The *C*-terminal domain of the GDP-mannose transporter, GmtA, is essential for localization to the Golgi apparatus in *Aspergillus nidulans*

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ABSTRACT

The *C*-terminal domain of the GDP-mannose transporter, GmtA, is essential for localization to the Golgi apparatus in *Aspergillus nidulans*

by

Laura Raquel Johnson

GDP-mannose transporters (Gmt) carry nucleotide sugars from the cytosol across the Golgi apparatus membrane in many eukaryotic organisms. Some fungal species like *Saccharomyces cerevisiae* express a single Gmt, while others including *A. nidulans* express two (GmtA and GmtB). We have shown that fluorescent versions of GmtA and GmtB (fusion with green fluorescent protein, GFP) localize to the Golgi apparatus. Gmt in *S. cerevisiae* localizes to the Golgi as well, and proper localization is dependent on homodimerization, which is facilitated by the *C*-terminus. Through localization studies of GmtA *C*-terminal truncations (fused to GFP), we determined that the region between 30 amino acids and 45 amino acids from the *C*-terminus is essential for proper localization to the Golgi. The full C-terminus truncated version of GmtA mislocalized to the Endoplasmic Reticulum. In addition, progress has been made toward determining if GmtA and GmtB form oligomers. Using split-YFP (Yellow Fluorescent Protein) to measure protein interaction, we did not observe heterodimerization of GmtA and GmtB or homodimerization of GmtA.

INTRODUCTION

The fungal cell wall is composed of polysaccharides and proteins having sugar constituents including mannose (De Groot *et al*., 2005). These sugars are added post-translationally to proteins in the endoplasmic reticulum (ER) and continued protein modification occurs in the Golgi apparatus (Gemmill & Trimble, 1999). Various numbers and combinations of sugar attachments signal the proteins to be transported to different places in the cell and are also signals for protein-to-protein recognition. The pathway of protein mannosylation is extensive and GDP-mannose transporters (Gmts) play a critical role in this process. Gmts transport GDP-mannose into the Golgi lumen where the mannose is then attached to the proteins being modifed (Gemmill & Trimble, 1999).

Gmts fall into a larger family of nucleotide-sugar transporters (NSTs), which are found largely in the Golgi but also to a lesser extent in the ER. Substrates for NSTs are sugars with nucleoside mono- or diphosphates (Reviewed in Handford *et al.*, 2006). These nucleotide sugars are synthesized in the cytosol, but utilized in the glycosylation of proteins in the ER and Golgi and thus must be transported from the cytosol into the ER and Golgi by NSTs (Reviewed in Handford *et al.*, 2006). The activity of NSTs can be the rate limiting step in glycosylation. Mutations lowering the activity of NSTs are detrimental not only to lower order eukaryotes but also to humans. There are several human diseases resulting from an inability or decreased ability to glycosylate proteins. One disorder, leukocyte adhesion deficiency type II, results from an absence of the fructose sugar in all glycoproteins and is characterized by retarded growth with weakened immune system and higher than normal levels of peripheral leukocytes (Jaeken & Carchon, 2004).

In the yeast *Saccharomcyes cerevisiae*, there is only one Gmt, VRG4. Mannosylation has proven to be important in *S. cerevisiae*, evidenced by the loss of normal endomembrane

morphology in *VRG4* mutant cells (Poster & Dean, 1996). At the cell wall level, *VRG4* mutants showed reduced mannosylation of cell wall polysaccharides and glycoproteins (Poster & Dean, 1996). There are two GDP-mannose transporters (Gmts), GmtA (AN8848.3) and GmtB (AN9298.3), in the filamentous fungus *Aspergillus nidulans* (Jackson-Hayes *et al.,* 2008). We have previously tagged GmtA and GmtB with both Green Fluorescent Protein (GFP) and Red Fluorescent Protein (RFP) and determined that both GmtA and GmtB localize to the Golgi apparatus (Jackson-Hayes *et al.,* 2008, 2009).

It has been shown that VRG4 uses GDP-mannose as a substrate. It also localizes to the Golgi and is predicted to contain six to eight transmembrane spanning domains (Gao and Dean, 2000). GmtA is predicted to have nine transmembrane domains (FIGURE 1A), while GmtB is predicted to have seven (FIGURE 2). Some common features of predicted nucleotide sugar transporters (NSTs) are that they are 300 to 350 amino acids in length with six to ten transmembrane domains (Reviewed in Handford *et al.*, 2006). VRG4, GmtA, and GmtB all fit these common NSTs features. Most predications of NSTs structures show an even number of transmembrane domains, indicating that the *N*- and *C*- termini would be on the same side of the membrane (Reviewed in Handford *et al.,* 2006). VRG4 and GmtA are predicated to have an even number of transmembrane domains, so their *N*- and *C*-termini are thought to be on the same side of the membrane. In contrast, GmtB has on odd number of transmembrane domains indicating a cytosolic *N*-terminus and a *C*-terminus inside the Golgi lumen.

The larger class of NSTs can be further differentiated into GDP-sugar transporters, UDP-sugar transporters, and ADP-sugar transporters (Reviewed in Handford *et al.*, 2006). VRG4, GmtA, and GmtB fit into the GDP-sugar transport category. Among GDP-sugar

transporters there is a conserved GALNK region (Reviewed in Handford *et al.*, 2006). VRG4, GmtA, and GmtB each contain this conserved region. Gao *et al*. (2001) found that when the GALNK region was mutated, VRG4 sugar transport ability was reduced. Therefore, they proposed that this region is responsible substrate binding. Studies have not yet been conducted on the effects of mutations in the GALNK region of GmtA and GmtB.

GmtA, GmtB, and VRG4 share significant homology. GmtA has 50% identity and 55% similarity to VRG4. GmtB has 43% identity and 50% similarity to VRG4 (Jackson-Hayes *et al*., 2008). GmtA and GmtB have 41% identity and 58% similarity to one another (Jackson-Hayes *et al*., 2008). A deletion of the VRG4 gene is lethal (Gao *et al*., 2001). Previous attempts to knockout *gmtA* and *gmtB* have been unsuccessful, thus their status as essential genes is unknown.

In *S. cerevisiae*, VRG4 homodimerizes before localizing to the Golgi (Gao and Dean, 2000). Triton X-100 interferes with VRG4 dimerization, suggesting hydrophobic interactions between the VRG4 monomers (Abe *et al*., 1999). Using *N*- and *C*- terminal truncations it was determined that the 44 *N*-terminal amino acids and the 34 *C*-terminal amino acids are required for proper VRG4 function (Gao and Dean, 2000). However, the loss of these two regions carried varying consequences. VRG4 proteins lacking the 44 *N*-terminal amino acids were able to form homodimers, but mislocalized to the ER. Proteins missing 34 amino acids from the *C*-terminus, the length of the *C*-terminal membrane-spanning domain, did not form dimers. These results suggest that the region responsible for dimer formation is at the *C*-terminus while the signal responsible for localization is near the *N*-terminus (Gao and Dean, 2000).

Gmt oligomerization has not been studied in *A. nidulans*. To investigate this possibility and its effect on Golgi localization we created GmtA *C*-terminal truncations and used Bimolecular Fluorescence Complementation (BiFC). There are several points of homology among VRG4, GmtA, and GmtB at their *C*-termini (FIGURE 3). If a *C*-terminal region responsible for formation exists in GmtA and GmtB, this region may be identified by removing portions of the *C*-termini of the GFP labeled versions of the proteins and observing localization of the fluorescent signal. Gao and Dean (2000) used co-immunoprecipitation and gel filtration chromatography to show homodimerization of VRG4 *in vitro*. Bimolecular Fluorescence complementation (BiFc) is an alternative to these methods and was used here because of its advantage of showing protein interaction *in vivo.*

The BiFc standard is split-YFP, in which a *N*- terminal portion of the YFP is fused to one protein of interest and the remaining *C*- terminal portion of YFP is fused to the potentially interacting protein of interest. If the proteins interact, such as in oligomer formation, yellow fluorescence is observed at the proper site of localization. Interacting proteins fused with a portion of the fluorescent protein, in this case a portion YFP, facilitate the union of the fluorescent protein fragments by providing thermodynamic stability (Reviewed in Kerppola, 2009). Intact YFP is composed of three anti-parrallel ß sheets that come together to form a ß barrel (Hu & Kerppola, 2003). Fragments of YFP that result in fluorescence when joined interrupt one of the three anti-parrallel ß sheets (Hu & Kerppola, 2003). These YFP fragments, when joined, interact at four separate sites with each site containing six to nine hydrogen bonds (Robida & Kerppola, 2009).

MATERIALS AND METHODS

Strains and media.–Strains used in this study are listed in TABLE I. Complete medium (CM) consisted of 1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, 5% nitrate salts, 1% trace elements, 0.1% vitamin mix, 1.2 mM L-arginine, 10 mM uracil and 5 mM uridine. Vitamin mix and nitrate salts are described in the appendix of Kafer (1977). Trace element solution is described in Hill & Kafer (2001). All cultures were incubated at 30°C.

Expression of GmtA *C-terminal truncations.*–PCR reactions were performed to amplify the 0.6 kb 5′- flanking region and coding region of *gmtA* (AN8848.3) using *Pfu* Turbo® (Stratagene), genomic DNA of strain GR5 as a template, and primers *gmtAT1* and *gmtAT2* (TABLE II). The two reverse primers were designed to sequentially omit 15 amino acids from the *C*-termini of the expressed proteins, creating GmtA T1 (GmtAΔ349-379) and GmtA T2 (GmtAΔ334-379). Neither of the 3' primers included a stop codon. All PCR products were column purified with the QIAquick PCR Purification Kit. The *gmtA* T1- and *gmtA* T2-containing PCR products were then digested with *Kpn*I and *Bam*HI. The pRG3-AMA-*Not*I-*gmtA*-GFP plasmid, which was designed to fuse GFP to proteins at their *C*-termini, (Jackson-Hayes *et al*., 2008) was digested with *Kpn*I and *Bam*HI to release full length *gmtA*. All digested products were separated on a 1% agarose gel, and the appropriately sized fragments were excised and gel-purified using the QIAquick Gel Extraction Kit (Qiagen). The truncated versions of *gmtA* were ligated into the *Kpn*I and *Bam*HI digested pRG3-AMA-*Not*I-*gmtA*-GFP plasmid creating plasmids pRG3-AMA-*Not*I-*gmtA T1*-GFP and pRG3-AMA-*Not*I-*gmtA T2*-GFP. Strain R205 (Jackson-Hayes *et al*., 2008) was transformed with pRG3-AMA-*Not*I-*gmtA T1*-GFP creating strain R501 and pRG3-AMA-*Not*I-*gmtA T2*-GFP creating strain R502. Both truncations were tested for functionality by comparing growth and

morphology of strains R501 and R502 to strain R653. See TABLE I for a description of strains used in this study.

YFP studies.– The previously created pRG3-AMA-*Not*I-*gmtA*-GFP plasmid (Jackson-Hayes *et al*., 2008) was used as a vector for cloning YFP segments. A *riboB* version of pRG3-AMA-*Not*I-*gmtA*-GFP was created so that one strain could be transformed with plasmids covering for both riboflavin and pyrimidine auxotrophies. The Af-*riboB* gene was amplified using *Aspergillus fumigatus* genomic DNA as a template. Primer sequences are listed in TABLE II. pRG3-AMA-*Not*I-*gmtA*-GFP was digested with *NarI* removing the *Pyr4* marker and Af-*riboB* containing *Nar*I overhangs was inserted creating plasmid pRG3-AMA-*Not*I-*riboB*-*gmtA*-GFP.

The *YFP* sequences were amplified using the *pYFP* plasmid as a template (Shiu *et al*., 2007; Fungal Genetics Stock Center). The *N*-terminal portion of YFP (YFP-N) contains the first 465 bp of *YFP*, encoding the first 155 amino acids. The *C*-terminal portion of YFP (YFP-C) contains the last 255 bp of YFP, encoding for the last 83 amino acids. Full length YFP (YFP) is 720 bp and 240 amino acids. The YFP-containing products were digested with *Bam*HI and *Not*I. The pRG3-AMA-*Not*I -*gmtA*-GFP plasmid was digested with *Bam*HI and *NotI* to release GFP. The YFP-C, YFP-N, and YFP were ligated into the *Bam*HI and *NotI* digested pRG3-AMA-*Not*I-*gmtA*-GFP plasmid creating plasmids pRG3-AMA-*Not*I-*gmtA*-YFP-C, pRG3-AMA-*Not*I-*gmtA*-YFP-N and pRG3-AMA-*NotI*-*gmtA*-YFP. The pRG3-AMA-*Not*I-*gmtB*-GFP plasmid (Jackson-Hayes *et al*., 2009) was digested with *Bam*HI and *NotI* to release GFP. The YFP-C, YFP-N, and YFP were ligated into the *Bam*HI and *NotI* digested pRG3-AMA-*Not*I-*gmtB*-GFP plasmid creating plasmids pRG3-AMA-*Not*I-*gmtB*-YFP-C, pRG3-AMA-*Not*I-*gmtB*-YFP-N and pRG3-AMA-*NotI*-*gmtB*-YFP.

To create YFP plasmids that could complement the *riboB* auxotrophy of strain A1145, pRG3-AMA-*Not*I-*riboB-gmtA*-GFP was digested with *Bam*HI and *NotI* to release GFP. YFP-C and YFP-N were ligated into the *Bam*HI and *NotI* digested pRG3-AMA-*Not*I-*riboB-gmtA*-GFP creating plasmids pRG3-AMA-*Not*I-*riboB-gmtA*-YFP-C and pRG3-AMA-*Not*I-*riboB-gmtA*-YFP-N. Heterodimerization was tested by transformation of strain A1145 with pRG3-AMA-*Not*I-*gmtB*-YFP-N and pRG3-AMA-*Not*I-*riboB-gmtA*-YFP-C creating strain R601. Homodimerization for GmtA was tested by transformation of strain A1145 with pRG3-AMA-*Not*I-*gmtA*-YFP-N and pRG3-AMA-*Not*I-*riboB-gmtA*-YFP-C creating strain R602.

Microscopic methods. – GmtA *C*-terminal truncations observations were made using an Olympus BX51 epifluorescence microscope in either fluorescence or transmitted-light mode, equipped with a 100× 1.35 N.A. objective and a SPOT RT-SE digital camera (Diagnostic Instruments, Inc). The U-MWB2 filter set was used for observation of the GFP signal. Observation of YFP strains were made using an Olympus 1X81 epifluorescence, inverted microscope equipped with a Plan APO 60X O-2 lens. The 41028 filter set (Chroma Technology Corp.) was used for observation of the YFP signal. For observation of cells expressing GFP or YFP chimeras, germlings were attached to coverslips that were transferred directly to slides and observed without fixation.

RESULTS

Localization of GmtA *C-terminal truncations.*– Full length GmtA-GFP fusion protein expressed in strain R653 displayed a punctate pattern of fluorescence consistent with fungal Golgi localization in *A. nidulans* (FIGURE 4A). The same pattern of localization has been shown in previous studies (Breakspear *et al*., 2007; Jackson-Hayes *et al.,* 2008; Pantazopoulou & Penalva, 2009). Strain R501 containing pRG3-AMA-*Not*I-*gmtA T1*-GFP (GmtAΔ349-379, a truncated version of GmtA missing 30 amino acids from the *C*-terminus) displayed the same punctate fluorescence localization that is well-characterized as Golgi localization (FIGURE 4B). This suggests that the first 30 amino acids from the *C*-terminus are not essential for proper localization of GmtA. However, strain R502 containing pRG3-AMA-*Not*I-*gmtA T2*-GFP (GmtAΔ334-379, a truncated version missing 45 amino acids from the *C*-terminus) did not show Golgi localization, but localized in ring-like structures (FIGURE 4C) that encompassed nuclei, which is consistent with a ER localization pattern (FIGURE 4D). Taken together these results suggest that the *C*-terminal region of GmtA from amino acids 334 to 349 is necessary for proper Golgi localization.

Suppression of the *calI*11 phenotype has been used as a test for functionality of wild type GmtA and GmtB (Jackson-Hayes *et al.,* 2008). The *calI*11 mutation is within the putative nucleotide-binding domain of *gmtA* and it conveys hypersensitivity to the chitin-binding agent, Calcofluor White (CFW) (Jackson-Hayes *et al.,* 2008). Functionality of the truncated GmtA was measured by the ability to complement *calI*11 mutation in the strain R205. Transformation of R205 with the plasmid pRG3-AMA-*Not*I-*gmtA*-GFP, a plasmid expressing a *C*-terminal GFP-labeled full length copy of *gmtA*, partially complemented the *calI*11 phenotype of R205 and allowed it to grow in the presence of CFW (FIGURE 5). The

C-terminal truncation, *gmtA* T1-GFP, partially complemented growth of R205 in the presence of CFW, but not to the same degree as wild type *gmtA* -GFP. Consistent with mislocalization of this protein to the ER, *gmtA* T2-GFP slightly complemented the growth of R205 but much less than *gmtA*-GFP or *gmtA* T1-GFP.

Gmt dimerization.– Based on the homodimerization of VRG4, which is mediated by the *C*-terminus in *S. cerevisiae,* and the homology among the *C*-termini of VRG4, GmtA, and GmtB, we studied GmtA and GmtB oligomerization. Before using split-YFP to determine protein interaction, GmtA and GmtB were both fluorescently tagged with full length YFP to confirm proper fluorescence. Transformation of strain A1145 with pRG3-AMA-*NotI-gmtA*-YFP created strain R603 and transformation of strain A1145 with pRG3-AMA-*NotI-gmtB*-YFP created strain R604. Both R603 and R604 displayed Golgi fluorescence, indicating that the YFP fluorescent tag did not interfere with GmtA and GmtB localization (FIGURE 6A and 6B). A1145 was also transformed with pRG3-AMA-*Not*I-*gmtA*-YFP-C to insure that YFP-C in the absence of YFP-N did not fluoresce (FIGURE 6D). In a similar fashion, A1145 was transformed with pRG3-AMA-*Not*I-*gmtB*-YFP-N confirming that YFP-N does not fluoresce in the absence of YFP-C (FIGURE 6C).

The mutant strain R205 was used to demonstrate the functionality of the GmtA –YFP and GmtB –YFP fusion proteins. pRG3-AMA-*NotI-gmtA*-YFP complemented the *calI*11 phenotype in R205 evident in the ability of the transformed strain to grow in the presence of CFW (FIGURE 7). The phenotype of R205 was also complemented by expression of pRG3-AMA-*NotI-gmtB*-YFP, but not to the same extent as transformation with pRG3-AMA-*NotI-gmtA*-YFP (FIGURE 7). The mutant strain R205 was also transformed individually with plasmids pRG3-AMA-*NotI-gmtA*-YFP-C and pRG3-AMA-*NotI-gmtB*-YFP-N. The result of

both transformations yielded complementation of R205 growth (Data not shown). These results indicate that all YFP chimeras are functional fusion proteins.

Heterodimerization of GmtA and GmtB was tested by transformation of strain A1145 with pRG3-AMA-*Not*I-*gmtB*-YFP-N and pRG3-AMA-*Not*I-*riboB-gmtA*-YFP-C creating strain 601. No distinct pattern of fluorescence was observed (FIGURE 6E), indicating that GmtA and GmtB do not heterodimerize. Homodimerization for GmtA was tested by transformation of strain A1145 with pRG3-AMA-*Not*I-*riboB-gmtA*-YFP-C and pRG3-AMA-*Not*I-*gmtA*-YFP-N creating strain R602. No distinct pattern of fluorescence was observed in this strain (FIGURE 6F), indicating that GmtA does not homodimerize.

DISCUSSION

The results of the current study support the conclusion that the 15 amino acid region between amino acids 334 and 349 from the *N*-terminus is essential for proper Golgi localization. Gao and Dean (2000) found that 34 amino acid *C*-terminal truncations of VRG4 were not able to form oligomers and also mislocalized to the ER. In VRG4 the *C*-terminal signal was responsible for oligomer formation (Gao and Dean, 2000). The accumulation of *C*-terminally truncated VRG4 in the ER was attributed to the misfolding of the truncated protein. In *A. nidulans*, GmtA T2, missing 45 amino acids from the *C*-terminus, localizes to the ER. The mutant strain with hypersensitivity to CFW, R205, supplied with GmtA T2-GFP on a plasmid did not show complementation of growth as it did when supplied with full length GmtA-GFP and GmtA T1-GFP. Based on its loss of ability to complement R205 growth in the presence of CFW, the 334 to 349 amino acid region of the GmtA protein is important for both localization to the Golgi and CFW resistance. GmtA oligomer formation might be a prerequisite for proper organelle targeting.

VRG4 cycles between the ER and the Golgi but predominantly is retained in the Golgi (Abe *et al.*, 2004). COPI retrograde transport, in part, is responsible for VRG4's ultimate Golgi localization. Abe *et al*. (2004) found the cytosolic *C*-terminus of VRG4 to bind to the COPI subunit. The consensus sequence for COP1 binding is known to be KKxxx or KxKxx (Cosson and Letourneur, 1994). VRG4 does not contain this consensus sequence but is known to bind COPI. However, Abe *et al*. (2004) speculated that the four lysine residues contained in the *C*-terminal cytosolic tail of VRG4 functioned for COP1 binding. GmtA also contains four lysine residues in its cytosolic *C*-terminal tail. In fact, GmtA contains two sequential KxKxx repeats. The KxKxx consensus sequence may serve for COPI

binding to GmtA to facilitate its localization to the Golgi. However GmtA T1, a truncated version of GmtA missing 30 amino acids from the *C*-terminus, still localized to the Golgi. Because this truncation includes the two KxKxxx repeats, Golgi localization of GmtA may be mediated by more than COPI binding, though COPI could be binding to another region on GmtA.

Although in *S. cerevisiae* VRG4 interacts with COPI, we found that in *A. nidulans* CopA, the COPI homolog, and GmtA reside in separate Golgi compartments (Jackson Hayes *et al*., 2008). CopA localized primarily to Golgi equivalents in the hyphal apex, while GmtA was visualized in Golgi equivalents nearer to basal regions (Jackson Hayes *et al*., 2008). Viewing live cells presented difficulties due to the mobility of the signal, thus observations of GmtA and CopA localization were made using fixed cells. New microscope technology allows for visualization of a fluorescent signal in a time lapse. We intend to view GmtA and CopA localization signals in a time lapse fashion to reaffirm previous data that did not support co-localization of GmtA and CopA.

Investigations into possible oligomer formation of GmtA and GmtB, using split-YFP, have yielded no positive results. We found that GmtA and GmtB do not heterodimerize and GmtA does not homodimerize. Further studies will be conducted using split-YFP to determine if Gmt B homodimerizes. Because this is a relatively new technique for measuring protein interaction *in vivo*, we will follow up our results by conducting a similar study using the proteins TeaA and TeaR. These proteins have been shown to heterodimerize in *A. nidulans* using split-YFP as the mode of detection (Takeshita *et al*., 2007). If we are able to replicate the TeaA and TeaR results, the lack of oligomer formation by GmtA will be further supported.

In addition, the region important for oligomer formation in VRG4 was at the *C*-terminus (Gao and Dean, 2000). In our studies GmtA and GmtB were both fluorescently tagged at the *C*-termini, so it is possible that the fluorescent tag may be interfering with the region responsible for oligomer formation. Because our BiFC studies were plasmid based, we cannot be certain of the plasmid concentrations. Low plasmid concentrations in the cells visualized could be responsible for a false negative YFP signal. Further studies could include genomic fluorescently tagged versions of GmtA and GmtB to avoid the problem of controlling concentrations. Takeshita *et al*. (2007) also conducted plasmid based BiFC studies on *teaA* and *teaR.* However, they placed *teaA* and *teaR* under the control of the inducible *alc*A promoter, which upregulates transcription on media containing glycerol. *gmtA* and *gmtB* were studied under their native promoters. Another option would be to place them under the control of an inducible promoter such as *alcA.*

The heterodimerization or homodimerization of GmtA and GmtB may be time dependent. Previously, we measured *gmtA* and *gmtB* mRNA expression using RT-PCR. Our previous work found that *gmtA* and *gmtB* are only expressed at the same level at eight hours after germination (Jackson-Hayes *et al*., 2009). Thus, this is the likely time point for GmtA and GmtB to heterodimerize. *gmtB* sustained expressions levels higher than *gmtA* for the entire time period measured, from zero to sixteen hours after germination. Thus, GmtB may be forming homodimers throughout vegetative growth of the fungus. Repetition of BiFC studies into *gmtA* and *gmtB* will consider time elapsed from germination since heterodimerization or homodimerization of the GmtA and GmtB proteins may be time dependent.

In addition, we have had difficulties fluorescently tagging GmtA and GmtB in the past in wild type strains. Fluorescence has been more easily visualized in mutant strains, such as R205, most likely due to overexpression of the *gmtA* or *gmtB* containing plasmids in these strains. It is possible that we are not able to visualize fluorescence of the rejoined fragments of YFP because our BiFC studies were conducted in the wild type strain, A1145. However we were able to visualize GmtA and GmtB fluorescently tagged with full length YFP. We did not have a control for plasmid concentration so strains expressing GmtA or GmtB fluorescently tagged with a fragment of YFP might have been at much lower concentrations than GmtA and GmtB fluorescently tagged with full length YFP.

Another option would be to change the fluorescent protein fragments used. Kerppola (2009) notes that the BiFC technique is most effective when using fragments of Venus. Venus is a fluorescent protein that is a variant of YFP. Using split-YFP has the advantage of low spontaneous association between the fragments proteins but has the disadvantage of weak fluorescent intensity (Reviewed in Kerppola, 2009). While Venus fragments showed higher specificity than YFP fragments and greater fluorescent intensity (Reviewed in Kerppola, 2009). Thus, GmtA and GmtB could be forming oligomers but at a low fluorescent intensity that we were not able to distinguish. A consideration for further studies would be to utilize Venus fragments instead of YFP fragments.

TABLE I. *Aspergillus nidulans* strains discussed in this study

Strain	**Genotype**
GR5*	*pyrG*89; *wA*3; *pyroA*4
R205†	*calI*11; *pyrG*89; *wA*3; *pyroA*4
A1145*	*pyrG*89; *pyroA*4; *nkuA*::*argB*; *riboB*2*; argB*2
R653†	*calI*11; *gmtA-GFP*::Nc*Pyr*4; *pyrG*89; *wA*3; *pyroA*4
R501‡	*calI*11; *gmtA T1-GFP*::Nc*Pyr*4; *pyrG*89; *wA*3; *pyroA*4
R502‡	*calI*11; *gmtA T2-GFP*::Nc*Pyr*4; *pyrG*89; *wA*3; *pyroA*4
R601‡	*gmtA-YFP-C*::Af*riboB*; *gmtB-YFP-N*::Nc*Pyr*4 *pyrG*89; *pyroA*4; *nkuA*::*argB*; *riboB*2*; argB*2
R602‡	*gmtA-YFP-C*::Af*riboB*; *gmtA-YFP-N*::Nc*Pyr*4, *pyrG*89; *pyroA*4; *nkuA*::*argB*; *riboB*2*; argB*2
R603‡	*gmtA-YFF*::Nc*Pyr*4; *pyrG*89; *pyroA*4; *nkuA*::*argB*; *riboB*2*; argB*2
R604‡	*gmtB-YFP*::Nc*Pyr*4; *pyrG*89; *pyroA*4; *nkuA*::*argB*; *riboB*2*; argB*2

Af = *Aspergillus fumigatus*

Nc = *Neurospora crassa*

* Available from Fungal Genetics Stock Center, University of Missouri, Kansas City, MO, USA. (McCluskey, 2003)

‡ Generated during this study

† Jackson-Hayes *et al.*, 2008

TABLE II. Primers used in this study

Primer name	Sequence†	Restriction site
Cloning primers		
gmtA T1 5′	TTGGTACCTTAGGCATAGAAGATGCACCG	*Kpn*I
gmtA T1 3′	TTGGATCCGATCTTGGCAACGGCGTA	*Bam*HI
gmtA T2 5′	TTGGATCCTTAGGCATAGAAGATGCACCG	*Kpn*HI
gmtA T2 3′	TTGGATCCGATGGCCGAAACGCTAGG	*Bam*HI
YFP primers		
YFP- *N* 5′	TTGGATCCATGGTGAGCAAGGGCGAG	*Bam*HI
YFP- *N* 3′	TTGCGGCCGCTGGCCATGATATAGACGTTGTGG	*Not*I
YFP- *C* 5′	TTGGATCCGACAAGCAGAAGAACGGCAT	*Bam*HI
YFP- *C* 3′	TTGCGGCCGCTTTACTTGTACAGCTCGTCCATGC	*Not*I
Af- *riboB* 5′	TTGGCGCCATCACATGGGATTAAAATATGGT	*Nar*I
Af- *riboB* 3′	TTGGCGCCTTACATGAGTGTGACGAGCATACA	*Nar*I

† The underlined sequence introduces the indicated restriction site.

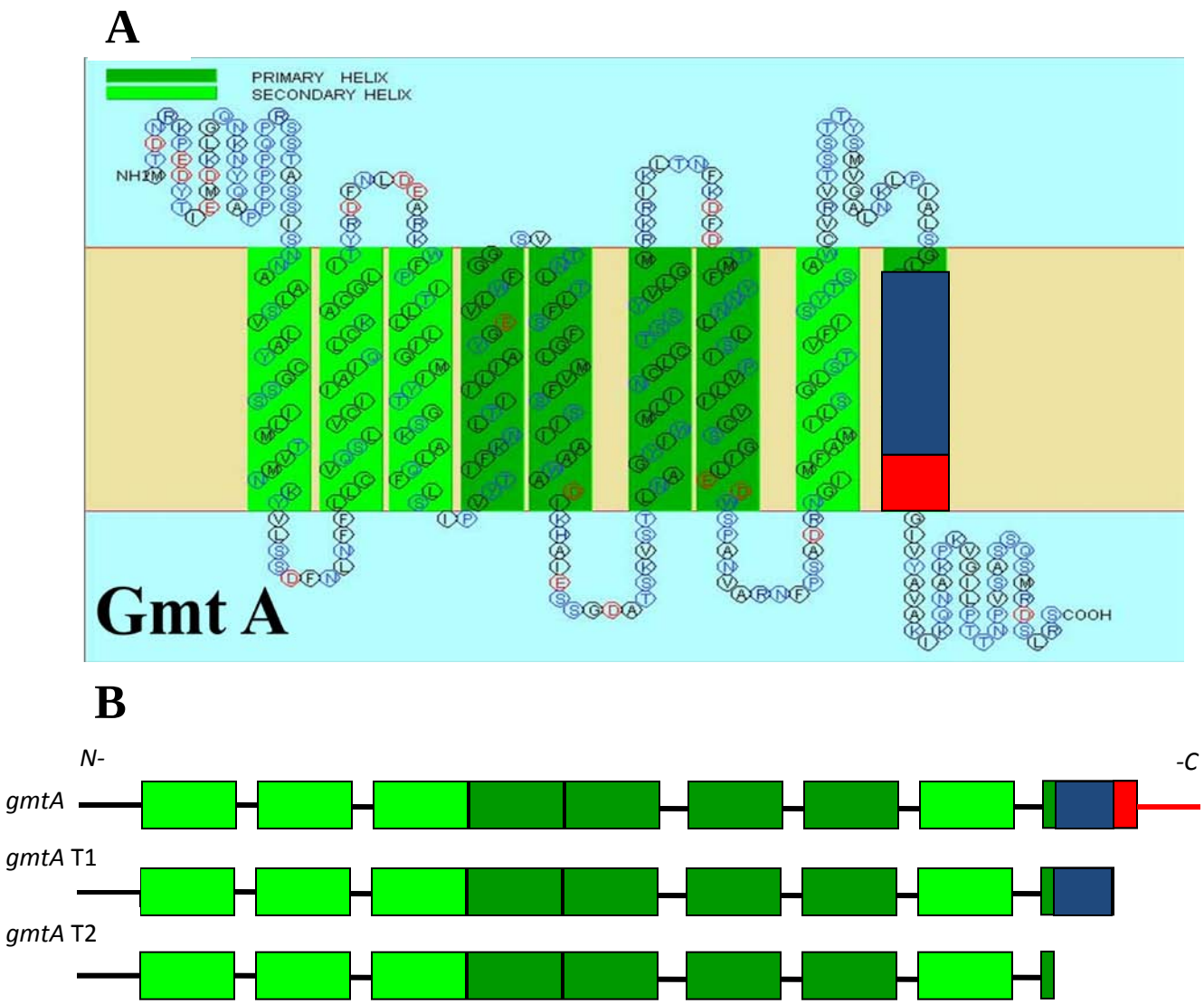

FIGURE 1. Putative GmtA Structure A. GmtA (AN8848.3) is a multi-pass transmembrane protein with nine membrane spanning domains. **B.** Two *C*-terminal truncations of GmtA were created. The first truncation, *gmtA* T1, is truncated 30 amino acids from the *C*-terminus of GmtA (shown in red). The second truncation, *gmtA* T2, is truncated 45 amino acids from the *C*-terminus of GmtA (shown in blue). Darker green indicates a primary helix while lighter green indicates a secondary helix. Amino acids colored blue are positively charged while amino acids colored red are negatively charged. Image was created using the SOSUI program.

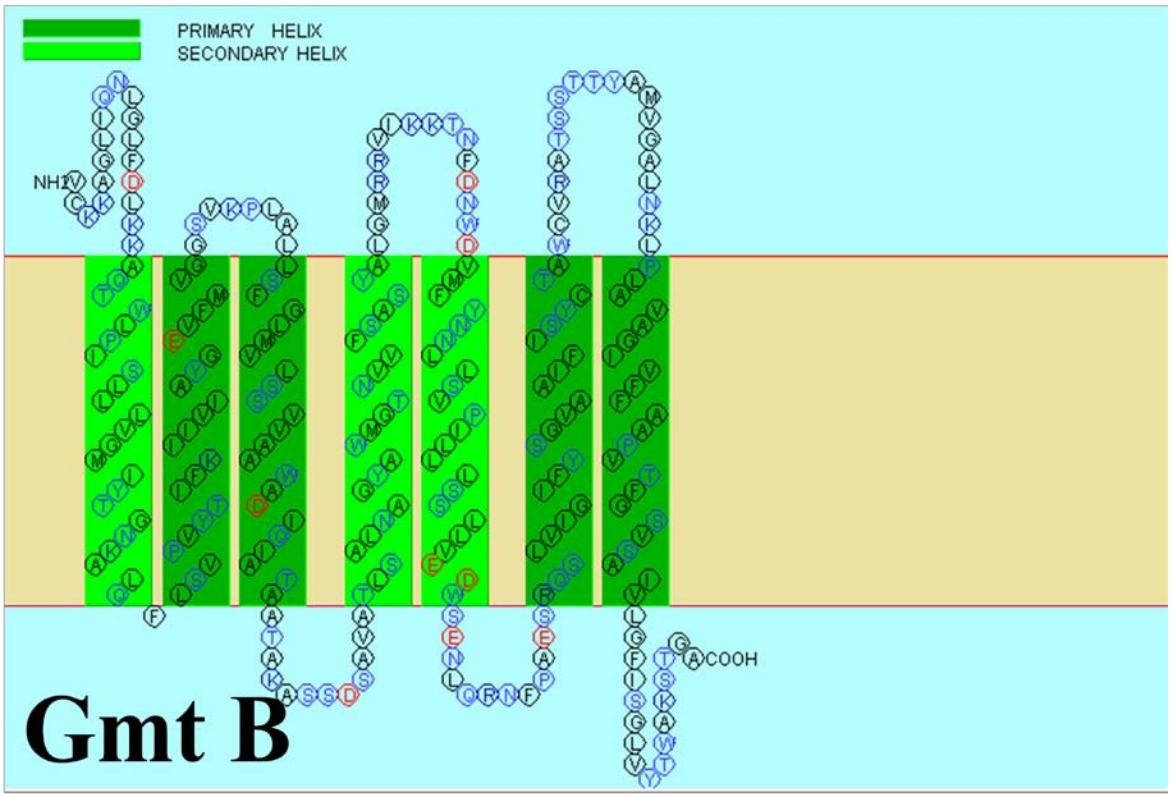

FIGURE 2. Putative GmtB Structure. GmtB (AN9298.3) is a multi-pass transmembrane protein with seven transmembrane domains. Darker green indicates a primary helix while lighter green indicates a secondary helix. Amino acids colored blue are positively charged while amino acids colored red are negatively charged. Image was created using the SOSUI program.

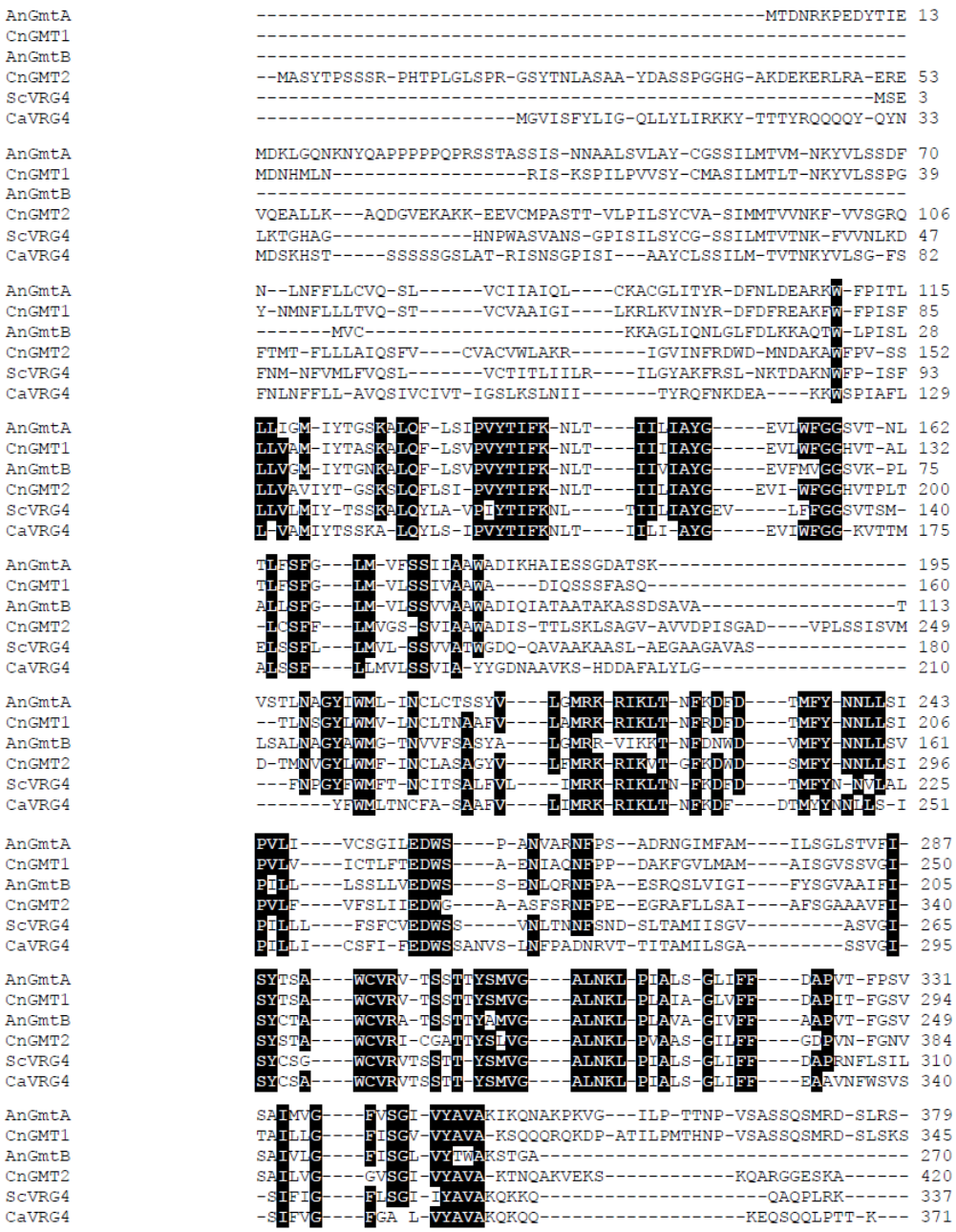

FIGURE 3. Homology between Gmts. AnGmtA refers to *Aspergillus nidulans* GmtA. CnGMT1 refers to GMT1 in *Crytococcus neoformans*. AnGmtB refers to *Aspergillus nidulans* GmtB. CnGMT2 refers to GMT2 in *Cyrtococcus neoformans*. ScVRG4 refers to VRG4 in *Saccharomyces cerevisiae*. CaVRG4 refers to VRG4 in *Candida albicans*. Shaded in black are points of homology.

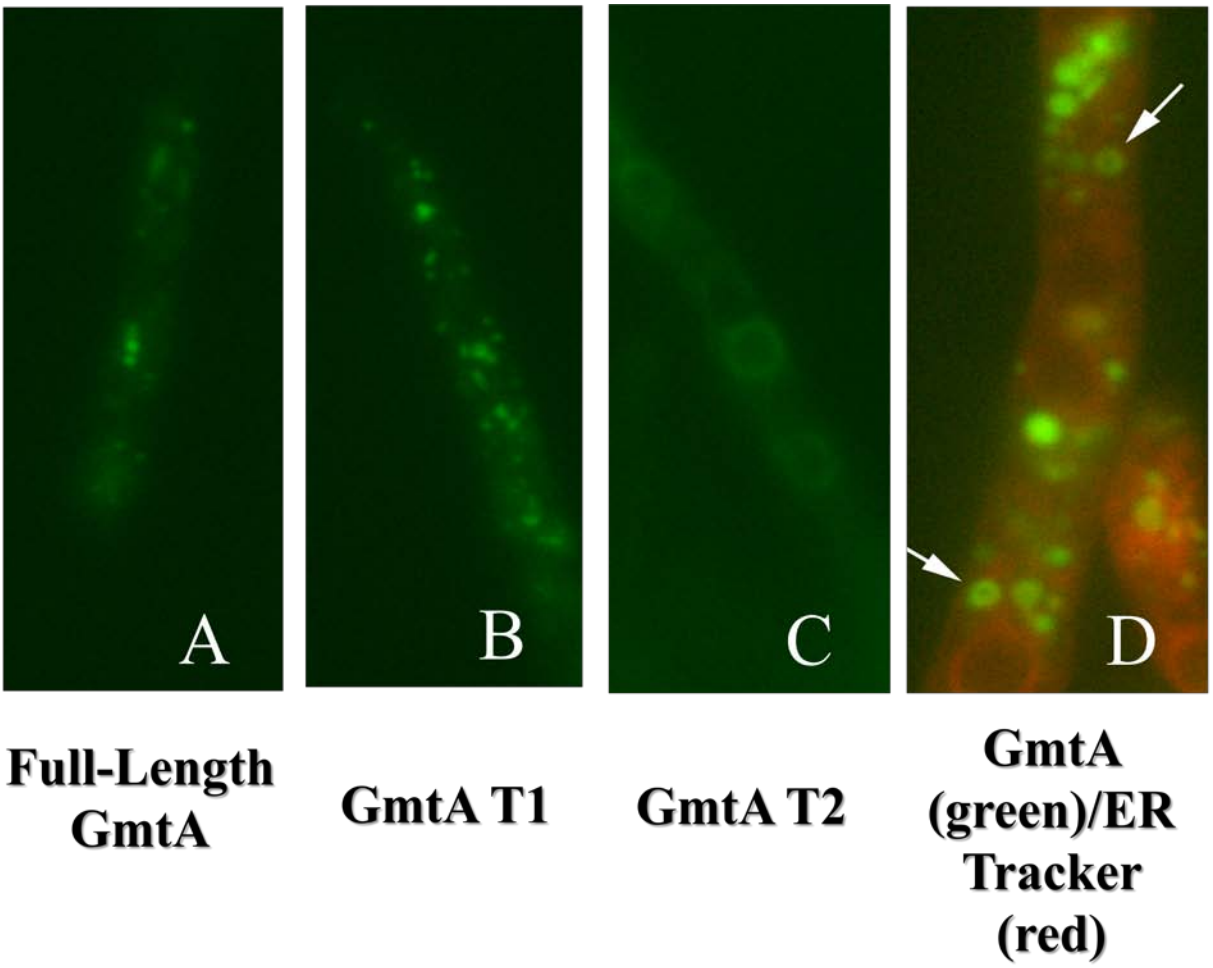

FIGURE 4. Subcellular localization of GmtA, GmtA T1, GmtA T2, and ER. A. Full length GmtA-GFP fusion protein expressed in strain R653 **B**. Strain R501 containing pRG3-AMA-*Not*I-*gmtA T1*-GFP (a truncated version of GmtA missing 30 amino acids from the *C*-terminus) **C**. Strain R502 containing pRG3-AMA-*Not*I-*gmtA T2*-GFP (a truncated version missing 45 amino acids from the *C*-terminus) **D**. The well-characterized Golgi punctuate pattern visualized by GFP label on full length GmtA alongside the ER-specific vital dye ER-Tracker in red (Jackson-Hayes *et al.*, 2008).

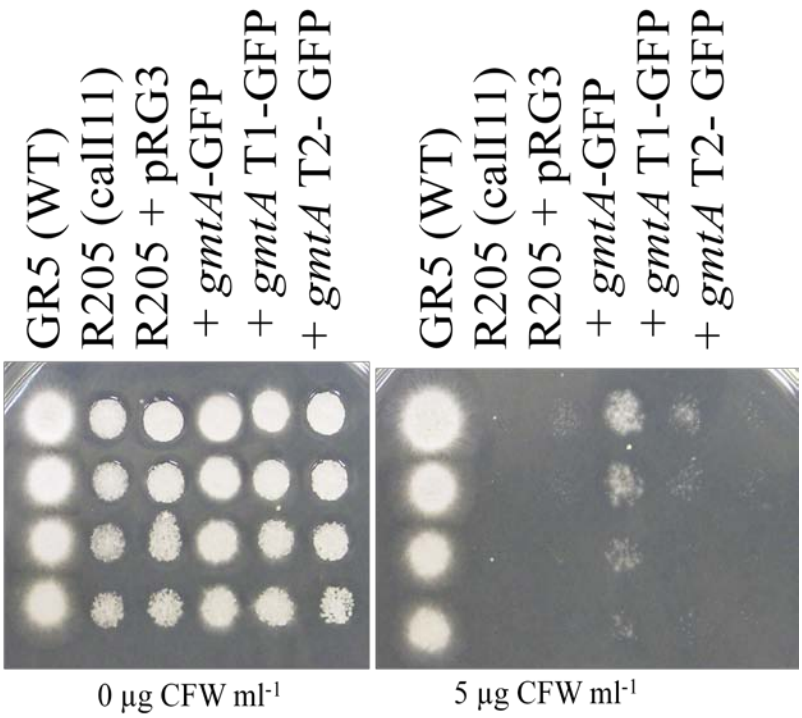

FIGURE 5. Sensitivities of mutant, wild-type and transformed strains to CFW. Colonies were grown for 36 hours at 30 °C on CM plus pyrimidines, containing either 5 or 0 µg CFW ml^{-1}. Each vertical column is a twofold dilution series, beginning with (top) 10,000 spores in 5 µg. R205 + *gmtA*- GFP has been transformed with plasmid pRG3-AMA-*Not*I-*gmtA*-GFP. R205 + *gmtA* T1-GFP has been transformed with plasmid pRG3-AMA-*Not*I-*gmtA T1*-GFP. R205 + *gmtA* T2-GFP has been transformed with plasmid pRG3-AMA-*Not*I-*gmtA T2*-GFP. R205 + pRG3 has been transformed with the empty pRG3 vector (control).

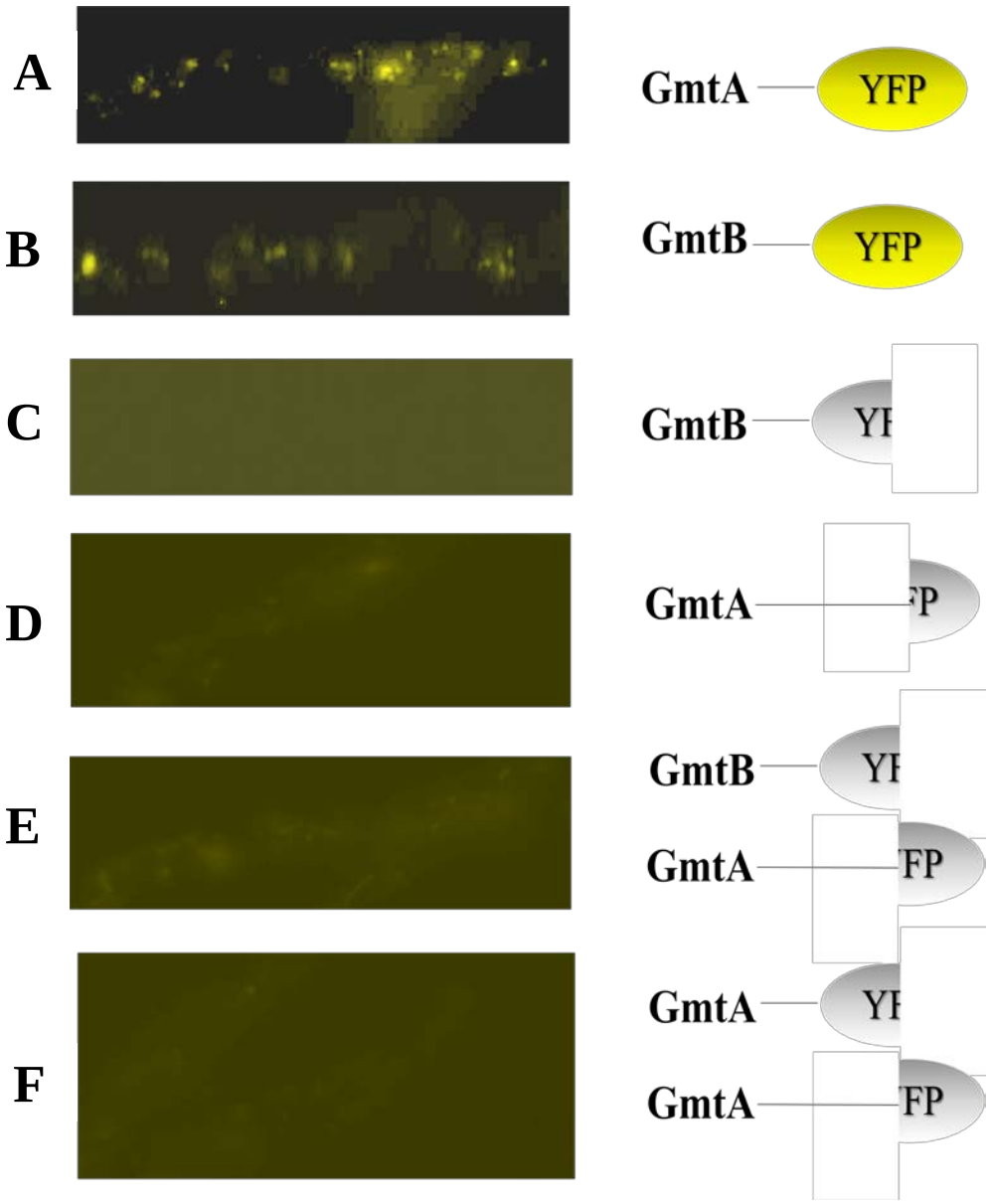

FIGURE 6. Subcellular localization of GmtA and GmtB with fluorescent tag YFP. A. Strain A1145 transformed with pRG3-AMA-*NotI-gmtA*-YFP, creating strain R603 **B**. Strain A1145 transformed with pRG3-AMA-*NotI-gmtB*-YFP, creating strain R604 ***C.*** A1145 transformed with pRG3-AMA-*Not*I-*gmtB*-YFP-N **D**. A1145 transformed with pRG3-AMA-*Not*I-*gmtA*-YFP-C **E**. Heterodimerization of GmtA and GmtB was tested by transformation of strain A1145 with pRG3-AMA-*Not*I-*gmtB*-YFP-N and pRG3-AMA-*Not*I-*riboB-gmtA*-YFP-C creating strain 601. **F**. Homodimerization for GmtA was tested by transformation of strain A1145 with pRG3-AMA-*Not*I-*riboB-gmtA*-YFP-C and pRG3-AMA-*Not*I-*gmtA*-YFP-N creating strain R602.

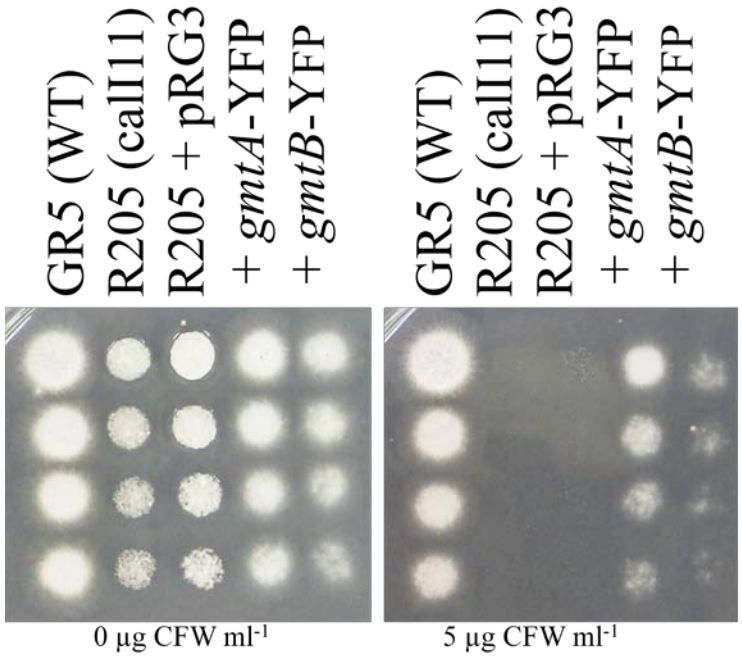

FIGURE 7. Sensitivities of mutant, wild-type and transformed strains to CFW. Colonies were grown for 36 hours at 30 °C on CM plus pyrimidines, containing either 5 or 0 µg CFW ml^{-1}. Each vertical column is a twofold dilution series, beginning with (top) 10,000 spores in 5 µg. R205 + *gmtA*-YFP has been transformed with plasmid pRG3-AMA-*NotI-gmtA*-YFP. R205 + *gmtB*-YFP has been transformed with plasmid pRG3-AMA-*NotI-gmtB*-YFP. R205 + pRG3 has been transformed with the empty pRG3 vector (control).

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