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A pheromone mutant of *Schizosaccharomyces pombe* displays nucleolar fragmentation

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Stresses and nutritional starvation are two main external signals for the induction of sex pheromones in the fission yeast *Schizosaccharomyces pombe*. In an attempt to identify the components involved in transduction of starvation signals, we screened 135 temperature-sensitive (ts) mutants and isolated 6 mutants that induced the pheromone even in the presence of a nitrogen source. These mutants exhibited two distict induction phenotypes: pheromone induction at restrictive but not at permissive temperatures; and pheromone induction at both permissive and restrictive temperatures. The times required for the maximum pheromone induction at the restrictive temperature differed slightly in each mutant. In addition to the pheromone induction phenotype, the *ts243* and *ts304* mutants exhibited cell-division-cycle defects. The *ts304* mutant cells showed an abnormal cytoplasmic DAPI staining pattern. The nucleolus of this mutant seemed to be fragmented, a phenomenon which is typically observed in aged yeast cells. The result of our genetic analysis indicated that the pheromone induction mutants belonged to 6 separate complementation groups. We designated these mutants *pws1* to *pws6*. [BMB reports 2008; 41(3): 248-253]

INTRODUCTION

The fission yeast *Schizosaccharomyces pombe* follows two alternative developmental pathways. Haploid cells divide mitotically while growing in rich medium. However, under nutrients depletion conditions, particularly nitrogen depletion, the cells cease to divide and conjugate with cells of the opposite mating type to form diploid zygotes. Prior to mating, the cells of the two mating types communicate via diffusible peptide hormones; cells of the h^+ mating type secrete P-factor and the h^- cells release M-factor (1). The pheromones induce mating- specific changes in the opposite mating type cells including altered pattern of gene transcription, a G1 arrest of cell growth, and unidirectional elongation of the cells (2).

Sexual differentiation in *S. pombe* occurs under nutritional starvation (3). Nitrogen starvation and carbon starvation are two major nutritional exhaustion signals which trigger sexual development. The cAMP cascade appears to play a central role in the transmission of a starvation signal to gene expression (4). A G-protein α_2 subunit, Gpa2, is likely to control the activity of adenylate cyclase according to the nutritional conditions (5). A G-protein coupled receptor, Stm1, is likely to function as a molecule which sense the nutritional state of cells and may couple with Gpa2 (6).

A reduction in the intracellular cAMP level results in the inactivation of cAMP dependent protein kinase A (PKA) (7). Inactivation of PKA induces expression of the *ste11* gene, a master regulator of the sexual differentiation program (8). Nitrogen starvation induces the transcription of ste11-controlled genes, including the mating-type specific genes (9). Ste11 encodes for a DNA-binding protein which contains a high-mobility group (HMG) motif that binds to the TR box in mating-type specific genes (8, 10). The mating-type loci, in turn, control the production of pheromones and their receptors (11). A zinc-finger protein, referred to as Rst2, directly regulates the transcription of the *ste11* gene by binding to the STREP motif (12).

In addition to the cAMP-PKA pathway, a stress-responsible MAP kinase cascade regulates the expression of *ste11* in response to a variety of stresses, including osmotic stress, heat stress, oxidative stress, nutritional limitation, UV radiation, and DNA damage (13). The signals of these stresses are believed to be transduced to the MAP kinase cascade. The two MAPK kinase kinases, Wak1 (also known as Wik1 or Wis4) and Win1 activate the MAPK kinase, Wis1, which in turn activates the MAP kinase *Spc1* (also known as Sty1 or Phh1) (14, 15). SpcI has been shown to phosphorylate a CRE-binding protein Atf1 (also known as Gad7) (16). Loss of function of Atf1 significantly reduces the level of *ste11* transcription (17). Another CRE-binding protein, Pcr1, is also required for the activation of *ste11* transcription (18). The CRE-Atf1-Pcr1 complex induces a local alteration of the chromatin structure (19). This alteration is regulated in response to environmental stresses (20). The DNA microarray analysis of *S. pombe* showed that a group of genes called core environmental stress response (CESR), are induced by multiple forms of stresses and the majority of these genes are regulated via the Spc1-Atf1 pathway (21).

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Despite these extensive studies, little remains known regarding the specific factors and mechanisms responsible for the pheromone induction triggered by nutritional starvation. To get a better understanding of the component required for the signal transduction of the nutritional starvation in *S. pombe*, we have initiated a search for mutants which induce pheromones in the absence of nutritional starvation. We identified six mutants that induce pheromones in nitrogen-rich media. In this work, we describe some characteristics of these mutants.

RESULTS

Isolation of pheromone mutants

In *S. pombe*, three genes (*mfm1*, *mfm2*, and *mfm3*) encoding for the M-factor have been sequenced (22, 23). Among these genes, we selected the *mfm1* gene as a probe for our Northern analysis, because its expression is greatly induced by nitrogen starvation (data not shown).

We generated a bank of 135 temperature-sensitive (ts^-) mutants by the EMS mutagenesis of the wild-type *S. pombe* strain 972 (h^-). Using antisense RNA transcribed from the *mfm1* gene, we screened the ts^- bank for the mutants that induce pheromones in the presence of a nitrogen source. The ts^- mutant cells were cultured to mid-log phase in YE and shifted to the restrictive temperature for 5 h. Total RNA was extracted and the transcript of the *mfm1* gene was examined by Northern analysis. As a control, wild-type cells cultured in PM or PM-N were also compared. The cells cultured in nitrogen-depleted PM medium showed a strong induction of the *mfm1* gene, whereas no transcript was observed in cells cultured in a nitrogen-containing medium (PM) (data not shown). This result was well-correlated with the results of Kjaerulff *et al*. (23) and those of our previous report (24), thereby confirming that the *mfm1* transcription was induced in the cells under nitrogen starvation conditions.

Of the 135 ts^- mutants screened, 18 mutants were initially selected as candidates for pheromone induction mutants. Among them, 6 mutants (*ts243*, *ts265*, *ts282*, *ts284*, *ts293*, and *ts304*) were confirmed to strongly induce pheromones in a second screening; thus, these mutants were subjected to further research. We examined the temperature sensitivity of pheromone induction in these mutants. Total RNA was prepared from cells grown at 23°C or after shifting to 37°C for 5 h and the pheromone mRNA was analyzed (Fig. 1). The six mutants exhibited two different phenotypes. In *ts243*, *ts284*, *ts293*, and *ts304*, pheromone induction was observed only at the restrictive temperature but not at the permissive temperature. In *ts265* and *ts282*, pheromones were synthesized at both permissive and restrictive temperatures. However, these mutants showed increased pheromone induction at 37°C.

Pheromone induction kinetics

To further characterize the temperature sensitivity of the pheromone mutants with regard to their pheromone induction abilities, the time-dependent variations in pheromone transcription at the restrictive temperature were examined. The RNA was prepared from each mutant that had been grown to mid-log phase at 23°C and shifted to 37°C for 2, 4, 6, 8, and 10 h and analyzed on Northern blots (Fig. 2). At 37°C, each of the mutants showed pheromone mRNA induction. The times required to reach the peak of the induction differed in each of the mutants as follows: *ts243* and *ts265* at 4 h; *ts282* at 2-4 h; *ts284* at 2-6 h; *ts293* at 6 h; *ts304* at 8-10 h. While *ts265*, *ts282* and *ts304* induce the pheromone at higher levels, *ts284* and *ts293* showed lower level of pheromone induction. An intermediate level of pheromone induction was observed in *ts243*. These results indicated that the kinetics of pheromone induction differs slightly in each of the mutants.

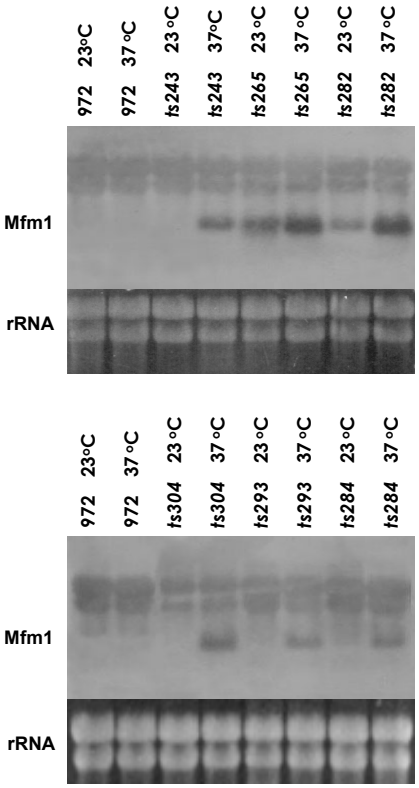

Fig. 1. Northern blot analysis of the pheromone induction mutants at both permissive and restrictive temperatures. Cells were cultured in minimal medium at 23°C until a concentration of 5×10^6 cells/ml was reached and then transferred to 23°C or 37°C for 5 h. Cells from prior and after the temperature were harvested and total RNA (10 μg) was analyzed via Northern analysis with single stranded *mfm1* RNA as a probe. The rRNAs were visualized with ethidium bromide.

Cell-division-cycle phenotypes

In addition to the pheromone induction phenotype, two of our mutants showed abnormal morphology. Cells from cultures

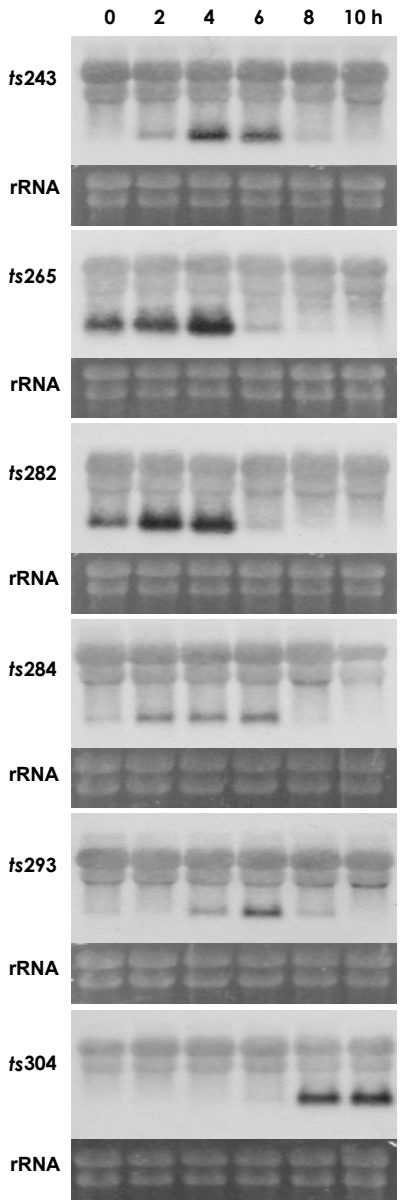

Fig. 2. Time-dependent variation of the pheromone induction. Cells were grown in minimal medium until mid-log phase and transferred to 37°C for the indicated time. The Northern blot analysis of total RNA was conducted as shown in Fig. 1.

grown at 23°C and then shifted to 37°C for 5 h were examined under the phase-contrast microscope (Fig. 3A). The wild-type cells maintained a normal size and shape, thereby implying that they had remained unaffected by the temperature shift. In contrast, in the *ts243*, swollen cells were seen after incubation at 37°C. The *ts304* showed elongated cell shape. The average length of the *ts304* mutant cells was about twice that of the wild-type cells, a classical indicator in fission yeast of a cdc^- defect (25). Both of these mutants appeared normal at 23°C. This result indicates that their cdc^- phenotypes are also temperature-sensitive. We observed no abnormal morphology with any of other mutants described herein.

To better understand the nature of these mutants, we conducted the chromatin staining with the DNA-binding fluorescent dye DAPI. The wild-type strain exhibited the most common nuclear morphology observed for growing *S. pombe* cells. The *ts243* mutant also expressed morphology identical to that of the wild-type strain (data not shown). However, in the *ts304* mutant, distinct morphology was observed at the restrictive temperature (Fig. 3B). The DAPI-stained materials were dispersed in the cytoplasm in addition to the nucleus. Such structures were typically observed in aged yeast cells in which the nucleoli were fragmented due to the accumulation of extrachromosomal rDNA circles (ERCs) (26, 27). Thus, it is likely that the *ts304* mutant may have a shorter life span and may display a premature aging phenotype. This abnormal structure was not observed at the permissive temperature, implying that such characteristics are also a temperature-sensitive phenotype.

Genetic characterization

To assay for genetic linkages between the new mutations, we crossed each mutant with itself or with all the others. Several hundred spores from each cross were germinated and tested for their temperature sensitivity. The self-crossed controls produced no ts^+ progeny, whereas the pair-wise crosses between the different mutants generated approximately 25-50 % progeny that grew well at 37°C (Table 1). This result suggested that the mutations in the six mutants lie at six independent, unlinked loci and fall into separate complementation groups. These mutations have been assigned the following names: *ts243, pws1; ts265, pws2; ts282, pws3; ts284, pws4; ts293, pws5;* and *ts304, pws6* (pheromone induction without starvation).

DISCUSSION

As a part of our investigations into starvation signal transduction, we have initiated an effort to identify genes involved in pheromone induction. The 135 ts^- mutant bank was screened for the mutants that could induce pheromones without nutritional starvation. Using a Northern blot assay designed to detect pheromone mRNA, six ts^- mutants, *ts243*, *ts265*, *ts282*, *ts284*, *ts293*, and *ts304*, were identified to induce pheromone mRNA at the restrictive temperature. This frequency of isolation of the pheromone induction mutants is slightly higher than what has been reported for the isolation of mRNA splicing mutants in *S. pombe* (28). The comparison of the frequency of pheromone induction mutants and that of the mRNA splicing mutants suggests that more than 20 genes might be involved in the pheromone induction in *S. pombe*.

The six mutants showed two distict pheromone induction phenotypes. In one group of mutants, the pheromone mRNA

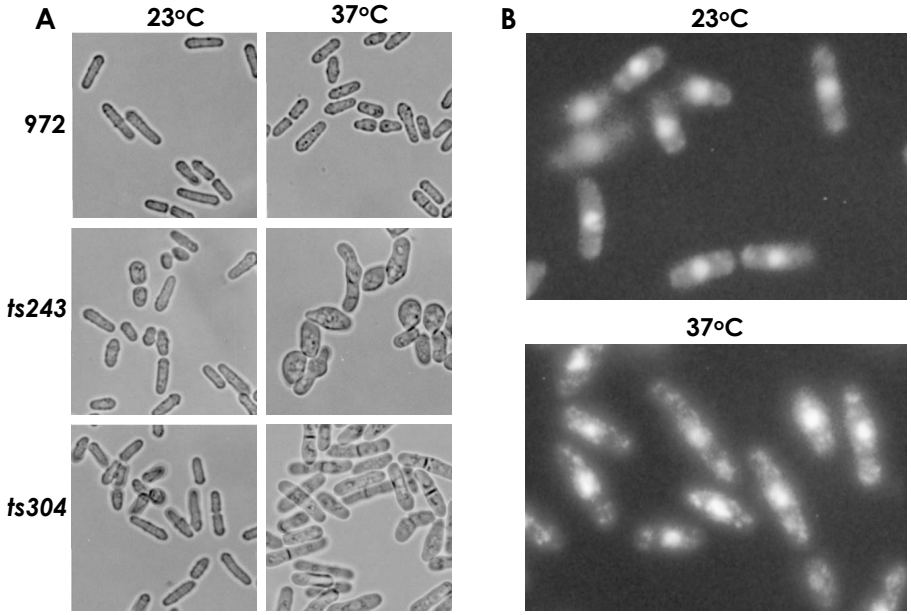

Fig. 3. The cellular morphology of the pheromone induction mutants. (A) Morphology of *ts243* and *ts304*. The cells were grown at 23°C and shifted to 37°C for 5 h. The pictures were taken with an Olympus BH-2 phase-contrast microscope at a final magnification of × 400. (B) Cell-division-cycle phenotype of *ts304*. Cells from prior to and after the temperature shift were stained with DAPI and examined with a fluorescence microscope (Olympus BH-2) under ultraviolet illumination. The photographs obtained at a magnification of × 400 were enlarged to better display the nuclear morphology.

Table 1. Genetic analysis of the pheromone induction mutants

	ts243	*ts265*	*ts282*	*ts284*	*ts293*	*ts304*
ts243	−					
ts265	+	−				
ts282	+	+	−			
ts284	+	+	+	−		
ts293	+	+	+	+	−	
ts304	+	+	+	+	+	−

−: no ts^+ progeny out of several hundred spores.
+: 25-50 % ts^+ progeny out of several hundred spores.

accumulated at 37°C but not at 23°C whereas the other group exhibited pheromone mRNA at both 37°C and 23°C. However, when we examined the time-dependent variations in pheromone induction, slight differences in the kinetics of pheromone induction were observed in each of the six mutants. These results suggest that the mutations of all six mutants reside on different genes. This was confirmed by our genetic analysis (Table 1).

Two of our pheromone induction mutants displayed a cdc^- phenotype. The mutant, *pws1*, accumulated swollen and aggregated cells at the restrictive temperature. Similar morphology was observed frequently in the cell division cycle mutants (25). The *pws6* mutant also displayed the characteristics of the cell division cycle mutants. At the restrictive temperature, the cells became elongated. However, their septa appear to be formed normally, although they showed a thick and elongated shape (Fig. 3A). These results suggest that the nuclei are segregated normally in these cells.

The nucleolus is a crescent-shaped region of the nucleus, which contains 100-200 copies of a 9.1 kb rDNA repeat and components for ribosome assembly. In aging yeast cells, the rDNA repeats are excised from the rDNA locus, replicate via their autonomous replicating sequence (ARS), and form the extrachromosomal RNA circles (ERCs) - a phenomenon called nucleolar fragmentation (27, 29). When we stained the *pws6* mutant cells with DAPI, we observed a dispersed DAPI staining in the cytoplasm (Fig. 3B), which is the characteristic staining pattern of nucleolar fragmentation (26). Thus, the *pws6* mutant may have a premature aging phenotype. Like the pheromone induction phenotype, the abnormal DAPI staining was not observed in the cells at the permissive temperature, suggesting that the single mutation may be responsible for both of these phenotypes. Further experiments including the measurement of the replicative life span may determine whether the *pws6* is a premature aging mutant.

The ts^- mutants in this report were obtained by random mutagenesis using 3.5 % EMS. In our experience, this EMS concentration generates mostly single mutation. However, to remove any possible multiple mutations, the pheromone mutants were backcrossed three times to the wild-type strain and the ts^- colony of the resulting progeny was selected. Northern analysis of cells from the third generation showed that they were still harboring the pheromone induction phenotype (data not shown), suggesting that the single mutation is responsible for both temperature-sensitive and pheromone induction phenotypes. We did not determine whether the mutations of the pheromone induction mutants were recessive or dominant. These mutations are, however, probably recessive, as the majority of the ts^- mutations generated by chemical mutagenesis

are recessive.

The synthesis of the pheromone is induced by nutritional starvation and controlled by the action of Ste11, a transcription factor essential for the activation of the many genes required for the conjugation and meiosis. Until now, two major pathways are known to regulate the expression of *ste11*. Nutritional starvation induces a decrease in the cAMP level which negatively regulates *ste11* expression. In addition, a stress signal is transduced to Ste11 via the Wis1-Spc1 MAP kinase cascade and the Atf1 transcription factor. We isolated mutants that induce pheromones without nutritional starvation from the temperature-sensitive mutant bank. Thus, any mutation that results in a reduction in cAMP level can activate Ste11 expression and induce pheromone expression. Alternatively, the mutation itself is likely to cause stress to the cells and activates the Wis1-Spc1 MAP kinase cascade. Therefore, mutations that can cause stress or decreases in cAMP concentration, rather than defects in the components of nutritional signal transduction, might be included in our pheromone mutants. The high frequency of the mutant isolation from our ts^- bank may be reflective of these possibilities. We have not assessed the effects of stress on our mutants. Hence, we are unable to dismiss the possibility that the phenotypes of the mutants represent the results of stress caused to the cells. Further experiments including the cloning and disruption of the wild-type gene that is defective in these strains may shed light on this issue.

MATERIALS AND METHODS

Yeast strains, media and growth conditions

All of the *S. pombe* strains used in this study were derived from the haploid wild-type strain of mating type h^- (972). Complete medium, YE, and minimal medium, PM, were prepared according to Alfa *et al.,* (30). As a nitrogen starvation medium, PM without nitrogen source was prepared by subtracting NH_4Cl. The wild-type strain was grown at 23°C. Temperature-sensitive strains were grown at 23°C as a permissive temperature or at 37°C as a restrictive temperature.

EMS mutagenesis and isolation of temperature-sensitive mutants

The mutagenesis of *S. pombe* strain 972 with 3.5 % ethylmethane sulfonate and the selection of temperature-sensitive mutants were conducted as previously described (31).

RNA preparation and Northern analysis

Cells grown to mid-log phase were harvested and the total RNA was prepared as described in Hwang and Kim, (31). Total RNA (10 μg) was analyzed via Northern hybridization as previously described (32, 33). Single-stranded RNA probes were transcribed from pSK2 (generously donated by Dr. Olaf Nielsen, University of Copenhagen). The pSK2 was composed of pGEM3 vector containing a 467-bp *Eco*RI-*Nhe*I fragment of the *S. pombe mfm1* gene (23). One microgram of pSK2 was linearized by digestion with *Eco*RI and transcribed with SP6 RNA polymerase in the presence of digoxigenin-11 UTP using a SP6/T7 transcription kit (Boehringer Mannheim). Non-incorporated ribonucleoside triphosphate was removed by repeated ethanol precipitations and the labeled RNA was used as a probe for Northern analyses (34).

Fluorescence microscopy

Cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) and visualized as described by Moreno *et al.* (35). The fluorescent images were produced on an Olympus BH2 microscope at a magnification of X400.

Genetic characterization

Standard fission yeast genetic techniques (35) were applied in this study. In order to test for linkages among the new mutations, we crossed each of the mutants with each of the others. The progeny spores were recovered on YE agar at 23°C and then replica-plated at 37°C. As controls, each of the strains was mated with itself. These control crosses produced no temperature-resistant (ts^+) progeny among several hundred random spores. Unlinked alleles generated 25-50 % ts^+ colonies. Tight linkage was indicated by the absence of ts^+ progeny as was seen in the self-mated control crosses, thereby indicating that the two mutations were allelic.

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