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MECHANISMS OF RESISTANCE

Vacuolar Sequestration of Azoles, a Novel Strategy of Azole Antifungal Resistance Conserved across Pathogenic and Nonpathogenic Yeast

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ABSTRACT Target alteration and overproduction and drug efflux through overexpression of multidrug transporters localized in the plasma membrane represent the conventional mechanisms of azole antifungal resistance. Here, we identify a novel conserved mechanism of azole resistance not only in the budding yeast *Saccharomyces cerevisiae* but also in the pathogenic yeast *Candida albicans*. We observed that the vacuolar-membrane-localized, multidrug resistance protein (MRP) subfamily, ATP-binding cassette (ABC) transporter of *S. cerevisiae*, Ybt1, could import azoles into vacuoles. Interestingly, the Ybt1 homologue in *C. albicans*, Mlt1p, could also fulfill this function. Evidence that the process is energy dependent comes from the finding that a Mlt1p mutant version made by converting a critical lysine residue in the Walker A motif of nucleotide-binding domain 1 (required for ATP hydrolysis) to alanine (K710A) was not able to transport azoles. Additionally, we have shown that, as for other eukaryotic MRP subfamily members, deletion of the conserved phenylalanine amino acid at position 765 (F765Δ) results in mislocalization of the Mlt1 protein; this mislocalized protein was devoid of the azole-resistant attribute. This finding suggests that the presence of this protein on vacuolar membranes is an important factor in azole resistance. Further, we report the importance of conserved residues, because conversion of two serines (positions 973 and 976, in the regulatory domain and in the casein kinase I [CKI] consensus sequence, respectively) to alanine severely affected the drug resistance. Hence, the present study reveals vacuolar sequestration of azoles by the ABC transporter Ybt1 and its homologue Mlt1 as an alternative strategy to circumvent drug toxicity among pathogenic and nonpathogenic yeasts.

KEYWORDS ABC transporter, *Candida albicans*, *Saccharomyces cerevisiae*, azole, drug resistance

Fungi are among the most prevalent opportunistic pathogens and thereby pose a persistent threat to human life. The human fungal pathogen *Candida albicans* is frequently found in the oral cavity, gastrointestinal tract, and genital area of many individuals. In immunocompromised patients, it can lead to superficial oral infections. However, it also can cause vaginal infections in immunocompetent females (1, 2). Moreover, *Candida* infections can at times be fatal after gaining access to vital organs via the bloodstream, with mortality rates nearing 50% (1, 3, 4). Candidiasis, predominantly caused by *C. albicans*, is the fourth most common basis of hospital-acquired disease (5–7).

The number of antifungal agents employed clinically is restricted because of the

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limited drug targets available, due to the close evolutionary relationship with human hosts (8). Most of the antifungal drug classes conventionally implemented target the biosynthesis of ergosterol, the main sterol of fungal membranes (8–10). Echinocandins are a new class of drugs currently employed clinically; they target the biosynthesis of (1,3)-β-D-glucan, a prime component of the fungal cell wall (8, 11).

Although modern medical practices have restricted the emergence and spread of pathogens, there has been a global increase in drug resistance. One of the most prevalent mechanisms of antifungal drug resistance, which is responsible for resistance to azoles as well as other classes of drugs, is the constitutive upregulation or overexpression of multidrug transporters, which most commonly belong to either the ATP-binding cassette (ABC) superfamily or the major facilitator superfamily (MFS) of transporters (10, 12–14). ABC transporters such as Cdr1 and Cdr2 from *C. albicans* (15, 16), Pdr5, Snq2, and Yor1 from *Saccharomyces cerevisiae* (17–19), CgCdr1 and CgCdr2 from *Candida glabrata* (20, 21), CnAfr1 and CnMdr1 from *Candida neoformans*, and many other ABC transporters from different fungi have been shown to be involved in antifungal drug efflux (22, 23). Interestingly, different ABC transporters within an organism and across species show sequence similarity and overlapping functions. For example, Cdr1 and Cdr2 from *C. albicans* show overlapping functions as drug and phospholipid transporters (24, 25). While the ABC transporters Cdr3 and Cdr4 are not drug transporters, they do translocate phosphoglycerides between the two monolayers of the lipid bilayer of the plasma membrane (PM) (26). Similarly, Pdr5 from *S. cerevisiae* shares common substrates, such as azole drugs and phosphoglycerides, with the Cdr1 and Cdr2 transporters of *C. albicans* (14, 26, 27) . Notably, most of the well-characterized ABC drug transporters in fungi are localized in the PM and belong to the PDR subfamily. In *S. cerevisiae* Yor1, a member of the multidrug resistance protein (MRP) subfamily has been shown to be involved in drug transport (28); however, it is also localized in the PM (29). This suggests that PM-localized ABC transporters are significantly involved in drug resistance. Of note, ABC transporters that are not PM localized show considerable sequence similarity to the PM-localized drug transporters, which suggests that these transporters may also identify drugs as substrates.

Vacuoles are organelles that are known to be involved in detoxification by sequestrating toxic compounds. Previously, a report suggested that vacuoles might take part in fluconazole (FCZ) resistance; among *C. albicans* clinical isolates from a myelofibrosis patient, the FCZ-resistant isolates were observed to possess many large vacuoles, compared to sensitive isolates (30). Recently, we and others showed that the vacuole-localized MRP subfamily member Ybt1 in *S. cerevisiae* and its homologue in *C. albicans* Mlt1 could transport phosphatidylcholine (PC) into the vacuolar lumen and the absence of these transporters rendered cells susceptible to methotrexate (MTX) (31, 32). This led us to the hypothesis that *S. cerevisiae* Ybt1 and its homologue Mlt1 from *C. albicans* might be involved in drug transport.

To explore this possibility, we attempted to further characterize the vacuolar transporter Mlt1 of *C. albicans* and its homologue Ybt1 in *S. cerevisiae*. We observed that *S. cerevisiae* Ybt1 could import azoles into the vacuoles and its deletion led to enhanced susceptibility to exposed drugs. Interestingly, Mlt1 of *C. albicans* could complement this function of Ybt1. Further, we showed that the conserved residues Ser-973 and Ser-976 in Mlt1 are critical for its function, since the replacement of conserved residues hampered its function. This study presents a novel conserved mechanism of azole resistance in both pathogenic and nonpathogenic yeasts.

RESULTS AND DISCUSSION

***YBT1* deletion in *S. cerevisiae* increases susceptibility to azoles.** Various ABC transporters in yeast and other organisms have been shown to share common substrates for which, in the absence of one transporter, another transporter can overcome or reduce the impact of the deleted transporter (24, 25, 27, 33, 34). Thus, for the functional study of a transporter through its deletion, use of a parental host strain lacking the other transporters that share the common substrates can provide better

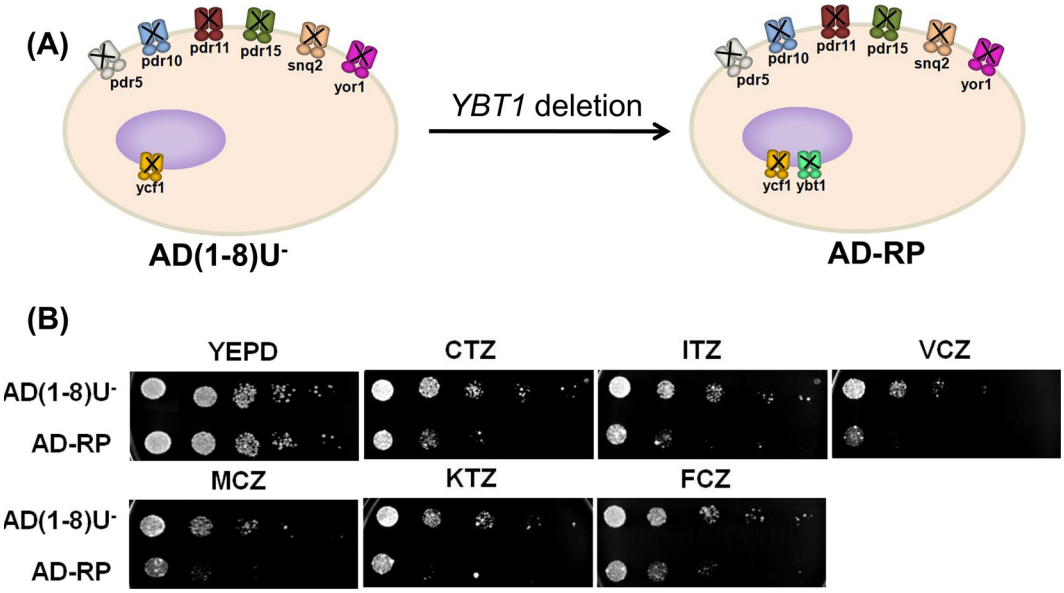

FIG 1 Deletion of *YBT1* in the *S. cerevisiae* AD(1–8)U$^-$ strain, resulting in enhanced susceptibility to azoles. (A) Cartoon depicting the *S. cerevisiae* strain AD(1–8)U$^-$ lacking seven ABC transporters, which was used as the parental strain, and deletion of *YBT1*, yielding the AD-RP strain. (B) Comparison of susceptibility, by spot tests, for the parental AD(1–8)U$^-$ strain and the AD-RP strain. Five-fold serial dilutions of each strain were spotted on YEPD agar plates with or without azole drugs (CTZ, 0.001 μg/ml; ITZ, 0.0008 μg/ml; VCZ, 0.002 μg/ml; MCZ, 0.0008 μg/ml; KTZ, 0.0012 μg/ml; FCZ, 0.1 μg/ml), and cells were grown for 48 h at 30°C.

functional insights. Considering the impact of overlapping substrates for transporters for functional characterization, we exploited *S. cerevisiae* AD(1–8)U$^-$, which lacks seven major ABC transporters, including Pdr5, Yor1, Pdr11, Snq2, Pdr10, Ycf1, and Pdr15, as the parental host strain (19) . *YBT1* was deleted in this parental host strain, and the resulting strain was designated as AD-RP (Fig. 1A) (32). Growth assays were performed in the presence of different azole drugs (clotrimazole [CTZ], itraconazole [ITZ], voriconazole [VCZ], miconazole [MCZ], ketoconazole [KTZ], and FCZ) with the *YBT1* knockout strain AD-RP, and results were compared with those for the parental host strain AD(1–8)U$^-$. As depicted in Fig. 1B, the *YBT1* knockout strain (AD-RP) showed enhanced susceptibility to the tested azoles, in comparison to the parental strain AD(1–8)U$^-$.

***C. albicans* Mlt1 could complement the function of *S. cerevisiae* Ybt1.** The *C. albicans* ABC transporter Mlt1 is a close homologue of the *S. cerevisiae* Ybt1 transporter, and both could transport 7-nitro-2,1,3-benzoxadiazol [NBD]-PC into vacuoles (31, 32). We explored whether the heterologous expression of Mlt1 could rescue the increased azole susceptibility phenotype in the YBT1 knockout (AD-RP) strain. For this, we used the AD-RP-Mlt1p-GFP strain (previously constructed by us), in which Mlt1 was heterologously hyperexpressed as a green fluorescent protein (GFP)-tagged fusion protein in the AD-RP strain (Fig. 2A) (32). We employed both AD-RP and AD-RP-Mlt1p-GFP strains and performed recovery assays in the presence of azole drugs. As depicted in Fig. 2B, the Mlt1p-GFP-hyperexpressing strain AD-RP-Mlt1p-GFP displayed better growth in the presence of the tested azoles than the AD-RP strain. This finding indicated that *C. albicans* Mlt1 could functionally complement the Ybt1 phenotype of *S. cerevisiae*.

Azole resistance elicited by the vacuolar transporter Mlt1 is energy dependent. Owing to the fact that Mlt1 is a member of the ABC superfamily, we checked whether ATP hydrolysis was linked to the observed azole resistance. For this, we used the AD-RP-K710AMlt1p-GFP strain, in which the critical conserved Lys-710 residue of the Walker A motif of nucleotide-binding domain 1 ($G^{704}KVGSGKS^{711}$) was replaced with alanine by site-directed mutagenesis (see Fig. S1 in the supplemental material). Previously, we showed that the Lys-710 residue in the Mlt1 transporter was critical for ATP

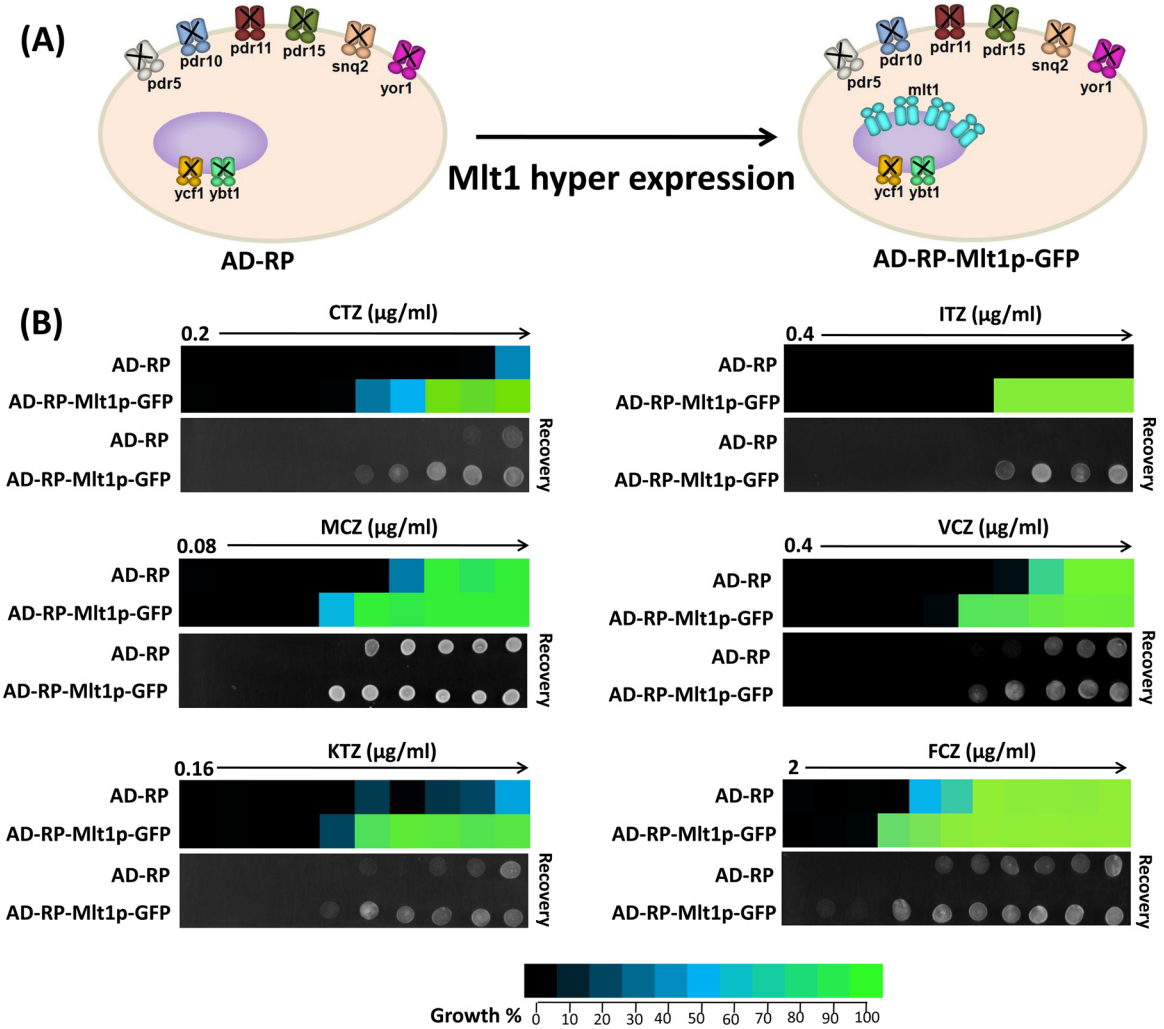

FIG 2 Heterologous hyperexpression of the *C. albicans* Mlt1 transporter in the *S. cerevisiae* AD-RP strain, increasing resistance to azoles. (A) Cartoon depicting the *S. cerevisiae* strain AD-RP lacking eight ABC transporters, which was used as the parental strain, and heterologous hyperexpression of the *C. albicans* Mlt1 transporter, yielding the AD-RP-Mlt1p-GFP strain. (B) Planktonic growth and recovery assays in YEPD medium, showing that, compared to the AD-RP strain, the AD-RP-Mlt1p-GFP strain was resistant to all tested azoles. Planktonic growth assays started with the indicated concentrations of azoles in YEPD medium in the first well, with subsequent 2-fold dilutions in the next wells, as described in Materials and Methods. (Upper) The OD_{600} values were determined as measurements of growth, and relative growth values (compared to drug-free growth) are indicated with colors. The color scale indicates growth percentages. (Lower) After 48 h of incubation, 2-μl aliquots of each well from the planktonic growth assay were spotted on fresh YEPD agar plates, and recovery of cells from the drug treatments was observed after incubation for 24 h at 30°C.

catalysis, since its replacement with alanine (K710A) resulted in a dramatic loss of ATPase activity (32).

To test the impact of this mutation on azole resistance, we performed disc diffusion assays using the AD-RP, AD-RP-Mlt1p-GFP, and AD-RP-K710AMlt1p-GFP strains and measured the zone of inhibition. The size of the zone of inhibition reflects the sensitivity level of the cells; larger size corresponds to greater susceptibility. As expected, the zone of inhibition for the AD-RP-Mlt1p-GFP strain was smaller than that for the AD-RP (*YBT1* null) strain (Fig. 3). However, the zones of inhibition for the AD-RP-K710AMlt1p-GFP strain in the presence of different azole drugs were similar to those for the AD-RP (*YBT1* null) strain, indicating that ATP hydrolysis is critical for the observed azole-resistant phenotype.

Conserved residues could affect the localization and function of Mlt1. Post-translational modifications are an important determinant for protein functionality. ABC transporters also undergo several protein modifications that could affect their function

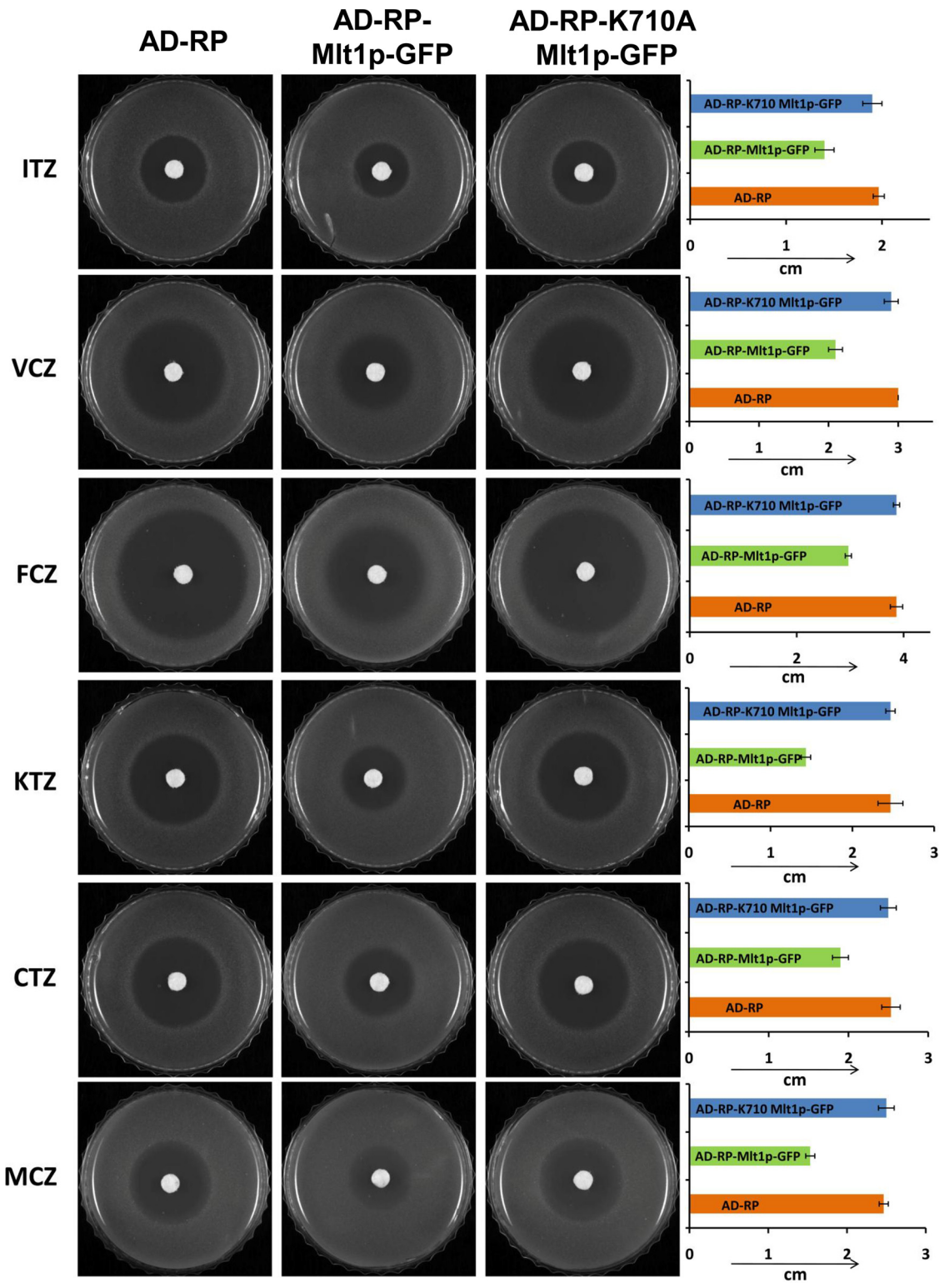

FIG 3 Drug resistance profiles of AD-RP, AD-RP-Mlt1p-GFP, and AD-RP-K710A-Mlt1p-GFP (expressing the ATPase null mutant [K710A] variant of Mlt1) strains by agar drug diffusion assays, as described in Materials and Methods. Susceptibility to the following azoles was tested: ITZ (0.08 μg), VCZ (0.1 μg), FCZ (14.7 μg), KTZ (0.08 μg), CTZ (0.1 μg), and MCZ (0.08 μg).

(35–39). Previously, it was shown that the function of the vacuolar transporter Ycf1, which belongs to the MRP subfamily in *S. cerevisiae*, was positively affected by phosphorylation at Ser-908 and Thr-911, while phosphorylation at Ser-251 had a negative effect (40, 41). However, a deletion mutation of a conserved phenylalanine residue in

an ABC transporter is known to affect the folding and endocytic processing of the transporter protein, resulting in improper localization (42–45). In *S. cerevisiae*, deletion of this conserved phenylalanine residue, which is at position 713, completely abolishes the function of Ycf1 (45).

To determine the position of these residues in the Mlt1 protein, we aligned the Mlt1 protein sequence with the *S. cerevisiae* Ycf1 protein using ClustalW, and we observed that Mlt1 Thr-270, Ser-973, and Ser-976 corresponded to Ycf1 Ser-251, Ser-908, and Thr-911, respectively (Fig. S2). Thr-270 is present within the casein kinase II (CKII) consensus sequence, while Ser-973 is present in the dibasic consensus sequence, which is a part of the regulatory domain, and Ser-976 is part of the CKI consensus sequence (Fig. S2). The conserved Phe-765 residue of Mlt1 corresponds to the Phe-713 residue of Ycf1 (Fig. S2).

To explore the impact of these residues on Mlt1-dependent azole resistance, we created mutant versions of the Mlt1 protein in which we replaced these conserved residues with alanine (T270A, S973A, and S976A); the strains expressing these variant proteins were designated AD-RP-T270AMlt1p-GFP, AD-RP-S973AMlt1p-GFP, and AD-RP-S976AMlt1p-GFP, respectively. Furthermore, we constructed a mutant strain, AD-RP-F765ΔMlt1p-GFP, in which Phe-765 was deleted by site-directed mutagenesis. Before examining the functional impact of these residues, we checked the localization of these variant proteins by exploiting the green fluorescence of the GFP-tagged Mlt1 protein. Notably, vacuolar membrane localization was confirmed by using the vacuolar-membrane-specific dye FM 4-64 [*N*-(3-triethylammoniumpropyl)-4-[6-[4-(diethylamino)phenyl]hexatrienyl] pyridinium dibromide], which provides red fluorescence. Confocal images of the strains AD-RP-T270AMlt1p-GFP, AD-RP-S973AMlt1p-GFP, and AD-RP-S976AMlt1p-GFP showed a rimmed appearance of the vacuolar membrane, similar to that of the wild-type (WT) strain, which merged with the red fluorescence of FM 4-64, thereby confirming that the T270A, S973A, and S976A mutations did not affect Mlt1 protein localization (Fig. 4A). However, the AD-RP-F765ΔMlt1p-GFP strain showed mislocalization of the mutant protein, which was evident from observed nonoverlapping fluorescence of GFP and FM 4-64 (Fig. 4A). The endoplasmic reticulum (ER) tracker dye revealed that the mislocalized variant in AD-RP-F765ΔMlt1p-GFP was trapped within the ER. As depicted in Fig. 4B, the fluorescence of the GFP-tagged variant merged well with that of the ER tracker dye, which suggested that the F765Δ mutation affected the endocytic processing of the protein. Our results are in good agreement with those of earlier studies in which deletion of the conserved Phe led to mislocalization of the human cystic fibrosis transmembrane conductance regulator (CFTR) and *S. cerevisiae* Ycf1 transporters (42–45).

To test the impact of these mutations on the functionality of the Mlt1 variant, we performed spot and MIC assays in the presence of different azoles. In these assays, we used MTX and $NiSO_4$ for the susceptibility tests, since it was shown previously that Mlt1 hyperexpression leads to enhanced resistance to MTX and $NiSO_4$ (32). Interestingly, the K710A and F765Δ versions of the protein were not able to function like the WT protein, not only in response to MTX and $NiSO_4$ but also in response to azoles. Notably, these variants phenocopied the parental AD-RP strain (Fig. 5; also see Fig. S3). These results suggest that the presence of Mlt1 at the vacuolar membrane and its ATPase activity are both required for the observed azole resistance.

We observed that mutation of the putative phosphorylation site T270A did not affect protein function, while the S973A and S976A mutations partially affected function negatively (Fig. 5; also see Fig. S3). Our results with the S973A and S976A mutations in Mlt1 are similar to those with the Ycf1 transporter mutations S908A and T911A (corresponding to S973A and S976A in Mlt1) (40). However, although the mutation of Ycf1 at the Ser-251 residue improved the functional capacity of Ycf1, replacement of the corresponding residue, Thr-270, in Mlt1 had no effect (46).

MTX transport by Ybt1 and Mlt1 is energy dependent. The transport of NBD-PC into the vacuoles by Ybt1 and Mlt1 is well established (31, 32). We explored whether

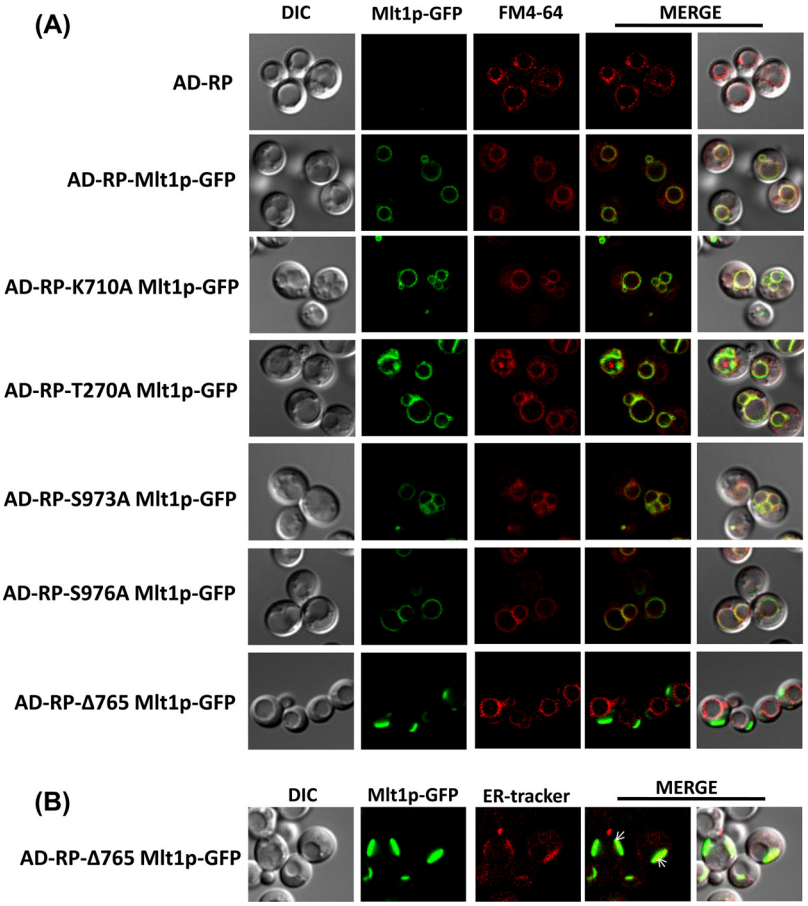

FIG 4 Localization of WT Mlt1 and its mutant variants in the *S. cerevisiae* AD-RP strain. (A) Confocal microscopic imaging, showing vacuolar membrane localization of Mlt1p-GFP and its mutant variants, with corresponding differential interference contrast (DIC) images, FM 4-64 staining images, and merged images. (B) Confocal microscopic imaging showing localization of the F765ΔMlt1p-GFP protein at the ER, with corresponding DIC images, ER tracker staining images, and merged images.

Ybt1 and Mlt1 could also accumulate MTX in the vacuoles. For this, we performed an *in vitro* vacuolar transport assay. In order to examine the MTX transport, we employed fluorescein-tagged MTX and exploited its green fluorescence to check the transport assay in a cell-free system using isolated vacuoles. Because fluorescein-tagged MTX and the GFP tag of Mlt1p both elicit green fluorescence, we performed the transport assays by using the Mlt1p-His-tagged heterologous hyperexpression strain (AD-RP-Mlt1p-HIS) that we had constructed previously (32). For this, we grew *S. cerevisiae* strains AD(1–8)U$^-$, AD-RP, and AD-RP-Mlt1p-HIS in yeast extract-peptone-dextrose (YEPD) medium and isolated vacuoles by Ficoll density gradient centrifugation (32, 47). FM 4-64 staining was employed to confirm the integrity of the vacuoles. As can be seen in Fig. 6A, vacuoles containing either Ybt1 protein [isolated from strain AD(1–8)U$^-$] or Mlt1 protein (isolated from strain AD-RP-Mlt1p-HIS) could accumulate MTX in the vacuolar lumen in the presence of ATP (Fig. 6A). However, these vacuoles could not accumulate MTX in the absence of ATP (Fig. 6A). As expected, the vacuoles without Ybt1 and Mlt1 (isolated from strain AD-RP) were not able to sequester MTX even in the presence of ATP, which was evident from a total lack of green fluorescence of the fluorescein-tagged MTX (Fig. 6A).

To test the function of the Mlt1 protein in its native host, we isolated vacuoles from *C. albicans* WT (SC5314) and Mlt1 null (*mlt1*Δ/Δ) strains. The MTX transport assay was performed as described above. As depicted in Fig. 6B, in the presence of ATP, the vacuoles isolated from the *C. albicans* WT strain could accumulate MTX in the lumen,

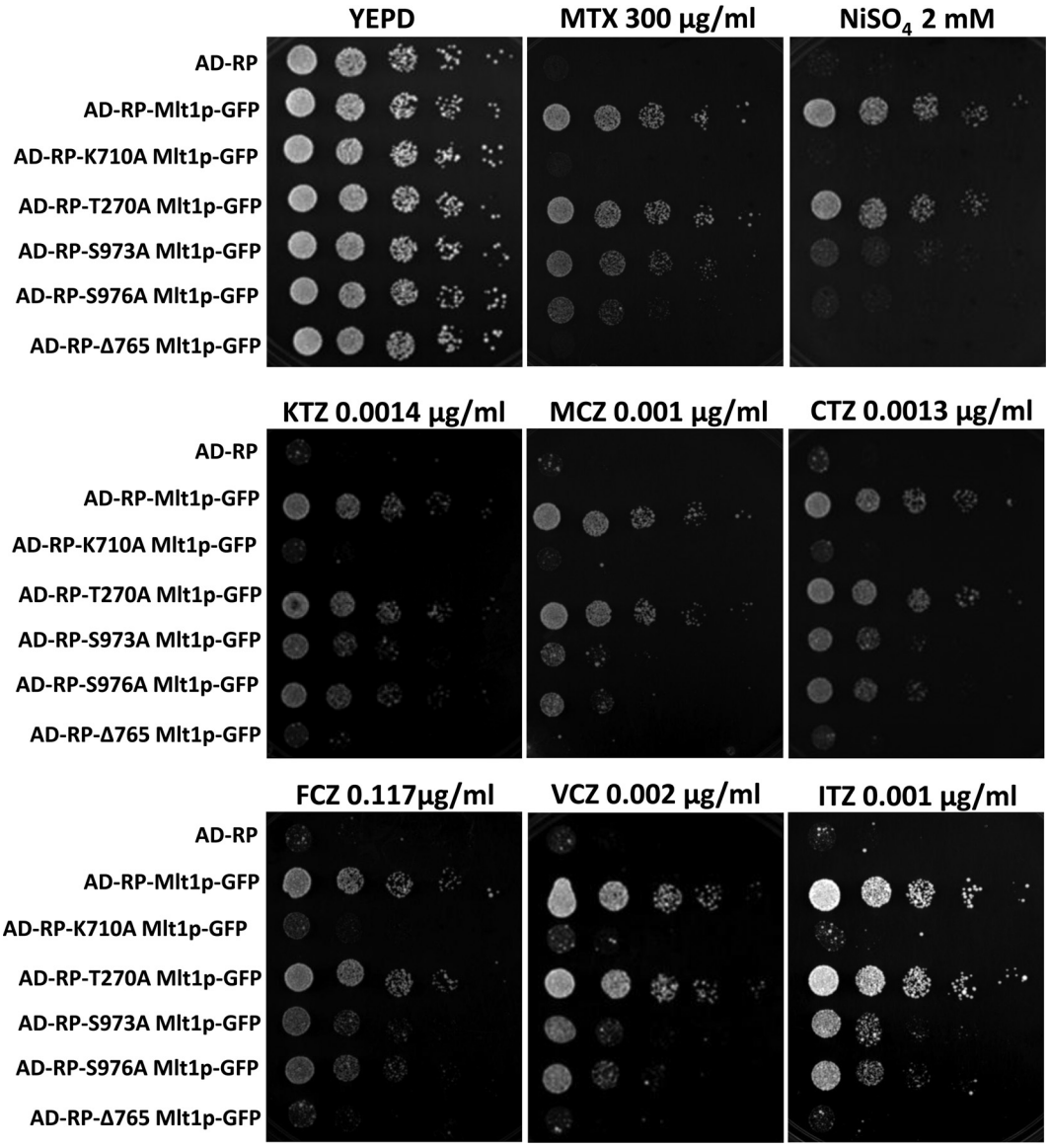

FIG 5 Drug resistance profiles of strains heterologously hyperexpressing WT Mlt1 and its mutant variants, along with the parental AD-RP strain, in the presence of different xenobiotics. Susceptibilities determined by spot dilution assays for the parental strain AD-RP and strains heterologously hyperexpressing WT and mutant variants of Mlt1, in the presence of azoles, MTX, and $NiSO_4$ at the indicated concentrations, were compared. Fivefold serial dilutions of each strain were spotted on YEPD agar plates and YEPD agar plates with xenobiotics, and cells were grown for 48 h at 30°C.

while vacuoles isolated from the *mlt1*Δ/Δ strain were not able to sequester MTX. As expected, in the absence of ATP, vacuoles from the *C. albicans* WT strain failed to accumulate MTX (Fig. 6B).

Ybt1 and Mlt1 could also transport azoles into the vacuolar lumen. For the analysis of azole sequestration in the vacuolar lumen, [^{3}H]FCZ was used and the transport assay was performed in a cell-free system using isolated vacuoles from *S. cerevisiae* [AD(1–8)U$^-$, AD-RP, and AD-RP-Mlt1p-HIS] and *C. albicans* [WT (SC5314) and *mlt1*Δ/Δ] strains, in the presence and absence of 5 mM ATP. As can be seen in Fig. 7A, significantly higher levels of [^{3}H]FCZ were accumulated in vacuoles isolated from the AD(1–8)U$^-$ and AD-RP-Mlt1p-HIS strains, as compared to the AD-RP strain. However, [^{3}H]FCZ accumulation in the vacuoles from the AD(1–8)U$^-$ and AD-RP-Mlt1p-HIS strains was dramatically reduced in the absence of ATP (Fig. 7A). The greatest amount of [^{3}H]FCZ was accumulated in the AD-RP-Mlt1p-HIS strain; this could be because Mlt1

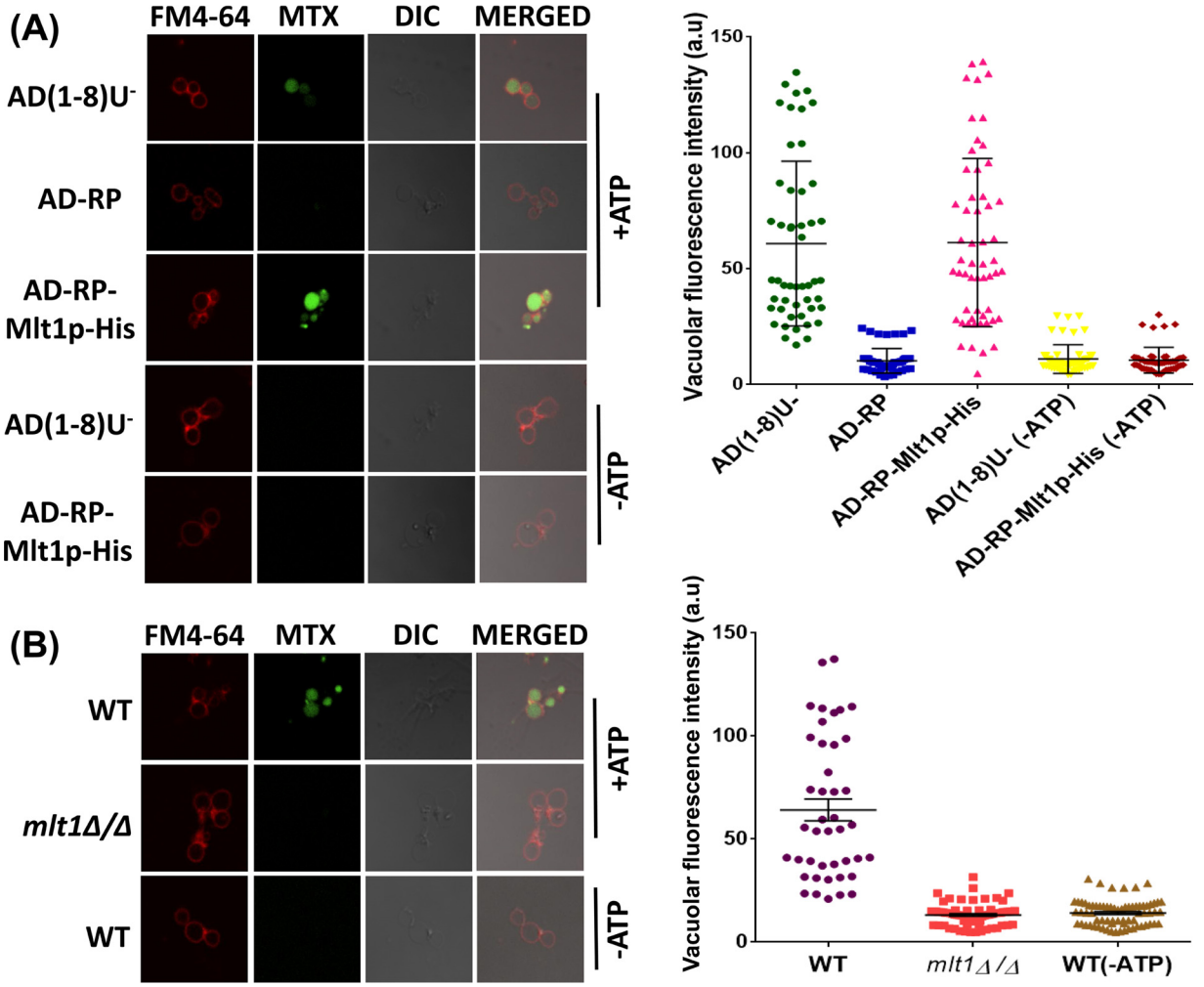

FIG 6 Ybt1- and Mlt1-dependent MTX transport into isolated vacuoles. (A) Energy-dependent MTX accumulation into the vacuolar lumen by Ybt1 and Mlt1. Vacuoles were isolated from *S. cerevisiae* AD(1–8)U$^-$, AD-RP, and AD-RP-Mlt1p-HIS strains, and 25 μg of purified vacuolar vesicles from each strain was incubated at 30°C for 30 min in buffer containing 25 μM fluorescein-tagged MTX and 10 μM FM 4-64. The vacuoles were washed and observed under a confocal microscope. (Left) The vacuoles isolated from the AD(1–8)U$^-$ and AD-RP-Mlt1p-HIS strains showed fluorescein-tagged MTX accumulation in the presence of ATP. There was no fluorescein-tagged MTX accumulation in the absence of ATP in AD(1–8)U$^-$ and AD-RP-Mlt1p-HIS or in vacuoles isolated from AD-RP. (Right) The intensities of the region of interest (ROI) (MTX and FM 4-64 merged channel), measured using ImageJ software, are plotted. Results are means ± standard errors of the means (SEMs). (B) MTX accumulation into vacuoles isolated from *C. albicans* WT and *mlt1*Δ/Δ strains. After the transport assay, as described in Materials and Methods, vacuoles were washed and observed under a confocal microscope. (Left) The vacuoles isolated from the *C. albicans* WT strain showed fluorescein-tagged MTX accumulation in the presence of ATP. MTX accumulation diminished in the absence of ATP. Of note, no MTX accumulation was observed in the vacuoles isolated from the *mlt1*Δ/Δ strain even in the presence of ATP. (Right) ROI intensities (MTX and FM 4-64 merged channel), measured using ImageJ software, are plotted. Results are means ± SEMs.

protein is hyperexpressed in this strain, which contributes to the amount of accumulation of the radiolabeled drug.

In the case of vacuoles isolated from *C. albicans* strains, the [^{3}H]FCZ accumulation was significantly higher in the WT (SC5314) strain, compared to the *mlt1*Δ/Δ strain, in the presence of ATP (Fig. 7B). In the absence of ATP, however, the difference was insignificant (Fig. 7B).

PM-localized azole ABC transporters mask the impact of the vacuolar transporters Ybt1 and Mlt1. To test the impact of PM-localized ABC transporters, which could efflux azoles and thus mask the Ybt1 phenotype, we performed spot assays in the presence of azoles with the *S. cerevisiae* WT strain BY4741 and a *ybt1*Δ strain in this parental background. To keep a positive control, we performed spot assays in the presence of $NiSO_4$, since it is exclusively a known substrate of Ybt1 (31). As depicted in Fig. 8, the BY4741 and *ybt1*Δ strains grew in a similar manner in the presence of the

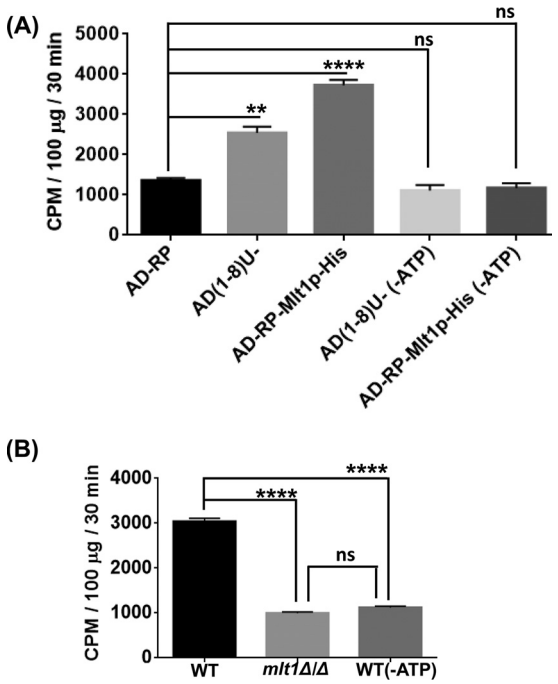

FIG 7 *In vitro* azole sequestration assay into the vacuolar lumen by Ybt1 and Mlt1. Vacuoles were isolated from *S. cerevisiae* [AD(1–8)U$^-$, AD-RP, and AD-RP-Mlt1p-HIS] and *C. albicans* [WT (SC5314) and *mlt1*Δ/Δ] strains. A total of 100 μg of purified vacuolar vesicles from each strain was incubated at 30°C for 30 min in buffer containing 100 nM [^{3}H]FCZ, in the presence and absence of ATP. The accumulated [^{3}H]FCZ radioactivity was measured using a liquid scintillation counter. (A) Transport assay with vacuoles isolated from *S. cerevisiae* AD(1–8)U$^-$, AD-RP, and AD-RP-Mlt1p-HIS strains. (B) Transport assay with vacuoles isolated from *C. albicans* WT (SC5314) and *mlt1*Δ/Δ strains. All experiments were performed with biological triplicates, and the results are shown as means ± SEMs. Statistical significance was determined using one-way analysis of variance followed by Tukey's multiple-comparison test. **, $P = 0.0012$; ****, $P < 0.0001$; ns, not significant.

azoles. As expected, however, the *ybt1*Δ strain became susceptible to $NiSO_4$. We also tested this phenotype in *C. albicans* WT and *mlt1*Δ/Δ strains. Similar to the observations made in the *S. cerevisiae ybt1*Δ strain, the absence of the homologous vacuolar transporter Mlt1 in *C. albicans* did not affect growth in the presence of azoles, and the cells displayed decreased resistance to $NiSO_4$ (Fig. S4). We can thereby conclude that we could not observe the impact of deletion of vacuolar transporters (Ybt1 in *S.*

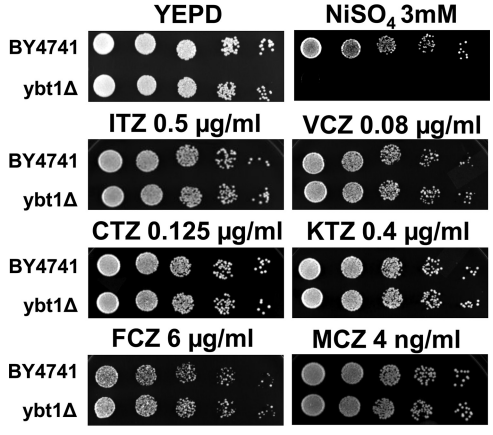

FIG 8 Drug resistance profiles of *S. cerevisiae* BY4741 and *ybt1*Δ strains, determined by spot test assays in the presence of azoles and $NiSO_4$ at the indicated concentrations. Fivefold serial dilutions of each strain were spotted on YEPD agar plates and YEPD agar plates with xenobiotics, and cells were grown for 48 h at 30°C.

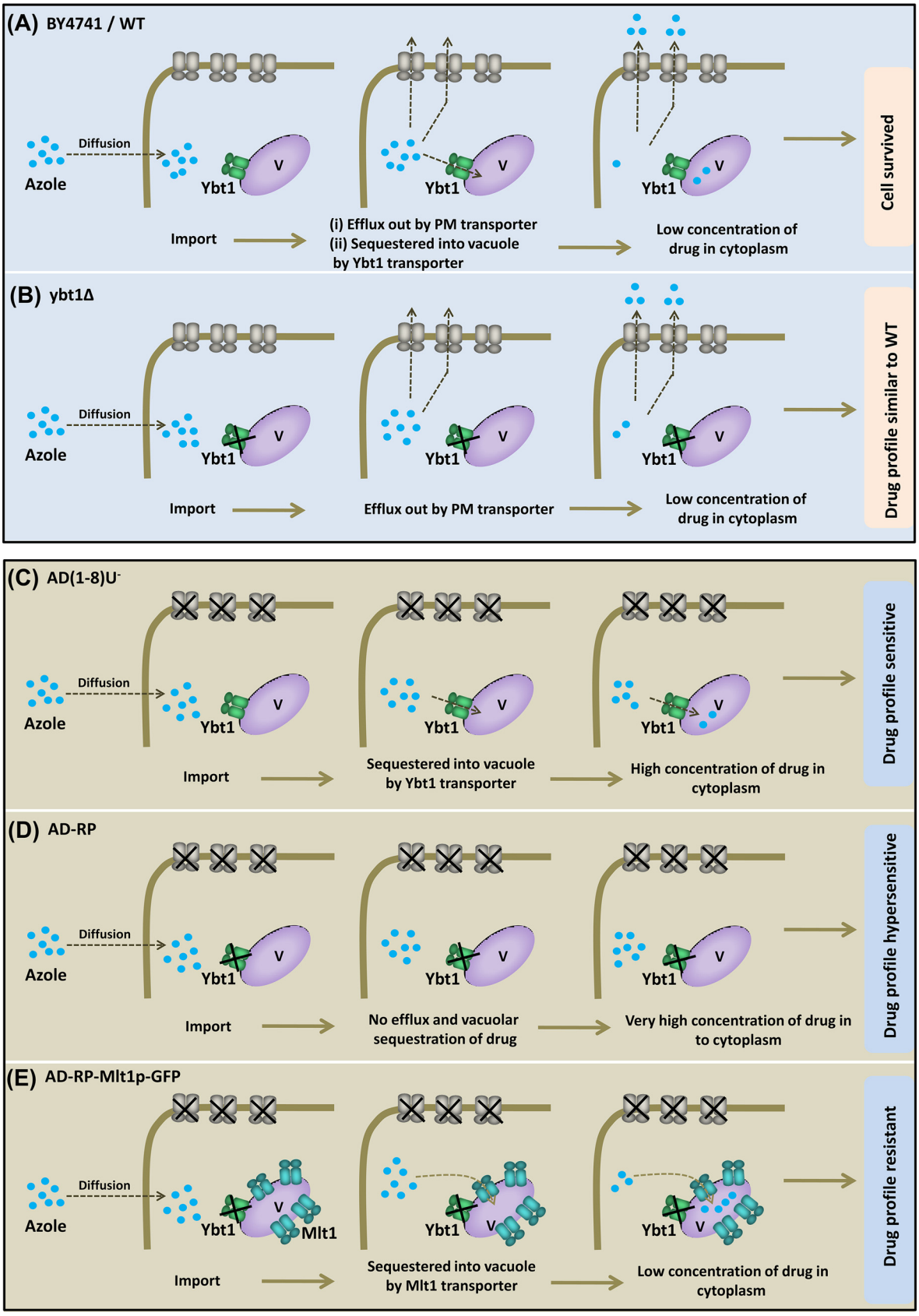

FIG 9 Proposed model for azole resistance dependent on the vacuolar transporters Ybt1 and Mlt1. (A) In the *S. cerevisiae* BY4741 WT strain, cytoplasmic azole accumulated in the cell by diffusion is a preferred substrate for PM-localized ABC transporters and export by them.

(Continued on next page)

cerevisiae and Mlt1 in *C. albicans*) on azole resistance, due to the presence of other PM-localized azole transporters. Of note, such was not the case with $NiSO_4$, since it is an exclusive substrate of Ybt1/Mlt1, and thus the presence of other PM-localized ABC transporters had no impact on its phenotype (Fig. 8; also see Fig. S4).

Conclusions. Yeast PM-localized ABC transporters are known to efflux various substrates, including antifungal agents, and their overexpression results in multidrug resistance. However, the impact on drug resistance of ABC transporters that are not present on the PM is not well explored. A study by Maebashi et al. with *C. albicans* clinical isolates from a myelofibrosis patient showed that the FCZ-resistant isolate TIMM 3318 accumulated significantly greater amounts of azoles in the vacuolar fraction, compared to sensitive isolates, suggesting that FCZ resistance observed for the resistant isolates is caused by the vesicular sequestration of FCZ in the cytoplasm (30). In the present study, we provide direct evidence that the vacuolar-membrane-localized ABC transporter in *S. cerevisiae* (Ybt1) and its homologue in *C. albicans* (Mlt1) could sequester the commonly used antifungal azoles in an energy-dependent manner. We speculate that Mlt1 overexpression could be the cause of the observed increased accumulation of azoles in the vacuoles of the azole-resistant isolate TIMM 3318. Expression analysis of the *MLT1* transcript in the clinical isolate TIMM 3318 could provide better insight about the role of the vacuolar ABC transporter Mlt1 in clinical azole resistance. We also observed that the function of these vacuolar-membrane-localized transporters remained masked by other PM-localized ABC transporters (Fig. 8; also see Fig. S4 in the supplemental material). This is mainly because several PM-localized ABC transporters use azoles as preferred substrates and can efflux them from the cells, resulting in decreased cytoplasmic concentrations of azoles in the cells (Fig. 9A). Thus, the deletion of *YBT1* in the presence of other PM-localized ABC transporters prevents the demonstration of any impact on azole resistance (Fig. 9B). However, the absence of PM-localized ABC transporters enables highlighting of the role of Ybt1. In a background in which major ABC transporters are absent, the role of Ybt1 becomes crucial, since its deletion in this background increases azole susceptibility (Fig. 9C and D). The *C. albicans* Mlt1 transporter is able to rescue the cells from the impact of the *S. cerevisiae* Ybt1 deletion on azole susceptibility (Fig. 9E). Together, our results reveal a novel conserved strategy of azole resistance in pathogenic and nonpathogenic yeast, in which vacuolar-membrane-localized transporters of the MRP subfamily actively sequester azoles in the vacuolar lumen.

MATERIALS AND METHODS

Materials. The growth media YEPD medium and LB broth were purchased from Himedia (Mumbai, India). The drugs FCZ, ITZ, KTZ, MCZ, VCZ, CTZ, and MTX and the molecular-grade chemicals Ficoll 400, creatine kinase, bovine serum albumin (BSA), sodium azide, phosphocreatine, ATP, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The vacuolar stain FM 4-64 and fluorescein-MTX were obtained from Life Technologies and Thermo Fisher Scientific, respectively. [^{3}H]FCZ was purchased from Moravek (Brea, CA, USA).

Media and strains. The bacterial strain *Escherichia coli* DH5α was used as a host for the construction and propagation of plasmids. *E. coli* cells were grown in LB medium containing 0.1 mg/ml ampicillin (Amresco). All of the yeast strains were grown and maintained in YEPD medium according to the experimental requirements. Glycerol stocks of all strains were prepared in 15% glycerol and maintained at −80°C. Table 1 lists all of the strains used.

FIG 9 Legend (Continued)
Additionally, the vacuolar-membrane-localized Ybt1 transporter sequesters some amount of cytoplasmic azole, resulting in decreased cytoplasmic concentrations and cell survival. (B) The deletion of *YBT1* in the *S. cerevisiae* BY4741 WT parental strain did not affect azole tolerance because of PM-localized ABC transporters that effluxed the cytoplasmic azole accumulated by diffusion, resulting in decreased cytoplasmic concentrations and cell survival. (C) The deletion of PM-localized ABC transporters in the *S. cerevisiae* BY4741 WT parental strain resulted in increased azole susceptibility in strain AD(1–8)U$^-$, in comparison to the BY4741 WT parental strain, because the absence of PM-localized ABC transporters resulted in high levels of cytoplasmic azole accumulation and azole susceptibility. (D) The deletion of *YBT1* in the *S. cerevisiae* AD(1–8)U$^-$ parental strain resulted in decreased azole tolerance in the AD-RP strain, in comparison to the AD(1–8)U$^-$ parental strain. The absence of the Ybt1 transporter in the AD-RP strain led to high levels of cytoplasmic azole accumulation, resulting in cell death. (E) Heterologous hyperexpression of the *C. albicans* Mlt1 transporter in the AD-RP parental strain resulted in increased azole sequestration in the vacuolar lumen and increased azole tolerance.

TABLE 1 Strains used in the present study

Strain	Genotype or description[a]	Source or reference
AD(1–8)U$^-$	MATα, *pdr1-3*, *ura3 his1*, Δ*yor1*::*hisG*, Δ*snq2*::*hisG*, Δ*pdr5*::*hisG*, Δ*pdr10*::*hisG*, Δ*pdr11*::*hisG*, Δ*ycf1*::*hisG*, Δ*pdr3*::*hisG*, Δ*pdr15*::*hisG*	19
AD-RP	AD(1–8)U$^-$ derivative; MATα, *pdr1-3*, *ura3 his1*, Δ*yor1*::*hisG*, Δ*snq2*::*hisG*, Δ*pdr5*::*hisG*, Δ*pdr10*::*hisG*, Δ*pdr11*::*hisG*, Δ*ycf1*::*hisG*, Δ*pdr3*::*hisG*, Δ*pdr15*::*hisG*, Δ*ybt1*::*kanMX*	32
AD-RP-Mlt1p-GFP	AD-RP cells harboring *MLT1* ORF fused with GFP and integrated at *PDR5* locus	32
AD-RP-K710A Mlt1p-GFP	AD-RP-Mlt1p-GFP cells harboring K710A mutation in *MLT1* ORF integrated at *PDR5* locus	32
AD-RP-Mlt1p-HIS	AD-RP cells harboring *MLT1* ORF fused with His tag and integrated at *PDR5* locus	32
AD-RP-K710A Mlt1p-HIS	AD-RP-Mlt1p-HIS cells harboring K710A mutation in *MLT1* ORF integrated at *PDR5* locus	32
AD-RP-T270AMlt1p-GFP	AD-RP-Mlt1p-GFP cells harboring T270A mutation in *MLT1* ORF integrated at *PDR5* locus	This study
AD-RP-S973AMlt1p-GFP	AD-RP-Mlt1p-GFP cells harboring S973A mutation in *MLT1* ORF integrated at *PDR5* locus	This study
AD-RP-S976AMlt1p-GFP	AD-RP-Mlt1p-GFP cells harboring S976A mutation in *MLT1* ORF integrated at *PDR5* locus	This study
AD-RP-F765ΔMlt1p-GFP	AD-RP-Mlt1p-GFP cells harboring F765Δ mutation in *MLT1* ORF integrated at *PDR5* locus	This study
S. cerevisiae BY4741 strain	MATα *his3*Δ::*leu2*Δ::*met15*Δ::*ura3*Δ	EUROSCARF
*S. cerevisiae ybt1*Δ strain	BY4741 derivative; *hisG*, Δ*ybt1*::*kanMX*	EUROSCARF
C. albicans SC5314 (WT) strain	WT strain	Laboratory stock
*C. albicans mlt1*Δ/Δ strain	SC5314 derivative; Δ*mlt1-1*::*hisG*/Δ*mlt1-2*::*hisG*	52

[a]ORF, open reading frame.

Construction of site-directed mutants. The previously designed plasmids pABC3-Mlt1-GFP and pABC3-Mlt1-His (32) were used for site-directed mutagenesis. Mutations were introduced in the plasmids using the primers mentioned in Table 2, with the help of the QuikChange site-directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA), according to the manufacturer's instructions, and the mutations were confirmed by DNA sequencing. The mutated plasmids were digested with the AscI enzyme, and the transformation cassettes were used to transform AD-RP cells using the lithium acetate method (32, 48).

Drug susceptibility assays. (i) Spot dilution assay. Spot dilution assays were performed using 5-fold serial dilutions, from an optical density at 600 nm (OD_{600}) of 0.1, in 0.9% saline; 5 μl of cells from each dilution was spotted on YEPD plates with or without drug (32).

(ii) Planktonic growth assay. The planktonic growth assay was performed in 96-well plates, in YEPD medium with an inoculum of 1×10^4 cells/ml, using the broth microdilution method described by the Clinical and Laboratory Standards Institute (49).

(iii) Recovery assay. The recovery assay was performed with planktonic growth culture plates. Briefly, the planktonic growth culture plates were carefully shaken, and a 2-μl aliquot of each well was spotted on fresh YEPD recovery plates, as described previously (50), to measure the extent to which cells recovered from the drug treatments.

(iv) Agar drug diffusion assay. The assay was performed using *S. cerevisiae* cells (1×10^5 cells/ml), as described previously (51). Images were captured with a Bio-Rad ChemiDoc XRS+ system, and the diameters of the zones of inhibition were measured using the quantification tool.

***In vitro* drug transport assay.** Vacuoles were purified from *S. cerevisiae* [AD(1–8)U$^-$, AD-RP, and AD-RP-Mlt1p-HIS] and *C. albicans* [WT (SC5314) and *mlt1*Δ/Δ] strains by using a Ficoll density gradient method described previously (47). The fluorescein-tagged MTX uptake assay was performed with freshly prepared vacuolar vesicles at 30°C, in Tris-sucrose (TS) buffer (250 mM sucrose, 25 mM Tris-morpholineethanesulfonic acid [MES] [pH 8.0]) containing 10 mM creatine phosphate, 20 U/ml creatine kinase, 25 μM fluorescein-tagged MTX, and 10 μM FM 4-64 dye. The experiments were performed in the presence or absence of 5 mM ATP in the buffer, as required (31, 32). Intact vacuoles were considered for the uptake assay, which were selected on the basis of limiting membrane staining with FM 4-64.

For azole accumulation assays, 100 μg vacuoles was used and assays were performed at 30°C in TS buffer, in the presence of [^{3}H]FCZ at a final concentration of 100 nM. After 30 min, the vacuoles were washed three times with ice-cold TS buffer. Finally, the vacuoles were transferred to a vial containing 5 ml scintillation mixture, and the radioactivity was measured using a liquid scintillation counter (Tri-Carb 2900TR liquid scintillation analyzer; Packard).

TABLE 2 Primers used in the present study

Primer name[a]	Primer sequence (5′ to 3′)
Orf19.5100T270A FP	TTGGGTAATATTGCTTGCGATGATACC
Orf19.5100T270A RP	GGTATCATCGCAAGCAATATTACCCAA
Orf19.5100S973A FP	TTGCGTCGTGCAGCTGTTGTTTCATAC
Orf19.5100S973A RP	GTATGAAACAACAGCTGCACGACGCAA
Orf19.5100S976A FP	GCAAGTGTTGTTGCATACGGTCATAATTAC
Orf19.5100S976A RP	GTAATTATGACCGTATGCAACAACACTTGC
Orf19.5100F765Δ FP	GGAGAATATTTTGGGTCACAAGTATGATGC
Orf19.5100F765Δ RP	GCATCATACTTGTGACCCAAAATATTCTCC

[a]FP, forward primer; RP, reverse primer.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01347-18.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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We have no conflicts of interest to declare. We alone are responsible for the content and writing of the paper.

N.K.K. and R.P. conceived of and designed the study, N.K.K. and M.G. constructed the mutants, N.K.K., N.A.G., and R.P. designed the transport assay, N.K.K., M.W., and M.K. performed the transport assay, N.K.K., M.W., and R.N. performed all of the screening assays, A.K.M., N.A.G., and R.P. provided the laboratory space and reagents, and N.K.K., R.N., M.G., and R.P. wrote the paper, with input from the other authors. All authors read and approved the final paper.

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