Synergistic roles of the phospholipase B homolog Plb1 and the cAMP-dependent protein kinase Pka1 in the hypertonic stress response of Schizosaccharomyces pombe

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Received: 7 July 2022 / Revised: 2 August 2022 / Accepted: 24 August 2022 / Published online: 16 September 2022
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Abstract
The phospholipase B homolog Plb1 and the cAMP-dependent protein kinase (PKA) pathway are required by fission yeast, also known as to Schizosaccharomyces pombe, to grow under KCl-stress conditions. Here, we report the relative contributions of Plb1 and the AMP/PKA pathway during the hypertonic stress response. We show that the plb1Δ, cyr1Δ, and pka1Δ single mutants are sensitive to high concentrations of KCl but insensitive to sorbitol-induced osmotic stress. In contrast, the plb1Δ cyr1Δ and plb1Δ pka1Δ double mutants are hypersensitive to KCl and sorbitol. The cyr1Δ pka1Δ double mutants showed the same phenotype of each single mutant. Growth inhibition due to hypertonic stress in the plb1Δ, plb1Δ cyr1Δ, and plb1Δ pka1Δ strains was partially rescued by cgs1 deletion—cgs1Δ has constitutively active Pka1—or by the deletion of transcription factor Rst2, which is negatively regulated by Pka1. Pka1-GFP localized in the nucleus and cytoplasm in plb1Δ, whereas it is localized only in the cytoplasm in cyr1Δ, indicating that Plb1 does not regulate Pka1 localization. Glucose limitation downregulates the PKA pathway, and it was accordingly observed that glucose limitation in plb1Δ further increased the strain’s sensitivity to KCl. Growth inhibition by KCl in plb1Δ under glucose-limited conditions was significantly rescued by cgs1Δ and slightly rescued by rst2Δ. These findings indicate that, in fission yeast, Plb1 and the glucose-sensing cAMP/PKA pathway play a synergistic role in responding to hypertonic stress.

Keywords Phospholipase B · cAMP-dependent protein kinase · Hypertonic stress response · Glucose limitation

Introduction
Signal transduction pathways play an important role in adapting to various environmental conditions. In the fission yeast, Schizosaccharomyces pombe, the stress-activated protein kinase (SAPK), the target of rapamycin (TOR), and the cAMP-dependent protein kinase (PKA) pathways respond to various environmental conditions. The SAPK pathway is activated in response to osmotic, oxidative, or heat stress (Shieh et al. 1997, 1998; Shiozaki and Russell 1996; Wilkinson et al. 1996). The TOR pathway is regulated in response to nutrient starvation, such as nitrogen or glucose limitation (Halova et al. 2013; Saitoh et al. 2015). The cAMP/PKA pathway mainly responds to glucose concentration (Hoffman 2005; Inamura et al. 2021; Welton and Hoffman 2000).

In the cAMP/PKA pathway, the G protein-coupled receptor Git3 receives the signal molecules and activates a G protein alpha subunit (Gpa2), by releasing the beta subunit (Git5) and the gamma subunit (Git11). Gpa2 then activates the adenylyl cyclase Cyr1, which synthesizes cAMP from ATP. In the absence of glucose signaling, cAMP-dependent protein kinase regulatory subunit (Cgs1) binds protein kinase A catalytic subunit (Pka1), thus keeping it inactive. Upon glucose signaling via Git3, the cAMP synthesized by Cyr1 binds to Cgs1, thereby releasing Pka1 in an activated form (DeVoti et al. 1991; Gupta et al. 2011a; Kawamukai...
Phospholipase B (Plb1) hydrolyses the acyl chains \( sn1 \) and \( sn2 \), and has fatty acid acyltransferase activity that converts lysophospholipids to phospholipids (Ansell and Hawthorne 1964). In \( S. pombe \), \( plb1 \Delta \) shows a high frequency of mated cells and a KCl-sensitive phenotype that is suppressed by \( Gtp3 \) or \( Gpa2 \) overexpression (Yang et al. 2003). Based on these results, Yang et al. suggested that Plb1 is a mediator of an osmotic stress response that is linked to the cAMP/PKA pathway. However, the relationship between Plb1 and the cAMP/PKA pathway is still unclear due to insufficient analyses.

In this study, we characterize the genetic relationship between Plb1 and the cAMP/PKA pathway in the context of the hypertonic stress response. We also show that Pka1 activation rescued the hypertonic-stress-induced growth defect of \( plb1 \Delta \). Glucose limitation enhanced the KCl-sensitive phenotype of \( plb1 \Delta \), and a gain-of-function of Pka1 suppressed this effect. Finally, we show that Plb1 is not involved in regulating Pka1 localization or phosphorylation. Together, our findings demonstrate that Plb1 and the cAMP/PKA pathway play a synergistic role in responding to hypertonic stress in \( S. pombe \).

### Materials and methods

#### Yeast strains, media, and genetic methods

The \( S. pombe \) strains used in this study are listed in Table 1. Standard yeast culture media and genetic methods were used (Murray et al. 2016; Petersen and Russell 2016). \( S. pombe \) cultures were grown on either YES medium (0.5% yeast extract, 3% glucose, 225 mg/L adenine, 225 mg/L uracil, 225 mg/L leucine, 225 mg/L histidine, and 225 mg/L lysine), YES (0.1% glucose) medium (0.5% yeast extract, 0.1% glucose, 3% glycerol, 225 mg/L adenine, 225 mg/L uracil, 225 mg/L leucine, 225 mg/L histidine, and 225 mg/L lysine), or synthetic minimal medium (EMM) with appropriate auxotrophic supplements (Petersen and Russell 2016).

#### Construction of the \( plb1 \Delta \) strain

A previously described recombinant polymerase chain reaction (PCR) method (Krawchuk and Wahls 1999) was used to generate the \( plb1::kanMX6 \) deletion cassette, in which the \( plb1 \) open reading frame sequence was replaced with the \( kanMX6 \) marker. To generate the \( plb1::kanMX6 \) cassette via PCR, the plasmid pFA6a-kanMX6 (Bahler et al. 1998) was used as the template DNA together with the oligonucleotide primer sets \( plb1-F1 \) (5′-CTT AAT CCA TCC AAG CGA -3′), \( plb1-R1 \) (5′-GGGGATCCGTCGACCTGCAG GTACGAAGATGCAAAAGATGTGC-3′), \( plb1-F2 \) (5′-GTTTAAACGAGCTGATCTCATGGAATCATTCAATGCAATTACAGG-3′), and \( plb1-R2 \) (5′-CAGTAAACTCAGTATCGC-3′). The PR109 strain was transformed with the \( plb1::kanMX6 \) cassette, and transformants were isolated on YES medium containing the antibiotic G418 to select for colonies containing \( kanMX6 \). Colony PCR was used to confirm that selected transformants lacked \( plb1 \) (Matsu et al. 2007).

The \( S. pombe \) strains \( plb1 \Delta \) cyr1 \( \Delta \) (YM628), \( plb1 \Delta pka1 \Delta \) (YM629), cyr1 \( \Delta \) pka1 \( \Delta \) (YM60), \( plb1 \Delta cg5 \Delta \) (YM761), \( plb1 \Delta cyr1 \Delta cg5 \Delta \) (YM807), \( plb1 \Delta rst2 \Delta \) (YM757), \( pka1 \Delta rst2 \Delta \) (YM220), and \( plb1 \Delta pka1 \Delta rst2 \Delta \) (YM809) were constructed by genetic crossing using standard yeast genetic techniques including random spore analysis (Ekwall and Thon 2017).
Fluorescence microscopy of GFP fusion protein

*S. pombe* cells were grown in YES liquid medium to the mid-log phase at 30 °C. GFP-tagged Plb1 and Pka1 were visualized in living cells, and images were taken by a BX51 microscope (Olympus) equipped with a DP74 digital camera (Olympus).

Preparation of cell lysates and detection of 13Myc fusion protein using immunoblotting

*S. pombe* cell lysates were prepared as previously described (Matsuo et al. 2004). Lysate proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), after which western blot analysis was performed using a Western Lightning enhanced chemiluminescence (ECL) Pro detection system (PerkinElmer) according to the supplier’s instructions. Mouse monoclonal anti-Myc (diluted 1:1000) and rabbit polyclonal anti-PSTAIRE (Cdc2; diluted 1:1000) antibodies were purchased from Santa Cruz Biotechnology. Horseradish-peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology) and anti-rabbit IgG antibody (Promega) were used as secondary antibodies.

Results

Pka1 overexpression rescues the KCl-sensitive phenotype of the *plb1Δ* strain

It has been proposed that the cAMP/PKA pathway is a downstream target of Plb1 during the osmotic stress response induced by a high concentration of KCl (Yang et al. 2003). To clarify how Plb1 might affect the cAMP/PKA pathway, we first analyzed cell growth in the presence of a high concentration of KCl in a *plb1Δ* strain with Pka1 overexpression and in a *pka1Δ* strain with Plb1 overexpression. Pka1 overexpression rescued the growth defect of *plb1Δ* in the presence of 1.2 M KCl (Fig. 1a), whereas Plb1 overexpression did not rescue the growth defect of *pka1Δ* (Fig. 1b). These results are compatible with the idea that the cAMP/PKA pathway is downstream of Plb1 in the context of responding to KCl stress.

*S. pombe* mutants lacking Plb1 and PKA are hypersensitive to hypertonic stress

We next analyzed the growth of single and double mutants under high-osmolarity stress. If Plb1 linearly affects the

| Table 1 *S. pombe* strains used in this study |
|-----------------------------------------------|
| **Strain** | **Genotype** | **Source** |
| PR109 | *h− leu1-32 ura4-D18* | P. Russell |
| YMP603 | *h− leu1-32 ura4-D18 plb1::kanMX6* | This study |
| YMP28 | *h− leu1-32 ura4-D18 cyr1::ura4* | Matsuo and Kawamukai (2017) |
| YMP36 | *h− leu1-32 ura4-D18 pka1::ura4* | Matsuo and Kawamukai (2017) |
| YMP628 | *h− leu1-32 ura4-D18 plb1::kanMX6 cyr1::ura4* | This study |
| YMP629 | *h− leu1-32 ura4-D18 plb1::kanMX6 pka1::ura4* | This study |
| YMP60 | *h− leu1-32 ura4-D18 cyr1::LEU2 pka1::ura4* | This study |
| YMP43 | *h− leu1-32 ura4-D18 git3::ura4* | This study |
| YMP886 | *h− leu1-32 ura4-D18 plb1::hphMX6 git3::ura4* | This study |
| YMP40 | *h− leu1-32 ura4-D18 cgs1::ura4* | Matsuo and Kawamukai (2017) |
| YMP58 | *h− leu1-32 ura4-D18 cyr1::LEU2 cgs1::ura4* | Matsuo and Kawamukai (2017) |
| YMP761 | *h− leu1-32 ura4-D18 plb1::kanMX6 cgs1::ura4* | This study |
| YMP807 | *h− leu1-32 ura4-D18 plb1::kanMX6 cyr1::LEU2 cgs1::ura4* | This study |
| YMP201 | *h− leu1-32 ura4-D18 rst2::natMX6* | This study |
| YMP757 | *h− leu1-32 ura4-D18 plb1::kanMX6 rst2::natMX6* | This study |
| YMP220 | *h− leu1-32 ura4-D18 pka1::ura4 rst2::natMX6* | This study |
| YMP809 | *h− leu1-32 ura4-D18 plb1::kanMX6 pka1::ura4 rst2::natMX6* | This study |
| YMP19 | *h− leu1-32 ura4-D18 plb1::GFP(S65T)-kanMX6* | Matsuo and Kawamukai (2017) |
| YMP893 | *h− leu1-32 ura4-D18 plb1::hphMX6 pka1-GFP(S65T)-kanMX6* | This study |
| YMP48 | *h− leu1-32 ura4-D18 cyr1::ura4 pka1-GFP(S65T)-kanMX6* | Matsuo and Kawamukai (2017) |
| YMP56 | *h− leu1-32 ura4-D18 cgs1::ura4 pka1-GFP(S65T)-kanMX6* | This study |
| YMP21 | *h− leu1-32 ura4-D18 pka1-13Myc-kanMX6* | This study |
| YMP915 | *h− leu1-32 ura4-D18 plb1::hphMX6 pka1-13Myc-kanMX6* | This study |
| YMP916 | *h− leu1-32 ura4-D18 cyr1::ura4 pka1-13Myc-kanMX6* | This study |
| YMP917 | *h− leu1-32 ura4-D18 cgs1::ura4 pka1-13Myc-kanMX6* | This study |
| YMP19 | *h− leu1-32 ura4-D18 plb1::hphMX6 git3::ura4* | This study |
| YMP893 | *h− leu1-32 ura4-D18 plb1::hphMX6 pka1-GFP(S65T)-kanMX6* | This study |
| YMP48 | *h− leu1-32 ura4-D18 cyr1::ura4 pka1-GFP(S65T)-kanMX6* | Matsuo and Kawamukai (2017) |
| YMP56 | *h− leu1-32 ura4-D18 cgs1::ura4 pka1-GFP(S65T)-kanMX6* | This study |
| YMP21 | *h− leu1-32 ura4-D18 pka1-13Myc-kanMX6* | This study |
| YMP915 | *h− leu1-32 ura4-D18 plb1::hphMX6 pka1-13Myc-kanMX6* | This study |
| YMP916 | *h− leu1-32 ura4-D18 cyr1::ura4 pka1-13Myc-kanMX6* | This study |
| YMP917 | *h− leu1-32 ura4-D18 cgs1::ura4 pka1-13Myc-kanMX6* | This study |
KCl (Fig. 2a). The double mutants KCl, it showed the KCl-sensitive phenotype on 1.2 M sorbitol, while plb1Δ and pka1Δ cells grew normally (Fig. S2). All the single and double mutants showed normal growth with 0.1 M CaCl₂ or 0.2 M NaCl (Fig. S2), indicating that Plb1 is not involved in responding to CaCl₂.

Gain of functional Pka1 partially recues growth defects of plb1Δ, cyr1Δ, and cyr1Δ plb1Δ under hypertonic stress conditions

It has been shown that cgslΔ rescued growth defects associated with the CaCl₂- and TBZ-sensitive phenotypes of the cyr1Δ strain (Matsuo and Kawamukai 2017; Tanabe et al. 2019). These results suggest that gain of functional Pka1 would rescue the stress-sensitive phenotype of cyr1Δ, and also of plb1Δ if Plb1 is indeed upstream of Pka1 in the context of hypertonic stress response. We investigated whether deleting cgsl suppresses the growth defects of plb1Δ, cyr1Δ, and plb1Δ cyr1Δ upon KCl stress. If Plb1 is required for modulating Pka1 activity upon KCl stress, deleting cgsl would completely rescue the hypertonic-stress-sensitive phenotype of plb1Δ. As could be expected, we found that cgslΔ significantly rescued the KCl-sensitive phenotype of cyr1Δ with both 1.2 and 1.5 M KCl (Fig. 3a). Additionally, cgslΔ rescued the growth defect of plb1Δ with 1.2 M KCl but not with 1.5 M KCl (Fig. 3b). Intriguingly, cgslΔ partially rescued the growth defect of plb1Δ cyr1Δ upon KCl stress and fully rescued the sorbitol-sensitive phenotype (Fig. 3b). These results indicate that while Pka1 activity is required for hypertonic stress response, Plb1 is not dispensable when responding to more serve hypertonic stress.
Fig. 2 Plb1 and the cAMP/PKA pathway have cooperative functions. 

**a** Wild type (PR109), \( plb1 \Delta \) (YMP603), \( cyr1 \Delta \) (YMP28), \( plb1 \Delta cyr1 \Delta \) (YMP628), \( pka1 \Delta \) (YMP36), \( plb1 \Delta pka1 \Delta \) (YMP629), and \( cyr1 \Delta pka1 \Delta \) (YMP60) cells were spotted onto the YES, YES + 0.5 M KCl, YES + 1.2 M KCl, and YES + 2 M sorbitol plates and incubated at 30 °C for 3–4 days (YES for 3 days and others for 4 days).

**b** Wild type (PR109), \( plb1 \Delta \) (YMP603), \( git3 \Delta \) (YMP43), and \( plb1 \Delta git3 \Delta \) (YMP886) cells were spotted onto the YES plate in the presence or absence of KCl and incubated at 30 °C for 3–5 days (YES for 3 days and YES + KCl for 5 days).
Loss of functional Rst2 rescues growth defects of \textit{plb1}\textsuperscript{Δ}, \textit{pka1}\textsuperscript{Δ}, and \textit{plb1}\textsuperscript{Δ} \textit{pka1}\textsuperscript{Δ} under hypertonic stress conditions

The transcription factor Rst2 is negatively regulated by Pka1 (Inamura et al. 2021; Takenaka et al. 2018). Loss of functional Rst2 rescues the phenotypes such as high expression of \textit{mug14} and \textit{ste11} mRNAs of the \textit{pka1}\textsuperscript{Δ} strain (Higuchi et al. 2002; Inamura et al. 2021). To investigate the role of Rst2 in hypertonic stress response, we next tested if \textit{rst2}\textsuperscript{Δ} rescues the growth defects of \textit{plb1}\textsuperscript{Δ}, \textit{pka1}\textsuperscript{Δ}, and \textit{plb1}\textsuperscript{Δ} \textit{pka1}\textsuperscript{Δ} under the hypertonic stress conditions. Our results showed that \textit{rst2}\textsuperscript{Δ} rescued the KCl-sensitive phenotype of \textit{plb1}\textsuperscript{Δ} with 1.0 M KCl but did not with 1.5 M KCl (Fig. 4a). Meanwhile, \textit{rst2}\textsuperscript{Δ} completely rescued the growth defect of \textit{pka1}\textsuperscript{Δ} on 1.5 M KCl (Fig. 4b). Finally, \textit{rst2}\textsuperscript{Δ} partially rescued the growth defects of \textit{plb1}\textsuperscript{Δ} \textit{pka1}\textsuperscript{Δ} on KCl-containing plates and fully rescued the growth defects on sorbitol-containing plates (Fig. 4c). These results indicate that Rst2 is the downstream target of the cAMP/PKA pathway in the context of responding to KCl stress, and that Plb1 is not dispensable when responding to higher levels of KCl.

\textbf{Plb1 does not regulate Pka1 localization}

We next analyzed the localization of Plb1 in the presence of KCl stress. We first made the \textit{plb1-GFP} strain by tagging GFP at the C-terminus of Plb1, but we did not observe GFP fluorescence (data not shown). To overcome this problem, we next constructed a GFP-Plb1-expressing plasmid, in which Plb1 tagged with GFP at its N-terminus is expressed under the \textit{nmt41} promoter. GFP-Plb1 rescued the growth of the \textit{plb1}\textsuperscript{Δ} strain upon KCl stress, indicating that GFP-Plb1 is functional (Fig. S3). Plb1 localized at the endoplasmic reticulum (ER) and highly accumulated as the dots under normal conditions. Upon KCl stress, Plb1 localization to the ER did not change, but its localization to the dots decreased (Fig. 5a).

Because the KCl-sensitive phenotype of \textit{plb1}\textsuperscript{Δ} is dependent on Pka1 activity, we next analyzed whether Pka1 localization is regulated by Plb1. Pka1-GFP mainly
localizes in the nucleus and diffusely in the cytoplasm in wild-type cells (Matsuo et al. 2008). In the presence of inactive endogenous Pka1 in the cyr1Δ strain, Pka1-GFP only localized to the cytoplasm. In the context of constitutive Pka1 activation in cgs1Δ under normal conditions, Pka1-GFP localized to the nucleus and diffusely to the cytoplasm as observed in the wild-type strain (Matsuo et al. 2008 and Fig. 5b). In plb1Δ, Pka1-GFP exhibited the same localization as in wild type and cgs1Δ cells. Under KCl stress, the localization of Pka1-GFP changed from the nucleus to the cytoplasm in wild-type cells, and still localized only in the cytoplasm in cyr1Δ. In cgs1Δ, the nuclear localization of Pka1-GFP decreased, and we observed accumulation as dots distributed through the cytoplasm. Under KCl-stress conditions in plb1Δ, Pka1-GFP changed its localization to the cytoplasm, as observed in wild-type

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**Fig. 4** The deletion of the rst2 gene rescues the plb1Δ, pka1Δ, and plb1Δ pka1Δ strains. a Wild type (PR109), plb1Δ (YMP603), rst2Δ (YMP201), and plb1Δ rst2Δ (YMP757) cells were spotted onto the YES in the presence or absence of KCl plate and incubated at 30 °C for 4–6 days (YES and YES + 1.0 M KCl for 4 days, YES + 1.2 M KCl and YES + 1.5 M KCl for 6 days). b Wild type (PR109), pka1Δ (YMP36), rst2Δ (YMP201), and pka1Δ rst2Δ (YMP220) cells were spotted onto the YES and YES + 1.5 M KCl plates and incubated at 30 °C for 4 to 6 days (YES for 4 days and YES + 1.5 M KCl for 6 days). c Wild type (PR109), plb1Δ (YMP603), pka1Δ (YMP36), plb1Δ pka1Δ (YMP629), rst2Δ (YMP201), and plb1Δ pka1Δ rst2Δ (YMP809) cells were spotted onto the YES in the presence or absence of KCl plate and incubated at 30 °C for 3–6 days (YES for 3 days, YES + 2 M sorbitol for 4 days, YES + 1.0 M KCl for 5 days, and YES + 1.2 M KCl and YES + 1.5 M KCl for 6 days).
Fig. 5 Plb1 does not affect the localization of Pka1. a Wild type (PR109) cells harboring pREP41GFP (vector) or pREP41GFP-plb1 were cultured for 1 day in EMMU at 30 °C to induce expression from the nmt1 promoter. Cells were resuspended into EMMU in the presence or absence of 1.2 M KCl, cultured for 8 h at 30 °C, and observed GFP-Plb1 by fluorescent microscopy. Scale bar: 10 µm. b Pka1-GFP (YMP19), plb1Δ Pka1-GFP (YMP893), cyr1Δ Pka1-GFP (YMP48), and cgs1Δ Pka1-GFP (YMP56) cells were cultured for 24 h in the presence or absence of 1.2 M KCl and observed by fluorescent microscopy. Scale bar: 10 µm. c Pka1-13Myc (YMP21), plb1Δ Pka1-13Myc (YMP915), cyr1Δ Pka1-13Myc (YMP916), and cgs1Δ Pka1-13Myc (YMP917) cells were cultured for 24 h in the presence or absence of 1.2 M KCl. Pka1-13Myc was detected with the anti-Myc antibody. The anti-PSTAIRE antibody was used as an internal loading control for Cdc2.
cells (Fig. 5b, S4). These results indicate that Plb1 does not regulate Pka1 localization, suggesting that Plb1 likely is not involved in the regulation of Pka1 activity.

We next tested whether deleting plb1 would affect Pka1 phosphorylation. Pka1 is phosphorylated at threonine 356 in cyr1Δ, whereas this phosphorylation site is not detected in wild type or cgs1Δ strains (Gupta et al. 2011a; McInnis et al. 2010). Under normal conditions, Pka1-13Myc was detected in a non-phosphorylated form in wild type and cgs1Δ cells, while it was phosphorylated in the cyr1Δ strain (Fig. 5c). However, Pka1-13Myc was not phosphorylated under normal conditions in plb1Δ (Fig. 5c). This result indicates that Plb1 does not affect Pka1 phosphorylation. We attempted to analyze the phosphorylation profile of Pka1 under KCl-stress

| No stress | +0.1 M KCl | +0.3 M KCl | +0.5 M KCl |
|-----------|------------|------------|------------|
| Wild type |            |            |            |
| plb1Δ     |            |            |            |
| pka1Δ     |            |            |            |
| plb1Δ pka1Δ |        |            |            |

| Wild type | 3% glucose | 0.1% glucose |
|-----------|------------|--------------|
| GFP-Plb1  | Green      | Green        |
| DIC       | DIC        | DIC          |

Fig. 6 Glucose limitation enhanced the KCl-sensitive phenotype of the plb1Δ strain. a Wild type, plb1Δ, pka1Δ, and plb1Δ pka1Δ cells were spotted onto YES (3% glucose) or YES (0.1% glucose) in the presence or absence of KCl plate and incubated at 30 °C for 4–6 days [YES (3% glucose) for 4 days and YES (0.1% glucose) for 6 days]. b Wild type (PR109) cells harboring pREP41GFP (vector) or pREP41GFP-plb1 were cultured for 1 day in EMMU at 30 °C to induce expression from the nmt1 promoter. Cells were resuspended into EMMU (3% glucose) or EMMU (0.1% glucose), cultured for 8 h at 30 °C, and observed GFP-Plb1 by fluorescent microscopy. Scale bar: 10 µm
conditions, but we were unable to detect Pka1 clearly, likely due to the instability of Pka1.

Glucose limitation induces growth defects in wild type and plb1Δ upon the KCl stress

Because the cAMP/PKA pathway is tightly regulated by extracellular glucose concentration (Byrne and Hoffman 1993; Inamura et al. 2021; Tanabe et al. 2020; Welton and Hoffman 2000), we next analyzed whether glucose limitation induces the KCl-sensitive phenotype. Wild type, plb1Δ, and pka1Δ grew, but plb1Δ pka1Δ did not grow, on glucose-rich (3% glucose) medium with 0.3 M KCl (Fig. 6a). Additionally, plb1Δ, pka1Δ, and plb1Δ pka1Δ exhibited a KCl-hypersensitive phenotype on low-glucose (0.1% glucose) medium than on normal glucose (3% glucose) medium (Fig. 6a).

Next, we analyzed the localization of GFP-Plb1 under the glucose-limited conditions. Localization of GFP-Plb1 showed the same pattern, localization at the ER and in the dots, in the glucose-rich (3% glucose) and glucose-limited (0.1% glucose) media, but the dots structure was decreased in the glucose-limited media (Fig. 6b), indicating that glucose levels affect Plb1 localization.

Gain of functional Pka1 rescues the KCl-sensitive phenotype of plb1Δ in glucose-limited media

We next tested whether gain of functional Pka1 affects growth during KCl stress under glucose-limited conditions. To do this, we compared the effect of cgs1 deletion in plb1Δ and plb1Δ cyr1Δ under glucose-limited conditions. While plb1Δ and plb1Δ cyr1Δ significantly exhibited the KCl-sensitive phenotype with 0.3 M KCl, plb1Δ cgs1Δ and plb1Δ cyr1Δ cgs1Δ grew on 0.5 M KCl under glucose-limited conditions (Fig. 7a). These results indicate that increased Pka1 activity overcomes sensitivity to KCl stress in plb1Δ under glucose-limited conditions.

We finally analyzed whether rst2Δ rescues the KCl-sensitive phenotype in the context of glucose limitation. The plb1Δ and plb1Δ pka1Δ strains exhibited the KCl-sensitive phenotype with 0.3 M KCl under glucose-limited conditions. Under the same conditions, the plb1Δ rst2Δ and plb1Δ pka1Δ rst2Δ strains grew, and the plb1Δ pka1Δ rst2Δ strain also grew with 0.5 M KCl under glucose-limited conditions (Fig. 7b). Therefore, loss of functional Rst2 rescued growth during KCl stress under glucose-limited conditions.

Discussion

Cells encounter various environmental stresses and use signal transduction systems to adapt to such stresses. In this study, we focused on the relationship between KCl stress and the cAMP/PKA pathway in fission yeast. Our findings show that Plb1 (a phospholipase B homolog) and the cAMP/PKA pathway play a cooperative role in the KCl-stress response. This idea contradicts the previously proposed idea that Plb1 acts upstream of the cAMP/PKA pathway (Yang et al. 2003), which was based on the observation that overexpression of Gpa2 or Git3 rescued the KCl-sensitive phenotype of plb1Δ. We also observed that Pka1 overexpression rescued the KCl-sensitive phenotype of both pka1Δ (Fig. 1). However, our observations that plb1Δ enhanced the KCl-sensitive phenotype of both pka1Δ (Fig. 2a) and of git3Δ (Fig. 2b) indicate that Plb1 and the cAMP/PKA pathway act separately to counteract KCl stress. Git3 is the G-protein-coupled receptor that senses extracellular glucose and transfers the signal through Gpa2 to the cAMP/PKA pathway (Fig. S1), which is downregulated by glucose limitation and upregulated by higher glucose concentration (Hoffman 2005; Inamura et al. 2021; Matsuo et al. 2008; Tanabe et al. 2020; Welton and Hoffman 2000). It is therefore unlikely that Plb1 interacts with the glucose-sensing cAMP/PKA pathway due to our observation that deletion of git3 enhanced the KCl-sensitive phenotype of plb1Δ. Growth inhibition by KCl in plb1Δ was also enhanced by glucose limitation (0.1% glucose) when compared to that by normal conditions (3% glucose) (Fig. 6a, 7) supporting the idea that the cAMP/PKA pathway works independently from Plb1.

Cgs1 negatively regulates Pka1 by forming a heterotrimer with Pka1, preventing it from being active. Therefore, deletion of cgs1 results in constitutive Pka1 activity (Gupta et al. 2011a). Our genetic observation regarding cgs1 further strengthen the idea that the glucose-cAMP-PKA pathway contributes to the KCl-stress response: (1) Deletion of cgs1 rescued the 1.5 M KCl sensitive phenotype of cyr1Δ to wild-type levels (Fig. 3a). (2) Growth defects by 1.2 M KCl of plb1Δ and plb1Δ cyr1Δ was alleviated but not to wild-type levels (Fig. 3b). (3) Deletion of cgs1 rescued the enhanced KCl-sensitive phenotype (0.5 M) of plb1Δ and plb1Δ cyr1Δ under glucose-limited conditions.
conditions (Fig. 7a). Together, these results support the idea that the glucose-cAMP-PKA pathway works independently of Plb1 to respond to KCl stress.

The transcription factor Rst2 is negatively regulated by Pka1 (Higuchi et al. 2002; Inamura et al. 2021). Our observation of rst2Δ combined with various other deletion mutants indicate that Rst2 is important for KCl tolerance. Deleting rst2 partially rescued the growth defects of plb1Δ and plb1Δ pka1Δ with 1.0 M KCl, whereas it fully rescued pka1Δ with 1.5 M KCl (Fig. 4). These observations are conserved under glucose limitation. We also observed that plb1Δ cyr1Δ cgs1Δ and plb1Δ pka1Δ rst2Δ exhibited the same growth rate under KCl stress as cgs1Δ and rst2Δ, respectively (Fig. 7). These results also support the idea that the glucose-cAMP-PKA pathway plays a major role in the KCl-stress response.

Localization of Pka1 changed from the nucleus to the cytoplasm with 1.2 M KCl in both wild type and plb1Δ (Fig. 5b). Loss of functional Plb1 did not affect the phosphorylation profile of Pka1 (Fig. 5c), consistent with the idea that Plb1 does not directly regulate the cAMP/PKA pathway. Pka1 is hyperphosphorylated in the cytoplasm when it is constitutively inactivated via deletion of cyr1 (Fig. 5b, c, and Gupta et al. 2011a, b; Matsuo et al. 2008; McInnis et al. 2010). In the Pka1-activated cgs1Δ strain, we could not detect phosphorylated Pka1 by western blotting, but we observed Pka1 localized to the cytoplasm and nucleus upon KCl stress (Fig. 5b, c), as observed previously (Matsuo et al. 2008; McInnis et al. 2010). Cytoplasmic localization of Pka1 prevents it from negatively regulating Rst2, and Rst2 therefore remains in an active form. Our results consistently indicate that the glucose-sensing pathway mediated by cAMP/PKA regulates Rst2 to respond to KCl stress.

We also would like to highlight our observation that glucose limitation enhanced the KCl-sensitive phenotype of pka1Δ and plb1Δ pka1Δ (Fig. 6a), suggesting that cAMP-PKA is not alone in responding to glucose limitation. Responding to glucose limitation is likely additionally mediated by other glucose-responding pathways such as the SAPK and TORC2 pathways (Cohen et al. 2014; Fraile et al. 2020; Ikai et al. 2011; Saitoh et al. 2015; Sanchez-Mir et al. 2018; Zuin et al. 2010). While we have not conducted any experiments investigating these pathways in the context of glucose limitation, many other works on the SAPK pathway clearly indicate it is important for the KCl-stress response (Matsuo and Kawamukai 2017; Matsuo et al. 2008; Shieh et al. 1997, 1998; Shiozaki and Russell 1996; Stiefel et al. 2004; Yang et al. 2003). Deleting styl causes sensitivity to 0.6 M KCl (Nunez et al. 2009), while cells lacking pka1 tolerates 0.7 M KCl (our unpublished data). The SAPK pathway is responsible for responding to lower concentrations of KCl, while cAMP/PKA is responsible for responding to higher concentrations (Matsuo and Kawamukai 2017; Matsuo et al. 2008). While loss of a functional SAPK pathway results in oxidative-, osmotic-, and heat-stress-sensitive phenotypes and decreases mating efficiency (Shieh et al. 1998; Shiozaki and Russell 1996), plb1Δ and pka1Δ do not show these phenotypes (Figs. 2a, 3b, 4c, S2 and Yang et al. 2003) and display a high frequency of mating and sporulation (Gupta et al. 2011a, b; Maeda et al. 1994; Yang et al. 2003). The SAPK pathway, cAMP/PKA pathway, and Plb1 are commonly involved in responding to only KCl stress. It is possible that the SAPK pathway is modulated by Plb1 since it has been shown that mRNA expression of gpd1 decreases in plb1Δ under KCl stress (Yang et al. 2003) and that it is upregulated by KCl through the SAPK pathway (Shieh et al. 1997; Wilkinson et al. 1996).

Our findings show that the glucose-activated cAMP/PKA pathway regulates Rst2 to produce a hypertonic stress response. In S. pombe, this signal transduction pathway is particularly important when responding to higher concentrations of KCl. Our study demonstrates that Plb1 and the cAMP/PKA pathway cooperatively but independently function to respond KCl-induced hypertonic stress.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00294-022-01253-z.

Acknowledgements The authors also thank all the members of the laboratory for helpful support and scientific advice. We would like to thank Editage (www.editage.com) for English language editing.

Author contributions YM planned this study, designed the experiments, carried out the experiments, made the yeast strains, and analyzed the data; SM planned this study; MK analyzed the data and provided advice. YM wrote the original draft. YM and MK reviewed and edited the original draft.

Funding The authors thank the faculty of Life and Environmental Sciences in Shimane University for help in financial support for publication. This work was supported by a JSPS KAKENHI Grant Number JP18K05438 (to YM) and JP19K222831 (to MK).

Declarations Conflict of interest The authors declare that they have no conflict of interest with the content of this article.

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