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MADS Box Transcription Factor Mbx2/Pvg4 Regulates Invasive Growth and Flocculation by Inducing *gsf2*$^+$ Expression in Fission Yeast

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The fission yeast *Schizosaccharomyces pombe* exhibits invasive growth and nonsexual flocculation in response to nitrogen limitation. Gsf2, a flocculin of fission yeast, is required not only for nonsexual flocculation but also for invasive growth through the recognition of galactose residues on cell surface glycoconjugates. We found that pyruvylation negatively regulates nonsexual flocculation by capping the galactose residues of *N*-linked galactomannan. We investigated whether pyruvylation also regulates invasive growth. The *pvg4*$^+$ gene originally was isolated as a multicopy suppressor of a *pvg4* mutant defective in the pyruvylation of *N*-linked oligosaccharides. However, we did not detect a defect in cell surface pyruvylation in the *pvg4*/*mbx2* deletion mutant, as assessed by alcian blue staining and a Q-Sepharose binding assay. Instead, the deletion prevented invasive growth under conditions of low nitrogen and high glucose, and it reduced the adhesion and flocculation of otherwise flocculent mutants by reducing *gsf2*$^+$ expression. *mbx2*$^+$-overexpressing strains exhibited nonsexual and calcium-dependent aggregation, which was inhibited in the presence of galactose but mediated by the induction of *gsf2*$^+$. These findings indicate that Mbx2 mediates invasive growth and flocculation via the transcriptional activation of *gsf2*$^+$ in fission yeast. In addition, we found that fission yeast Mbx2 induces the nonsexual flocculation of budding yeast by the activation of *FLO1*.

Yeasts propagate in a unicellular fashion by budding or by binary fission. However, many types of yeast can switch growth modes, changing from unicellular growth to filamentous branching multicellular hyphae. This transition can be induced by a wide variety of environmental changes, ranging from pH to the nature of the carbon source. Many species of dimorphic yeasts are pathogenic toward humans and plants in hyphal form (19, 32). Invasive filamentous growth has been reported in *Schizosaccharomyces pombe*, and *S. pombe* cells grow as long and branched invasive structures under conditions of low nitrogen and high glucose (LNB medium) (1).

We recently reported that *gsf2*$^+$, encoding a flocculin that binds to galactose residues located on cell surface glycoconjugates, is essential not only for nonsexual flocculation but also for filamentous invasive growth in *S. pombe* (7, 20). Cell surface glycoproteins play a key role in flocculation and filamentous invasive growth in yeasts. The budding yeast *S. cerevisiae* extends the core oligosaccharide with an α1,6-linked mannose backbone, which is further modified by the addition of α1,2- and α1,3-linked mannose side chains (3). Flo1, a lectin-like cell surface protein that aggregates cells into "flocs," binds to mannose sugar chains on the surface of other cells (16, 38, 39). In contrast, the glycoproteins of fission yeast *S. pombe* contain large amounts of α-linked galactose residues in addition to α-linked mannose residues (4). The galactosylation of glycoproteins is a unique feature of fission yeast, and Gsf2 binds to galactose-containing sugar chains on the surfaces of other cells. *N*-linked galactomannans of *S. pombe* have pyruvylated galactose (PvGal) caps on a portion of the galactose residues in their outer chains (12). PvGal biosynthesis has been investigated by ethyl methanesulfonate mutagenesis in *S. pombe*, followed by the isolation of cells devoid of negatively charged *N*-glycans by Q-Sepharose exclusion and by the failure of such cells to bind the human serum amyloid P component, which acts as a lectin for terminal PvGal residues (2). Andreishcheva et al. isolated mutants that lack PvGal in cell surface glycoconjugates (*pvg* mutants) (2). The restoration of a negative charge to the cell surface by complementation with an *S. pombe* genomic library led to the identification of five genes involved in PvGal biosynthesis, designated *pvg1*$^+$ to *pvg5*$^+$. We previously reported that pyruvylation negatively regulates nonsexual flocculation by capping the galactose residues in *N*-linked galactomannan (20). However, it was not clear whether PvGal is involved in filamentous invasive growth.

In this study, we report that the deletion of the MADS box gene *mbx2*$^+$/*pvg4*$^+$ prevents invasive growth and flocculation and is associated with a decrease in *gsf2*$^+$ mRNA levels. Mbx2/Pvg4 was found to induce nonsexual flocculation in both fission and budding yeast by upregulating flocculins.

MATERIALS AND METHODS

Strains and media. The strains used in this study are listed in Table 1. Wild-type *S. pombe* strains ARC039 (*h*$^-$ *ura4-C190T leu1-32*) and ARC001 (*h*$^-$ *leu1-32*) were obtained from the Yeast Genetic Resource Center of Japan, which is supported by the National BioResource Project (NBRP). Rich YES medium (3% glucose, 0.5% yeast extract with minimal medium [MM] supplements), and synthetic MM for *S. pombe* were used as described previously (23). For filamentous growth, LNB medium was used (0.067 g/liter yeast nitrogen base without amino acids [Bacto], 20 g/liter glucose, and salts and vitamins as in MM). A previously described modified lithium acetate method was used for plasmid transformation

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TABLE 1 *S. pombe* and *S. cerevisiae* strains used in this study

Strain	Genotype	Reference or source
Schizosaccharomyces pombe		
ARC039	*h*$^-$ *leu1-32 ura4-C190T*	NBRP[a]
ARC001	*h*$^-$ *leu1-32*	NBRP[a]
*gsf2*Δ	*h*$^-$ *leu1-32 ura4-C190T gsf2*::*ura4*	22
*pvg1*Δ	*h*$^-$ *leu1-32 ura4-C190T pvg1*::*ura4*	22
*pvg2*Δ	*h*$^-$ *leu1-32 ura4-C190T pvg2*::*ura4*	This study
*pvg3*Δ	*h*$^-$ *leu1-32 ura4-C190T pvg3*::*ura4*	This study
*mbx2*Δ/*pvg4*Δ	*h*$^-$ *leu1-32 ura4-C190T mbx2/pvg4*::*ura4*	This study
*pvg5*Δ	*h*$^-$ *leu1-32 ura4-C190T pvg5*::*ura4*	This study
*lkh1*Δ	*h*$^-$ *leu1-32 ura4-C190T lkh1*::*ura4*	22
*lkh1*Δ *mbx2*Δ	*h*$^-$ *leu1-32 ura4-C190T lkh1 mbx2/pvg4*::*ura4*	This study
gsf1	*h*$^-$ *leu1-32 ura4-C190T*	41
*gsf1 mbx2*Δ	*gsf1 mbx2/pvg4*::*ura4*	This study
Saccharomyces cerevisiae		
BY4742	*MAT***a** *leu2D0 ura3D0 his3D1 met15D0*	NBRP[a]
*flo1*Δ	*MAT***a** *leu2D0 ura3D0 his3D1 met15D0 flo1*::*kanMX4*	Open Biosystems
*flo10*Δ	*MAT***a** *leu2D0 ura3D0 his3D1 met15D0 flo10*::*kanMX4*	Open Biosystems
*flo11*Δ	*MAT***a** *leu2D0 ura3D0 his3D1 met15D0 flo11*::*kanMX4*	Open Biosystems

[a] These strains were obtained from the Yeast Genetic Resource Center of Japan, which is supported by the National BioResource Project (NBRP).

and *S. pombe* gene disruption (24). The *Escherichia coli* strain used for all cloning procedures was XL1-Blue (Stratagene, CA).

Gene disruptions. *pvg2*$^+$, *pvg3*$^+$, *mbx2*$^+$, and *pvg5*$^+$ were disrupted using *ura4*$^+$ as a selectable marker. *pvg2*$^+$ was amplified by PCR using the primers 5′-GCAGCGTAGTTGTAATCACAAAGACGCGG-3′ and 5′-GAGAGAGAGAACAAACGGATGCTTAGGAG-3′ and was cloned into the pGEM T vector (Promega, Madison, WI). The *ura4*$^+$ marker was inserted at the BamHI site. The wild-type strain ARC039 was transformed with the PCR products amplified from this construct. Gene disruption was confirmed by PCR using appropriate primers: 5′-GCCCTGCTCATTTCCTAGGAAAACCATGAC-3′ and 5′-GCTTCAGCTGCATCAAGACCATGATAGGGC-3′.

pvg3$^+$ was amplified by PCR using the primers 5′-GTTACCAACGCTATGAGGGCATTAAGGG-3′ and 5′-GCTTTTAAGCTTATGGACGGCAATGGCGTC-3′ and was cloned into the pGEM T vector. The *ura4*$^+$ marker was inserted at the SnaBI and BglII sites. The wild-type strain ARC039 was transformed with the PCR products amplified from this construct. Gene disruption was confirmed by PCR using appropriate primers: 5′-CTCTGAGATAAAGAAATGTAGCAACCCGG-3′ and 5′-CCAATTGCTTACCGTCGACAAGAGAGGG-3′.

mbx2$^+$ was amplified by PCR using the primers 5′-CACGCACTTATAAACGCACACAGACAAC-3′ and 5′-GATAGTAGTAAGGAGCGAAGTGCGTTTGCC-3′ and cloned into the pGEM T vector. The *ura4*$^+$ marker was inserted at the HpaI and NheI sites. The wild-type strain ARC039 was transformed with the PCR products amplified from this construct. Gene disruption was confirmed by PCR using appropriate primers: 5′-GTTGCGGTTTCCTAGTTATTGCTATCCCTG-3′ and 5′-GACTGGTCTTGTTGCAGTACATGAGTACCC-3′.

The *pvg5*$^+$ upstream region and downstream region were cloned into the pBluescript *ura4*$^+$ vector. pBluescript *ura4*$^+$ contains the *ura4*$^+$ marker at the ClaI site. The *pvg5*$^+$ upstream region was amplified by PCR using the following primers, including the indicated restriction sites (underlined): 5′-GTTTTGGTACCGTGATTTCGTGAGACCGAAG-3′ (KpnI) and 5′-GTTTCTCGAGCCGATACCATTACCAGC-3′ (XhoI). Similarly, the *pvg5*$^+$ downstream region was amplified using the primers 5′-GTTTTGGATCCTTTGCCCCTGATGCGTC-3′ (BamHI) and 5′-GTTTGAGCTCATAACGGTGTAAGAGG-3′ (SacI). The *pvg5*$^+$ upstream region was digested with KpnI and XhoI, the *pvg5*$^+$ downstream region was digested with BamHI and SacI, and the fragments were cloned into the corresponding sites of pBluescript *ura4*$^+$. The wild-type strain ARC039 was transformed with the PCR products amplified from this construct. Gene disruption was confirmed by PCR using appropriate primers: 5′-CATCTTCCAAGTATAAATGCTCTGAGCCCC-3′ and 5′-CGACAGCATAAGAGGAGTAGAAATTGCCGC-3′.

lkh1 mutant (20) cells were plated on FOA-supplemented YNB medium (6.7 g/liter yeast nitrogen base without amino acids, 20 g/liter glucose with MM supplements) to select for the loss of *ura4*$^+$ activity. The *ura4*$^-$ cells then were used as parent cells to generate an *lkh1 mbx2* double mutant.

Plasmids. To create the pTN197-*mbx2*$^+$ plasmid, *mbx2*$^+$ was amplified by PCR using the following primers, including the indicated restriction sites (underlined): 5′-GTTTTCTCGAGAATGGGGCGCAAAAAAATCTCCAT-3′ (XhoI) and 5′-GTTTTGCGGCCGCCGTATTTGACATCACTGAGTGA-3′ (NotI). The fragment was digested with XhoI and NotI and cloned into the corresponding sites of pTN197 (25).

To create the pREP1-*mbx2*$^+$ plasmid, *mbx2*$^+$ was amplified by PCR using the following primers, including the indicated restriction sites (underlined): 5′-GTTTTGTCGACATGGGGCGCAAAAAAATCTCC-3′ (SalI) and 5′-GTTTTGGATCCTTAGTATTTGACATCACTGAG-3′ (BamHI). The fragment was digested with SalI and BamHI and cloned into the corresponding sites of pREP1 (25). pTN197-*mbx2*$^+$ plasmid was used for the expression of Mbx2-GFP, and the pREP1-*mbx2*$^+$ plasmid was used for the overexpression of *mbx2*$^+$ in *S. pombe*. These vectors include *LEU2* as a nutritional marker for selection.

To create pBP73G-*mbx2*$^+$ and pBP73G-*FLO8*, *mbx2*$^+$ and *FLO8* were amplified by PCR using the following primers, including the indicated restriction sites (underlined): for *mbx2*$^+$, 5′-GTTTTTCTAGAATGGGGCGCAAAAAAATCTCCA-3′ (XbaI) and 5′-GTTTTGGATCCTTAGTATTTGACATCACTGAGTG-3′ (BamHI); for *FLO8*, 5′-GTTTTTCTAGAATGAGTTATAAAGTGAATAGTTCG-3′ (XbaI) and 5′-GTTTTGGATCCTCAGCCTTCCCAATTAATAAAATTG-3′ (BamHI). The amplified fragments then were digested and cloned into the corresponding sites of pBP73G (6). The pBP73G-*mbx2*$^+$ and pBP73G-*FLO8* plasmids were used for the overexpression of *mbx2*$^+$ and *FLO8*, respectively, in *S. cerevisiae*. These vectors include *URA3* as a nutritional marker for selection.

Alcian blue staining. Standard methods for the alcian blue staining of yeast cells were used (10, 28). Cells were cultivated at 30°C, harvested while in logarithmic growth, and washed with 0.02 N HCl. Washed cells were suspended in 1 ml of 50 μg/ml alcian blue solution (Nacalai Tesque, Inc., Japan). The cells were allowed to settle for 10 min and then were collected by centrifugation at 25°C for 5 min at 15,000 rpm. The optical density at 600 nm (OD_{600}) of the supernatant (OD_{600} sup) was measured,

and the difference between the OD_{600} of the original solution (OD_{600} ori) and that of the supernatant was calculated using the following formula: $T = 61.3 \times (OD_{600}\ \text{ori} - OD_{600}\ \text{sup})$, where T is the total amount (in μg) of absorbed alcian blue. Alcian blue binding (in $\mu g/OD_{600}$ unit) is represented by the following equation: alcian blue binding $= T \times d/OD_{600}$ c.c., where d is a dilution ratio to measure the diluted cell density (OD_{600} c.c.).

Q-Sepharose binding assay. The Q-Sepharose binding assay has been described already (2, 11). Q-Sepharose fast flow (GE Healthcare, Japan) beads were sterilized by suspension in 95% ethanol. The beads then were washed five times with double-distilled water (DDW) to remove the ethanol. Cells were collected by centrifugation, washed three times with DDW, and resuspended in DDW. The sterile Q-Sepharose beads were added to the cell suspension, and the mixture was allowed to settle at room temperature for 5 min. Q-Sepharose and cells were observed by microscopy.

Fluorescence microscopy. Cells were collected by centrifugation, resuspended with 5 μl of culture, and then placed on a slide glass and visualized. Fluorescent images of living cells were taken with a cooled charge-coupled device camera and stored digitally using MetaMorph software (Universal Imaging, Downingtown, PA). For fixed samples, the cultured cells were suspended in 70% ethanol, washed with phosphate-buffered saline, and suspended in 5 μl of phosphate-buffered saline containing Hoechst 33342 dye (0.1 mg/ml).

Northern blot analysis. Total RNA from cultures with an OD_{600} of 0.8 to 1.0 was extracted by the glass bead method as described previously (31). Total RNA (30 μg) was separated on a 1% (wt/vol) agarose gel containing 20% formaldehyde. Subsequently, RNA was blotted onto Hybond-N membranes in 10× SSC buffer, pH 7 (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and hybridized with gene-specific probes.

RT-PCR. Total RNA from cultures with an OD_{600} of 0.8 to 1.0 was extracted by the glass bead method using the RNeasy kit (Qiagen, CA) with DNase treatment. A ReverTra Ace quantitative RT kit (Toyobo, Japan) was used for cDNA synthesis. Reverse transcription-PCR (RT-PCR) was performed using the following *FLO*- and *ACT1*-specific primer sets: *FL01*-specific primer set, 5′-CTACTTCTACCGAATTGACCACAGTCA CTGGC-3′ and 5′-GCCAGCAATAAGGACGCAATGAAGACACTTAA AC-3′; *FLO10*-specific primer set, 5′-GCCTGTGGCTGCTCGATATAT ATTTTTGACCG-3′ and 5′-GACCCCTTTATGTCGGTAGGTGCATCT GCG-3′; *FLO11*-specific primer set, 5′-GTCACGACGGCTATTCCAAC CACAGTTATTACC-3′ and 5′-GAATACAACTGGAAGAGCGAGTAG CAACCAC-3′; and *ACT1*-specific primer set, 5′-GACTCCTACGTTGG TGATGAAGCTCAA-3′ and 5′-GGAGGAGCAATGATCTTGACCTTC ATGGA-3′.

RESULTS

Disruption of *mbx2*$^+$/*pvg4*$^+$ causes a defect in invasive growth. Andreishcheva et al. isolated *pvg* mutants and identified five genes (*pvg1*$^+$ to *pvg5*$^+$) involved in PvGal biosynthesis (2). Pvg1 was predicted to be a pyruvyltransferase, because it shares an ~300 to 350-amino-acid stretch of about 30% identity and about 45% similarity with nearly 20 identified or predicted pyruvyltransferases, including PssK exopolysaccharide polymerization proteins of *Rhizobium leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *niciae* (30). Pvg3 was predicted to be a galactosyltransferase (2). This corresponds with the finding that galactomannans purified from the *pvg3* mutant lack both pyruvate and β1,3-linked galactose. Pvg2 and Pvg5 have no apparent orthologs, and Pvg4 (also called Mbx2) has an MEF2-type MADS box in its N-terminal region (2). The MADS box genes encode a eukaryotic family of transcriptional regulators involved in diverse and important biological functions. This class of proteins has been identified in yeasts, plants, insects, nematodes, lower vertebrates, and mammals (22). These proteins contain a conserved DNA binding and dimerization domain, which was named the MADS box after the five founding members of the family: Mcm1 (yeast) (29), Arg80 (yeast) (9), Agamous (plant) (41), Deficiens (plant) (34), and SRF (human) (26).

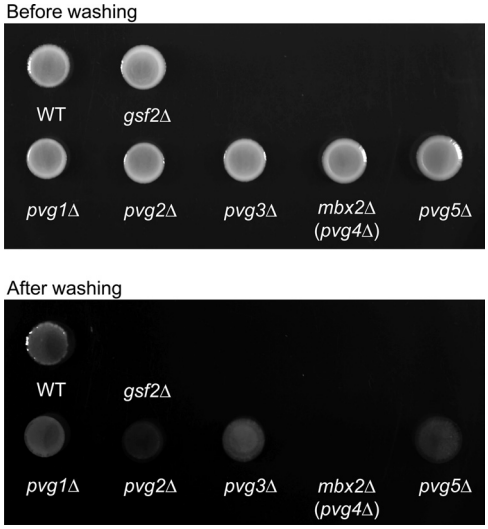

FIG 1 *mbx2*$^+$/*pvg4*$^+$ is essential for invasive growth. Wild-type, *gsf2*Δ, *pvg1*Δ, *pvg2*Δ, *pvg3*Δ, *mbx2*Δ/*pvg4*Δ, and *pvg5*Δ cells were spotted onto LNB medium, which was incubated at 30°C for 14 days (Before washing). Spots of growth were washed with tap water to score for invasive growth (After washing). *leu1-32* and *ura4-C190T* strains were complemented with *leu1*$^+$ and *ura4*$^+$, respectively, to allow growth on LNB medium.

We examined whether *pvg1*Δ to *pvg5*Δ strains exhibited invasive growth on LNB plates and on a low-nitrogen and high-glucose plate relative to levels for the wild type. We constructed *pvg1*, *pvg2*, *pvg3*, *mbx2*/*pvg4*, and *pvg5* deletion mutants and spotted them together with the wild type and a *gsf2*Δ mutant on LNB plates incubated at 30°C for 14 days. All strains grew equally well. The wild-type, *pvg1*Δ, *pvg2*Δ, *pvg3*Δ, and *pvg5*Δ cells remained on the agar surface after the plates were washed, while *gsf2*Δ and *mbx2*Δ/*pvg4*Δ cells were removed by washing (Fig. 1). This result indicates that pyruvylated galactose is not necessary for invasive growth, whereas Mbx2/Pvg4 is essential.

Andreishcheva et al. isolated *pvg* mutants and obtained strains *pvg1*$^+$ to *pvg5*$^+$ by complementation. However, they did not construct deletion strains for each *pvg* gene. We therefore constructed *pvg1*Δ, *pvg2*Δ, *pvg3*Δ, *mbx2*Δ/*pvg4*Δ, and *pvg5*Δ mutants and examined whether their respective *N*-glycans were negatively charged by alcian blue staining and Q-Sepharose binding. Because no phosphate or sulfate has previously been detected in *S. pombe* glycans, pyruvate appears to be the only negatively charged functional group on the cell surface (11). Alcian blue can bind to negatively charged glycans, such as pyruvylated galactose of *S. pombe* and mannosylphosphate of *S. cerevisiae* (28). Q-Sepharose beads are anion-exchange resins that bind to negatively charged cells to form a fluffy precipitate that quickly settles.

Whereas wild-type cells bound alcian blue at a ratio of about 19 $\mu g/OD_{600}$ cells, *pvg1*Δ, *pvg2*Δ, *pvg3*Δ, and *pvg5*Δ cells did not bind alcian blue (Fig. 2A). In contrast, *mbx2*Δ/*pvg4*Δ cells bound alcian blue as well as wild-type cells did (Fig. 2A). In addition, wild-type and *mbx2*Δ/*pvg4*Δ cells adhered to Q-Sepharose beads, but *pvg1*Δ, *pvg2*Δ, *pvg3*Δ, and *pvg5*Δ cells did not (Fig. 2B). These results indirectly suggest that the biosynthesis of pyruvylated galactose

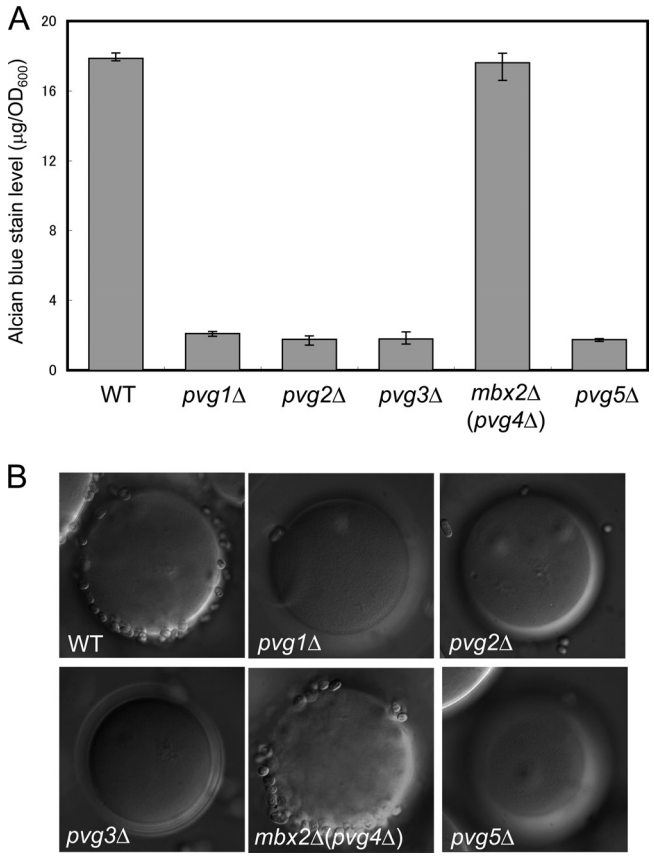

FIG 2 Alcian blue and Q-Sepharose binding assays of *pvg*Δ cells. (A) Alcian blue binding assay of *pvg*Δ cells. Alcian blue binding (in μg/OD unit) was scored based on the amount of dye binding to cells. Values are means ± SEM ($n = 3$). (B) Q-Sepharose binding assay of *pvg*Δ cells. Negatively charged cells adhere tenaciously to positively charged beads, whereas uncharged cells do not bind the beads.

does not require Mbx2/Pvg4, therefore we designated the gene Mbx2.

Mbx2 is a nuclear-localized MEF2-type MADS box transcription factor. Mbx2 is a member of the MADS box protein family and is highly similar to Rlm1 in budding yeast. Here, we found that Mbx2-GFP localized to the nucleus (Fig. 3B). Rlm1 has been reported to be a key transcription factor that acts downstream of Slt2/Mpk1 mitogen-activated protein kinase (MAPK) to regulate cell wall integrity signaling (40). We compared the sequence of the putative DNA binding domain of Mbx2 to those of human MEF2C and *S. cerevisiae* Rlm1 (Fig. 3A). These putative DNA binding domains show significant similarity to that of Mbx2, and most importantly, MADS box residues 1 and 11 to 15, which together determine DNA binding specificity (27, 33), share significant conservation among Mbx2, Rlm1, and MEF2C. Although Mbx2 exhibits significant similarity to Rlm1, Takada et al. reported that Mbx2 seems to play only a minor role in cell wall integrity signaling (36). This indicates that MADS box domain sequence similarity is not necessarily linked to functional similarity. Interestingly, Dodou and Treisman reported that the microscopic examination of *S. cerevisiae rlm1*Δ cells grown in liquid medium limited for nitrogen revealed an absence of the cell aggregates observed for wild-type cells, suggesting that *rlm1*Δ cells are defective in flocculation (8).

Mbx2 is essential for adhesion and flocculation. The fission yeast LAMMER kinase homolog, Lkh1, regulates Tup transcriptional repressors through phosphorylation, and an *lkh1* null mutant flocculates upon reaching stationary phase in liquid media and adheres to agar surfaces (15). In addition, we previously isolated a fission yeast *gsf1* mutant that flocculates constitutively during growth in liquid media and also adheres to agar (20, 37). The ability of cells to adhere to various substrates, such as host extracellular matrix, is considered a critical factor for many unicellular pathogenic organisms to establish an infection. A number of ad-

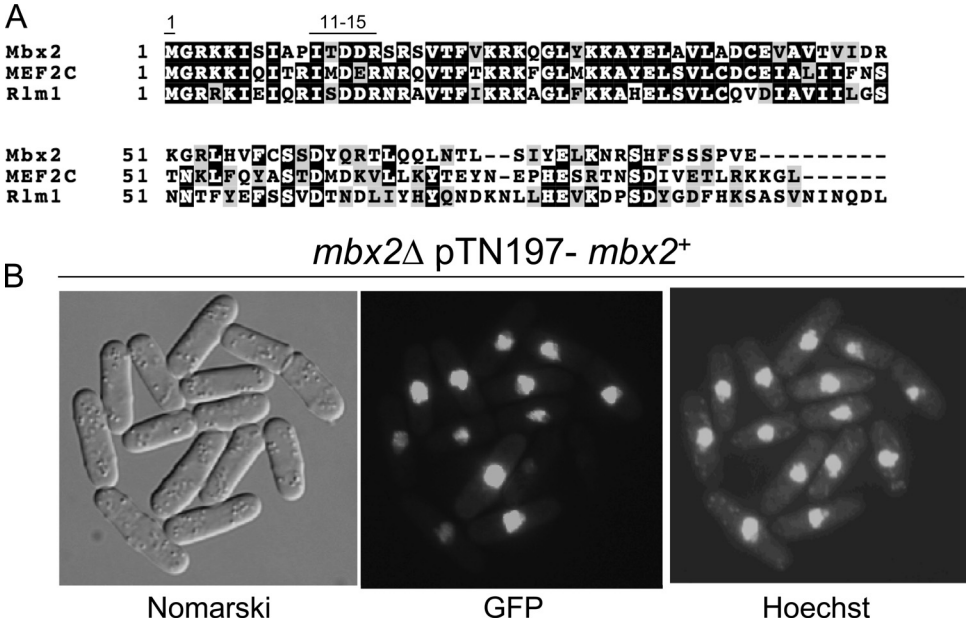

FIG 3 Mbx2 is an MEF2-like MADS box transcription factor. (A) Comparison of MADS box domains of Mbx2, Rlm1, and MEF2C. MADS box residues 1 and 11 to 15 are shown. (B) Localization of Mbx2-GFP. Nomarski differential interference contrast (Nomarski) microscopy, GFP fluorescence (GFP), and Hoechst 33324 staining (Hoechst) are shown.

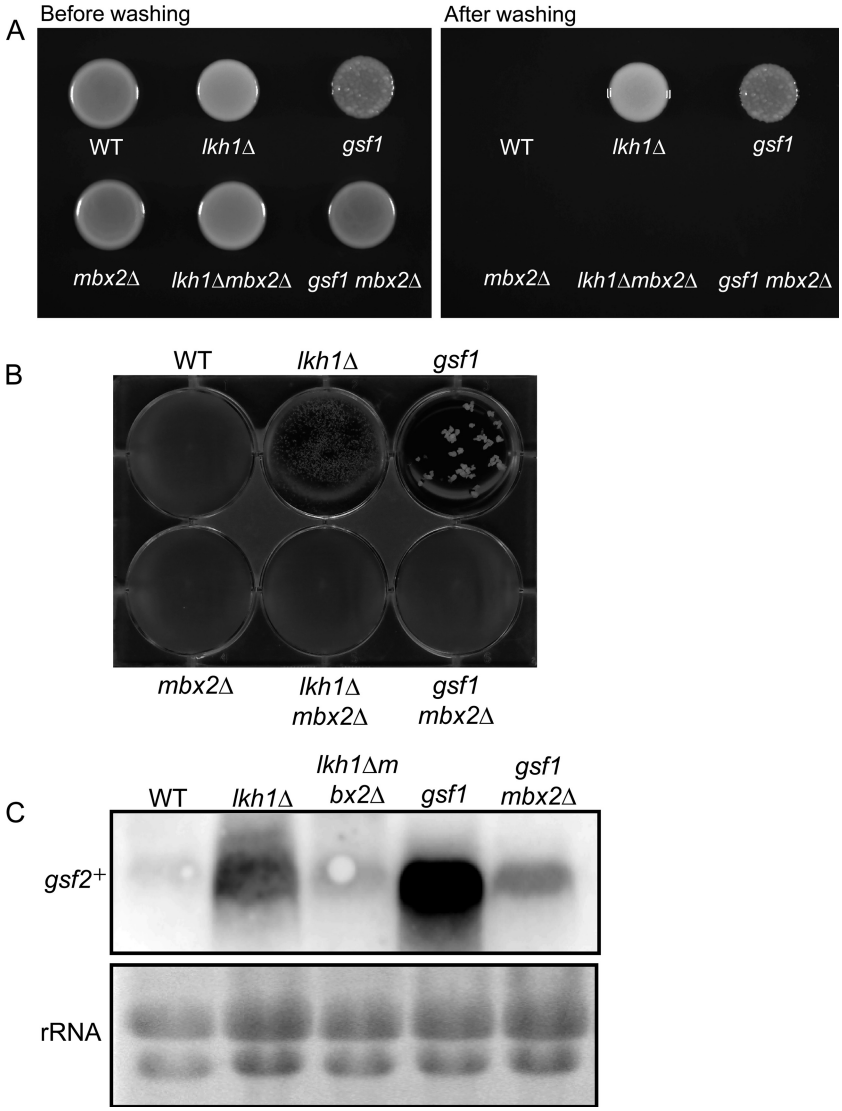

FIG 4 Mbx2 is required for *gsf2*$^+$-dependent adhesion and nonsexual flocculation. (A) Wild-type, *lkh1*Δ, *gsf1*, *mbx2*Δ, *lkh1*Δ *mbx2*Δ, and *gsf1 mbx2*Δ cells were spotted onto YES plates and incubated at 30°C for 5 days, after which the spots were washed with a stream of water to score for adhesion. (B) Flocculation phenotypes of wild-type, *lkh1*Δ, *gsf1*, *mbx2*Δ, *lkh1*Δ *mbx2*Δ, and *gsf1 mbx2*Δ cells cultured on YES medium. (C) mRNA levels of *gsf2*$^+$ in *lkh1*Δ, *gsf1*, *mbx2*Δ, *lkh1*Δ *mbx2*Δ, and *gsf1 mbx2*Δ cells. Cells were cultured in YES medium at 30°C to an OD_{600} of 1.0, at which time total RNA was extracted. Each RNA sample was separated on a 1% agarose gel in the presence of formaldehyde.

hesive proteins have been identified in several fungal pathogens, including *Candida albicans* and *Aspergillus fumigatus*, and their expression has been shown to be an important virulence trait (21). The adhesion and nonsexual flocculation phenotypes of *gsf1* and *lkh1*Δ mutants are completely abolished by the deletion of *gsf2*$^+$, indicating their dependence on *gsf2*$^+$ in *S. pombe* (20).

We found that adhesion and nonsexual flocculation by *gsf1* and *lkh1*Δ mutants were completely abolished by the deletion of *mbx2*$^+$ (Fig. 4). Wild-type, *lkh1*Δ, *gsf1*, *mbx2*Δ, *lkh1*Δ *mbx2*Δ, and *gsf1 mbx2*Δ cells were spotted on YES plates and incubated at 30°C for 5 days. Cells of the *gsf1* and *lkh1*Δ mutants adhered to the agar surface after the plates were washed, but the disruption of *mbx2*$^+$ in the *lkh1*Δ and *gsf1* mutants completely abolished adherence (Fig. 4A). In addition, the nonsexual flocculation of the *lkh1*Δ and *gsf1* mutants also disappeared after the deletion of *mbx2*$^+$ (Fig. 4B). Based on Northern analysis, *gsf2*$^+$ was highly expressed in *lkh1*Δ and *gsf1* mutants grown in YES medium, whereas its expression decreased remarkably after the deletion of *mbx2*$^+$ (Fig. 4C). *mbx2*Δ cells transformed with the *nmt41-gsf2*$^+$ construct, which produces a high level of *gsf2*$^+$ mRNA under the control of the thiamine-repressible *nmt41* promoter (20), flocculated as well as wild-type cells transformed with the *nmt41-gsf2*$^+$ construct (data not shown), indicating that the abolition of flocculation in the *mbx2*$^+$ deletion mutants was linked to a reduction in *gsf2*$^+$ mRNA levels.

Overexpression of *mbx2*$^+$ induces flocculation. We next overexpressed *mbx2*$^+$ in wild-type and *gsf2*Δ cells under the control of the *nmt1* promoter. Wild-type and *gsf2*Δ cells transformed with pREP1-*mbx2*$^+$ were cultured on MM-leucine medium at 30°C for 18 h with or without 20 μg/ml of thiamine. Flocculation

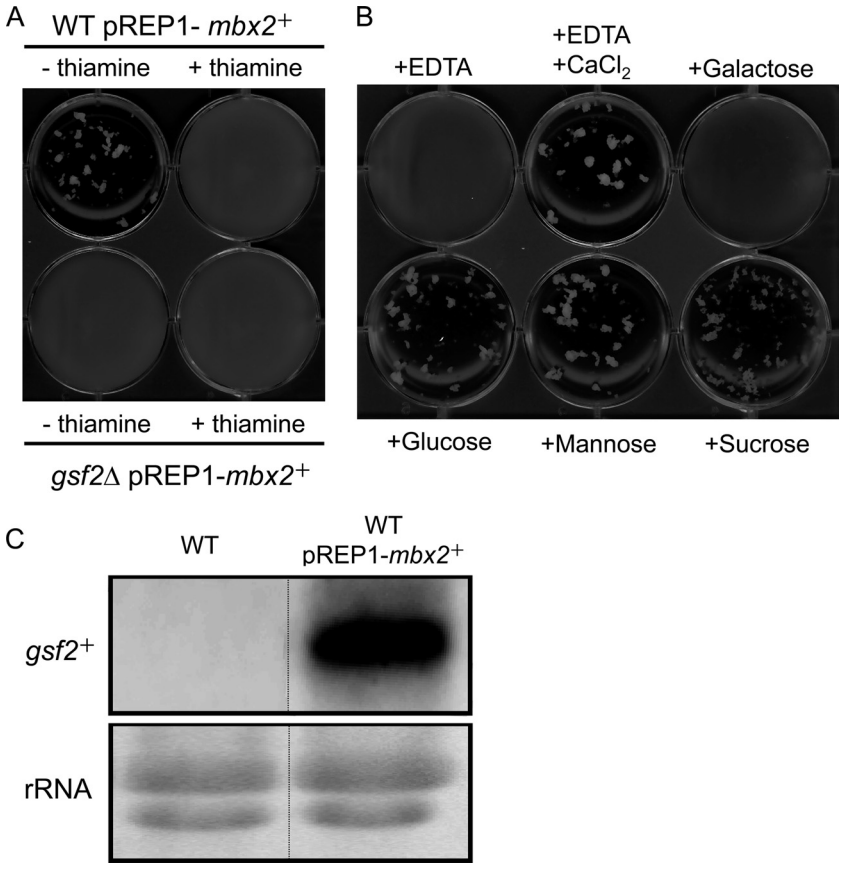

FIG 5 *mbx2*$^+$ is a dominant flocculation gene. (A) Wild-type and *gsf2*Δ cells transformed with pREP1-*mbx2*$^+$ were cultured on MM-leucine medium at 30°C for 18 h with or without 20 μg/ml of thiamine, after which flocculation was assessed. (B) Effect of various sugars on flocculation of the *mbx2*$^+$-overexpressing strain. EDTA (10 mM final concentration), $CaCl_2$ (200 mM final concentration), and sugars (galactose, mannose, or sucrose; 200 mM final concentration) were added to *mbx2*$^+$-overexpressing cells. (C) Northern analysis of the *mbx2*$^+$-overexpressing strain. RNA was extracted from wild-type cells transformed with pREP1-*mbx2*$^+$ and grown on MM-leucine medium at 30°C for 18 h.

was observed in the wild-type cells transformed with pREP1-*mbx2*$^+$ but not in the *gsf2*Δ cells cultured without thiamine (Fig. 5A). The flocculation of the wild-type cells transformed with pREP1-*mbx2*$^+$ was abolished by growth with thiamine, indicating that *mbx2*$^+$ expression was required for flocculation. Flocculation in the wild-type strain overexpressing *mbx2*$^+$ was inhibited by the addition of EDTA or galactose but was not inhibited by the addition of glucose, mannose, or sucrose (Fig. 5B). These flocculation phenotypes are consistent with the flocculation of *gsf2*$^+$-overexpressing cells. Based on Northern analysis, no detectable expression of *gsf2*$^+$ was observed in wild-type cells, whereas *gsf2*$^+$ was highly expressed in *mbx2*$^+$-overexpressing cells (Fig. 5C). These results indicate that Mbx2 mediates flocculation via the transcriptional activation of *gsf2*$^+$ in fission yeast. Nonsexual flocculation induced by Mbx2 overexpression was abolished by deletion or point mutations of the MADS box domain of Mbx2, whereas the nuclear localization of Mbx2-GFP was unaffected by these alterations (data not shown), indicating that the MADS box domain is essential for function as a dominant flocculation gene but not for nuclear localization.

Expression of *S. pombe mbx2*$^+$ induces nonsexual flocculation of *S. cerevisiae*. The budding yeast *FLO8* gene, encoding a transcriptional activator of the dominant flocculation genes *FLO1* and *FLO11*, induces nonsexual flocculation in *S. cerevisiae* (17). Interestingly, we found that nonsexual flocculation in *S. cerevisiae* haploid cells was induced by the expression of *S. pombe mbx2*$^+$ under the control of the GPD promoter (Fig. 6A). The *mbx2*$^+$-dependent flocculation of *S. cerevisiae* was inhibited by the addition of mannose or EDTA but was not inhibited by the addition of galactose, glucose, fructose, or sucrose (Fig. 6B). We also expressed *mbx2*$^+$ and *FLO8* in *flo1*Δ, *flo10*Δ, and *flo11*Δ cells under the control of the GPD promoter. Flocculation was observed in *flo10*Δ and *flo11*Δ cells expressing *mbx2*$^+$ or *FLO8* but not in *flo1*Δ cells (Fig. 6C). To clarify which *FLO* genes are induced by *mbx2*$^+$ overexpression, the expression of the *FLO* genes was assayed by RT-PCR (Fig. 6D). Whereas *FLO1* was induced in both *mbx2*$^+$- and *FLO8*-expressing cells, *FLO11* was induced highly in *FLO8*-expressing cells but only slightly by *mbx2*$^+$. In addition, the adhesive growth of haploid cells and pseudohypha formation by diploid cells were observed in *FLO8*-expressing haploid and diploid cells, but these growth forms were not observed in *mbx2*$^+$-expressing cells (data not shown). These results indicate that Mbx2 mediates flocculation in both *S. pombe* and *S. cerevisiae* via the transcriptional activation of *gsf2*$^+$ and *FLO1*, respectively.

DISCUSSION

Previously, we characterized dominant genes involved in flocculation in fission yeast and cloned the *gsf2*$^+$ gene that encodes a

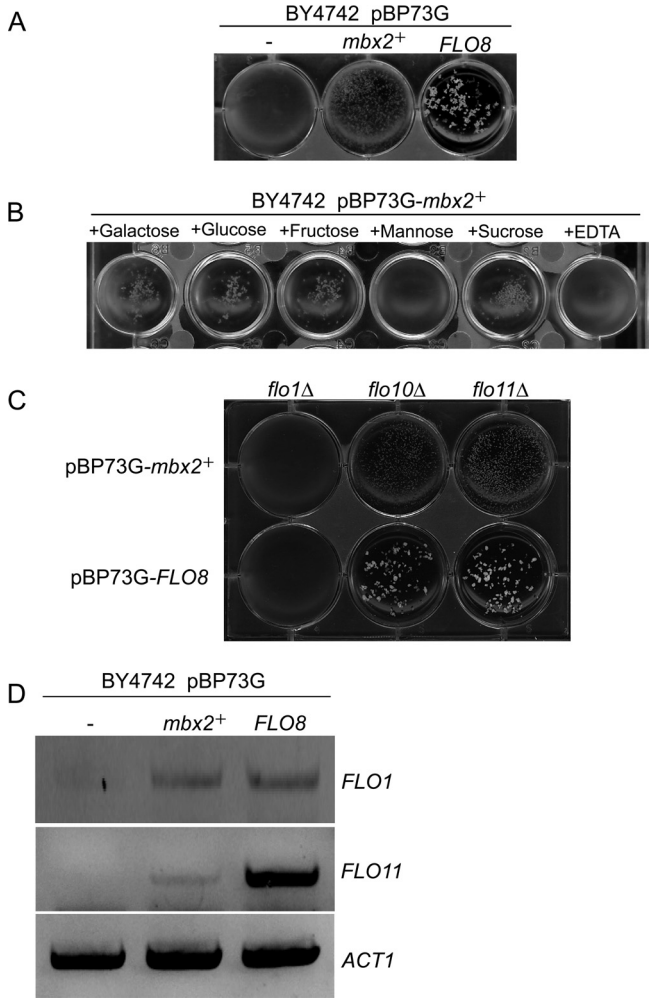

FIG 6 *S. pombe mbx2*$^+$ induces nonsexual flocculation in *S. cerevisiae*. (A) Nonsexual flocculation of *S. cerevisiae* cells expressing *S. pombe mbx2*$^+$ and *S. cerevisiae FLO8*. pBP73G, containing the GPD promoter, was used as an overexpression vector. Wild-type (BY4742) cells transformed with pBP73G (−), pBP73G-*mbx2*$^+$ (*mbx2*$^+$), and pBP73G-*FLO8* (*FLO8*) cells were cultured in SD-uracil medium at 30°C for 24 h, after which flocculation was assessed. (B) Effects of various sugars on flocculation of the *mbx2*$^+$-overexpressing strain. EDTA (10 mM final concentration) and sugars (galactose, glucose, fructose, mannose, and sucrose; 200 mM final concentration) were added to the *mbx2*$^+$-overexpressing cells. (C) Assessment of flocculation of *flo1*Δ, *flo10*Δ, and *flo11*Δ strains transformed with pBP73G-*mbx2*$^+$ and pBP73G-*FLO8*. (D) mRNA levels of *FLO1*, *FLO11*, and *ACT1* in cells transformed with pBP73G (−), pBP73G-*mbx2*$^+$ (*mbx2*$^+$), and pBP73G-*FLO8* (*FLO8*). Total RNA was extracted from transformants, and gene expression was monitored by RT-PCR.

flocculin (20). In the present study, we cloned a new dominant flocculation gene, *mbx2*$^+$. Mbx2 has a MADS box domain, induces nonsexual flocculation via the induction of *gsf2*$^+$ (Fig. 5), and is essential for invasive growth (Fig. 1). Andreishcheva et al. gave Mbx2 the designation Pvg4, because the multicopy expression of *mbx2*$^+$ complemented the defective pyruvylation phenotype of *pvg4* mutants (2). However, we did not detect a difference between wild-type and *mbx2*Δ cells in alcian blue staining or Q-Sepharose binding assays (Fig. 2). Because Andreishcheva et al. did not carry out a genetic analysis and did not determine the mutation responsible for the pyruvylation defect in the *pvg4* mutant (2), it is still unclear why the multicopy expression of *mbx2*$^+$ complemented this mutation. We tested whether Mbx2 induced the expression of genes known to be involved in pyruvylation and glycosylation, including *pvg1*$^+$, *pvg2*$^+$, *pvg3*$^+$, and *pvg5*$^+$ and genes encoding glycosyltransferases, but we found no evidence for induction in *mbx2*$^+$-overexpressing cells (data not shown).

In fungi, two distinct types of MADS box genes have been identified, the SRF-like class, including Mcm1 and Arg80, and the MEF2-like class, including Rlm1 and Smp1 (22). *S. pombe* has two MADS box-type transcription factors, Mbx1 and Mbx2. Mbx1, an *S. cerevisiae* Mcm1 homologue in fission yeast, belongs to the SRF-like class and is involved in regulating *ecm33*$^+$, encoding a glycosyl-phosphatidylinositol (GPI)-anchored cell surface protein involved in calcium ion homeostasis as a transcriptional target of Pmk1 and Atf1 (35). Mbx2, a homologue of *S. cerevisiae* Rlm1 that is involved in the maintenance of cell integrity (13, 14), belongs to the MEF2-like class (Fig. 3A). Although Mbx2 shares sequence similarity with Rlm1, it has been reported that Mbx2 seems to play only a minor role in cell wall integrity signaling (36), suggesting that the S. *pombe* MEF2-like class transcription factor, Mbx2, is specialized in the induction of flocculation.

We previously reported that *S. cerevisiae FLO8*, encoding a transcriptional activator of the dominant flocculation genes *FLO1* and *FLO11* (5, 17, 18), induces nonsexual flocculation in both *S. cerevisiae* and *S. pombe* (20). In the present study, we demonstrated that *S. pombe mbx2*$^+$ also induced nonsexual flocculation in *S. cerevisiae* via the induction of *FLO1*, although Mbx2 does not share sequence similarity with Flo8. To investigate possible functional differences between these transcription factors in fission yeast, microarray analysis was performed in wild-type and *mbx2*$^+$-expressing cells. We showed that only eight genes, *gsf2*$^+$, SPCC1450.08c, SPAC977.14, *spn3*$^+$, SPAC22A12.04c, SPBC211.03c, *bms1*$^+$, and *adh4*$^+$, were upregulated in *FLO8*-expressing cells (20). In contrast, at least 50 genes, including *gsf2*$^+$, were induced by the overexpression of *mbx2*$^+$ (data not shown). *gsf2*$^+$, SPCC1450.08c, and SPAC977.14 were induced by both *FLO8* and *mbx2*$^+$, and five other genes were specific for *FLO8*. The analysis of the promoter regions of *gsf2*$^+$, SPCC1450.08c, and SPAC977.14 of *S. pombe* and *FLO1* of *S. cerevisiae* and the binding specificities of Mbx2 and Flo8 in *S. pombe* will be addressed in a future study.

The major environmental signal for *S. pombe* to differentiate into hyphae appears to be a lack of nitrogen, provided that cells are within a fairly narrow temperature range (~30°C) and a preferred carbon source is present (1). Our ongoing efforts are focused on analyzing the regulatory machinery that controls Mbx2, including the identification of upstream regulators.

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