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A Measurable Activation of the bZIP Transcription Factor Atf1 in a Fission Yeast Strain Devoid of Stress-activated and Cell Integrity Mitogen-activated Protein Kinase (MAPK) Activities*[S]

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Background: Activation of Atf1 is regulated by the Sty1 MAPK under multiple stresses, and the Pmk1 MAPK is also suggested to regulate the activity of Atf1.
Results: We obtained a measurable activation of Atf1 in Δ*sty1*Δ*pmk1* cells.
Conclusion: Activation of Atf1 is not fully dependent on Sty1 and Pmk1.
Significance: The *Renilla* reporter assay identified a Sty1-independent activation of Atf1.

In *Schizosaccharomyces pombe*, the stress-activated Sty1 MAPK pathway is essential for cell survival under stress conditions. The Sty1 MAPK regulates Atf1 transcription factor to elicit stress responses in extreme conditions of osmolarity and reactive oxygen species-generating agents such as hydrogen peroxide, heat, low glucose, and heavy metal. Herein, using a newly developed *Renilla* luciferase reporter assay with enhanced detection sensitivity and accuracy, we show that distinct signaling pathways respond to cadmium and other reactive oxygen species-generating agents for the activation of Atf1. Also, surprisingly, a measurable activation of Atf1 transcription factor was still observed devoid of Sty1 MAPK activity. Further genetic and biological analyses revealed that the residual activation is caused by the activation of the cell wall integrity Pmk1 MAPK pathway and a redox-mediated activation of Atf1.

The mitogen-activated protein kinase (MAPK) signaling pathways are critical for the response of cells to changes in their external environment. Such responses involve changes in the gene expression pattern of the cell, leading to increased levels and activities of proteins that have stress-protective functions (1). In mammalian cells, these stress-activated MAP kinases (SAPKs) fall into two distinct classes based on sequence and are termed the c-Jun N-terminal kinase (JNK) and p38 kinase (2). Both of these two SAPKs phosphorylate the activating transcription factor 2 (ATF2), which plays an important role in the cellular stress response (3, 4).

In *Schizosaccharomyces pombe*, the Sty1 MAPK (also known as Spc1 or Phh1), homologous to the mammalian JNK and p38 (5), plays a major role in response to ROS[2]-generating agents and many other stress factors (6–9). Activated Sty1 accumulates in the nucleus (10, 11), where it activates transcription factors, such as Atf1. As a bZIP transcription factor with strong homology to the mammalian factor ATF2, Atf1 regulates the expression of many genes that are relevant to the stress adaptation. Thus, the Sty1/Atf1-dependent core environmental stress response has been identified as being important for protection against different stress conditions, including osmolarity and some ROS-generating agents (12).

Our previous results of the firefly luciferase reporter assay led us to the conclusion that KCl stimulates the Sty1 MAPK pathway through MAPKKKs, whereas $CdCl_2$ and LG stimulate the pathway through the inhibition of Pyp1 independently of MAPKKKs (13). However, the firefly luciferase requires a relatively abundant and continual supply of ATP that might lead to inaccurate or misleading conclusions in investigating the cellular responses that may consume ATP. Therefore, we newly developed a *Renilla* luciferase reporter system that is barely influenced by the level of ATP in living cells to avoid artifacts due to ATP consumption in response to stimuli such as H_2O_2. Here we show the results of *S. pombe* haploid strains obtained by the new *Renilla* luciferase reporter system. We also improved the assay by using a new type of luminometer (AB-2350; ATTO Co., Tokyo, Japan) with enhanced detection accuracy and sensitivity. The results show that distinct signaling pathways respond to cadmium and other ROS-generating agents for the activation of Atf1 and, surprisingly, that Atf1 transcription factor can still be activated by different extracellular stress agents independently of the activation of the Sty1 MAPK pathway. Further genetic and biological analyses revealed that the cell wall integrity Pmk1 MAPK pathway also activates Atf1 in a parallel manner and that there exists a redox-mediated activation of Atf1 in cells devoid of Sty1 and Pmk1 MAPK activities.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic and Molecular Biology Methods—*S. pombe* haploid strains used in this study are listed in Table 1.

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[S] This article contains supplemental Figs. 1 and 2.
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[2] The abbreviations used are: ROS, reactive oxygen species; LG, low glucose; EMM, Edinburgh minimal medium; NAC, *N*-acetyl-L-cysteine.

TABLE 1
***S. pombe* haploid strains used in this study**

Strain	Genotype	Reference
HM123	*h⁻ leu1-32*	Our stock
KP208	*h⁻ leu1-32 ura4-D18 pmk1::ura4⁺*	Our stock
KP226	*h⁻ leu1-32 his1-102 wis1::his1⁺*	29
KP471	*h⁻ leu1-32 ura4-D18 sty1::ura4⁺*	29
KP495	*h⁻ leu1-32 ura4-D18 atf1::ura4⁺*	30
KP2729	*h⁻ leu1-32 mcs4::KanMX₆*	This study
KP2730	*h⁻ leu1-32 ura4-D18 wis4::ura4⁺*	31
KP2731	*h⁻ leu1-32 ura4-D18 win1::ura4⁺*	32
KP2773	*h⁻ leu1-32 ura4-D18 wis4::ura4⁺ win1::ura4⁺*	This study
KP2858	*h⁻ leu1-32 ura4-D18 pyp1::ura4⁺*	33
KP2941	*h⁻ leu1-32 ura4-D18 wis4::ura4⁺ mcs4::KanMX₆*	This study
KP2943	*h⁻ leu1-32 ura4-D18 win1::ura4⁺ mcs4::KanMX₆*	This study
KP4330	*h⁻ leu1-32 lys3-1 ura4-D18 sty1::lys3⁺ pmk1::ura4⁺*	This study

The medium, notation, and genetic methods have been described previously (14, 15).

Construction of cAMP-response Element (CRE) Reporter Plasmids—The 3xCRE::luc (R2.2) reporter vector (pKB5760 for the firefly luciferase reporter assay) was constructed as described previously (13). The 3xCRE::*Renilla* reporter vector (pKB5878 for the *Renilla* luciferase reporter assay) was constructed as follows. A 1176-bp fragment (86–1226 bases) containing *Renilla*-type luciferase gene was cut with HindIII and XbaI from a Rapid Response reporter vector phRG(R2.2)-basic (Promega) and subcloned into the HindIII/XbaI sites of pBluescript II SK(+) (pKB1019) to give pKB5836. Again, a *Renilla*-type luciferase gene-containing fragment was cut with NcoI and NotI from pKB5836 and subcloned into three tandem repeats of CRE-like sequence containing pKB5760 to displace the firefly-type luciferase gene.

Real-time Monitoring Assays of Atf1-mediated Transcriptional Activity—The method of real-time monitoring of Atf1-mediated transcriptional activity by using the firefly luciferase reporter assay has been described previously (13). The method of the 3xCRE::*Renilla* reporter assay is similar to that of the firefly luciferase reporter assay except that the substrate luciferin was displaced by coelenterazine. The experiments with $CdCl_2$ and LG were performed as described previously (13). For the experiments with heat shock, cells were cultured at 27 °C in EMM containing 2% glucose overnight to mid-log phase and incubated in 42 or 34 °C for 10 min just before detection. *N*-Acetyl-L-cysteine (NAC; NACALAI TESQUE, Inc.) was added to the cells and incubated for 2 min before the addition of $CdCl_2$ and H_2O_2. Emission of light was detected at 1-min intervals and reported as relative light units using a new type of luminometer (AB-2350; ATTO Co.).

Real-time Reverse Transcription-PCR (RT-PCR)—The total RNA was isolated by QIAzol (Qiagen) extraction. DNA was removed from RNA preparations using recombinant DNase I (RNase-free; TaKaRa). RNA quantitation assays were performed by a two-step method. In the first step, cDNA synthesis was performed using High Capacity cDNA reverse transcription kits (Applied Biosystems). Hsp9 primers for RT-PCR were 5′-ACC CTC GAC AAG GCC AAG GAA-3′ and 5′-TGT CAT CAA CAA AGC GTT GAG CCT-3′. Arp3 was used as an internal control, and Arp3 primers for RT-PCR were 5′-TTT CAG CTA TGC TGG GAA CGA TGC-3′ and 5′-GCT TGA GAG ATG CCC ACT GCC-3′. In the second step, SYBR® Green PCR master mix (Applied Biosystems) was used, and real-time PCR was run on Applied Biosystems 7500.

RESULTS AND DISCUSSION

Our previous data obtained by using the firefly luciferase reporter assay suggest that the protein tyrosine phosphatase Pyp1 is responsible for sensing and transmitting the signals to Sty1 MAPK upon treatment with $CdCl_2$ or LG (13). These results led us to look into the Atf1 transcriptional activities induced by other ROS-generating agents such as heat shock and H_2O_2. Upon heat shock (42 °C, 10 min), in wild-type cells, the Atf1 transcriptional activity showed a steady increase, with a peak rise at about 80 min; in contrast, in Δ*pyp1* cells, the Atf1 activity was barely observed and even lower than the basal level after the peak rise (Fig. 1*A*). Upon the elevated extracellular H_2O_2 (1.0–4.0 mM H_2O_2), in wild-type cells, the Atf1 transcriptional activity showed the steady increases with the dose-dependent delay of the peak. On the other hand, in Δ*pyp1* cells, unexpectedly, a series of three doses of H_2O_2 caused dose-dependent suppression of the peak (Fig. 1*A*). These results suggest that ATP consumption in cells treated by H_2O_2 (16) may lead to an insufficient ATP supply to ATP-dependent D-luciferin oxidation of the firefly luciferase reporter assay, thereby resulting in apparently low Atf1 transcriptional activity. It should be noted that although we use the same firefly luciferase reporter vector, the levels of the Atf1 transcriptional activities detected by the new type of luminometer (AB-2350) were much higher (Fig. 1*A*) than those detected by the previous type (AB-2300) (13).

In view of the facts that the transcriptional activity of Atf1 upon H_2O_2 treatment in living cells is not capable of being monitored accurately by the firefly luciferase reporter assay, we constructed the 3xCRE::*Renilla* reporter vector for the ATP-independent *Renilla* luciferase reporter system. The wild-type, Δ*atf1*, and Δ*pyp1* cells were subjected to heavy metal stress (2 mM $CdCl_2$), oxidative stress (2 mM H_2O_2), heat shock (42 °C, 10 min), and LG (0.1% glucose), respectively. In wild-type cells, various kinds of stresses caused various patterns of increase in response, and in Δ*atf1* cells, there was no response upon any treatment (Fig. 1*B*), indicating that the 3xCRE::*Renilla* reporter assay also reflects the Atf1 activity.

Similar to the results obtained with the firefly luciferase reporter assay (Fig. 1*A*, basal), Δ*pyp1* cells still showed a higher basal reporter activity than wild-type cells with the *Renilla* reporter (Fig. 1*C*), suggesting that a higher level of Atf1 transcriptional activity in the absence of extracellular stresses was induced by the relief of phosphatase Pyp1 inhibition of the Sty1 MAPK. However, in Δ*pyp1* cells treated with ROS-generating agents, the results obtained with the *Renilla* reporter were completely different from those obtained with the firefly luciferase reporter assay (Fig. 1*B*). Upon treatment with $CdCl_2$, H_2O_2, heat shock, or LG, in Δ*pyp1* cells, an increase in Atf1 transcriptional activity was observed, similarly to wild-type cells (Fig. 1*B*), suggesting that Pyp1 does not function as a sensor for these ROS-generating agents in the Sty1 MAPK pathway. This is quite in contrast with our previous hypothesis obtained accord-

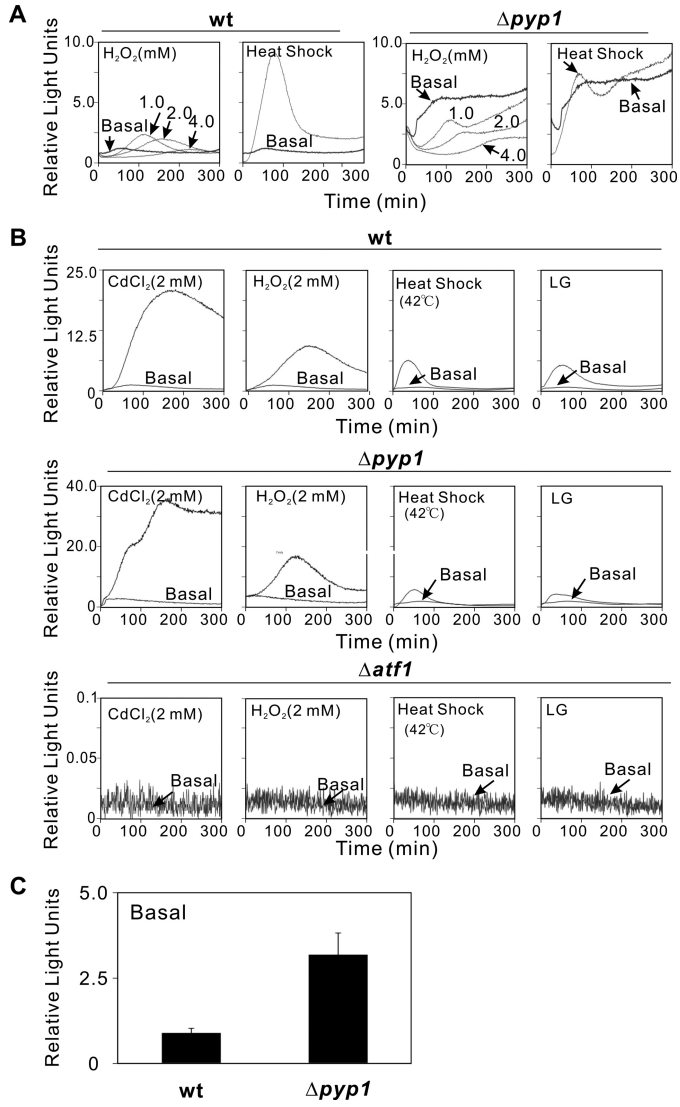

FIGURE 1. **Real-time monitoring of Atf1 activity in living cells.** *A*, live cell monitoring of Atf1 activity in wild-type (*wt*) and Δ*pyp1* cells in response to H_2O_2 and heat shock by using the firefly reporter assay. Wild-type cells and Δ*pyp1* cells transformed with the firefly reporter plasmid (3xCRE::luc(R2.2)) were incubated with D-luciferin and treated with H_2O_2 (1–4 mM) or incubated in 42 °C for 10 min before detection. Relative light units are expressed as the ratio of light emission of each sample to the basal (without stimulation) light emission of wild-type cells in EMM at 120 min. The data shown are representative of multiple experiments. *B*, live cell monitoring of Atf1 activity in wild-type, Δ*pyp1*, and Δ*atf1* cells in response to ROS-generating agents by using the *Renilla* reporter assay. The cells as indicated were transformed with the 3xCRE::*Renilla* reporter plasmid, incubated with coelenterazine, and treated with $CdCl_2$ (2 mM), H_2O_2 (2 mM), heat shock (42 °C, 10 min), or LG (0.1% glucose), as described under "Experimental Procedures." *C*, the Δ*pyp1* cells showed a higher basal reporter activity than wild-type cells. Values are from at least three independent experiments. *Error bars* indicate means ± S.D.

ing to the results obtained with the firefly luciferase reporter assay (13). We reasoned that changes in ATP consumption upon treatment with these ROS-generating agents (16–20) and high basal transcriptional activity of Atf1 in Δ*pyp1* cells might lead to incomplete response of the firefly luciferase that is ATP-dependent.

The Sty1/Atf1-dependent core environmental stress response is directly regulated by a MAPKK, Wis1, which is phosphorylated by two redundant MAPKKKs, Wis4 and Win1 (21). Also, a response regulator protein, Mcs4, associates with Wis4, and probably with Win1, to regulate MAPKKK activity in response to several forms of stress (8, 22). Then, we monitored Atf1 transcriptional activity using the *Renilla* reporter in the following cells: Δ*mcs4* (response regulator), Δ*wis4* and Δ*win1* (MAPKKK), Δ*wis4*Δ*mcs4*, Δ*win1*Δ*mcs4* or Δ*wis4*Δ*win1* double mutants, and Δ*wis1* (MAPKK) and Δ*sty1* (MAPK).

These cells showed varying degrees of the *Renilla* reporter response upon treatment with the ROS-generating agents (Fig. 2, *A* and *B*). It should be noted that the Atf1 transcriptional activities in Δ*wis4* and Δ*win1* cells induced by the ROS-generating agents are similar, whereas the reporter activities in Δ*win1*Δ*mcs4* double mutants were markedly lower than that in Δ*wis4*Δ*mcs4* double mutants and were almost at the same level as the reporter activities in Δ*wis4*Δ*win1* double mutants (Fig. 2*A*). These results suggest that the interaction of MAPKKK Win1 with either the response regulator Mcs4 or MAPKKK Wis4 is important for the signal transduction induced by the ROS-generating agents.

Our previous results obtained with the firefly luciferase reporter assay showed that a measurable Atf1 activation was observed in the MAPKKK double mutant Δ*wis4*Δ*win1* by stimulation with ROS-generating agents, whereas it was abolished in Δ*sty1* or Δ*wis1* (13). These results led us to conclude that $CdCl_2$ and LG stimulate the Sty1 pathway independently of MAPKKKs. Using the *Renilla* luciferase reporter assay, under treatment with the ROS-generating agents, a measurable Atf1 activation was observed not only in Δ*wis4*Δ*win1*, but also in Δ*wis1* or Δ*sty1* cells, although it was extremely low (Fig. 2, *B* and *C*), suggesting that activation of Atf1 in response to these ROS-generating agents is not fully dependent on Sty1 MAPK. Moreover, the Atf1 transcriptional activities in Δ*wis4*Δ*win1* double mutants induced by H_2O_2, heat shock, or LG are not much different from those in Δ*wis1* or Δ*sty1* cells, whereas $CdCl_2$-induced reporter activity in Δ*wis4*Δ*win1* double mutants is much higher than that in Δ*wis1* or Δ*sty1* cells (Fig. 2*B*). These results suggest that there exist distinct signaling pathways in response to cadmium and other ROS-generating agents upstream of the Wis1 MAPKK for the activation of Atf1.

It should be noted that Δ*wis1* and Δ*sty1* cells were subjected to a lower concentration of H_2O_2 (0.2 mM) (Fig. 2*C*) and a lower temperature (34 °C, 10 min) because the response curves of these cells quickly decreased to almost zero under H_2O_2 treatment (2 mM) and heat shock (42 °C, 10 min) (data not shown). Presumably, the transcriptional activity of Atf1 in these vulnerable cells may be turned off upon severe oxidative stress (2 mM H_2O_2) or heat shock (42 °C, 10 min).

To validate the results obtained by our reporter assay, Δ*sty1* and Δ*atf1* cells treated or untreated with 2 mM $CdCl_2$ and 0.2 mM H_2O_2 were isolated, and mRNA levels of Atf1-dependent transcripts *hsp9*$^+$ (23) were quantified by real-time RT-PCR. There was almost no increase of *hsp9*$^+$ mRNA in Δ*atf1* cells upon stress, and under the same conditions, significant increases were observed in Δ*sty1* cells (supplemental Fig. S1). These results again proved the existence of some activators of Atf1 other than Sty1 MAPK.

In fission yeast, the Pmk1 MAPK signaling pathway is also activated under multiple stresses, including heat shock, cell wall damage, and low glucose (15, 24, 25), and recent studies have

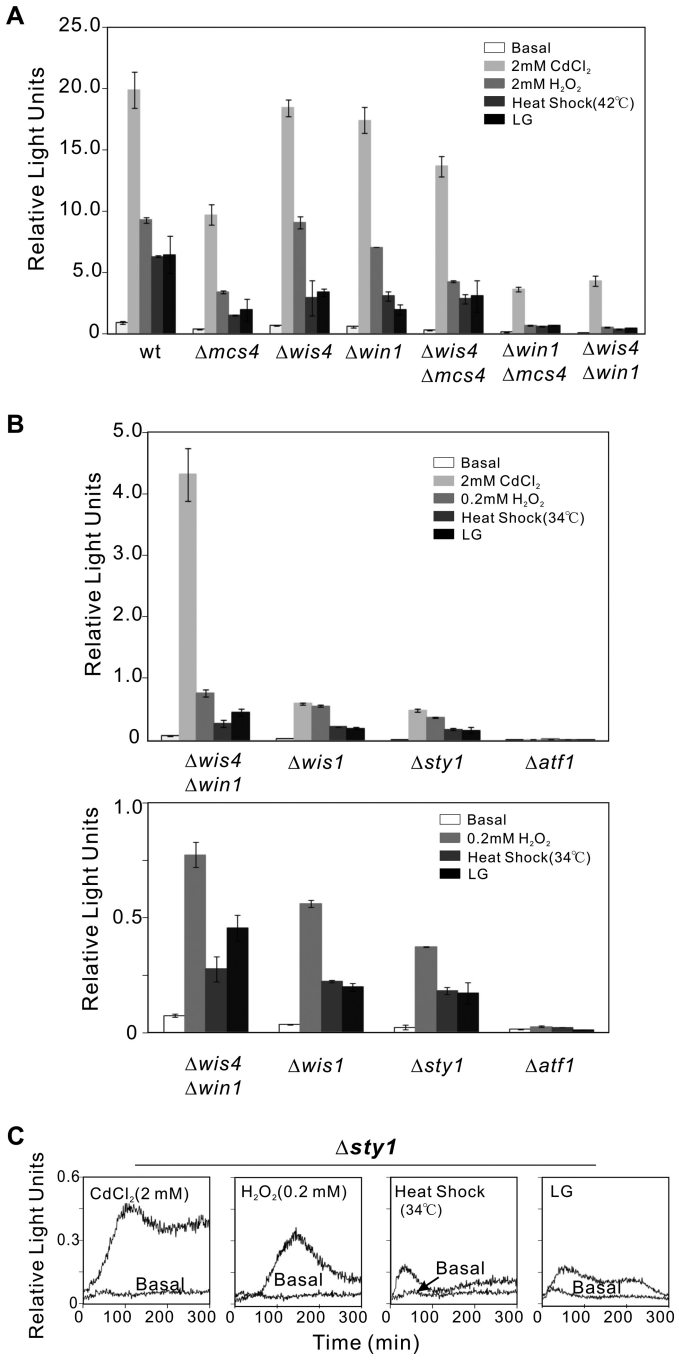

FIGURE 2. **Detection of Atf1 transcriptional activity in various cells of the Sty1 MAPK pathway.** *A*, interaction of MAPKKK Win1 with either the response regulator Mcs4 or MAPKKK Wis4 plays an important role for the activation of Atf1. Wild-type, Δ*mcs4*, Δ*wis4*, Δ*win1*, Δ*wis4*Δ*mcs4*, Δ*win1*Δ*mcs4*, or Δ*wis4*Δ*win1* cells transformed with the *Renilla* reporter plasmid were treated as indicated. In each case, the peak height of each response was compared with the basal (without stimulation) light emission of wild-type cells in EMM at 120 min. Data were analyzed and plotted as in Fig. 1. *B*, distinct signaling pathways exist in response to cadmium and other ROS-generating agents upstream of the Wis1 MAPKK for the activation of Atf1. The Δ*wis4*Δ*win1*, Δ*wis1*, or Δ*sty1* cells transformed with the *Renilla* reporter plasmid were treated as indicated. Data were analyzed and plotted as in Fig. 1. *Error bars* in *A* and *B* indicate means ± S.D. *C*, activation of Atf1 in response to ROS-generating agents is not fully dependent on Sty1. The Δ*sty1* cells were transformed with the 3xCRE::*Renilla* reporter plasmid, incubated with coelenterazine, and treated with $CdCl_2$ (2 mM), H_2O_2 (0.2 mM), heat shock (34 °C, 10 min), or LG (0.1% glucose), as described under "Experimental Procedures."

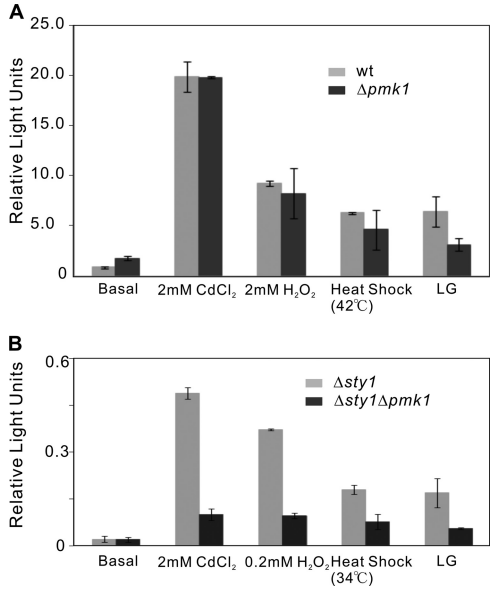

FIGURE 3. **Detection of Atf1 transcriptional activity in Δ*pmk1* and Δ*sty1*Δ*pmk1* cells.** *A*, activation of Atf1 in response to ROS-generating agents is not dependent on Pmk1 MAPK in the presence of Sty1 MAPK. Wild-type and Δ*pmk1* cells transformed with the *Renilla* reporter plasmid were treated with $CdCl_2$ (2 mM), H_2O_2 (2 mM), heat shock (42 °C, 10 min), or LG (0.1% glucose) as indicated. *B*, Pmk1 MAPK turns into one of the regulatory factors of Atf1 in the absence of Sty1 MAPK. The Δ*sty1* and Δ*sty1*Δ*pmk1* cells transformed with the *Renilla* reporter plasmid were treated with $CdCl_2$ (2 mM), H_2O_2 (0.2 mM), heat shock (34 °C, 10 min), or LG (0.1% glucose) as indicated. Data were analyzed and plotted as in Fig. 1. *Error bars* in *A* and *B* indicate means ± S.D.

reported that Pmk1 MAPK pathway plays a key role in cell wall integrity by regulating Atf1 (26). These results led us to investigate the *Renilla* reporter response upon treatment with the ROS-generating agents in Δ*pmk1* single and Δ*sty1*Δ*pmk1* double mutants. The single mutant Δ*pmk1* cells showed a similar reporter activity with wild-type cells (Fig. 3*A*), suggesting that the absence of Pmk1 MAPK barely affects the Atf1 transcriptional activity in the presence of the Sty1 MAPK. However, in Δ*sty1*Δ*pmk1* double mutants, significantly lower but still measurable reporter activities were observed than those observed in Δ*sty1* cells (Fig. 3*B*). Also, the Δ*sty1*Δ*pmk1* cells showed synergism in the sensitivities to osmotic and oxidative stress when compared with parental cells (supplemental Fig. S2*A*). We further examined the effect of the overexpression of $pmk1^+$ in Δ*sty1* under these stress conditions (supplemental Fig. S2*B*) and found that Pmk1 cannot rescue the phenotypes of Δ*sty1*. These results indicate that Sty1 and Pmk1 MAPK regulate Atf1 activity in a parallel manner.

NAC, a well known thiol antioxidant, could increase intracellular cysteine pools to provide a source of sulfhydryl groups as an acetylated precursor of reduced GSH and also directly interact with ROS. NAC can block ROS-generating agent-induced changes in signal transduction and suppress activation of MAPKs (27, 28). Upon $CdCl_2$ (2 mM) and H_2O_2 (0.2 mM) addition, NAC caused dose-dependent decrease of reporter activation in wild-type and Δ*sty1* cells (Fig. 4, *left* and *middle panels*), and these responses still remained higher than the activity in the presence of 0.5 mM NAC alone. However, in Δ*sty1*Δ*pmk1* cells, the addition of 0.1 mM NAC abolished $CdCl_2$- and H_2O_2-induced Atf1 reporter activity (Fig. 4, *right panel*), suggesting

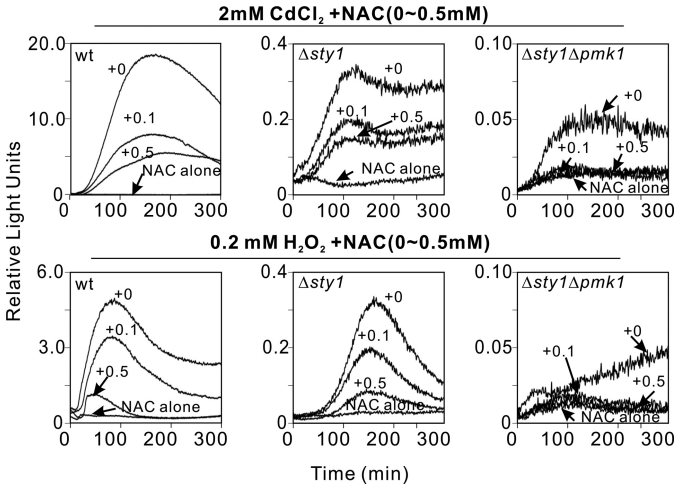

FIGURE 4. **The measurable Atf1 activity in Δ*sty1*Δ*pmk1* cells is scavenged by NAC.** Wild-type, Δ*sty1*, and Δ*sty1*Δ*pmk1* cells transformed with the *Renilla* reporter plasmid were treated with or without NAC (0.1 or 0.5 mM) before $CdCl_2$ (2 mM) and H_2O_2 (2 mM) were added as described under "Experimental Procedures." The reporter activities in cells were compared with the activity in the presence of 0.5 mM NAC alone.

that the residual activity in Δ*sty1*Δ*pmk1* cells may be mediated by redox reactions.

We also investigated the *Renilla* reporter response under treatment with NAC alone. By adding different doses of NAC, Atf1 activity showed dose-dependent decrease in various cells (data not shown), suggesting that even in the absence of $CdCl_2$ and H_2O_2, cellular redox products may cause the increase of reporter activity.

In conclusion, here we have presented data that establish the *Renilla* luciferase reporter assay as a novel semiquantification method for monitoring the Atf1 transcriptional activity in living cells upon treatment with ROS-generating agents including $CdCl_2$, H_2O_2, heat shock, and LG. By using the new reporter assay and the luminometer with enhanced detection sensitivity and accuracy, a measurable activation of Atf1 transcription factor induced by ROS-generating agents was clearly observed in Δ*wis1*, Δ*sty1*, as well as in Δ*sty1*Δ*pmk1* cells. This measurable activity was abolished by the addition of a thiol antioxidant NAC.

We also obtained similar increased reporter activities in Δ*wis4*Δ*win1*, Δ*wis1*, and Δ*sty1* cells induced by osmotic stress (0.3 M KCl) by using both the firefly and the *Renilla* luciferase reporter assays with the new type of luminometer (data not shown). These unexpected results not only evidenced that the Pmk1 MAPK also takes part in regulating Atf1 activity in the absence of the Sty1 MAPK, but also suggested that the redox state of cells may directly regulate Atf1 upon exposure to many environmental stresses, and future work will be continued to test this hypothesis.

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