germlings, consistent with the suppression of the cell fusion defect of the Δgt69-2 cells by deletion of rfw-1 (Fig. 5E).
using the gt69-2 or rfw-1 native promoters. The ccc-1-regulated gfp-gt69-2 construct fully complemented the growth and cell fusion defects of the ∆gt69-2 mutant (Fig. S3E). Both GFP-GT69-2 and GFP-RFW-1 showed a similar subcellular localization pattern as numerous fluorescent punctate structures in hyphal compartments (Fig. 6A and B),

**FIG 5** Fusion phenotype of ∆gt69-2 germlings shows a block in cell wall dissolution. (A) Transmission electron microscopy of FGSC2489 or ∆gt69-2 germlings undergoing self-fusion. FP, fusion pore; CW, cell wall. (B) Microscopic and graphic representation of MAK-2-GFP localization in FGSC2489 in germling pairs undergoing chemotropic interactions. (C) Microscopic and graphic representation of MAK-2-GFP localization in ∆rfw-1 germling pairs undergoing chemotropic interactions. (D) Microscopic and graphic representation of MAK-2-GFP localization in ∆gt69-2 germling pairs undergoing chemotropic interactions. (E) Microscopic and graphic representation of MAK-2-GFP localization in ∆rfw-1-Δgt69-2 germling pairs undergoing chemotropic interactions. T1 = CAT tip of germling one; T2 = CAT tip of germling two. The y axis shows the ratio of relative fluorescence intensity (R.F.I.) in the interaction zone compared to background. The x axis shows time (min). Panels show representative experiments; n = 4.
FIG 6 Cellular localization of GT69-2 and RFW-1 and phenotype of strains overexpressing rfw-1. (A) Upper panel shows confocal images of heterokaryons coexpressing GFP-GT69-2 and the ER marker mCherry-ERV-25; bottom panel shows confocal images of heterokaryons coexpressing GFP-GT69-2 and the Golgi marker mCherry-VPS-52 imaged by confocal microscopy. (B) Cellular localization of RFW-1. Upper panel shows confocal images of heterokaryons coexpressing GFP-RFW-1 and ER marker mCherry-ERV-25; bottom panel shows confocal images of heterokaryons coexpressing GFP-RFW-1 and the Golgi marker mCherry-VPS-52. (C) Slant tube phenotype of the indicated strains grown for 7 days. (D) Strains overexpressing rfw-1 (pccg-1gfp-rfw-1) showing a conidial separation defect. Left panel shows free conidia from FGSC2489. Middle panel shows the conidial separation defect observed in the Δrfw-1 (pccg-1gfp-rfw-1) strain. Right panel shows conidial septa stained by calcofluor white. Arrows show the double-doublet staining of septa between conidia. (E) Frequency of conidial chains in cultures of the indicated strains; for example, 13% conidial chains means that 13 conidial chains were observed in a population of 100 conidia. n = 4. (F) Percentage of germination of conidia at 3 h after conidial suspensions from FGSC2489 and Δrfw-1 (pccg-1gfp-rfw-1) were inoculated on VMM plates. (G) Microscopic analyses of cell fusion of Δrfw-1 or Δrfw-1 (pccg-1gfp-rfw-1) paired with an FGSC2489 strain expressing histone 1-GFP (H1-GFP). Δrfw-1 and Δrfw-1 (pccg-1gfp-rfw-1) germinals were stained with FM4-64. Cytoplasmic mixing was observed in Δrfw-1 + FGSC2489 (H1-gfp) pairings (left panel) but not in Δrfw-1 (pccg-1gfp-rfw-1) + FGSC2489 (H1-gfp) pairings (right panel). Calcofluor white staining showed undissolved cell wall (arrowheads) at the contact point of Δrfw-1 (pccg-1gfp-rfw-1) and FGSC2489 (H1-gfp) cells.
with a similar localization pattern in germlings (Fig. S4). It is likely that increased protein levels from \textit{ccg-1}-driven \textit{gt69-2} and \textit{rfw-1} expression resulted in a more abundant localization to Golgi. Localization of GFP-GT69-2 or GFP-RFW-1 to puncta within the cell did not change in germlings undergoing chemotropic interactions or cell fusion. To determine which organelles the puncta were, we coexpressed GFP-GT69-2 or GFP-RFW-1 with the Golgi marker mCherry-VPS-52 or the ER marker mCherry-ERV-25 in heterokaryotic strains. Colocalization of GFP-GT69-2 or GFP-RFW-1 with the ER marker ERV-25 was not observed, however, many of the GFP-GT69-2 and GFP-RFW-1 puncta colocalized with mCherry-VPS-52 (Fig. 6A and B). These data suggested that the punctate structures to which GFP-GT69-2 and GFP-RFW-1 localized were Golgi compartments.

The \textit{\textit{\Delta.rfw-1}} mutant did not show obvious growth or cell fusion defects. However, when GFP-RFW-1 driven by the \textit{\textit{ccg-1}} promoter was expressed in \textit{\textit{\Delta.rfw-1}} or \textit{\textit{\Delta.rfw-1.gt69-2}} cells, the resulting strains \textit{\textit{\Delta.rfw-1}} (\textit{\textit{pccg-1.gfp-rfw-1}}) and \textit{\textit{\Delta.rfw-1.gt69-2}} (\textit{\textit{pccg-1.gfp-rfw-1}}) showed significantly less and shorter aerial hyphae and numerous conidial chains with unreleased conidia (Fig. 6C to E). Calcofluor white staining showed the unreleased conidia were separated by two complete septa (Fig. 6D), suggesting that the conidial chains were caused by failure of the digestion of the connective material between these two septa. The \textit{\textit{\Delta.rfw-1}} (\textit{\textit{pccg-1.gfp-rfw-1}}) and \textit{\textit{\Delta.rfw-1.gt69-2}} (\textit{\textit{pccg-1.gfp-rfw-1}}) strains were also delayed in conidial germination. Three hours after plating a conidial suspension onto VMM agar plates, the majority of FGSC2489 conidia germinated, while the majority of \textit{\textit{\Delta.rfw-1}} (\textit{\textit{pccg-1.gfp-rfw-1}}) and \textit{\textit{\Delta.rfw-1.gt69-2}} (\textit{\textit{pccg-1.gfp-rfw-1}}) conidia remained ungerminated (Fig. 6F). When GFP-RFW-1 was driven by its native promoter in \textit{\textit{\Delta.rfw-1}} cells, a GFP signal was not detected, nor were conidial separation and germination defects observed in the \textit{\textit{\Delta.rfw-1}} (\textit{\textit{prfw-1.gfp-rfw-1}}) strain, in contrast to the \textit{\textit{\Delta.rfw-1}} (\textit{\textit{pccg-1.gfp-rfw-1}}) and \textit{\textit{\Delta.rfw-1.gt69-2}} (\textit{\textit{pccg-1.gfp-rfw-1}}) strains (Fig. S4).

To test whether overexpression of \textit{rfw-1} also resulted in cell fusion defects, we paired FM4-64-stained \textit{\textit{\Delta.rfw-1}} (\textit{\textit{pccg-1.gfp-rfw-1}}) cells with FGSC2489 expressing histone 1-GFP. As shown in Fig. 6G, cytoplasmic mixing was not observed between \textit{\textit{\Delta.rfw-1}} (\textit{\textit{pccg-1.gfp-rfw-1}}) cells + FGSC2489 expressing histone 1-GFP (Fig. 6G). The cell wall, as shown by staining with calcofluor white, was also observed at the contact points. In contrast, cytoplasmic mixing and cell wall breakdown occurred in pairings between the \textit{\textit{\Delta.rfw-1}} mutant and FGSC2489 (H1-GFP) (Fig. 6G). These data indicated that, in addition to a conidial separation defect, cell fusion between \textit{\textit{\Delta.rfw-1}} (\textit{\textit{pccg-1.gfp-rfw-1}}) and \textit{\textit{\Delta.rfw-1.gt69-2}} (\textit{\textit{pccg-1.gfp-rfw-1}}) strains (Fig. S4).

The \textit{\textit{gt69-2}} locus encodes an alpha-1,3-mannosyltransferase predicted to transfer a mannosyl group to either a carbohydrate or a lipid. We therefore hypothesized that loss of \textit{\textit{gt69-2}} might affect aspects of the cell wall biosynthesis. To test this hypothesis, we assessed growth of \textit{\textit{\Delta.rfw-1}}, \textit{\textit{\Delta.gt69-2}}, and \textit{\textit{\Delta.rfw-1.gt69-2}} mutants on agar media containing different cell wall stress drugs, including the \textit{\textit{\beta.-1,3-glucan synthase inhibitor caspofungin}} and two different anionic dyes that bind chitin and block chitin-glucan cross-linking, calcofluor white and Congo red. Similar to the parental strain FGSC2489, the \textit{\textit{\Delta.rfw-1}} and \textit{\textit{\Delta.rfw-1.gt69-2}} mutants were mildly sensitive to all three drugs (Fig. S5). Consistent with conidial inoculations, the \textit{\textit{\Delta.gt69-2}} mutant showed a slight growth defect in drug-free medium. However, these defects were not exacerbated on caspofungin, calcofluor white, or Congo red, indicating that the absence of \textit{\textit{gt69-2}} did not result in major cell wall defects.

\textbf{Alleles at \textit{\textit{gt69-2}} and \textit{\textit{rfw-1}} show evidence of balancing selection.} Genes that regulate allorecognition, such as the major histocompatibility complex (MHC) in humans, the \textit{\textit{S}} locus in plants, allorecognition loci in colonial ascidians, and heterokaryon incompatibility loci in fungi, often show evidence of balancing selection, which includes the presence of discrete haplotypes in populations, nearly equal frequency of allelic classes in population samples, and transspecies polymorphisms (26, 34–36). In \textit{\textit{N. crassa}} populations, \textit{\textit{gt69-2}} alleles fell into two discrete haplotypes, suggesting a role in
allorecognition (Fig. 2A). In strains containing rfw-1, the gene was always linked with gt69-2 and was highly conserved among isolates. Phylogenetic trees were constructed to test whether allelic polymorphisms at rfw-1 (NCU05915) and gt69-2 (NCU05916) were retained among different Neurospora species. Consistent with their potential role in allorecognition, the gt69-2 alleles clustered by haplogroup rather than by species (Fig. 7B). The gt69-2 alleles from Neurospora discreta and Neurospora tetrasperma isolates grouped into the same two N. crassa haplogroups. Similar to N. crassa, the haplogroup I gt69-2 alleles in both N. discreta and N. tetrasperma were linked to rfw-1, while species of all strains within haplogroup II lacked rfw-1. The transspecies polymorphisms observed in the gt69-2 alleles suggested that this locus was under balancing

**FIG 7** Haplotypes of gt69-2 alleles in populations of Neurospora and cell fusion frequency of germling pairs containing alternate gt69-2 alleles. (A) Flow cytometry results of sec9swap strains with alleles from the different haplogroups of gt69-2. WT (FGSC2489) + WT (FGSC2489) pairings were a positive control for cell fusion and showed a high cell death score; WT (FGSC2489) + Δgt69-2 pairings were the negative control and showed a low cell death score due to a block cell fusion. *P value < 0.0001 versus negative control; #, P value < 0.001 versus positive control; n = 3. (B) Phylogenetic analyses of gt69-2 orthologs in Neurospora species show transspecies polymorphisms. Amino acid sequences of gt69-2 from indicated isolates were used to build a maximum-likelihood phylogenetic tree. Results from 100 bootstrap replicates are shown beside branches. Strains of the same species are shown in identical colors. Nc, Neurospora crassa; Nd, Neurospora discreta; Nt, Neurospora tetrasperma. Light blue boxed gt69-2 alleles have linked rfw-1 alleles.
Regulation of Cell Fusion in Neurospora crassa

March/April 2021 Volume 12 Issue 2 e00307-21 mbio.asm.org

selection and that allelic polymorphisms at this locus predates divergence of these species. We tested this hypothesis by calculating the Tajima’s D values for the gt69-2 alleles. The high, positive, and significant Tajima’s D values calculated for gt69-2 (Tajima’s D = 2.07708; P < 0.05), but not NCU05914 (Tajima’s D = 0.73738; P > 0.1) or NCU05917 (Tajima’s D = 1.07540; P > 0.1), indicated that gt69-2 is under balancing selection in Neurospora species.

To assess whether allelic polymorphisms were present in other species of fungi, we analyzed the gt69-2 and rfw-1 homologs among various species of Fusarium, in particular, Fusarium oxysporum, as genome sequences for multiple isolates are available (Table S3). In Fusarium species, most strains have more than one paralog of gt69-2 and rfw-1 (Fig. S6). However, in strains of different species of Fusarium, if rfw-1 was present, it was always linked with gt69-2, although gt69-2 loci were identified that lacked linked rfw-1. In a sample of F. oxysporum isolates, although variation was observed in the number of gt69-2 and rfw-1 homologs in these isolates, allelic polymorphisms and discrete haplotypes were not observed (Fig. S6B).

In N. crassa, to determine if gt69-2 plays a role in allorecognition, we cloned the haplogroup II allele of gt69-2 (haplotype group II isolates lack rfw-1) from isolate JW224 (gt69-2 J W224) driven by a tef-1 promoter, tagged it with GFP, and introduced this construct into the Δgt69-2 and Δrfw-1Δgt69-2 mutants. The resulting strains Δgt69-2 (gfp-gt69-2 J W224) and Δrfw-1Δgt69-2 (gfp-gt69-2 J W224) were used to test the growth and cell fusion phenotype. The Δgt69-2 (gfp-gt69-2 J W224) and Δrfw-1Δgt69-2 (gfp-gt69-2 J W224) strains showed similar growth phenotypes to FGSC2489 (Fig. S3E), suggesting that the introduction of gt69-2 J W224 into the Δgt69-2 mutant restored cell fusion.

Consistent with this observation, use of flow cytometry to quantify cell fusion frequencies in pairings between Δgt69-2 (gfp-gt69-2 J W224) + gfp-gt69-2 J W224 rfw-1FGSC2489 or Δrfw-1Δgt69-2 (gfp-gt69-2 J W224) + gtf69-2 J W224 rfw-1FGSC2489 showed a high frequency of cell fusion (Fig. 7A). Identical to results of pairings between Δgt69-2 (gfp-gt69-2) + Δgt69-2 (Fig. 4), the Δgt69-2 (gfp-gt69-2 J W224) strain failed to fuse with Δgt69-2 cells. We also tested whether coexpression of gfp-gt69-2 J W224 and gtf69-2 J W224 rfw-1FGSC2489 in the same cells would affect growth or cell fusion. However, a gt69-2 J W224 rfw-1FGSC2489 (gfp-gt69-2 J W224) strain showed no obvious defects in growth or cell fusion (Fig. 57). These data indicated that the introduction of the gt69-2 allele from a different haplogroup was sufficient to complement both the growth and cell fusion defects of the Δgt69-2 mutant, but was not sufficient to induce allorecognition and a restriction of cell fusion.

DISCUSSION

In this study, we identified a linked gene pair, gt69-2 and rfw-1, that functions to regulate somatic cell fusion in N. crassa. The gt69-2 locus is predicted to encode a CAP59-like α-1,3-mannosyltransferase and, based on its similarity to C. neoformans CMT1, to catalyze the transfer of mannose from GDP-mannose to α-1,3-linked mannose disaccharides (31). A paralog of CMT1 in C. neoformans, CAP59, is required for capsule synthesis by playing a role in the export of the capsular polysaccharide glucuronoxylanmannan (31). Both gt69-2 and CAP59 orthologs belong to glycosyltransferase family 69 and contain the conserved CAP59 family alpha-1,3-mannosyltransferase catalytic domain. In Aspergillus fumigatus, the Golgi-localized protein ClpA adds an alpha-1,3-linked mannose to glycosylphosphatidylinositol (GPI) anchors (37); clpA is a homolog of Cap59. GPI anchors are important for anchoring cell surface proteins to the plasma membrane/cell wall (38). The attachment of the GPI anchor occurs in the ER, but the understanding of the maturation of the GPI anchor that occurs in the Golgi is limited.

We hypothesized that GT69-2 functions to modify secreted protein(s), such as GPI-anchored proteins, destined for the cell wall or plasma membrane, or that a small fraction of GT69-2 is trafficked to the cell surface during chemotropic interactions, modifying proteins important for late stages of MAK-2 signaling and cell wall remodeling/dissolution during the process of cell fusion. A wrinkle in this hypothesis was the observation that loss-of-function mutations in rfw-1 suppressed the cell fusion defect...
of the \( \Delta gt69-2 \) mutant; \( \Delta gt69-2\Delta rfw-1 \) mutants were fusion competent. These data indicated that neither GT69-2 nor RFW-1 are essential for cell fusion in \( N.\ crassa \), but rather, in the absence of GT69-2, RFW-1 functions to block cell fusion. We predict that in the absence of GT69-2, RFW-1 may inappropriately modify a protein or block secretion of a protein needed for mediating the transition from chemotropic interactions to cell wall dissolution, resulting in the loss of MAK-2 localization at cell contact sites and cessation of the cell fusion process. Localization of MAK-2 to the fusion pore as cell wall dissolution and membrane merger are occurring has been reported previously (20), and MAK-2 kinase activity is required for cell wall dissolution (39).

Consistent with the above hypothesis, overexpression of \( rfw-1 \) resulted in a block in cell fusion, even in the presence of \( gt69-2 \). The overexpression \( rfw-1 \) strain also showed a conidial separation deficiency associated with an inability to remove cell wall material at the double-doublet stage of conidial development. The phenotype of the \( rfw-1 \) overexpression strain most closely resembles the \( csp-2 \) mutant in \( N.\ crassa \), where \( csp-2 \) encodes a homolog of grainy head-like transcription factors (40). An inability to remove the thin connectives between adjacent conidia has been associated with a decrease in autocatalytic activity of the cell wall, hypothesized to be due to a lack of secreted enzymes, such as chitinases (41); a gene encoding a chitinase and additional proteins associated with cell wall structure were identified as transcriptional targets of \( csp-2 \) (40). Two cell wall glycosyl hydrolases, the \( CGL-1 \) \( \beta\)-1,3-glucanase and the \( NAG-1 \) exochitinase, function in remodeling the cell wall between adjacent conidia to facilitate conidia formation and dissemination (42). Two additional predicted GPI-anchored proteins, BGT-1 and BGT-2, encoding predicted \( \beta\)-1-3 endoglucanases (GH17 family) (43), localize to double-doublets in developing conidia and also to fusion points of germlings and hyphae (44). The \( \Delta bgt-1 \) and \( \Delta bgt-2 \) mutants display a deficiency in conidial separation, but do not display a cell fusion defect (44). Other mutants in \( N.\ crassa \) that show defects in conidial separation do show defects in cell fusion, however, including loss-of-function mutations in \( whi-2 \), \( csp-6 \), and \( amph-1 \) (23, 32). CSP-6 and WHI-2 physically interact (45) and WHI-2, which localizes to the cell periphery, is required for signaling during chemotropic interactions via the MAK-2 MAPK pathway (23). Future studies to identify targets of RFW-1 and GT69-2 should help to understand the molecular basis of the cell wall remodeling process regulated by the RFW-1/GT69-2 system.

In the genomes of \( Fusarium \) and \( Neurospora \) species, all predicted \( rfw-1 \) genes were always linked to \( gt69-2 \) genes, although homologs of \( gt69-2 \) occurred without a linked \( rfw-1 \) gene (Fig. S6). These observations suggest that GT69-2 and RFW-1 also function as a pair in species other than in \( N.\ crassa \). Coevolution of linked genes to maintain physical or functional interactions of their products occurs via coordinated sequence changes between the gene pairs (46). In \( Neurospora \) species, \( gt69-2 \) orthologs found in two haplogroups showed evidence of balancing selection, similar to other systems regulating allorecognition (25, 27, 29, 30, 47). However, expression of a \( \text{gt69-2}^{\text{csp2c489}} \) (haplogroup II allele) in a \( \text{gt69-2}^{\text{csp2c489}} \) (haplogroup I allele) strain was insufficient to activate allorecognition and block cell fusion. The \( \text{gt69-2}^{\text{csp2c489}} \) allele was fully functional, as it fully complemented the fusion-deficiency phenotype of a \( \Delta gt69-2 \) mutant. One possible explanation is that the \( gt69-2 \) alleles from haplogroup II have adapted to the loss of \( rfw-1 \), while haplogroup I strains need both \( gt69-2 \) and \( rfw-1 \) to correctly modify their targets in the Golgi. Alternatively, it is possible that the evolutionary forces driving balancing selection at \( gt69-2/\text{rfw-1} \) do not reflect the function of these two proteins in cell fusion/conidial separation. Further work to identify the targets of the \( GT69-2/RFW-1 \) pair from haplogroup I relative to \( GT69-2 \) from haplogroup II will help to resolve this question, in addition to identifying cell membrane/cell wall-associated proteins required for late functions of MAK-2 signaling involved in cell wall dissolution and membrane merger during somatic cell fusion.

**MATERIALS AND METHODS**

**Strains and growth conditions.** Standard procedures and protocols for \( N.\ crassa \) can be found on the \( Neurospora \) homepage at the Fungal Genetics Stock Center (FGSC, www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm). Vogel’s minimal medium (VMM) (with supplements, if required) was
used to culture all strains (48). Crosses were performed on Westergaard’s synthetic crossing medium (49). All the strains used in this study are listed in Table S1 in the supplemental material. The wild N. crassa isolates from a Louisiana population have been previously described (25, 26, 50). FGSC2489 served as the wild-type (WT) control for all experiments and the parental strain for gene engineering, unless stated otherwise.

**Strain construction.** All gene deletion constructs were generated by double-joint PCR (25, 51). The deletion mutants were obtained as described (25, 29). For the Δrfw-1Δgt69-2 double mutant, the whole region containing both NCU05915 and NCU05916 was replaced with the hygromycin B-resistance cassette in FGSC2489. For the independently derived Δncu05915 Δgt69-2 double mutant, rfw-1 was replaced with the nourseothricin-resistance cassette (52) in the Δgt69-2 mutant. Putative deletion mutants were screened for drug resistance and further confirmed by PCR (Fig. S3A and B). The primers are listed in Table S2.

To generate the Δgt69-2 gfp-gt69-2 strain, superfolder-gfp-fused gt69-2 was cloned into a pGM272-derived vector to create gfp fusions (25) using HiFi DNA assembly (New England BioLabs) under the regulation of the ccq-1 promoter (53), and introduced in the his-3 locus (25, 54) of a Δgt69-2 strain. Positive transformants were backcrossed to a Δgt69-2 mutant of the opposite mating type to obtain homokaryotic strains that were subsequently confirmed by PCR (Fig. S3A and B). Similar approaches were used to generate Δgt69-2 (gfp-gt69-2 Δrfw-1), Δrfw-1 (gfp-rfw-1), and Δrfw-1Δgt69-2 (gfp-rfw-1) strains.

The FGSC2489 sec-9swap strain, which was engineered to carry sec-gfp at the native sec-9 locus, has been previously described (30). The Δrfw-1 and/or Δgt69-2 mutants were crossed with FGSC2489 sec-9swap to obtain the resulting sec-9swap strains.

**Bulk segregant analysis.** Bulk segregant analysis (BSA) followed by whole-genome resequencing was performed as previously described (25). Approximately 60% of genome DNA from ∼49 progeny strains in each DNA pool was used for library preparation and sequencing. All paired-end libraries were sequenced on a HiSeq2000 sequencing platform using standard Illumina operating procedures (QB3 Genomics Lab, University of California, Berkeley).

**Microscopy.** Cell fusion experiments were performed as described (25). Cytoplasmic or histone 1-tagged GFP-expressing cells and FM-64-stained (Thermo Fisher Scientific) cells were mixed in a 1:1 proportion and incubated on VMM plates at 30°C in the dark for 4 h. Cytoplasmic mixing was examined with a Zeiss Axioskop 2 microscope equipped with a Q Imaging Retiga-2000R camera (Surrey) using a 40×/1.3 Plan-Neofluar oil immersion objective and the iVision Mac 4.5 software.

Heterokaryotic strains bearing both GFP and mCherry fluorescent proteins were prepared as described (25) for colocalization analysis. Images were taken with a Leica SD6000 confocal microscope equipped with a Yokogawa CSU-X1 spinning disk head, and a 488-nm or 561-nm laser controlled by Metamorph software. For MAK-2 oscillation experiments, conidia from strains expressing MAK-2-GFP were prepared for microscopy as described (25). Time-lapse microscopy was performed using the confocal microscope system as described above. Images were captured at 30 s intervals. The software ImageJ was used for image processing. Fluorescence signals were quantified as previously described (20).

**Transmission electron microscopy.** Conidia were inoculated in 100 ml of liquid VMM at a final concentration of 10⁶ conidia/ml for 5 h at 30°C (shaking at 220 rpm for 2.5 h and standing for 2.5 h). Cells were harvested by centrifugation and then fixed with electron microscopy fix buffer (2% glutaraldehyde, 4% paraformaldehyde, 0.04 M phosphate buffer [pH 7.0]), followed by 2% KMnO₄ treatment. Samples were dehydrated using a graded ethanol series before embedding the samples in resin.

**Flow cytometry.** Flow cytometry was performed as described (29). For each experiment, 20,000 events per sample were recorded on a BD LSR Fortessa X-20 (BD Biosciences, Franklin Lakes, NJ, USA). Cell death frequencies were analyzed with a specific designed MATLAB script (29). Each experiment was performed at least three times.

**Growth assays.** To evaluate growth rate, a hyphal plug (1 mm²) or 5 μl of a conidial suspension (10⁶ conidia/ml) was inoculated onto 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.

Cell wall stress assays were conducted on VMM + FG5 with 1.3 μg/ml caspofungin, 1.5 mg/ml calcium fluoride white, or 1 mg/ml Congo red as described (55). A 1:5 dilution series was prepared starting with a concentration of 10⁶ conidia/ml. Conidial solutions were then spotted onto freshly poured plates at 5 μl per spot.

**Phylogenetic analysis.** The sequences of gt69-2 and rfw-1 orthologs were obtained by a BLAST search using NCU05915 and NCU05916 from FGSC2489 as a query against sequence database of *Neurospora* (26, 56–58) and *Fusarium* (http://fungi.ensembl.org/index.html) species. Amino acid alignments were carried out using MAFFT alignments (59) and phylogenetic trees were constructed using MEGAX (60). Tajima’s D tests were processed using DnaSP6 (61).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1** EPS file, 1.6 MB.
**FIG S2** EPS file, 0.2 MB.
**FIG S3** EPS file, 1.1 MB.
**FIG S4** EPS file, 0.4 MB.
**FIG S5** EPS file, 0.3 MB.
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