Vacuolar MDR Transporters Nft1p and Vmr1p Contribute to Malathion Tolerance in *Saccharomyces cerevisiae*

การทำงานร่วมกันของโปรตีนขนส่งดื้อยาหลายชนิดของแวคิวโอล ชนิด Nft1p และ Vmr1p ต่อการทนทานมาลาไธออนในยีสต์ แซกคาโรไมซีส เซเรวิซิอี

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ABSTRACT

Organophosphate (OP) insecticides malathion is widely used for insects control due to their low toxicity in mammalian species but high toxicity in insects. However, insecticide misapplication and overuse has resulted in the development of resistance in insect species, leading to increased crop losses. Utilizing the baker's yeast *Saccharomyces cerevisiae* as a model organism we have screened strains deleted for members of the multi-drug resistance (MDR) transporter family for malathion sensitivity. Deletion of genes encoding the vacuolar MDRs, *VMR1* and *NFT1*, sensitized yeast to malathion under respiratory conditions. Yeast lacking Vmr1p and Nft1p were not sensitized to osmotic or oxidative stress conditions. Lipid droplet content was found to be altered in yeast deleted for *VMR1* and *NFT1* suggesting a possible link between malathion sensitivity and lipid droplets.

บทคัดย่อ

ยาฆ่าแมลงกลุ่มออร์กาโนฟอสเฟตนิยมใช้ในการจัดการศัตรูพืชแบบผสมผสาน มาลาไทออนถูกใช้กันอย่างแพร่หลายในการควบคุมแมลง เนื่องจากมีความเป็นพิษต่ำในสัตว์เลี้ยงลูกด้วยนมแต่มีความเป็นพิษสูงในแมลง อย่างไรก็ตาม การใช้ยาฆ่าแมลงแบบผิดวธิ ีและการใช้ปริมาณมากเกินไปส่งผลให้เกิดการดื้อยาในแมลง นำไปสู่ความเสียหายต่อการ เพาะปลูกพืชผลที่เพิ่มขึ้น การศึกษานี้ใช้ยีสต์สายพันธ์ ยีสต์ขนมปัง ซึ่งอยู่ในขนมปังเป็นแบบจำลอง โดยศึกษาสายพันธุ์ที่อยู่ในกลุ่มโปรตีนขนส่งที่ดื้อยาหลายชนิด (MDR) ต่อความไวของมาลาไธออน พบว่ายีสต์ที่ถูกลบยีน ชนิด *VMR1* และ *NFT1* ไวต่อมาลาไธออนภายใต้สภาวะการหายใจ แต่ไม่ไวต่อสภาวะความเครียดออสโมติกหรือออกซิเด ชัน พบว่ามีการเปลี่ยนแปลงของปริมาณหยดไขมันในยีสต์ที่ถูกลบยีน *VMR1* และ *NFT1* ซึ่งชี้ให้เห็นถึงความเชื่อมโยงที่ เป็นไปได้ระหว่างความไวต่อมาลาไทออนกับหยดไขมัน

Keywords: Malathion, Vacuole, Multi-drug resistant transporter

คำสำคัญ: มาลาไธออน แวคิวโอล สารขนส่งดื้อยาหลายชนิด

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Introduction

Application of insecticides has been beneficial for limiting crop loss and reducing post-harvest damage in agricultural countries, such as Thailand. The increased productivity due to the use of insecticides has benefited farmers; however, over-use and misapplication has led to elevated human exposure and development of resistance in insects. Data from the International Rice Research Institute reported that Thailand uses the largest amount of insecticides among countries in South-Asian such as Indonesia, Philippines, and Vietnam (data from the International Rice Research Institute Study; http://www poobokto.blogspot.com). The over-use of pesticides has led to concerns about poisoning of farmers and environmental contamination. Adverse effects on the health of people living in agricultural areas has been documented with 3,067 patient treated for insecticide poisoning and 407 deaths reported between October 2018 to July 2019 (data from Nation Health Security Office; http://www.nhso.go.th). Among members of OP insecticides, malathion (MA) is widely used to control insects because of low toxicity and persistence in mammalian but high toxicity in insects (Gupta 2006). These compounds have high neurotoxicity on non-target organisms (Sandoval-Herrera et al. 2019).

To overcome the problems of over-use and human exposure there is significant interest in the search for strategies that delay or avoid development of insecticide resistance. We focused on the study of possible insecticide toxicity to find alternative targets to insecticide sensitivity, as potential targets for synergist development. Synergists are chemicals that make insecticide ingredients more effective at killing insects. The role of synergists in resistance is related to restoring the susceptibility of insects to the chemical (Bernard and Philogene 1993) which require higher levels of the toxic substance for their control. For example, the addition of piperonyl butoxide (PBO) and S,S,S- tributyl phosphorotrithioate (DEF) on the toxicity of carbamate insecticides against *Blattella germanica* in Tehran city (Sanei Dehkordi et al. 2017).

Due to limitations in genetic tools for insect systems, we selected the Baker' s yeast *Saccharomyces cerevisiae* as a model organism to investigate the genetics of insecticide resistance. *S. cerevisiae* is a single cell eukaryote and has been widely used as an experimental system for biological studies due to its high conservation of metabolic and regulatory mechanisms with other eukaryotic cells. Yeast cells also grow rapidly, are non-pathogenic, and many tools exist for genetic manipulation (Mohammadi et al. 2015). Many discoveries, such as the treatment of human diseases have been made using yeast system, highlighting the utility of this model organism (Nielsen 2019). In addition, yeast do not contain cholinesterase enzymes, the primary target of malathion. The lack of cholinesterase enzymes allows for the examination of off-target effects using the yeast model.

The genome of *S. cerevisiae* contains more than 30 distinct genes encoding Multi-Drug Resistance (MDR) proteins, members of the ABC drug efflux pump family. These efflux transporters are known to aid in translocation of structurally and functionally unrelated xenobiotics. The MDR system in yeast includes transporters localized to the plasma membrane, vacuole, mitochondria, and peroxisome.

Interestingly, resistance to many chemical compounds including insecticides is mediated by MDR transporter system (Klein, Kuchler, and Valachovic 2011; Chang 2003). Lipid droplets (LDs), also called lipid particles, lipid bodies, fat bodies, oil bodies, or adiposomes, are found in almost all cells. LDs play an essential role in intracellular lipid mobilization and are also important for response to xenobiotic stress due to their ability to sequester hydrophobic compounds within the lipid core (Jarc and Petan 2019; Rajakumar et al. 2020; Shpilka et al. 2015).

The aim of this study was to examine members of the *S. cerevisiae* MDR transporters for their contribution to malathion tolerance. Screening yeast deleted for the MDR genes revealed two genes encoding vacuolar MDR proteins, Vmr1p and Nft1p, that were sensitized to malathion. Sequence homologues of Vmr1p and Nft1p are present in insects. Yeast Vmr1p displays 34% identity and 55% similarity to *Drosophila melanogaster* Multidrug-Resistance like protein 1 (Mrp1) isoform M. Nft1p is homologous (27% identity and 46% similarity) to another splice variant of *D. melanogaster* Mrp1 isoform I. Substrates for the different *D. melanogaster* Mrp1 isoforms have not been well characterized.

Vmr1p has been implicated in transport of cadmium- and lead-GSH conjugates into the vacuole (Wawrzycka et al. 2010; Sousa, Hanselaer, and Soares 2015) but a role for this transporter in insecticide tolerance has not been previously reported. The Nft1p protein is essentially uncharacterized and no substrate for this transporter has been described. Examining levels of LDs indicated that both Vmr1p and Nft1p contributed to LD formation or accumulation. These findings suggest that tolerance to malathion requires LD accumulation and provides for a new role for the vacuolar MDR transporters in lipid homeostasis.

Materials and Methods

Yeast strains and medium

Saccharomyces cerevisiae strains used in this study were derived from BY4742 (Mat α*, leu2Δ0, lys2Δ 0, ura3Δ 0, his3Δ 1). Single deletion strains were obtained from Open Biosystems and include pd5Δ, pdr10Δ, pdr11Δ, pdr12Δ, pdr15Δ, pdr18Δ, snq2Δ, yor1Δ, aus1Δ, vmr1Δ, and nft1Δ.*

Yeast transformations were performed using the lithium acetate procedure (Gietz and Schiestl 1991). Cells were propagated at 30 °C either in rich medium (YPD; 1% yeast extract, 2% bacteriological peptone, 2% D-glucose) or synthetic complete media (0.17% yeast nitrogen base, 2% glucose, 0.5% ammonium sulfate and amino acids) with 3% glycerol (SCG) or 2% D-glucose (SC) lacking the appropriate nutrients. For solid media 2% agar was included.

Plasmids

The plasmid for expression of the Erg6p-GFP fusion was generated by PCR amplifying the *ERG6* promoter and coding sequence (-900 to +1149). The PCR introduced a 5' *HindIII* site and a 3' *NotI* site, in addition the *ERG6* stop codon was revoved to allow in frame fusion with GFP. The *ERG6* PCR produce was digested with *HindIII* and *NotI* and ligated into plasmid pAA1 (Hobbs et al. 2001) cut with the same

enzymes. The candidate clones were verified by restriction mapping and the resulting plasmid was named pMD001.

Sensitivity assays

Malathion was obtained from PATO Chemical Industry Public Co., Ltd. (Bangkok, Thailand). Commercial grade malathion utilized in these studies contains 57% malathion, 30% petroleum solvent, and 13% emulsifier. Malathion was dissolved in sterile distilled water and mixed vigorously for 20 minutes. Tween 80 was added as a solubilizer at final concentration of 0.2%. Various concentrations of MA were added while preparing synthetic complete with 3% glycerol (SCG) media.

WT and mutant yeasts were pre-cultured on YPD agar enriched medium for 3 days at 30°C. Cells were diluted in dH_2O before being sequentially diluted. Cells (10^4, 10^3, or 10^2) were spotted onto SCG agar supplemented with vehicle or the indicated stressor. Stress conditions include malathion at 100 and 200 µg/mL, 10% ethanol, 500 and 750 mM NaCl, and 0.5 and 1 mM H_2O_2. Plates were incubated at 30°C for 7 days and photographed.

Fluorescence Imaging

Yeast strains were transformed with an *ERG6*-GFP expressing plasmid. Cells were cultured on SC-Leu media for 3 days. For microscopy experiments, cells were grown in 10 mL of SC-Leu medium with or without malathion overnight at 30° C in a rotary shaker for 16 hours.

Pads were prepared using 20 µL of SC-Leu medium containing 0.1% agarose by placing the preheated solution onto a glass slide, covering with a second slide, and then cooled on ice. Cells were placed on the agarose pad, covered with a cover slip and sealed with a thin layer of nail polish to prevent dehydration. Cells were observed using a confocal laser scanning microscope (Olympus FV10i-DOC), Olympus Bioimaging Center, Mahidol University. A magnification of 60X was used with a universal plan super apochromat phase- contrast oil- immersion objective. The induction of lipid droplet accumulation was monitored with excitation wavelength of 488 nm with an emission wavelength of 550 nm (Rostron and Lawrence 2017; Wolinski and Kohlwein 2008). The presence of LDs in yeast was monitored by counting 200 cells per sample in each condition. Cells were scores as either LD containing or LD absent.

Results and Discussion

Screening the collection of yeast deletion strains for those with increased susceptibility to malathion revealed several candidate genes with roles in malathion tolerance. Yeast lacking genes encoding the vacuolar ABC transporters Vmr1p and Nft1p were among strains that exhibited malathion sensitivity. To better judge the role of *VMR1* and *NFT1* in malathion resistance the full set of ABC transporter deletion mutants was tested for malathion sensitivity.

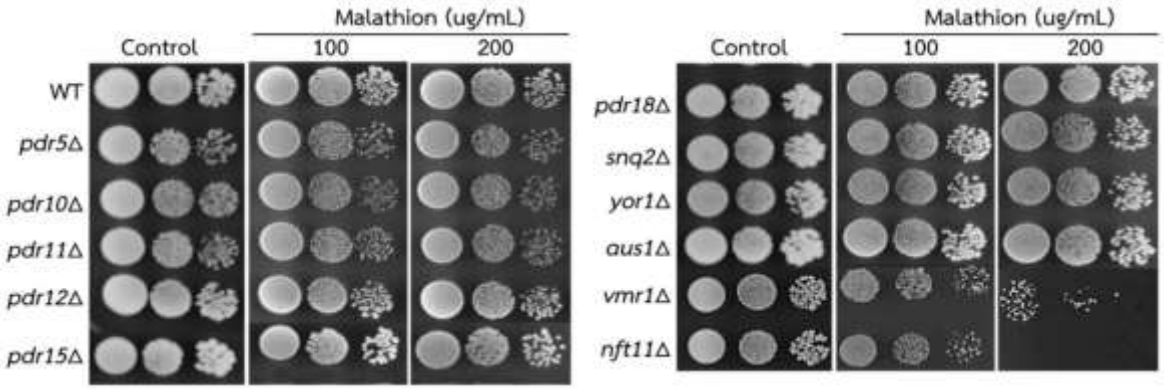

Figure 1. Sensitivity of yeast deleted for MDR genes on respiratory medium (SCG) containing vehicle or malathion. Deletion strains *pd5Δ, pdr10Δ*, *pdr11Δ*, *pdr12Δ*, *pdr15Δ, pdr18Δ*, *snq2Δ, yor1Δ*, and *aus1Δ* lack the indicated plasma membrane MDR transporter. The *vmr1Δ* and *nft1Δ* strains are deleted for the indicated vacuolar MDR transporters, Plates were incubated at 30°C for 7 days.

As seen in Figure 1, deletion of genes for plasma membrane ABC transporters implicated in multi-drug resistance (MDR) did not sensitize yeast to malathion. This result was unexpected as efflux of hydrophobic compounds, such as OP insecticides, is mediated primarily by the MDR transporters. Consistent with findings from the original screen for yeast sensitized to malathion both, *nft1*Δ, and *vmr1*Δ were unable to grow when challenged with a dose of malathion insecticide that was not toxic to WT cells.

The Vmr1p and Nft1p transporters are not well characterized and may be important for detoxification or sequestration of malathion molecules inside of cells, alternatively these transporters may be involved in a general response to stress conditions. To examine the selectivity of malathion on impaired survival of the MDR deletion strains tolerance to several other stress condition was examined. While the *nft1*Δ and *vmr1*Δ strains were sensitive to heat stress (37°C) relative to WT cells, these strains displayed similar resistance to other environmental stresses (Figure 2).

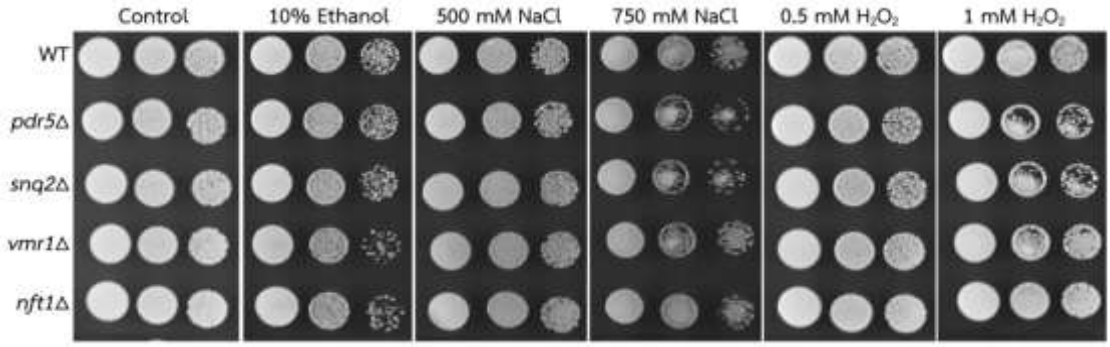

Figure 2. Sensitivity of MDR transporter deletion strains to environmental stresses. Plates were incubated at 30°C for 7 days.

Expansion of the ER was observed following malathion exposure (Thosapornvichai unpublished). One consequence of ER expansion is altered lipid metabolism and accumulation of sterol esters and triacylglycerides in lipid droplets. We investigated whether lipid droplet formation was altered in the *nft1*Δ and *vmr1*Δ strains. Following exposure to malathion for 16 hours, lipid droplets were viewed using Erg6p-GFP, a resident LD protein that has been utilized previously to identify the number and structure of lipid droplets (Na et al. 2015; Olzmann and Carvalho 2019). It appears that malathion exposure leads to a significant decrease in lipid droplets in the *nft1*Δ and *vmr1*Δ strains compared to the vehicle control (Figure 3). This suggests that a connection may exist between LD content and malathion sensitivity. However, it is also possible that the petroleum solvent contained in the commercial grade malathion may contribute to the observed effects on lipid droplet formation.

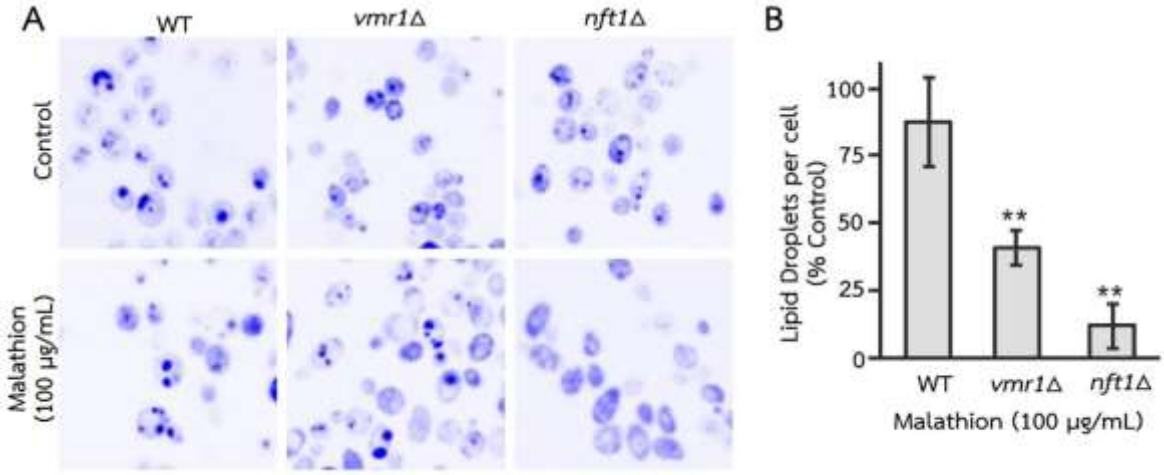

Figure 3. Examination of lipid droplet accumulation in yeast exposed to malathion under respiration. A) Erg6-GFP localization, a marker for LDs, was visualized using fluorescence microscopy in yeast exposed to vehicle (control) or 100 µg/mL malathion. B) Quantitation LD numbers in yeast cells exposed to malathion. Results are expressed as average ± standard deviation. $**P > 0.01$ determined using Student's t-test. Yeast cells cultured for 16 hours prior to visualization.

Conclusions

The substrate for Vmr1p is not known and we speculate that the molecule moved either into or out of the vacuole by this ABC transporter is important for lipid droplet formation, either directly or indirectly. A possible indirect effect would be the inability to sense malathion or molecules that are capable of inducing lipid droplet production. Further experiments that test whether genes that are required for lipid droplets are altered by loss of Vmr1p or Nft1p. Understanding how *vmr1*Δ and *nft1*Δ cells are impaired for LD accumulation following malathion exposure should facilitate identification of pathway(s) that are important for insecticide tolerance.

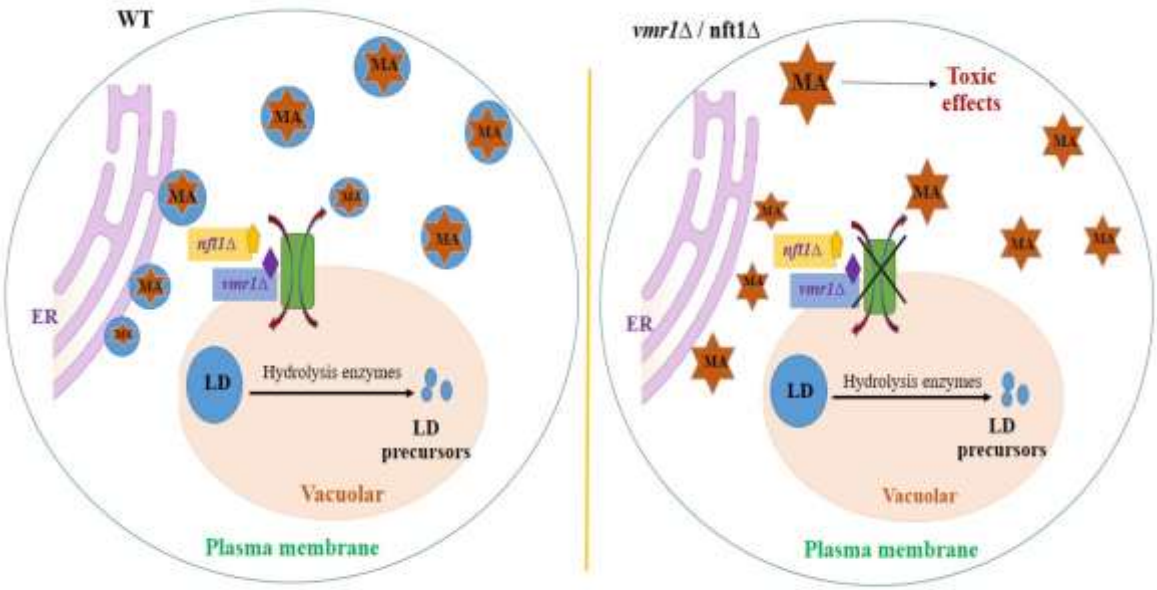

Figure 4. Model for malathion resistance due to sequestration in lipid droplets. Malathion is absorbed by lipid droplets in WT yeast protecting against toxicity. In contrast, *vmr1Δ* and *nft1Δ* cells accumulate reduced levels of lipid droplets resulting in a higher concentration of free malathion, leading to sensitivity.

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