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**The fission yeast stress MAPK cascade regulates the *pmp3*⁺ gene
that encodes a highly conserved plasma membrane protein**

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Abstract

In eukaryotic organisms, stress-activated MAPKs play crucial roles in transmitting environmental signals to regulate gene expression for cellular stress adaptation. Here we report that, in the fission yeast *Schizosaccharomyces pombe*, Spc1/Sty1 MAPK and the Atf1 transcription factor regulate the stress-induced expression of Pmp3, a ubiquitous small membrane protein implicated in the modulation of the plasma membrane potential. The *pmp3* null mutant, as well as the *spc1* and *atf1* mutants, is hypersensitive to the cationic antibiotic hygromycin B. Transcriptional regulation of the Pmp3-like genes by the stress-activated MAPK may also be conserved in other eukaryotes, including plants.

Keywords:

environmental stress; hygromycin B; MAP kinase; plasma membrane; *Schizosaccharomyces pombe*; stress response.

1. Introduction

Adaptation to fluctuations in the environmental conditions, such as temperature, osmolarity, pH and concentration of nutrients and toxic compounds, are essential cellular functions, particularly in unicellular organisms. Eukaryotes from yeast to humans utilize a highly conserved signaling module, a mitogen-activated protein kinase (MAPK) cascade, to transmit extracellular stimuli to the nucleus and regulate gene expression for appropriate cellular responses. MAPKs dedicated for environmental stress signaling are also called stress-activated protein kinases (SAPKs).

In the fission yeast *Schizosaccharomyces pombe*, Spc1 (also known as Sty1) MAPK is activated in response to diverse forms of stress, including high osmolarity, oxidative stress, heat stress, UV irradiation and nutritional limitation [1]. In fact, the *spc1* null mutant ($\Delta spc1$) is hypersensitive to these conditions, indicating that the function of Spc1 is crucial for cellular resistance to those stresses [2-4]. Activated Spc1 enters the nucleus [5] and regulates gene expression through Atf1, a bZIP transcription factor [3,6]. Recent DNA microarray analysis in *S. pombe* revealed that ~140 genes induced by multiple forms of stress, named the core environmental stress response (CESR) genes, are mostly under the regulation of the Spc1–Atf1 pathway [7]. The CESR genes include those involved in carbohydrate and lipid metabolism, signaling, transcription, antioxidants, DNA repair, protein folding/degradation, and transporters, indicating that a wide spectrum of physiological activities is reprogrammed in stressed cells through the Spc1 MAPK cascade.

In this study, we have demonstrated *pmp3*⁺ as a new stress response gene, whose expression is induced by diverse stress stimuli through the Spc1–Atf1 pathway. Pmp3 belongs to

an evolutionarily conserved family of small plasma membrane proteins, whose members include budding yeast Pmp3p/Sna1p that regulates the membrane potential [8,9]. Like the *pmp3* mutant in budding yeast, *S. pombe* cells lacking the *pmp3*⁺ gene, as well as the *spc1* and *atf1* mutants, are hypersensitive to the cationic antibiotic hygromycin B. We propose that induced expression of *pmp3*⁺ is part of the cellular stress response mediated by the SAPK cascade.

2. Materials and methods

2.1. Yeast strains and cultures

S. pombe strains used in this study are CA101, *h⁻ leu1-32*; KS1366, *h⁻ leu1-32 ura4-D18 spc1::ura4⁺* [10]; KS1376, *h⁻ leu1-32 ura4-D18 spc1:HA6H(ura4⁺)* [10]; KS1497, *h⁻ leu1-32 ura4-D18 atf1::ura4⁺* [3]; CA3944, *h⁻ leu1-32 pmp3::kanR*; CA3946, *h⁻ leu1-32 pmp31::kanR*; CA3972, *h⁻ leu1-32 pmp3::kanR pmp31::kanR*. Growth media and basic techniques for *S. pombe* have been described [11]. Sensitivity of *S. pombe* strains to the stress conditions was determined by spotting onto agar media saturated liquid cultures diluted to OD₆₀₀ 1.0 and subsequent 10-fold serial dilutions. The DiSC₃(3) uptake assay was performed as described [12].

2.2. Gene disruption

The *pmp3* null mutant (*pmp3::kanR*) was constructed by the two-step PCR-based method [13] using primers, U5pmp3, U3pmp3, D5pmp3 and D3pmp3, as well as the plasmid template pFA6a-KanMX6 [14]. Similarly, the *pmp31* null mutant (*pmp31::kanR*) was constructed with PCR primers, U5pmp31, U3pmp31, D5pmp31 and D3pmp31. The gene disruptions were confirmed by Southern hybridization. The sequences of the PCR primers are available upon

request.

2.3. Northern analysis

S. pombe cells were cultured at 30°C in YES medium to the mid-log phase, followed by treatment of sorbitol, H₂O₂, heat shock or hygromycin B. Total RNA was isolated and analyzed by the standard method. As hybridization probes, the *pmp3*⁺ and *pmp31*⁺ open reading frames were amplified by PCR, using primer pairs, 5'NdeI-pmp3 and pmp3-NotIc for *pmp3*⁺ and 5'NdeI-pmp31 and 3'pmp31-NotI for *pmp31*⁺, respectively. The hybridization signals were quantified by PhosphorImager (Molecular Dynamics) and normalized against the level of *leu1*⁺ signals.

2.4. Analysis of Spc1 MAPK activation

Strain KS1376, which expresses Spc1 tagged with the HA epitope followed by six His residues, was grown at 30°C in YES medium and treated with 10 µg/ml anisomycin or 20 µg/ml Hygromycin B. The Spc1HA6H protein was purified under the denaturing condition and analyzed by immunoblotting with anti-HA and anti-phospho-p38 antibodies as described previously [15].

3. Results and discussion

3.1. Two PMP3-like genes in fission yeast

Spc1 MAPK and its downstream transcription factor, Atf1, are involved in cellular resistance to toxic cations, such as Na^+ and Li^+ , through the transcriptional regulation of *sod2*⁺, which encodes a Na^+/Li^+ exporter in the plasma membrane [16]. However, we noticed that, unlike the *sod2* mutant (data not shown), the null mutants of Spc1 (Δspc1) and Atf1 (Δatf1) are sensitive also to the antibiotic hygromycin B (Fig. 1). Hygromycin B is an aminoglycoside active against prokaryotic and eukaryotic ribosomes and inhibits protein synthesis. Due to its cationic property, cellular uptake of hygromycin B is significantly affected by the electro gradient across the plasma membrane, and increased sensitivity to hygromycin B is suggestive of hyperpolarized membrane potential [17-20]. Therefore, it is likely that the Spc1–Atf1 pathway regulates additional cation resistance genes other than *sod2*⁺.

In the budding yeast *Saccharomyces cerevisiae*, a 55 amino-acid, hydrophobic protein (proteolipid) in the plasma membrane, Pmp3p/Sna1p, is known to modulate the membrane potential [8,9]. The Δpmp3 mutant is sensitive to both Na^+ and hygromycin B due to membrane hyperpolarization. Pmp3p-like proteins are highly conserved from bacteria, fungi to nematode and higher plants, and the orthologs in barley, rice, *Arabidopsis* and a halophyte *Aneurolepidium chinense* are transcriptionally induced in response to stress, such as low temperatures, salinity, and H_2O_2 [9,21-24]. Thus, we hypothesized that a *S. pombe* PMP3 ortholog may also be important for cellular hygromycin B-resistance and transcriptionally regulated by the stress MAPK cascade. Our search for PMP3 orthologs in the *S. pombe* genome database

(http://www.sanger.ac.uk/Projects/S_pombe/) identified two open reading frames, SPBC713.11c

and SPCC1183.09c, which we named *pmp3*⁺ and *pmp31*⁺, respectively. *pmp3*⁺ encodes a 50-amino acid, 5.4-kDa protein with 47% identity and 76% similarity to *S. cerevisiae* Pmp3p in the amino acid sequence (Fig. 2A). Following a probable signal peptide at the N-terminus, *S. pombe* Pmp3 contains a single predicted transmembrane helix at residues 26-48. On the other hand, *pmp31*⁺ encodes a 105-amino acid, 11.4-kDa protein, of which N-terminal half (residues 12-58) shows 40% identity and 70% similarity to *S. cerevisiae* Pmp3p. Pmp31 contains two probable transmembrane helices at residues 7-29 and 34-56, with a unique 47-amino acid extension at the C-terminus (Fig. 2A).

3.2. *pmp3*⁺, but not *pmp31*⁺, is essential for cellular resistance to hygromycin B

To examine whether the *pmp3*⁺ and *pmp31*⁺ genes are involved in cellular cation resistance, gene disruption experiments were performed (Materials and methods). Both genes are not essential for viability and the haploid $\Delta pmp3$ and $\Delta pmp31$ strains showed no apparent growth defect under normal growth conditions (data not shown). However, like the $\Delta pmp3$ mutant in *S. cerevisiae*, the *S. pombe* $\Delta pmp3$ mutant exhibited hypersensitivity to hygromycin B (Fig. 1), implying that the orthologs in these yeast species have a similar function in the plasma membrane. On the other hand, the sensitivity of the $\Delta pmp3$ mutant to other diverse cations, Na⁺, Li⁺, Cs⁺, Tl⁺, Ca²⁺, tetramethylammonium and norspermidine is similar to that of the wild-type strain (data not shown), while the $\Delta pmp3$ mutation in budding yeast also brings about hypersensitivity to Na⁺ [8,9]. The $\Delta pmp31$ mutation, in contrast, did not affect the cellular resistance to hygromycin B both in the *pmp3*⁺ and $\Delta pmp3$ backgrounds (Fig. 1).

Together, these data indicate that Pmp3, but not Pmp31, are required for cellular resistance to the cationic antibiotic hygromycin B. As proposed for budding yeast Pmp3p, Pmp3

in *S. pombe* might also affect the plasma membrane potential; indeed, the $\Delta pmp3$ phenotype was complemented by the *S. cerevisiae* *PMP3* gene (Fig. 2B). However, the cation sensitivity of the *S. pombe* $\Delta pmp3$ mutant is limited to hygromycin B. Furthermore, unlike the $\Delta hal4$ mutant that shows membrane hyperpolarization [25], $\Delta pmp3$ cells did not exhibit an obvious increase in the uptake of a potentiometric fluorescent dye, DiSC₃(3) (data not shown). It is possible that the loss of Pmp3 changes the plasma membrane permeability to hygromycin B by a different mechanism.

3.3. *pmp3*⁺ is induced by the *Spc1–Atf1* pathway in response to diverse stress stimuli

While neither *pmp3*⁺ nor *pmp31*⁺ were listed as the core environmental stress response (CESR) genes [7], gene expression analysis by *S. pombe* DNA microarrays suggested increased expression of these genes under some environmental stress conditions (http://www.sanger.ac.uk/PostGenomics/S_pombe/projects/). In order to test whether *pmp3*⁺ and *pmp31*⁺ are among the stress response genes induced by the Spc1 MAPK cascade and the Atf1 transcription factor, we performed northern analysis with wild-type, $\Delta spc1$ and $\Delta atf1$ strains. In response to high osmolarity, heat shock, and oxidative stress, the conditions known to activate the Spc1 cascade [2], 3-5 fold increases in the *pmp3*⁺ expression was detected with the wild-type strain (Fig. 3). In contrast, no induction of *pmp3*⁺ was observed in the $\Delta spc1$ and $\Delta atf1$ strains, indicating that the stress-induced expression of *pmp3*⁺ is mediated by the Spc1–Atf1 pathway. Expression of *pmp31*⁺ was also induced to a lesser extent after osmotic stress and heat shock, but no significant induction was detected under oxidative stress (Fig. 3). Although $\Delta spc1$ cells expressed a lower level of *pmp31*⁺ mRNA in the absence of stress (time 0), the increased expression of *pmp31*⁺ after stress appeared to be largely independent of Spc1 and Atf1.

Because of the stress-inducible expression of *pmp3*⁺ and *pmp31*⁺, we examined whether these genes are involved in cellular stress resistance. Growth of the $\Delta pmp3$, $\Delta pmp31$ and $\Delta pmp3 \Delta pmp31$ mutants under high temperature (37°C), high osmolarity (2 M sorbitol) and oxidative stress (0.5 mM H₂O₂) was comparable to that of wild-type cells under the same conditions (Fig. 1).

3.4. Hygromycin B induces activation of Spc1 MAPK and expression of *pmp3*⁺

Since $\Delta spc1$ cells are hypersensitive to hygromycin B (Fig. 1), we became interested in whether Spc1 MAPK is activated when cells are exposed to hygromycin B. Other types of translation inhibitors, anisomycin and cycloheximide, are also known to activate SAPKs in both mammalian and fission yeast cells [26,27]. By immunoblotting with antibodies that recognize the phosphorylated, active form of Spc1, we detected Spc1 activation in cells treated by 20 μ g/ml hygromycin B, though the activation level is less than that by anisomycin (Fig. 4A). Consistently, we observed that expression of *pmp3*⁺ is induced by hygromycin B in a manner dependent on Spc1 and Atf1 (Fig. 4B). Thus, in the presence of hygromycin B, Spc1 MAPK is activated and promotes expression of *pmp3*⁺ gene through Atf1, resulting in increased resistance to this antibiotic.

In conclusion, our data indicate that Pmp3, an evolutionarily conserved proteolipid in the plasma membrane, is transcriptionally regulated by the Spc1 stress MAPK pathway. *pmp3*⁺ is induced by diverse forms of environmental stress as well as hygromycin B treatments. Also in plants, expression of Pmp3 orthologs are induced upon diverse environmental stimuli [9,21-24], though the signaling pathways responsible for such regulations remain to be determined [28].

Our finding that *S. pombe* Pmp3 expression is regulated by the stress-activated MAPK may provide a clue for identification of the transcriptional regulations for this highly conserved gene family in other eukaryotes.

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Figure Legends

Fig. 1. Spc1 MAPK, its downstream transcription factor Atf1, and the Pmp3 protein are required for cellular resistance to hygromycin B. Serial dilutions of wild-type, $\Delta spc1$, $\Delta atf1$, $\Delta pmp3$, $\Delta pmp31$ and $\Delta pmp3 \Delta pmp31$ strains were spotted on YES agar plates with various stress treatments: 30 $\mu\text{g/ml}$ of hygromycin B (Hyg B); incubation at 37°C for heat stress; 2 M sorbitol for high osmolarity; 0.5 mM H₂O₂ for oxidative stress.

Fig. 2. Pmp3-like proteins are highly conserved in bacteria, yeast, nematode and plants. (A) Amino acid sequence alignment between Pmp3 orthologs from *E. coli* P77240, *C. elegans* CEESP35F, *P. infestans* Ric1, *A. thaliana* RCI2A and RCI2B, *S. cerevisiae* Pmp3p, and *S. pombe* Pmp3, Pmp31. Black boxes represent identical residues and similar amino acid residues are shown in grey. (B) The *S. pombe* $\Delta pmp3$ mutation is complemented by the *S. cerevisiae* PMP3 gene. $\Delta pmp3$ cells transformed with the *S. pombe* expression vector pREP1 [29] and the same plasmids carrying the *S. pombe* $pmp3^+$ or *S. cerevisiae* PMP3 genes were spotted onto YES agar plates with or without 30 $\mu\text{g/ml}$ of hygromycin B. Wild-type cells transformed with pREP1 served as control.

Fig. 3. Expression of $pmp3^+$ is induced by Spc1 MAPK and Atf1 transcription factor under stress conditions. Wild-type, $\Delta spc1$ and $\Delta atf1$ strains grown at 30°C were treated with high osmolarity stress of 1 M sorbitol (A), heat stress at 39°C (B) and oxidative stress by 0.5 mM H₂O₂ (C). Aliquots were harvested at the indicated times for northern blot analysis of $pmp3^+$ and

pmp31⁺. For the bar graphs shown on the right, signal levels were quantified and normalized with control hybridization by the *leu1*⁺ probe.

Fig. 4. Hygromycin B activates the Spc1–Atf1 pathway and induces *pmp3*⁺ expression. (A) The *spc1:HA6H* strain was treated with 10 µg/ml anisomycin or 20 µg/ml hygromycin B, and cellular lysate was analyzed by immunoblotting with anti-HA (Spc1) and anti-phospho-p38 (Spc1-P) antibodies, which specifically recognize the phosphorylated, active form of Spc1 MAPK. (B) Northern analysis of *pmp3*⁺ and *pmp31*⁺ was performed as in Fig. 3, with cell cultures treated with 20 µg/ml hygromycin B.

Figure 1 (new)

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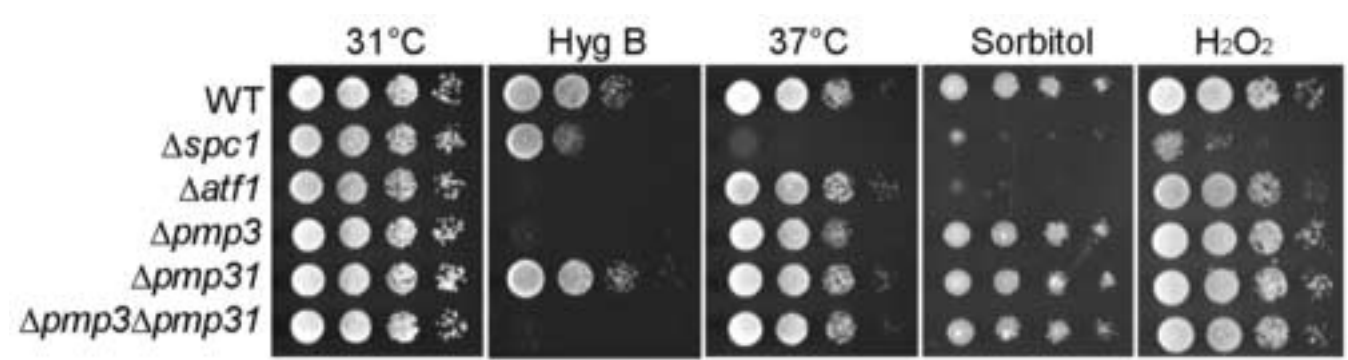


Figure 2 (new)

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A

E.col	(1)	-----MGFWRI	VIT	IILPPLGVLLG	GF	GWAFI	INILLT	LG	YIPGI	IHA	FW																																		
C.ele	(1)	-MALTCTDIPK	F	CALLLPP	IGVWLE	RGCTYHLA	INILLT	LG	YIPGI	IHA	CY																																		
P.inf	(1)	-MPITCGDIPR	ICS	VII	PPVGVFFQ	VGCTK	LAIN	CLLTVL	GYIPG	VI	HAVY																																		
A.tal A	(1)	---MSTATF	V	DI	I	AILLPP	LG	VFLR	FG	CG	VEFWICLVLT	LG	YIPGI	I	YAY																														
A.tal B	(1)	---MSTATF	V	E	I	AILLPP	LG	VFLK	FG	CK	VEFWIC	IL	T	LG	YIPGI	I	YAY																												
S.cer	(1)	---MDSAKI	I	N	I	S	IFLPP	V	V	FL	ARG	NG	D	C	I	M	I	IL	T	L	A	W	E	P	G	M	L	Y	A	Y															
S.pom 3	(1)	-----MTQ	V	I	F	A	I	L	P	P	L	G	V	F	L	E	R	G	C	G	A	D	V	I	N	I	L	L	C	C	L	G	Y	P	G	I	I	H	A	L	Y				
S.pom 3l	(1)	MSNVTLSD	F	L	L	I	V	L	S	F	F	V	E	F	V	V	G	I	R	R	G	F	C	T	A	D	F	I	N	I	C	L	C	A	L	G	I	P	G	I	I	H	A	L	Y

E.col	(48)	VQTRD	-----
C.ele	(53)	VILAY	-----
P.inf	(53)	ILIKE	-----
A.tal A	(51)	VLTK	-----
A.tal B	(51)	IITK	-----
S.cer	(51)	IVLQD	-----
S.pom 3	(48)	IILKY	-----
S.pom 3l	(54)	IVIKYPR	TVRLDIENSPNDPLVRYTPNPEHAVSPHSGPAPPSYSSSLASNGMP

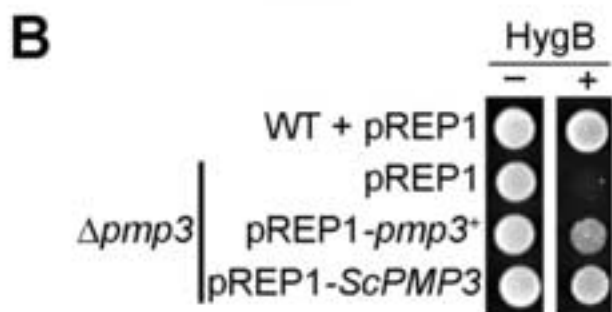


Figure 3
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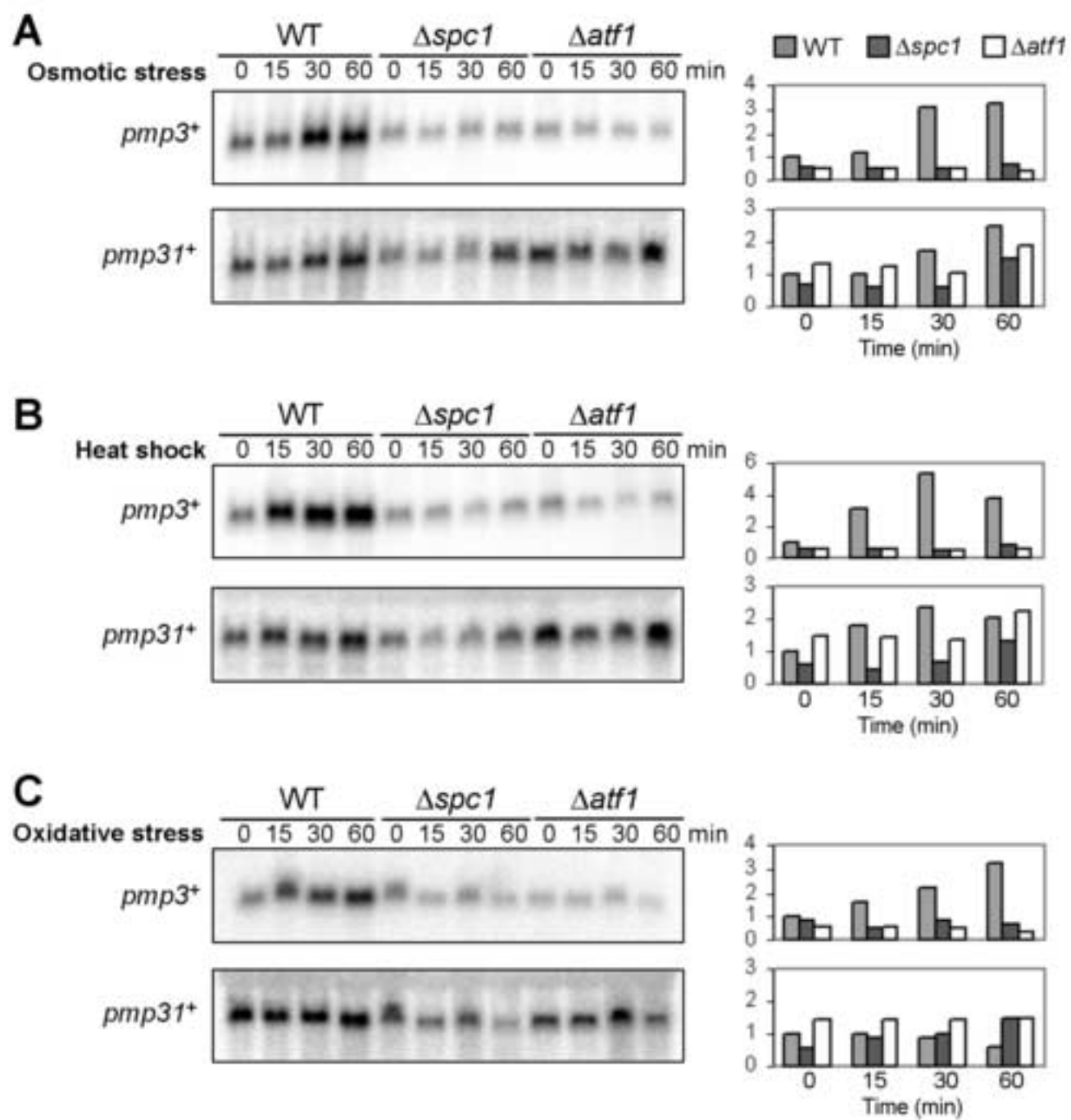


Figure 4

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