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RESEARCH ARTICLE

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LAMMER Kinase Lkh1 Is an Upstream Regulator of Prk1-Mediated Non-Sexual Flocculation in Fission Yeast

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ABSTRACT

The cation-dependent galactose-specific flocculation activity of the *Schizosaccharomyces pombe* null mutant of *lkh1*$^+$, the gene encoding LAMMER kinase homolog, has previously been reported by our group. Here, we show that disruption of *prk1*$^+$, another flocculation associated regulatory kinase encoding gene, also resulted in cation-dependent galactose-specific flocculation. Deletion of *prk1* increased the flocculation phenotype of the *lkh1*$^+$ null mutant and its overexpression reversed the flocculation of cells caused by *lkh1* deletion. Transcript levels of *prk1*$^+$ were also decreased by *lkh1*$^+$ deletion. Cumulatively, these results indicate that Lkh1 is one of the negative regulators acting upstream of Prk1, regulating non-sexual flocculation in fission yeast.

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1. Introduction

Flocculation is a well-known natural phenomenon of active aggregation, and is defined as the reversible aggregation of cells into flocs [1,2]. This process is important in industrial processes related to fermentation technology such as brewing, wine-making, and bioconversion; however, it is also a nuisance in many industrial processes, clinical settings, and in the laboratory [3]. Flocculation is due to divalent-cation-dependent bonding between the cell-surface proteins (lectins) of flocculent cells and specific sugar residues of the mannans embedded in the yeast cell wall [4].

Shankar and Umesh-Kumar [5] purified a cell-surface lectin from a flocculent strain of *Saccharomyces cerevisiae*, which, in the presence of Ca^{2+}, binds specifically to the mannose and mannan residues isolated from yeast cell walls belonging to intact cells. Several dominant flocculation genes have been identified in *Sc. cerevisiae*: *FLO1*, *FLO5*, *FLO8*, *FLO9*, *FLO10*, *FLO11*, and *FLO2* [6–10]. These genes are now considered a part of the 'flocculin family' and encode highly homologous proteins [8,11]. Different FLO genes confer variable degrees of flocculation, or encode proteins that have different responses to proteases and heat treatment or sugar binding capabilities [12].

Heterothallic haploid strains of the fission yeast *Schizosaccharomyces pombe* are usually non-flocculent when cultured independently [11]. However, the deletion or mutation of some negative regulators of flocculin expression, such as Ume5 kinase homolog Prk1 [13], LAMMER kinase homolog Lkh1 [14], transcriptional repressor Tup12 [15], and ribosomal protein Rpl32 [16,17], and the overexpression of some adhesins [18] induced non-sexual flocculation in *Sch. pombe*. Earlier, we reported that Lkh1-mediated flocculation is a cation-dependent, galactose-specific process [14], and that Lkh1 phosphorylates global transcription repressor Tup12, deletion of which showed asexual flocculation [15]. As of now, no report on the physicochemical properties of Prk1-dependent flocculation, or genetic interaction between the two protein kinases, Lkh1 and Prk1, in the asexual flocculation of *Sch. pombe* has been published. In order to explore the possible association of these two kinases with flocculating ability, we investigated ion-dependence and sugar-specificity of flocculation in the Prk1 null mutant, and genetic interaction between *prk1*$^+$ and *lkh1*$^+$.

In this study, we found that disruption of *prk1*$^+$ produced cation-dependent galactose-specific flocculation similar to that of *lkh1*$^+$. Also, the overexpression of Prk1 reversed the flocculation phenotype of the *lkh1*$^+$ null mutant. Moreover, we found a significant decrease in the transcriptional level of *prk1*$^+$ in the *lkh1*$^+$ null mutant.

2. Materials and methods

2.1. Strains, media, and vectors

The strains used in this study are listed in Table 1. The rich medium was YE medium, and the selective

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Table 1. *Schizosaccharomyces pombe* strains used in this study.

Strain	Genotype	Source
ED665	*h- ade6-M201 leu1-32 ura4-D18*	Lab. Collection
PHM5L	*h- ade6-M201 leu1-32 ura4-D18 lkh1::leu2*	Kim et al. [14]
SP107	*h- ade6-M201 leu1-32 ura4-D18 prk1::ura4*	This study
SP109	*h- ade6-M201 leu1-32 ura4-D18 lkh1::leu2 prk1::ura4*	This study
SP202	*h- ade6-M201 leu1-32 ura4-D18 lkh1::leu2 pREP2-prk1:ura4*	This study

medium was Edinburgh synthetic minimal medium with appropriate supplements. Yeasts were grown at 30 °C. Standard techniques for fission yeast molecular genetics were used following the methods described in the books written by Moreno *et al.* [19] and Alfa [20]. *Escherichia coli* and *Sch. pombe* cells were transformed by the ultra-competent and the lithium acetate method, respectively [20,21]. pREP2 (100X), a *Sch. pombe* expression vector containing the thiamine-repressible $nmt1^+$ promoter was used for overexpression of $prk1^+$ in the $lkh1^+$ null mutant. Thiamine (2 μM) was added to the medium to repress transcription from the $nmt1^+$ promoter [22].

2.2. Disruption and cloning of prk1$^+$

The $prk1^+$ gene was replaced with the 1.8-kb *Sch. pombe* $ura4^+$ cassette [23]. To construct the $prk1^+$ disruption cassette, the upstream non-coding region was amplified by PCR using primers Prk-uf and Prk-ur. The downstream non-coding region was amplified using primers Prk-df and Prk-dr (Table 2). Transformation was performed using the lithium acetate method [20,24]. Stable Ura^+ transformants were initially screened by PCR, and the replacement of the $prk1^+$ loci was confirmed by Southern blot analysis. To amplify the $prk1^+$ gene, the sense primer Prk-F, and the antisense primer Prk-R were used. The pREP2-Prk1 vector was constructed by cloning a *Sal*I–*Bam*HI fragment containing the full-length $prk1^+$ coding sequence into pREP2.

2.3. Flocculation assay

The effects of metal ions and sugars on flocculation were tested by employing the previously described methods [13,14,19]. Briefly, cells grown to early stationary phase ($\sim 10^8$ cells/ml) in YE medium were washed with 10 mM EDTA, followed by excess deionized water to repress flocculation activity, and resuspended in deionized water. Ions or sugars were added to the cell suspension, and then $CaCl_2$ was added to a final concentration of 10 mM to initiate the flocculation reaction. Percentage flocculation was estimated as described previously [25]. Briefly, the yeast suspension (20 ml) was vigorously shaken in 100-ml flasks and the optical density (OD_{600}) (A1) was measured subsequently. The suspension was left for 30 min at room temperature and the optical density of the upper layer of the cell suspension (A2) was measured again. Percentage flocculation was calculated according to an equation: $\%Floc = [(A1 - A2)/A1] \times 100$.

2.4. RNA preparation and qRT-PCR

Cells at early stationary phase were harvested and total RNAs were prepared with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer. Relative mRNA levels were determined by real-time quantitative reverse transcription (qRT) PCR using the specific primer sets (Table 2). Briefly, total RNA was reverse transcribed into cDNA using the M-MLV Reverse Transcriptase (Elpis Biotech, Daejeon, Korea) according to the manufacturer's instructions. Each gene-specific primer was optimized for expression on a CFX96 Real-Time PCR (Bio-Rad Laboratories, USA) using TOPreal^{TM} qPCR 2X PreMIX (Enzynomics, Daejeon, Korea). The PCR cycling parameters were 5 min at 95 °C, 45 cycles of 10 sec at 95 °C, 15 sec at 60 °C, and 30 sec at 72 °C, and 10 sec at 95 °C. A melting curve analysis to confirm specificity was performed for each primer set. β -actin gene was used for normalization and the relative expression was calculated according to a previously reported method [26].

3. Results and discussion

3.1. Deletion of prk1$^+$ slightly increases the flocculating ability of the lkh1$^+$ null mutant

We previously reported that Lkh1, a LAMMER kinase homolog, negatively regulates asexual flocculation in fission yeast by showing flocculation of the $lkh1^+$ null mutant, which is cation-dependent and galactose-specific in liquid culture [14]. Next, to determine the physicochemical properties of non-sexual flocculation caused by the $prk1^+$ deletion, and the genetic interaction between $lkh1^+$ and $prk1^+$ during flocculation, we constructed Δ*lkh1*, Δ*prk1*, and Δ*lkh1*Δ*prk1* double mutants, and then assayed them for flocculation.

As reported [13,14], ED665 (wild-type) cells remained in suspension during the course of the assay, whereas PHM5L (Δ*lkh1*) and SP107 (Δ*prk1*) cells flocculated and settled rapidly. Interestingly, while the disparity in flocculation was not much, the additional

Table 2. Primers used in this study.

Name	Sequence (5′ to 3′)*	Mutagenic information
<Deletion & Cloning>		
Prk-uf	TA**GAATTC**TGGCATACTTCTAAAGTGCTATTG	*Eco*RI site
Prk-ur	TA**GGATCC**TTTCATGCTGAAATGACCCTA	*Bam*HI site
Prk-df	TA**GTCGAC**AACTCACTTTTTAGCCCATTTTT	*Sal*I site
Prk-dr	TA**AAGCTT**TCGCTAGAGTTATCAGCCTCA	*Hind* III site
Prk-F	CT**GTCGAC**TATGAAAGACGGTTATAAAATTATTG	*Sal*I site
Prk-R	CT**GGATTC**TTATTAAAAATGGGCTAAAAAGTG	*Bam*HI site
<qRT-PCR>		
Act1-RT-F	CACCCTTGCTTGTTGACTGA	
Act1-RT-R	ATTTACGCTCAGGAGGAGCA	
Prk1-RT-F	GGAAAGGTTTATAAAGCGG	
Prk1-RT-R	TTCAAAGCTGAGTCAAAGC	

*The initiator ATG and the stop anticodon TTA are underlined and restriction enzyme sites included are emboldened.

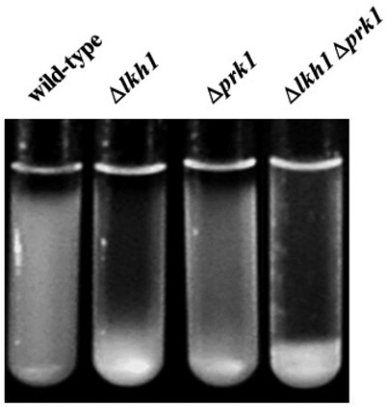

Figure 1. Effect of *prk1*$^{+}$ deletion on the flocculation phenotype of the *lkh1*$^{+}$ null mutant. Cells grown to early stationary phase (~10^8 cells/ml) in YE medium were washed with 10 mM EDTA and excess water. $CaCl_2$ was added to the cell suspension to initiate the flocculation reaction. Each culture tube was photographed 30 min after initiation of the flocculation reaction. ED665 (wild-type), PHM5L (Δ*lkh1*), SP107 (Δ*prk1*), and SP109 (Δ*lkh1*Δ*prk1*) cells were used.

deletion of the *prk1*$^{+}$ gene (SP109, Δ*lkh1*Δ*prk1*) nonetheless increased the flocculation of the *lkh1*$^{+}$ null mutant (PHM5L) (Figure 1), clearly suggesting that Prk1 is functionally linked to the Lkh1-mediated asexual flocculation in fission yeast.

3.2. prk1^{+} also acts as a negative regulator of divalent-cation-dependent asexual flocculation in fission yeast

Watson and Davey [13] reported the involvement of the Prk1 protein kinase in *Sch. pombe* flocculation. However, a substantial number of features of *prk1*$^{+}$-mediated flocculation are unknown. Therefore, we tested the consequences of using metal ions and sugars on the flocculation activity of *prk1*$^{+}$ null mutants. The flocculation activities of SP107 (Δ*prk1*) and SP109 (Δ*lkh1*Δ*prk1*) cells were completely repressed upon washing them with 10 mM EDTA (data not shown), but restored by the subsequent addition of divalent cations. As in the *lkh1*$^{+}$ null mutant PHM5L [14], the flocculation activity of SP107 (Δ*prk1*) cells was restored by the addition of Ca^{2+}, Mg^{2+}, Li^{2+}, Zn^{2+}, Mn^{2+}, or Co^{2+}, but not by Cu^{2+} or Ni^{2+} (Figure 2(A)). However, Zn^{2+}, Mn^{2+}, and Co^{2+} were more effective in restoring the flocculation activity in SP107 rather than in PHM5L. It is also noteworthy that SP109 cells were similar to PHM5L cells in terms of their divalent-cation requirements for flocculation activity. On the other hand, the effect of metal ions on the flocculation of SP107 cells differed slightly relative to the *Sch. pombe* AAD-1 mutant, for which the Ca^{2+}, Mn^{2+}, Li^{2+}, Cu^{2+}, and Zn^{2+} ions proved to be effective, unlike Mg^{2+} and Co^{2+} ions [27]. The requirement for different cations might be due to the involvement of different flocculins. Interestingly, several cations, including Li^{2+}, were almost equally efficient in the flocculation of kinase gene disruptants, in contrast to Ca^{2+}, which was most effective in the flocculation of *Sc. cerevisiae* and *Sch. pombe* AAD-1 mutants. Our results also indicate that flocculation of the *prk1*$^{+}$ null mutants is divalent-cation-dependent, rather than Ca^{2+}-specific, which is the case with the *lkh1*$^{+}$ null mutant.

3.3. Prk1-dependent asexual flocculation is galactose-specific

Sugar-specificity for the asexual flocculation of SP107 (Δ*prk1*) and SP109 (Δ*lkh1*Δ*prk1*) cells was determined. After suppressing the flocculation activity of cells by washing with 10 mM EDTA, sugar moieties were added to saturate the sugar-specific lectins. Then, $CaCl_2$ was added to initiate flocculation. The repression of flocculation, when a specific sugar is added, indicates that this sugar has saturated the lectins. These lectins interact with the same kinds of sugars on other cell surfaces, implying that this sugar-specific interaction is involved in the flocculation process. Galactose and lactose significantly inhibited the flocculation activity of SP107 and SP109 cells. On the other hand, glucose and maltose had no effect (Figure 2(B)). These results accord well with previous results for AAD-1 [27] and PHM5L [14], indicating that galactose residues, the major components of the cell-wall galactomannoproteins of *Sch.*

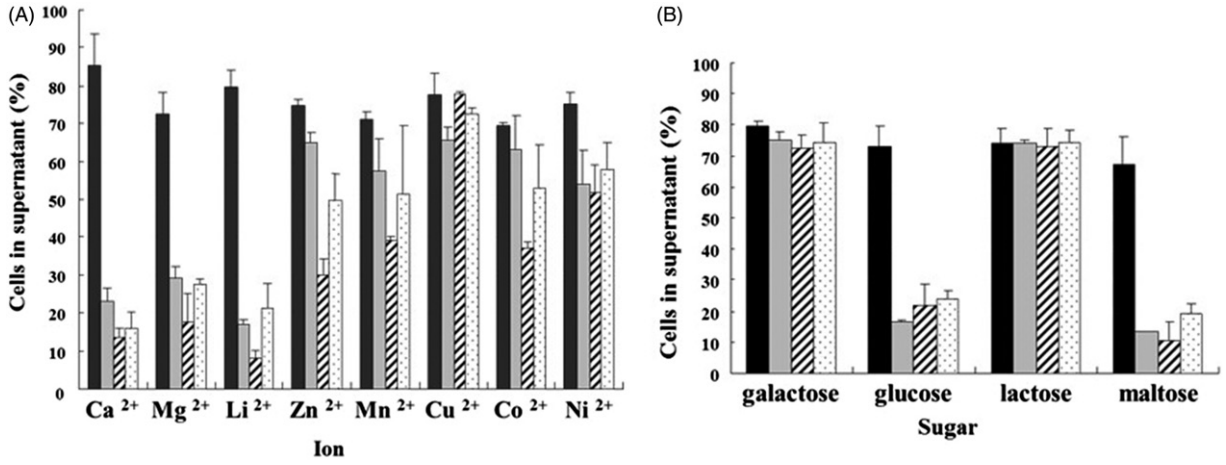

Figure 2. Effect of divalent cations and sugars on flocculation. Stimulatory effect of divalent cations (A) and inhibitory effect of sugars (B) on flocculation. Cells in suspension represent the percentage of cells remaining in the upper layer 30 min after initiation of the flocculation reaction. The number of cells in suspension was determined by measuring optical density (OD 600_{nm}). The bars indicate the standard deviation of four independent measurements for ED665 (wild-type, black bar), PHM5L (Δ*lkh1*, gray bar), SP107 (Δ*prk1*, oblique line bar), SP109 (Δ*lkh1*Δ*prk1*, dotted bar) cells. Each of the measurement was made in triplicates.

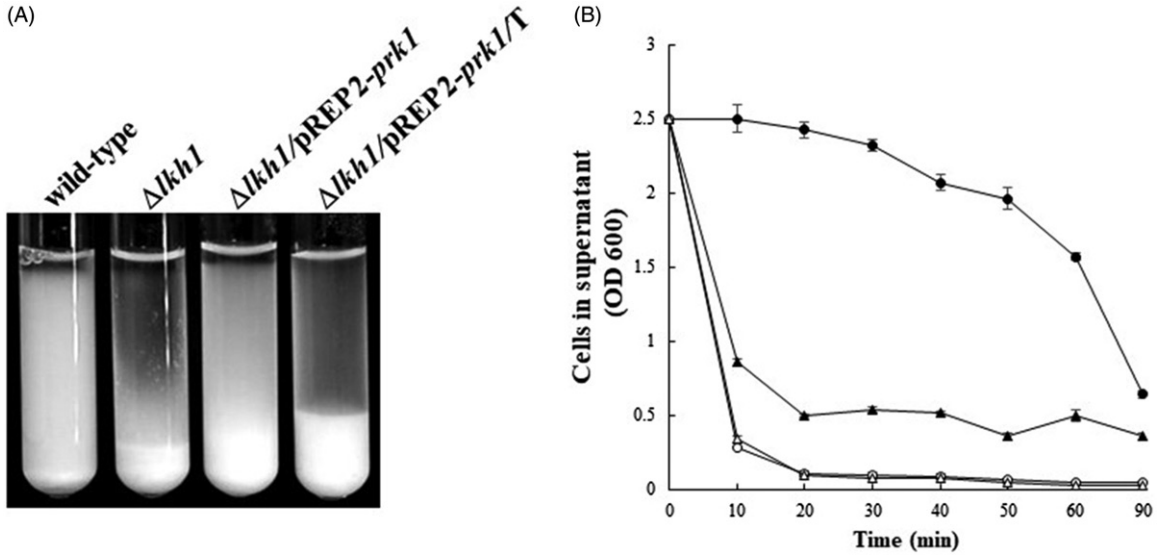

Figure 3. Reversal of the *lkh1*$^+$ null mutant phenotype by *prk1*$^+$. Cells were grown to early stationary phase (~10^8 cells/ml) in YE medium. Culture tubes containing ED665 (wild-type, tube 1), PHM5L (Δ*lkh1*, tube 2), SP202 (pREP2-*prk1*$^+$, tube 3), and SP202 with 2 μM thiamine (tube 4) were photographed 30 min after initiation of the flocculation reaction (A). Absorbance of culture supernatants of ED665 (closed circle), PHM5L (open circle), SP202 (closed triangle), and SP202 with 2 μM thiamine (open triangle) was measured at 10-min intervals (B).

pombe, may act as receptors for the divalent-cation-dependent aggregation of cells into floccules.

3.4. Lkh1 acts at the upstream of the Prk1 for non-sexual flocculation in fission yeast

Because *prk1*$^+$ and *lkh1*$^+$ are involved in the asexual flocculation of fission yeast [13,14], we constructed a *lkh1*$^+$ null mutant transformed with a Prk1 overexpression vector, and tested whether Prk1 reverses the flocculation phenotype of the *lkh1*$^+$ null mutant. The overexpression vector was constructed by inserting the ORF of *prk1*$^+$ (1.2 kb) into the multicloning site of pREP2, under the control of the thiamine-repressible *nmt1*$^+$ promoter. Cells were cultured in defined minimal medium in the absence or presence of 2 μM thiamine. ED665 (wild-type) cells showed no significant flocculation (Figure 3(A), tube 1) but PHM5L (Δ*lkh1*) cells flocculated and settled rapidly (tube 2). Overexpression of *prk1*$^+$ partially reversed the flocculation phenotype of the *lkh1*$^+$ null mutant (tube 3), but repression of *prk1*$^+$ by thiamine had no effect on the flocculation phenotype of PHM5L cells transformed with the Prk1 overexpression vector (tube 4).

Absorbance values (OD_{600}) for the supernatants of ED665, PHM5L, and SP202 (Δ*lkh1*-pREP2-*prk1*) cell cultures were 2.5, 0.28, and 0.87, respectively, after 10 min at rest. This tendency was maintained for 30 min, after which the wild-type cells started to settle

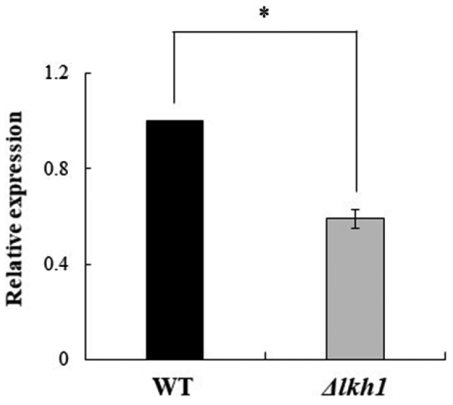

Figure 4. Relative expression level of *prk1*$^+$. The expression level of *prk1*$^+$ in cells of ED665 (wild-type) and PHM5L (Δ*lkh1*) was analyzed by qRT-PCR. Three biological samples were taken for the first cDNA synthesis, and subsequent quantitative real-time PCR analysis was performed using specific primer set for *prk1*$^+$. The levels of *prk1*$^+$ expression in both strains were standardized to that of beta-actin gene and calculated by the $2-\Delta\Delta Ct$ method. The asterisk indicates statistically significant change due to the deletion of LAMMER kinase (Δ*lkh1*) as calculated with the Student's t-test for $p \leq .02$.

slowly. In the presence of thiamine, the absorbance of SP202 cells was similar to that of PHM5L cells. These results indicate that the overexpression of *prk1*$^+$ partially reversed the flocculation phenotype of null mutant. In the previous section 3.3, it was shown that the flocculation activity of the *prk1*$^+$ null mutant differed slightly from that of the *lkh1*$^+$ null mutant in terms of its requirement for divalent cations, and Δ*lkh1*Δ*prk1* double mutant cells also flocculated and settled rapidly (see Figure 1).

Taken together, these results indicate that Lkh1 acts upstream of Prk1 for non-sexual flocculation. To explore the connection between *lkh1*$^+$ and *prk1*$^+$ further, effect of *lkh1*$^+$ on transcription of *prk1*$^+$ was tested via qRT-PCR. The expression levels of *prk1*$^+$ were observed to be significantly diminished (41%, *p-value* = .013) in the *lkh1*$^+$-null mutant compared to those in wild type (Figure 4). Although it remains to be demonstrated conclusively, it can be inferred from our results that Prk1 is functionally correlated with Lkh1 through unidentified signaling pathways involved in the non-sexual flocculation of fission yeast.

Although more experimental evidence is required, these results allow us to better understand the molecular mechanisms of flocculation in *Sch. pombe*.

Disclosure statement

No potential conflict of interest was reported by the author.

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