Full Paper

Vacuolar amino acid transporters upregulated by exogenous proline and involved in cellular localization of proline in *Saccharomyces cerevisiae*

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In the budding yeast *Saccharomyces cerevisiae*, the AVT genes (*AVT1*–*7*), which encode vacuolar amino acid transporters belonging to the amino acid vacuolar transport (AVT)-family, were significantly upregulated in response to exogenous proline. To reveal a novel role of the Avt proteins in proline homeostasis, we analyzed the effects of deletion or overexpression of the AVT genes on the subcellular distribution of amino acids after the addition of proline to the cells grown in minimal medium. Among seven AVT gene disruptants, *avt1Δ* and *avt7Δ* showed the lowest ratios of vacuolar proline. Consistently, overexpression of the *AVT1* gene specifically enhanced the vacuolar localization of proline. Since double disruption of the *AVT1* and *AVT7* genes did not completely abrogate vacuolar accumulation of proline, it is presumed that Avt1 has a dominant role, and Avt7 and other Avt proteins have redundant functions, in the localization of proline into the vacuolar lumen. In contrast, deletion of the *AVT3* gene increased vacuolar proline, although the highly expressed *AVT3* gene interfered with the accumulation of proline in the vacuole. Based on these results, it appears that Avt3 is the major protein involved in the export of proline from the vacuole. We also observed vacuolar membrane localization of GFP-fused Avt1, Avt3, and Avt7 proteins. Taken together, our data suggest that the AVT genes induced by exogenous proline are involved in the bidirectional transport of proline across the vacuolar membrane.

Key Words: amino acid localization; AVT family; proline; *Saccharomyces cerevisiae*; vacuolar transporter

Introduction

Proline serves multiple functions *in vitro*, including the stabilization of protein and membrane, the inhibition of dehydration and ice nucleation, the reduction of DNA melting temperature, and the scavenging of reactive oxygen species (Takagi, 2008). In earlier studies, we established an *in vivo* role of proline as a stress protectant in the budding yeast *Saccharomyces cerevisiae*. Yeast cells that accumulate proline were artificially constructed by disrupting the *PUT1* gene (*put1Δ*) encoding proline oxidase in the degradation pathway and by expressing the mutant *PRO1* gene (*PRO1*^{I150T} or *PRO1*^{D154N}) encoding the feedback inhibition-less sensitive γ -glutamate kinase to enhance the biosynthetic activity. The engineered strains which include industrial yeasts successfully showed enhanced tolerance toward various environmental stresses during fermentation processes (Kitagaki and Takagi, 2014; Takagi, 2008). Intriguingly, deletion of the *PEP3* gene that encodes a vacuolar membrane protein required for vacuolar biogenesis led to stress sensitivity of proline-accumulating cells (Matsuura and Takagi, 2005). These results suggest that a specific vacuolar function is involved in the stress-protective effects of proline. Thus, it is worth analyzing the vacuolar transport of proline and its related transporter(s) in *S. cerevisiae*.

As compared with well-studied plasma-membrane pro-

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line permeases Gap1, Gnp1, Agp1, and Put4 (Andréasson et al., 2004; Lasko and Brandriss, 1981), there are only a few reports of vacuolar proline transporters, although multiple vacuolar membrane proteins have been so far identified as amino acid transporters. Among seven vacuolar amino acid transporters, which belong to the amino acid vacuolar transport (AVT)-family, Avt1 imports several neutral amino acids including glutamine, asparagine, leucine, isoleucine, and tyrosine into the vacuole, whereas the closely related Avt3 and Avt4 proteins redundantly export them out of the vacuole (Russnak et al., 2001; Tone et al., 2015b). Avt6 mediates the extrusion of acidic amino acids, glutamate and aspartate (Russnak et al., 2001). More recently, Avt3, Avt4, and Avt7 were shown to mediate the efflux of proline under nitrogen starvation conditions (Sekito et al., 2014; Tone et al., 2015a). Although the proline import activity of the purified vacuolar membrane has not been detected (Ohsumi and Anraku, 1981; Sato et al., 1984; Tone et al., 2015b), the *in vitro* export of proline from the vacuole occurs in an ATP-dependent manner (Ishimoto et al., 2012). Atg22 mediates the efflux of leucine and other amino acids resulting from autophagic degradation, and also redundantly functions with Avt3 and Avt4 (Yang et al., 2006). In the homeostasis of cationic amino acids, the vacuolar basic amino acid transporter (VBA)-family member proteins Vba1, Vba2, and Vba3 (Shimazu et al., 2005) and the PQ-loop proteins Ypq1, Ypq2, and Ypq3 (Jézégou et al., 2012) are specifically involved in the import and export of basic amino acids, respectively. Since proline is one of the nonpreferred nitrogen sources, the analysis of intracellular localization of proline has been difficult under typical nutrient conditions. To find clues to novel mediators of proline homeostasis, we examined here the amino acid distribution in the presence of a high concentration of exogenous proline.

It would be reasonable if proline itself regulates the expression of the genes involved in proline-mediated cellular functions. The *PUT1* and *PUT2* genes, both of which encode the enzymes in the proline utilization pathway (proline oxidase and Δ^1 -pyrroline-5-carboxylate dehydrogenase, respectively), are upregulated by Put3, which is a member of the Zn_2Cys_6 binuclear cluster family of transcription factors (Siddiqui and Brandriss, 1989; Walters et al., 1997). Put3 is hyperphosphorylated and maximally activated in the absence of preferred nitrogen sources (e.g. ammonium) and in the presence of proline (Huang and Brandriss, 2000). Furthermore, Put3 was also reported to undergo conformational changes in response to proline *in vitro* (Des Etages et al., 2001). These indicate that proline, in part, directly induces the gene expressions and physiological changes related to proline metabolism. However, exogenous proline to minimal medium containing glutamate as a nitrogen source does not enhance the intracellular uptake of proline via a high-affinity proline permease Put4 and other known proline permeases on the plasma membrane (Lasko and Brandriss, 1981). Genome-wide analyses have revealed that the Put3-binding sites are identified in hundreds of genes (Harbison et al., 2004; Reimand et al., 2010), many of which are not directly involved in proline metabolism or utilization. Thus, neither the regulations of proline-related genes nor the global ef-

fects of exogenous proline on the gene expression are fully understood to date. To find clues to proline-induced mechanisms involved in intracellular amino acid homeostasis, we examined transcriptomic changes of *S. cerevisiae* cells upon the addition of proline to the growth environments.

Materials and Methods

Strains, plasmids, and culture media. *S. cerevisiae* strain MB329-17C (*MAT α trp1 ura3–52 put1–54*) was supplied by M. C. Brandriss (Wang and Brandriss, 1986). MB329-17C cells harboring plasmids pTV3 (2 μ *TRP1*) and pUV2 (2 μ *URA3*) (supplied by J. Nikawa; Rose and Broach, 1991) to complement the auxotrophic markers were used for DNA microarray analysis. Strain BY4741 (*MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0*) and its single deletion mutants (*avt1–7 Δ ::kanMX4*) were provided by EUROSCARF (Germany). The ORF of the *AVT1* gene in the *avt7 Δ ::kanMX4* strain was substituted by the *hphNT1* marker to construct the *avt1 Δ ::hphNT1 avt7 Δ ::kanMX4* double disruptant using a PCR-based method (Goldstein and McCusker, 1999) with primers AVT1-S1 (5'-GTA GAC TTA CGT ATT CTG TAT AAC TGA TTC CGA GAC GCA ACG TAC GCT GCA GGT CGA C-3') and AVT1-S2 (5'-GTA AAT GAA TTT TAA GTA GAG TAA GTA TGC CCC TCG TCG AAT CGA TGA ATT CGA GCT CG-3') and plasmid pFA6a-hphNT1 (Janke et al., 2004). Deletion of the *AVT1* gene was confirmed by PCR. If appropriate, the auxotrophy of each strain was complemented by introducing the empty vectors, pAD4 (2 μ *LEU2 P_{ADHI}*; supplied by J. Nikawa), pRS415-CgHIS3-MET15 (*CEN LEU2 CgHIS3 MET15*), and/or pRS416-CgHIS3-MET15 (*CEN URA3 CgHIS3 MET15*). *Escherichia coli* strains DH5 α and DB3.1 (Invitrogen) were used to construct vectors or subclone the yeast genes.

For overexpression of the *AVT1*, *AVT3*, or *AVT7* gene, we subcloned these genes into the vector pV208 (*CEN URA3 P_{tetO7}*) (Van Mullem et al., 2003) using Gateway cloning technology (Invitrogen). The ORFs of the *AVT1*, *AVT3*, and *AVT7* genes without stop codons flanked with attB1 and attB2 sites were cloned into pDONR221 (Invitrogen) via standard BP reactions. The cloned sequences were then transferred into pV208 by LR reactions. Correct insertion was confirmed by PCR. For the expression of the Avt1 protein fused with GFP at the amino termini, the two-step PCR method was adopted (Krawchuk and Wahls, 1999). In the first PCR, GFP-coding sequence without a stop codon was amplified with oligonucleotide primers HindIII-GFP-N-F (5'-AGC TAA GCT TAT GAG TAA AGG AGA AGA ACT TTT-3') and GFP-C-R (5'-CTA TTT GTA TAG TTC ATC CAT GCC A-3') and plasmid pFA6a-GFP-kanMX6 (Bähler et al., 1998) as a template. The *AVT1* gene was also amplified with primers GFP-C-AVT1 (5'-ACA TGG CAT GGA TGA ACT ATA CAA AAT GCC TGA GCA AGA ACC ATT-3') and SacI-AVT1-R (5'-AGC TGA GCT CTT ATG AAA TAA TTG CAG CAC-3'). In the second PCR, both PCR fragments were fused with primers HindIII-GFP-N-F and SacI-AVT1-R. This PCR product was digested with *HindIII* and *SacI*, and then ligated into the *HindIII*-*SacI* site of pAD4 to obtain the pAD4-GFP-AVT1 plasmid. Similarly, for the construction

of the pAD4-GFP-AVT3 plasmid, the GFP-coding sequence was amplified with oligonucleotide primers PstI-GFP-N-F (5'-AGC TCT GCA GAT GAG TAA AGG AGA AGA ACT TTT-3') and GFP-C-R from plasmid pFA6a-GFP-kanMX6. The *AVT3* gene was amplified with primers GFP-C-AVT3 (5'-ACA TGG CAT GGA TGA ACT ATA CAA AAT GAA TGG AAA AGA GGT TTC-3') and SacI-AVT3-R (5'-AGC TGA GCT CTT ACT GGC TCC ACA TTT TAA-3'). Both PCR fragments were fused with the primers PstI-GFP-N-F and SacI-AVT3-R. This PCR product was ligated into the *PstI*-*SacI* site of pAD4. For the construction of the pAD4-GFP-AVT7 plasmid, the GFP-coding sequence was amplified with oligonucleotide primers HindIII-GFP-N-F and GFP-C-start-AVT7-N-R (5'-ACG AAA GAG CAC TTG ATG TAG CCT CCA TTT TGT ATA GTT CAT CCA TGC CAT GT-3') from plasmid pFA6a-GFP-kanMX6. The *AVT7* gene was amplified with primers start-AVT7-N-ORF1-F (5'-ATG GAG GCT ACA TCA AGT GCT CT-3') and *AVT7*-stop-SacI-R (5'-AGC TGA GCT CTT ATT AGG CTT CAT CTG AAC GGT TGA-3'). Both PCR fragments were fused with the primers HindIII-GFP-N-F and *AVT7*-stop-SacI-R. This PCR product was ligated into the *HindIII*-*SacI* site of pAD4.

The media used for the growth of *S. cerevisiae* were a nutrient medium YPD (1% yeast extract, 2% peptone, and 2% glucose) and a synthetic defined minimal medium SD (0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories), 0.5% ammonium sulfate, and 2% glucose) with required amino acids or antibiotics, if necessary. *E. coli* cells were grown in Luria-Bertani (LB) medium containing 100 µg/mL ampicillin or 50 µg/mL kanamycin.

DNA microarray analysis. MB329-17C cells carrying pTV3 and pUV2 were inoculated to 200 ml of SD + 50 mM proline medium at an initial optical density at 600 nm (OD_{600}) of approximately 1. During the growth phase, aliquots of cells were taken for measuring OD_{600} , the quantification of the intracellular proline levels was determined with an amino acid analyzer (AminoTac JLC-500/V; JEOL), and the isolation of the total RNA for transcriptomic analysis was achieved by using the RNeasy Mini Kit (Qiagen). Subsequent cRNA preparation and microarray analysis were conducted by Bio Matrix Research (Chiba, Japan). Cy3-labeled cRNA was prepared with 500 ng of total RNA using the Quick Amp Labeling kit (Agilent). Fragmentation of labeled cRNA and hybridization onto the Yeast Oligo Microarray 4x44K (Agilent) were done using the Gene Expression Hybridization kit (Agilent) according to the manufacturer's instructions. The hybridized array was scanned with an Agilent Microarray Scanner (Agilent), and the data of the fluorescent intensities were extracted with Feature Extraction Software version 9.5.3.1 (Agilent).

Differential determination of the cytosolic and vacuolar amino acid pools. Yeast cells were precultured in 5 ml of SD medium at 30°C for 1 d by shaking in test tubes. Cells were inoculated to 50 ml of SD medium at an initial OD_{600} of 0.1. When the culture reached an OD_{600} of approximately 1 (about 10 h after inoculation), cells were harvested and adjusted to an OD_{600} of 1.0 in SD + 50 mM proline medium. After 2 h, early-log phase cells corre-

Fig. 1. Growth curve (A) and intracellular proline content (B) of strain MB329-17C (*put1-54*) after the addition of 50 mM proline to SD medium.

sponding to an OD_{600} of 20 were harvested and washed three times with distilled water. The cytosolic and vacuolar amino acid pools were extracted by a modified procedure of Ohsumi et al. (1988). In brief, cells were suspended in 0.6 ml of a 2.5 mM potassium phosphate buffer (pH 6.0) containing 0.6 M sorbitol, 10 mM glucose, and 0.2 mM copper(II) chloride. After incubation for 20 min at 30°C, the cell suspension was centrifuged and the cells were washed twice with 0.15 ml of the solution described above but without 0.2 mM copper(II) chloride. The combined supernatants (0.9 ml) were used as the cytosolic extract. The pellets were then suspended in 0.45 ml of distilled water and boiled for 20 min. After centrifugation (5 min at 14,000 × *g*), the supernatant was used as the vacuolar extract. All amino acids in the samples were quantified with an amino acid analyzer.

Fluorescent microscopy. GFP-Avt1, GFP-Avt3, and GFP-Avt7-overexpressing cells were grown to the exponential growth phase in SD medium at 25°C. Cells were subjected to 40 µM FM 4-64 (PromoCell) for 15 min to visualize the vacuolar membrane, harvested, washed with cold SD medium three times, incubated 30 min at 25°C, and concentrated about 10-fold by centrifugation. Cells were viewed immediately without fixation using a fluorescence microscope Axiovert 200M (Carl Zeiss). Images were captured with an HBO 100 Microscope Illuminating System (Carl Zeiss) digital camera.

Results and Discussion

Identification of AVT1 to AVT7 as novel proline-responsive genes

To explore novel genes that are involved in cellular responses to proline, we performed, in the presence of exogenous proline, a DNA microarray analysis of strain MB329-17C, which carries a loss-of-function mutation in the *PUT1* gene encoding proline oxidase (*put1-54*) and is deficient in proline degradation (Wang and Brandriss, 1986). As shown in Fig. 1, this strain exhibited exponential growth until 12 h after the addition of 50 mM proline to the minimal medium, and the intracellular proline level linearly increased for 9 h. This increase is likely to be associated with the transport activity of proline across the

Fig. 2. Transcriptional changes of the genes involved in proline biosynthesis, degradation, and intracellular uptake, as well as the AVT genes, in strain MB329-17C (*put1–54*) after the addition of 50 mM proline to SD medium.

The horizontal axis of each graph indicates the time (h) after the addition of proline. The vertical axis of each graph indicates the relative expression level of each gene that was normalized per chip (to the median signal of expressed genes within each sample).

plasma membrane. The well-defined proline-inducible genes, *PUT1* and *PUT2*, on the proline-degradation pathway (Brandriss and Magasanik, 1979; Siddiqui and Brandriss, 1989) were markedly upregulated for 5 h (Fig. 2). After 5 h, the *PUT1* mRNA level continued to be elevated, while the expression of *PUT2* was slightly repressed. It should be noted that the *put1–54* mutation site was not contained in the *PUT1* gene-specific probe on the microarray slide used in this study. Based on this result, we focused on transcriptional changes within 5 h after the addition of proline. Besides *PUT1* and *PUT2*, downstream genes including *GDH2* (Miller and Magasanik, 1991) and tricarboxylic acid (TCA) cycle genes were also upregulated for 5 h. The *GAP1*, *GNP1*, *AGP1*, and *PUT4* genes, which encode known proline permeases on the plasma membrane (Andréasson et al., 2004), also showed similar increases in transcription. In contrast, the *PRO1*, *PRO2*, and *CAR1* genes, which encode the upstream enzymes of the proline biosynthetic pathways (Brandriss and Magasanik, 1980; Tomenchok and Brandriss, 1987), were downregulated throughout the growth in the presence of proline. These results suggest that exogenous proline induces intracellular uptake and subsequent degradation of proline, and represses *de novo* biosynthesis of proline at the transcriptional level. Since the 5'-UTR regions of these genes, except *PUT1* and *PUT2*, do not contain the recognition site of the proline-dependent transcriptional acti-

vator Put3 (searched in the YEASTRACT database; <http://www.yeasttract.com/>), another unknown regulatory mechanism might mediate effective proline assimilation.

Does exogenous proline affect the expression of other genes? In this study, we focused on the AVT genes because they constitute a family of vacuolar transporters that mediate bidirectional transport of neutral amino acids (Rusnak et al., 2001), and, thus, these genes might also participate in the maintenance of intracellular proline homeostasis via its import and/or export across the vacuolar membrane. As we expected, the mRNA levels of all seven AVT genes were elevated by 2% (*AVT3*) to 37% (*AVT6*) after 1 h, and about 1.5-fold (*AVT1–AVT5*), 3-fold (*AVT7*), or 5-fold (*AVT6*) after 5 h (Fig. 2). These trends were also visualized by the scatter plots presented in Fig. 3A. The *p*-values obtained from the Wilcoxon rank sum test using the open-source R statistical analysis language indicate that the transcriptional changes of the AVT genes were significantly greater than those of all genes tested ($p < 10^{-15}$, Fig. 3B) even after a 1-h exposure to exogenous proline. Based on these results, the *AVT1* to *AVT7* genes were identified as novel proline-responsive genes. Unlike the AVT genes, other vacuolar transporter genes, such as the *VBA* and *YPQ* genes, were not commonly induced by proline (data not shown). Since the 5'-UTR regions of the AVT genes do not contain any typical Put3-recognition sites (searched in the YEASTRACT database; [http://](http://www.yeasttract.com/)

Fig. 3. Transcriptional changes of the AVT genes in strain MB329-17C (*put1–54*) after the addition of 50 mM proline to the minimal medium.

(A) Scatter plots of the expression levels of all the genes tested at 0 h versus 1 h (left) and 5 h (right). Light gray diamond symbols indicate the data of the AVT genes, and numbers correspond to *X* of the AVTX gene names. All the data were normalized per chip (to the median signal of expressed genes within each sample). (B) Box plots of the relative expression levels of the AVT genes and all the genes tested at 1 h (left) or at 5 h (right) standardized by the values at 0 h. An asterisk (*) indicates a significant increase from all genes ($p < 10^{-15}$ by the Wilcoxon rank sum test).

www.yeasttract.com/), the *cis* and *trans* elements responsible for the proline-dependent induction of the AVT genes should be further explored in future studies.

Effects of *Avt1* to *Avt7* on the subcellular distribution of proline

To reveal the physiological roles of the AVT-family vacuolar transporters in the presence of exogenous proline, we examined the effects of the AVT genes on the vacuolar localization of proline. As shown in Fig. 4, the intracellular proline contents of the wild-type strain BY4741 drastically increased 2 h after the addition of proline to the culture in the minimal SD medium, whereas other amino acids, such as glutamate (exclusively localized in the cytosol) and arginine (accumulated in the vacuole) (Kitamoto et al., 1988), were not affected. In our preliminary experiments, it should be noted that the percentage of vacuolar proline content was the highest between 1 to 3 h after the addition of proline, and decreased thereafter. This increase was more prominent in the vacuolar fraction (27-fold) than in the cytosolic fraction (11-fold), and, thus, the percentage of proline in the vacuolar fraction was significantly

Fig. 4. Cytosolic and vacuolar amino acid contents of strain BY4741 (wild-type) before, and 2 h after, the addition of 50 mM proline to SD medium.

The percentage indicates the ratios of amino acid contents in the vacuolar fraction. The values are the means and standard deviations of the results from three independent experiments. An asterisk (*) indicates a significant increase from the control ($p < 0.01$ by Student's *t* test).

Fig. 5. The ratios of amino acid contents in the vacuolar fraction of the *AVT* gene disruptant cells 2 h after the addition of 50 mM proline to SD medium.

The values are the means and standard deviations of the results from three independent experiments. Asterisks (*) and **) indicate a significant increase and decrease, respectively, from strain BY4741 (WT) ($p < 0.01$ by Student's t test). NS, not significant.

Fig. 6. The ratios of amino acid contents in the vacuolar fraction of the *AVT* gene-overexpressing cells 2 h after the addition of 50 mM proline to the minimal medium.

The values are the means and standard deviations of the results from three independent experiments. Asterisks (*) and **) indicate a significant increase and decrease, respectively, from strain BY4741 (+vector) ($p < 0.05$ by Student's t test).

elevated (14% to 29%). This may indicate that a larger part of exogenously added proline was accumulated into the vacuole than in the cytosol, providing evidence for the active proline import system on the vacuolar membrane. Next, we performed the same experiments using the *AVT* gene-deleted strains, and found that the loss of the *Avt1* or *Avt7* proteins decreased the ratio of vacuolar proline (Fig. 5). Deletion of both the *AVT1* and *AVT7* genes did not give any apparent additive effect on the amino acid localization. In contrast, the *avt3*Δ strain exhibited an enhanced vacuolar localization of proline. Single deletion of *AVT2*, *AVT4*, *AVT5*, or *AVT6* did not clearly alter the distribution of proline. None of the *AVT* genes affected the cytosolic and vacuolar localization of glutamate and arginine, respectively. Furthermore, when the *AVT1* gene was overexpressed, proline localization into the vacuole was specifically enhanced, although the overexpression of the *AVT3* gene decreased vacuolar proline (Fig. 6).

Fig. 7. Subcellular localization of the GFP-tagged *Avt1*, *Avt3*, and *Avt7* proteins.

GFP-fluorescent signals (GFP), vacuolar membrane (FM 4–64), and differential interference contrast images of cellular morphology (DIC) are shown. Bar, 2 μ m.

Unexpectedly, highly expressed *AVT7* did not increase the vacuolar localization of proline, but rather decreased the ratio of vacuolar proline, as well as those of glutamate and arginine. Consistent with previous studies (Chardwiriyaapreecha et al., 2015; Russnak et al., 2001; Tone et al., 2015a, b), our fluorescent microscopic observations confirmed that GFP-fused *Avt1*, *Avt3*, and *Avt7* expressed under the control of a constitutive *ADHI* promoter were mainly located on the vacuolar membrane, as well as in the endoplasmic reticulum (ER; cortical ER and nuclear envelope) to a lesser extent, in SD medium (Fig. 7). Their localization at the vacuolar membrane was maintained in SD + 50 mM proline medium (data not shown).

All these results suggest that *Avt1* and *Avt3* directly, or at least indirectly, mediate the import and export of proline across the vacuolar membrane, respectively. The data

of *avt7Δ* suggest that Avt7 is also necessary for the effective vacuolar localization of proline. The nonspecific decreases in amino acid levels in the vacuolar fraction observed in *AVT7*-overexpressing cells might be explained as artifacts caused by a reduction in the size of the vacuoles, or by perturbation of the vacuolar storage functions. Taken together, it appears that both vacuolar proteins involved in the import and export of proline are concurrently active in the presence of exogenous proline. The physiological significance and regulatory mechanisms of dynamic intracellular proline transport are of great interest.

Based on our results, it appears that Avt1 plays a pivotal role in the vacuolar localization of proline (Figs. 5 and 6), as well as of other large neutral amino acids, such as glutamine, asparagine, leucine, isoleucine, and tyrosine (Russnak et al., 2001; Tone et al., 2015b). We previously reported that the vacuolar function is essential for maintaining cell viability under stress conditions in a proline-accumulating mutant (Matsuura and Takagi, 2005). Thus, when cells are exposed to high concentrations of exogenous proline and/or when cells cannot degrade proline, Avt1-mediated sequestration of proline into the vacuole might play physiologically important roles. A recent report on the extension of replicative lifespan via Avt1 (Hughes and Gottschling, 2012) was shown to spotlight a general significance of vacuolar amino acid uptake. Although previous reports have not detected the *in vitro* ATP-dependent import activity at the vacuolar membrane (Ohsumi and Anraku, 1981; Sato et al., 1984; Tone et al., 2015b), our results showed that proline is significantly accumulated in the vacuole *in vivo* at least in the presence of exogenous proline (Fig. 4). These data suggest that the *in vitro* proline uptake activity of the vacuolar membrane is very low because unidentified essential factors in the cytosol might be eliminated during the isolation of the vacuolar membrane.

Proline accumulated in the vacuole might be returned to the cytosol via Avt3 to meet metabolic or other cellular demands. Avt3 appears to be the only major member involved in the efflux of proline from the vacuole in response to the addition of proline. In a previous report, Avt3 and another member Avt4, which share about a 40% amino acid sequence identity, act synergistically in the export of several neutral amino acids (Russnak et al., 2001). These two *AVT* proteins were also reported to be involved in the excretion of proline under nitrogen starvation (Sekito et al., 2014). In contrast, we showed that Avt3 has a dominant role in vacuolar proline efflux in response to exogenous proline (Figs. 5 and 6), indicating a preference of Avt3 and Avt4 for amino acid substrates. Since the *in vitro* ATP-dependent activity of ¹⁴C-labelled proline efflux at the vacuolar membrane has been already detected (Ishimoto et al., 2012), it should be further determined whether Avt3 is fully responsible for this activity. Our result does not conflict with a finding in *Schizosaccharomyces pombe* that the SpAvt3 protein, an ortholog of Avt3 and Avt4, mediates the vacuolar export of proline and other various amino acids (Chardwiriapreecha et al., 2015).

Although the target amino acids of Avt7 have been un-

identified, this study provides evidence of a possible function of Avt7 as a proline transporter into the vacuolar lumen in the presence of exogenous proline (Fig. 5). Avt1 and Avt7 are, however, not sufficient to explain the full activity of the vacuolar proline importer, and, thus, other Avt proteins and/or other vacuolar transporters might have redundant functions with Avt1 and Avt7 in the vacuolar distribution of proline. Interestingly, an earlier study revealed that Avt7 mediates the efflux of proline and glutamine from the vacuole under nitrogen-starved conditions (Tone et al., 2015a). Taken together, Avt7 might have a bidirectional transport activity of proline across the vacuolar membrane, which can be supported by the fact that both deletion and overexpression of the *AVT7* gene decreased vacuolar-localized proline (Figs. 5 and 6). The direction of proline transport by Avt7 in response to intracellular and extracellular cues is one of the important issues to be addressed.

In terms of biotechnological applications, proline is expected to contribute to yeast-based industries by improving the production of frozen dough and alcoholic beverages, or breakthroughs in bioethanol production (Kitagaki and Takagi, 2014; Takagi, 2008). We have reported that proline is toxic to cells only when accumulated in the cytosol and that the vacuolar function is involved in the protective effect of proline on ethanol stress (Matsuura and Takagi, 2005). Excess proline might also delay cell growth in the presence of ethanol (Takagi et al., 2007). These results suggest that an appropriate proline level and localization in yeast cells is important for its stress-protective effect.

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References

- Andréasson, C., Neve, E. P., and Ljungdahl, P. O. (2004) Four permeases import proline and the toxic proline analogue azetidine-2-carboxylate into yeast. *Yeast*, **21**, 193–199.
- Bähler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., 3rd, et al. (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast*, **14**, 943–951.
- Brandriss, M. C. and Magasanik, B. (1979) Genetics and physiology of proline utilization in *Saccharomyces cerevisiae*: mutation causing constitutive enzyme expression. *J. Bacteriol.*, **140**, 504–507.
- Brandriss, M. C. and Magasanik, B. (1980) Proline: an essential intermediate in arginine degradation in *Saccharomyces cerevisiae*. *J. Bacteriol.*, **143**, 1403–1410.
- Chardwiriapreecha, S., Manabe, K., Iwaki, T., Kawano-Kawada, M., Sekito, T. et al. (2015) Functional expression and characterization of *Schizosaccharomyces pombe* Avt3p as a vacuolar amino acid exporter in *Saccharomyces cerevisiae*. *PLoS One*, **10**, e0130542.
- Des Etages, S. A., Saxena, D., Huang, H. L., Falvey, D. A., Barber, D. et al. (2001) Conformational changes play a role in regulating the

- activity of the proline utilization pathway-specific regulator in *Saccharomyces cerevisiae*. *Mol. Microbiol.*, **40**, 890–899.
- Goldstein, A. L. and McCusker, J. H. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast*, **15**, 1541–1553.
- Harbison, C. T., Gordon, D. B., Lee, T. I., Rinaldi, N. J., Macisaac, K. D. et al. (2004) Transcriptional regulatory code of a eukaryotic genome. *Nature*, **431**, 99–104.
- Huang, H. L. and Brandriss, M. C. (2000) The regulator of the yeast proline utilization pathway is differentially phosphorylated in response to the quality of the nitrogen source. *Mol. Cell. Biol.*, **20**, 892–899.
- Hughes, A. L. and Gottschling, D. E. (2012) An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast. *Nature*, **492**, 261–265.
- Ishimoto, M., Sugimoto, N., Sekito, T., Kawano-Kawada, M., and Kakinuma, Y. (2012) ATP-dependent export of neutral amino acids by vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem.*, **76**, 1802–1804.
- Janke, C., Magiera, M. M., Rathfelder, N., Taxis, C., Reber, S. et al. (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast*, **21**, 947–962.
- Jézégou, A., Llinares, E., Anne, C., Kieffer-Jaquinod, S., O'Regan, S. et al. (2012) Heptahelical protein PQLC2 is a lysosomal cationic amino acid exporter underlying the action of cysteamine in cystinosis therapy. *Proc. Natl. Acad. Sci. USA*, **109**, E3434–E3443.
- Kitagaki, H. and Takagi, H. (2014) Mitochondrial metabolism and stress response of yeast: Applications in fermentation technologies. *J. Biosci. Bioeng.*, **117**, 383–393.
- Kitamoto, K., Yoshizawa, K., Ohsumi, Y., and Anraku, Y. (1988) Dynamic aspects of vacuolar and cytosolic amino acid pools of *Saccharomyces cerevisiae*. *J. Bacteriol.*, **170**, 2683–2686.
- Krawchuk, M. D. and Wahls, W. P. (1999) High-efficiency gene targeting in *Schizosaccharomyces pombe* using a modular, PCR-based approach with long tracts of flanking homology. *Yeast*, **15**, 1419–1427.
- Lasko, P. F. and Brandriss, M. C. (1981) Proline transport in *Saccharomyces cerevisiae*. *J. Bacteriol.*, **148**, 241–247.
- Matsuura, K. and Takagi, H. (2005) Vacuolar functions are involved in stress-protective effect of intracellular proline in *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.*, **100**, 538–544.
- Miller, S. M. and Magasanik, B. (1991) Role of the complex upstream region of the *GDH2* gene in nitrogen regulation of the NAD-linked glutamate dehydrogenase in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **11**, 6229–6247.
- Ohsumi, Y. and Anraku, Y. (1981) Active transport of basic amino acids driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **256**, 2079–2082.
- Ohsumi, Y., Kitamoto, K., and Anraku, Y. (1988) Changes induced in the permeability barrier of the yeast plasma membrane by cupric ion. *J. Bacteriol.*, **170**, 2676–2682.
- Reimand, J., Vaquerizas, J. M., Todd, A. E., Vilo, J., and Luscombe, N. M. (2010) Comprehensive reanalysis of transcription factor knockout expression data in *Saccharomyces cerevisiae* reveals many new targets. *Nucleic Acids Res.*, **38**, 4768–4777.
- Rose, M. D. and Broach, J. R. (1991) Cloning genes by complementation in yeast. *Methods Enzymol.*, **194**, 195–230.
- Russnak, R., Konczal, D., and McIntire, S. L. (2001) A family of yeast proteins mediating bidirectional vacuolar amino bidirectional vacuolar amino acid transport. *J. Biol. Chem.*, **276**, 23849–23857.
- Sato, T., Ohsumi, Y., and Anraku, Y. (1984) Substrate specificities of active transport systems for amino acids in vacuolar-membrane vesicles of *Saccharomyces cerevisiae*. Evidence of seven independent proton/amino acid antiport systems. *J. Biol. Chem.*, **259**, 11505–11508.
- Sekito, T., Chardwiriyaapreecha, S., Sugimoto, N., Ishimoto, M., Kawano-Kawada, M. et al. (2014) Vacuolar transporter Avt4 is involved in excretion of basic amino acids from the vacuoles of *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem.*, **78**, 969–975.
- Shimazu, M., Sekito, T., Akiyama, K., Ohsumi, Y., and Kakinuma, Y. (2005) A family of basic amino acid transporters of the vacuolar membrane from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **280**, 4851–4857.
- Siddiqui, A. H. and Brandriss, M. C. (1989) The *Saccharomyces cerevisiae* *PUT3* activator protein associates with proline-specific upstream activation sequences. *Mol. Cell. Biol.*, **9**, 4706–4712.
- Takagi, H. (2008) Proline as a stress protectant in yeast: physiological functions, metabolic regulations and biotechnological applications. *Appl. Microbiol. Biotechnol.*, **81**, 211–223.
- Takagi, H., Matsui, F., Kawaguchi, A., Wu, H., Shimoi, H. et al. (2007) Construction and analysis of self-cloning sake yeasts that accumulate proline. *J. Biosci. Bioeng.*, **103**, 377–380.
- Tomenchok, D. M. and Brandriss, M. C. (1987) Gene-enzyme relationships in the proline biosynthetic pathway of *Saccharomyces cerevisiae*. *J. Bacteriol.*, **169**, 5364–5372.
- Tone, J., Yamanaka, A., Manabe, K., Murao, N., Kawano-Kawada, M. et al. (2015a) A vacuolar membrane protein Avt7p is involved in transport of amino acid and spore formation in *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem.*, **79**, 190–195.
- Tone, J., Yoshimura, A., Manabe, K., Murao, N., Sekito, T. et al. (2015b) Characterization of Avt1p as a vacuolar proton/amino acid antiporter in *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem.*, **79**, 782–789.
- Van Mullem, V., Wery, M., De Bolle, X., and Vandenhaute, J. (2003) Construction of a set of *Saccharomyces cerevisiae* vectors designed for recombinational cloning. *Yeast*, **20**, 739–746.
- Walters, K. J., Dayie, K. T., Reece, R. J., Ptashne, M., and Wagner, G. (1997) Structure and mobility of the *PUT3* dimer. *Nat. Struct. Biol.*, **4**, 744–750.
- Wang, S. S. and Brandriss, M. C. (1986) Proline utilization in *Saccharomyces cerevisiae*: analysis of the cloned *PUT1* gene. *Mol. Cell. Biol.*, **6**, 2638–2645.
- Yang, Z., Huang, J., Geng, J., Nair, U., and Klionsky, D. J. (2006) Atg22 recycles amino acids to link the degradative and recycling functions of autophagy. *Mol. Biol. Cell*, **17**, 5094–5104.