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 decreasing *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 (PyGal) caps on a portion of the galactose residues in their outer chains (12). PyGal 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 PyGal residues (2). Andreishcheva et al. isolated mutants that lack PyGal 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 PyGal 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 PyGal 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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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′-GACGGGTAGTTGTAACGACAGGCCG-3′ and 5′-GAGAGAGAGCACGCTACCC-3′ and was cloned into the pGem T vector (Promega, Madison, WI). The ura4 marker was inserted at the BamHI and SacI 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′-GCGCTGCTATTCTTACAGAACAAGACAC-3′ and 5′-GCTTCAGCTGCATCAAGAC-3′.

pvg3 was amplified by PCR using the primers 5′-GTTTTGGATCCTTAGTATTTGACATC-3′ and 5′-GCTTTTAAGCTTATGGACGG-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′-GTTTTGGATCCTTTGCCCCTGATGCGTC-3′ and 5′-GCCCTGCTACATTATCGCAAAAAATCTCCA-3′ and 5′-GCTTCAGCTGCATCAAGACAC-3′.

mbx2 was amplified by PCR using the primers 5′-CAGCCACTTATAACGGCAACAGACACAC-3′ and 5′-GATAGTGTAAGGAGGCGAAGTGCGT-3′ and was cloned into the pGem T vector. The ura4 marker was inserted at the BamHI 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′-GTTTTCTCGAGAATGGGGCGCAAAAAAA-3′ and 5′-GCTTCAGCTGCATCAAGACAC-3′.

pvg5 was amplified by PCR using the primers 5′-GTTTTCTCGAGAATGGGGCGCAAAAAAA-3′ and 5′-GCTTCAGCTGCATCAAGACAC-3′ and was 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′-CATCTTCCAGATATAAATGCTCGAGC-3′ and 5′-CGACAGCATAAGAGGAGTAGAAGAAATGTCCGCG-3′.

**Plasmids.** To create the pTN197-mbx2 plasmid, mbx2 was amplified by PCR using the following primers, including the indicated restriction sites (underlined): 5′-GTTTTTCTCGAGAATGGGGCGCAAAAAAA-3′ and 5′-GCCCTGCTATTCTTACAGAACAAGACAC-3′. This fragment was digested with NdeI and NotI and cloned into the corresponding sites of pTN197 (25).

To create the plasmid constructs mbx2, and mbx2, and pvg5 were amplified by PCR using the following primers, including the indicated restriction sites (underlined): 5′-GTTTTTCTCGAGAATGGGGCGCAAAAAAA-3′ and 5′-GCCCTGCTATTCTTACAGAACAAGACAC-3′. This fragment was digested with NdeI and NotI and cloned into the corresponding sites of plasmid pREP1 (25). pTN197-mbx2 plasmid was used for the overexpression of mbx2 and the plasmid pREP1-mbx2 plasmid was used for the overexpression of mbx2 in S. pombe. These vectors include LEU2 as a nutritional marker for selection.

**Plasmids.** To create plasmids pREP1-mbx2 and pBP73G-mbx2 and pFLO8-mbx2 and pFLO8 were amplified by PCR using the following primers, including the indicated restriction sites (underlined): 5′-GTTTTTCTCGAGAATGGGGCGCAAAAAAA-3′ and 5′-GCCCTGCTATTCTTACAGAACAAGACAC-3′. This fragment was digested with NdeI and NotI and cloned into the corresponding sites of plasmid pREP1 (25). pTN197-mbx2 plasmid was used for the overexpression of mbx2 and the plasmid pREP1-mbx2 plasmid was used for the overexpression of mbx2 in S. pombe. These vectors include LEU2 as a nutritional marker for selection.

**Alican blue staining.** Standard methods for the alican 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 alican 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 (OD600) of the supernatant (OD600 sup) was measured,
and the difference between the $OD_{600}$ of the original solution ($OD_{600 \text{ ori}}$) 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 (\( \mu \text{g} \)) of absorbed alcian blue. Alcian blue binding (in \( \mu \text{g}/OD_{600} \text{ unit} \)) is represented by the following equation: alcian blue binding = \( T \times d/OD_{600} \text{ c.c.} \), where \( d \) is a dilution ratio to measure the diluted cell density (\( OD_{600} \text{ c.c.} \)).

Q-Sepharose binding assay. The Q-Sepharose fast flow assay has been described already (2, 11). Q-Sepharose fast flow (GE Healthcare, Japan) beads were sterilized by suspension in 95% ethanol. 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 \( \mu \text{g} \) of culture, and then placed on a slide glass and visualized. Fluorescent images of living cells were taken with a cooled charged-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 \( \mu \text{g} \) 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 \( \mu \text{g} \)) was separated on a 1% (wt/vol) agarose gel containing 20% formaldehyde. Subsequently, RNA was blotted onto Hybond-N membranes in 10\( \times \) SSC buffer, pH 7 (1\( \times \) SSC = 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: FLO1-specific primer set, 5'-CTATCTTCTACGGAATTTGACCCACGTCACGTGCC-3' and 5'-GCCAGACAAATTAGGAGGCATAGAACACCTCAA-3'; FLO10-specific primer set, 5'-GGCTTGTGCGCTGCTGATATATATTGTTGCGC-3' and 5'-GGACCCTTTTATGTCGGTAGGTGCATCTATTTGTTGCGC-3'; FLO11-specific primer set, 5'-GTCAGCCGGCTGATCTTCCCAACACAGGTATTACC-3' and 5'-GATACACCTAGGAGAGGAGTACGACACACC-3'; and ACT1-specific primer set, 5'-GACTCCCTAGCTTGCTGATGAGCTCAA-3' and 5'-GGAGGAGCAGATGCTTTGACGTTCC-3'.

RESULTS

Disruption of mxb2\textsuperscript{Δ}/pvg4\textsuperscript{Δ} causes a defect in invasive growth. Andreishcheva et al. isolated pvg mutants and identified five genes (pvg1\textsuperscript{Δ} to pvg5\textsuperscript{Δ}) involved in PvgGal biosynthesis (2). Pvg1 was predicted to be a pyruvyltransferase, because it shares an \( \sim 300 \) 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. nictia (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 \( \beta1,3 \)-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), Ama1 (plant) (41), Deficiens (plant) (34), and SRF (human) (26).

We examined whether pvg1\textsuperscript{Δ} to pvg5\textsuperscript{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\textsuperscript{Δ}, pvg2\textsuperscript{Δ}, pvg3\textsuperscript{Δ}, mxb2\textsuperscript{Δ}/pvg4\textsuperscript{Δ}, and pvg5\textsuperscript{Δ} deletion mutants and spotted them together with the wild type and a gfs2\textsuperscript{Δ} mutant on LNB plates incubated at 30°C for 14 days. All strains grew equally well. The wild-type, pvg1\textsuperscript{Δ}, pvg2\textsuperscript{Δ}, pvg3\textsuperscript{Δ}, and pvg5\textsuperscript{Δ} cells remained on the agar surface after the plates were washed, while gfs2\textsuperscript{Δ} and mxb2\textsuperscript{Δ}/pvg4\textsuperscript{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\textsuperscript{Δ} to pvg5\textsuperscript{Δ} by complementation. However, they did not construct deletion strains for each pvg gene. We therefore constructed pvg1\textsuperscript{Δ}, pvg2\textsuperscript{Δ}, pvg3\textsuperscript{Δ}, mxb2\textsuperscript{Δ}/pvg4\textsuperscript{Δ}, and pvg5\textsuperscript{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 \( \mu \text{g}/OD_{600} \text{ cells} \), pvg1\textsuperscript{Δ}, pvg2\textsuperscript{Δ}, pvg3\textsuperscript{Δ}, and pvg5\textsuperscript{Δ} cells did not bind alcian blue (Fig. 2A). In contrast, mxb2\textsuperscript{Δ}/pvg4\textsuperscript{Δ} cells bound alcian blue as well as wild-type cells did (Fig. 2A). In addition, wild-type and mxb2\textsuperscript{Δ}/pvg4\textsuperscript{Δ} cells adhered to Q-Sepharose beads, but pvg1\textsuperscript{Δ}, pvg2\textsuperscript{Δ}, pvg3\textsuperscript{Δ}, and pvg5\textsuperscript{Δ} cells did not (Fig. 2B). These results indirectly suggest that the biosynthesis of pyruvylated galactose
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 rlm1Δ* cells grown in liquid medium limited for nitrogen revealed an absence of the cell aggregates observed for wild-type cells, suggesting that *rlm1Δ* 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-
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*/H9004 mutants are completely abolished by the deletion of *gsf2*/H11001, indicating their dependence on *gsf2*/H11001 in *S. pombe* (20).

We found that adhesion and nonsexual flocculation by *gsf1* and *lkh1Δ* mutants were completely abolished by the deletion of *mbx2Δ* (Fig. 4). Wild-type, *lkh1Δ*, *gsf1*, *mbx2Δ*, *lkh1Δ mbx2Δ*, and *gsf1 mbx2Δ* cells were spotted on 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, *lkh1Δ*, *gsf1*, *mbx2Δ*, *lkh1Δ mbx2Δ*, and *gsf1 mbx2Δ* cells cultured on YES medium. (C) mRNA levels of *gsf2* in *lkh1Δ*, *gsf1*, *mbx2Δ*, *lkh1Δ mbx2Δ*, and *gsf1 mbx2Δ* cells. Cells were cultured in YES medium at 30°C to an OD₆₀₀ 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.

**FIG 4** Mbx2 is required for *gsf2Δ*-dependent adhesion and nonsexual flocculation. (A) Wild-type, *lkh1Δ*, *gsf1*, *mbx2Δ*, *lkh1Δ mbx2Δ*, and *gsf1 mbx2Δ* 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, *lkh1Δ*, *gsf1*, *mbx2Δ*, *lkh1Δ mbx2Δ*, and *gsf1 mbx2Δ* cells cultured on YES medium. (C) mRNA levels of *gsf2* in *lkh1Δ*, *gsf1*, *mbx2Δ*, *lkh1Δ mbx2Δ*, and *gsf1 mbx2Δ* cells. Cells were cultured in YES medium at 30°C to an OD₆₀₀ 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.

We next overexpressed *mbx2Δ* in wild-type and *gsf2Δ* cells under the control of the thiamine-repressible *nmt41* promoter (20), flocculated as well as wild-type cells transformed with the *nmt41-gsf2Δ* construct (data not shown), indicating that the abolition of flocculation in the *mbx2Δ* deletion mutants was linked to a reduction in *gsf2Δ* mRNA levels.

**Overexpression of mbx2Δ induces flocculation.** We next overexpressed *mbx2Δ* in wild-type and *gsf2Δ* cells under the control of the *nmt1* promoter. Wild-type and *gsf2Δ* cells transformed with pREP1-*mbx2Δ* were cultured on MM-leucine medium at 30°C for 18 h with or without 20 μg/ml of thiamine. Flocculation
was observed in the wild-type cells transformed with pREP1-
mbx2+/H11001 but not in the gsf2+/H9004 cells cultured without thiamine (Fig. 5A). The flocculation of the wild-type cells transformed with
mbx2+/H11001 was abolished by growth with thiamine, indicating that
mbx2+/H11001 expression was required for flocculation. Flocculation
in the wild-type strain overexpressing mbx2+/H11001 was inhibited by the
addition of EDTA or galactose but was not inhibited by the addi-
tion of glucose, mannose, or sucrose (Fig. 5B). These flocculation
phenotypes are consistent with the flocculation of
gsf2+/H11001-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 floccu-
culation induced by Mbx2 overexpression was abolished by dele-
tion 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 floccula-
tion 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 addi-
tion of mannose or EDTA but was not inhibited by the addition of
galactose, glucose, fructose, or sucrose (Fig. 6B). We also ex-
pressed mbx2+ and FLO8 in flo1Δ, flo10Δ, and flo11Δ cells under
the control of the GPD promoter. Flocculation was observed in
flo10Δ and flo11Δ cells expressing mbx2+ or FLO8 but not in flo1Δ
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 adhe-
sive growth of haploid cells and pseudohypha formation by
diploid cells were observed in FLO8-expressing haploid and dip-
loid 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 floccu-
lation in fission yeast and cloned the gsf2+ gene that encodes a
flocculin (20). In the present study, we cloned a new dominant flocculation gene, \textit{mbx2}^+ . \textit{Mbx2} has a MADS box domain, induces nonsexual flocculation via the induction of \textit{mbx2}^+ expressed in \textit{S. cerevisiae} \textit{FLO8}, \textit{pBP73G}, containing the GPD promoter, was used as an overexpression vector. Wild-type (BY4742) cells transformed with \textit{pBP73G} (−), \textit{pBP73G-\textit{mbx2}^+} (\textit{mbx2}^+), and \textit{pBP73G-\textit{FLO8}} (\textit{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 \textit{mbx2}^+ -overexpressing strain. EDTA (10 mM final concentration) and sugars (galactose, glucose, fructose, mannose, and sucrose; 200 mM final concentration) were added to the \textit{mbx2}^+ -overexpressing cells. (C) Assessment of flocculation of \textit{flo1}Δ, \textit{flo10}Δ, and \textit{flo11}Δ strains transformed with \textit{pBP73G-\textit{mbx2}^+} and \textit{pBP73G-\textit{FLO8}}. (D) mRNA levels of \textit{FLO1}, \textit{FLO11}, and \textit{ACT1} in cells transformed with \textit{pBP73G} (−), \textit{pBP73G-\textit{mbx2}^+} (\textit{mbx2}^+), and \textit{pBP73G-\textit{FLO8}} (\textit{FLO8}). Total RNA was extracted from transformants, and gene expression was monitored by RT-PCR.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{\textit{S. pombe} \textit{mbx2}^+ induces nonsexual flocculation in \textit{S. cerevisiae}. (A) Nonsexual flocculation of \textit{S. cerevisiae} cells expressing \textit{S. pombe} \textit{mbx2}^+ and \textit{S. cerevisiae} \textit{FLO8}. \textit{pBP73G}, containing the GPD promoter, was used as an overexpression vector. Wild-type (BY4742) cells transformed with \textit{pBP73G} (−), \textit{pBP73G-\textit{mbx2}^+} (\textit{mbx2}^+), and \textit{pBP73G-\textit{FLO8}} (\textit{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 \textit{mbx2}^+ -overexpressing strain. EDTA (10 mM final concentration) and sugars (galactose, glucose, fructose, mannose, and sucrose; 200 mM final concentration) were added to the \textit{mbx2}^+ -overexpressing cells. (C) Assessment of flocculation of \textit{flo1}Δ, \textit{flo10}Δ, and \textit{flo11}Δ strains transformed with \textit{pBP73G-\textit{mbx2}^+} and \textit{pBP73G-\textit{FLO8}}. (D) mRNA levels of \textit{FLO1}, \textit{FLO11}, and \textit{ACT1} in cells transformed with \textit{pBP73G} (−), \textit{pBP73G-\textit{mbx2}^+} (\textit{mbx2}^+), and \textit{pBP73G-\textit{FLO8}} (\textit{FLO8}). Total RNA was extracted from transformants, and gene expression was monitored by RT-PCR.}
\end{figure}

\textit{Mbx2} Regulates Invasive Growth and Flocculation

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

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

The major environmental signal for \textit{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 \textit{Mbx2}, including the identification of upstream regulators.

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