Slt2 MAPK association with chromatin is required for transcriptional activation of Rlm1 dependent genes upon cell wall stress

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Abstract

The regulation of gene expression through the cell wall integrity (CWI) pathway in yeast is mainly coordinated by the MAPK Slt2 and the transcription factor Rlm1. In this work, we elucidate a new role for Slt2 as a part of the transcriptional activation machinery that regulates CWI gene expression in response to cell wall stress. We show that Slt2 is recruited to promoters and coding regions of CWI Rlm1-dependent genes in response to stress. This phenomenon is dependent both on the activation of the MAPK and its kinase activity. Slt2 binding is also dependent on Rlm1 and SWI/SNF and SAGA complexes. During the initial steps of transcription, the catalytic activity of Slt2 on Rlm1 is critical for the binding of the activator to promoters in response to stress. In addition, Slt2 itself acts as a transactivator, as it is able to induce the transcription of CWI responsive genes when it is bound to promoters through the Rlm1 binding domain independently of its catalytic activity. Slt2 interacts with RNA Pol II in a Rlm1-dependent manner to provide further support to a role of this MAPK as an integral component of the transcriptional complexes under cell wall stress. Selective recruitment and progression of the complex Slt2-RNA Pol II from the promoters to the coding regions of Rlm1-dependent genes does not rely on Paf1, suggesting a different mechanism from that which is exerted by Slt2 on the Swi4/Swi6 (SBF)-regulated genes.

Introduction

Signal transduction pathways allow cells to sense and respond to extracellular stimuli through multiple mechanisms, particularly by the regulation of gene expression [1, 2]. Development of yeast genome-wide analysis has provided a global view of the changes in gene expression in response to a variety of environmental stresses [3]. The regulation of specific transcriptional programs is achieved through different MAPK routes with the participation of specific transcription factors [4-7]. However, the role of the MAPKs in the regulation of these responses is not only restricted to the phosphorylation of the transcription factors but also to the association with chromatin for the recruitment of different regulatory elements [8-10]. In this context, the osmostress-related MAPK Hog1 has been largely studied [11]. Once bound to the promoters of its target genes, Hog1 mediates the assembly of the pre-initiation complex and the recruitment of RNA Pol II and different coactivators, including chromatin-modifying activities [12-16]. In addition to regulating transcriptional initiation, Hog1 is also associated with the coding regions of osmo-dependent genes, in which it behaves as a transcriptional elongator factor [17, 18]. Activated Hog1 plays a role in elongation by direct phosphorylation of Spt4 elongation factor to regulate the activity of RNA Pol II [19]. Hog1 also interacts with the RSC chromatin remodeling complex to direct its association with the coding regions and modify nucleosome organization at this level [20]. In addition to Hog1, other yeast-signaling kinases, such as Fus3, Kss1, PKA, or Slt2/Mpk1, have also been reported to associate with transcribed genes [9, 10, 17, 21].

Cell wall stress adaptive responses are regulated in yeast by the cell wall integrity (CWI) MAPK pathway (as reviewed in [7]), which includes the conserved MAPK module comprising the MAPKKK Bck1, the redundant MAPKKs Mkk1/Mkk2, and the MAPK Slt2. CWI signaling is induced by a variety of cell wall stressors, including elevated temperature, pheromone-induced morphogenesis, hypo-osmotic shock, and cell wall-interfering compounds [7]. The activation of the MAPK Slt2 leads to the activation of two transcription factors, namely, Swi4/Swi6 (SBF) [22] and Rlm1 [23] by different mechanisms. SBF, which is essential for G1-specific transcription [24], also drives gene expression in response to cell wall stress. While Rlm1 is activated through direct phosphorylation [25, 26], SBF is activated by the MAPK Slt2 and by the pseudokinase Mlp1/Kdx1 using a non-catalytic mechanism [10, 21]. This mechanism

requires an activated Slt2 or Mlp1 to form a complex with Swi4, which associates with the SBF-binding sites in the *FKS2* gene in response to the cell wall stress that is induced by elevated growth temperature [10, 27]. In a second step, Swi6 is recruited to the *FKS2* promoter for transcriptional activation. In addition to its role in this mechanism for the regulation of transcriptional initiation, Slt2 also mediates a function at the coding regions in transcription elongation, which is also independent of its catalytic activity [21]. This function is mediated by the interaction of Slt2 with the Paf1 elongation complex. Interestingly, Slt2 interaction with Paf1, which requires Swi4 and Swi6 but not Rlm1, allows transcription elongation of the *FKS2* gene by preventing premature transcription termination by the Sen1-Nrd1-Nab3 complex [21].

While these non-catalytic mechanisms mediated by Slt2 and SBF only regulate the transcription of a very small subset of CWI-responsive genes (*FKS2*, *CHA1*, *YLR042C*, and *YKR013W*) [10, 28], Rlm1 is responsible for the majority of the global transcriptional output that is mediated by the CWI pathway upon subjection to cell wall stress [29-31]. Rlm1 is a MADS-box transcription factor related to members of the mammalian MEF2 family of transcriptional regulators that share the same DNA-binding specificity *in vitro* (CTA[T/A]$_4$TAG; [23, 25]). In response to cell wall stress, activated Rlm1 binds *in vivo* to exposed Rlm1-binding sites at the promoters of CWI-responsive genes. The SWI/SNF ATP-dependent chromatin remodeling complex is also recruited to these promoters through direct interaction with Rlm1 [31]. The activity of SWI/SNF is necessary to alter chromatin organization at promoters and surrounding regions facilitating the entry of Rlm1 to binding sites previously occluded by positioned nucleosomes and Pol II to initiate transcription [31]. In this context, SAGA complex acetylates histones at the promoter level to facilitate this process [32]. Moreover, signaling through the CWI pathway is critical for Rlm1 recruitment following stress, as this binding is completely dependent on the presence of Slt2, and it requires both the phosphorylation of the MAPK by Mkk1/2 and the catalytic activity of the MAPK [31]. In agreement, mutations in Rlm1 at putative Slt2 phosphorylation sites, namely, Ser-427 and Thr-439, are important for stress-induced transcription of cell wall stress genes, suggesting that Slt2 regulates the transcriptional activity of Rlm1 by direct phosphorylation of these residues [26].

In this work, we analyzed the association of the MAPK Slt2 with Rlm1-dependent genes and its role in the transcriptional regulation of these genes. Slt2 binds to the chromatin at the promoter and coding regions of Rlm1-dependent genes in response to cell wall stress working as a part of the transcriptional activation machinery. This process includes the selective recruitment of the RNA Pol II by Slt2 at initiation and progress of the complex Slt2-RNA Pol II to the coding region during elongation.

Material and Methods

Yeast strains and growth conditions

Experiments were performed with the *S. cerevisiae* strain BY4741 (*MAT*a; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and mutant derivatives provided by Euroscarf (Frankfurt, Germany). The tagged strain in BY4741 background WT *SLT2-HA*::*HIS3*, and the corresponding mutants (*rlm1*Δ, *snf2*Δ, and *gcn5*Δ) were obtained by using the one-step PCR-mediated technique for gene modification [33]. The fragment, including the 3xHA-His3, was amplified by PCR using the primers that are described in the study of Longtine *et al.* [33] with the corresponding gene-specific sequences and the pFA6a-3HA-His3MX6 plasmid as template. The resulting fragment was integrated by homologous recombination into the *SLT2* locus and correct integration was confirmed with a PCR-based strategy. WT *RLM1-HA::HIS3* was previously described [31]. The double mutant *slt2*Δ *rlm1*Δ (*slt2*::Kan^R, *rlm1*::*HIS3)* was obtained, replacing the gene *RLM1* in the *slt2*Δ mutant with the *HIS3* marker using the SFH PCR-based method that was described by Wach *et al*. [34].

Routinely, cells were grown overnight in liquid media YPD (1% yeast extract, 2% peptone, and 2% glucose) or SD (0.17% yeast nitrogen base, 0.5% ammonium sulphate, 2% glucose, and supplemented with the required amino acids) in the case of cells bearing plasmids at 220 rpm and 24°C to an optical density of 0.8-1 (A_{600}). The culture was refreshed to 0.2 (A_{600}) in YPD, grown for additional 2.5 hours, and then divided into two parts. One part continued growing under the same conditions (non-treated culture), whereas the other one was supplemented with Congo red (CR) (30 µg/ml; Merck, Darmstadt, Germany). Cells were collected at the indicated times and processed depending on the experimental approach.

Plasmids

The plasmids pRS315-SLT2-HA (p2188), pRS315-*slt2*T190A/Y192F-HA (p2190), and pRS315-*slt2*K54R-HA (p2193) that were described by Kim *et al.* [10] along with pRS314-RLM1-HA (p1826), pRS314 *rlm1*-3m-HA (including the following three point mutations: S374A, S427A, and T439A), and pRS314-*rlm1* L324A/V326A-HA (p1821) that were described by Jung *et al.* [26] were kindly provided by Dr. David Levin (Department of Moleculat and Cell Biology, Boston University Goldman School of Dental Medicine, Boston, MA, USA).

The plasmids pRS315-RLM1DBD-SLT2-HA, pRS315-RLM1DBD-*slt2*T190A/Y192F-HA, and pRS315-RLM1DBD-*slt2*K54R-HA were constructed by PCR amplification of the DNA-binding domain (DBD) of *RLM1* (1–438 bp) flanked by NcoI restriction sites. This fragment was then cloned into a NcoI restriction site that was previously created in plasmids p2188, p2190, and p2193 by site-directed mutagenesis just before the ATG of *SLT2*, allowing the fusion of the Rlm1 DBD in frame with native *SLT2* or the corresponding variants.

To obtain the plasmid pRS314-*rlm1*-10m-3HA, a DNA fragment of 2101 bp, including the sequence of *RLM1* from +280 (HpaI site) to the STOP codon fused to the HA epitope sequence and mutations to substitute the ten potential MAPK phosphorylation sites of Rlm1 to Ala (S234, S261, T276, S299, S374, S427, T439, S518, T646 and T654), was chemically synthesized by GenScript (NJ, USA) and cloned (KpnI/PstI) into pUC57 vector to obtain the plasmid pUC57-RLM1-10m. An HpaI/Pst1 DNA fragment of this plasmid substituted the corresponding HpaI/Pst1 DNA fragment from the plasmid pRS314-RLM1-HA (p1826) to obtain the plasmid pRS314-*rlm1*-10m-3HA. Plasmid p704S is a derivative of plasmid pLG669-Z [35], in which a SmaI/SmaI fragment of the *CYC1* promoter was deleted and re-ligated to obtain a construction in which *lacZ* is under the control of a UAS element.

Plasmid p*MLP1*-LacZ has been described previously [36] and p*PIR3*-LacZ was obtained following a similar strategy but including the promoter region of *PIR3* (from -1193 to -6) that was obtained from genomic DNA by PCR amplification.

Quantitative RT-PCR Assays

Total RNA isolation and purification was conducted as detailed elsewhere [30]. Real-time quantitative RT-PCR (RT-qPCR) assays were performed, as previously detailed [30]. For quantification, the abundance of each mRNA was determined relative to the standard transcript of *ACT1* for input cDNA normalization, and the final data on relative gene expression between the conditions tested were calculated following the $2^{-\Delta\Delta Ct}$ method [37]. Primer sequences are available upon request.

β-galactosidase reporter assays

Cells from 5 ml of an exponential growing culture were recovered by centrifugation and the assay was performed as described [38].

Western Blotting Assays

The procedures used for immunoblot analyses, including cell collection and lysis, collection of proteins, fractionation by SDS-PAGE, and transfer to nitrocellulose membranes, have been described previously [39]. Quantification of protein bands was determined by densitometry analysis using Image Studio Lite 5.0 software from LI-COR Biosciences (Lincoln, Nebraska USA).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was performed as previously described [31]. The antibodies used in these experiments were polyclonal anti-HA (Clone ab9110, Abcam, Cambridge, UK) and monoclonal anti-Pol II (Clone 8WG16, 920101 BioLegend, CA, USA). The immunoprecipitated DNA was quantified by qPCR using primers that amplified the following regions (locations are indicated by the distance from the respective ATG initiation codon): MLP1BOX1: -453/-313; MLP1POLII: -143/+56; MLP1ORF2: +555/+687; MLP1ORF3: +1210/+1359; SRL3BOX: -265/-135; SRL3PolII: -142/+43; SRL3ORF1: +73/+223; SRL3ORF3: +631/+772; YLR194CBOX: -254/-123; YLR194CORF1: +87/+239; YLR194CORF3: +666/+805; PIR3BOX: -301/-160; PIR3ORF2: +518/+666; PIR3ORF3: +878/+1022 and VMA8: -321/-191. The primers used to amplify a region of the *lacZ* ORF have been previously described [40]. The fold enrichment (FE) at specific DNA regions was calculated using the Comparative Ct Method [41] and the promoter region of the *VMA8* gene as

sequence control. Data represent the mean and standard deviation of at least three independent experiments.

Co-immunoprecipitation assay

Cells were grown at 24ºC in YPD or in the corresponding SC medium and subjected to CR treatment (3 h, 30 μg/ml), collected by centrifugation at 4ºC, and disrupted in lysis buffer (50 mM Tris-HCl [pH 7.5], 10% glycerol, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 50 mM NaF, 1 mM sodium orthovanadate, 50 mM β-glycerol phosphate, 5 mM sodium pyrophosphate, supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitors). Then, 0.5 mg of protein extract was incubated with monoclonal anti-Pol II antibody (Clone 8WG16, 920101 BioLegend, CA, USA) overnight at 4ºC. Next, rProtein A-Sepharose Fast Flow beads (GE Healthcare Bio-Sciences AB, Sweden) were added and incubated for two hours at 4ºC. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of samples from the input and immunoprecipitated material was followed by immunoblotting analysis using anti-HA (Clone 16B12, 9015014, BioLegend, CA, USA) and anti-Pol II monoclonal antibodies.

Results

Slt2 binds in vivo to CWI-responsive genes in a Rlm1, Snf2 and Gcn5-dependent manner

To explore the putative role of Slt2 as a transcriptional co-activator regulating the transcriptional response mediated by Rlm1, the interaction of Slt2 with genes induced under cell wall stress mediated by Congo red (CR) was analyzed by chromatin immunoprecitation (ChIP) assays. This compound binds to chitin interfering with proper cell wall assembly [42]. Chromatin from a wild-type strain expressing a functional HA epitope-tagged Slt2 was immunoprecipitated with anti-HA antibody and analyzed by qPCR to check occupation at the *MLP1* (*KDX1*) gene. *MLP1* was used as a reference gene, as it shows high levels of Rlm1 recruitment [31] and is highly expressed under cell wall stress, being this induction largely dependent on Slt2 and Rlm1 [30]. As shown in Fig. 1, and in contrast to Rlm1 [31], occupation by Slt2 was not observed in the untreated cells. However, Slt2 bound to the *MLP1* promoter (specifically at the

Rlm1-binding sites) in a stress-dependent manner, reaching maximum values after 2 to 4 hours of CR treatment, coincident with the kinetics of Rlm1 and RNA Pol II binding as well as *MLP1* gene expression [31]. Interestingly, Slt2 was also found at the *MLP1* coding regions in stressed cells and this binding was associated with RNA Pol II enrichment at the same DNA regions (Fig. 1). Slt2 was also recruited to other Rlm1-dependent genes that are highly induced during cell wall stress, such as *YLR194C*, *SRL3,* and *PIR3* (Fig. 2). Slt2 association to the promoter and coding regions of these genes followed a similar pattern to that found in *MLP1*, indicating a general role for Slt2 in the regulation of Rlm1-dependent genes. In contrast with Slt2 binding, we have previously observed that the Rlm1 transcription factor was bound only at the promoter region in agreement with its established role in transcription initiation [31].

We have previously described the necessary cooperation between SAGA and SWI/SNF complexes for efficient transcriptional responses regulated by Slt2 [32]. In response to cell wall stress, the SWI/SNF complex is recruited to CWI-dependent genes, through the interaction with Rlm1, for nucleosome displacement. This displacement is facilitated by the SAGA complex, which also binds to the promoters of these genes in a Slt2-, Rlm1-, and SWI/SNF-dependent manner. As shown in Fig. 3, Slt2 recruitment mediated by CR stress was largely dependent on Rlm1, the ATPase of the SWI/SNF complex Snf2, and, to a lesser extent, on the histone acetyltransferase Gcn5 of the SAGA complex. We further analyzed the requirements for the binding of Slt2 to CWI genes in response to stress to know whether the catalytic activity of the MAPK and its phosphorylation by Mkk1/2 were required. To this end, ChIP experiments were performed using a *slt2*Δ mutant that expressed versions of Slt2 carrying mutations within the activation loop of the MAPK (*slt2*T190A/Y192F) or within the ATP-binding site (*slt2*K54R). As deduced from Fig. 4, Slt2 phosphorylation by Mkk1/2 was a precondition for the association with both regions of the *MLP1* gene, namely, promoter and ORF. Moreover, the MAPK catalytic activity was also necessary for this recruitment (Fig. 4). All these results indicate that the association of Slt2 with the promoter region relies on the interaction with the Rlm1 transcription factor, and it requires an active Slt2 MAPK and the participation of the chromatin-modifying complexes that open the chromatin promoter architecture.

Catalytic role elicited by Slt2 at initial transcriptional events

The interdependent recruitment of Slt2 and Rlm1 to CWI gene promoters makes it difficult to separate the contribution by the transcription factor from that of the kinase in the recruitment of RNA Pol II. Rlm1 mediates an essential role in eliciting gene expression under cell wall stress. However, it is unknown how Slt2 participates at this level beyond the phosphorylation of Rlm1. In previous studies, analysis of double and triple *rlm1* mutant alleles in the three potential target sites for MAPK phosphorylation (Ser/Thr-Pro motif), namely, S374, S427, and T439, concluded that Rlm1 phosphorylation by Slt2 at Ser427 and Thr439 residues was responsible for the majority of the transcriptional activation of the *YIL117C*-LacZ reporter [26]. However, we show here that although at lower levels than the wild-type Rlm1, the triple mutant Rlm1-3m still had the ability to induce the transcription of *MLP1*-LacZ significantly in response to CR (Fig. 5A). Moreover, Rlm1-3m protein levels were also increased upon stress as a consequence of the Rlm1 auto-regulatory positive transcriptional feedback mechanism [43] (Fig. 5B) and, apparently, it was hyper-phosphorylated, as deduced from the Rlm1 shift observed under stress conditions (Fig. 5B). On the basis of these results, in addition to S374, S427, and T439, we decided to mutate to Ala the seven additional potential target sites of Rlm1 for MAPK phosphorylation, excluding those located in the DNA-binding domain. Mutation of the ten putative phosphorylation sites of Rlm1 for the MAPK completely abrogated its transcriptional activation capacity, as deduced from the complete lack of *MLP1*-LacZ induction (Fig. 5A) and the loss of the increase of the Rlm1 protein levels upon stress (Fig. 5B). Therefore, in addition to S427, T439, and S374, other residues of Rlm1 that are potentially phosphorylatable by Slt2 are necessary for its transcriptional activation function.

We further analyzed the effect of Rlm1 phosphorylation by Slt2 on its DNA-binding activity by checking the ability of the non-phosphorylatable Rlm1-10m mutant to bind the *MLP1* promoter. ChIP experiments revealed that recruitment of this version, as well as a variant of Rlm1 unable to interact with the MAPK Slt2 (Rlm1-L324A/V326A) [26], were severely compromised with respect to the wild-type Rlm1 binding upon stress (Fig. 5C). The residual recruitment of inactive Rlm1 versions observed (Fig. 5C), was not sufficient to induce *MLP1* (Fig. 5A). All these results confirm that an active Slt2 phosphorylates Rlm1 in response to stress to direct both elements at the target promoters of Rlm1-dependent genes to drive gene expression.

Additional non-catalytic role for Slt2 in transcriptional initiation

We then asked whether Slt2 was recruited to CWI-dependent genes to regulate Rlm1-mediated transcription by a mechanism other than direct phosphorylation of the activator. It has been described that Slt2 possesses a transcriptional activation domain within its C-terminal extension that is atypical of MAP kinases [44, 45]. Moreover, in an artificial system, when Slt2 is fused to the Gal4DBD, it can activate *GAL1*-LacZ transcription in response to cell wall stress [46]. These observations are difficult to interpret in a more physiological situation due to the interdependency in the promoter binding of Slt2 and Rlm1, which impedes to separate the contribution by the DNA-binding factor from that of the kinase in the recruitment of the RNA Pol II and transcriptional activation. To overcome this situation, we fused the DNA binding domain of Rlm1 to the Slt2 protein and studied the activation of an *MLP1*-LacZ reporter. As shown in Fig. 6A, the expression of a native version of Slt2 was able to induce high levels of expression of the reporter system in a *slt2*Δ mutant upon cell wall stress, whereas transcriptional activation was blocked in cells expressing versions of Slt2 carrying mutations within the ATP-binding site (Slt2-K54R) or within the activation loop of the MAPK (Slt2-T190A/Y192F), supporting the catalytic role of Slt2 in the transcriptional regulation of CWI-responsive genes. Moreover, in agreement with the requirement on Rlm1 for Slt2 binding to promoters, in the absence of Rlm1 (*slt2*Δ *rlm1*Δ strain), Slt2 was not able to drive gene expression of the reporter system in response to stress (Fig. 6A). However, when the version of Slt2 fused to the DNA-binding domain of Rlm1 was expressed in the *slt2*Δ *rlm1*Δ strain, the MAPK elicited some *MLP1*-LacZ reporter induction under non-stressed conditions and the system still exhibited stress-regulated properties, as deduced from the transcriptional activation of *MLP1*-LacZ (Fig. 6A) and the phosphorylation of the MAPK under stress conditions (Fig. 6B). Moreover, under these circumstances, Rlm1DBD-Slt2 protein and RNA Pol II are recruited to the promoters and to a lesser extent to coding regions of CWI responsive genes in a stress dependent manner (Fig. S1). The moderate levels of transcriptional induction compared with those observed for the native Slt2 could be a consequence of the lower levels of expression of the Rlm1DBD-Slt2 protein (Fig. 6B), although a partial defective progression cannot be ruled out. As shown in Fig. 6A, the induction of *MLP1*-LacZ under non-stressed conditions was independent of the phosphorylation of Slt2 by Mkk1/2 and also of its kinase activity. Interestingly, the

expression of the Rlm1DBD-Slt2-T190A/Y192F version rendered the system insensitive to stress, as deduced from the fact that *MLP1* expression levels were similar under stressed and unstressed conditions, whereas the Rlm1DBD-Slt2K54R version exhibited similar behaviors to that of Rlm1DBD-Slt2. In conclusion, all these results suggest that Slt2 is able to drive the expression of Rlm1-dependent genes, such as *MLP1*, by itself and independently of Rlm1. Moreover, at this level, Slt2 mediates a non-catalytic mechanism that seems to be dependent on the phosphorylation of Slt2 by cell wall stress through the CWI pathway but which is independent of the Slt2 kinase activity.

Slt2 interacts with RNA Pol II upon cell wall stress

Our results suggested that the interaction of Slt2 with CWI genes is functionally significant not only because Slt2 activates and recruits Rlm1 to the promoters but also as it works as a co-activator itself. Trying to understand how Slt2 functions as a co-activator, we analyzed a possible interaction between Slt2 and RNA Pol II by *in vivo* co-immunoprecipitation (CoIP) assays, in yeast cells expressing a functional HA epitope-tagged Slt2 grown in the presence or absence of cell wall stress. As shown in Fig. 7A, Slt2 was able to co-precipitate with RNA Pol II (Rpb1 subunit) in response to CR stress. This interaction was dependent on Rlm1 (Fig. 7A) in agreement with the dependence on Rlm1 for Slt2 recruitment and the dependence on Slt2 and Rlm1 for binding of RNA Pol II to the promoter regions in response to cell wall stress [31]. We performed similar CoIP experiments in cells expressing a functional HA epitope-tagged Rlm1 to study the possible interaction of the RNA Pol II-Slt2 with Rlm1. The results revealed that there is no interaction between the polymerase and Rlm1 (Fig. 7B). Therefore, in response to cell wall stress, Slt2 mediates the interaction necessary for the recruitment of the RNA Pol II transcriptional machinery to the Rlm1-dependent genes.

RNA Pol II progression from the promoter to ORF in Rlm1-dependent genes does not rely on Paf1

Previous works have established a role for Slt2 in the transcriptional elongation of CWI responsive genes regulated by SBF [21]. As Slt2 interacts with the RNA Pol II and localizes to the promoter regions of Rlm1-targeted genes as well as ORFs, we wanted to analyze the relationship between these two events by analyzing whether the

promoter regions were sufficient or not to direct Slt2 to the coding regions. For this purpose, we placed the promoters of *MLP1* or *PIR3* upstream of the *lacZ* gene on a plasmid and tested them for the presence of Slt2 and RNA Pol II in the *lacZ* ORF by ChIP assays (Fig. 8). We found that the promoters of both genes were sufficient to recruit both Slt2 (Fig. 8A) and RNA Pol II (Fig. 8B) to the *lacZ* ORF in a stress-dependent manner, suggesting that Slt2 is first recruited to promoters, moving from the transcription initiation complex to the transcription elongation complex at the coding region in a second step.

Heat stress induction of *FKS2* by Swi4/Swi6 is mediated through a Slt2 non-catalytic mechanism that is necessary for the recruitment of Pol II to the *FKS2* promoter. This recruitment still occurs in a *paf1*Δ mutant strain; although, in these circumstances, the polymerase does not progress to the coding region [21]. However, we found that the RNA Pol II recruitment induced by CR-mediated stress to the promoter and ORF of *MLP1* is not affected in a *paf1*Δ mutant strain (Fig. 9A). In agreement, the lack of Paf1 almost did not affect the induction of *MLP1*, *YLR194C*, and *CRG1* genes by cell wall stress (Fig. 9B), in contrast to the blockage of heat-stress transcriptional induction of *FKS2* in a *paf1*Δ mutant [21]. All these results suggest a different mechanism for the control of transcriptional elongation of Rlm1-dependent genes. To identify other mutations in components of the Poll II elongation machinery affecting gene expression of Rlm1-dependent genes upon stress, we analyzed mRNA levels of *MLP1*, *YLR194C*, and *CRG1* genes in response to CR treatment in mutants of the THO (*rlr1*Δ), CCR4-NOT (*ccr4*Δ), and SPT4/5 (*spt4*Δ) complexes. As shown in Fig. 9B, the expression of these genes was significantly affected in these mutant strains, indicating that these elongation factors are important for the transcriptional activation of Rlm1-dependent genes in response to cell wall stress.

Discussion

Gene expression changes are crucial events of the adaptive stress responses. The regulation of gene expression is based on the control by different signal transduction pathways of several steps during mRNA biogenesis from chromatin dynamics, transcriptional initiation, and elongation to mRNA stability and transport [2, 47]. In addition to regulation of transcription factors, yeast and mammalian MAPKs have been shown to interact with chromatin at regulatory and coding regions of its target genes [6,

8-10, 48, 49], suggesting that they could function in chromatin regulation as integral components of the transcriptional regulatory machinery in response to stress.

In response to cell wall stress, the MAPK Slt2 has also been shown to interact with chromatin at the promoters and ORFs of a small subset of CWI-responsive genes that are regulated by SBF (Swi4/Swi6) through a non-catalytic mechanism that is able to regulate both transcriptional initiation [10, 27] and elongation [21]. However, the majority of the global transcriptional output mediated by the CWI pathway upon cell wall stress is regulated by Rlm1 [29-31]. Previous efforts that utilized ChIP did not find evidence for Slt2 gene occupancy under specific conditions of Slt2 activation (cell cycle and pheromone exposure) [9]. Here, we show that in addition to the recruitment of Rlm1 to CWI-promoter genes [31], the MAPK Slt2 also binds to the chromatin at the promoter and coding regions of Rlm1-dependent genes in response to cell wall stress. This recruitment is higher at the Rlm1-binding sites, and it requires the presence of the transcription factor Rlm1, suggesting that the activator labels the genes to which Slt2 is delivered. This binding also requires the co-activators SWI/SNF and SAGA complexes. Once recruited to the promoters of these genes, Slt2 might function at several steps in transcriptional activation, modulating promoter activity through the following different mechanisms: delivery and/or activation of the transcription factor Rlm1, acting as a structural adaptor, or by modulation of the general transcription machinery.

It has been suggested that the phosphorylation of Rlm1 by Slt2 at Ser427 and Thr439 is important for its transcriptional activation function [26]. Moreover, as *RLM1* is one of the transcriptional targets regulated by the CWI pathway, Rlm1 also mediates a positive transcriptional feedback mechanism that is critical for an efficient transcriptional response under cell wall stress [43]. Here, we show that a version of Rlm1 containing mutations to Ala of these two residues reduces the transcriptional activation of the Rlm1-dependent gene *MLP1,* but it is still functional in response to CR stress. In contrast, a version of Rlm1 that is mutated at the ten potential phosphorylation sites for MAPKs resulted in the lack of *MLP1* induction. Thus, as the first step in transcription, phosphorylation of Rlm1 at these additional residues is necessary for its binding at the upstream regulatory regions of the CWI-dependent genes, such as *MLP1* and, consequently, for transcriptional activation. Therefore, phosphorylation of Rlm1 by Slt2 is an essential mechanism to control gene expression through the CWI pathway.

During association to chromatin, MAPKs can also play a structural role by facilitating the formation of transcriptional complexes [47]. Here, we show that when Slt2 is artificially targeted to the promoter of *MLP1* by the fusion of the MAPK to the Rlm1 DNA binding domain, it is able to interact with promotor and coding regions and induce certain levels of *MLP1* gene expression in the absence of Rlm1. Thus, Slt2, by itself, is able to activate the transcription of CWI responsive genes that are regulated by Rlm1. In these circumstances, under non-stress conditions, Slt2 could interact with RNA Pol II and constitutively activates transcription, independently of its phosphorylation and kinase activity. Remarkably, the transcriptional induction of *MLP1* upon stress depends on the phosphorylation of the MAPK but not on its kinase activity. These data support the possibility that the kinase contributes to the transcriptional activation as a structural component, as previously suggested for Hog1 [8].

The interdependent recruitment of Slt2 and Rlm1 to CWI gene promoters makes it difficult to separate the contribution by the transcription factor from that of the kinase in the recruitment of RNA Pol II. However, the fact that Slt2 interacts with the RNA Pol II in response to cell wall stress explains the ability of Slt2 to drive gene expression by itself and suggests a model in which, once targeted to the promoter regions of target genes by Rlm1, Slt2 recruits Pol II in the initial steps of transcription. Supporting this mechanism, Slt2 interacts with RNA Pol II in a Rlm1-dependent manner, but Rlm1 does not interact with the polymerase in response to stress. Similarly, activated Hog1 tethered to the promoters of Hog1-responsive genes by the Hot1 transcription factor also mediates the recruitment and activation of RNA Pol II and other components of the general transcriptional machinery [8, 13]. The interaction between Slt2 and RNA Pol II is not necessarily direct and we cannot exclude the possibility that it is mediated by additional factors, such as the elongation factors described in this work affecting CWI-gene activation.

In addition to their role in the initiation of transcription, MAPKs are also critical for transcriptional elongation of stress-responsive genes [18]. The MAPK Slt2 modulates transcriptional elongation in the context of CWI-dependent genes that are regulated by SBF (Swi4/Swi6), such as *FKS2*, through a non-catalytic mechanism. The results shown here suggest that Slt2 regulates both transcriptional initiation and elongation of Rlm1-dependent genes through mechanisms that differ from those

developed by the MAPK to regulate the expression of genes controlled by SBF. In response to cell wall stress, Rlm1 and Slt2 bind to the promoter of CWI-responsive genes at regions containing Rlm1-binding sites, and Rlm1 recruitment following stress requires it be activated by the MAPK [31]. The interdependence of Slt2 and Rlm1 binding at the CWI promoters would support a model in which an activated Slt2-Rlm1 complex is preferentially recruited to the promoter of target genes. Once recruited to the chromatin of Rlm1-dependent genes, the MAPK Slt2 would recruit RNA Pol II in the initial steps of transcription. In this context, nucleosome displacement, elicited by SWI/SNF and SAGA complexes, facilitates this process. Slt2 is also recruited, in response to stress, to the coding regions of genes regulated by Rlm1, such as *MLP1*, *SRL3*, *PIR3* and *YLR194C*. This recruitment is not associated with Rlm1, as Rlm1 binds to the promoters of CWI-regulated genes but not to the ORFs [31]. Moreover, the promoter regions of Rlm1-dependent genes contain enough information to direct Slt2 and Pol II to coding regions. An opposite situation has been described for regulation of *STL1* upon osmotic stress in which the recruitment of Hog1 to the coding region does not require the native *STL1* promoter, but it needs the 3' non-coding region [17]. However, a large-scale localization of RNA Pol II and Hog1 upon osmotic stress also indicated that most ORFs occupied by Hog1 are marked by the presence of Hog1, Hot1 and Sko1 transcription factors in their regulatory regions [40]. Our data points to the selective recruitment of the RNA Pol II by Slt2 and the progress of the complex Slt2-RNA Pol II to the coding region during elongation. Slt2 should move from the transcription initiation complex to the transcription elongation complex, leaving Rlm1 behind. In agreement with this mechanism, in the absence of Rlm1, Slt2 tethered to CWI gene promoters through the Rlm1 DBD and the RNA Pol II are recruited to coding regions. For regulation of *FKS2*, Slt2 also moves from the initiation to the elongation complex but in a different scenario, as, in that case, the activity of the MAPK is not necessary for its recruitment. Moreover, Paf1 does not regulate RNA Pol II progression from the promoter of *MLP1* to the ORF upon stress and deletion of *PAF1* does not affect the transcriptional activation of *MLP1*, *YLR194C* and *CRG1*. Therefore, our results point to a different mechanism for regulation, by the MAPK Slt2, of transcriptional elongation of Rlm1-dependent genes. Interestingly, transcriptional induction of these genes is affected in mutants of other elongation factors, such as Rlr1, Ccr4, or Spt4, suggesting that they could be involved in this mechanism. However,

further studies are needed to elucidate the mechanisms by which these factors and the MAPK regulate transcriptional elongation.

Conclusions

Transcriptional reprogramming of yeast cells in response to cell wall stress is regulated mostly by the transcription factor Rlm1. We describe here a role for the yeast MAPK Slt2 as a part of the transcriptional activation machinery necessary for the regulation of Rlm1-dependent genes. Slt2 associates to the chromatin at the promoter and coding regions of these genes in response to stress and this association relies on the interaction with Rlm1 and it requires an active MAPK and the participation of chromatin-remodeling complexes. Slt2 is essential to phosphorylate and activate Rlm1 triggering its binding to DNA. However, we also demonstrated that Slt2 is able to drive gene expression by itself and independently of Rlm1 when tethered to Rlm1-dependent promoters. Our data point to a mechanism for regulation of Rlm1-dependent genes by Slt2, which differs from the one developed by Slt2 to regulate the expression of genes controlled by SBF. It includes the selective recruitment of the RNA Pol II by Slt2 to the promoters and the progress of the complex Slt2-RNA Pol II to the coding regions during elongation in a Paf1-independent manner.

Highlights

-Slt2 is part of the transcriptional activation machinery.
-Slt2 binds to CWI-responsive genes in a Rlm1, Snf2 and Gcn5-dependent manner.
-Slt2 mediates catalytic and non-catalytic mechanisms to activate transcription.
-Slt2 mediates the recruitment of the RNA Pol II to Rlm1-dependent genes.
-Slt2-RNA Pol II progression along Rlm1-dependent genes does not rely on Paf1.

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Figure Legends

Figure 1. Slt2 associates with the *MLP1* promoter and coding region. Schematic representation of the *MLP1* gene is shown. White boxes at the promoter mark the Rlm1-binding sites. Regions amplified for ChIP analysis along the gene are shown as horizontal black lines. Recruitment of the MAPK Slt2 (top panel) and RNA Pol II (bottom panel) at the promoter (BOX1) and coding (ORF2 and ORF3) regions of *MLP1* gene was determined by ChIP in a WT strain expressing Slt2-3xHA subjected or not to CR treatment (30 µg/ml) at the indicated times. Data represent the mean and standard deviation of at least three independent experiments. Statistical analysis was carried out with a two-tailed, unpaired, Student's t-test calculated between treated (CR+) and non-treated (CR-) strains. Only significant differences are indicated; 0.0001<***P<0.001; 0.001<**P<0.005; 0.005<*P<0.05.

Figure 2. Slt2 associates with the chromatin of other Rlm1-dependent genes. Binding of the MAPK Slt2 to promoter (BOX) and coding regions of *SRL3* (ORF1 and ORF3), *YLR194C* (ORF1 and ORF3) and *PIR3* (ORF2 and ORF3) genes was determined by ChIP analysis in a WT strain expressing Slt2-3xHA after CR treatment (30 µg/ml) at the indicated times. Schematic representations of the corresponding genes include the Rlm1-binding sites (labeled with boxes) and the regions amplified for ChIP analysis that are shown as horizontal black lines. Data represent the mean and standard deviation of at least three independent experiments. Student's t-test was calculated between treated (CR+) and non-treated (CR-) strains. Only significant differences are indicated; 0.001<**P<0.005; 0.005<*P<0.05.

Figure 3. Slt2 association with chromatin at *MLP1* and *SRL3* genes depends on Rlm1, SWI/SNF, and SAGA complexes. Recruitment of Slt2 to the promoter and coding regions of *MLP1* and *SRL3* was analyzed by ChIP in a WT strain and the corresponding mutants (*rlm1*Δ, *snf2*Δ and *gcn5*Δ) expressing Slt2-3xHA, subjected or not to CR treatment (30 µg/ml) for 3 hours. The regions amplified for ChIP analysis were the same as the ones detailed in Figures 1 and 2. Data correspond to the mean and standard deviations of at least three independent biological replicates. Only significant differences calculated using Student's t-test are indicated; 0.001<**P<0.005; 0.005<*P<0.05.

Figure 4. Binding of Slt2 to the promoter and coding regions of *MLP1* gene requires both phosphorylation of the MAPK and its catalytic activity. Slt2 association with the promoter (BOX1) and coding region (ORF2) of *MLP1* was analyzed by ChIP in *slt2*Δ cells transformed with the empty vector (pRS315) or plasmids pRS315-SLT2-HA, pRS315-*slt2*T190A/Y192F-HA or pRS315-*slt2*K54R-HA after 3 hours of CR treatment (30 µg/ml). Data correspond to the mean and standard deviations of at least three independent biological replicates. Student's t-test was calculated between treated (CR+) and non-treated (CR-) strains. Only significant differences are indicated; 0.001<**P<0.005; 0.005<*P<0.05.

Figure 5. Phosphorylation of Rlm1 by Slt2 is required for the recruitment of the transcription factor and expression of *MLP1* upon cell wall stress. (A) β-galactosidase activity was measured in a *rlm1*Δ strain that were co-transformed with the p*MLP1*-LacZ reporter plasmid and each of the following alleles of *RLM1* in the centromeric vector pRS314: RLM1-HA, *rlm1*-3m-HA, *rlm1*-10m-HA and *rlm1*-L324A/V326A-HA after 3 hours of CR treatment (30 µg/ml). (B) Levels of Rlm1 were measured in the strains expressing Rlm1 variants analyzed in A treated or not with CR (30 µg/ml for 3hours) by immunoblotting using HA polyclonal antibody. G6PDH was used as loading control. Quantification of the Rlm1 protein levels is shown below. Relative amounts of Rlm1 were normalized with respect to the G6PDH and relativized with respect to the levels of the wild-type Rlm1 version in the absence of stress (first lane: value 1). Data represent the media and standard deviation of three independent experiments. (C) Recruitment of Rlm1 to BOX1 region at the *MLP1* promoter was determined by ChIP in the indicated strains. Data correspond to the mean and standard deviations of three independent biological replicates. Statistical analysis was carried out with a two-tailed, unpaired, Student's t-test calculated between treated and non-treated strains. Only significant differences are indicated; 0.0001<***P<0.001; 0.001<**P<0.005; 0.005<*P<0.05.

Figure 6. Recruitment of Slt2 to the *MLP1* promoter by fusion of the Rlm1 DNA binding domain to the MAPK promotes transcriptional activation of *MLP1* under stress conditions. (A) β-galactosidase activity was measured in the indicated strains co-transformed with the p*MLP1*-LacZ reporter plasmid and different versions of Slt2 in the centromeric vector pRS315: SLT2-HA, *slt2*T190A/Y192F-HA, *slt2*K54R-HA,

Rlm1DBD-SLT2-HA, Rlm1DBD-*slt2*T190A/Y192F-HA and Rlm1DBD-*slt2*K54R-HA that were grown in the absence or presence of CR (30 µg/ml for 3 hours). Data correspond to the mean and standard deviations of at least three independent biological replicates. (B) Activation of Slt2 in indicated strains grown to midlog phase and exposed to CR (30 µg/ml for 3 hours) was analyzed by immunoblotting using antibodies to detect Slt2 and the phosphorylated form of Slt2 (P-Slt2). G6PDH was used to monitor protein load. Graphics indicate the relative amounts (quantified by densitometry analysis) of phosphorylated Rlm1DBD-Slt2 (up) and Rlm1DBD-Slt2 (down) with respect to the first lane (value 1) and normalized with respect to G6PDH levels. Data represent the media and standard deviation of three independent experiments. Statistical analysis was carried out with a two-tailed, unpaired, Student's t-test calculated between treated (CR+) and non-treated (CR-) strains. Only significant differences are indicated; 0.0001<***P<0.001; 0.001<**P<0.005; 0.005<*P<0.05.

Figure 7. Slt2 but not Rlm1 physically interacts with the RNA Pol II *in vivo*. (A) Co-immunoprecipitation of Slt2-HA and RNA Pol II. *slt2*Δ mutant and WT and *rlm1*Δ strains expressing Slt2-3xHA were grown to midlog phase and treated or not with CR (30 µg/ml for 3 hours). Cell extracts (input) and immunoprecipitates (IP) by anti-Pol II antibody were analyzed by immunoblotting using anti-HA or anti-Pol II antibodies. (B) Co-immunoprecipitation of Rlm1-HA and RNA Pol II. WT strain expressing Rlm1-3xHA was grown and treated as in (A). Cell extracts (Input) and immunoprecipitates (IP) with anti-Pol II antibody were analyzed by immunoblotting using anti-HA or anti-Pol II antibodies. The input protein represents 10% of the total extract.

Figure 8. *MLP1* and *PIR3* promoters are sufficient to recruit Slt2 and RNA Pol II to the *lacZ* ORF in a stress-specific manner. Binding of the MAPK Slt2 (A) and RNA Pol II (B) to the promoters (BOX1 and BOX regions) and coding regions (ORF-*lacZ*) of p*MLP1*-LacZ and p*PIR3*-LacZ constructions, respectively, were analyzed by ChIP in WT *SLT2-HA* cells transformed with plasmids p704S, p*MLP1*-LacZ, or p*PIR3*-LacZ after CR treatment (30 µg/ml for 3 hours). Statistical analysis was carried using Student's t-test calculated between treated and non-treated strains. Only significant differences are indicated; 0.0001<***P<0.001; 0.001<**P<0.005; 0.005<*P<0.05.

Figure 9. Slt2 regulates transcriptional elongation of Rlm1-dependent genes. (A) Pol II progression from the promoter of *MLP1* to the coding region does not require

Paf1 complex. Recruitment of RNA Pol II at the promoter (POL II) and ORF (ORF2) regions of *MLP1*, which has been labelled as horizontal lines in the schematic representation of the *MLP1* gene, was analyzed by ChIP in WT and *paf1*Δ strains expressing Slt2-3xHA after three hours of CR treatment (30 µg/ml). (B) Gene expression of CWI responsive genes *MLP1*, *YLR194C* and *CRG1* was analyzed by RT-qPCR in WT and mutant strains deleted in several components of the transcriptional elongation machinery. Values represent the expression ratio between CR-treated and non-treated cells. Data correspond to the mean and standard deviation of three independent experiments. Statistical analysis was carried out using Student's t-test calculated between treated (CR+) and non-treated (CR-) strains. Only significant differences are indicated; 0.0001<***P<0.001; 0.001<**P<0.005; 0.005<*P<0.05.

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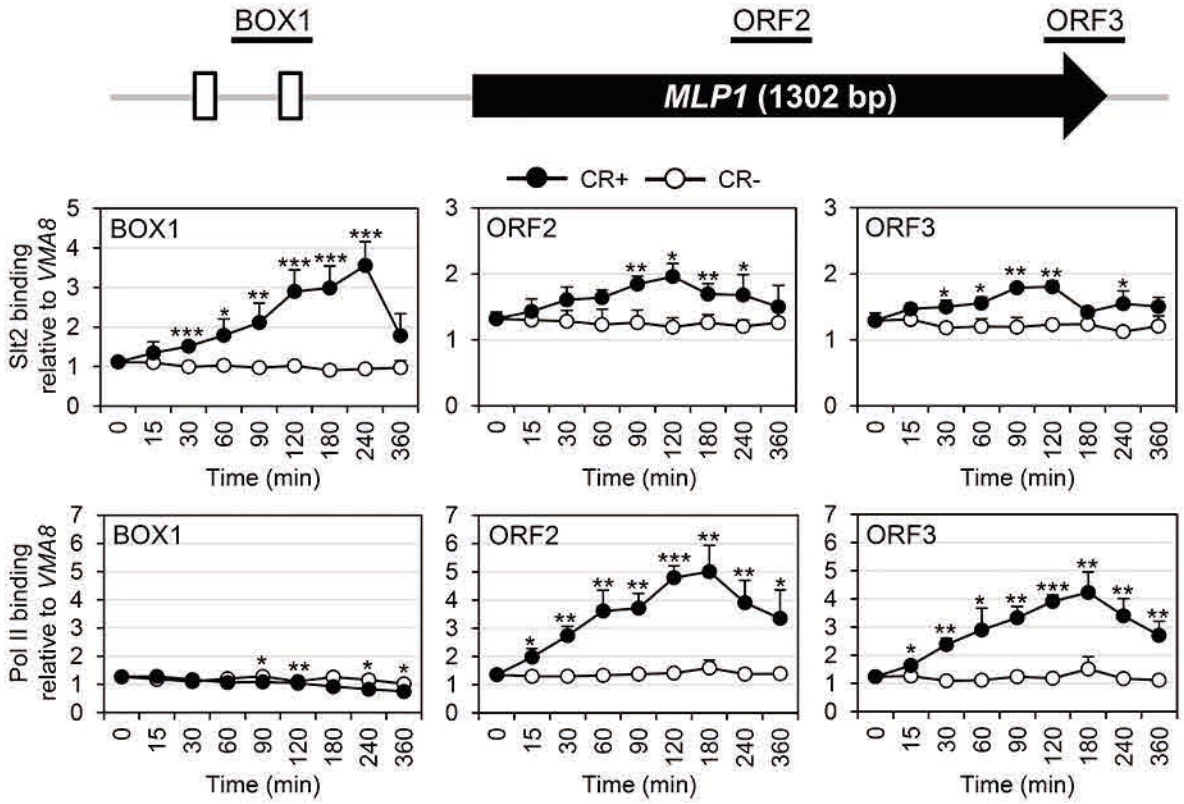
Figura 1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CR-
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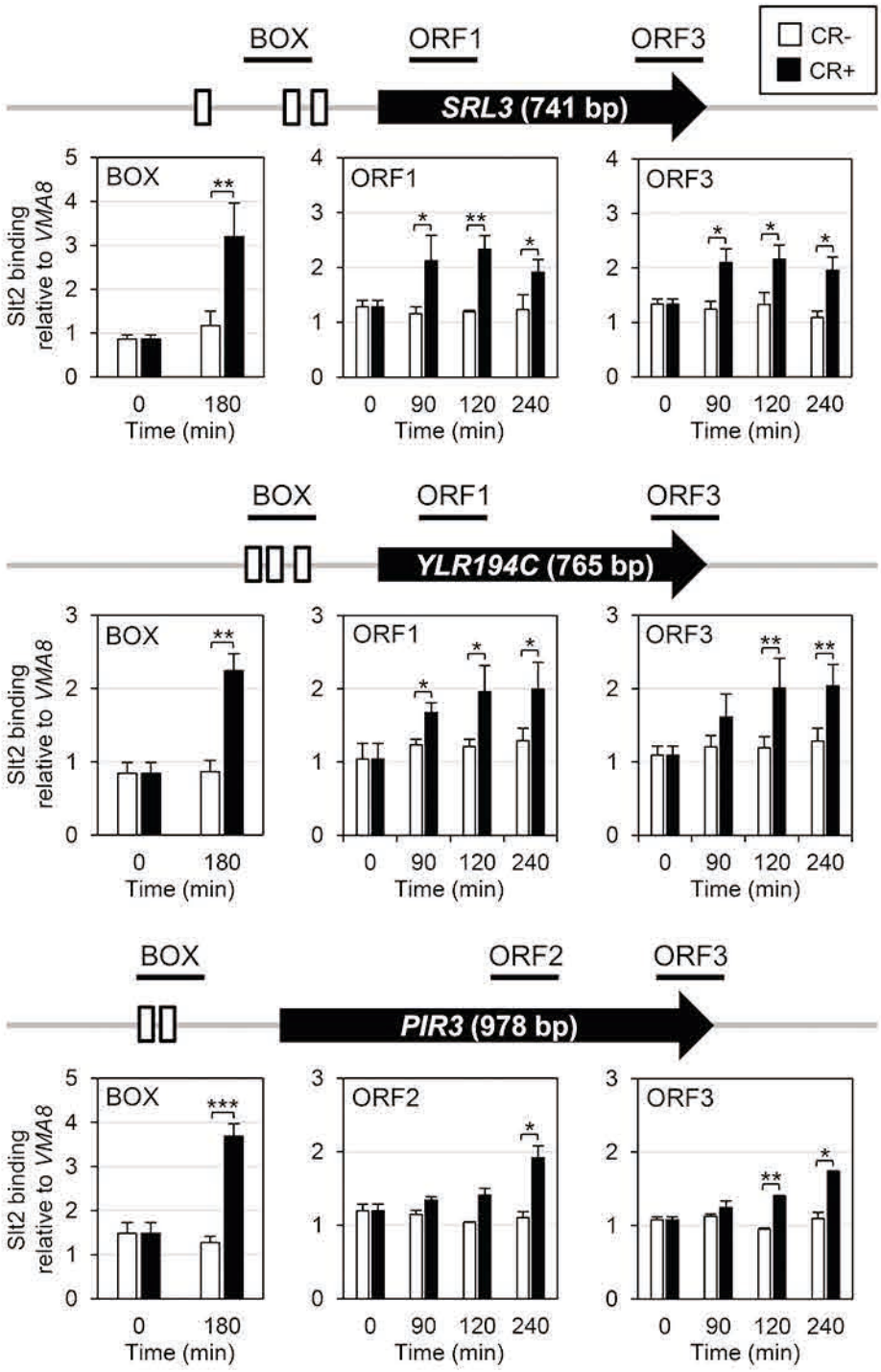
Pol II binding relative to VMA8
Time (min)

Figura 2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CR-
CR+
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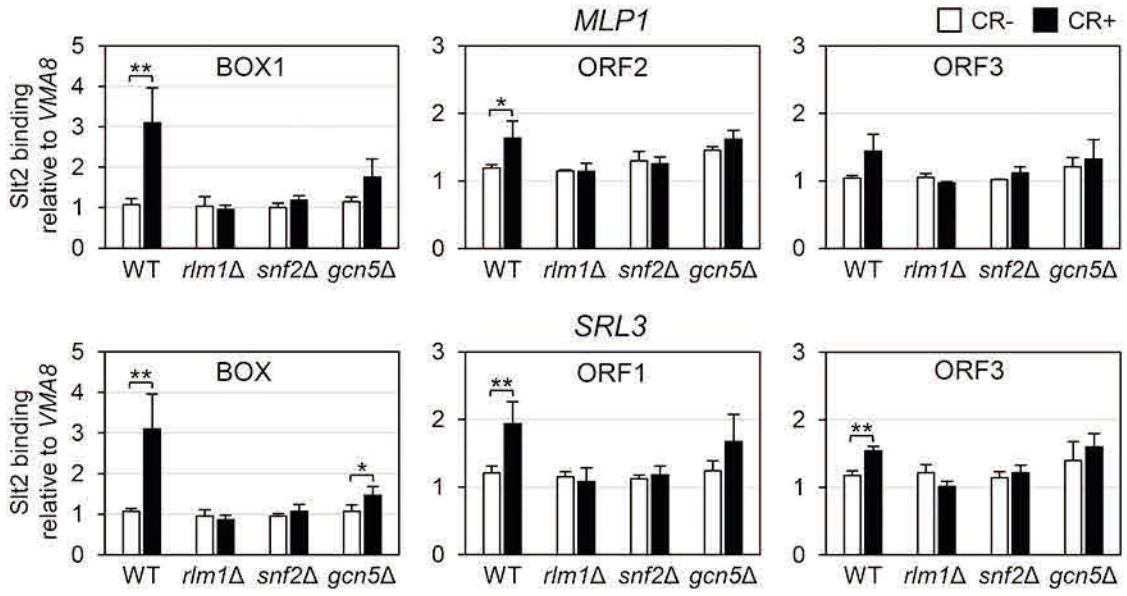
Figura 3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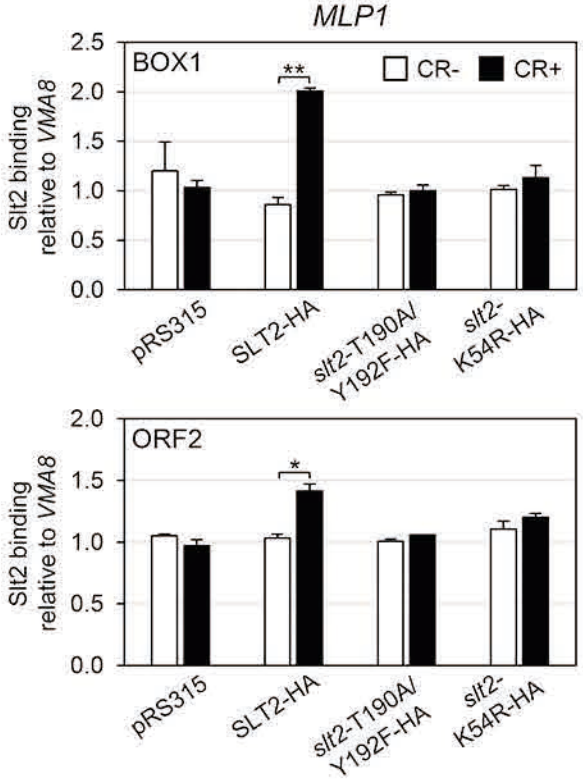
SRL3
BOX
ORF1
ORF3

Figura 4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BOX1
CR-
CR+
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Slt2 binding relative to VMA8
2.5
2.0
1.5
1.0
0.5
0.0
pRS315
SLT2-HA
slt2-T190A/ Y192F-HA
slt2-K54R-HA
ORF2
*
Slt2 binding relative to VMA8
2.0
1.5
1.0
0.5
0.0
pRS315
SLT2-HA
slt2-T190A/ Y192F-HA
slt2-K54R-HA

Figura 5

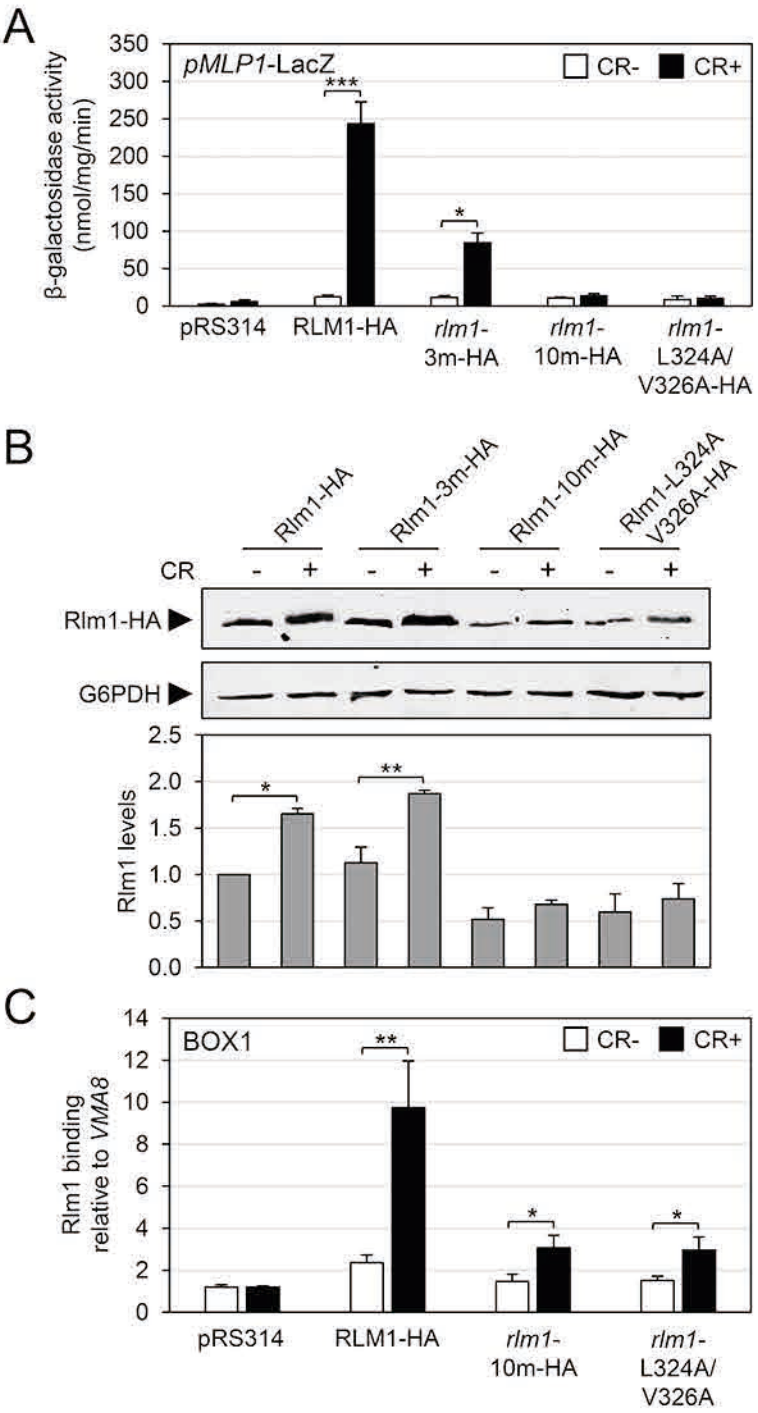
A
pMLP1-LacZ
CR-
CR+
β-galactosidase activity (nmol/mg/min)
350
300
250
200
150
100
50
0
***
*
pRS314
RLM1-HA
rlm1-3m-HA
rlm1-10m-HA
rlm1-L324A/V326A-HA
B
Rlm1-HA
Rlm1-3m-HA
Rlm1-10m-HA
Rlm1-L324A V326A-HA
CR
- + - + - + - +
Rlm1-HA
G6PDH
Rlm1 levels
2.5
2.0
1.5
1.0
0.5
0.0
*
**
C
BOX1
CR-
CR+
Rlm1 binding relative to VMA8
14
12
10
8
6
4
2
0
**
*
*
pRS314
RLM1-HA
rlm1-10m-HA
rlm1-L324A/V326A

Figura 6

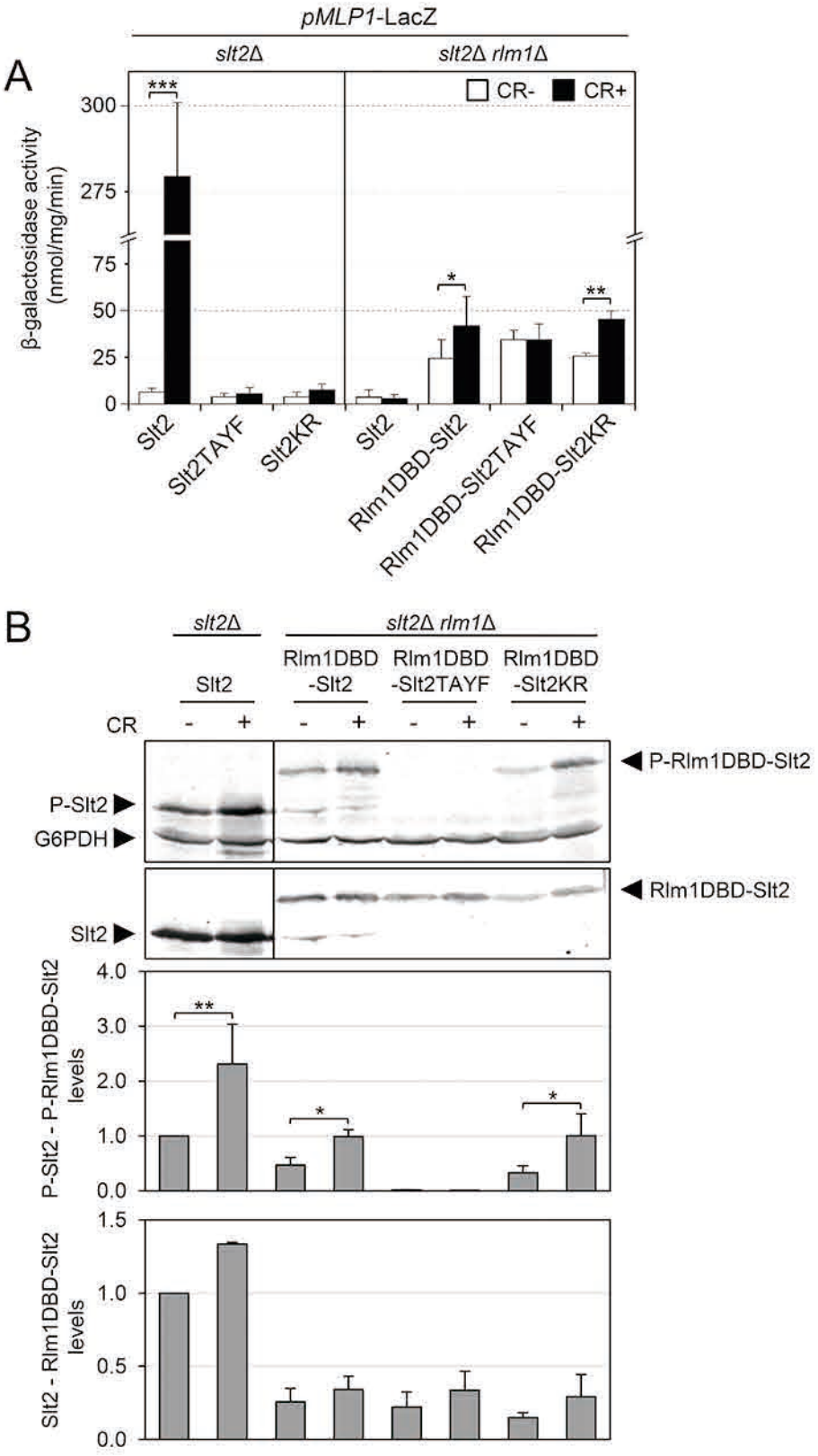
A
pMLP1-LacZ
slt2Δ
slt2Δ rlm1Δ
CR-
CR+
β-galactosidase activity (nmol/mg/min)
300
275
75
50
25
0
***
*
**
Slt2
Slt2TAYF
Slt2KR
Slt2
Rlm1DBD-Slt2
Rlm1DBD-Slt2TAYF
Rlm1DBD-Slt2KR
B
slt2Δ
slt2Δ rlm1Δ
Slt2
Rlm1DBD -Slt2
Rlm1DBD -Slt2TAYF
Rlm1DBD -Slt2KR
CR
- + - + - + - +
P-Slt2
G6PDH
P-Rlm1DBD-Slt2
Slt2
Rlm1DBD-Slt2
P-Slt2 - P-Rlm1DBD-Slt2 levels
4.0
3.0
2.0
1.0
0.0
**
*
*
Slt2 - Rlm1DBD-Slt2 levels
1.5
1.0
0.5
0.0

Figura 7

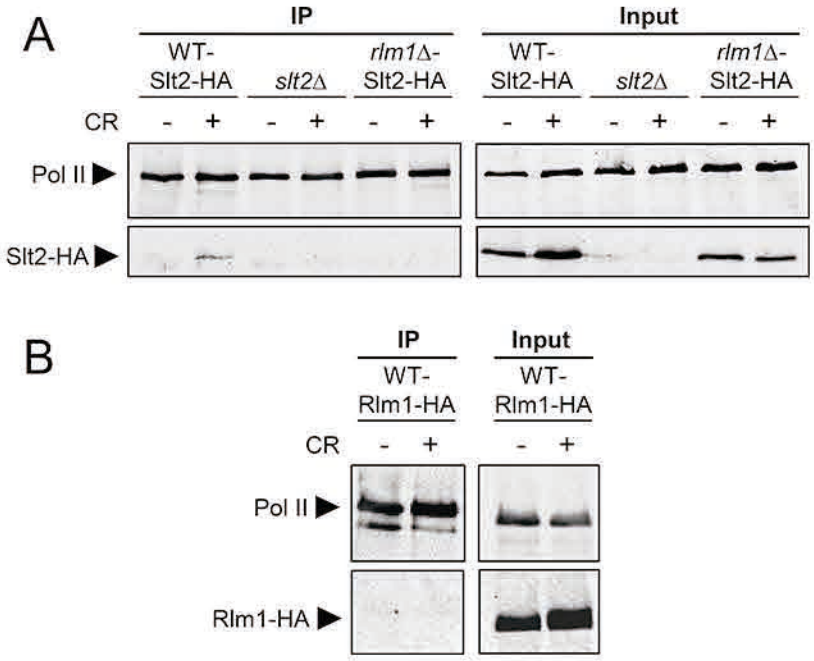
A
IP
Input
WT-Slt2-HA
slt2Δ
rlm1Δ-Slt2-HA
WT-Slt2-HA
slt2Δ
rlm1Δ-Slt2-HA
CR
- + - + - + - + - + - +
Pol II
Slt2-HA
B
IP
Input
WT-Rlm1-HA
WT-Rlm1-HA
CR
- + - +
Pol II
Rlm1-HA

Figura 8

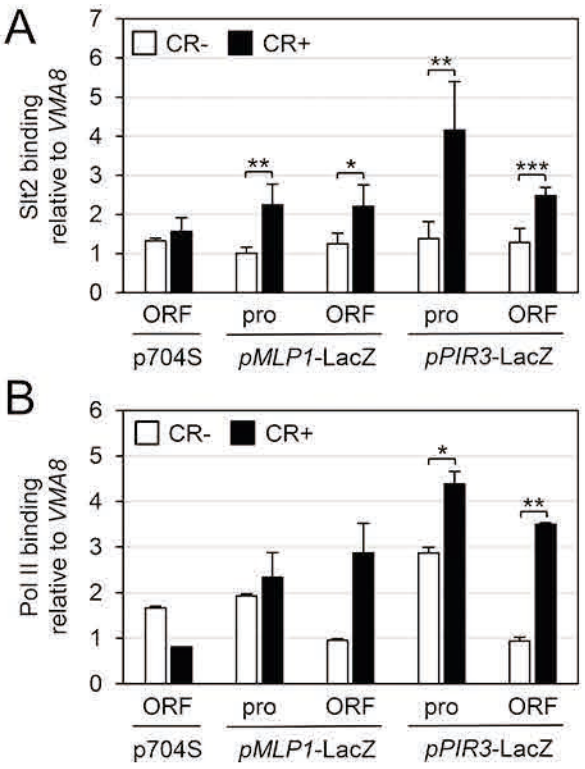
A
CR-
CR+
Slt2 binding relative to VMA8
0
1
2
3
4
5
6
7
**
*
**
***
ORF
pro
ORF
pro
ORF
p704S
pMLP1-LacZ
pPIR3-LacZ
B
CR-
CR+
Pol II binding relative to VMA8
0
1
2
3
4
5
6
*
**
ORF
pro
ORF
pro
ORF
p704S
pMLP1-LacZ
pPIR3-LacZ

Figura 9

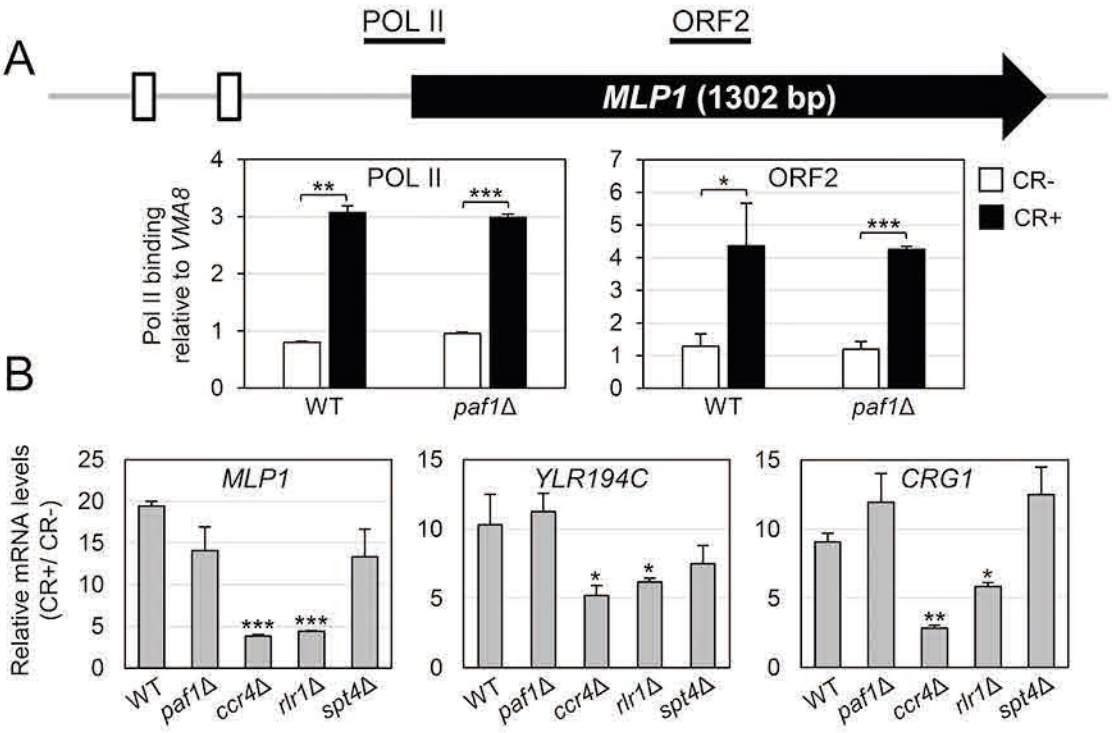
A
POL II
ORF2
MLP1 (1302 bp)
B
Pol II binding relative to VMA8
POL II
ORF2
CR-
CR+
WT
paf1Δ
Relative mRNA levels (CR+/ CR-)
MLP1
YLR194C
CRG1
WT
paf1Δ
ccr4Δ
rlr1Δ
spt4Δ

Fig. S1

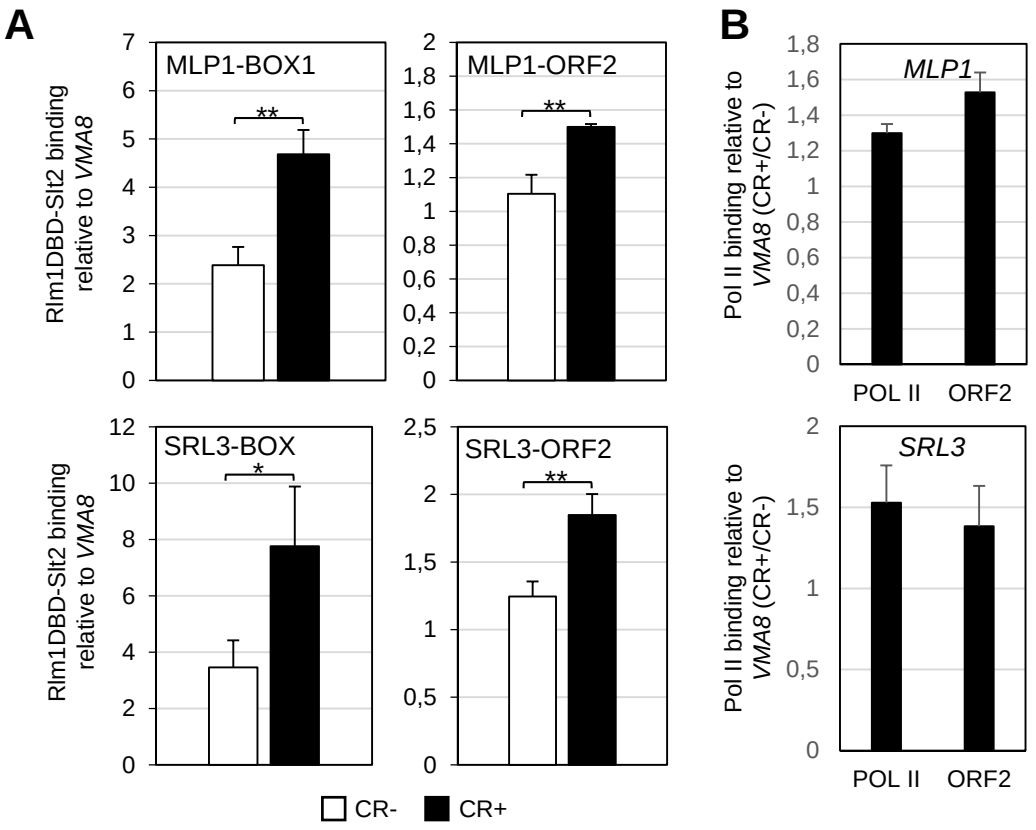

Fig. S1. Rlm1DBD-Slt2 and RNA Pol II bind to CWI-regulated genes in the absence of Rlm1 in a stress dependent-manner. Recruitment of Rlm1DBD-Slt2 (A) and RNA Pol II (B) to the indicated regions of *MLP1* and *SRL3* genes was analyzed by ChIP in a double mutant *rlm1*Δ *slt2*Δ transformed with the plasmid pRS315-RLM1DBD-SLT2-HA that were grown in the absence or presence of CR (30 µg/ml for 2 hours). Pol II binding is represented as the ratio between CR+ and CR at the indicated regions. Data correspond to the mean and standard deviations of at least three independent biological replicates. Only significant differences calculated using Student's t-test are indicated; 0.001<**P<0.005; 0.005<*P<0.05.