Reduction of Ribosome Level Triggers Flocculation of Fission Yeast Cells

Rongpeng Li, Xuesong Li, Lei Sun, Feifei Chen, Zhenxing Liu, Yuyu Gu, Xiaoyan Gong, Zhonghua Liu, Hua Wei, Ying Huang, Sheng Yuan

Jiangsu Key Laboratory for Microbes and Functional Genomics, Jiangsu Engineering and Technology Research Center for Industrialization of Microbial Resources, College of Life Science, Nanjing Normal University, Nanjing, People’s Republic of China

Deletion of ribosomal protein L32 genes resulted in a nonsexual flocculation of fission yeast. Nonsexual flocculation also occurred when two other ribosomal protein genes, rpl21-2 and rpl9-2, were deleted. However, deletion of two nonribosomal protein genes, mpg/h and fbp/h, did not cause flocculation. Overall transcript levels of rpl32 in rpl32-1Δ and rpl32-2Δ cells were reduced by 35.9% and 46.9%, respectively, and overall ribosome levels in rpl32-1Δ and rpl32-2Δ cells dropped 31.1% and 27.8%, respectively, compared to wild-type cells. Interestingly, ribosome protein expression levels and ribosome levels were also reduced greatly in sexually flocculating diploid YHL6381/WT (h+/h−) cells compared to a mixture of YHL6381 (h+) and WT (h−) nonflocculating haploid cells. Transcriptome analysis indicated that the reduction of ribosomal levels in sexual flocculating cells was caused by more-extensive suppression of ribosomal biosynthesis gene expression, while the reduction of ribosomal levels caused by deleting ribosomal protein genes in nonsexual flocculating cells was due to an imbalance between ribosomal proteins. We propose that once the reduction of ribosomal levels is below a certain threshold value, flocculation is triggered.

MATERIALS AND METHODS

Strains, media, and culture conditions. The fission yeast strains used in this study are listed in Table 1. The YEPD medium consists of yeast extract (10 g/liter), tryptone (20 g/liter), and glucose (20 g/liter). The YEMP medium was derived from YEPD medium (16) by replacing glucose with malt extract (containing ~50% maltose) (1, 17). The synthetic Edinburgh minimal medium (EMM2) and LB medium were made as described by Kim et al. (2001) (12). Different antibiotics were added when needed and were specified in each experiment. Fission yeast cells were usually cultured in 100 ml of medium in an Erlenmeyer flask at 30°C and 220 rpm.

To determine growth curves, cells were cultured in YEPD medium, sampled every 2 h, harvested by centrifugation, washed twice in 100 mM EDTA to disperse floc (18), and suspended in the same volume of solution for determination of the cell density at an optical density of 600 nm (OD600), which was converted into the number of cells based on the blood cell count standard curve.

For nonsexual flocculation analysis, haploid yeast cells were cultured to the late exponential phase (about 48 h; OD600 of 5.0) in YEPD medium.
TABLE 1 Schizosaccharomyces pombe strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type SPQ01 (WT)</td>
<td>h−, leu1-32</td>
<td>Stratagene</td>
</tr>
<tr>
<td>WT SPQ01/pESP3</td>
<td>h−, leu1-32/pESP3</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−Δ</td>
<td>h−, rpl32-2::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−Δ</td>
<td>h−, rpl32-2::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−Δ/pESP3</td>
<td>h−, rpl32-2::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−2Δ/pESP3</td>
<td>h−, rpl32-2::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−2Δ</td>
<td>h−, rpl32-2::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−2Δ</td>
<td>h−, rpl32-2::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−2Δ</td>
<td>h−, rpl32-2::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−2</td>
<td>h−, rpl32-2::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−1</td>
<td>h−, rpl32-1::pESP3-rpl32−1 (leu )</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−2</td>
<td>h−, rpl32-2::pESP3-rpl32−2 (leu )</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl21−Δ</td>
<td>h−, rpl21-2::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 rpl32−2</td>
<td>h−, rpl32-2::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 mpgΔ</td>
<td>h−, mpg::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 hpaΔ</td>
<td>h−, hpa::kanmx6+, leu1-32</td>
<td>This report</td>
</tr>
<tr>
<td>SPQ01 kanmx6</td>
<td>h−, leu1-32::pESP3-kanmx6</td>
<td>This report</td>
</tr>
<tr>
<td>YHL6381</td>
<td>h−, his3-D1, leu1-32, ura4-D18, ade6-M210</td>
<td>19</td>
</tr>
<tr>
<td>YHL6381/WT</td>
<td>h−/h+, his3-D1, leu1-32, ura4-D18, ade6-M210/h+, leu1-32</td>
<td>This report</td>
</tr>
</tbody>
</table>

For complementation experiments, the haploid yeast cells of deletion mutants transformed with a wild-type (WT) ribosomal protein gene were precultured to the late exponential phase (about 48 h; OD600 of 5.0) in EMM2 medium and then harvested by centrifugation. The WT strain or deletion mutants were transformed with the constructs pESP3 or pESP3-rpl32−2, which expresses RPL32−2 as an N-terminal 6His fusion protein. The plasmid was transformed into Escherichia coli strain BL21 (CodonPlus (DE3) Rosetta (Novagen)). The transformants were grown to an OD600 of 0.6 in LB broth containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37°C. Synthesis of RPL3−2 was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to reach a final concentration of 1 mM. Cells were harvested 6 h after the induction. The pellet was suspended in 10 mM Tris-HCl (pH 8.0) buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 μg/ml of lysozyme and sonicated. The cell debris was removed by centrifugation, and the extract (10 ml, ~20 mg) was diluted with an equal volume of binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.9) and loaded onto 1 ml-ml-nickel-nitrilotriacetic acid (Ni-NTA) agarose columns (Qiagen). After being rinsed with wash buffer (20 mM Tris-HCl, 0.5 M NaCl, 40 mM imidazole, pH 7.9), RPL3−2 protein was eluted from the column with elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 M imidazole, pH 7.9) (21). Eluted RPL3−2 proteins were dialyzed in 10 mM Tris-HCl (pH 7.9) buffer and then sent to Yingli Corporation (Shanghai, China) for anti-RPL3−2 antibody preparation in rabbits.

Before use, anti-RPL3−2 antibody was purified by an affinity purification method (22). Briefly, recombinant RPL3−2 proteins were separated using SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was then incubated with RPL3−2 polyclonal antibody (1:3,000 dilution) in TBS-T buffer (25 mM Tris, 150 mM NaCl, 0.5% Tween 20, pH 7.5) at room temperature for 4 h. The PVDF membrane strips containing RPL3−2 band were cut out, put into 10 ml of 0.2 M glycine–HCl solution (pH 2.7), and subjected to a vortex procedure for 10 min at 4°C. The anti-RPL3−2 antibody was eluted from PVDF strips, and the elution was immediately neutralized with NaOH to pH 8.0. The anti-RPL3−2 antibody can recognize both RPL3−2 and RPL3−1 proteins, since there are only four amino acid differences (96.85% homology in amino acid sequences) between these two proteins.

Western blot analysis. Denatured protein extracts were prepared by bead-beating cells directly in a solution containing 10 mM Tris–HCl (pH 7.5, 0.5 mM PMSF, and 0.5 mM protease inhibitors, followed by adding the same volume of SDS sample buffer (10 mM Tris–HCl [pH 7.5], 2% SDS, 10% glycerol, 100 mM dithiothreitol [DTT], 0.25% bromophenol blue) and heating for 5 min at 100°C. Proteins were subjected to SDS-PAGE on 12% gradient gels and transferred to a PVDF membrane. Membranes were probed with anti-beta-actin or anti-RPL3−2 antibody at 37°C for 4 h, followed by alkaline phosphatase (AP)-conjugated secondary antibody, in TBS–2% milk–0.5% Tween 20 at 37°C for 2 h. Signal was detected using an alkaline phosphatase detection kit (Qiagen) according to the manufacturer’s instructions (23).
some lysis buffer (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.1 mg/ml cycloheximide, 0.2 mg/ml heparin). The cell pellets were resuspended in 1 ml of polysome lysis buffer supplemented with protease inhibitors (Roche, Indianapolis, IN) and RNase inhibitors (Promega). Cell suspensions were transferred to prechilled 15-ml conical tubes containing 0.5 g of glass beads and subjected to 6 to 10 pulses of full-speed vortexing for 100 s followed by 100-s pauses. After the glass beads and cell debris were removed by centrifugation at 3,500 rpm for 5 min at 4°C, extracts were transferred to prechilled microcentrifuge tubes and centrifuged for an additional 15 min at 14,000 rpm and 4°C. A total of 15 A₂₆₀ units of extracts was layered onto 5% to 50% sucrose gradients in polysome lysis buffer and centrifuged for 4 h at 44,000 rpm and 4°C in a Beckman SW41 rotor. The gradients were then fractionated by upward displacement with microtubes using a peristaltic pump, and the absorbance at 254 nm was continuously monitored using a UV detector (24).

Polyribosomes were measured by calculating the total absorbance tracing of the peak area (25, 26).

Observation and analysis of flocculation. For observation of flocculation, 2 ml of cell cultures was placed in a petri dish for 10 min. Images were then taken with a Nikon D3100 camera. For micrograph imaging, 5 μl of culture was placed between the slide and coverslip and was observed under a Zeiss Axio Imager A1 microscope (Zeiss, Jena, Germany) with a 63× oil immersion objective differential interference contrast (DIC) lens (numerical aperture [NA], 1.4). Images were taken using Senisacam, a quantum efficiency (QE) cooled digital camera system (Cooke Corp., Romulus, MI).

For determination of flocculation activity, 2 ml of cell culture was transferred to a 2-ml centrifuge tube. Two identical samples and tubes were prepared in this way for each culture. After thorough mixing, the optical density (OD₆₀₀) of 400 μl of the upper layer of the cells in one of the tubes for each culture was carefully measured as the initial cell density, or A₁₀₀. After 10 min of settling, the optical density (OD₆₀₀) of 400 μl of the upper layer of the cells from the other tube of each culture was measured as the end cell density, or A₁₀₀. Cell sedimentation rate was calculated as V = 1 - A₁₀₀ / A₁₀₀ (4).

Analysis of sexual mating diploid cells. Mixed YHL6381 (h⁻) and WT (h⁺) cultures in YEPME mating medium were streaked on YEPME solid mating medium to form individual colonies. The cells from individual colonies were inoculated into 5 ml of YEPD liquid medium and cultured at 30°C and 220 rpm until the OD₆₀₀ reached 2.0. YHL6381 (h⁻) or WT (h⁺) cells were also separately cultured in 5 ml YEPD liquid medium as controls. Genomic DNA extracted from different types of cells was used for PCR amplification using primers (see Table S2 in the supplemental material) specific for M and P mating factor genes at the MatI locus (27).

Microarray analysis using yeast gene chip. For transcriptome analysis of nonsexual flocculation, haploid rpl32-1Δ rpl32-2Δ cells cultured in YEPD medium were used as experiment groups whereas haploid WT cells cultured in YEPD medium were used as a control. For transcriptome analysis of sexual flocculation, diploid YHL6381/WT cells cultured and induced in YEPME mating medium were used as experiment groups, and mixed haploid WT and YHL6381 cells cocultivated in YEPD medium were used as a control. The above-described cells were harvested by centrifugation and directly frozen and stored at −80°C. For RNA isolation, 5 × 10⁶ cells were disrupted in the presence of liquid nitrogen using a mortar and pestle. RNA isolation, cDNA synthesis, and microarray analysis were done as a fee-based service by Gene-BioTech, Shanghai, China. Microarray analysis was replicated three times using cells from independent biological samples. GeneChip Yeast Genome 2.0 arrays (Affymetrix, Santa Clara, CA) were used, and the bioin-labeled cDNA hybridization products were washed by the use of streptavidin, R-phycocerythrin (SAPE) conjugate. Arrays were scanned after hybridization, and data were collected by the use of a Scanner 3000 7G system (Affymetrix). All 15 raw expression files were normalized using a GC-RMA processor. All normalized expression data were analyzed using the PARTEK genomic suite 6.5 software tool. A t test was applied to detect differences in gene expression between each experimental group and the control group. Two criteria were used to determine whether a gene was differentially expressed: fold change of ≥ 1.2 and P value < 0.05 (using a two-tailed distribution). The value of 1.2 is an accepted cutoff which, with statistical significance, is likely to be validated by real-time PCR (28). Gene ontology and cluster analysis was performed by the use of the dChip software tool.

Microarray data accession numbers. All the microarray data from these independent fission yeast samples were divided into two sets, a nonsexual flocculation series and a sexual flocculation series, and have been uploaded into the NCBI Gene Expression Omnibus (GEO) database. The GEO accession number for the nonsexual flocculation series is GSE43248, and the GEO accession number for the sexual flocculation series is GSE43250.

RESULTS

Deletion of rpl32 genes reduces the ribosome level and induces nonsexual flocculation in haploid mutant cells. RPL32 proteins in S. pombe are encoded by two duplicated homologue genes, rpl32-1 (SPB16C6.11) and rpl32-2 (SPAC3H5.10). Deletion mutants were created for individual genes from the wild-type (WT) SPQ01 strain (h⁺) using the gene replacement method (Fig. 1A) and were confirmed using PCR (Fig. 1B). QPCR analysis showed that the expression of rpl32-1 in rpl32-1Δ cells and the expression of rpl32-2 in rpl32-2Δ cells were completely abolished (Fig. 1C). The total reductions of rpl32 (rpl32-1 + rpl32-2) transcript levels were 35.9% and 46.9% in rpl32-1Δ and rpl32-2Δ cells, respectively, compared to WT cells. Western blot analysis also showed a significant decrease in the total amount of RPL32 protein in each deletion mutant (Fig. 1D), consistent with the QPCR results. Reduction of RPL32 protein levels affected ribosome assembly and structure (Fig. 1E). Compared to the WT strain, the rpl32-1Δ and rpl32-2Δ deletion mutants both showed a lowered accumulation of free 60S subunits. This ribosomal subunit imbalance led to 31.1% and 27.8% reductions, respectively, in overall ribosome levels (80s monosome and polysomes) in rpl32-1Δ and rpl32-2Δ cells compared to WT cells (Fig. 1E).

rpl32-1Δ and rpl32-2Δ cells grew much slower, reaching only half of the growth rate of WT cells (Fig. 2A). Surprisingly, when the deletion mutant cells were cultured in a nutrient-rich YEPD medium, they aggregated to form flocculations (Fig. 2B). Flocculation was not observed in WT cells grown in the same medium. Quantitative analysis showed that WT cells in liquid culture came out of the medium slowly with 10.4% sedimentation after 10 min of settling, while rpl32-1Δ and rpl32-2Δ cells showed 48.5% and 49.9% sedimentation, respectively (Fig. 2C).

Microarray analysis of free 60S subunits. This ribosomal subunit imbalance led to 31.1% and 27.8% reductions, respectively, in overall ribosome levels (80s monosome and polysomes) in rpl32-1Δ and rpl32-2Δ cells compared to WT cells (Fig. 1E).

To eliminate the possibility that the flocculation may be caused by the kanmx6 antibiotic resistance gene introduced during the mutant generation as a selection marker (G418 resistance), we expressed the kanmx6 gene in the SPQ01 strain.
No flocculation was observed in this recombinant strain expressing the \textit{kannx6} gene (Fig. 2D). Further, we randomly selected two other duplicated ribosomal protein genes, \textit{rpl21-2} (SPAC959.08) and \textit{rpl9-2} (SPCC613.06), and two nonribosomal protein genes, the mannos-1-phosphate guanylytransferase gene, or \textit{mpg} (SPBC13G1.02), and the fructose-2,6-bisphosphate 2-phosphatase gene, or \textit{fbp} (SPAC732.02c), and generated deletion mutants for these genes. \textit{rpl21-2}/H9004 and \textit{rpl9-2}/H9004 deletion mutants also showed flocculation in YEPD medium whereas \textit{mpg}/H9004 and \textit{fbp}/H9004 deletion mutants did not (Fig. 2E), suggesting that the nonsexual flocculation was not caused specifically by reduction of the RPL32 protein level but was rather a general response to the reduction of the overall ribosome level.

Sexual flocculation fission yeast cells display a reduction of ribosomal protein expression and ribosomal biogenesis during mating. Since flocculation in the fission yeast \textit{S. pombe} is an essential step required for mating and ascospore formation in sexual reproduction (2, 29), we examined whether sexually flocculating yeast cells also exhibited a reduction of ribosomal protein expression and ribosomal biogenesis during mating. The WT \textit{S. pombe} SPQ01 strain used in this study is a haploid strain of the \textit{h}+ mating type, so an \textit{h}− strain, YHL6381, was used as the mating partner for the WT strain. We used YEPD medium, which is YEG medium (29) plus peptone, as a non-flocculation-inducing medium, and YEPME mating medium, in which malt extract containing \(50\%\) maltose (17) replaced glucose in YEPD, as a flocculation-inducing medium (16).

We first established a sexually flocculating cell system for \textit{S. pombe}. When haploid WT and YHL6381 cells precultured separately in YEPD medium were mixed at a 1:1 cell number ratio and transferred into YEPME mating medium, flocculation occurred after 90 min of inoculation (Fig. 3A, lower panel, column 3), whereas when the same mixed cells were transferred into YEPD medium, no flocculation was observed (Fig. 3A, upper panel, column 3), indicating that it was only when they were cocultured in

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.pdf}
\caption{Deletion of \textit{rpl32} genes reduced ribosome levels in haploid cells. (A) Deletion of \textit{rpl32} paralogous genes in \textit{S. pombe} using a PCR-based approach with long DNA tracts of flanking homology to the target locus. (B) Deletion mutants were confirmed by PCR with genomic DNA as a template and locus-specific primers flanking the target sites. (C) QPCR quantification of \textit{rpl32-1} and \textit{rpl32-2} transcript abundance in deletion mutants and WT cells cultured in YEPD medium. \textit{beta-actin} was used to standardize transcript levels of paralog \textit{rpl32-1} and \textit{rpl32-2}. (D) Western blot analysis of RPL32 in deletion mutants and WT cells cultured in YEPD medium. (E) Polysome profiles of deletion mutants and WT cells cultured to the late exponential phase (OD\textsubscript{600} of 5.0) in YEPD medium. A total of 15 \(A_{260}\) units of extracts from each culture was analyzed on 5% to 50% sucrose gradients centrifuged for 4 h at 44,000 rpm (25).}
\end{figure}
YEPME mating medium that heterothallic strains of *S. pombe*, *h*− and *h*+, were able to be sexually coflocculated. When haploid YHL6381 and WT cells precultured separately in YEPD medium were transferred into YEPME mating medium for further individual cultivation, flocculation did not occur either (Fig. 3A, lower panel, columns 1 and 2), indicating that after being cultured separately in YEPME mating medium, the heterothallic strains, *h*− and *h*+, were nonflocculent. When WT cells that overexpressed *rpl32*-1 or *rpl32*-2 as a consequence of harboring the constructs of *pESP3*-rpl32-1 and *pESP3*-rpl32-1 were, respectively, mixed with YHL6381 cells and cocultured in YEPME mating medium, flocculation still took place, indicating that high levels of *RPL32* alone did not interfere with sexual flocculation (Fig. 3A, lower panel, columns 4 and 5). When haploid YHL6381 and WT cells were precultured separately in the YEPME mating medium first and then the two culture broths were mixed together, no flocculation was observed (Fig. 3A, column 6), indicating that heterothallic haploid cells, in the absence of opposite mating type cells, did not trigger flocculation even in YEPME mating medium. We were able to amplify only a 729-bp M factor DNA band from the haploid SPQ01 cells and a 987-bp P factor DNA band from the YHL6381 cells, while both 729-bp and 987-bp DNA bands were amplified from each single colony isolated on the plate from YHL6381 (*h*+)/WT (*h*−) cells cocultivated in the YEPME mating medium (Fig. 3B), further proving that mating had occurred and that the cells in those colonies were *h*+/*h*− diploid.

The QPCR analysis showed that the transcript levels of *rpl32* genes in nonflocculating haploid YHL6381 (*h*+) and WT (*h*−) cells grown in YEPME mating medium were reduced by 12.1% and 23.5%, respectively, compared to the same type of haploid cells grown in YEPD medium. Interestingly, the overall transcript levels of *rpl32* genes in the nonflocculating mixed cells of haploid YHL6381 (*h*+) and WT (*h*−) cells cocultured in YEPD medium were also reduced by 16.3% and 10.8%, respectively, compared to those of the haploid YHL6381 (*h*+) and WT (*h*−) cells cultured separately in the same medium. However, the flocculating diploid YHL6381/WT (*h*+/h−) cells formed by being cocultured in YEPME mating medium showed a 42.1% decrease in the total *rpl32* transcript levels compared to mixed haploid cells of YHL6381 (*h*+) and WT (*h*−) cells cocultured in YEPD medium (Fig. 3C). We also studied the expression of other ribosomal protein genes. As shown in Fig. 3C, the total transcript levels of the *rpl30* and *rps10* paralogous genes were reduced by only approximately 13% to 23% in haploid YHL6381 (*h*+) and WT (*h*−) cells separately grown in YEPME mating medium compared to the same haploid cells grown in YEPD medium. Similarly, the overall transcript levels of *rpl30* and *rps10* paralogous genes in the nonflocculating mixed cells of the haploid YHL6381 (*h*+) and WT...
strains cocultured in YEPD medium were also decreased only by 6% and 11%, respectively, compared to those of the haploid YHL6381 (h<sup>+</sup>) and WT (h<sup>+</sup>) cells cultured separately in the same medium, while in diploid YHL6381/WT (h<sup>+</sup>/h<sup>-</sup>) cells cocultivated in YEPD medium. Polyribosomal analysis demonstrated that flocculating diploid cells cultured in YEPME mating medium showed a 37.2% decrease in the total ribosome level compared to the nonflocculating haploid cells from a mixed YHL6381 (h<sup>+</sup>) and WT (h<sup>+</sup>) culture cogrown in YEPD medium (Fig. 3D). These results indicated a general trend of reduction in the ribosomal protein gene expression level as well as in the ribosome level during sexual flocculation, as shown in nonsexually flocculating haploid rpl32<sup>-1</sup>/H9004 and rpl32<sup>-2</sup>/H9004 cells.

Transcriptome analysis indicates that similar molecular mechanisms are involved in nonsexual and sexual flocculation in fission yeast. Since both nonsexual and sexual flocculations are associated with the reduction of ribosomal protein gene expression and ribosome level, we wondered whether nonsexual and sexual flocculation shared similar molecular mechanisms. Thus, we compared the transcriptome of nonsexually flocculating haploid rpl32<sup>-1</sup>/H9004 and rpl32<sup>-2</sup>/H9004 cells to that of nonflocculating haploid WT SPQ01 cells and compared the transcriptome of sexually flocculating diploid YHL6381/WT (h<sup>+</sup>/h<sup>-</sup>) cells cocultivated in YEPD medium. Figure 4 presents the comparative transcriptomic patterns of 85 genes which showed a change in expression of over 1.2-fold (P < 0.05) between flocculating and nonflocculating cells. As shown in Fig. 4, the transcription pattern of nonflocculating mixed haploid cells of YHL6381 (h<sup>+</sup>) and WT (h<sup>-</sup>) strains was almost as the same as that of nonflocculating WT cells, while the transcription pattern of nonsexually flocculating haploid rpl32<sup>-1</sup>Δ and rpl32<sup>-2</sup>Δ cells was similar to that of sexually flocculating diploid YHL6381/WT (h<sup>+</sup>/h<sup>-</sup>) cells. Most of genes which
were minimally expressed in nonflocculating cells were upregulated in expression level in both nonsexually and sexually flocculating cells, while some parts of genes which were highly expressed in nonflocculating cells were downregulated in both nonsexually and sexually flocculating cells and the other parts of those genes were downregulated only in sexually flocculating cells.

From the supporting information presented in Table S1 in the supplemental material, we found that 20 different ribosomal protein genes, including \( rpl32-1 \) and \( rpl32-2 \), showed reduced expression in sexually flocculating diploid \( YHL6381/WT \) relative to nonflocculating mixed haploid cells of \( YHL6381 \) and WT strains cocultivated in YEPD medium and that only 4 of those genes showed reduced expression in both nonsexually flocculating haploid \( rpl32-1 \Delta \) and \( rpl32-2 \Delta \) mutant cells. And 17 ribosomal biogenesis genes showed reduced expression in sexually flocculating diploid cells; among those, only 2 genes showed decreased expression in both \( rpl32-1 \Delta \) and \( rpl32-2 \Delta \) cells. In contrast, expression of \( sfp1 \) and \( crf1 \), both which are involved in repression of ribosomal biogenesis gene expression (GeneDB), was increased in sexually flocculating diploid \( YHL6381/WT \) cells. However, expression of these two genes was not affected in the nonsexually flocculating haploid cells. It was noticed that \( rpl21-2 \) and \( rpl9-2 \), deletion of which resulted in nonsexual flocculation of haploid cells (Fig. 2E), were not included in the above-mentioned 20 downregulated ribosomal protein genes.
We found that expression of seven regulatory factor genes (STE11, pmk1, cmk2, byr1, srk1, STE12, and sin1) in the mitogen-activated protein kinase (MAPK) pheromone response pathway (30) was upregulated in sexually flocculating diploid YNL6381/WT (h+ / h−) cells and that four of those genes (STE11, pmk1, cmk2, and byr1) were also upregulated in nonsexually flocculating rpl32-1Δ and rpl32-2Δ haploid cells. Moreover, expression of the pkal gene which encodes a key factor in the cyclic AMP (cAMP)/protein kinase A (PKA) pathway was downregulated in both nonsexually and sexually flocculating cells. Reduced expression of pkal is often accompanied by increased expression of STE11, which is a key regulatory factor in sexual flocculation (31). Among the downstream genes that are regulated by STE11, 12 genes were upregulated in sexually flocculating diploid cells (YHL6381/WT) and 9 genes were upregulated in nonsexually flocculating rpl32-1Δ and rpl32-2Δ haploid cells. Four of these upregulated genes, mug37, mug97, mei4, and SPCC1442.04c, were common to both sexually and nonsexually flocculating cells. In addition, seven genes involved in sexual spore formation and differentiation were upregulated in the sexual flocculation diploid YHL6381/WT cells, and three genes, meu14, mde10, and ADY3, were also upregulated in nonsexually flocculating rpl32-1Δ and rpl32-2Δ haploid cells.

As shown in Table S1 in the supplemental material, 12 putative cell wall adhesin genes, including known adhesin protein Mam3 and Map4 genes, were upregulated in sexually flocculating diploid YHL6381/WT cells, and 8 putative cell wall adhesin genes were upregulated in nonsexually flocculating haploid rpl32-1Δ and rpl32-2Δ cells; of those, 6 were also upregulated in sexually flocculating cells.

We randomly chose 20 genes with an average transcription fold change > 1.2 in microarray analysis between flocculating and nonflocculating cells from different catalogs for QPCR analysis and proved that these differentially expressed genes really were upregulated or downregulated in flocculating cells compared to nonflocculating cells, consistent with the results from microarray analysis (see Fig. 4 legend; see also Table S1 in the supplemental material).

**DISCUSSION**

This report shows that deletion of ribosomal proteins as well as a corresponding reduction of overall ribosome levels leads to a nonsexual flocculation of fission yeast. A downregulation of ribosomal protein synthesis and reduction of the ribosomal level were also detected in sexually flocculating diploid YHL6381/WT (h+ / h−) cells. A logical interpretation is that nonflocculation caused by deletion of ribosomal proteins may exploit the regulation of the ribosomal level in the onset of sexual flocculation. The transcriptome analysis provided additional data to support this hypothesis. The transcript levels of 20 ribosomal protein genes and 17 ribosomal biogenesis genes in the yeast genome were reduced during sexual flocculation, while expression levels of two known ribosome synthesis repressor genes were increased in sexually flocculating diploid cells. Thus, in sexual flocculation cells, reduction of the ribosome level was caused by extensive downregulation of expression of ribosomal protein genes and ribosomal biogenesis genes and by upregulation of the ribosomal biogenesis gene repressors. This point is supported by the fact that sexual flocculation could not be blocked by overexpression of one specific ribosomal protein alone such as RPL32-1 or RPL32-2 in mating cells (Fig. 3A, lower panel, columns 4 and 5). It is known that ribosome synthesis depends on a specific ratio of each of the ribosome proteins and rRNAs in the ribosome (24, 25). Deletion of individual ribosomal protein genes broke this balance between ribosomal proteins and therefore led to a reduction in the overall ribosome level, as indicated by the fact that polyribosome analysis (see Results) triggered a nonsexual flocculation of haploid cells. Furthermore, rpl21-2 and rpl9-2, deletion of which resulted in nonsexual flocculation of haploid cells (Fig. 2E), were not included among the 20 downregulated ribosomal protein genes in sexually flocculating cells (see Table S1 in the supplemental material), further implying that as long as ribosomal protein levels as well as the corresponding ribosomal levels were reduced, flocculation might be triggered in haploid cells, as occurred in sexual flocculation cells. Therefore, deleting ribosomal protein genes may lead to nonsexual flocculation as a consequence of mimicking the reduction of the ribosomal level in the sexual flocculation process.

However, though the haploid YHL6381 (h+) and WT (h−) cells grown in YEPME mating medium showed overall rpl32 gene transcription that was reduced by 12.1% and 23.5%, respectively, compared to the levels seen with the same haploid cells grown in YEPD medium, and overall rpl32 gene transcription of the mixed haploid cells of YHL6381 (h+) and WT (h−) cocultured in YEPD medium also decreased by 16.3% and 10.8%, respectively, compared to the transcription levels seen with the haploid YHL6381 (h+) and WT (h−) cells cultured separately in the same medium, none of these cells formed flocculates. This may be related to the extent of reduction of the ribosomal protein transcript level in fission yeast, since the flocculating diploid YHL6381/WT (h+/h−) cells formed in YEPME mating medium showed a 42.1% decrease in the overall rpl32 transcript level compared to nonflocculating mixed haploid YHL6381 (h+) and WT (h−) cells cocultivated in YEPD medium. This idea is further supported by the nonsexual flocculation of haploid rpl32-1Δ and rpl32-2Δ deletion mutant cells grown in YEPD medium, which showed 35.9% and 46.9% reductions in the overall rpl32 transcript level, respectively, compared to nonflocculating WT cells grown in the same medium. Therefore, flocculation may occur only when the reduction of ribosomal protein levels is below a certain threshold value (probably below 35%, based on the present study); that is, triggering of flocculation may be dependent on the extent of the reduction of the transcript level of the ribosome proteins.

Current data suggest that, once past the ribosome checkpoint level, nonsexually and sexually flocculating cells share some common mechanism for initiation of flocculation. First, deleting either of the ribosomal protein L32 paralogs upregulated the expression level of putative adhesin genes in nonssexual flocculating cells, while expression levels of putative adhesin genes were enhanced in sexual flocculating cells also, accompanied by a decrease in the ribosomal protein expression level. And six upregulated adhesin genes were shared by the nonsexually and sexually flocculating cells. These results suggest that a decrease in expression of ribosomal proteins stimulates expression of adhesin proteins, leading to flocculation. Second, both nonsexually and sexually flocculating haploid cells of rpl32 deletion mutants and sexually flocculating diploid cells showed that some major components in the MAPK pheromone response pathway, such as STE11, Mei4, Byr1, and Pka1, were significantly upregulated (STE11, Mei4, and Byr1) or downregulated (Pka1) at the transcript level. In S. pombe, STE11 is a key transcription factor in sexual reproduction (31); Mei4 is a meiosis-specific transcription factor that plays an important role.
in the progression of meiosis and sporulation (32); byr1 resembles byr2 in many respects, overexpression of byr2 increases cell agglutinability, and interfering forms of byr2 block the increased agglutinability (33); and a defect of the pka1 gene readily initiates sexual development in rich medium (34, 35). It has been reported that the MAPK pheromone response pathway can trigger adhesin FLO11-mediated cell surface flocculation in S. cerevisiae cells, such as pseudohyphal development in diploids, or filament and invasive growth or mating in haploids, in response to stress and nutrient limitation (36–38). Taking these data together, reductions of ribosomal protein levels may trigger adhesin synthesis for flocculation and mating via the STE11-mediated MAPK pheromone response pathway. Third, genes involved in meiosis and spore formation, such as meu14 (39), mde10 (40), and ADY3 (41), were also upregulated in both nonsexually and sexually flocculating environments and initiate sexual reproduction on time. Taking these results together, we propose that, by linking the ribosome level to the onset of flocculation, yeast cells can sense environmental changes and initiate sexual reproduction on time.

**ACKNOWLEDGMENTS**

This research is supported by the National Science Foundation of China (no. 30670025 and no. 31070060) and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

**REFERENCES**

23. Powers T, Walter P. 1999. Regulation of ribosome biogenesis by the


