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Pka1 contributes to the canavanine response and controls E3 ubiquitin ligase Pub1 localization

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Abstract

Background

The *Schizosaccharomyces pombe* Pka1 pathway is a major glucose response signaling pathway. Although Pka1 is involved in multiple cellular processes, its role in the response to canavanine, a structural analog of arginine, remains unclear. In this study, we investigated the role of Pka1 in canavanine tolerance and its relationship with amino acid transporters and the Any1-Pub1 regulatory system.

Results

Loss of Pka1 function increases sensitivity to canavanine. The *pka1Δ* strain exhibited reduced intracellular canavanine accumulation and abnormal Cat1 and Aat1 localization, indicating that increased canavanine uptake is not responsible for the canavanine-sensitive phenotype. We found that overexpression of Any1, an adaptor protein of Pub1, rescued the growth defect of the *pka1Δ* strain with canavanine. Pub1, an E3 ubiquitin ligase, and functional Any1 were required for suppression of the *pka1Δ* phenotype by Any1 overexpression. Canavanine sensitivity of *pka1Δ* was enhanced by deletion of *any1* or *pub1*, indicating that Pka1 and the Any1-Pub1 regulatory system contribute in parallel to canavanine tolerance. Golgi localization of Pub1 was decreased in the *pka1Δ* strain, whereas Pub1 expression, Any1 localization, and Any1 expression were unchanged. Any1 overexpression did not rescue reduced canavanine accumulation or abnormal Cat1 and Aat1 localization, whereas it restored Pub1 localization.

Conclusions

Our findings demonstrate that Pka1 contributes to canavanine tolerance through mechanisms that cannot be explained solely by the regulation of canavanine uptake. They further reveal a role for Pka1 in the regulation of Pub1 localization, providing new insight into the cellular response to canavanine in fission yeast.

Introduction

Canavanine is a structural analog of arginine and becomes toxic after being taken up by cells [1]. Because canavanine is not synthesized intracellularly, it has been widely used as a tool to analyze amino acid uptake from the extracellular environment and the regulation of amino acid transport [2]. In the fission yeast *Schizosaccharomyces pombe*, canavanine uptake and the cellular responses to canavanine are regulated by the plasma membrane arginine/lysine transporter Cat1 [3–5], the plasma membrane amino acid transporter Aat1 [6], the arrestin-family adaptor Any1 [4–6], and the HECT-type ubiquitin E3 ligase Pub1 [4, 6]. Any1 forms a complex with Pub1, and this Any1-Pub1 complex regulates the plasma membrane localization of Cat1 and Aat1 [4, 6]. Deletion of *cat1* confers canavanine tolerance [3], whereas deletion of *any1* or *pub1* results in canavanine sensitivity [4, 5, 7]. Any1 mutants, including K263R, PWGW (P116W and G117W), and PFPY/4A (P348A, F350A, P358A, and Y360A), also exhibit canavanine sensitivity. However, overexpression of Any1 and the Any1 (R175C) mutant (also known as *can1-1*) confer canavanine tolerance [4, 8]. In canavanine-tolerant cells, Cat1 predominantly localizes to the Golgi apparatus, whereas in canavanine-sensitive cells, Cat1 localizes to the plasma membrane. Thus, the cellular response to canavanine depends on the function of Any1, Pub1, and Cat1.

The cAMP-dependent protein kinase (PKA) pathway is known as the glucose-response pathway in *S. pombe*. Under glucose starvation, the PKA pathway is inactivated [9, 10]. Extracellular glucose is sensed by the G protein-coupled receptor Git3 and transmitted through the heterotrimeric G protein composed of Gpa2 (G α), Git5 (G β), and Git11 (G γ). When glucose is abundant, Gpa2 activates the adenylate cyclase Cyr1, which synthesizes cAMP from ATP [11]. In the inactivated state, two cAMP-dependent protein kinase regulatory subunits (Cgs1) associate with two protein kinase A catalytic subunits (Pka1) to form a heterotetrameric complex [12]. When cAMP binds to Cgs1, Pka1 is released and activated [13–16]. The PKA pathway also plays a role in the salt stress

response [16–19], maintenance of microtubule structure [20], and the regulation of proper chromosome segregation [21–23].

In this study, we found that the *pka1Δ* strain exhibits canavanine sensitivity despite reduced intracellular canavanine accumulation. We further showed that the localization of Cat1, Aat1, and Pub1 is altered in *pka1Δ* cells. Overexpression of Any1 partially restored Pub1 localization and suppressed the canavanine-sensitive phenotype of the *pka1Δ* strain. These findings indicate that Pka1 contributes to the regulation of the intracellular localization of proteins involved in amino acid transport and that altered Pub1 localization contributes, at least in part, to canavanine sensitivity in *pka1Δ* cells.

Materials and methods

Yeast strains, media, and genetic methods

The *S. pombe* strains used in this study are listed in Supplementary Table S1. Standard yeast culture media and genetic techniques were used [24, 25]. Cells were grown in YES medium (0.5% yeast extract, 3% glucose, and 225 mg/L each of adenine, uracil, leucine, histidine, and lysine) or Edinburgh Minimal Medium (EMM) supplemented with the appropriate auxotrophic requirements [24].

Construction of knockouts and GFP-tagging strains

The oligonucleotide primers used for strain and plasmid construction are listed in Supplementary Table S2. Recombinant polymerase chain reaction (PCR) [26] was used to construct YMP860 (*any1Δ*), YMP878 (*any1-GFP*), YMP923 (*cat1-GFP*), AKP1 (*aat1-GFP*), and AKP5 (*aat1Δ*). PR109 was used as the parent strain. Correct integration was verified by colony PCR [27].

The strains *cgs1Δ cat1-GFP-FLAG* (YMP935), *pka1Δ cat1-GFP-FLAG* (YMP938), *cgs1Δ aat1-GFP-FLAG* (AKP2), *pka1Δ aat1-GFP-FLAG* (AKP4), *cat1Δ* (YMP1012), *pka1Δ cat1Δ* (YMP1014), *pka1Δ aat1Δ* (AKP6), *pka1Δ pub1Δ* (YMP887 and YMP890), *pka1Δ*

any1 Δ (YMP883), *cgs1* Δ *any1-GFP* (YMP891), *pka1* Δ *any1-GFP* (YMP884), *pub1-GFP* (YMP908), *cgs1* Δ *pub1-GFP* (YMP909), and *pka1* Δ *pub1-GFP* (YMP910) were generated by genetic crossing followed by random spore analysis [28].

Plasmid construction and induction of expression under the *nmt1* promoter

To construct pREP3X-Any1, primers ANY1-SF and ANY1-BR (Supplementary Table S2) were used to amplify a 1,086 bp fragment containing the *any1* coding sequence from genomic DNA. The amplified fragment was digested with *Sall* and *Bam*HI and cloned into pREP3X [29]. To generate pREP3X-Any1 (PWGW), pREP3X-Any1 (K263R), and pREP3X-Any1 (PY Δ), pREP3X-Any1 was used as the template with primers listed in Supplementary Table S2. To construct pREP41X-pub1, pREP1-pub1 (FYP1468) was digested with *Nde*I and *Sma*I and cloned into pREP41X [29].

Wild-type and mutant cells were transformed with the indicated pREP-derived plasmids (*LEU2* marker), and transformants were selected on the appropriate EMM medium supplemented with 15 μ M thiamine to repress expression from the *nmt1* promoter. For induction of expression, cells were first grown on EMMU with 15 μ M thiamine for 2 days at 30°C, then transferred to EMMU lacking thiamine, and incubated for 1 day at 30°C. Cells were then spotted onto EMMU plates with or without 15 μ M thiamine and incubated for 5-6 days at 30°C.

Measurement of intracellular canavanine levels

Cells were grown in EMMLU liquid medium to mid-log phase at 30°C. For the experiment shown in Fig. 1C, cells were resuspended in EMMLU containing 20 μ M canavanine and incubated at 30°C for 24 h. For experiments involving Any1 overexpression, cells were treated as described in the corresponding figure legend. Cells were harvested by centrifugation, washed three times with water, and resuspended in 300 μ L of water. Samples were boiled at 100°C for 15 min with brief vortexing every 5 min. After centrifugation for 3 min, the supernatants were collected. Intracellular canavanine

concentrations were determined using an amino acid analyzer (LaChrom Elite; Hitachi High-Technologies Corp.).

Fluorescence microscopy of GFP fusion proteins

S. pombe cells were grown in EMM liquid medium to mid-log phase at 30°C. Cells were resuspended in the indicated medium with or without 20 µM canavanine and incubated as described in the corresponding figure legends. GFP-tagged Cat1, Aat1, Any1, and Pub1 were visualized in living cells. Images were acquired using a BX51 microscope (Olympus) equipped with a DP74 digital camera (Olympus). Approximately 300 cells were analyzed for each experiment.

Preparation of cell lysates and immunoblotting

S. pombe cell lysates were prepared as described previously [27]. Lysate proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blot analysis using the Western Lightning enhanced chemiluminescence (ECL) Pro detection system (PerkinElmer) according to the manufacturer's instructions. Primary and secondary antibodies were used at 1:1,000 dilution: mouse monoclonal anti-GFP (Roche Life Science), mouse monoclonal anti-FLAG (M2; Sigma-Aldrich Merck), mouse monoclonal anti-HA (F-7; Santa Cruz Biotechnology), and mouse monoclonal anti-Cdc2 (Y100.4; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology) was used as the secondary antibody.

Statistical analysis

Statistical analyses were performed using EZR (Jichi Medical University, Saitama, Japan), a graphical user interface for R. Comparisons between two groups were performed using a two-tailed Student's *t*-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple-

comparisons test. For Dunnett's test, the wild-type strain under normal conditions was used as the control group unless otherwise indicated. $P < 0.05$ was considered statistically significant.

Results

Loss of Pka1 function causes canavanine sensitivity

To investigate additional functions of the cAMP/PKA pathway in *S. pombe*, we examined the sensitivity of mutants lacking components of the pathway to various stresses. Among the mutants examined, *git3* Δ , *gpa2* Δ , *cyr1* Δ , and *pka1* Δ cells were sensitive to 30 μ M canavanine, whereas the wild-type and *cgs1* Δ cells grew well (Fig. 1A). To determine whether Pka1 activity influences canavanine sensitivity, we examined the *cyr1* Δ *cgs1* Δ strain. Since Cgs1 functions downstream of Cyr1 and negatively regulates Pka1 activity, deletion of *cgs1* results in constitutive activation of Pka1. Deletion of *cgs1* rescued the canavanine-sensitive phenotype of the *cyr1* Δ strain (Fig. 1B). Because canavanine enters cells through arginine transporters, we next measured intracellular canavanine concentration in wild-type and *pka1* Δ cells. Unexpectedly, intracellular canavanine concentrations were reduced in *pka1* Δ cells despite increased canavanine sensitivity (Fig. 1C). These results indicate that loss of Pka1 function causes canavanine sensitivity despite reduced intracellular canavanine accumulation, suggesting that mechanisms other than increased canavanine uptake contribute to the canavanine-sensitive phenotype of *pka1* Δ cells.

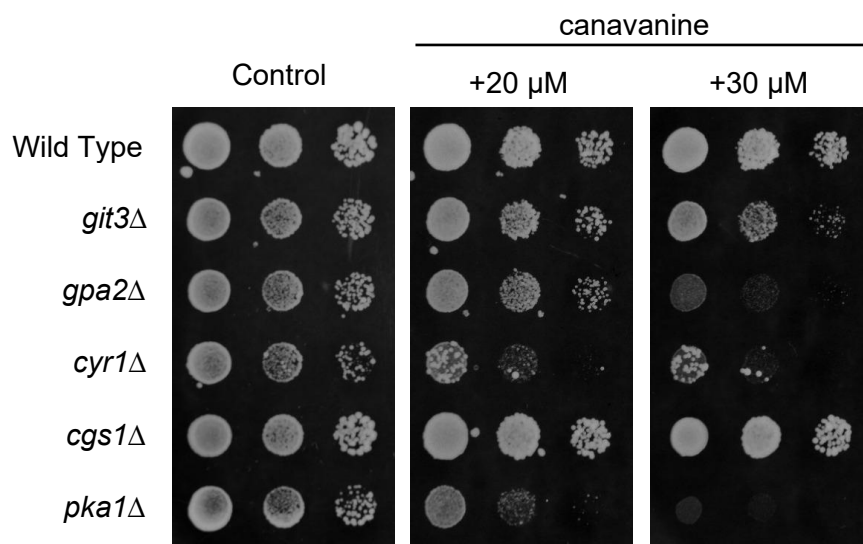
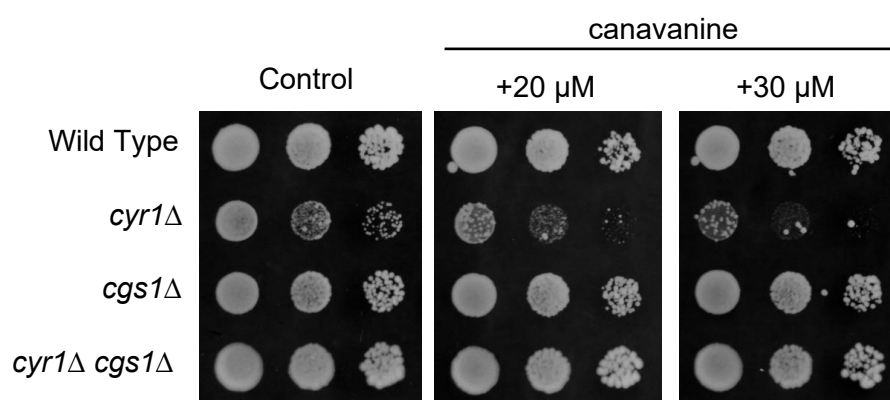
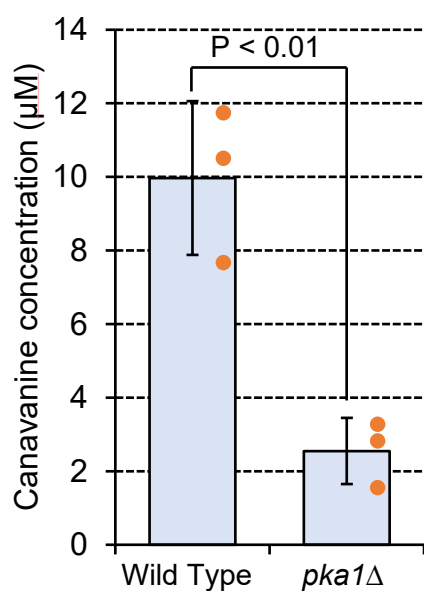
A**B****C**

Fig. 1 Loss of Pka1 function causes the canavanine-sensitive phenotype.

(A) Wild-type (PR109), *git3Δ* (YMP43), *gpa2Δ* (YMP39), *cyr1Δ* (YMP28), *cgs1Δ* (YMP40), and *pka1Δ* (YMP36) *S. pombe* strains were grown on YES medium for 1 day at 30°C. Cells were harvested and resuspended in water at 1×10^7 cells/mL. Cell suspensions were serially diluted (1:10), and each dilution was spotted onto EMMLU plates with or without canavanine. All plates were incubated for 5 days at 30°C. **(B)** Wild-type (PR109), *cyr1Δ* (YMP28), *cgs1Δ* (YMP40), and *cyr1Δ cgs1Δ* (YMP58) strains were grown on YES medium for 1 day at 30°C. Culture dilutions were prepared as described in Fig. 1A, and each dilution was spotted onto EMMLU plates with or without canavanine. All plates were incubated for 5 days at 30°C. **(C)** Wild-type (PR109) and *pka1Δ* (YMP36) strains were grown in EMMLU medium for 24 h at 30°C, resuspended into EMMLU with 20 μM canavanine, and incubated for 24 h. Intracellular canavanine levels were measured using an amino acid analyzer. Data are presented as the mean $\pm$ S.D. of three independent experiments (n = 3), with individual data points shown. Statistical significance was assessed using a two-tailed Student's *t*-test.

Loss of Pka1 function causes abnormal localization of the arginine transporter Cat1 and amino acid transporter Aat1

Because intracellular canavanine accumulation was reduced in the *pka1Δ* strain, we examined the arginine/lysine transporter Cat1. Cat1 normally localizes to the plasma membrane [3, 4]. Indeed, Cat1 localized to the plasma membrane under normal conditions in the wild-type and *cgs1Δ* strains, whereas Cat1 was mislocalized to intracellular compartments in the *pka1Δ* strain (Fig. 2A). In the presence of canavanine, plasma membrane localization of Cat1 was no longer detected in the wild-type, *cgs1Δ*, and *pka1Δ* strains (Fig. 2A and B). Cat1 protein levels were unchanged among these strains (Supplementary Fig. S1). These results indicate that deletion of *pka1* severely impairs plasma membrane localization of Cat1. Since deletion of *cat1* confers tolerance to canavanine [3, 5], we analyzed whether *cat1* deletion rescues the canavanine-sensitive

phenotype of the *pka1Δ* strain. The *pka1Δ cat1Δ* strain was tolerant to canavanine, whereas *pka1Δ* cells remained sensitive (Fig. 2C). These results suggest that Cat1 contributes to the canavanine-sensitive phenotype of the *pka1Δ* strain.

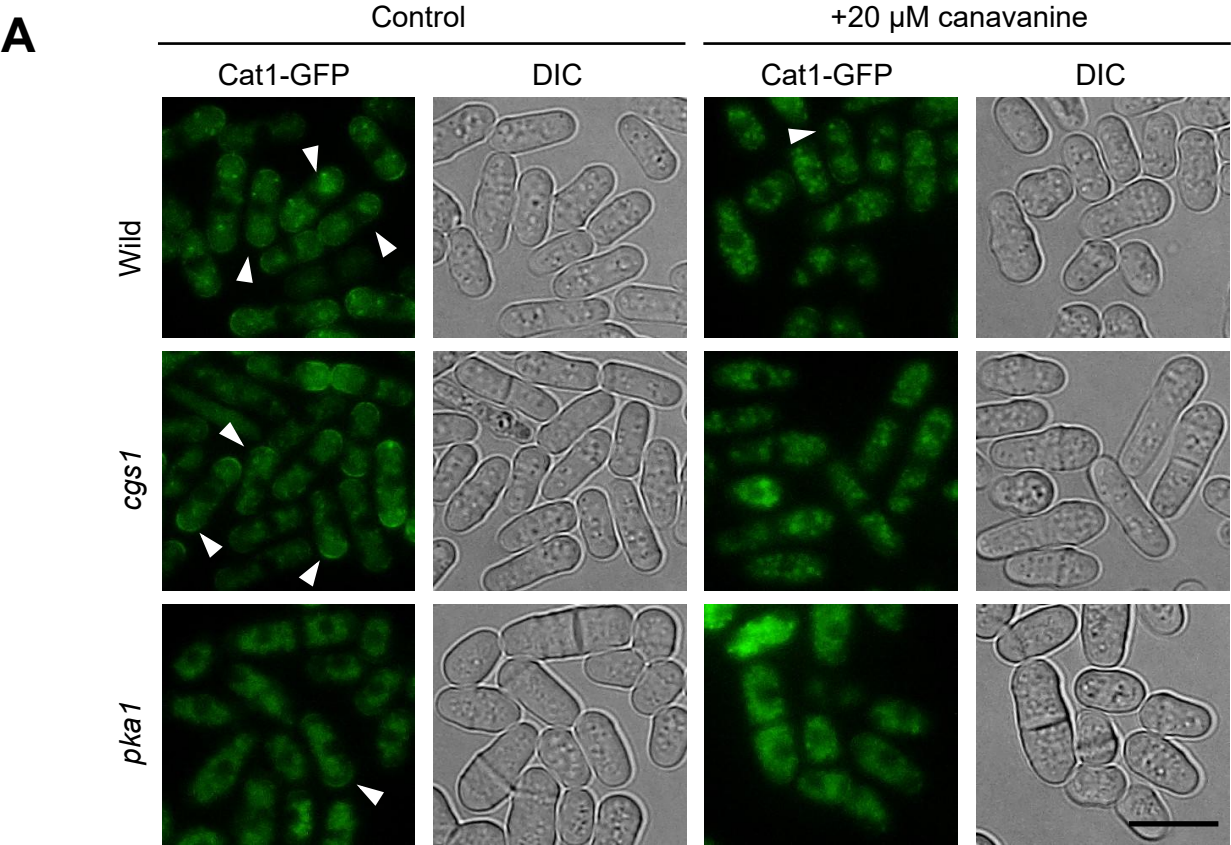
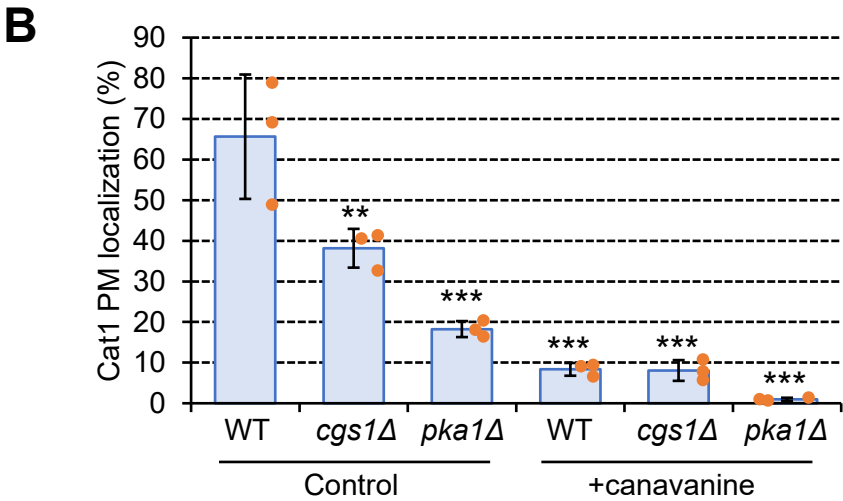
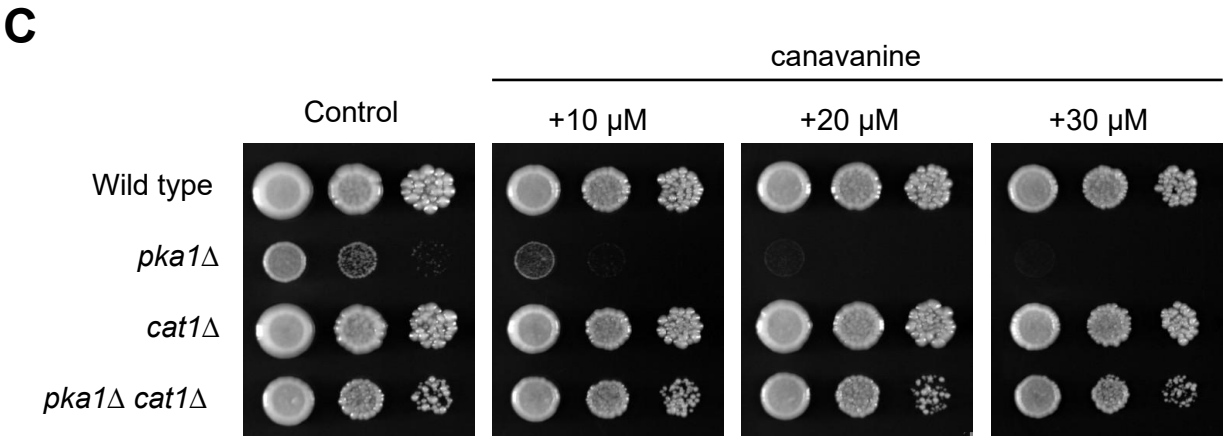


Fig. 2 Plasma membrane localization of Cat1 is decreased in the *pka1Δ* strain.

(A) Cat1-GFP (YMP923), *cgs1Δ* Cat1-GFP (YMP935), and *pka1Δ* Cat1-GFP (YMP938) cells were cultured to mid-log phase, resuspended into EMMLU with or without 20 μ M canavanine, and incubated for 8 h. Cat1-GFP localization was observed by fluorescence microscopy. Scale bar, 10 μ m. **(B)** Approximately 300 cells were counted in each independent experiment, and the percentage of cells showing plasma membrane localization of Cat1-GFP is shown. Data are presented as the mean $\pm$ S.D. of three independent experiments (n = 3), with individual data points shown. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple-comparisons test. Double and triple asterisks indicate $P < 0.01$ and $P < 0.001$, respectively, compared with the wild-type strain under normal conditions. **(C)** Wild-type (PR109), *pka1Δ* (YMP36), *cat1Δ* (YMP1012), and *pka1Δ cat1Δ* (YMP1014) strains were grown on YES for 1 day at 30°C. Cells were harvested, and cell suspensions were prepared as described in Fig. 1A. Each dilution was spotted onto EMMLU plates with or without canavanine. All plates were incubated for 7 days at 30°C.

The amino acid transporter Aat1 also localizes to the plasma membrane and responds to canavanine [6]. Similar to Cat1, Aat1 localized to the plasma membrane in wild-type and *cgs1Δ* strains, but plasma membrane localization of Aat1 was markedly reduced in the *pka1Δ* strain under normal conditions (Fig. 3A and 3B). When cells were exposed to canavanine, plasma membrane localization of Aat1 further decreased in the wild-type and *cgs1Δ* strains and was not detected in the *pka1Δ* strain (Fig. 3A and B). Aat1 protein levels were not altered in these strains (Supplementary Fig. S2). Because the role of Aat1 in canavanine sensitivity remains unclear, we examined whether deletion of *aat1* affects the canavanine-sensitive phenotype of the *pka1Δ* strain. The *aat1Δ* strain grew normally, and *aat1* deletion did not affect the growth of the *pka1Δ* strain under canavanine conditions

(Fig. 3C). These results indicate that Pka1 contributes to proper plasma membrane localization of Aat1.

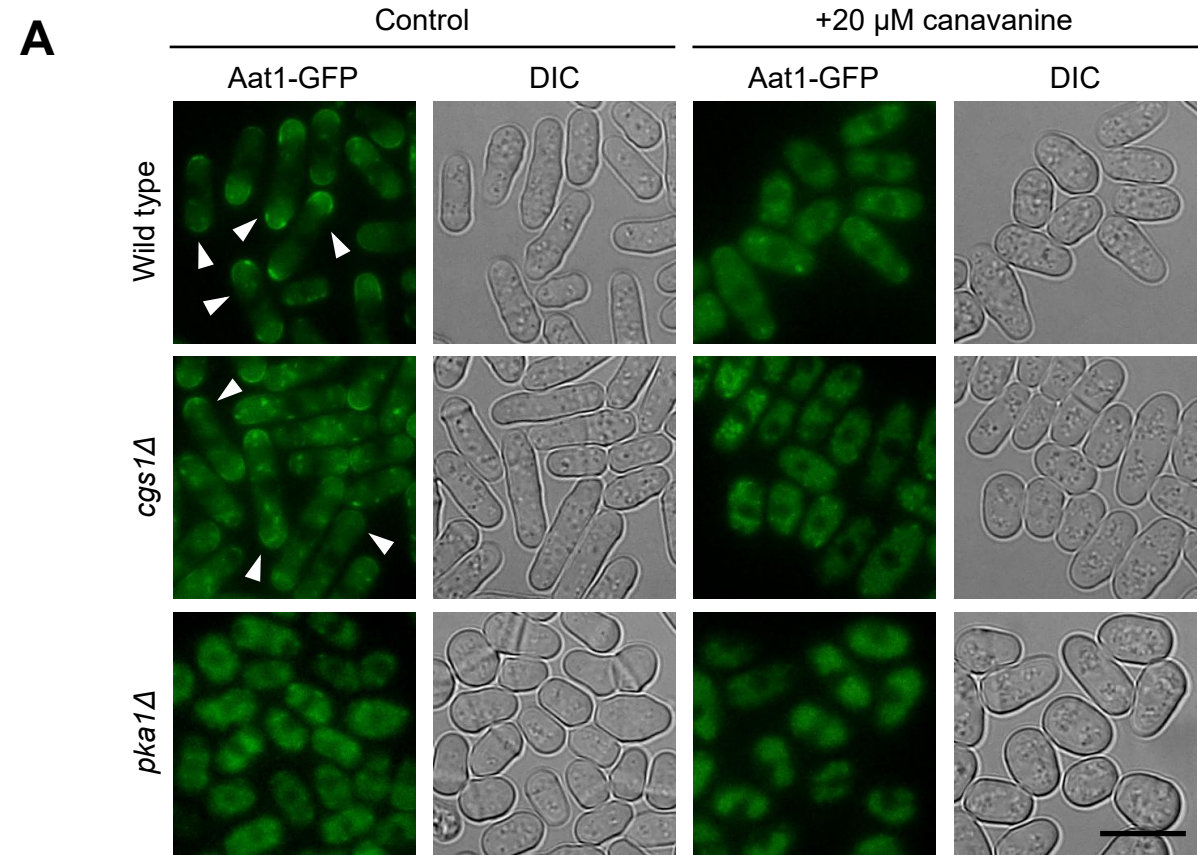
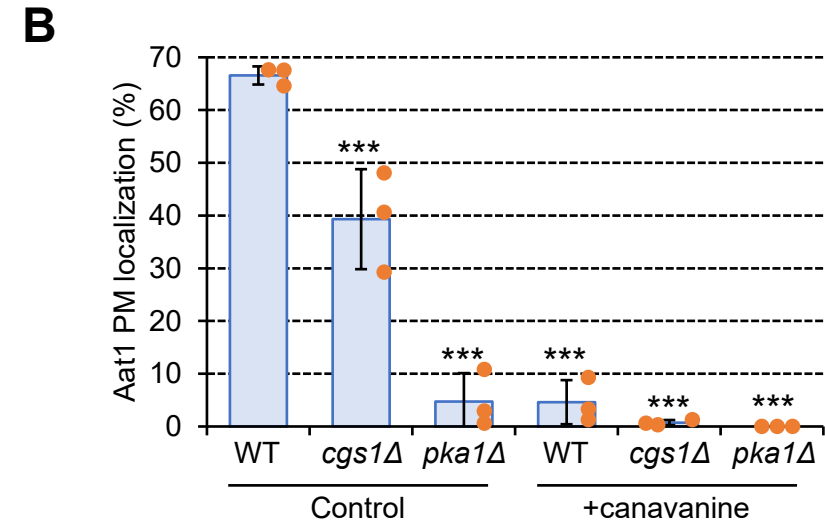
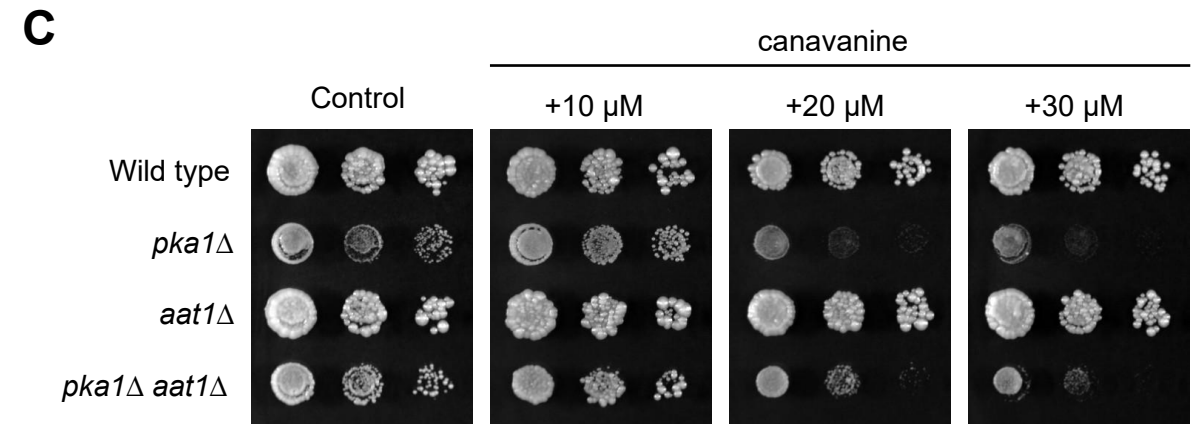


Fig. 3 Plasma membrane localization of Aat1 is decreased in the *pka1*Δ strain.

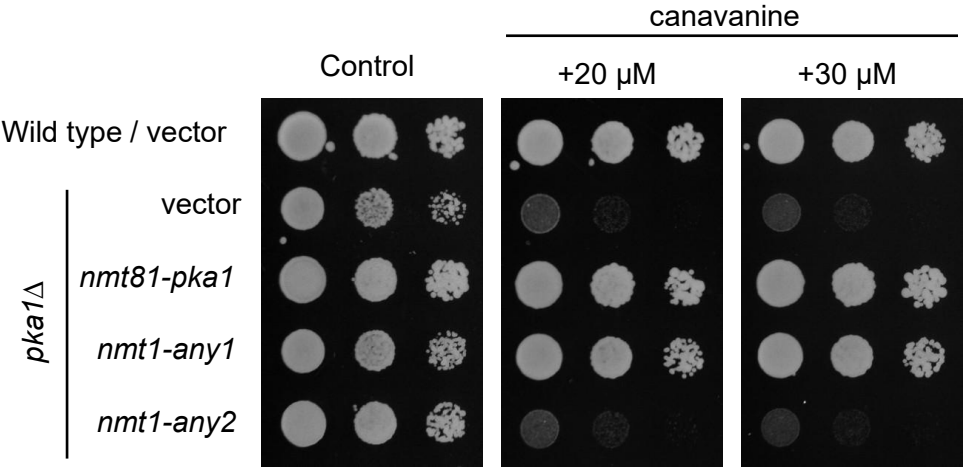
(A) Aat1-GFP (AKP1), *cgs1*Δ Aat1-GFP (AKP2), and *pka1*Δ Aat1-GFP (AKP4) cells were cultured to mid-log phase, resuspended in EMLU with or without 20 μM canavanine, and incubated for 8 h. Aat1-GFP localization was observed by fluorescence microscopy. Scale bar, 10 μm. **(B)** Approximately 300 cells were counted in each independent experiment, and the percentage of cells showing plasma membrane localization of Aat1-GFP is shown. Data are presented as the mean ± S.D. of three independent experiments (n = 3), with individual data points shown. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple-comparisons test. Triple asterisks indicate P < 0.001 compared with the wild-type strain under normal conditions. **(C)** Wild-type (PR109), *pka1*Δ (YMP36), *aat1*Δ (AKP5), and *pka1*Δ *aat1*Δ (AKP6) strains were grown on YES medium for 1 day at 30°C. Cells were harvested, and cell suspensions were prepared as described in Fig. 1A. Each dilution was spotted onto EMLU plates with or without canavanine. All plates were incubated for 7 days at 30°C.

Any1 suppresses the canavanine-sensitive phenotype of the *pka1*Δ strain

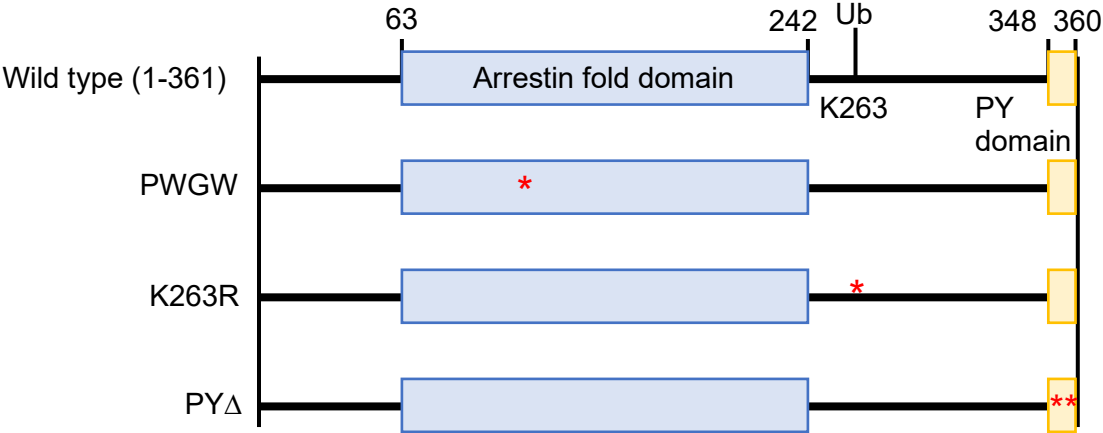
The plasma membrane localization of Cat1 and Aat1 is regulated by the Pub1 E3 ubiquitin ligase and its adaptor protein Any1 [4, 6]. We therefore examined whether Pka1 affects the function of Any1 and Pub1. To determine whether the Any1-Pub1 regulatory mechanism contributes to the *pka1*Δ phenotype, we examined genetic interactions among Pka1, Any1, and Pub1. Overexpression of Any1 rescued the canavanine-sensitive phenotype of the *pka1*Δ strain, whereas overexpression of Any2, a homolog of Any1, did not (Fig. 4A). Any1 contains an arrestin fold domain (residues 63-242), a putative ubiquitination site (K263), and a PY domain (X1-Pro-X2-Tyr: residues 348-360) [4] (Fig. 4B). The arrestin and PY domains mediate interactions with G protein-coupled receptors [30, 31] and the WW (Trp-Trp) domains of Nedd4-family HECT-type E3 ubiquitin ligases [32, 33], respectively. Overexpression of Any1 mutants (PWGW, K263R, and PYΔ) did not

rescue the canavanine-sensitive phenotype of the *pka1* Δ strain in the presence of canavanine (Fig. 4C). These results suggest that functional Any1 is required to suppress the canavanine-sensitive phenotype of the *pka1* Δ strain.

A



B



C

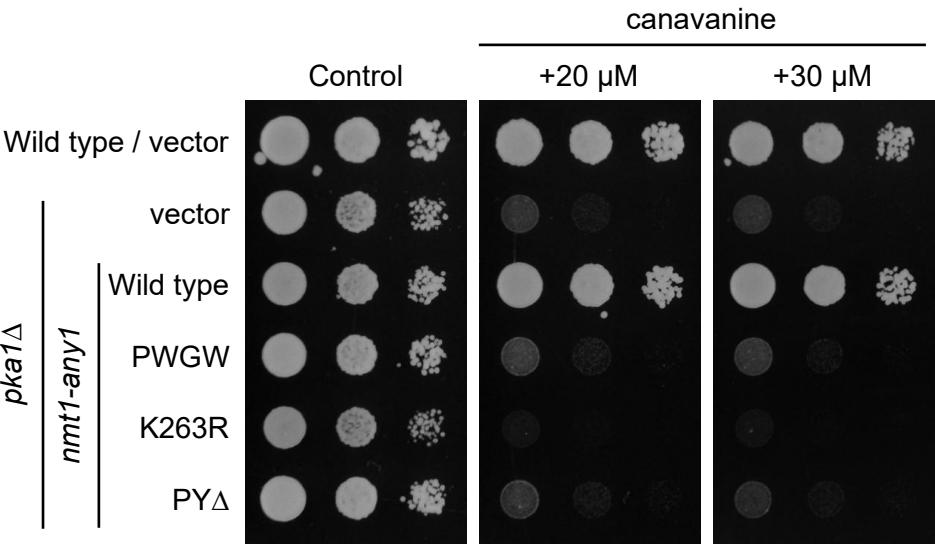


Fig. 4 Any1 overexpression rescues the canavanine-sensitive phenotype of the *pka1*Δ strain.

(A) Wild-type (PR109) and *pka1*Δ (YMP36) cells harboring pREP3X (vector), pREP81-*pka1*, pREP3X-any1, or pREP3X-any2 were cultured in EMMU medium for 1 day at 30°C to induce expression from the *nmt* promoter. Culture dilutions were prepared as described in Fig. 1A, and each dilution was spotted onto EMMU plates with or without canavanine. All plates were incubated for 6 days at 30°C. **(B)** Schematic diagram of the domain structure of Any1. The arrestin fold domain (amino acids 63-242) and the PY domain (amino acids 348-360) are indicated by blue and yellow boxes, respectively. The mutation sites in the Any1 mutants (PWGW, K263R, and PYΔ) are indicated by red asterisks. **(C)** Wild-type (PR109) and *pka1*Δ (YMP36) cells harboring pREP3X (vector), pREP3X-any1 (wild-type), pREP3X-any1 (PWGW), pREP3X-any1 (K263R), or pREP3X-any1 (PYΔ) were cultured in EMMU medium lacking thiamine for 1 day at 30°C to induce expression from the *nmt1* promoter. Culture dilutions were prepared as described in Fig. 1A, and each dilution was spotted onto EMMU plates with or without canavanine. All plates were incubated for 6 days at 30°C.

Because overexpression of Any1 rescues the canavanine-sensitive phenotype of the *pka1*Δ strain, we analyzed the localization of Any1 in the presence or absence of canavanine. Any1 localizes to the Golgi apparatus [6]. Any1 localization was not altered among the wild-type, *cgs1*Δ, and *pka1*Δ strains under either condition (Supplementary Fig. S3A). Any1 protein levels were also unchanged among these strains (Supplementary Fig. S3B and S3C). These results indicate that loss of Pka1 function does not alter the localization or expression of Any1.

Any1-mediated suppression of canavanine sensitivity is independent of canavanine uptake and transporter localization

384 Because overexpression of Any1 rescued the canavanine-sensitive phenotype of the
385 *pka1Δ* strain, we next examined whether Any1 restores reduced canavanine accumulation
386 and defects in Cat1 and Aat1 localization. Any1 overexpression did not restore reduced
387 canavanine accumulation (Fig. 5A) or impaired plasma membrane localization of Cat1
388 (Fig. 5B). The decreased plasma membrane localization of Aat1 in the *pka1Δ* strain was
389 partially restored under normal conditions but not in the presence of canavanine (Fig. 5C).
390 These results indicate that Any1-mediated suppression of the canavanine-sensitive
391 phenotype of the *pka1Δ* strain is independent of restoration of canavanine accumulation
392 and Cat1 and Aat1 localization.

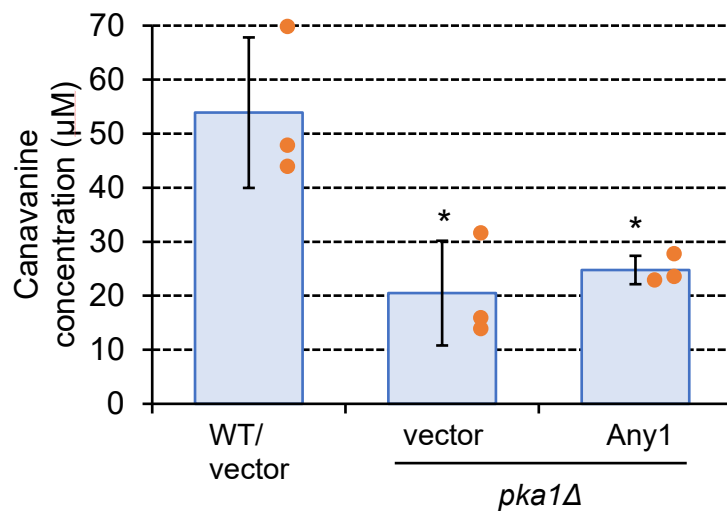
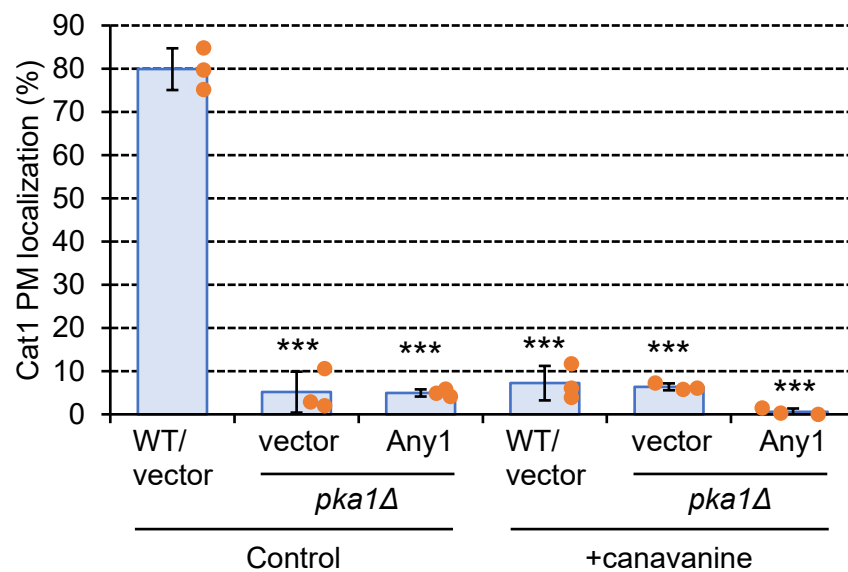
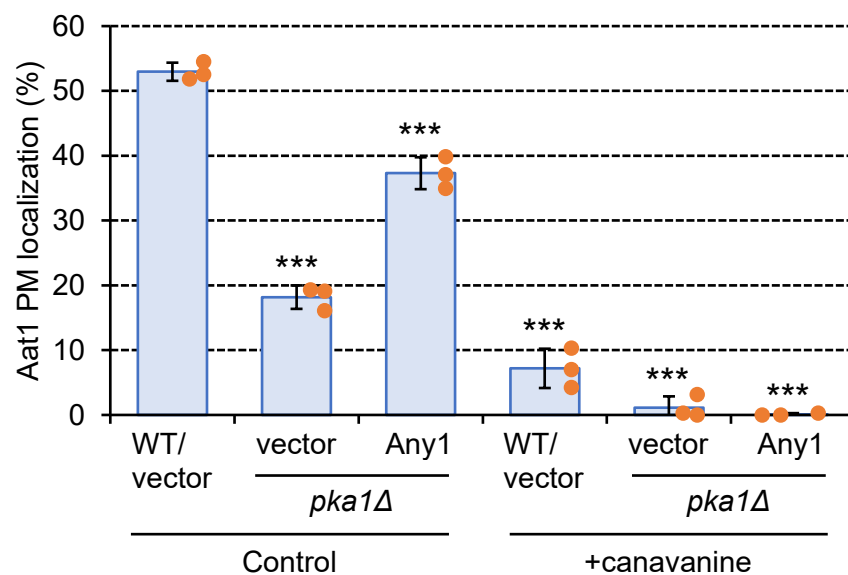
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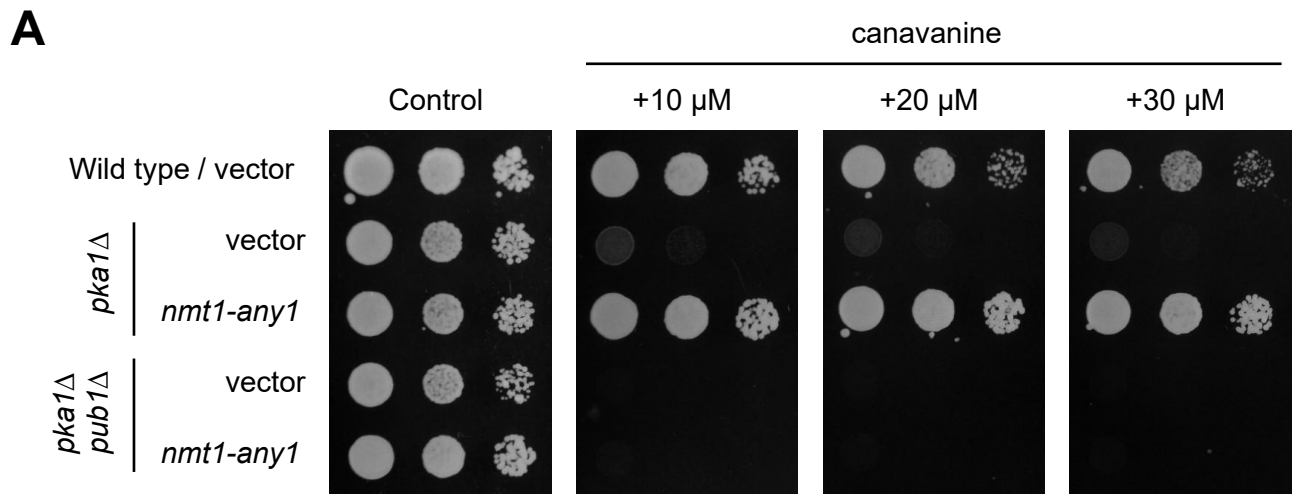
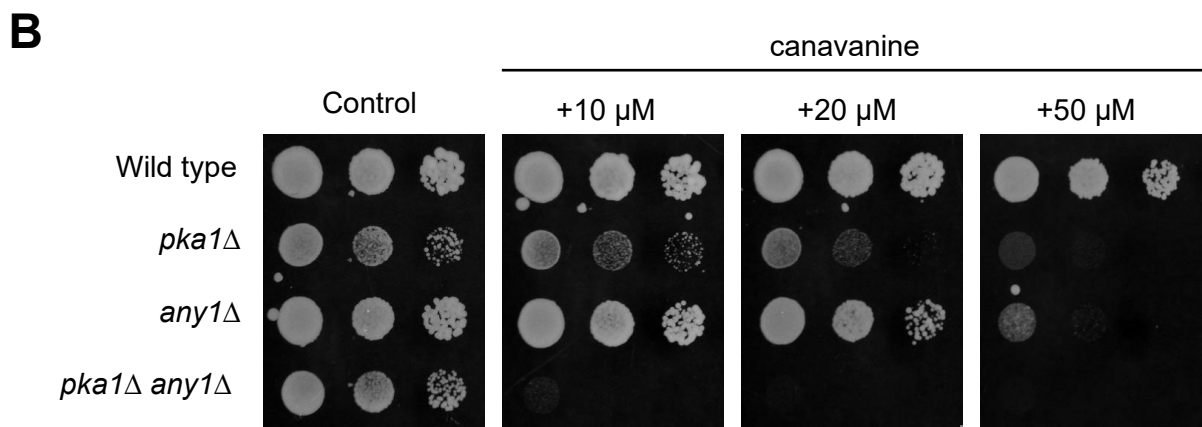
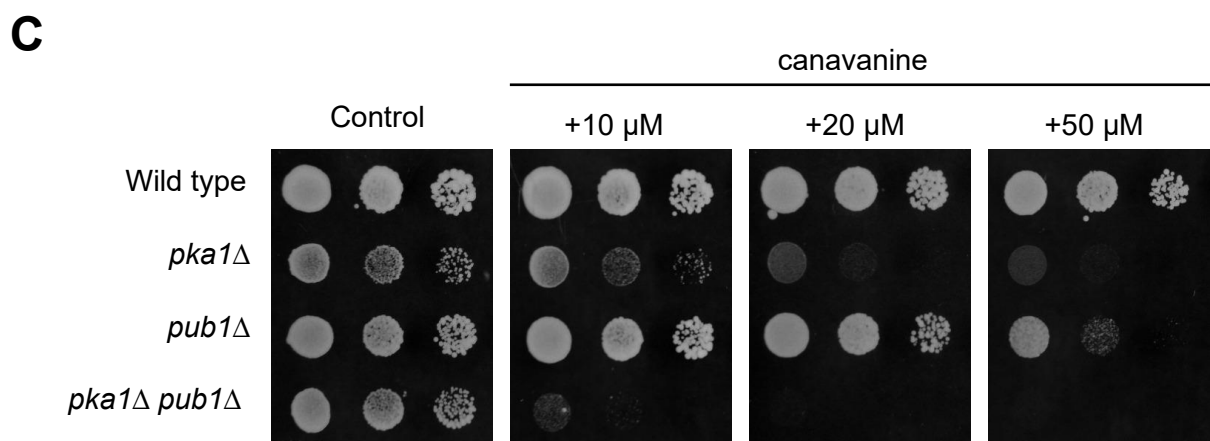
Fig. 5 Any1 overexpression does not rescue reduced canavanine uptake or abnormal localization of Cat1 and Aat1 in the *pka1*Δ strain.

(A) Wild-type (PR109) and *pka1*Δ (YMP36) cells harboring pREP3X (vector) or pREP3X-any1 were grown in EMMU medium for 12 h at 30°C to induce expression from the *nmt1* promoter, resuspended in EMMU lacking thiamine and containing 20 μM canavanine, and incubated for 8 h. Intracellular canavanine levels were measured. Data are presented as the mean ± S.D. of three independent experiments (n = 3), with individual data points shown. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple-comparisons test. An asterisk indicates P < 0.05 compared with the wild-type strain under normal conditions. **(B)** Cat1-GFP (YMP923) and *pka1*Δ Cat1-GFP (YMP938) cells harboring pREP3X (vector) or pREP3X-any1 were cultured for 1 day in EMMU containing thiamine, washed three times with water, resuspended in EMMU lacking thiamine and containing 20 μM canavanine, and cultured for 18 h at 30°C. Approximately 300 cells were counted in each independent experiment, and the percentage of cells showing plasma membrane localization of Cat1-GFP is shown. Data are presented as the mean ± S.D. of three independent experiments (n = 3), with individual data points shown. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple-comparisons test. Triple asterisks indicate P < 0.001 compared with the wild-type strain under normal conditions. **(C)** Aat1-GFP (AKP1) and *pka1*Δ Aat1-GFP (AKP4) cells harboring pREP3X (vector) or pREP3X-any1 were cultured as described in Fig. 5B. Approximately 300 cells were counted in each independent experiment, and the percentage of cells showing plasma membrane localization of Aat1-GFP is shown. Data are presented as the mean ± S.D. of three independent experiments (n = 3), with individual data points shown. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple-comparisons test. Triple asterisks indicate P < 0.001 compared with the wild-type strain under normal conditions.

Pub1 is required for Any1-mediated suppression of the canavanine-sensitive phenotype of the *pka1*Δ strain

Any1 functions as an adaptor protein for the E3 ubiquitin ligase Pub1 [4, 6]. To determine whether Pub1 is required for Any1-mediated suppression of the canavanine-sensitive phenotype of the *pka1*Δ strain, we analyzed the *pka1*Δ *pub1*Δ double mutant. Any1 overexpression rescued the *pka1*Δ strain but not the *pka1*Δ *pub1*Δ strain in the presence of canavanine (Fig. 6A), indicating that Pub1 is required for Any1-mediated suppression of the canavanine-sensitive phenotype of the *pka1*Δ strain.

We next analyzed whether deletion of *any1* or *pub1* enhances the canavanine-sensitive phenotype of the *pka1*Δ strain. The *any1*Δ strain exhibited sensitivity to 50 μM canavanine. Deletion of *any1* dramatically enhanced the canavanine-sensitive phenotype of the *pka1*Δ strain (Fig. 6B). Similarly, the *pub1*Δ strain exhibited sensitivity to 50 μM canavanine, and deletion of *pub1* dramatically enhanced the canavanine-sensitive phenotype of the *pka1*Δ strain (Fig. 6C). We next analyzed whether Pub1 overexpression restores growth of the *pka1*Δ strain. Because high-level Pub1 expression causes a growth defect under normal conditions (Supplementary Fig. S4), we used the weaker *nmt41* promoter. Overexpression of Pub1 did not rescue the growth defect of the *pka1*Δ strain in the presence of canavanine (Supplementary Fig. S5). This result may reflect growth inhibition caused by Pub1 overexpression. These results indicate that the Any1-Pub1 regulatory mechanism contributes to the canavanine response of the *pka1*Δ strain.



468

469 **Fig. 6 Pub1 is required for suppression of the canavanine-sensitive phenotype of**

470 **the *pka1* Δ strain by *Any1* overexpression.**

(A) Wild-type (PR109), *pka1Δ* (YMP36), and *pka1Δ pub1Δ* (YMP887) cells harboring pREP3X (vector) or pREP3X-*any1* were cultured in EMMU medium for 1 day at 30°C to induce expression from the *nmt1* promoter. Culture dilutions were prepared as described in Fig. 1A, and each dilution was spotted onto EMMU plates with or without canavanine. All plates were incubated for 5 days at 30°C. (B) Wild-type (PR109), *pka1Δ* (YMP178), *any1Δ* (YMP860), and *pka1Δ any1Δ* (YMP883) strains were grown on YES medium for 1 day at 30°C. Cells were harvested, and cell suspensions were prepared as described in Fig. 1A. Each dilution was spotted onto EMMLU plates with or without canavanine. All plates were incubated for 7 days at 30°C. (C) Wild-type (PR109), *pka1Δ* (YMP178), *pub1Δ* (KNP63), and *pka1Δ pub1Δ* (YMP890) strains were grown on YES medium for 1 day at 30°C. Cells were harvested, and cell suspensions were prepared as described in Fig. 1A. Each dilution was spotted onto EMMLU plates with or without canavanine. All plates were incubated for 7 days at 30°C.

Pub1 localization is regulated by the cAMP/PKA pathway

Because the Any1-Pub1 regulatory mechanism is required for suppression of the canavanine-sensitive phenotype of the *pka1Δ* strain, we examined Pub1 localization and expression in the *pka1Δ* strain. Pub1 localizes to the cytoplasm and Golgi apparatus [6, 34]. The punctate Pub1-GFP signals partially colocalized with the Golgi marker Gms1-RFP (Supplementary Fig. S6), consistent with previous reports that Pub1 localizes to the Golgi apparatus [6]. Therefore, punctate Pub1-GFP signals were considered to represent Golgi localization in this study. Pub1 localized to the cytoplasm and Golgi apparatus under normal conditions in each strain (Fig. 7A). The proportion of cells showing Golgi localization of Pub1 was similar between the wild-type and *cgs1Δ* strains under normal conditions (Fig. 7B). In the presence of canavanine, Golgi localization of Pub1 was maintained in the wild-type and *cgs1Δ* strains but was reduced in the *pka1Δ* strain (Fig. 7A and B). Pub1 protein levels, however, were unchanged among wild-type, *cgs1Δ*, and

pka1Δ strains regardless of canavanine (Fig. 7C and D). These results indicate that Pka1 function contributes to the regulation of Pub1 localization in response to canavanine.

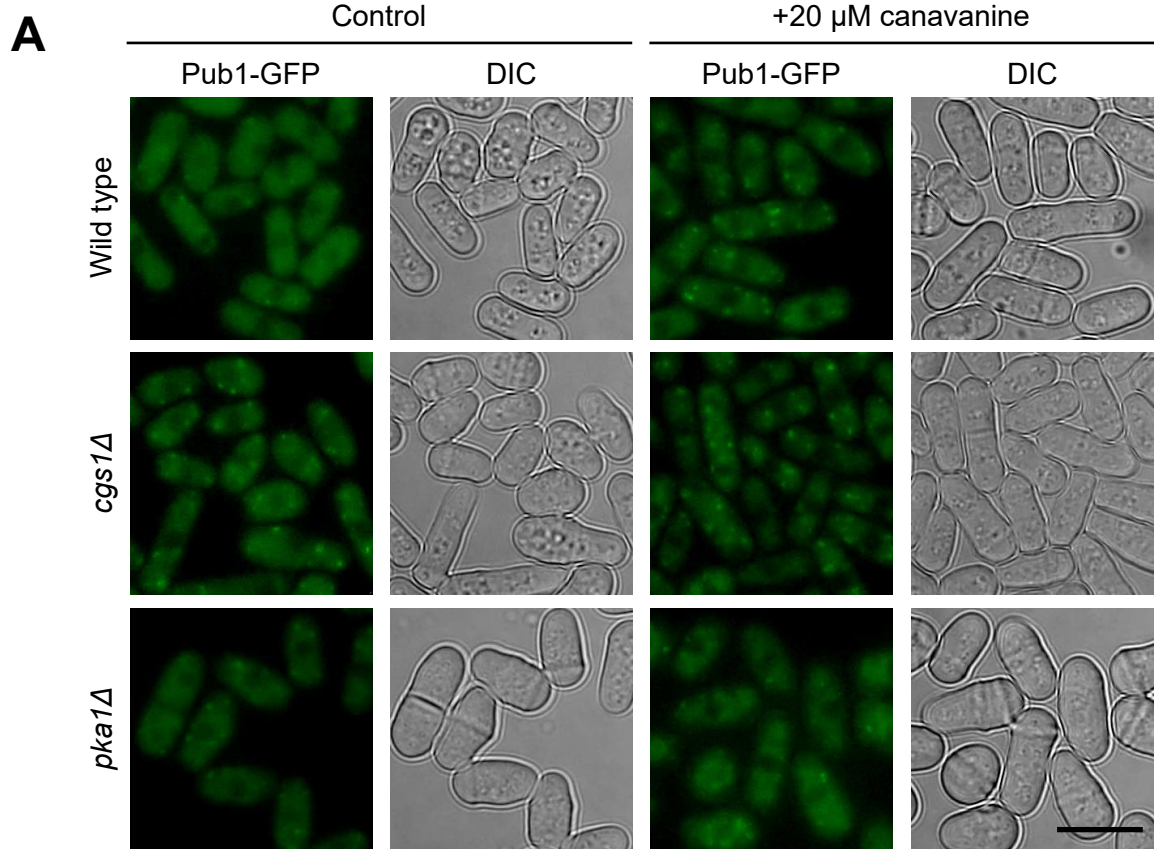
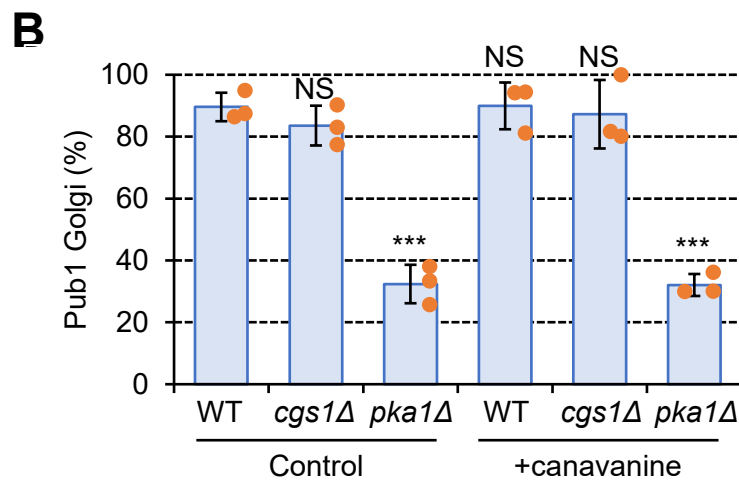
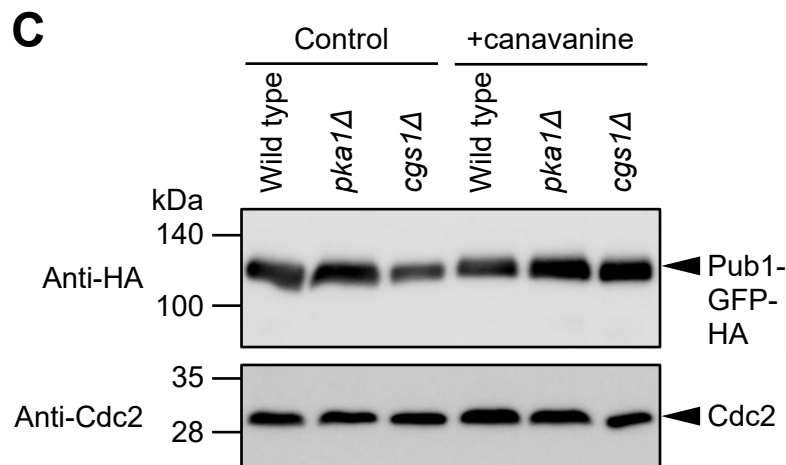
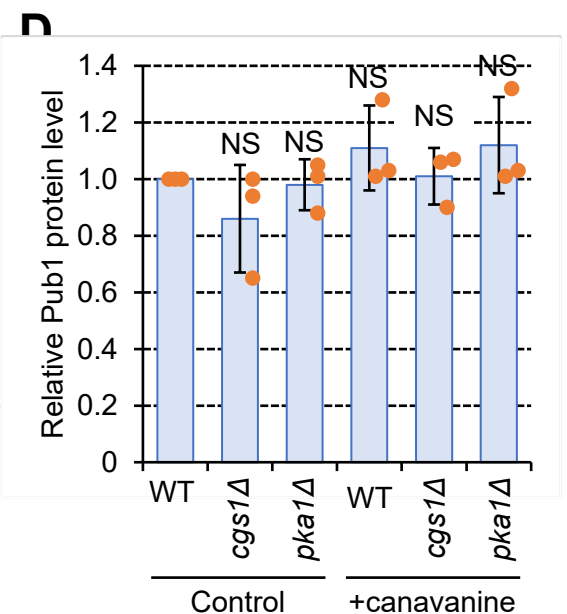


Fig. 7 Pub1 localization is regulated by Pka1 in response to canavanine.

(A) Pub1-GFP (YMP908), *cgs1* Δ Pub1-GFP (YMP909), and *pka1* Δ Pub1-GFP (YMP910) cells were cultured as described in Fig. 2A. Pub1-GFP localization was observed by fluorescence microscopy. Scale bar, 10 μ m. **(B)** Approximately 300 cells were counted in each independent experiment, and the percentage of cells showing Golgi localization of Pub1-GFP is shown. Data are presented as the mean $\pm$ S.D. of three independent experiments ($n = 3$), with individual data points shown. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple-comparisons test. NS and triple asterisks indicate no significant difference and $P < 0.001$, respectively, compared with the wild-type strain under normal conditions. **(C)** Pub1-GFP-HA (YMP908), *cgs1* Δ Pub1-GFP-HA (YMP909), and *pka1* Δ Pub1-GFP-HA (YMP910) cells were cultured as described in Fig. 2A. Pub1-GFP-HA was detected using an anti-HA antibody. An anti-Cdc2 antibody was used as an internal loading control. Full-length blots are presented in Supplementary Fig. S7. **(D)** Expression levels of Pub1-GFP-HA were quantified and normalized to the wild-type strain under normal conditions. Data are presented as the mean $\pm$ S.D. of three independent experiments ($n = 3$), with individual data points shown. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple-comparisons test. NS indicates no significant difference compared with the wild-type strain under normal conditions.

Any1 overexpression rescues abnormal Pub1 localization in the *pka1* Δ strain

Finally, we examined whether Any1 overexpression restores Golgi localization of Pub1 in the *pka1* Δ strain. Under normal conditions, Golgi localization of Pub1 was reduced in *pka1* Δ cells carrying the empty vector, whereas it was maintained in wild-type cells carrying the empty vector. Any1 overexpression restored Golgi localization of Pub1 in the *pka1* Δ strain (Fig. 8A and B). Similarly, in the presence of canavanine, Any1 overexpression restored Golgi localization of Pub1 in the *pka1* Δ strain, whereas Golgi

localization remained reduced in *pka1Δ* cells carrying the empty vector (Fig. 8A and B). These results suggest that restoration of Pub1 localization contributes to Any1-mediated suppression of the canavanine-sensitive phenotype of the *pka1Δ* strain.

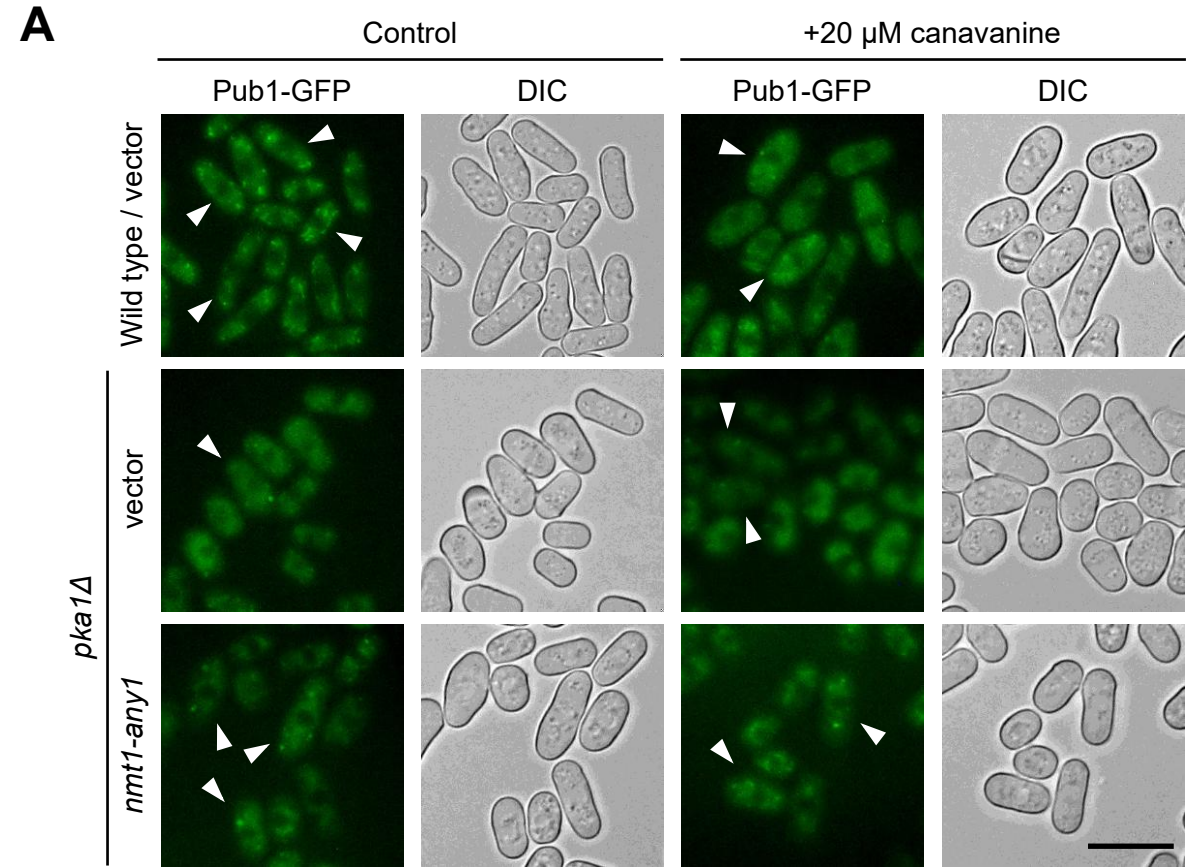
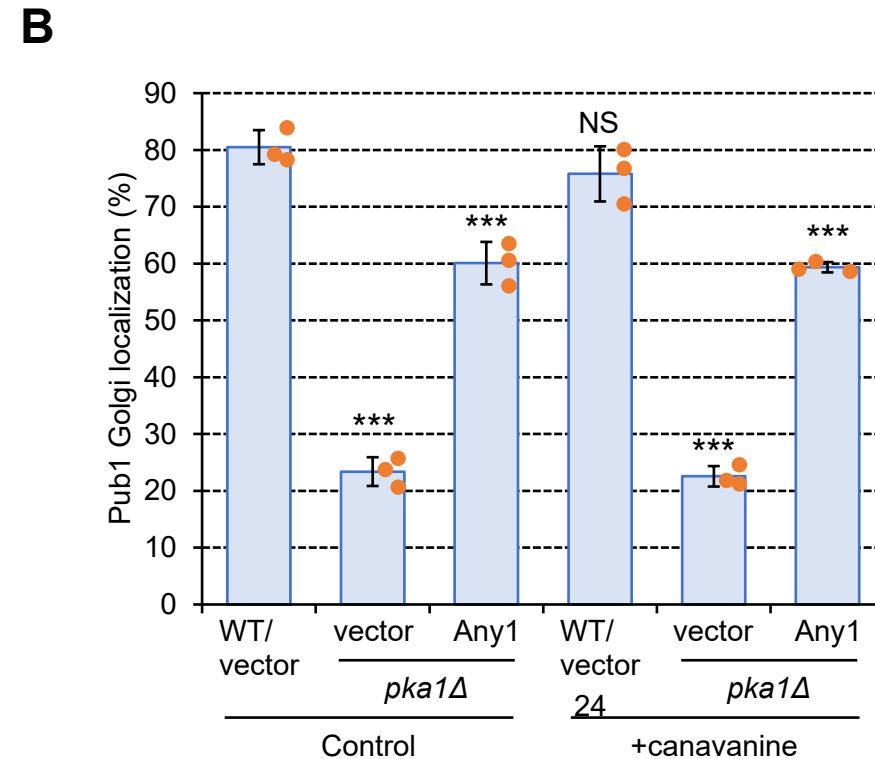


Fig. 8 Any1 overexpression restores Pub1 Golgi localization in the *pka1* Δ strain.

(A) Pub1-GFP (YMP908) and *pka1* Δ Pub1-GFP (YMP910) cells harboring pREP3X (vector) or pREP3X-any1 were cultured as described in Fig. 5A. Pub1-GFP localization was observed by fluorescence microscopy. Scale bar, 10 μ m. **(B)** Approximately 250 cells were counted in each independent experiment, and the percentage of cells showing Golgi localization of Pub1-GFP is shown. Data are presented as the mean $\pm$ S.D. of three independent experiments (n = 3), with individual data points shown. Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple-comparisons test. NS and triple asterisks indicate no significant difference and $P < 0.001$, respectively, compared with the wild-type strain under normal conditions.

Discussion

Canavanine is a toxic analog of arginine that is taken up through amino acid transport systems. Once inside cells, canavanine can be incorporated into proteins in place of arginine, resulting in the production of dysfunctional proteins and cellular toxicity [1, 2]. Loss of Pka1 function caused growth inhibition in the presence of canavanine (Fig. 1A and B). However, *pka1* Δ cells exhibited reduced intracellular canavanine accumulation (Fig. 1C), suggesting that increased intracellular canavanine accumulation is not responsible for the canavanine-sensitive phenotype of the *pka1* Δ strain. Because plasma membrane localization of Cat1 and Aat1 is closely associated with canavanine sensitivity [4, 6], we hypothesized that Pka1 regulates amino acid transport through the regulation of these transporters. Intriguingly, Cat1 and Aat1 accumulated in intracellular compartments in *pka1* Δ cells (Fig. 2A, 2B, 3A, and 3B), whereas deletion of *cat1* suppressed the canavanine-sensitive phenotype of the *pka1* Δ strain. These results suggest that Cat1 contributes to the canavanine-sensitive phenotype in the *pka1* Δ strain and that Pka1 regulates the localization of Cat1 and Aat1. These findings also suggest that abnormal localization of Cat1 and Aat1 alone cannot fully explain the canavanine-sensitive

phenotype of the *pka1Δ* strain. Although the present study demonstrates that Pka1 function contributes to the regulation of plasma membrane localization of Cat1 and Aat1, the underlying molecular mechanism remains unknown. Any1 is a well-characterized regulator of Cat1 and Aat1 [4, 6] and overexpression of Any1 suppressed the canavanine-sensitive phenotype of the *pka1Δ* strain, but Any2 did not (Fig. 4A). Any1 mutants (PWGW, K263R, and PYΔ) were not able to suppress the canavanine-sensitive phenotype of the *pka1Δ* strain under canavanine conditions, whereas Any1 localization and expression were unchanged in the *pka1Δ* strain (Fig. 4B, 4C, and Supplementary Fig. S3), indicating that functional Any1 is required for suppression of the *pka1Δ* phenotype. However, Any1 overexpression did not restore canavanine accumulation or the plasma membrane localization of Cat1 and Aat1 in *pka1Δ* cells (Fig. 5). These findings suggest that Any1 suppresses the canavanine-sensitive phenotype of *pka1Δ* cells through a mechanism independent of canavanine accumulation and Cat1/Aat1 localization. Indeed, suppression of the *pka1Δ* phenotype by Any1 overexpression requires Pub1 function in the presence of canavanine (Fig. 6A). This result indicates that Any1-mediated suppression depends on the Any1-Pub1 regulatory mechanism. The *pka1Δ* strain exhibited stronger canavanine sensitivity than the *any1Δ* and *pub1Δ* single mutants, suggesting that Pka1 regulates additional mechanisms involved in canavanine tolerance besides the Any1-Pub1 regulatory system. These genetic interactions suggest that Pka1 and the Any1-Pub1 regulatory system contribute to canavanine tolerance through mechanisms that are not completely overlapping. Supporting this idea, the *pka1Δ any1Δ* and *pka1Δ pub1Δ* double mutants exhibited stronger canavanine sensitivity than the corresponding single mutants (Fig. 6B and C). Pub1 overexpression did not rescue the canavanine-sensitive phenotype of *pka1Δ* cells (Supplementary Fig. S5). This finding may be explained by two possibilities: high-level Pub1 expression may cause growth defects, or Any1-mediated regulation of Pub1 may be required for suppression of the *pka1Δ* phenotype. Pub1 localizes to the Golgi apparatus [6], and the proportion of cells showing Golgi localization of Pub1 was decreased in the *pka1Δ* strain, whereas Pub1 expression levels were similar to those in

the wild-type strain (Fig. 7). Under Any1 overexpression, the reduced Golgi localization of Pub1 was almost restored in *pka1Δ* cells (Fig. 8). Importantly, Any1 overexpression restored Pub1 localization without restoring intracellular canavanine accumulation or the plasma membrane localization of Cat1 and Aat1. These findings suggest that restoration of Pub1 localization contributes to Any1-mediated suppression of the canavanine-sensitive phenotype and represents a regulatory mechanism that is not simply explained by restoration of Cat1 and Aat1 localization.

Although our results indicate that Pka1 and the Any1-Pub1 regulatory system contribute to canavanine tolerance, several questions remain unresolved. The mechanisms underlying the reduced intracellular canavanine accumulation and decreased plasma membrane localization of Cat1 and Aat1 in *pka1Δ* cells remain unclear. Furthermore, the relationship between these phenotypes and canavanine sensitivity needs to be elucidated. In addition, the molecular mechanism by which Pka1 and Any1 regulate Pub1 localization remains unknown. Further studies will be required to clarify how Pka1 and the Any1-Pub1 regulatory system control amino acid transport and cellular adaptation to canavanine toxicity.

Conclusions

Our findings demonstrate that Pka1 contributes to canavanine tolerance and regulation of Pub1 localization. The genetic and cell biological analyses further indicate that Pka1 and the Any1-Pub1 regulatory system contribute to the cellular response to canavanine. Together, these findings reveal a previously unrecognized role of Pub1 localization in adaptation to canavanine stress.

Supplemental Information

The online version contains supplementary material available.

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Author contributions

Conceptualization: Y.M., Methodology: A.K. and Y.M., Formal analysis and investigation: A.K. and Y.M., Data curation: A.K. and Y.M., Funding acquisition: Y.M., Resources: Y.M., Supervision: Y.M., Writing – original draft: Y.M., Writing – review and editing: A.K. and Y.M.

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Data Availability

The data supporting the findings of this study are available within the article and its Supplementary Information. Additional data are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

696 Not applicable.

697

698 **Competing interests**

699 The authors declare that they have no competing interests.

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