Korean Journal of Microbiology (2018) Vol. 54, No. 4, pp. 309-319
DOI https://doi.org/10.7845/kjm.2018.8068

pISSN 0440-2413
eISSN 2383-9902

A putative prolyl tRNA synthetase is involved in pheromone induction in *Schizosaccharomyces pombe*

Daemyung Kim*

Department of Biomedical Science, Cheongju University, Cheongju 28160, Republic of Korea

*Schizosaccharomyces pombe*의 pheromone 유도와 연관된 prolyl tRNA synthetase

김대명*

청주대학교 바이오메디컬학과

(Received September 14, 2018; Revised October 1, 2018; Accepted October 3, 2018)

Previously, six *Schizosaccharomyce pombe* mutants that induce pheromone even in the presence of nitrogen source were isolated from a bank of temperature sensitive mutants. In this report, one of these mutants, *pws6* was further characterized. The pheromone induction in *pws6* mutant cells was specific to nutrient: the M-factor pheromone was induced without nitrogen starvation but not without glucose starvation. This result suggests that the *pws6* mutant might have a specific defect in the pathway for nitrogen starvation. The *pws6* mutant induces P-factor pheromone as well as M-factor without starvation of nitrogen in temperature sensitive mode, suggesting that the pheromone induction phenotype of *pws6* mutation is not cell-type specific. From cloning of the $pws6^+$ gene by complementation of the temperature sensitive growth defect, three plasmids containing 8.1 kb, 3.3 kb, and 4.8 kb yeast DNA were recovered. These plasmids complement the growth defect of the *pws6* mutant by 100%, 70%, and 10~20%, respectively. The abilities of these plasmids to complement pheromone induction phenotype of *pws6* mutant cells were correlated well with the efficiencies of complementation of the growth defect. With comparison of their open reading frames to the complementation efficiencies, it is concluded that the open reading frame, SPBC19C7.06 is responsible for the complementation of temperature sensitive phenotype of the *pws6* mutant. This open reading frame, named *prs1*, contains one long exon with no intron and encodes a putative prolyl tRNA synthetase. The putative Prs1 protein exhibits significant similarities to the prolyl tRNA synthetases of other species.

Keywords: *Schizosaccharomyces pombe*, nitrogen starvation, pheromone induction, prolyl tRNA synthetase

***For correspondence.** E-mail: kim204@gmail.com;
Tel.: +82-43-229-8563; Fax: +82-43-232-8799

Fission yeast *Schizosaccharomyces pombe* is a unicellular organism that has two alternative developmental programs. Under nutrient-rich condition, the fission yeast normally proliferates by mitotic division in haploid state. When they are starved for nutrients, especially nitrogen, *S. pombe* cells initiate sexual development. Cells cease to divide and haploid cells of the two opposite mating types, termed h^+ and h^-, conjugate and form zygotes. The zygotes proceed to meiosis and form four haploid spores. If the zygotes are shifted to rich medium before they are committed to meiosis, they can proliferate as diploid cells. The sexual development process is controlled by two diffusible peptide hormones; P-factor and M-factor are produced by h^+ and h^- cells, respectively.

M-factor is a nonapeptide whose C-terminal residue is carboxymethylated and S-farnysylated (Davey, 1992; Wang *et al.*,

1994). Three genes, *mfm1*, *mfm2*, and *mfm3*, that have strong sequence similarity, encode M-factor as a large precursor that contains intron (Davey, 1992; Kjaerulff *et al.*, 1994). P-factor, encoded by *map2* gene, is an unmodified peptide of 23 amino acids (Imai and Yamamoto, 1994). Receptors for M-factor and P-factor are encoded by *map3* and *mam2* gene located on the h^+ and h^- cells, respectively (Kitamura and Shimoda, 1991; Tanaka *et al.*, 1993).

Nutritional starvation is required for induction of sexual differentiation. Nitrogen and carbon sources are two major signals for nutritional starvation. For carbon starvation, cAMP plays a critical role for the transmission of the starvation signal to gene expression (Watanabe, 1988). The presence of glucose is monitored by Git3, a G-protein coupled receptor at the plasma membrane (Welton and Hoffman, 2000). Gpa2, a G-protein α-subunit, is likely to mediate nutritional signal to cAMP (Isshiki *et al*., 1992). cAMP is synthesized by adenylate cyclase and degraded by cAMP phosphatase. Both adenylate cyclase (Git2/Cyr1) and cAMP phosphatase (Pde1/Cgs2) regulate the cAMP signaling (Kawamukai *et al.*, 1991; Mochizuki and Yamamoto, 1992; Byrne and Hoffman, 1993). The intracellular level of cAMP is kept high when glucose is sufficient.

Carbon starvation causes a lowered intracellular cAMP level and results in down-regulation of cAMP dependent protein kinase A (PKA) (Maeda *et al*., 1994). PKA inhibits the transcription factor Rst2 by phosphorylation and exclusion of Rst2 from the nucleus (Kunitomo *et al.*, 2000; Higuchi *et al.*, 2002). Thus, a decrease of PKA activity leads to the translocation of this zinc finger protein into nucleus and bind the STREP motif in the *ste11* promoter to induce expression (Higuchi *et al.*, 2002). *Ste11* encodes HMG-type transcription factor which binds to the TR box found in mating-type specific genes (Sugimoto *et al*., 1991; Aono *et al*., 1994). Ste11 acts as a developmental switch for sexual differentiation and directly stimulates production of pheromones and their receptors (Davey, 1998).

Nitrogen availability is another determinant of cell growth and differentiation. Nitrogen starvation also causes transcriptional activation of *ste11* gene and induces sexual differentiation (Mata and Bahler, 2006). In this case, the highly conserved Target of Rapamycin (TOR) pathway plays a central role in the regulation of cell growth and differentiation. TOR exists in two distinct complexes, termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (Loewith *et al.*, 2002; Wullschleger *et al*., 2006). Disruption of TORC1 in many eukaryotes, including yeast, results in cellular phonotype that resembles nitrogen starvation (Zoncu *et al.*, 2011; Kupiec and Weisman, 2012). TORC1 plays a major role in mediating environmental nitrogen insufficiency to the starvation responsive processes.

As in mammalian cells and budding yeast, *S. pombe* has two TOR complexes (Kawai *et al.*, 2001; Weisman and Choder, 2001). TORC1 is composed of Tor2 kinase and Mip1 (Raptor), whereas Tor1 kinase interacts with Ste20 (Rictor) and Sin1 to form TORC2 (Hayashi *et al*., 2007; Matsuo *et al.*, 2007). TORC1 is essential for vegetative growth and represses sexual differentiation under nitrogen-rich conditions (Alvarez and Moreno, 2006; Hayashi *et al.*, 2007; Weisman *et al.*, 2007). Inactivation of the Tor2 kinase (TORC1) mimics nitrogen starvation and activates the sexual development pathway (Matsuo *et al.*, 2007; Weisman *et al.*, 2007). On the other hand, TORC2 is not essential for growth but is required for survival under stress conditions, including nutritional starvation, high temperatures, oxidative or osmotic stress, and DNA damage or replication stresses (Kawai *et al.*, 2001; Weisman and Choder, 2001; Schonbrun *et al.*, 2009, 2013). Thus, Tor2 in TORC1 normally mediates a signal of nitrogen availability to sexual differentiation in *S. pombe* (Matsuo *et al*., 2007).

A number of downstream factors of *S. pombe* TORC1 have been identified. Among these, Mei2 and Ste11 are the direct phosphorylation targets of TORC1 that regulate sexual differentiation. Mei2, a master regulator of meiosis, which is involved in the regulation of G1 arrest and conjugation, interacts physically with Tor2 (Alvarez and Moreno, 2006). Mip1, an essential component of mTORC1 was originally isolated as a genetic interactor of Mei2 and facilitates function of Mei2 (Shinozaki-Yabana *et al.*, 2000). Tor2 impairs meiosis by phosphorylation of Mei2 and interfering with its function. Phosphorylation of Mei2 induces its polyubiquitination and proteasomal degradation, leading to suppression of sexual differentiation (Otsubo *et al.*, 2014).

As well as Mei2, Tor2 exists in complex with Ste11, a developmental switch for sexual differentiation and phosphorylates these two essential regulators of different phases of sexual differentiation (Alvarez and Moreno, 2006). N-terminal fragment of Ste11 was phosphorylated by immune-precipitated TORC1

(Otsubo *et al.*, 2017). Inactivation of TORC1 causes nuclear accumulation of Ste11, suggesting that TORC1 regulates nuclear localization of Ste11 by phosphorylation (Valbuena and Moreno, 2010).

In addition to Mei2 and Ste11, TORC1 positively regulates the phosphorylation of GATA transcription factor Gaf1 by inhibiting the PP2A-like phosphatase Ppe1 (Laor *et al.*, 2015). When TORC1 is inactivated under nitrogen starvation condition, Gaf1 is dephosphorylated and translocated into the nucleus. Gaf1 positively regulates genes that are induced early in response to nitrogen stress, such as the 2-oxoglutarate-Fe(II) dependent oxygenase isp7, and inhibits later response genes, such as ste11 (Kim *et al.*, 2012; Laor *et al.*, 2015). However, this nucleus localization of Gaf1 is transient. Relocalization of Gaf1 into the cytoplasm may induce the transcription of ste11 thereby allowing sexual differentiation (Laor *et al.*, 2015).

Meanwhile, how the information of nitrogen availability is transmitted to TORC1 is largely unknown. In mammalian cells, the Ras superfamily G-protein, Rheb, regulates mTORC1 positively whereas the tumor suppressor proteins, TSC1 and TSC2, negatively control the functions through inactivation of Rheb (Corradetti and Guan, 2006; Huang and Manning, 2008). Functional homologues of TSCs and Rheb have been identified in fission yeast. Like the mammalian system, fission yeast TSC proteins (Tsc1 and Tsc2) negatively regulate TORC1 activity via inhibition of fission yeast Rheb protein (Rhb1) (van Slegtenhorst *et al.*, 2005; Uritani *et al.*, 2006; Urano *et al.*, 2007). Disruption of Tsc1 or Tsc2 leads to impaired amino acid uptake and causes defect in sexual development upon nitrogen starvation (Matsumoto *et al.*, 2002; van Slegtenhorst *et al.*, 2004). Thus, the Tsc1-Tsc2 complex and Rhb1 act as an upstream player of TORC1 in fission yeast. Recently, it was shown that Gtr1 and Gtr2, the Rag GTPase in *S. pombe*, function downstream of Vam6, a GTP-exchange factor, and upstream of TORC1 (Valbuena *et al.*, 2012a). The conserved pathway with Vam6 and Gtr1-Gtr2 induces cellular growth and represses sexual differentiation by activating the TORC1 in response to amino acids. The Vam6 and Gtr1-Gtr2 pathway may be another upstream pathway of *S. pombe* TORC1.

To gain insights into the question of how nutritional starvation in *S. pombe* causes induction of the pheromone, my laboratory has previously isolated temperature sensitive (ts^-) mutants that induce pheromone without nutritional starvation and named these mutants *pws1* to *pws6* (Jun and Kim, 2008). In this study, one of these mutants, *pws6,* was further characterized. Cloning of the $pws6^+$ gene by complementation of the growth defect showed that the cloned gene is encoding prolyl tRNA synthetase, implying the significance of tRNA metabolism for nutrient-dependent signal transduction in fission yeast.

Materials and Methods

Yeast strains, media and growth conditions

S. pombe standard wild type strains, 972 (h^-) and 975 (h^+) were used in this study. All of the ts^- mutants were derived from the wild type strains, 972. For recombination of *leu1-32*$^-$ mutation, ED666 (h^+, *leu1-32*$^-$, *ura4-D18*$^-$, *ade6-M210*$^-$) was used. *E. coli* strain JA226 (*RecBC*, *leuB6*, *trpES*, *hsdR*$^-$, *hsdm*$^+$, *lacYc600*) was used to recover the plasmid from yeast. Complete medium YE and minimal medium PM were prepared according to Alfa *et al.* (1993). MB medium, prepared according to Okazaki *et al.* (1990), was used for yeast transformation. In sporulation experiments, SPA (1% glucose, 0.1% $KHPO_4$, vitamin mixture, and 1.5% agar) was used. Wild type was grown at 23°C. *Ts*$^-$mutants were grown at 23°C as a permissive temperature and 37°C as a non-permissive temperature.

Northern blot analysis

Yeast cells were grown to mid-log phase and the total RNA was prepared as described in Nischt *et al*. (1986). Total RNA was analyzed via Northern hybridization as previously described (Jun and Kim, 2008). To detect transcription of the M-factor pheromone (*mfm1*), single stranded RNA probe was transcribed from pSK2 as previously described (Jun and Kim, 2008). For detection of the P-factor pheromone transcription, *map2* gene (from pMap2, kind donation by Dr. Yamamoto, Tokyo University) was amplified by PCR using KBK-F (5'-CGAAGCTTGCTCT CAAATTTGGCAGT-3') and KBK-R (5'-CGGGATCCATG AAGATCACCGCTGT-3') primers. The amplified fragment was subcloned into *Hin*dIII and *Bam*HI sites of pBluescript SK+, generating a plasmid pKBK20. The plasmid was linearized by digestion with *Xba*I and transcribed with SP6 RNA polymerase in the presence of digoxigenin-11 UTP according to the manual

of the manufacturer (Boehringer Mannheim). Not incorporated ribonucleotide triphosphate was removed by repeated ethanol precipitations and the labeled RNA was used as a probe for the transcription of P-factor pheromone.

Yeast transformation

S. pombe cells were transformed with plasmids as described in Okazaki *et al.* (1990). Briefly, yeast cells were grown in MB medium to mid-log phase, harvested and resuspended in 0.1 M lithium acetate. The resuspension was incubated at 23°C for 60 min and plasmid DNA (1 μg) was added. The tube was mixed by gentle vortexing and added 290 μl of 50% PEG. After incubation at 23°C for 60 min, cells were heat-shocked at 43°C for 15 min. Cells were harvested and resuspended in 10 ml of YE medium and incubated for 2 h. Aliquots were plated onto selective PM plates supplemented with 150 μg adenine and incubated 23°C for 7 days.

Plasmid recovery from yeast

Plasmid of yeast was recovered by preparing total DNA from yeast, transforming recombination deficient *E. coli* with the total DNA, and preparing plasmid DNA from the transformed *E. coli* cells. Total DNA from *S. pombe* cells was isolated as described in Hoffman and Winston (1987). In short, yeast cells were cultured at 23°C to mid-log phase. Cells were harvested and resuspended in breaking buffer (10 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% SDS, 2% Triton X-100). An equal volume of phenol/chloroform (1:1 v/v) and 0.3 g of glass bead were added to the resuspension. The whole mixture was vortexed vigorously for 2 min and centrifuged for 5 min. The aqueous phase was collected and DNA was precipitated with ethanol. The nucleic acid pellet was resuspended with TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The DNA was used to transform *E. coli* JA226 and plasmid was prepared in mini-scale using standard methods (Sambrook *et al.*, 1989).

Cloning of *pws6*$^+$ gene

S. pombe genomic DNA library, constructed in pWH5 plasmid (Wright *et al.*, 1986), was used to clone the *pws6*$^+$ gene. The library of genomic DNA was prepared by partial *Hin*dIII digestion of *S. pombe* DNA and ligation of DNA fragment into *Hin*dIII site of pWH5. *S. cerevisiae* LEU2 gene in pWH5 complements *S. pombe leu1*$^-$ mutation and can be used as a selection marker. For this purpose, *leu1-32*$^-$ mutation was introduced to the *pws6* mutant (*h*$^-$, *ts*$^-$) by crossing to the strain carrying *leu1-32*$^-$ mutation (JHJ136: *h*$^+$, *leu1-32*$^-$) and selecting the recombinant carrying both *ts*$^-$ and *leu1-32*$^-$. The *S. pombe* genomic DNA library was introduced into these mutant cells and the transformed colonies were selected on the leucine deficient plates. The ability to complement temperature sensitivity was monitored by the growth at non-permissive temperature. The transformation plates were incubated at 23°C for 24 h and then shifted to 37°C for 5 days. Plasmids were isolated from the surviving colonies.

Analysis of DNA sequence

To determine the DNA sequence, the yeast DNA fragments in pWH5 were subcloned into *Hin*dIII site of pBluescript SK+. DNA sequencing was performed in Korea Research Institute of Bioscience and Biotechnology with forward primer (5'-GTAA AACGACGGCCAGT-3') and reverse primer (5'-AACAGCT ATGACCTG-3') of pBluescript SK+. DNA sequencing was performed in both directions. The sequenced region was analyzed and compared with the database in National Center for Biotechnology Information to search for the open reading frame and homology.

Results

Effect of nutrient on pheromone induction

To analyze the effect of the nutrients on M-factor pheromone induction in *pws6* mutant, cells were grown in minimal medium to mid-log phase at 23°C, shifted to the medium containing various concentration of nitrogen or carbon, and incubated at 37°C for 6 h. The RNA was prepared and the mRNA of M-factor (*mfm1*) was analyzed on Northern blots (Fig. 1).

When nitrogen concentration was varied from 0 to 1%, in the *pws6* cells, the pheromone induction was observed in all ranges of nitrogen concentration. The extent of induction was gradually decreased as concentration of nitrogen increased. In normal concentration of nitrogen (0.5%) and above (1%), significant pheromone was induced in *pws6*, implying that the *pws6* mutant induces M-factor pheromone without nitrogen starvation.

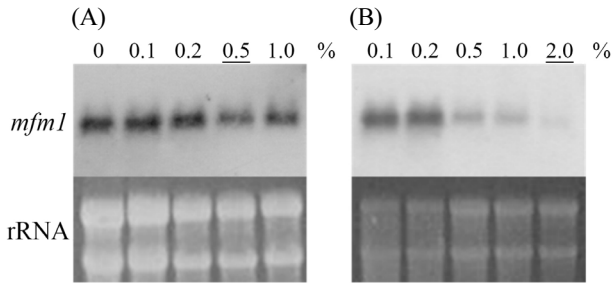

Fig. 1. Effect of nutrient on the M-factor pheromone induction in *pws6* mutant cells. (A) Effect of nitrogen. (B) Effect of glucose. Cells were grown at 23°C and shifted to 37°C for 6 h in the minimal medium containing indicated concentration of the nutrient. Cells were harvested and total RNA was analyzed on the Northern blot with antisense *mfm1* RNA as a probe. The rRNA visualized with ethidium bromide was used as a loading control. Normal concentrations of nitrogen (0.5%) and glucose (2%) were underlined.

The effect of carbon starvation to the pheromone induction was also analyzed. Strong pheromone induction was observed in the *pws6* cells at 0.1 and 0.2% glucose concentration and very weak induction at 0.5 and 1.0%. However, in 2% glucose concentration (normal glucose concentration), the pheromone induction was not observed (Fig. 1). This result implies that the *pws6* cells do not induce the pheromone without glucose starvation. Taken together, these results imply that the *pws6* cells induce the pheromone without nitrogen starvation but not without glucose starvation.

Induction of P-factor pheromone

Prior to analysis of P-factor, the ts^- mutation in the *pws6* (h^-) cells was moved into h^+ cells by crossing the mutant with the strain 975 (h^+) and selecting the recombinant carrying both h^+, ts^-. It has been reported that *S. pombe* P-factor is encoded by *map2* gene (Imai and Yamamoto, 1994). To detect the induction of P-factor, the antisense RNA of the *map2* gene was used as a probe for Northern analysis. Wild type cells 975 (h^+) induced P-factor mRNA very strongly after transfer to the nitrogen starvation media (Fig. 2A), confirming that *S. pombe* wild type cells induce pheromone when the cell is starved for nitrogen. In case of *pws6* mutant, cells were cultured in the medium of normal nitrogen concentration. Induction of P-factor mRNA was observed only when the cells were shifted to 37°C (Fig. 2A). P-factor induction was not observed in the cells at 23°C. This result indicates that the *pws6* mutant induces P-factor

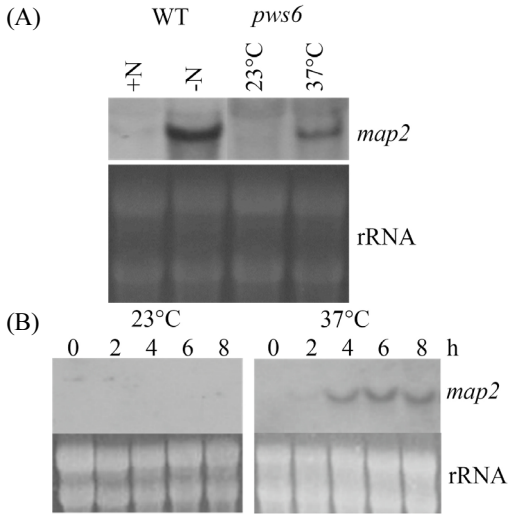

Fig. 2. P-factor pheromone induction in *pws6* mutant cells. (A) P-factor inductions in wild type and *pws6* mutant cells. Wild type cells (975) were grown at 23°C and then transferred to either PM-N (-N) or PM (+N) for 6 h. Cells of *pws6* mutant pre-grown in PM at 23°C were shifted to 37°C for 6 h. (B) Time-dependent variation of P-factor induction in *pws6* cells. Cells were grown in PM medium at 23°C and shifted to 37°C for indicated time. Cells were harvested and total RNA was analyzed on the Northern blot with antisense *map2* RNA as a probe. The rRNA visualized with ethidium bromide was used as a loading control.

pheromone as well as M-factor without starvation of nitrogen and its P-factor induction phenotype is also temperature sensitive.

The temperature sensitivity of P-factor induction was further characterized as the incubation time increased. The induction of P-factor was observed after shift to 37°C in normal nitrogen concentration but no P-factor induction was detected in the cells at 23°C (Fig. 2B). The induction signals were slightly but gradually escalated as the time of incubation increased starting from 4 h up to 8 h after shift to non-permissive temperature. These results confirm that the mutation of P-factor induction in *pws6* mutant cells is temperature sensitive.

Cloning by complementation of the growth defect

S. pombe genomic DNA library constructed in pWH5 plasmid was used to clone the $pws6^+$ gene by complementation of the growth defect. The *pws6* mutant cells (ts^-, *pws6*) were transformed with *S. pombe* genomic DNA library and the colonies that can grow at non-permissive temperature were selected. Of 36,000 transformed colonies, 29 ts^+ clones were identified (Table 1). From these ts^+ clones, three different plasmids, named pJHJ716, pJHJ722, and pKBK102, were recovered. Restriction digestion

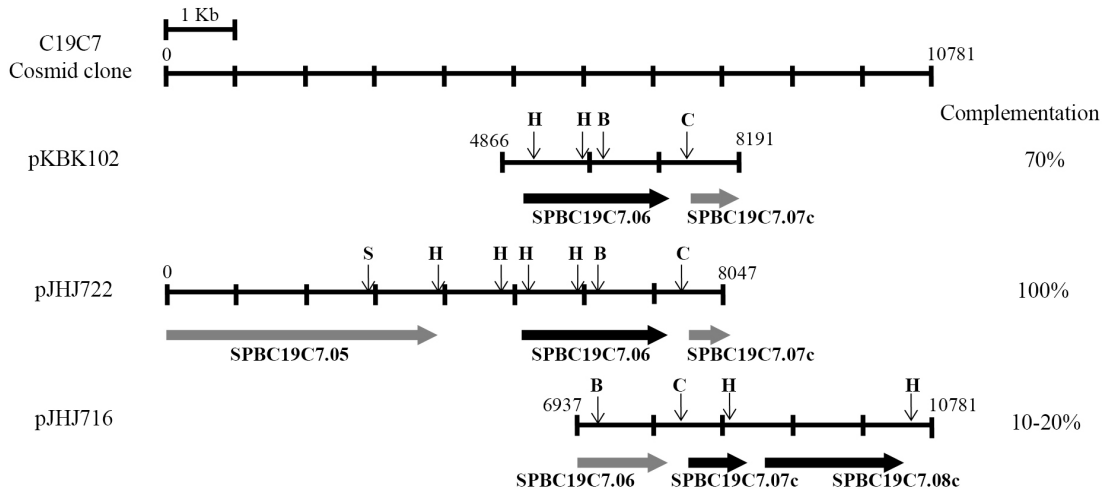

Fig. 3. Restriction map and complementation analysis of the *pws6* clones. The extent and direction of the open reading frame (ORF) is shown by arrow. The complete ORF is indicated by the solid arrow. The partial ORF is indicated by the shaded arrow. Restriction sites shown are *Bam*HI (B), *Cla*I (C), *Hin*dIII (H), and *Sal*I (S).

Table 1. Transformation of the *pws6* cells with genomic DNA

Description	Number of colony	
	Leu^+	Ts^+, Leu^+
Water	0	0
pWH5*	~3,600	0
Genomic DNA	~36,000	29

* indicates the empty vector

analyses showed that 4.8 kb, 8.1 kb, and 3.3 kb yeast DNA fragments were inserted in pJHJ716, pJHJ722, and pKBK102, respectively.

The abilities of these plasmids to complement the growth defect were accessed by retransforming the *pws6* mutant cells with the plasmids (Fig. 3). The transformant with pJHJ722 showed 100% complementation of ts^- phenotype whereas the pKBK102 transformants were observed to complement 70% of growth defect. The complementation efficiency was reduced to 10−20% upon the transformation with pJHJ716. These results suggest that the 8.1 kb insert in pJHJ722 contain a full sized open reading frame to complement the growth defect whereas partial open reading frames may be included in 3.3 kb fragment of pKBK102 or 4.8 kb fragment of pJHJ716.

Complementation of pheromone induction phenotype

We analyzed the pheromone induction phenotypes in the *pws6* cells retransformed with the plasmids (Fig. 4). While the

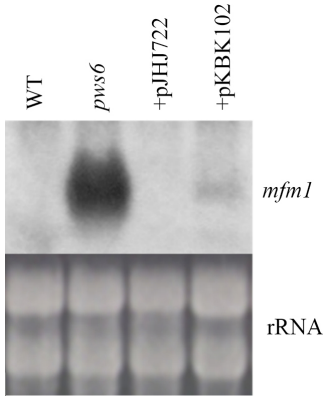

Fig. 4. Complementation of pheromone induction phenotype. Cells were cultured in PM to mid-log phase at 23°C and then shifted to 37°C for 6 h. Northern blot analyses of the total RNA in the wild type cells (WT), the *pws6* mutant cells (*pws6*), the *pws6* cells transformed with the plasmids, pJHJ722 (+pJHJ722), or pKBK102 (+pKBK102) were performed with antisense *mfm1* RNA probe. The rRNA stained with ethidium bromide was used as a loading control.

control *pws6* cells accumulated intensive amount of M-factor mRNA, no significant accumulation was observed in the transformants of pJHJ722, indicating that M-factor pheromone is not induced in the absence of nitrogen starvation. In the transformant with pKBK102, we observed a slight and significant accumulation of the pheromone mRNA. Thus, pJHJ722 complements the pheromone induction phenotype of *pws6* cells almost completely, whereas pKBK101 complements the phenotype partially. These results are well correlated with 100% and 70% complementation of the growth defect by pJHJ722 and

pKBK101, respectively, and indicate that the yeast DNA fragments in these plasmids complement not only the growth defect of *pws6* mutant cells but also the pheromone induction phenotype.

DNA sequence analysis

In order to get insight on the function of *pws6*$^+$ gene, the yeast DNA fragments of the complementing clones were sequenced. The yeast DNA fragments in pJHJ716, pJHJ722, and pKBK102 were subcloned into pBluescript vector and their DNA sequences were determined in both directions. The sequences of these DNAs were identical to the part of chromosome II sequence of *S. pombe* in National Center for Biotechnology Information (NCBI), deposited in the GenBank under accession number NC_003423.

Examination of the sequences revealed that these yeast DNA fragments contain many open reading frames (Fig. 3). The yeast DNA of pJHJ722 contains one complete open reading frame (SPBC19C7.06) and two truncated open reading frames (SPBC19C7.05 and SPBC19C7.07c). The yeast DNA of pKBK102 contains a complete SPBC19C7.06 open reading frame and truncated open reading frame of SPBC19C7.07c. In the yeast DNA of pJHJ716, one partial open reading frame (SPBC19C7.06) and two complete open reading frames (SPBC19C7.07c and SPBC19C7.08c) were found.

These open reading frames were compared to the complementation efficiencies. The complete open reading frame SPBC19C7.06, found in pJHJ722, was present partially (about 60%) in pJHJ716 and these plasmids complement the growth defect of *pws6* mutant with 100% and 10~20% efficiencies, respectively (Fig. 3). These results suggest that the open reading frame, SPBC19C7.06 is responsible for the complementation of *ts*$^-$ phenotype of *pws6* mutant. The full length of SPBC19C7.06 open reading frame was also found in the yeast DNA of pKBK102. However, the 5'-flanking sequence of this open reading frame was shortened in pKBK102. The reduced complementation efficiency of *ts*$^-$ phenotype of this plasmid (70%) might be due to the lack of this regulatory sequence present in the 5'-flanking region. These results are also well correlated with complementation of the pheromone induction phenotype by these plasmids (Fig. 4).

The open reading frame SPBC19C7.06 contains one long exon with no intron and encodes a polypeptide composed of 716 amino acids with a molecular weight of 79 kDa. The protein is a putative prolyl tRNA synthetase and its sequence has been deposited in the GenBank database under the accession number of O60155. The enzyme is encoded by *prs1* gene located in *S. pombe* chromosome II. Using BLAST algorithm of NCBI, the protein sequence data base was searched for the homology of Prs1 protein. The Prs1 protein exhibits strong similarities to the prolyl tRNA synthetases of *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Homo sapiens*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*, especially in the class II core catalytic domain. These results imply that the *pws6*$^+$ gene may encode a putative the prolyl tRNA synthetase, a protein that is conserved during evolution.

Discussion

To investigate the pathway by which the signal of nutritional starvation is transduced into pheromone secretion, an effort to identify genes involved in this pathway was initiated. For this purpose, six *ts*$^-$ mutants that induce pheromone even in the absence of nutritional starvation were isolated in previous study. In this study, the gene of one of these mutants, *pws6*, was cloned by the complementation of the growth defect. The cloned gene encodes a putative prolyl tRNA synthetase which exhibits a strong sequence homology with those of other organisms. There is a possibility that the cloned prolyl tRNA synthetase gene is a multi-copy suppressor of the *pws6* mutation. Determining the position of mutation by sequencing the prolyl tRNA synthetase gene in the *pws6* mutant can answer this question.

Carbon and nitrogen are two major sources for nutritional starvation. The signals of these nutrients appear to be transduced through the different pathways; carbon starvation is transduced through cAMP-PKA pathway and nitrogen starvation is transferred via TOR pathway. Both of these two pathways activate the expression of a transcription factor Ste11, leading to the induction of mating type specific genes. Examinations of the pheromone induction in *pws6* mutant cells in different concentrations of nutrients reveal that the pheromone was not induced in normal concentration of carbon source whereas significant induction of pheromone was detected in the typical concentration of

nitrogen (Fig. 1). These results suggest that the *pws6* mutant might have a specific defect in the pathway for nitrogen starvation, but not in the pathway for carbon starvation.

P-factor is encoded by *map2* gene that expressed specifically in the h^+ cells (Imai and Yamamoto, 1994). The sexual background of *pws6* mutant is h^- because it was generated originally by random mutagenesis of wild type h^- cells, 972. In order to examine the expression of P-factor in *pws6* mutant cells, the sexual background of *pws6* mutation was converted to h^+ by recombination. Without starvation of nutrients, the h^+, *pws6* mutant cells induce P-factor with maximum induction at 8 h after shift to non-permissive temperature (Fig. 2). In our previous research, the h^-, *pws6* mutant showed similar pattern of M-factor pheromone induction reaching to maximum induction at 10 h (Jun and Kim, 2008). These results imply that the pheromone induction phenotype of *pws6* mutation is not cell-type specific and suggest that a component common to both cell types might be defected in the *pws6* mutant cells.

Unanswered question is how prolyl tRNA synthetase is involved in induction of pheromone even in the presence of nutrients. Thus far, it remains largely unknown how the information of extracellular nutrient level is transmitted to TORC1. It has been reported in mammalian cells that leucyl tRNA synthetase senses amino acid concentrations and activates mTORC1 by acting as a GTPase-activating protein (GAP) for RagD GTPase (Han *et al.*, 2012). In *S. cerevisiae*, leucyl tRNA synthetase interacts with the Rag GTPase Gtr1 in leucine dependent manner and this interaction is mediated by the editing domain (Bonfils *et al.*, 2012). As in mammalian cells and budding yeast, Gtr1 and Gtr2, the Rag GTPase in *S. pombe*, function upstream of TORC1 and downstream of a GTP-exchange factor, Vam6 in response to amino acid (Valbuena *et al.*, 2012a). Recently, it has been reported that inactivation of leucyl tRNA synthetase and other aminoacyl tRNA synthetases of *S. pombe* initiates sexual differentiation under nutrition-rich condition via the down-regulation of TORC1 activity (Otsubo *et al.*, 2018). It is intriguing to test whether the prolyl tRNA synthetase described in this report interacts with Gtr1-Gtr2 or Vam6 in *S. pombe*.

In *Saccharomyces cerevisiae*, highly conserved Gcn2 (general control of nonderepressible 2) is a well-described example to sense the condition of amino acids (Hinnebusch, 1994; Kim and Guan, 2011; Jewell *et al.*, 2013). Deficiency of amino acids or defect in aminoacyl tRNA synthetase causes accumulation of free tRNA. The uncharged tRNA binds to the regulatory region of Gcn2 thereby activates its protein kinase (Rodland *et al.*, 2014; Lageix *et al.*, 2015). This amino acid sensor protein phosphorylates the α-subunit of translation initiation factor eIF2α, resulting in repression of general protein synthesis (Wek *et al*., 1990; Zaborske *et al.*, 2009). It has been recently reported that Gcn2 also phosphorylates Kog1, the regulatory subunit of TORC1 of *Saccharomyces cerevisiae* in response to amino acid starvation, thereby inhibiting TORC1 kinase activity (Yuan *et al.*, 2017). Like the budding yeast, fission yeast Gcn2 is activated in response to nutrient starvation (Valbuena *et al.*, 2012b; Rodland *et al.*, 2014). Thus, it is likely that the fission yeast Gcn2 might inhibit TORC1 activity by phosphorylating Mip1 as in the budding yeast. Further studies are needed to test whether the accumulated proline tRNA in *pws6* cells interacts with Gcn2 and thereby down-regulates TORC1 activity.

Another possibility is the hypothesis proposed by Otsubo *et al.* (2018). Based on the observations that expression of pre-tRNAs is reduced upon nitrogen starvation and overexpression of pre-tRNAs prevents TORC1 downregulation, they proposed that pre-tRNA is operating as a switch turning from vegetative to reproductive growth. It will be also interesting to examine the expression of pre-tRNA in the *pws6* mutant cells and its association with TORC1 activity.

Recent evidences showed the importance of prolyl tRNA synthetase in Gcn2 activation. Inhibition of prolyl tRNA synthetase induced cell death in several tumor cell lines through activation of the Gcn2-Atf4 pathway (Arita *et al.*, 2017). Proline addition to the embryonic stem cell down-regulates 77% of genes that are direct targets of Atf4, the main downstream effector of Gcn2 (D'Aniello *et al.*, 2015). This down-regulation of the genes is specific to proline. None of the amino acids other than proline reduced the expression of the target genes, concluding that the proline is a unique growth limiting amino acid for cultured embryonic stem cells. They also showed that pharmacological inhibition of prolyl tRNA synthetase by halofuginone antagonizes the effects of exogenous proline (D'Aniello *et al.*, 2015) suggesting that proline metabolism is important in the pathway for induction of the amino acid starvation response. In this aspect, the defect in prolyl tRNA synthetase in *pws6* cells

could produce more immediate effect in triggering the starvation response than the defects in other aminoacyl tRNA synthetases. Further studies described above may shed a light to answer the question on how the signal of nutritional starvation is transduced into pheromone secretion via aminoacyl tRNA synthetase.

적 요

이전의 연구에서 질소원이 존재하여도 페로몬을 유도하는 6개의 *Schizosaccharomyces pombe* 돌연변이체를 온도민감성 돌연변이체들의 저장고로부터 분리하였음이 보고된 바 있다. 본 연구에서는 이들 중 하나인 *pws6* 돌연변이체의 특성을 더 연구하였다. 이 돌연변이체는 영양물질에 특이적으로 페로몬 유도를 나타내었다. 즉 질소의 고갈은 없어도 M-factor 페로몬을 유도하였으나 탄소의 고갈이 없으면 유도되지 않았다. 이러한 결과는 *pws6* 돌연변이체가 질소 고갈을 전달하는 경로에 특이한 결함을 가지고 있음을 시사한다. 이 돌연변이체는 M-factor 페로몬뿐만 아니라 P-factor 페로몬도 온도에 민감한 양식으로 질소의 고갈 없이 유도함을 보여 주어 이 돌연변이체의 페로몬 유도는 세포 유형에 특이적이지 않음을 시사하였다. 이 돌연변이체의 온도 민감성 성장 결함의 상보적 보완에 의해 $pws6^{+}$ 유전자를 클로닝하여 8.1 kb, 3.3 kb, 그리고 4.8 kb 효모 DNA를 가진 3개의 플라스미드가 분리되었다. 이 플라스미드들은 *pws6* 돌연변이체의 성장 결함을 각각 100%, 70%, 그리고 10–20% 보완하였다. 또한 이 플라스미드들은 *pws6* 돌연변이체의 페로몬 유도 특성을 보완하는 능력을 가지고 있었으며 이는 돌연변이체의 성장 결함 보완 효율과 밀접한 연관성이 있음을 보여 주었다. 이들의 오픈 리딩 프레임을 성장 결함의 보완 효율과 비교하여 오픈 리딩 프레임 SPBC19C7.06이 *pws6* 돌연변이체의 온도 민감성 특성을 상보적으로 보완하는데 원인이 되는 리딩 프레임으로 결론 내렸다. 이 오픈 리딩 프레임은 *prs1*으로 명명되었으며 인트론이 없이 하나의 긴 엑손을 가지고 있는 추정된 prolyl tRNA synthetase를 암호화한다. 추정된 Prs1 단백질은 다른 종의 prolyl tRNA synthetase와 상당한 유사성을 보여 주었다.

Acknowledgements

This work is supported by the research grant of Cheonju University (2018.03.01~2020.02.29).

References

Alfa C, Fantes P, Hyams J, McLeod M, and Warbrick E. 1993. Experiments with fission yeast: a laboratory course manual, pp. 133–136, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Alvarez B and Moreno S. 2006. Fission yeast Tor2 promotes cell growth and represses cell differentiation. *J. Cell Sci.* **119**, 4475–4485.

Aono T, Yanai H, Miki F, Davey J, and Shimoda C. 1994. Mating pheromone-induced expression of the *MAT1-Pm* gene of *Schizosaccharomyces pombe*: identification of signaling components and characterization of upstream controlling elements. *Yeast* **10**, 757–770.

Arita T, Morimoto M, Yamamoto Y, Miyashita H, Kitazawa S, Hirayama T, Sakamoto S, Miyamoto K, Adachi R, Iwatani M, *et al.* 2017. Prolyl-tRNA synthetase inhibition promotes cell death in SK-MEL-2 cells through GCN2-ATF4 pathway activation. *Biochem. Biophys. Res. Commun.* **488**, 648–654.

Bonfils G, Jaquenoud M, Bontron S, Ostrowicz C, Ungermann C, and De Virgilio C. 2012. Leucyl-tRNA synthetase control TORC1 via the EGO complex. *Mol. Cell* **46**, 105–110.

Byrne SM and Hoffman CS. 1993. Six *git* genes encode a glucose-induced adenylate cyclase activation pathway in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **105**, 1095–1100.

Corradetti MN and Guan KL. 2006. Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? *Oncogene* **25**, 6347–6360.

D'Aniello C, Fico A, Casalino L, Guardiola O, Di Napoli G, Cermola F, De Cesare D, Tate R, Cobellis G, Patriarca EJ, *et al.* 2015. A novel autoregulatory loop between the Gcn2-Atf4 pathway and L-Proline metabolism controls stem cell identity. *Cell Death Differ*. **22**, 1094–1105.

Davey J. 1992. Mating pheromones of the fission yeast *Schizosaccharomyces pombe:* purification and structural characterization of M-factor and isolation and analysis of two genes encoding the pheromone. *EMBO J.* **11**, 951–960.

Davey J. 1998. Fusion of a fission yeast. *Yeast* **14**, 1529–1566.

Han JM, Jeong SJ, Park MC, Kim G, Kwon NH, Kim HK, Ha SH, Ryu SH, and Kim S. 2012. Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. *Cell* **149**, 410–424.

Hayashi T, Hatanaka M, Nagao K, Nakaseko Y, Kanoh J, Kokubu A, Ebe M, and Yanagida M. 2007. Rapamycin sensitivity of the *Schizosaccharomyces pombe tor2* mutant and organization of two highly phosphorylated TOR complexes by specific and common subunits. *Genes Cells* **12**, 1357–1370.

Higuchi T, Watanabe Y, and Yamamoto M. 2002. Protein kinase A regulates sexual development and gluconeogenesis through phosphorylation of the Zn finger transcriptional activator Rst2p in fission yeast. *Mol. Cell. Biol.* **22**, 1–11.

Hinnebusch AG. 1994. The eIF-2 kinases: regulators of protein

synthesis in starvation and stress. *Semin. Cell Biol.* **5**, 417–426.

Hoffman CS and Winston F. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**, 267–272.

Huang J and Manning BD. 2008. The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. *Biochem. J.* **412**, 179–190.

Imai Y and Yamamoto M. 1994. The fission yeast mating pheromone P-factor: its molecular structure, gene structure, and physiological activities to induce gene expression and Gl arrest in the mating partner. *Genes Dev.* **8**, 328–338.

Isshiki T, Mochizuki N, Maeda T, and Yamamoto M. 1992. Characterization of a fission yeast gene, *gpa2*, that encodes a G alpha subunit involved in the monitoring of nutrition. *Genes Dev*. **6**, 2455–2462.

Jewell JL, Russell RC, and Guan KL. 2013. Amino acid signaling upstream of mTOR. *Nat. Rev. Mol. Cell Biol.* **14**, 133–139.

Jun J and Kim D. 2008. A pheromone mutant of *Schizosaccharomyces pombe* displays nucleolar fragmentation. *BMB Rep.* **41**, 248–253.

Kawai M, Nakashima A, Ueno M, Ushimaru T, Aiba K, Doi H, and Uritani M. 2001. Fission yeast Tor1 functions in response to various stresses including nitrogen starvation, high osmolality, and high temperature. *Curr. Genet*. **39**, 166–174.

Kawamukai M, Ferguson N, Wigler M, and Young D. 1991. Genetic and biochemical analysis of adenylyl cyclase of *Schizosaccharomyces pombe*. *Cell Regul*. **2**, 155–164.

Kim J and Guan KL. 2011. Amino acid signaling in TOR activation. *Annu. Rev. Biochem.* **80**, 1001–1032.

Kim L, Hoe K., Yu YM, Yeon JH, Maeng PJ. 2012. The fission yeast GATA factor, Gaf1, modulates sexual development via direct downregulation of *ste11*$^{+}$ expression in response to nitrogen starvation. *PLoS One* **7**, e42409.

Kitamura K and Shimoda C. 1991. The *Schizosaccharomyces pombe mam2* gene encodes a putative pheromone receptor which has significant homology with the *Saccharomyces cerevisiae* Ste2 protein. *EMBO J.* **10**, 3743–3751.

Kjaerulff S, Davey J, and Nielsen O. 1994. Analysis of the structural genes encoding M-factor in the fission yeast *Schizosaccharomyces pombe*: identification of a third gene, *mfm3*. *Mol. Cell. Biol*. **14**, 3895–3905.

Kunitomo H, Higuchi T, Iino Y, and Yamamoto M. 2000. A zinc-finger protein, Rst2p, regulates transcription of the fission yeast *ste11*$^{+}$ gene, which encodes a pivotal transcription factor for sexual development. *Mol. Biol. Cell* **11**, 3205–3217.

Kupiec M and Weisman R. 2012. TOR links starvation responses to telomere length maintenance. *Cell Cycle* **11**, 2268–2271.

Lageix S, Zhang J, Rothenburg S, and Hinnebusch AG. 2015. Interaction between the tRNA-binding and C-terminal domains of yeast Gcn2 regulates kinase activity *in vivo*. *PLoS Genet.* **11**, e1004991.

Laor D, Cohen A, Kupiec M, and Weisman R. 2015. TORC1 regulates developmental responses to nitrogen stress via regulation of the GATA transcription factor Gaf1. *mBio* **6**, e00959-15.

Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, and Hall MN. 2002. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol. Cell* **10**, 457–468.

Maeda T, Watanabe Y, Kunitomo H, and Yamamoto M. 1994. Cloning of the *pka1* gene encoding the catalytic subunit of the cAMP-dependent protein kinase in *Schizosaccharomyces pombe*. *J. Biol. Chem*. **269**, 9632–9637.

Mata J and Bahler J. 2006. Global roles of Ste11p, cell type, and pheromone in the control of gene expression during early sexual differentiation in fission yeast. *Proc. Natl. Acad. Sci. USA* **103**, 15517–15522.

Matsumoto S, Bandyopadhyay A, Kwiatkowski DJ, Maitra U, and Matsumoto T. 2002. Role of the Tsc1-Tsc2 complex in signaling and transport across the cell membrane in the fission yeast *Schizosaccharomyces pombe*. *Genetics* **161**, 1053–1063.

Matsuo T, Otsubo Y, Urano J, Tamanoi F, and Yamamoto M. 2007. Loss of the TOR kinase Tor2 mimics nitrogen starvation and activates the sexual development pathway in fission yeast. *Mol. Cell Biol.* **27**, 3154–3164.

Mochizuki N and Yamamoto M. 1992. Reduction in the intracellular cAMP level triggers initiation of sexual development in fission yeast. *Mol. Gen. Genet.* **233**, 17–24.

Nischt R, Thuroff E, and Kufer NF. 1986. Molecular cloning of a ribosomal protein gene from the fission yeast *Schizosaccharomyces pombe*. *Curr. Genet.* **10**, 365–370.

Okazaki K, Okazaki N, Kume K, Jinno S, Tanaka K, and Okayama H. 1990. High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **18**, 6485–6489.

Otsubo Y, Matsuo, T, Nishimura A, Yamamoto M, and Yamashita A. 2018. tRNA production links nutrient conditions to the onset of sexual differentiation through the TORC1 pathway. *EMBO Rep.* **19**, e44867.

Otsubo Y, Nakashima A, Yamamoto M, and Yamashita A. 2017. TORC1-dependent phosphorylation targets in fission yeast. *Biomolecules* **7**, 50.

Otsubo Y, Yamashita A, Ohno H, and Yamamoto M. 2014. *S. pombe* TORC1 activates the ubiquitin-proteasomal degradation of the meiotic regulator Mei2 in cooperation with Pat1 kinase. *J. Cell Sci.* **127**, 2639–2646.

Rodland GE, Tvegard T, Boye E, and Grallert B. 2014. Crosstalk between the Tor and Gcn2 pathways in response to different stresses. *Cell Cycle* **13**, 453–461.

Sambrook J, Fritsh EF, and Maniatis T. 1989. Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Schonbrun M, Kolesnikov M, Kupiec M, and Weisman R. 2013. TORC2 is required to maintain genome stability during S phase

in fission yeast. *J. Biol. Chem.* **288**, 19649–19660.

Schonbrun M, Laor D, Lopez-Maury L, Bahler J, Kupiec M, and Weisman R. 2009. TOR complex 2 controls gene silencing, telomere length maintenance, and survival under DNA-damaging conditions. *Mol. Cell. Biol.* **29**, 4584–4594.

Shinozaki-Yabana S, Watanabe Y, and Yamamoto M. 2000. Novel WD repeat protein Mip1p facilitates function of the meiotic regulator Mei2p in fission yeast. *Mol. Cell. Biol.* **20**, 1234–1242.

Sugimoto A, Iino Y, Maeda T, Watanabe Y, and Yamamoto M. 1991. *Schizosaccharomyces pombe ste11*$^{+}$ encodes a transcription factor with an HMG motif that is a critical regulator of sexual development. *Genes Dev*. **5**, 1990–1999.

Tanaka K, Davey J, Imai Y, and Yamamoto M. 1993. *Schizosaccharomyces pombe map3* encodes the putative M-factor receptor. *Mol. Cell. Biol.* **13**, 80–88.

Urano J, Sato T, Matsuo T, Otsubo Y, Yamamoto M, and Tamanoi F. 2007. Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells. *Proc. Natl. Acad. Sci. USA* **104**, 3514–3519.

Uritani M, Hidaka H, Hotta Y, Ueno M, Ushimaru T, and Toda T. 2006. Fission yeast Tor2 links nitrogen signals to cell proliferation and acts downstream of the Rheb GTPase. *Genes Cells* **11**, 1367–1379.

Valbuena N and Moreno S. 2010. TOR and PKA pathway synergized at the level of the Ste11 transcription factor to prevent mating and meiosis in fission yeast. *PLoS One* **5**, e11514.

Valbuena N, Guan KL, and Moreno S. 2012a. The Vam6 and Gtr1-Gtr2 pathway activates TORC1 in response to amino acids in fission yeast. *J. Cell Sci.* **125**, 1920–1928.

Valbuena N, Rozalen AE, and Moreno S. 2012b. Fission yeast TORC1 prevents eIF2α phosphorylation in response to nitrogen and amino acids via Gcn2 kinase. *J. Cell Sci.* **125**, 5955–5959.

van Slegtenhorst M, Carr E, Stoyanova R, Kruger WD, and Henske EP. 2004. *Tsc1*$^{+}$ and *tsc2*$^{+}$ regulate arginine uptake and metabolism in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **279**, 12706–12713.

van Slegtenhorst M, Mustafa A, and Henske EP. 2005. Pas1, a G1 cyclin, regulates amino acid uptake and rescues a delay in G1 arrest in Tsc1 and Tsc2 mutants in *Schizosaccharomyces pombe*. *Hum. Mol. Genet.* **14**, 2851–2858.

Wang SH, Xue CB, Nielsen O, Davey J, and Naider F. 1994. Chemical synthesis of the M-factor mating pheromone from *Schizosaccharomyces pombe. Yeast* **10**, 595–601.

Watanabe Y, Iino Y, Furuhata K, Shimoda C, and Yamamoto M. 1988. The *S. pombe mei2* gene encoding a crucial molecule for commitment to meiosis is under the regulation of cAMP. *EMBO J.* **7**, 1197–1214.

Weisman R and Choder M. 2001. The fission yeast tor homolog, *tor1*$^{+}$, is required for the response to starvation and other stresses via a conserved serine. *J. Biol. Chem*. **276**, 7027–7032.

Weisman R, Roitburg I, Schonbrun M, Harari R, and Kupiec M. 2007. Opposite effects of Tor1 and Tor2 on nitrogen starvation responses in fission yeast. *Genetics* **175**, 1153–1162.

Wek RC, Ramirez M, Jackson BM, and Hinnebusch AG. 1990. Identification of positive-acting domains in GCN2 protein kinase required for translational activation of GCN4 expression. *Mol. Cell. Biol*. **10**, 2820–2831.

Welton RM and Hoffman CS. 2000. Glucose monitoring in fission yeast via the Gpa2 Gα, the git5 Gβ and the git3 putative glucose receptor. *Genetics* **156**, 513–521.

Wright A, Maundrell K, Heyer WD, Beach D, and Nurse P. 1986. Vectors for the construction of gene banks and the integration of cloned genes in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. *Plasmid* **15**, 156–158.

Wullschleger S, Loewith R, and Hall MN. 2006. TOR signaling in growth and metabolism. *Cell* **124**, 471–484.

Yuan W, Guo S, Gao J, Zhong M, Yan G, Wu W, Chao Y, and Jiang Y. 2017. General control nonderepressible 2 (GCN2) kinase inhibits target of rapamycin complex 1 in response to amino acid starvation in *Saccharomyces cerevisiae*. *J. Biol. Chem*. **292**, 2660–2669.

Zaborske JM, Narasimhan J, Jiang L, Wek SA, Dittmar KA, Freimoser F, Pan T, and Wek RC. 2009. Genome-wide analysis of tRNA charging and activation of the eIF2 kinase Gcn2p. *J. Biol. Chem*. **284**, 25254–25267.

Zoncu R, Efeyan A, and Sabatini DM. 2011. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat. Rev. Mol. Cell. Biol.* **12**, 21–35.