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Deletion of the Golgi Ca^{2+}-ATPase *PMR1* gene potentiates antifungal effects of dodecanol that depend on intracellular Ca^{2+} accumulation in budding yeast

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One sentence summary: Deletion of the Golgi Ca^{2+}-ATPase *PMR1* potentiates antifungal effects of dodecanol that depend on intracellular Ca^{2+} accumulation in budding yeast.

ABSTRACT

One strategy for overcoming infectious diseases caused by drug-resistant fungi involves combining drugs rendered inactive by resistance with agents targeting the drug resistance mechanism. The antifungal activity of *n*-dodecanol disappears as incubation time passes. In *Saccharomyces cerevisiae,* anethole, a principal component of anise oil, prolongs the transient antifungal effect of dodecanol by downregulating genes of multidrug efflux pumps, mainly *PDR5*. However, the detailed mechanisms of dodecanol's antifungal action and the anethole-induced prolonged antifungal action of dodecanol are unknown. Screening of *S. cerevisiae* strains lacking genes related to Ca^{2+} homeostasis and signaling identified a *pmr1Δ* strain lacking Golgi Ca^{2+}-ATPase as more sensitive to dodecanol than the parental strain. Dodecanol and the dodecanol+anethole combination significantly increased intracellular Ca^{2+} levels in both strains, but the mutant failed to clear intracellular Ca^{2+} accumulation. Further, dodecanol and the drug combination reduced *PMR1* expression and did not lead to specific localization of Pmr1p in the parental strain after 4-h treatment. By contrast with the parental strain, dodecanol did not stimulate *PDR5* expression in *pmr1Δ.* Based on these observations, we propose that the antifungal activity of dodecanol is related to intracellular Ca^{2+} accumulation, possibly dependent on *PMR1* function, with anethole enabling Ca^{2+} accumulation by restricting dodecanol efflux.

KEYWORDS

ABC transporter, anethole, calcium, dodecanol, Golgi Ca^{2+}-ATPase, Pmr1p

INTRODUCTION

Drug-resistant pathogens frequently emerge because of inappropriate use of antibiotics. Fungal clinical isolates show resistance to antifungal drugs, e.g., azoles, including fluconazole (Whalay et al. 2017) and 5-fluorocytosine (Vu et al. 2018). The occurrence of human pathogenic clinical isolates of *Candida* with reduced susceptibility to echinocandins has also been reported (Perlin 2015). Because of drug resistance, the development of antifungal drugs with novel modes of action and fewer adverse effects in human is urgently required. However, it is difficult to develop such antifungals because human and fungi are eukaryotic organisms, with similar metabolisms. Therefore, strategies for overcoming drug resistance are needed, to improve the antifungal chemotherapy.

trans-Anethole (anethole; Fig. 1), a principal component of anise and fennel oils, exhibits a broad antimicrobial activity against a spectrum of microorganisms, from bacteria to fungi (Hitokoto et al. 1980; Kosalec et al. 2005; Yutani et al. 2011). We previously reported that anethole inhibits hyphal growth accompanying morphological alterations in a filamentous fungus, *Mucor mucedo*, by inducing cell wall fragility, and that the effect is dependent on the inhibition of chitin synthase (Yutani et al. 2011). In addition, we found that anethole induces apoptotic-like fungal cell death accompanied by the production of reactive oxygen species (ROS) and DNA fragmentation in the human opportunistic pathogenic fungus *Aspergillus fumigatus* and the budding yeast *Saccharomyces cerevisiae* (Fujita et al. 2014). Although the antifungal potency of anethole is weaker than that of marketed antifungals, it synergistically potentiates the antifungal effect of *n*-dodecanol (dodecanol; Fig. 1), *trans*-2-undecenal, polygodial, and nagilactone E in *S. cerevisiae* and in the human pathogenic fungus *Candida albicans*

(Fujita et al. 2007; Kubo et al. 1995; Kubo and Himejima. 1992; Kubo and Kubo 1995).

The antifungal effects of a model antifungal drug, dodecanol, against *S. cerevisiae* disappear over incubation time (Fujita et al. 2007; Fujita et al. 2017). The effect in which the antifungal action disappears as incubation time passes is partly associated with drug efflux via an overexpressed multi-drug ATP-binding cassette (ABC) transporter, *PDR5* (Fujita et al. 2017). Anethole restricts *PDR5* expression, which is regulated by the transcription factor Pdr3p, thereby inhibiting drug efflux, and enhances and prolongs the antifungal effect of dodecanol (Fujita et al. 2017). However, the antifungal mechanism of dodecanol alone is also unclear, as well as the mechanisms by which dodecanol stress stimulates *PDR3* expression and anethole causes dodecanol's durable antifungal effect due to the inhibition of *PDR3* expression.

Anethole inhibits the activity of sarco/endoplasmic reticulum (ER) Ca^{2+}-ATPase (SERCA) in pig (Sárközi et al. 2007). Although SERCA homologs are not encoded by the *S. cerevisiae* genome, Degand et al. (Degand et al. 1999) reported that rabbit SERCA can be used to replace yeast vacuole and Golgi Ca^{2+}-ATPases to support cell viability and calcineurin-dependent regulation of Ca^{2+} tolerance. Further, disturbance of Ca^{2+}-regulated signaling affects the activities of several antifungals (Edlind et al. 2002). Specifically, *S. cerevisiae* and *Candida glabrata* cells treated with FK506 and cyclosporine A, which inhibit calcineurin in Ca^{2+}-regulated signaling, are hypersensitive to several antifungal drugs, such as miconazole, fluconazole, itraconazole, and amphotericin B (Larsen et al. 2004). In addition, FK506 enhances the antifungal activity of ketoconazole against *C. albicans* (Iwasaki 2007). Furthermore, the sensitivity to antifungal drugs, such as miconazole, terbinafine, and cycloheximide, is enhanced in *S. cerevisiae* deletion mutants of *cnb1* (regulatory subunit of calcineurin), *cna1*, and *cmp2*

(catalytic subunit of calcineurin) (Edlind et al. 2002). On the other hand, Tanabe et al. (Tanabe et al. 2018) proposed that FK506 directly inhibits Pdr5p drug efflux by slowing the transporter opening and/or substrate release, and that FK506 resistance of Pdr5p is achieved by modifying critical intramolecular contact points in the protein that, when altered, enable the co-transport of FK506 with other pump substrates.

To gain further insight into the antifungal mechanism of dodecanol and the synergistic mechanism of anethole in combination with dodecanol, we investigated the intracellular Ca^{2+} levels in *S. cerevisiae* treated with dodecanol and the drug combination. A screen of strains lacking genes related to calcium signaling and the regulation of intracellular Ca^{2+} levels revealed durable antifungal effects of dodecanol in deletion mutant cells of *pmr1* encoding a major Golgi membrane P-type ATPase that transports cytosolic Ca^{2+} into the Golgi apparatus (Sorin et al. 1997). An increase in intracellular Ca^{2+} levels continued in *pmr1Δ* cells treated with dodecanol and its parental cells treated with the drug combination. In addition, dodecanol and the drug combination restricted *PMR1* expression. These results indicate that such elevated Ca^{2+} levels are involved in the antifungal effects of dodecanol and that the duration of the effect is dependent on the inhibition of the calcium efflux to Golgi.

MATERIALS AND METHODS

Strains and culture conditions

The parental strain *S. cerevisiae* BY4741 (*MATa, ura3-Δ0, leu2-Δ0, met15-Δ0,* and *his3-Δ1*) and derived knockout strains (*cna1*Δ/YLR433C, *cmp2*Δ/YML057W, *cnb1*Δ/YKL190W, *pmr1*Δ/YGL167C, *pmc1*Δ/YGL006W, *cch1*Δ/YGR217W, *mid1*Δ/YNL291C, *ecm7*Δ/YLR443W, *vcx1*Δ/YDL128W, and *yvc1*Δ/YOR087W) were

obtained from the Yeast Knockout Strain Collection (Thermo Scientific Open Biosystems, Waltham, MA). The yeast cells were grown in 2.5% malt extract (ME; w/v; Oriental Yeast, Tokyo, Japan) broth at 30°C, unless otherwise stated.

Chemicals

n-Dodecanol (purity 97% by gas chromatography (GC)) was purchased from Kishida Chemical (Osaka, Japan). *trans*-Anethole (purity >99.0% by GC) was purchased from Sigma-Aldrich (St. Louis, MO). 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl)ester (BAPTA-AM) (purity >95% by high-performance liquid chromatography) was obtained from Tokyo Chemical Industry (Tokyo, Japan). *N,N*-Dimethylformamide (DMF, purity >99.0% by GC) was purchased from Wako Pure Chemicals (Osaka, Japan). Fluo-4-acetoxymethyl ester (Fluo-4 AM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Anethole and dodecanol were diluted using DMF before addition to cell suspensions.

Antifungal susceptibility assay

The antifungal susceptibility assay was performed using previously described methods (Fujita et al. 2002). Typically, serial two-fold dilutions of tested compounds were prepared in DMF, and 30 μL of the 100-fold dilution was added to 3 mL of ME broth in a test tube (diameter of 10 mm). The exponentially growing yeast cells were inoculated in the medium for a final cell density of 1.0×10^6 colony-forming units (CFU) mL^{-1}. The cultures were incubated without shaking at 30°C for up to 96 h. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the test compound at which no visible yeast growth was detected.

Time-kill assay

Exponentially growing yeast cells (1.0×10^6 CFU mL^{-1}) were incubated without shaking at 30°C for up to 72 h in ME broth containing dodecanol, anethole, or their combination. Aliquots of cell culture were withdrawn, diluted and then plated on agar plates containing 1% Bacto yeast extract (BD; Franklin Lakes, NJ), 2% Bacto peptone (BD), and 2% D-glucose (w/v). Viable cell numbers were determined based on CFU, after cultivation for 48 h at 30°C.

Assessment of intracellular Ca^{2+} levels

Intracellular Ca^{2+} levels were evaluated using a Ca^{2+}-specific fluorescent dye, Fluo-4 AM (Gee et al. 2000). The yeast cells (2.0×10^5 CFU mL^{-1}) were treated in ME broth for 4 h or 48 h with the indicated drugs, and then centrifuged at 9600 × g at 4°C for 10 min. The harvested cells were washed twice with phosphate-buffered saline (PBS), and then incubated at 30°C for 1 h in PBS containing 18 μM Fluo-4 AM accompanied by ultrasonic treatment using ULTRASONIC CLEANER (Iwaki Glass Corporation, Chiba, Japan). After incubation, the cells were washed twice with PBS and then resuspended in 25 μL of Recording Medium (Dojindo Laboratories, Kumamoto, Japan) prior to observation under a BX51 microscope (OLYMPUS, Tokyo, Japan). Excitation and emission wavelengths of 495 and 518 nm, respectively, were used to visualize the fluorescence.

RNA extraction and RT-qPCR

Total RNA was extracted from the yeast cells using an RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, the yeast cells treated with

dodecanol, anethole, or their combination, as indicated, were collected by centrifugation at 5000 × g for 5 min, and then lysed by using 6.2 mg mL^{-1} zymolyase 20T (Nacalai Tesque, Kyoto, Japan). The RNA samples were purified using the columns provided in the kit, and then treated with DNase. The RNA samples were reverse-transcribed to generate complementary DNA using ReverTra Ace (TOYOBO, Osaka, Japan). The reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was conducted using SsoAdvanced Universal SYBR Green Supermix (Bio Rad, Hercules, CA) using complementary DNA as a template, by using a CFX Connect Real-Time PCR Detection System (Bio Rad). The 20-μL qPCR reaction mixtures contained 10 μL of 2 × Supermix, 5 ng template, and 5 pM each of forward and reverse primers. The cycling profile was 3 min at 95°C; followed by 40 cycles of 10 s at 95°C and 30 s at 55°C. The relative expression of *PDR5* and *PMR1* was normalized to the expression of the housekeeping gene *ACT1*, as an internal positive control. The primers used in the current study are listed in Supplementary Table 1.

Flow cytometry

The yeast cells (1.0×10^6 CFU mL^{-1}) were incubated in ME broth with the indicated drugs for 4 or 48 h and then centrifuged at 9600 × *g* at 4°C for 5 min. The harvested cells were washed twice with PBS. Cell suspensions were filtered through a cell-strainer cap. Cells with fluorescence derived from green fluorescent protein (GFP)-fusion proteins were analyzed using a BD Accuri C6 flow cytometer (Becton–Dickinson), and the data were analyzed with BD Accuri C6 software (Oshiro and Takagi 2014; Brink et al. 2016). A laser with a 488 nm excitation wavelength and 533/30 bandpass filter were used.

Visualization of GFP-Pmr1p fusion protein

The yeast cells (2.0×10^5 CFU mL^{-1}) were incubated in ME broth with the indicated drugs. After incubation, the cell suspension was centrifuged at 9600 × g at 4°C for 10 min. The harvested cells were washed twice with PBS, and then observed under a BX51 microscope (OLYMPUS). The excitation and emission wavelengths used to visualize the fluorescence derived from GFP were 488 and 507 nm, respectively.

Quantitation of dodecanol

The yeast cells (1.0×10^7 CFU mL^{-1}) were incubated in ME broth with the indicated drugs for 4 and 48 h and centrifuged at 9600 × *g* at 4°C for 5 min. Total lipid fractions were obtained as follows based on the method of Kaneko and Ito (1971). The harvested cells were washed twice with PBS and then suspended in 1:2 chloroform:methanol. The cells were broken by a ShakeMan (Biomedicalscience, Tokyo, Japan). Dodecanol in the supernatant was analyzed by GC using a Shimadzu GC-8APF (Shimadzu, Kyoto, Japan) with a silicon OV-17 column (i. d. 2.6 mm × 1.5 m, GL Sciences, Tokyo, Japan) (Hirosawa et al. 2019).

Statistical analysis

Statistical evaluation was performed using Student's *t*-test. Results with *p*-values below 0.05 were considered statistically significant.

RESULTS

PMR1 deletion increases susceptibility to dodecanol and delays cell recovery after dodecanol treatment

First, we examined the MIC of dodecanol in *S. cerevisiae* mutants lacking genes related to calcium signaling and the regulation of intracellular Ca^{2+} levels (Table 1). After 72-h incubation in ME broth, MICs of dodecanol exceeded 2500 μM for all strains tested, including the parental strain, except for the strain *pmr1Δ*, for which the MIC was 78–156 μM. This indicated that strain *pmr1Δ* was the most sensitive to dodecanol after a 72-h incubation among the strains tested. We therefore focused on the *PMR1* gene, as a possible candidate involved in the mechanism inducing drug resistance to dodecanol and enhancing the antifungal effect of dodecanol by anethole.

Dodecanol at 250 μM rapidly reduced cell viability of the parental strain within 4 h, as determined based on the number of CFU after the treatment (Fig. 2A). However, after a 4-h incubation, cell viability was gradually recovered. After 72 h, the number of viable cells exceeded that in the initial inoculum size. These observations reconfirmed the fungistatic effect of dodecanol against the parental strain, as previously reported (Fujita et al. 2007). By contrast, cell growth was completely inhibited over 72 h when the cells were treated with 250 μM dodecanol in combination with 312.5 μM anethole (Fig. 2A). We then examined the effect of *PMR1* deletion on the fungistatic activity of dodecanol (Fig. 2B). The deletion of *PMR1* did not alter the effect of anethole or dodecanol in combination with anethole on cell viability. However, the recovery of cell viability was greatly delayed upon treatment with 250 μM dodecanol as compared with the parental strain. These results indicate that *PMR1* deletion potentiated the antifungal effect of dodecanol.

Drugs induce an increase of intracellular Ca^{2+} levels

We then evaluated the intracellular Ca^{2+} levels in cells of the parental and *pmr1Δ* strains

treated with dodecanol, anethole, or their combination (Fig. 3). We used Fluo-4 AM, which is taken up by the cell, deacetylated, and binds to Ca^{2+}, producing fluorescence at 518 nm upon excitation at 480–500 nm (Gee et al. 2000). Dye fluorescence intensity increases with increasing intracellular levels of Ca^{2+} (Gee et al. 2000). In the parental strain, 4-h treatment with 312.5 μM anethole did not induce an increase in intracellular Ca^{2+} levels (Fig. 3A, left). However, treatment with 250 μM dodecanol, and 250 μM dodecanol in combination with 312.5 μM anethole apparently induced an increase in intracellular levels of Ca^{2+} (Fig. 3A, left). After a 48-h treatment with dodecanol, the intracellular levels of Ca^{2+} were reduced to control levels (Fig. 3A, right). By contrast, the Ca^{2+} levels remained elevated upon treatment with a combination of dodecanol and anethole, indicating that anethole sustains dodecanol-induced intracellular Ca^{2+} accumulation. Similar changes in intracellular Ca^{2+} levels were apparent in strain *pmr1Δ*, except for the dodecanol treatment (Fig. 3B). After 48-h incubation, the Ca^{2+} levels remained elevated in the presence of dodecanol. These observations suggested that the drug-induced decrease in cell viability was linked to the elevation of intracellular Ca^{2+} levels.

Effect of a membrane-permeable Ca^{2+} chelator on the viability of cells treated with dodecanol

To confirm the contribution of an increase in intracellular Ca^{2+} levels to the enhancement of dodecanol fungicidal activity by anethole, we next used BAPTA-AM, a membrane-permeable Ca^{2+} chelator, to passively reduce intracellular accumulation of Ca^{2+} (Hong et al. 2010). BAPTA-AM did not affect the vegetative growth of anethole-treated and untreated control cells (Fig. 4), and dodecanol exerted a temporary fungicidal activity

regardless of the BAPTA-AM treatment (compare Figs. 2A and 4). However, the viability of dodecanol-treated cells was restored more rapidly in the presence of BAPTA-AM than in its absence, suggesting that Ca^{2+} accumulation partly contributes to the fungicidal activity of dodecanol (Fig. 4). Furthermore, in the presence of BAPTA-AM, the viability of cells incubated with the combination of dodecanol and anethole, or dodecanol alone, was gradually restored after 24 h. These observations supported the involvement of elevated Ca^{2+} levels in a drug-induced reduction in cell viability.

Dodecanol inhibits *PMR1* expression

We showed that dodecanol temporarily induces an increase in intracellular Ca^{2+} levels (Fig. 3A, left). In addition, strain *pmr1Δ* was hypersensitive to dodecanol (Table 1), which accompanied intracellular accumulation of Ca^{2+} (Fig. 3B, right). Together with the vacuolar Ca^{2+}-ATPase Pmc1p, Pmr1p also plays a crucial role in detoxifying the cytosol in the presence of high extracellular Ca^{2+} concentrations, allowing the maintenance of low intracellular Ca^{2+} levels (Gunningham and Fink 1996). Further, *pmr1* and *pmc1* mutants show increased sensitivity to high levels of extracellular Ca^{2+} (Miseta et al. 1999; Szigeti et al. 2005). Based on these reports and the results of the current study, we proposed that dodecanol affects the expression *PMR1* and/or Pmr1p activity, ultimately leading to intracellular accumulation of Ca^{2+}. We used RT-qPCR analysis to quantify the relative expression of *PMR1* in BY4741 parental cells treated with 250 μM dodecanol and/or 312.5 μM anethole for 4 and 48 h (Fig. 5). *PMR1* expression in treatment without drugs (None) was normalized as 1.0 against *ACT1* expression. Following treatment with drugs for 4 h (Fig 5, left), anethole increased *PMR1* expression up to 120% as compared with the treatment without drugs, while dodecanol reduced the expression by 39%.

Dodecanol in combination with anethole inhibited *PMR1* expression to a similar degree (31%) as dodecanol treatment. Treatment for 48 h (Fig. 5, right) returned similar results to treatment for 4 h. These observations suggested that dodecanol and the drug combination suppress *PMR1* expression.

Effects on expression of Pmr1p

After treatment with dodecanol for 48 h, cell viability was restored (Fig. 2A), and intracellular Ca^{2+} accumulation disappeared (Fig. 3A, right). If Pmr1p contributes to calcium efflux, we may expect *PMR1* expression to be restored to control levels. However, such restoration was not observed (Fig. 5, right). Therefore, we estimated protein levels of Pmr1p using a yeast strain expressing GFP-Pmr1p with endogenous promoters (Fig. 6A). Following treatment for 4 h, dodecanol and the drug combination showed reduced GFP-Pmr1p expression compared to that of treatment without drugs (Fig. 6A, left). Anethole slightly reduced the expression. These results were more or less consistent with the gene expression level of *PMR1,* except for in the case of anethole. In contrast to the results obtained with 4-h treatment, protein expression levels were not proportional to gene expression levels after 48-h treatment (Figs. 5, right and 6, right). Namely, dodecanol and the drug combination increased the protein expression levels compared with those observed following treatment with anethole and without drugs. This indicates that the regulation of Pmr1p expression may involve other processes, such as translation and protein degradation, after 48-h treatment.

Dodecanol induces abnormal localization of Pmr1p

Pmr1p localizes at the membrane of the Golgi apparatus (Rudolph et al. 1989). We

investigated the localization of Pmr1p in cells expressing a chromosomal fusion of Pmr1p tagged with GFP (Huh et al. 2003), and treated cells with 250 μM dodecanol and/or 312.5 μM anethole (Fig. 6B). Without drug treatment, the fusion protein localized to the membranes of vesicles; this suggests that Pmr1p was localized and functioned normally on membranes. Although the Golgi localization of Pmr1p has been confirmed based on the subcellular fractionation of yeast lysates (Mandal et al. 2000), our results indicated that Pmr1p localizes to vesicle membranes, similar to proteins that localize to the membranes of the ER (Huh et al. 2003; Zhu et al. 2019). A similar localization of Pmr1p has been reported previously (Büttner et al. 2013). Anethole did not affect fusion protein localization after 4- and 48-h treatment. In contrast, the fusion protein did not show specific localization in cells treated with dodecanol or the drug combination after 4-h treatment. These observations indicate a possibly disordered architecture of the vesicles—not the Golgi but possibly the ER—in dodecanol- and drug combination-treated cells. After 48-h treatment with dodecanol, localization of Pmr1p was restored to vesicle membranes. These results suggest that the dodecanol-induced reduction in cell viability was at least partly related to the abnormal localization of Pmr1p.

Although the combination of dodecanol and anethole completely inhibited cell viability, supplementation with BAPTA-AM partly restored cell viability (Fig. 4). In the presence of BAPTA-AM and the drug combination, the localization of Pmr1p to the vesicle membranes was partially restored, resembling that in cells treated without drugs (Fig. 6C). This indicates that Ca^{2+} accumulation may affect the architecture of vesicles such as the Golgi and/or ER and their functions.

Dodecanol inhibits *PDR5* expression in *pmr1Δ* strain

When *S. cerevisiae* cells were treated with dodecanol, we observed a rapid decrease in cell viability within a 4-h incubation (Fig. 1). However, cell viability was gradually recovered after 4 h (Fig. 1). This recovery partly depends on the efflux of dodecanol by a fungal multidrug efflux pump, Pdr5p (Fujita et al. 2017). In this study, dodecanol efflux was newly confirmed using GC in the total lipid fraction derived from yeast cells. In the fractions of cells treated without dodecanol, dodecanol was not detected at all. After 4-h treatment, dodecanol had accumulated at 556 nmol per 10^8 cells. After 48-h treatment with dodecanol, accumulation of dodecanol decreased to 44% of that after 4-h treatment, indicating a role for dodecanol efflux in restoring cell viability. The rate of recovery in the *pmr1Δ* strain was slower than that in the parental strain (Fig. 2B), suggesting that the deletion of *PMR1* was involved in the suppression of *PDR5* expression. Therefore, we compared the relative transcript levels of *PDR5* in the *pmr1Δ* and parental strains treated with 250 μM dodecanol, using RT-qPCR. *PMR1* expression in treatment without drugs (None) was normalized as 1.0 against *ACT1* expression in *pmr1Δ* and its BY4741 parental strains. *PDR5* expression in the dodecanol-treated parental cells was increased 2.4-fold in comparison with that in the untreated cells (Fig. 7). However, *PDR5* expression in strain *pmr1Δ* only increased 1.2-fold compared with the untreated cells (Fig. 7), indicating that dodecanol did not stimulate *PDR5* expression in the *pmr1Δ* strain. This raised the possibility that dysfunction of the Ca^{2+}-ATPase Pmr1p in the Golgi apparatus is indirectly involved in the attenuation of dodecanol resistance.

DISCUSSION

Dodecanol exerts a transient fungicidal effect against such fungi as *S. cerevisiae* and *C.*

albicans (Fujita et al. 2017). As incubation time passes, the effect disappears (Fujita et al. 2017). However, prior to the current study, its fungicidal mechanism remained unclear. In the current study, we investigated the hypothesis that the antifungal activities of dodecanol is involved in disorder of calcium metabolism, judging from the durable antifungal effect in a deletion strain of *pmr1*, encoding Golgi Ca^{2+}-ATPase. We demonstrated the intracellular accumulation of Ca^{2+} in dodecanol-treated cells (Fig. 3) and the recovery from dodecanol-caused restriction of cell viability by the membrane-permeable Ca^{2+} chelator BAPTA-AM in cells treated with dodecanol and dodecanol in combination with anethole (Fig. 4).

n-Alkanols (ca. C11), including dodecanol, inhibit oxygen respiration, e.g., state III respiration and uncoupled respiration in intact mitochondria isolated from the rat liver (Hammond and Kubo 2000). Since *S. cerevisiae* is a facultative anaerobe, such inhibition is not directly involved in the fungicidal activity (Fujita et al. 2007). However, dodecanol induces the production of cellular ROS in *S. cerevisiae* (Fujita et al. 2007). ROS are mainly produced by the mitochondrial complexes I and III during oxygen respiration (Machida et al. 1998). Hence, ROS production is not associated with the fungicidal effect of dodecanol (Edelfors and Ravn-Jonsen 1990). On the other hand, another *n*-alkanol, octanol, increases the fluidity of plasma membranes in *S. cerevisiae* (Fujita et al. 2007). In addition, dodecanol increases the fluidity of the brain synaptosomal plasma membrane in rat and stimulates membrane Ca^{2+}/Mg^{2+}-ATPase activity (Edelfors and Ravn-Jonsen 1990), highlighting the possibility that the compound modulates Ca^{2+} transport in yeast membranes, thus exerting antifungal activity. Further, amiodarone toxicity is mediated by a disruption of Ca^{2+} homeostasis, causing an immediate and extensive Ca^{2+} influx into *S. cerevisiae* cells (Gupta et al. 2003), and supporting the notion that the dodecanol-induced

fungicidal effect is linked to intracellular Ca^{2+} accumulation.

Anethole enhances the fungicidal effect of dodecanol against *S. cerevisiae* and sustains the effect of dodecanol exposure (Fujita et al. 2017). Dodecanol is most likely removed from the cell by fungal multidrug-resistant pumps, mainly Pdr5p, explaining deterioration of its antifungal effect with an increasing incubation time. The expression of ABC transporter genes, including *PDR5*, is regulated by a complex regulatory network of transcription factors, and zinc finger regulators Pdr1p and Pdr3p are predominantly responsible for *PDR5* regulation (Jungwirth and Kuchler 2006). Anethole probably inhibits drug efflux by inhibiting the Pdr3p-dependent expression of *PDR5* (Fujita et al. 2017). Loss of mitochondrial DNA leads to a strong induction of *PDR5* expression that is strictly Pdr3p-dependent (Song and Ahn 2010). Further, *LGE1* deletion fails to fully induce *PDR5* transcription in mitochondrial DNA-depleted (ρ^0) cells (Zhang et al. 2005). This is supported by the lack of growth recovery of *lge1Δ* cells treated with dodecanol (Fujita et al. 2017), and also suggests that dodecanol stress might partly affect the mitochondria. However, the detailed signaling pathway by which drug stress stimulates *PDR5* expression remains to be elucidated.

Pdr5p belongs to the family of asymmetric ABC transporters, which also includes the cystic fibrosis transmembrane conductance regulator, multidrug resistance-associated protein 1, ABCG5/ABCG8, and others (Jee et al. 2016; Sorum et al. 2017; Yang et al. 2003). The expression of mammalian ABC transporters, such as P-glycoprotein and breast cancer resistant protein/ABC family G2, is upregulated by several pathways involving p38 mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2, and nuclear factor-κB/p65 (Chen et al. 2016). In hepatocellular carcinoma cells, doxorubicin, hypoxia, and ionizing radiation stimulate the induction of multidrug

resistance, which is accompanied by intracellular Ca^{2+} accumulation (Wen et al. 2016). Such accumulation is involved in the modulation of transient receptor potential canonical (TRPC) 6 (Wen et al. 2016). TRPC6 is a cation channel located in the plasma membrane, which mainly contributes to Ca^{2+} influx (Dietrich and Gudermann 2014). Another type of TRP channel, TRPC5, is essential for P-glycoprotein induction in drug-resistant cancer cells (Ma et al. 2012). In *S. cerevisiae*, a TRPC homolog, Yvc1p, mediates the release of Ca^{2+} from the vacuoles in response to hyperosmotic shock (Denis et al. 2002; Palmer et al. 2001). In the current study, *YVC1* deletion did not affect the antifungal activity of dodecanol (Table 1). By contrast, *PMR1* deletion and anethole prolonged the transient antifungal effect of dodecanol and the accompanying intracellular Ca^{2+} accumulation, although the effect of *PMR1* deletion was weaker than that of anethole. Yadav et al. (Yadav et al. 2007) reported that *pmr1Δ* cells are sensitive to multiple drugs, such as amiodarone, wortmannin, sulfometuron methyl, and tunicamycin. The targets of these drugs are diverse and unrelated. This suggests that Ca^{2+} transport plays a major role in mediating the requirement for Pmr1p in the cellular response to multiple drugs. The findings of the current study support the results that were obtained in previous reports. In fact, the growth recovery of dodecanol-treated *pmr1Δ* cells was delayed compared with that of the parental strain, indicating that the intracellular Ca^{2+} levels probably affect the rate of dodecanol efflux. Furthermore, anethole prolonged intracellular Ca^{2+} accumulation, thereby maintaining the antifungal activity of dodecanol. This effect also depends on the restriction of drug efflux.

To date, the target of anethole that inhibits drug efflux-associated drug resistance remains unknown. Anethole inhibits the activity of pig SERCA (Sárközi et al. 2007) and rabbit SERCA can replace yeast Ca^{2+}-ATPases (Degand et al. 1999). Pmr1p differs from

the previously characterized SERCA and plasma membrane Ca^{2+}-ATPases with respect to the sensitivity to inhibitors, such as vanadate, thapsigargin, and cyclopiazonic acid, and affinity to substrates, such as MgATP and Ca^{2+} (Sorin et al. 1997). Hence, anethole might directly inhibit Ca^{2+} transport dependent on Pmr1p in addition to sustaining *PMR1* downregulation. On the other hand, although dodecanol increases the fluidity of the nerve plasma membrane and stimulates Ca^{2+}/Mg^{2+} ATPase activity in the membranes (Gupta et al. 2003), it might also inhibit Ca^{2+} transport via Pmr1p. The Ca^{2+} accumulation induced by dodecanol and the drug combination may depend on this response, in addition to the inhibition of *PMR1* expression, at least after 4-h treatment. After 4-h treatment with dodecanol and the drug combination, stress responses, such as upregulation of Pmr1p levels, may have occurred (Fig. 6A).

In the current study, we observed that Pmr1p did not show specific localization in cells treated with dodecanol and the drug combination when cell viability was inhibited (Fig. 6B). Although Pmr1p has been previously shown to localize to the Golgi apparatus membrane (Rudolph et al. 1989), our results instead showed that Pmr1p rather localizes to the ER membrane (Fig. 6B). Therefore, this abnormal localization suggests that drug stress affects other Golgi and/or ER functions and/or architecture, in addition to the dysfunction of Ca^{2+} transport into the internal Golgi apparatus. Gdt1p, a Golgi-localized Ca^{2+}/H^{+} antiporter, controls cellular Ca^{2+} stores and plays a major role in Ca^{2+} response induced by osmotic shock in the absence of Pmr1p (Colinet et al. 2016). In *C. albicans*, five genes related to the glycosylation pathway and one gene related to the cell wall integrity pathway are upregulated after a deletion of *GDT1* and/or *PMR1* (Jiang et al. 2018). Jiang et al. (Jiang et al. 2018) concluded that Pmr1p is the major player in the regulation of Ca^{2+} homeostasis in *C. albicans*. On the other hand, Salaroglio et al.

(Salaroglio et al. 2017) reported that adaptation to ER stress, which activates the so-called unfolded protein response (UPR), leads to the acquisition of a multidrug resistance phenotype, e.g., overexpression of the plasma-membrane ABC transporter multidrug resistance-associated protein 1 in different tumor types. The authors concluded that the gene for the UPR sensor protein kinase, RNA-like ER kinase, is the only gene significantly upregulated in both cells selected for chemoresistance and cells selected for ER stress resistance (Salaroglio et al. 2017). Therefore, in *S. cerevisiae*, the antifungal activity of dodecanol is probably attributed to signaling pathways initiated by the Golgi and/or ER stresses, in addition to intracellular Ca^{2+} accumulation.

The dodecanol-induced intracellular Ca^{2+} accumulation and the prolonged Ca^{2+} accumulation caused by anethole might also extend to lipophilic antifungal drugs, in addition to medium chain *n*-alkanols around C_{12}. Namely, as anethole shows synergistic antifungal effects in combination with several lipophilic drugs that are unrelated in their antifungal mechanisms (Fujita et al. 2007; Kubo et al. 1995; Kubo and Himejima et al. 1992; Kubo and Kubo 1995), drugs whose antifungal action involve membrane permeability may also cause Ca^{2+} accumulation.

The deletion of *PMR1* encoding Golgi Ca^{2+} ATPases disabled the induction of *PDR5* expression in cells treated with dodecanol (Fig. 7). Therefore, *PMR1* itself in addition to intracellular Ca^{2+} accumulation is a potential candidate gene for developing antifungal drugs overcoming drug resistance.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

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Conflict of interest. None declared.

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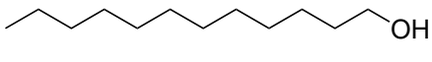
Table 1. MIC of dodecanol in *S. cerevisiae* mutants lacking genes related to Ca^{2+} signaling and regulation of intracellular Ca^{2+} levels

Strain	MIC (μM), as assessed after incubation for the indicated time (h)			Gene function
	48	72	96	
BY4741	72	>2500	>2500	Parental strain (Winston et al. 1995)
CNA1	39	>2500	>2500	Calcineurin related (Cyert et al. 1991; Liu et al. 1991; Kingsbury 2000)
CMP2	78	>2500	>2500	
CNB1	39	>2500	>2500	
RCN1	39	>2500	>2500	
PMR1	39	78	156	Golgi apparatus Ca^{2+}-ATPase (Rudolph et al. 1989)
PMC1	39	>2500	>2500	Vacuolar Ca^{2+}-ATPase (Cunningham et al. 1994)
CCH1	78	>2500	>2500	Ca^{2+} channel in the plasma membrane (Paidhungat and Garrett 1997; Iida et al. 1994; Martin et al. 2011)
MID1	78	>2500	>2500	
ECM7	156	>2500	>2500	

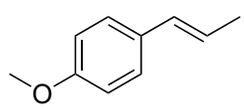
VCX1	>2500	>2500	>2500	Vacuolar membrane antiporter with Ca^{2+}/H^{+} (Cunningham et al. 1996)
YVC1	78	>2500	>2500	Vacuolar Ca^{2+}, K^{+}, and Na^{+} channel (Palmer et al. 2001)

Figure legends

Fig. 1. Chemical structures of *n*-dodecanol and *trans*-anethole.



n-Dodecanol



trans-Anethole

Fig. 1. Oyama et al.

Fig. 2. Effect of anethole on dodecanol-induced fungistatic action against parental (A) and *pmr1Δ* (B) strains of *S. cerevisiae* BY4741. Exponentially growing yeast cells were grown at 30°C in 2.5% malt extract (ME) broth supplemented with 250 μM dodecanol (▲), 312.5 μM anethole (◆), or 250 μM dodecanol + 312.5 μM anethole (○). Closed circles (●) denote no drug treatment. Data are expressed as the mean ± s.d. (n = 3).

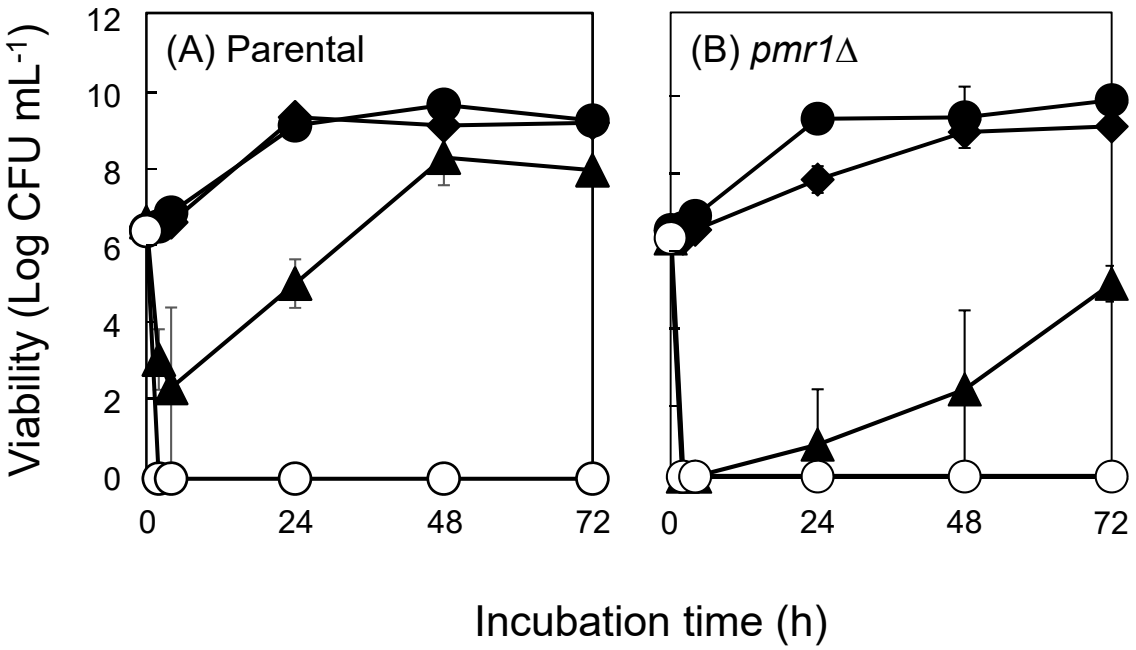


Fig. 2. Oyama et al.

Fig. 3. Phase-contrast and fluorescent micrographs of Fluo-4 AM-stained cells. Exponentially growing cells of parental (A) and *pmr1Δ* (B) strains were incubated at 30°C in ME broth supplemented with 312.5 μM anethole (Ane), 250 μM dodecanol (Dode), or their combination (Comb), for the indicated times prior to Fluo-4 AM staining. None, no drug treatment. To observe fluorescence of the Ca^{2+} and Fluo-4 AM complexes, the images were acquired using Ex and Em wavelengths of 495 and 518 nm, respectively. The images are representative results. More than 200 cells were selected at random to calculate the percentage of Fluo 4-stained cells among the total cells. Data are expressed as the mean ± s.d. (n = 3).

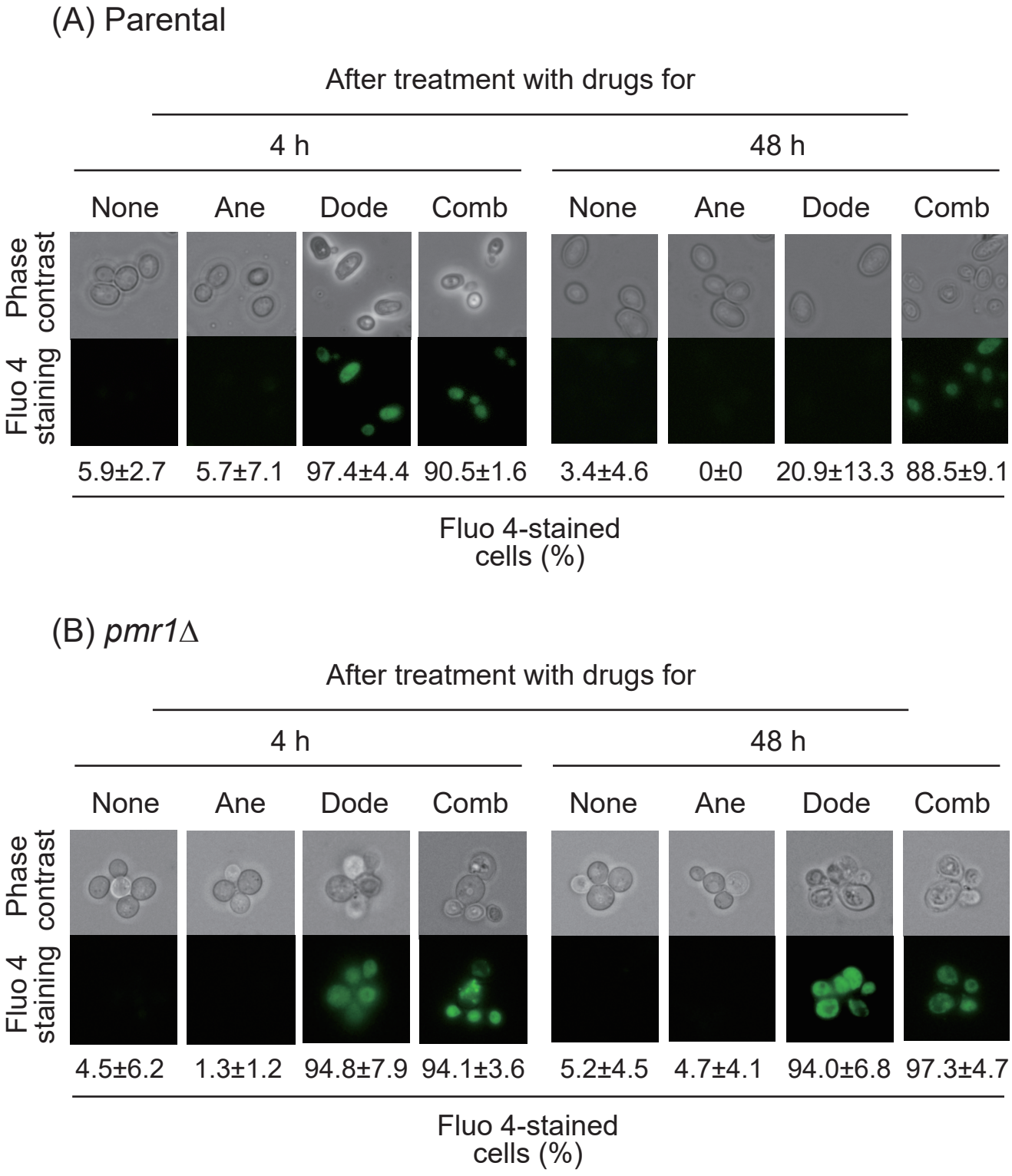


Fig. 3. Oyama et al.

Fig. 4. Effect of BAPTA-AM on the viability of *S. cerevisiae* BY4741 cells treated with dodecanol and/or anethole. Exponentially growing yeast cells were pre-incubated in 2.5% ME broth supplemented with 50 μM BAPTA-AM at 30°C for 30 min. Following the pre-incubation, the following drugs were added to the culture medium: 250 μM dodecanol (▲), 312.5 μM anethole (◆), or their combination (○), and the cells were then grown. Closed circles (●) denote no drug treatment. Data are expressed as the mean ± s.d. (n = 3).

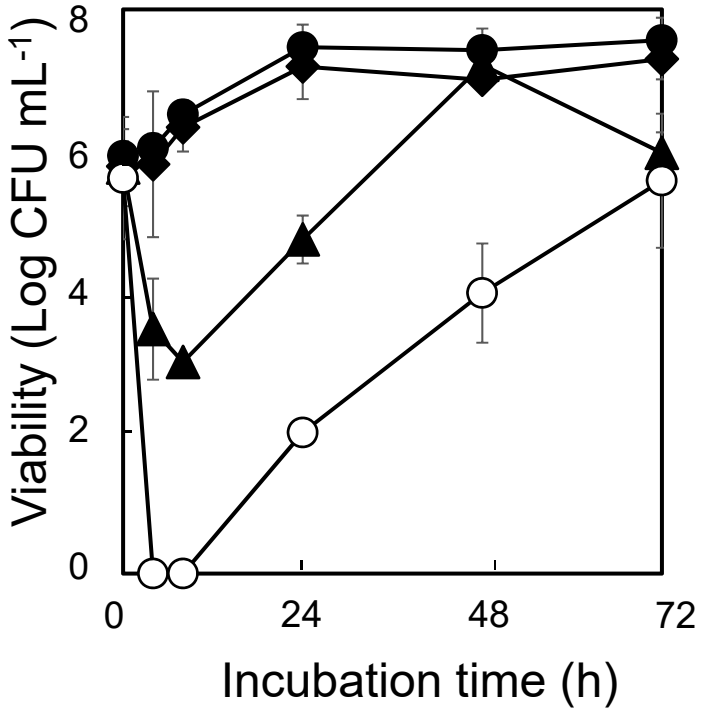


Fig. 4. Oyama et al.

Fig. 5. Relative expression of the *PMR1* gene in the presence of drugs. Exponentially growing *S. cerevisiae* BY4741 cells were incubated in 2.5% ME broth supplemented with 250 μM dodecanol and/or 312.5 μM anethole at 30°C for 4 and 48 h. None, no drug treatment. Total RNA was then extracted and analyzed by RT-qPCR. Gene expression is shown relative to *ACT1* expression. Data are expressed as the mean + s.d. (n = 3). * p < 0.05.

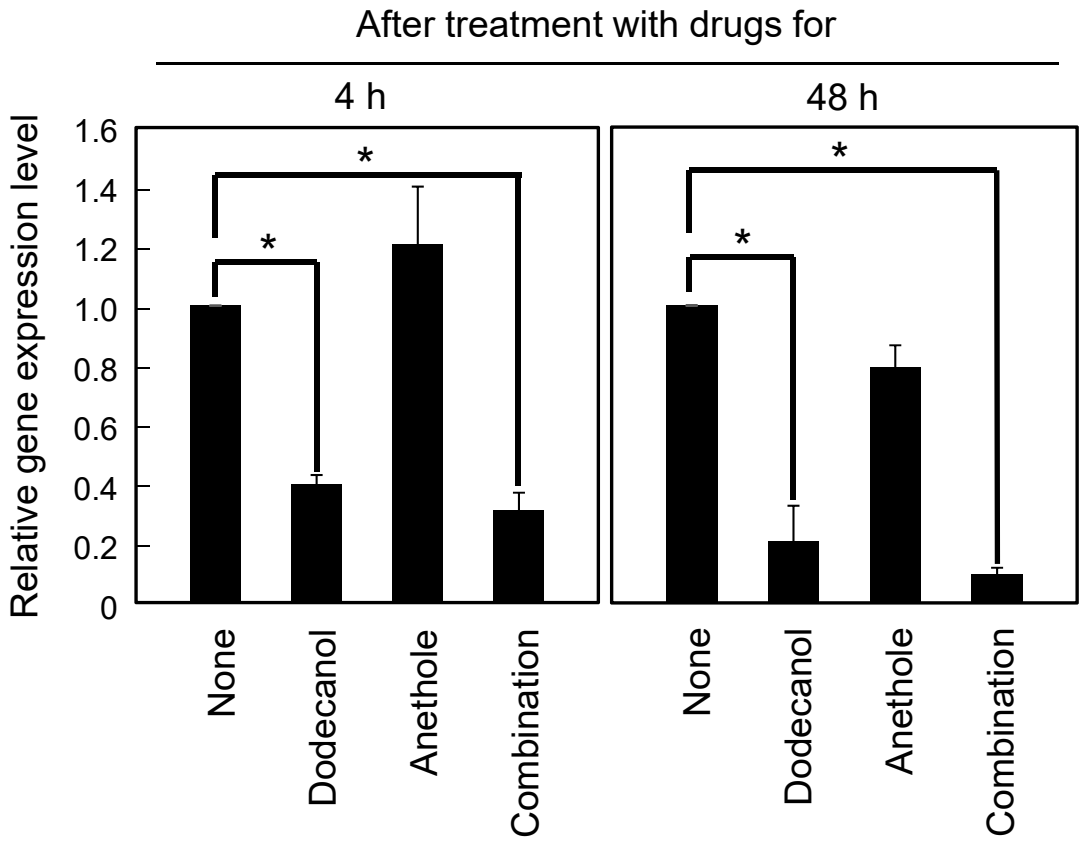


Fig. 5. Oyama et al.

Fig. 6. Flow cytometry analysis of cells expressing GFP-Pmr1p with endogenous promoter *PMR1* (A) and localization of GFP-Pmr1p in *S. cerevisiae* BY4741 (B and C). Exponentially growing *S. cerevisiae* PMR1-GFP cells were incubated in 2.5% ME broth supplemented with 250 μM dodecanol (Dode), 312.5 μM anethole (Ane), or their combination (Comb) at 30°C. None, no drug treatment. (A) Particle count versus GFP area is plotted for yeast cells. Flow cytometry was independently performed on 10 000 cells. (B and C) The cells were incubated without (B) or with (C) 50 μM BAPTA-AM. The GFP fluorescence was detected at Ex and Em wavelengths of 488 and 507 nm, respectively. The images are representative results. More than 200 cells were selected at random to calculate the percentage of cells with abnormal localization of Pmr1p among the total cells. Arrows indicate localization of Pmr1p on membranes of vesicles. Data are expressed as the mean + s.d. (n = 3).

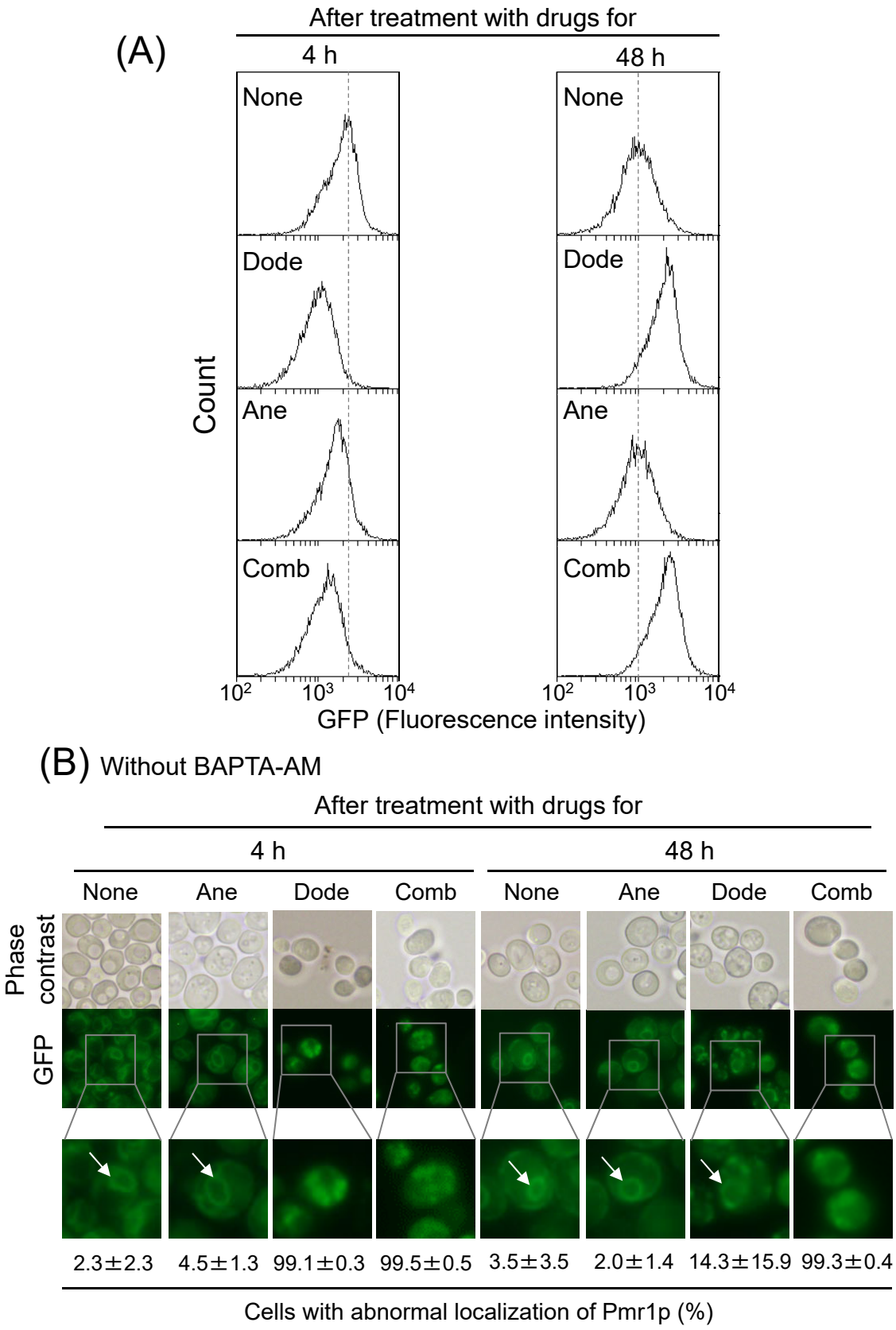


Fig. 6. Oyama et al.

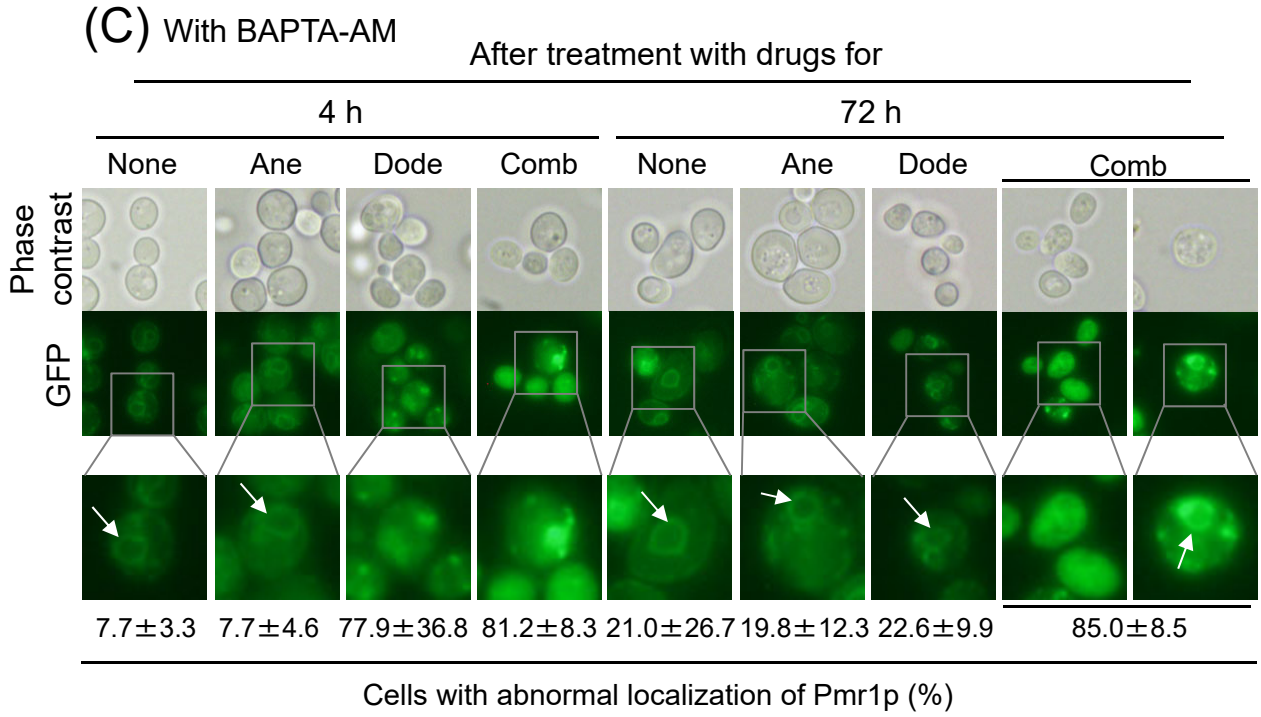


Fig. 6 C. Oyama et al.

Fig. 7. Relative expression of the *PDR5* gene in *pmr1Δ* and parental strain in the presence of dodecanol. Exponentially growing cells of *S. cerevisiae* BY4741 (parental) and *pmr1Δ* were incubated in 2.5% ME broth supplemented with 250 μM dodecanol at 30°C for 4 h. Total RNA was extracted and then analyzed by RT-qPCR. Gene expression is shown relative to *ACT1* expression. Data are expressed as the mean ± s.d. ($n = 3$). * $p < 0.05$.

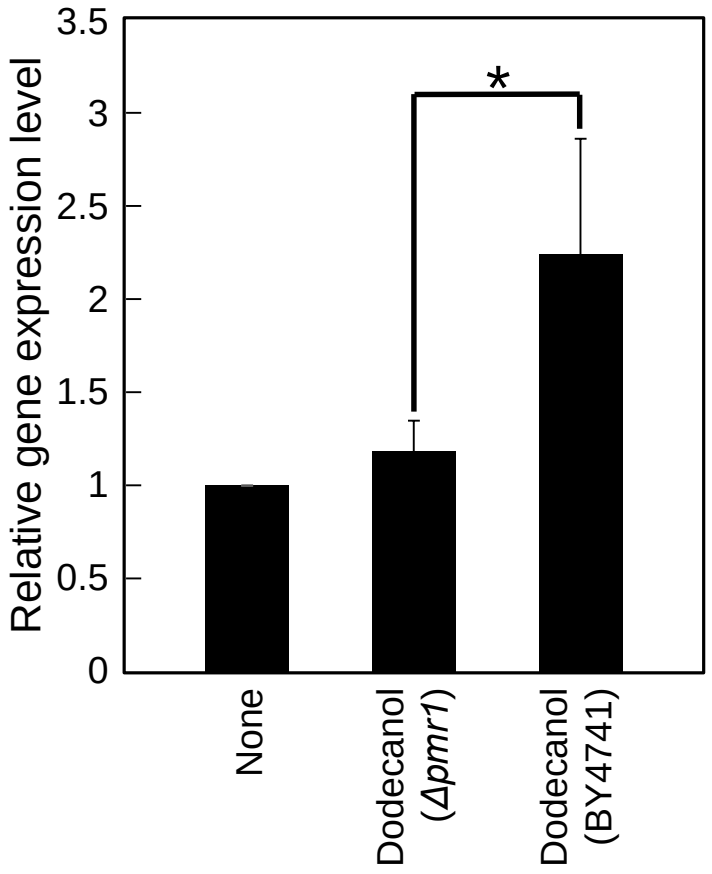


Fig. 7. Oyama et al.

Supplementary data:

Deletion of the Golgi Ca^{2+}-ATPase *PMR1* gene potentiates antifungal effects of dodecanol that depend on intracellular Ca^{2+} accumulation in budding yeast

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Supplementary Table 1. Primers used for reverse-transcription quantitative polymerase chain reaction

Primer name	Sequence (5′–3′)
ACT1-F	ATGGTCGGTATGGGTCAAAA
ACT1-R	AACCAGCGTAAATTGGAACG
PMR1-F	CCTTAGCGGTTGCTGCTATT
PMR1-R	ACCTTCTCACGATGGCTTTAC
PDR5-F	GTTGCCTAAACCCAGGTGAA
PDR5-R	ATTGCTACTTCCGCCAAATG