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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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Name

学位論文題目：
Title of Dissertation

Characterization of the VBA transporter family in yeast
(酵母液胞膜トランスポーターVBAファミリーの機能解析)

学位論文要約：
Dissertation Summary

Vacuoles are the largest organelles in the budding yeast *Saccharomyces cerevisiae*, occupying about 25% of the cell volume that serve as a storage compartment for a variety of amino acids (1). In *S. cerevisiae*, majority of the cellular basic amino acids are accumulated in vacuoles, whereas acidic amino acid is almost excluded from vacuoles. While other amino acids are also compartmentalized in vacuoles, their concentrations are lower than those of the basic amino acids (1, 2). These differences in concentration between vacuolar and cytosolic pools imply the presence of active amino acid transport systems on the vacuolar membrane. The ATP-dependent amino acid uptake, which is driven by the proton electrochemical gradient generated by the action of the proton-pumping V-ATPase, has been investigated in purified vacuolar membrane vesicles (3, 4). Subsequently, two gene families, AVT (amino acid vacuolar transport) and VBA (vacuolar basic amino acid transporter), were reported to be involved in amino acid transport across the vacuolar membrane of *S. cerevisiae* (5, 6, Sekito *et al.*, in press) (Table 1). Avt1p in the AVT family is involved in vacuolar uptake of glutamine, isoleucine and tyrosine, whereas three export systems, Avt3p, Avt4p, and Avt6p, are concerned with the extrusion of amino acids from vacuoles (5, 7, Sekito *et al.*, in press). Transport substrates of Avt2p, Avt5p, and Avt7p have been unclear so far. The VBA family, which belongs to the major facilitator superfamily (MFS), comprises of seven members, Vba1p, Vba2p, Vba3p, Vba4p, Vba5p, Azr1p, and Sge1p. The Vba1p, Vba2p, and Vba3p are involved in ATP-dependent uptake of basic amino acids: arginine, histidine and lysine, into *S. cerevisiae* vacuole (6). *VBA4* gene expression was reported to decrease in response to nitrogen starvation. Other members of the VBA family, Azr1p and Sge1p are localized to the plasma membrane and involved in resistance to several drugs (8, 9). However, the functions of Vba4p and Vba5p have not been well characterized.

Vba3p (458 residues) and Vba5p (582 residues) are predicted to have 11 and 14 transmembrane domains, respectively (*Saccharomyces* genome database; www.yeastgenome.org/). Based on the alignment of amino acid sequences of Vba5p and Vba3p, most of their sequences are conserved except for the extra 124 amino acid residues in N-terminal segment of Vba5p and five amino acids in the transmembrane region. To investigate intracellular localization, green fluorescence protein (GFP) was tagged to the N-terminus of Vba5p, and GFP-Vba5p clearly localized to the plasma membrane (Fig.1). Since Vba5p is the closest to the Vba3p in the VBA family, and the Vba3p is the vacuolar membrane protein, the difference between Vba5p and Vba3p in intracellular localization can be determined by the existence of an extra N-terminal sequence. To investigate intracellular localization, the extra N-terminal sequences of Vba5p was transferred into Vba3p to make a chimera protein designed as Vba5(N)-Vba3p. As shown in Fig.1, GFP-Vba5(N)-Vba3p clearly localized to the plasma membrane of *S. cerevisiae*, indicating that the extra residues in the N-terminus of Vba5p was important for its intracellular localization.

Table 1 Vacuolar amino acid transporters and related proteins in *S. cerevisiae*

Family	Subfamily	Protein	Selectivity	Subcellular localization
AAAP	AVT	Avt1p	Neutral amino acids	Vacuolar membrane
		Avt2p	-	-
		Avt3p	Neutral amino acids	Vacuolar membrane
		Avt4p	Neutral and basic amino acids	Vacuolar membrane
		Avt5p	-	-
		Avt6p	Glu, Asp	Vacuolar membrane
		Avt7p	-	-
MFS	VBA	Vba1p	His, Lys	Vacuolar membrane
		Vba2p	His, Lys, Arg	Vacuolar membrane
		Vba3p	His, Lys	Vacuolar membrane
		Vba4p	-	Vacuolar membrane
		Vba5p	-	-
		Azr1p	Azole	Plasma membrane
		Sge1p	Crystal violet	Plasma membrane

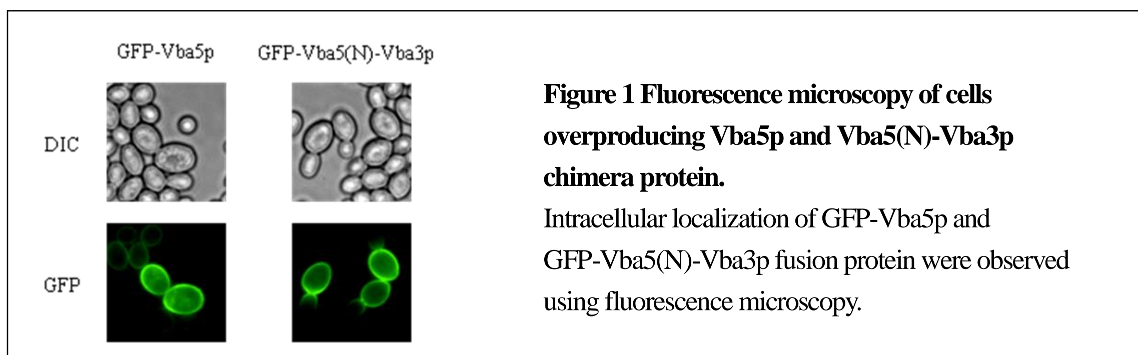

Figure 1 Fluorescence microscopy of cells overproducing Vba5p and Vba5(N)-Vba3p chimera protein.
Intracellular localization of GFP-Vba5p and GFP-Vba5(N)-Vba3p fusion protein were observed using fluorescence microscopy.

Next the effect of Vba5p on amino acid uptake by whole cells was examined. I could not detect any differences in the uptake activities of basic amino acids between wild-type and *vba5*Δ strain. It is probably due to the existence of other transporters including the unknown systems in *S. cerevisiae* cells. The uptake of arginine and lysine, but not of histidine, was significantly stimulated by overexpression of the *VBA5* gene (data not shown). The arginine uptake activity was determined by using *can1*Δ strain to suppress basal uptake activity of arginine in *S. cerevisiae* cells. As shown in Fig.2, arginine uptake activity was stimulated by overexpression of the *VBA5* gene when compared with the *can1*Δ (vehicle) cells. To confirm the arginine uptake by Vb5p, the role of Vba5p in the sensitivity to canavanine, an analog of arginine, was examined by spot assay. As shown in Fig.3, the growth of wild type cells was inhibited by 150 μg/mL canavanine. The inhibitory effect was attenuated by disruption of the *CAN1* gene. The overexpression of the *VBA5* gene slightly reduced the tolerance of *can1*Δ strain to canavanine. These results suggest that Vba5p is involved in arginine uptake across the plasma membrane in *S. cerevisiae* cells.

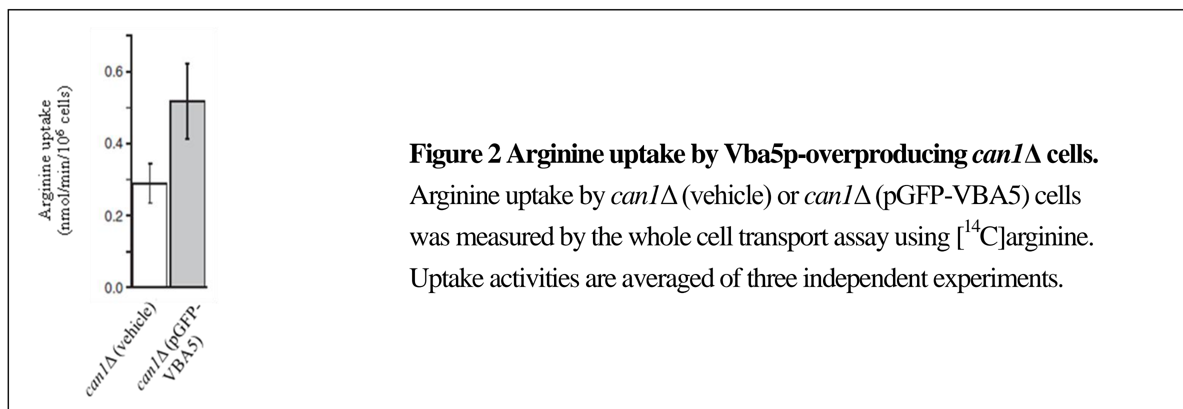

Figure 2 Arginine uptake by Vba5p-overproducing *can1*Δ cells.
Arginine uptake by *can1*Δ (vehicle) or *can1*Δ (pGFP-VBA5) cells was measured by the whole cell transport assay using [^{14}C]arginine. Uptake activities are averaged of three independent experiments.

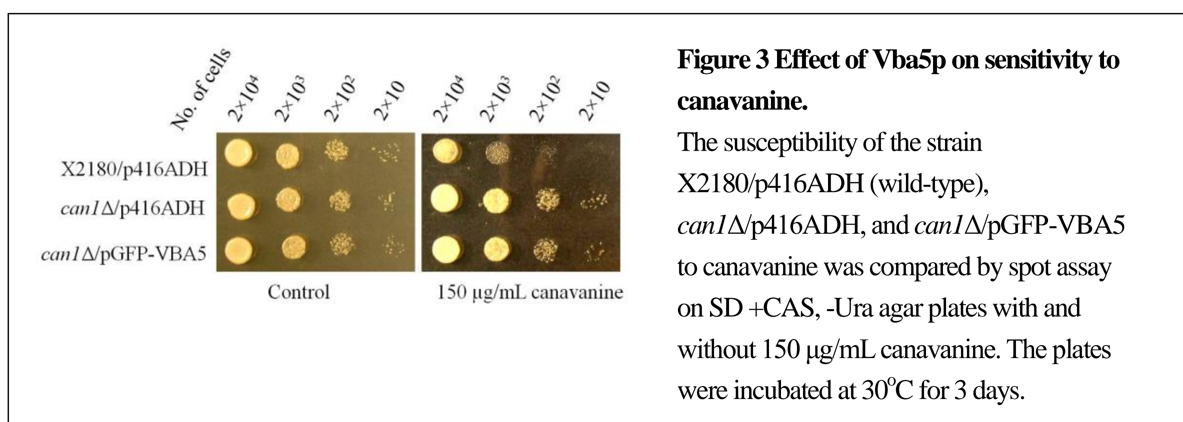

Figure 3 Effect of Vba5p on sensitivity to canavanine.
The susceptibility of the strain X2180/p416ADH (wild-type), *can1*Δ/p416ADH, and *can1*Δ/pGFP-VBA5 to canavanine was compared by spot assay on SD +CAS, -Ura agar plates with and without 150 μg/mL canavanine. The plates were incubated at 30°C for 3 days.

Two other genes in the VBA family, *SGE1* and *AZR1*, have been reported to be involved in resistance to cationic dyes, such as 10-*N*-nonyl acridine orange, and azoles, such as ketoconazole and fluconazole, respectively (8, 10). To examine the role of Vba5p in drug sensitivity, the susceptibility of BY4741 strain (wild-type), *vba5*Δ, and Vba5p-overproducing cells (pGFP-VBA5) was evaluated using 4-nitroquinoline *N*-oxide (4-NQO) (Fig.4). The wild-type and *vba5*Δ cells were almost equally tolerant to 4-NQO. The inhibitory effect of 4-NQO on cell growth was accelerated by overexpression of the *VBA5* gene. The addition of arginine improved the growth of these strains in the presence of 5 and 10 μM of 4-NQO, suggesting that Vba5p might be a site of competition of arginine with 4-NQO into cells.

All these results suggest that Vba5p is a plasma membrane protein involved in arginine uptake and drug sensitivity. The N-terminus extra sequence of Vba5p was important for its intracellular localization. To precisely evaluate the mechanism, substrate specificity and regulation of Vba5p, it is also necessary to investigate the role of five different amino acid residues in the transmembrane domain.

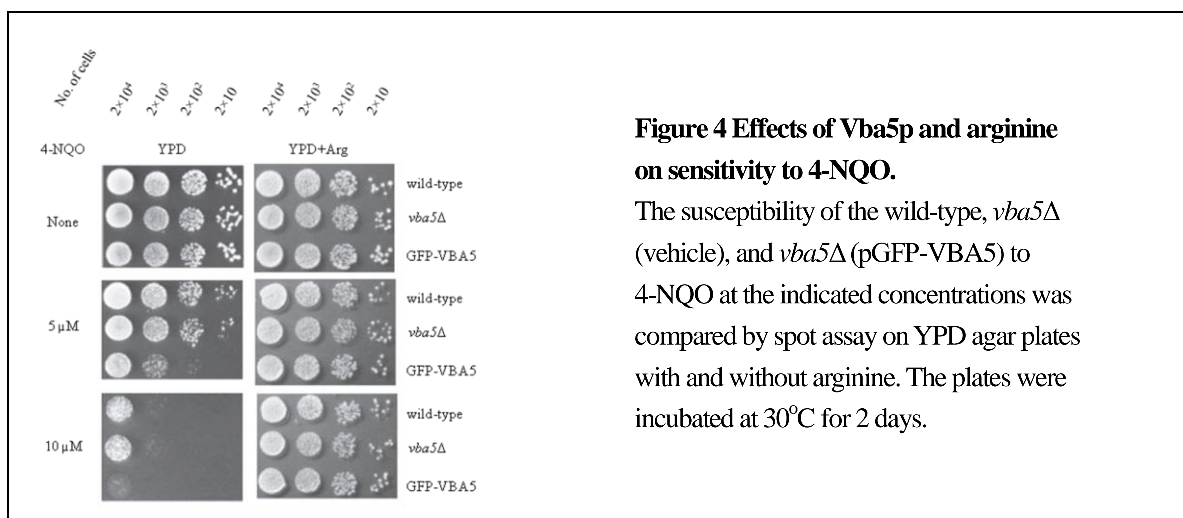

Figure 4 Effects of Vba5p and arginine on sensitivity to 4-NQO.
The susceptibility of the wild-type, *vba5*Δ (vehicle), and *vba5*Δ (pGFP-VBA5) to 4-NQO at the indicated concentrations was compared by spot assay on YPD agar plates with and without arginine. The plates were incubated at 30°C for 2 days.

In the fission yeast *Schizosaccharomyces pombe*, large amounts of basic amino acids are also compartmentalized in the vacuoles, suggesting the existence of transporter(s) for these amino acids on the vacuolar membrane, as in the *S. cerevisiae* cells (11). However, little is known about vacuolar amino acid transport in *S. pombe* at the molecular level, because a method for the purification of vacuoles from *S. pombe* cells has not been verified due to difficulty in the separation of small-sized vacuoles from other organelles, such as endosomes.

In the fission yeast *S. pombe*, Fnx1p, Fnx2p, and Vba2p, phylogenetically related to the Vba2p of *S. cerevisiae*, were reported to be involved in vacuolar amino acid uptake (6, 12, 13). These proteins were localized to the vacuolar membrane of *S. pombe* cells, and previously their roles in amino acid uptake were investigated by intact cells. Uptake activities for several amino acids, including lysine, histidine, and arginine, were impaired upon disruption of *S. pombe vba2*$^{+}$ (13). The *fnx1*$^{+}$ and *fnx2*$^{+}$ were involved in vacuolar amino acid uptake of asparagine, especially for isolecine and lysine (12). To characterize these *S. pombe* transporters biochemically and more precisely, it is important to establish an *in vitro* membrane vesicle assay system. Therefore, I expressed *S. pombe vba2*$^{+}$ in *S. cerevisiae* cells to examine transport activity of amino acids using isolated vacuolar membrane vesicles. *S. cerevisiae* Avt1p, Avt3p, and Avt4p are the vacuolar transporters critically involved in vacuolar compartmentalization of basic and neutral amino acids. Therefore, to minimize the background activity of vacuolar amino acid transport in *S. cerevisiae*, plasmid pGFP-VBA2 was introduced into the strain X2180 *avt1*Δ *avt3*Δ *avt4*Δ. GFP fused Vba2p specifically localized at the vacuolar membrane in *S. cerevisiae* cells, merged with the signal of red fluorescent probe FM4-64 (Fig.5).

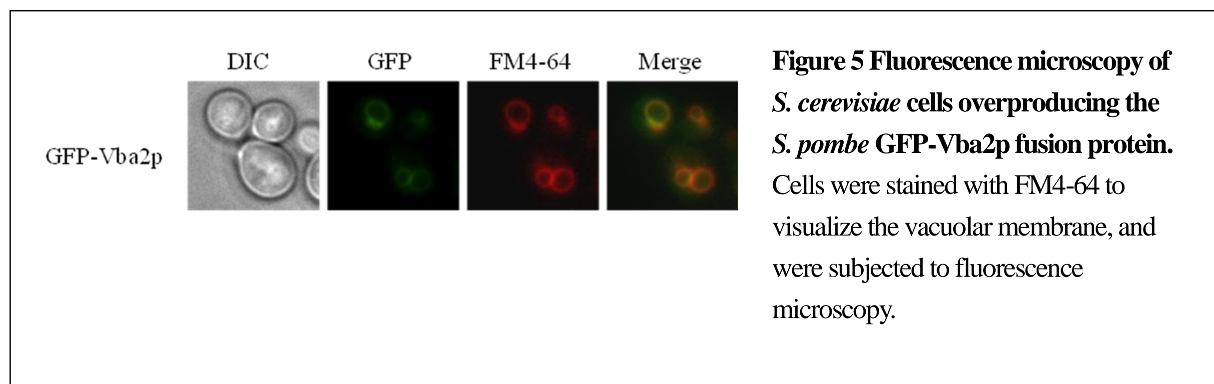

Figure 5 Fluorescence microscopy of *S. cerevisiae* cells overproducing the *S. pombe* GFP-Vba2p fusion protein. Cells were stained with FM4-64 to visualize the vacuolar membrane, and were subjected to fluorescence microscopy.

The effect of overproduction of *S. pombe* Vba2p on the vacuolar amino acid composition of the *S. cerevisiae* cells was examined. The vacuolar levels of lysine and arginine were increased by overexpression of *vba2*$^{+}$ gene, and that of histidine was slightly affected (data not shown). The amounts of neutral amino acids, alanine, threonine and glutamine, and acidic amino acids, glutamate were also increased by the expression of Vba2p. The amounts of amino acids in the whole cell fraction were not significantly affected by overproduction of Vba2p. These results suggest that Vba2p is involved in the uptake of amino acid into the vacuole, particularly in uptake of lysine and arginine.

Next I isolated vacuolar membrane vesicles from the strain X2180 *avt1*Δ*avt3*Δ*avt4*Δ overproducing *S. pombe* Vba2p, and examined transport activity. The initial rate and the accumulation level for lysine uptake by the vacuolar membrane vesicles were significantly increased by Vba2p overproduction (Fig.6). The uptake activity of arginine was also enhanced, but that of histidine was less affected. ATP-dependent uptake of lysine by Vba2p was inhibited almost completely by the addition of a specific V-ATPase inhibitor, concanamycin A, and of a protonophore CCCP (Fig.7), suggesting that the activities of *S. pombe* Vba2p was dependent on the proton electrochemical gradient across the vacuolar membrane. These results suggest that *S. pombe* Vba2p is a vacuolar proton-coupled importer, probably a proton/amino acid antiporter for lysine and arginine.

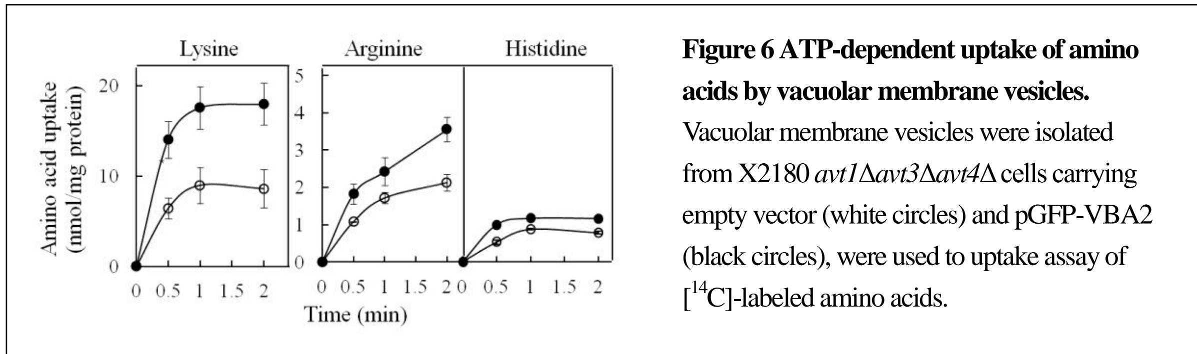

Figure 6 ATP-dependent uptake of amino acids by vacuolar membrane vesicles. Vacuolar membrane vesicles were isolated from X2180 *avt1*Δ*avt3*Δ*avt4*Δ cells carrying empty vector (white circles) and pGFP-VBA2 (black circles), were used to uptake assay of [^{14}C]-labeled amino acids.

The VBA transporters belong to the MFS transporters, and many drug/H^+ antiporters are included in the MFS. I also examined the susceptibility of *S. pombe* wild-type and *vba2*Δ cells to 4-NQO and quinidine. The growth of the wild-type *S. pombe* cells was strongly inhibited by addition of 0.5 µM 4-NQO (data not shown) and 2 mM quinidine, and the inhibitory effect was attenuated by disruption of the *vba2*$^+$ gene (Fig.8). The addition of 2 mM lysine improved the growth of both wild-type and *vba2*Δ strains on quinidine-containing media (Fig.8). The sensitivity to quinidine was slightly enhanced by overexpression of *S. pombe vba2*$^+$ gene in *S. cerevisiae* cells (data not shown), probably due to the existence of others drug transporters in *S. cerevisiae*. These results suggest that *S. pombe vba2*$^+$ was also responsible for the sensitivity to quinidine and 4-NQO.

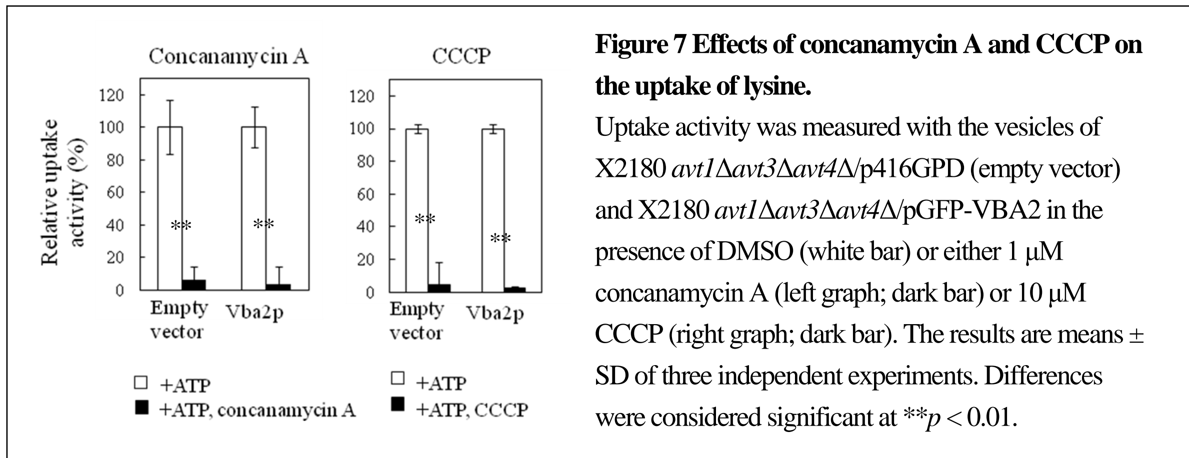

Figure 7 Effects of concanamycin A and CCCP on the uptake of lysine. Uptake activity was measured with the vesicles of X2180 *avt1*Δ*avt3*Δ*avt4*Δ/p416GPD (empty vector) and X2180 *avt1*Δ*avt3*Δ*avt4*Δ/pGFP-VBA2 in the presence of DMSO (white bar) or either 1 µM concanamycin A (left graph; dark bar) or 10 µM CCCP (right graph; dark bar). The results are means ± SD of three independent experiments. Differences were considered significant at $^{**}p < 0.01$.

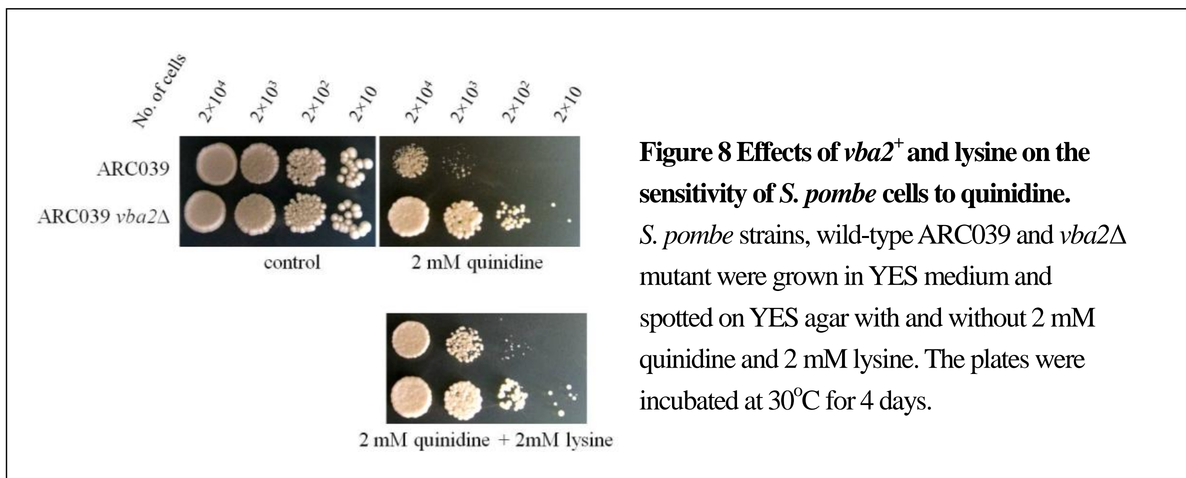

Figure 8 Effects of *vba2*$^+$ and lysine on the sensitivity of *S. pombe* cells to quinidine. *S. pombe* strains, wild-type ARC039 and *vba2*Δ mutant were grown in YES medium and spotted on YES agar with and without 2 mM quinidine and 2 mM lysine. The plates were incubated at 30°C for 4 days.

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These results suggest that *S. pombe* Vba2p is involved in uptake of basic amino acids into vacuoles and susceptibility to 4-NQO and quinidine. It is possible that *S. pombe* Vba2p mediates the uptake of quinidine into the vacuole, like lysine, and enhances the penetration of the quinidine into cells. Determination of the quinidine concentration in the cytosol and the vacuoles with and without lysine is required in further investigation.

This study is to characterize the VBA family transporters in *S. cerevisiae* and *S. pombe*. These transporters are considerable to be important not only for transport of basic amino acids, but also for drug sensitivity. Characterization of these transporters at the molecular level, especially with regard to its substrate specificity, multiplicity, and regulatory mechanism, should be required for further understanding of their physiological roles in yeast.

References

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