

Weak Organic Acids Trigger Conformational Changes of the Yeast Transcription Factor War1 *in Vivo* to Elicit Stress Adaptation^{*[S]}

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The *Saccharomyces cerevisiae* zinc cluster regulator War1 mediates an essential transcriptional and adaptive response to weak organic acid stress. Here we investigate the mechanism of War1 activation upon weak acid stress. We identified several gain-of-function *WAR1* alleles mapping to the central War1 region. These mutations constitutively increase levels of the plasma membrane ABC transporter Pdr12, the main War1 target mediating stress adaptation. Functional analysis of War1 reveals that the central region and its C-terminal activation domain are required for function. Notably, the native DNA-binding and dimerization domains appear dispensable for War1 activity, because they can be replaced by a LexA DNA-binding domain. Chromatin immunoprecipitation demonstrates elevated promoter affinity of activated War1, because its *PDR12* promoter association increases upon stress. Hyperactive *WAR1* alleles have constitutively high *PDR12* promoter association. Furthermore, fluorescence resonance energy transfer of functional CFP-War1-YFP proteins also demonstrates conformational changes of stress-activated War1 *in vivo*. Our results suggest a mechanism whereby War1 activation is accompanied by conformational changes enhancing promoter association, thus initiating the adaptation process.

Weak organic acids, such as potassium sorbate, calcium propionate, and sodium benzoate, are commonly used to preserve foods and beverages (1). In *Saccharomyces cerevisiae*, uncharged weak acids diffuse across the plasma membrane and dissociate inside the cell, causing intracellular acidification, free radical production, and oxidative stress (1), as well as inhibition of several metabolic processes (2). The yeast ABC transporter Pdr12 confers resistance to monocarboxylic acids with chain lengths up to C₇ (3, 4). Only organic acids but not other stresses

cause a dramatic induction of *PDR12* transcription and a concomitant increase of Pdr12 (4, 5). A physiological role of Pdr12 is suggested from its proposed ability to extrude amino acid catabolism by-products (6). Weak acid regulation of *PDR12* occurs at the level of transcription and requires the Zn(II)₂Cys₆ zinc cluster transcription factor War1, which recognizes and binds response elements within the *PDR12* promoter (7). War1 is functionally conserved among several yeast species, including fungal pathogens. An orthologue mediating sorbate tolerance was identified in the distantly related human fungal pathogen *Candida albicans* (8), but the mechanism by which weak organic acids cause War1 activation is currently an open question.

In *S. cerevisiae*, some 55 members of the binuclear zinc cluster regulator family exist (for a review see Ref. 9). Most exhibit a similar molecular architecture and domain organization. The N-terminal DNA-binding domain is followed by a coiled-coil dimerization domain, by an extended region of limited homology referred to as the “middle homology region” (MHR)³ and finally by a short acidic stretch encompassing the C-terminal activation domain (10). Fungal zinc cluster proteins are involved in diverse cellular processes, including carbon and amino acid metabolism, stress response, mitochondrial signaling, peroxisome biogenesis, and ergosterol biosynthesis (9).

A common principle as to the function of these regulators is only beginning to emerge. Like War1 (7), many other zinc cluster proteins permanently localize to the nucleus and constitutively bind their target promoters. Some of them are activated by small molecule stimuli such as drugs, fatty acids, and metabolic intermediates. For example, Pdr1 and Pdr3 directly bind structurally diverse xenobiotics to induce transcription of drug transporter genes (11). Put3 regulates proline utilization genes and is controlled by direct interaction with proline (12). Similarly, Leu3, a regulator of leucine biosynthesis, binds its target promoters and is only activated in the presence of the leucine precursor α -isopropyl malate (13–15).

Mutations within the MHR can lead to hyperactive alleles, as shown for Leu3, Pdr1/Pdr3, as well as Hap1, indicating a crucial regulatory function of this domain (16–18). Several zinc cluster proteins are activating their target genes in response to a signal generated through the interaction with small ligand molecules

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^[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table 5.

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³ The abbreviations used are: MHR, middle homology region; ChIP, chromatin immunoprecipitation; FRET, fluorescence resonance energy transfer; ORF, open reading frame; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; WARE, weak acid response element; 3-AT, 3-amino-1,2,4-triazole; ABC, ATP-binding cassette.

TABLE 1

Yeast strains used in this study

Strains	Genotype	Ref.
W303-1A	<i>MATa ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100</i>	49
FY1679-28c	<i>MATa ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63</i>	42
YAK100	<i>MATa war1Δ::His3-Mx6</i> (isogenic to FY1679-28c)	7
YAK120	<i>MATa war1Δ::His3-Mx6</i> (isogenic to W303-1A)	7
YCS42-D4	<i>MATa leu2-Δ1 his3-Δ200 trp1-Δ1 ade2-10oc lys2-801a ura3-52::pCS12ZI URA3 war1-42</i>	21
YAK2	<i>MATa ura3::PDR12-lacZ</i> (isogenic to W303-1A)	7
YBB27	<i>MATa ura3::PDR12-lacZ war1Δ::HIS3MX6</i> (isogenic to W303-1A)	22
YCG7	<i>MATa URA3-P_{PDR12}HIS3</i> (isogenic to W303-1A)	This study
YCG8	<i>MATa war1Δ::Kan-Mx4</i> (isogenic to YCG7)	This study
L40	<i>MATa his3-200 trp1-901 leu2-3, 112 ade2 LYS2:::(lexAop)4-HIS3 URA:::(lexAop)s-lacZ GAL4 gal80</i>	Stan Hollenberg

(19, 20). This may also be true for War1, which is tightly regulated by weak acids, but high level War1 overexpression is not sufficient for increased *PDR12* transcription. Nevertheless, regulation of War1-like zinc cluster factors is a rapid and highly dynamic process, despite constitutive War1 localization in the nucleus and the apparent absence of stress-transducing mechanisms such as mitogen-activated protein kinase pathways, for example. Notably, as for War1, activation of several zinc cluster factors triggers appearance of phosphorylated forms, and it is not entirely clear whether phosphorylation is the cause or consequence of activation. The rapid weak acid-triggered activation of War1 is accompanied by the concomitant appearance of several phosphorylated War1 isoforms (7, 21, 22). As reported recently, exposure to the drug fluphenazine causes transient activation of the zinc cluster proteins Pdr1/Pdr3 on a similar time scale (23).

Here we investigated how weak acids change the activity of War1 *in vivo*. To identify regulatory domains and residues of War1, we devised a random mutagenesis screen to isolate hyperactive alleles and performed deletion analysis. Two lines of evidence suggest dynamic changes of War1 structure in response to weak acid stress. First, chromatin immunoprecipitation assays (ChIP) show increased and strong recruitment of War1 to its target promoter, suggesting a higher promoter affinity of activated War1. Second, FRET analysis suggests that conformational changes of War1 occur in stressed cells *in vivo*. Furthermore, inactive and hyperactive War1 mutant alleles appear locked in the inactive or the activated conformation, respectively. The latter War1 variants also show constitutive behavior regarding promoter binding. Taken together, these data suggest a mechanism by which War1 activation is triggered by stress, leading to the adaptive transcriptional response triggering overexpression of the Pdr12 pump.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Growth Conditions—Strains used in this study are listed in Table 1 and Table 2. Rich medium (YPD) and synthetic medium (SC) were prepared as described elsewhere (24). All yeast strains were grown at 30 °C. For weak acid susceptibility, exponentially growing cultures were adjusted to A_{600} of 0.2 and diluted 1:10, 1:100, and 1:1,000, and equal volumes were spotted onto YPD, pH 4.5, plates containing different sorbate concentrations (7). The strain YCG8 used for the mutant screen was constructed as follows. A 1000-bp PCR fragment of the *PDR12* promoter was cloned into the KpnI and EcoRI sites of pRS306 (25) giving pRS306-*PDR12*. Then a PCR fragment, including the *HIS3* ORF plus 160 bp of its 3'

TABLE 2

Plasmids used in this study

Name	Relevant DNA insert	Ref.
pGalWAR1	<i>GAL1-10Pro-WAR1</i>	This study
pGalWAR1-K762R	<i>GAL1-10Pro-WAR1-K762R</i>	This study
pBTM116	<i>lexA</i>	Stan Hollenberg
pBWAR	<i>lexA-WAR1</i>	This study
pBWARΔ300	<i>lexA-WAR1Δ1-300</i>	This study
pBWARΔ762	<i>lexA-WAR1Δ1-762</i>	This study
pBWARΔ1950	<i>lexA-WAR1Δ1-1950</i>	This study
pBWARΔ2460	<i>lexA-WAR1Δ1-2460</i>	This study
pCGWAR1	<i>WAR1</i>	21
pCGWAR1-S368L	<i>WAR1-S368L</i>	This study
pCGWAR1-K762R	<i>WAR1-K762R</i>	This study
pCGWAR1-K762D	<i>WAR1-K762D</i>	This study
pCGWAR1-K762F	<i>WAR1-K762F</i>	This study
pCGWAR1-R764Δ	<i>WAR1-R764Δ</i>	21
pAWY	<i>ADHPro-WAR1-YFP</i>	This study
pAWC	<i>ADHPro-WAR1-CFP</i>	This study
pACWY	<i>ADHPro-CFP-WAR1-YFP</i>	This study
pACWY-S368L	<i>ADHPro-CFP-WAR1-S368L-YFP</i>	This study
pACWY-K762R	<i>ADHPro-CFP-WAR1-K762R-YFP</i>	This study
pACWY-42	<i>ADHPro-CFP-WAR1-42-YFP</i>	This study
pPS1887	<i>NLS-CFP</i>	27
pPS1888	<i>NLS-YFP</i>	27
pPS1889	<i>NLS-CFP-YFP</i>	27

region amplified from pRS303 (25), was cloned into the EcoRI and XbaI sites of pRS306-*PDR12*. The resulting plasmid pCG4 containing the *HIS3* gene under the control of the *PDR12* promoter was linearized with EcoRV and integrated into the *ura3-1* locus of W303-1A, creating strain YCG7. Correct integration was verified by genomic PCR. For strain YCG8, a 1880-bp fragment containing a KAN^R deletion cassette, flanked by 170 bp of the promoter and 175 bp of the terminator region of *WAR1*, was amplified from the strain Y06734 (*war1Δ::Kan-Mx4* EURO-SCARF collection) and used to delete the *WAR1* gene from YCG7. Correct integration was verified by PCR, and loss of War1 function was tested by growth inhibition assays on sorbate.

Plasmid pGalWAR1 is a derivative of pAMG (26). The *ADH1* promoter was replaced by the *GAL1-10* promoter using the KspI and SalI restriction sites. The resulting plasmid pGMG (kindly provided by W. Reiter) was used for replacement of the *MSN2-GFP* construct with the entire *WAR1* gene, including 0.5-kb 3' region via the SalI and NotI restriction sites. pGalWAR1-K762R was generated by replacing a 2440-bp NsiI fragment of pGalWAR1 by the corresponding fragment from pCGWAR1-K762R.

To construct plasmids expressing LexA-War1, the *WAR1* ORF was amplified from W303-1A genomic DNA introducing a 5' EcoRI site, followed by an NcoI site at the *WAR1* start codon and a PstI site after the stop codon, and finally cloned into pBTM116 (Stan Hollenberg, Vollum Institute, Portland, OR) creating plasmid pBWAR1. Further N-terminal deletion

derivatives were generated taking advantage of the unique NcoI site of pBWAR1.

Plasmids for FRET Analysis—pAWY was created in two steps from pAMG (26) by replacing the NcoI-HindIII green fluorescent protein fragment with YFP, and the *MSN2* sequences by a *WAR1* fragment flanked by SalI and NcoI giving pYCplac111-AWY. The entire construct, including the *ADHI* promoter, was cloned as a KspI-HindIII fragment into pRS316. pAWC was created from pAMG by replacing the NcoI-HindIII green fluorescent protein fragment with CFP and the *MSN2* sequences by *WAR1* (SalI and NcoI). For pACWY, an XhoI-SalI CFP fragment was introduced into the SalI site of pYCplac111-AWY. pACWY-K762R, pACWY-S368L, and pACWY-42 are derivatives of pACWY encoding the corresponding *WAR1* gain-of-function variants, as well as the loss-of-function mutant *war1-42* (21) generated by replacing an internal NsiI fragment. Plasmids pPS1887 (NLS-CFP), pPS1888 (NLS-YFP), and pPS1889 (NLS-CFP-YFP) encoding fluorescent proteins were kindly provided by Pamela Silver (27) and are referred to as pNLS-CFP, pNLS-YFP, and pNLS-CFP-YFP in this study.

Random Mutagenesis and Screening for Constitutively Active War1 Mutants—The plasmid pCGWAR1 (21) was mutagenized *in vitro* (28). Briefly, about 10 μ g of plasmid DNA were dissolved in 0.5 ml of hydroxylamine solution (90 mg of NaOH, 350 mg of hydroxylamine-HCl in 5 ml of water, pH 6.5, freshly prepared before use) and incubated for 20 h at 37 °C. The reaction was terminated by adding 10 μ l of 5 M NaCl, 50 μ l of bovine serum albumin (1 mg/ml), and 1 ml of ethanol. After ethanol precipitation at -80 °C, the plasmid DNA was re-precipitated three times with ethanol and then dissolved in water. As a second method for mutagenesis, pCGWAR1 was passed through mutator strain XL1red (Stratagene) according to the protocol supplied by the manufacturer. Finally, the pool of mutagenized plasmid DNAs obtained by both methods was transformed into yeast recipient strain YCG8. Transformants growing on medium lacking leucine were washed from agar plates, and ~100 colonies from each transformation were plated on selective plates containing 50 mM 3-amino-1,2,4-triazole (3-AT) (Sigma). Growing colonies were selected for further analysis.

Preparation of Cell Extracts and Immunoblotting—For immunoblotting, cell-free extracts were prepared by the trichloroacetic acid method exactly as described previously (29). Strains were grown to an A_{600} of 1; the cultures were split, and one-half was stressed with 8 mM potassium sorbate. Cell lysates equivalent to 0.5 A_{600} units were resolved by 7.5% SDS-PAGE. War1 and Pdr12 were visualized by immunoblotting using ECL (Amersham Biosciences) and the polyclonal anti-War1 (21) and anti-Pdr12 antisera (4). β -Galactosidase assays were carried out in triplicate using total cell-free crude glass bead extracts as described previously (24).

RNA Isolation and Northern Analysis—Total yeast RNA was isolated by the hot phenol method. About 20 μ g of total RNA were separated on a 1.4% agarose gel and transferred to nylon membranes (Amersham Biosciences). Northern blots were hybridized with PCR-amplified probes and radiolabeled by incorporation of [α -³²P]dCTP and a MegaPrime labeling kit (Amersham Biosciences). Methylene blue staining was used as

control for equal RNA loading. The radiolabeled probes were added to the prehybridization solution after purification on a NICK column (Amersham Biosciences). Membranes were washed at 65 °C three times in 2 \times SSC, 1% SDS, and three times in 1 \times SSC, 1% SDS, and then exposed to x-ray films at -70 °C or analyzed with a PhosphorImager (Storm 1840; GE Healthcare).

DNA Microarray Experiments—50-ml cultures of YAK120 either transformed with pGalWAR1 or pGalWAR1-K762R were grown in YP + 2% raffinose to an A_{600} of about 0.8 before adding galactose at a final concentration of 2% w/v. After 1 h, cells were harvested, washed, and immediately frozen. Total RNA was prepared by hot phenol method. RNA concentration was measured at 260 nm in TE, pH 7. Samples of 20 μ g of total RNA were used for cDNA synthesis using 200 units of Superscript II reverse transcriptase (Invitrogen) with either Cy3-dCTP or Cy5-dCTP. Labeled cDNAs were pooled, and RNA was hydrolyzed for 20 min in 50 mM NaOH at 65 °C, followed by neutralization with acetic acid.

Hybridization to whole genome cDNA microarrays (Ontario Cancer Institute, Toronto, Canada) was done in DigEasyHyb (Roche Applied Science) solution at 37 °C overnight with 70 μ g of salmon sperm DNA/ml as a carrier. Microarrays were washed three times in 1 \times SSC, 0.1% SDS at 50 °C, followed by a 1-min wash in 1 \times SSC at room temperature. Glass slides were spun dry for 5 min at 500 rpm in a tabletop centrifuge, scanned on an Axon 4000B scanner (Molecular Devices), and analyzed and normalized using Gene Pix Pro4.1 software (Axon). DNA microarrays and protocols were obtained from the Ontario Cancer Institute. All microarray experiments were carried out with independent RNA preparations. Microarray data sets are MIAME-compliant and have been submitted to Gene Expression Omnibus (GSE8399).

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation PCR assays were performed as described elsewhere (30). A 50-ml yeast culture in YPD medium was grown to A_{600} of 0.4–0.6, treated with 8 mM sorbate for 20 min, or left untreated. For cross-linking, yeast cells were treated with 1% formaldehyde for 20 min at room temperature. Cross-linked protein-DNA complexes were immunoprecipitated by incubation with polyclonal anti-War1 antibodies (21) for 1 h at 4 °C, and co-precipitated DNA was amplified by PCR and analyzed by agarose gel electrophoresis. The oligonucleotides Pdr12s 5'-TTTCACCTGTGATGGTGGAC-3' and Pdr12as 5'-TGAGC-CATAGCCGAAAGATC-3' binding in the *PDR12* promoter region were used, giving a 320-bp PCR product covering both WAREs identified previously (7). Primers specific for *CLB2* promoter served as internal control; the primers Clb2-up 5'-ACTC-CTTCTAATCAAACACGCG-3' and Clb2-low 5'-CTGAAAC-TCTATGCCCATGC-3' giving a 255-bp PCR product were used.

FRET Imaging and Data Analysis—The fluorescence imaging workstation consisted of a Zeiss AxioplanII upright microscope, a Quantix CCD 5-MHz camera (Photometrics), z-step motor, and filter cubes with CFP, YFP, and FRET filter sets (Zeiss), all fully controlled by MetaFluor software (Molecular Devices). Three images were acquired in the same order in all experiments as follows: first, the FRET filter set CFP/YFP (excitation bp 436/25, BS FT 455, emission bp 535/30), second, the

YFP filter set (excitation bp 500/25, BS FT 515, emission bp 535/30), and third, the CFP filter set (excitation bp 436/25, BS FT 455, emission bp 480/40). YFP fluorescence visible through the CFP filter set and vice versa was negligible in our experiments. The acquisition cycle was controlled by a MetaFluor software. Pictures were captured with 1-s exposures using the $\times 63$ lens and 2×2 binning to achieve the necessary signal intensity above background. The mercury lamp was pre-heated for at least 20 min for equilibration.

All fusion constructs expressed produced nuclearly localized fluorescence. Living unfixed cells were viewed on pre-warmed slides, and not more than three pictures were recorded per slide within 5 min. Sorbate and propionate treatment was limited to a maximum of 15 min in 50 mM potassium phosphate-buffered synthetic medium, pH 6.5. A minimum of three sets of pictures per condition was recorded. Nuclei (5–30) and three control spots from an empty part in each microscopic picture were tagged with a 6×6 pixel field by hand for quantification, because the automatic capturing methods did not provide satisfactory results because of stochastic signal level variations.

Intensity values were recorded with Meta Fluor software and transformed to a spreadsheet-compatible format. Background was subtracted, and the ratio of FRET to donor fluorescence was calculated. Because of different fluorophores present in the same molecule, the direct corrected ratio of FRET *versus* CFP channel is possible (31). Measurements with intensity below 1.5-fold over background and values caused by movement of the cells during the picture acquisition cycle were excluded. Data from several series are included in the supplemental Table 5. Variance analysis of the data revealed significant differences at the 0.05α level between the different series. Confidence intervals (95%) of the mean FRET value between wild type C-War1-Y and mutated alleles of War1 and treated *versus* untreated were estimated assuming a normal distribution of FRET values. The difference (d_j) of the observed FRET values (x_{2i} and x_{1i}) of two constructs (1 and 2) is within the confidence interval with 95% probability (Equations 1–3),

$$\mu_2 - \mu_1 < x_2 - x_1 \pm 1.96 s \quad (\text{Eq. 1})$$

$$S^2 = \frac{s_1^2}{n_1} + \frac{s_2^2}{n_2} \quad (\text{Eq. 2})$$

$$\sum_{j=1}^m a_j (d_j - \Delta) \quad (\text{Eq. 3})$$

To summarize the results of several series, we calculated the weighted difference between FRET values of War1 alleles and propionate treatment, respectively. We assume the difference (Δ) is the same for all series (Equation 4).

$$\sum_{j=1}^m a_j s_j^2 \quad (\text{Eq. 4})$$

We obtained a confidence interval for Δ based on the weighted average of the observed differences d_j (Equation 5),

$$a_j = \frac{s_j^{-2}}{\sum_{e=1}^m s_e^{-2}} \quad (\text{Eq. 5})$$

and the weighted variance (Equation 6),

$$\sum_{j=1}^m a_j d_j \pm 1.96 \sqrt{\sum_{j=1}^m a_j^2 s_j^2} \quad (\text{Eq. 6})$$

The weights a_i ($\sum a_i = 1$) were chosen according to Equation 5 to obtain an interval that is as small as possible. The resulting minimal confidence intervals for the weighted mean differences between constructs between C-War1-Y and its derivatives were calculated according to Equation 6 and included in Fig. 6, C and E, and Tables 3–5.

RESULTS

Mutagenesis of WAR1 and Selection of Constitutively Active Alleles—To identify potential regulatory domains in War1, we set up a mutagenesis screen for isolation of constitutively active WAR1 alleles. The reporter strain YCG8 (P_{PDR12} -*HIS3* *war1* Δ) transformed with the plasmid pCGWAR1 (21) containing wild type WAR1 was unable to grow on SC-His in the presence of 50 mM 3-AT. Addition of 1 mM sorbate to the same plates increased *PDR12* promoter-driven *HIS3* expression to a level that allowed for growth on this concentration of 3-AT (data not shown). Constitutively active mutant alleles of WAR1 should hence induce the P_{PDR12} -driven reporter expression even in the absence of weak acid stress, conferring cell growth on 3-AT plates without sorbate. To introduce mutations, the pCGWAR1 plasmid DNA was treated with hydroxylamine or propagated in the bacterial XL1-red mutator strain and transformed into strain YCG8. Transformants harboring gain-of-function WAR1 alleles were then selected on 3-AT plates and tested by immunoblotting for elevated Pdr12 levels in the presence and absence of stress. To exclude genomic mutations, plasmids were rescued from YCG8 and transformed into strain YAK100 (*war1* Δ) for further characterization.

WAR1 Hyperactive Mutant Alleles—Sequencing of both strands of the entire WAR1 ORF in all isolated alleles identified seven distinct mutations, each of them causing constitutive War1 activity (Fig. 1). The mutant alleles carried the following residue changes: S368L, Y452C, Y463C, A640T, S703P, and K762R. Furthermore, the G to A transition at nucleotide position 2808 introduced a stop codon, resulting in a truncated War1 protein lacking nine amino acids at the C terminus of the protein. Plasmids isolated during the screen were named according to the amino acid substitutions pCGWAR1-S368L, pCGWAR1-Y452C, pCGWAR1-Y463C, pCGWAR1-A640T, pCGWAR1-S703P, pCGWAR1-K762R, and pCGWAR1-936. Except for the stop mutation at the end of the activation domain, all identified mutations are localized in the region between the putative dimerization and the activation domain of War1, representing a putative functional analogue of the middle homologous region present in other yeast zinc finger regulators. Sequence alignment of *S. cerevisiae* War1 and War1

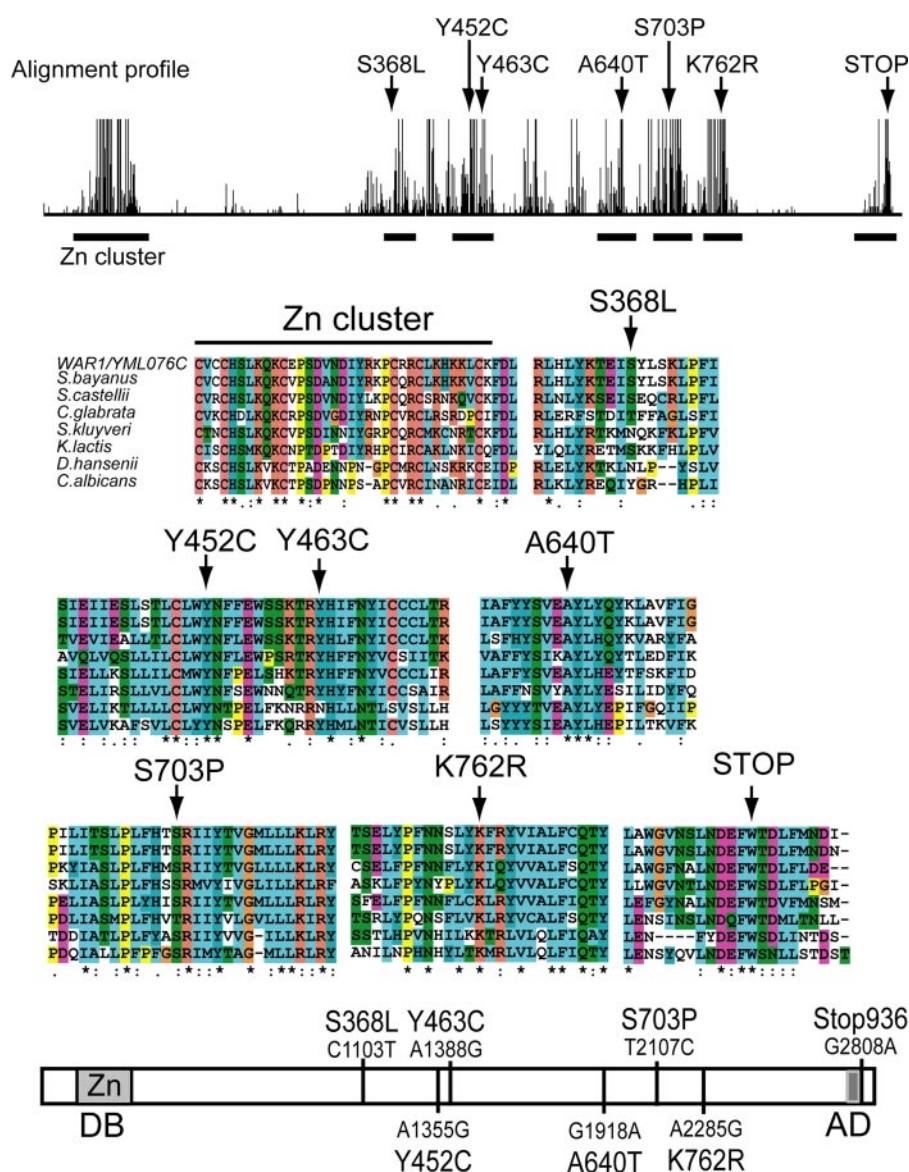


FIGURE 1. Localization of mutations within *WAR1* causing hyperactivity. Multiple sequence alignments from selected *War1* homologues were done with ClustalX 1.83 using default parameters. The alignment quality profile is indicated as local similarity (y axis) along the aligned sequence positions (x axis), and the selected regions around the mutant alleles are indicated on the top panel. Parts of the multiple sequence alignment indicated (black bars) are shown in detail. The schematic depicts the location of mutations in *WAR1* isolated during the genetic screen. Zn, zinc cluster; DB, DNA-binding domain; AD, activation domain.

orthologues from several other fungal species indeed identified conserved stretches within this internal domain (Fig. 1). The profile of the alignment is shown in Fig. 1, upper panel, indicating regions of high similarity. The hyperactive mutant alleles and the zinc cluster region are marked. Notably, two alleles replace a tyrosine, and three others are neighboring a tyrosine residue. The mutation leading to stop after amino acid 935 also disrupts a conserved motif invariant in all selected proteins.

Characterization of Constitutively Active *WAR1* Alleles—Next, we analyzed expression levels and phosphorylation status of the hyperactive *War1* gain-of-function mutants. Cultures of YAK100 transformed with mutant or wild type *WAR1* plasmids were stressed with potassium sorbate, and protein extracts were analyzed by immunoblotting. Detection of *War1* variants revealed similar expression levels of wild type and most

mutant proteins in both stressed and unstressed cells (Fig. 2A). Only the *War1*-A640T variant showed slightly decreased protein levels.

War1 isolated from unstressed cells migrates as a typical double band because of phosphorylation (7). The faster migrating band was reduced in the mutants *War1*-936, *War1*-K762R, *War1*-Y463C, *War1*-Y452C, and *War1*-S368L (Fig. 2A). The lack of the faster migrating band was already observed for another constitutively active mutant *War1*-K762N as reported earlier (21). In contrast, the mutants *War1*-A640T and *War1*-S703P migrated as double bands with a more pronounced difference when compared with the wild type.

The previously identified *WAR1* gain-of-function mutant exchanges Lys-762 by asparagine (21). The mutant independently isolated in this screen encodes arginine at position 762. Thus, replacement of Lys-762 by polar residues such as arginine or asparagine leads to constitutive *War1* activity.

To test the effect of acidic or nonpolar amino acids at the same position, we introduced either aspartic acid or phenylalanine instead of lysine 762 via site-directed mutagenesis giving plasmids pCGWAR1-K762D and pCGWAR1-K762F. High Pdr12 expression levels in a *war1Δ* background expressing these mutants demonstrated that they are also constitutive active alleles (data not shown). *War1* activation correlated with a change in phosphorylation resulting in a shift to a slower migrating isoform (7). For all isolated gain-of-function variants, slower migrating species appeared in response to stress, suggesting changes in the phosphorylation status of the hyperactive proteins. Pdr12 levels were not further up-regulated upon weak acid stress, indicating full activity of the mutant alleles even in the unstressed state (Fig. 2A).

We next asked whether constitutive high Pdr12 levels confer higher tolerance of weak acid stress. Growth inhibition assays on YPD plates supplemented with sorbate, propionate, and benzoate suggested no enhanced resistance (Fig. 2B) of mutants expressing hyperactive *War1* alleles. The *War1*-(1–936) mutant exhibited even slightly reduced weak acid tolerance when compared with wild type. Likewise, slightly impaired growth was observed for *War1*-S703P on sorbate. No growth

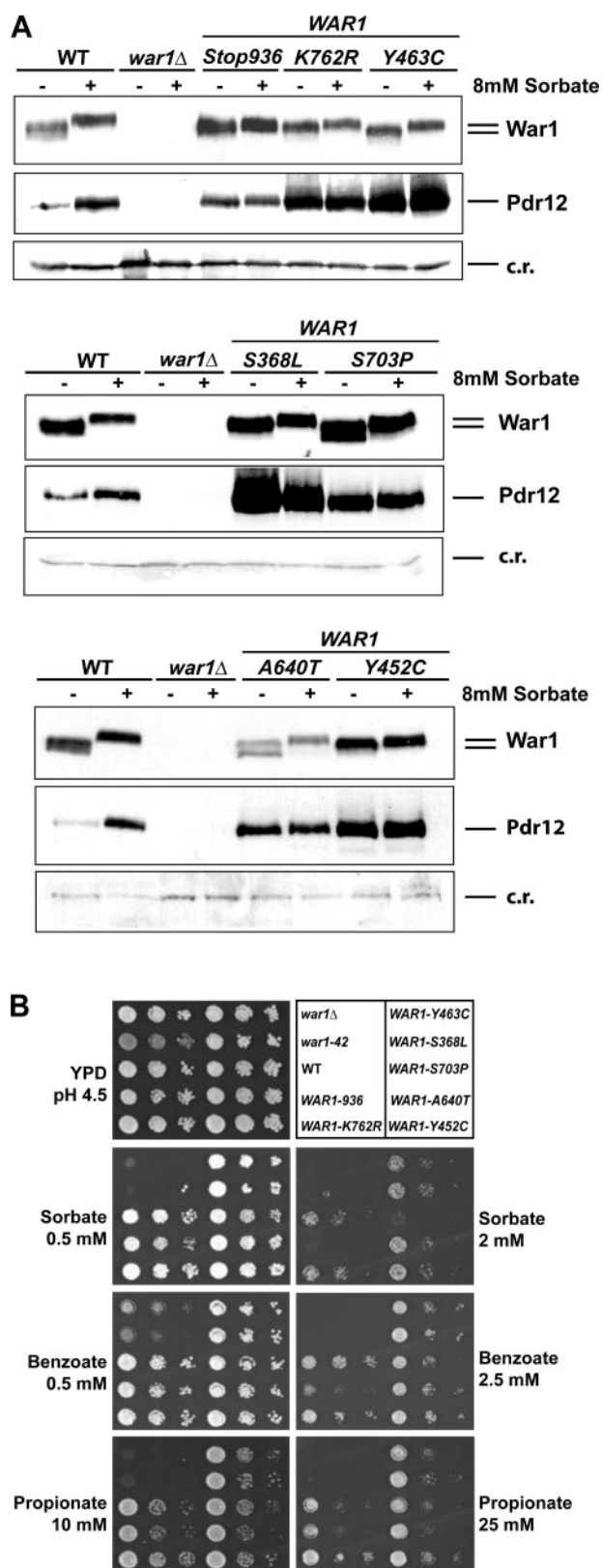


FIGURE 2. WAR1 mutants up-regulating Pdr12 levels in the absence of stress. A, strains YAK100 (*war1Δ*) and YAK100 harboring a plasmid expressing either wild type WAR1 or the indicated gain-of-function allele were grown in YPD to an A_{600} of about 1; the cultures were split, and one-half was stressed with 8 mM sorbate for 40 min. Cell extracts equivalent to 0.5 A_{600} were separated in 7% SDS-polyacrylamide gels, and the immunoblots were decorated with polyclonal anti-War1 and anti-Pdr12 antisera. A cross-reaction (c.r.) to

difference to the wild type was observed on YPD, pH 4.5, without weak acid stress.

To confirm that the mutant alleles are acting at the level of *PDR12* transcription, the activity of a *lacZ* reporter gene under the control of the *PDR12* promoter was analyzed (Fig. 3A). We performed β -galactosidase activity measurements in the *war1Δ* strain background YBB27 expressing the mutant alleles War1-S368L and War1-K762R. In the absence of sorbate, the War1 mutants conferred a more than 5-fold higher *PDR12* promoter activity when compared with the wild type protein (Fig. 3A). Addition of sorbate did not lead to any further induction of *PDR12* promoter activity in cells expressing War1-S368L or War1-K762R.

Hyperactive War1 Alleles Retain Target Specificity—Most residue changes in mutant alleles (except one) map to an internal region, leaving the DNA-binding domain intact. Nevertheless, other War1 regions may still contribute to DNA targeting. To exclude that constitutive activity correlates with changes in DNA-binding specificity, we compared the transcriptional profiles of cells expressing an inducible hyperactive variant with wild type War1. Galactose-induced expression of War1 variants allowed for the detection of changes in transcriptional responses that are directly associated with occurrence of hyperactive War1 rather than resulting from long term expression of a mutated protein. Northern blotting analysis revealed induction of *PDR12* transcription that was specific for the constitutively active War1 protein and cannot be observed after galactose-induced expression of wild type War1 (Fig. 3B). Microarray analysis of mRNA isolated 60 min after galactose addition revealed only a limited set of genes that were significantly induced or repressed in strains expressing a War1 variant when compared with the same strain expressing wild type War1. Strikingly, two “marker” genes already identified as weak acid-inducible (*ATO2/FUN34*, *BDH2/YAL061W*) were more than 3-fold up-regulated in cells expressing constitutively active War1. *ATO2* was reported to be War1-regulated, and our results demonstrate that *BDH2* expression is also at least partially dependent on War1 (22). The up-regulation of the putative medium chain alcohol dehydrogenase *BDH2* in War1-K762R mutant cells was also confirmed by Northern analysis (Fig. 3B), revealing a novel gene potentially belonging to the War1 regulon.

Genes differentially expressed in War1-K762R were compared with microarray data from sorbate-stressed cells (22). Using cluster analysis, we found that genes significantly up-regulated by War1-K762R were also found to be induced by sorbate stress in wild type cells (Fig. 3C). Only a very small set of genes was exclusively up-regulated in cells expressing the constitutively active transcription factor, demonstrating that hyperactive War1 alleles do not dramatically alter target specificity or drive expression of other genes lacking the WARE.

the War1-antibodies served as loading control. WT, wild type. B, strains YAK100 (*war1Δ*) and YAK100 carrying either a plasmid expressing wild type WAR1 or the indicated gain-of-function allele were cultured to the exponential phase and diluted to A_{600} of 0.2. Cells were spotted along with 1:10 serial dilutions onto YPD agar plates, pH 4.5, containing the indicated amounts of weak organic acids. Strain YCS42-D4 (*war1-42*) was included as an additional control. Growth was inspected after 48 h of incubation at 30 °C.

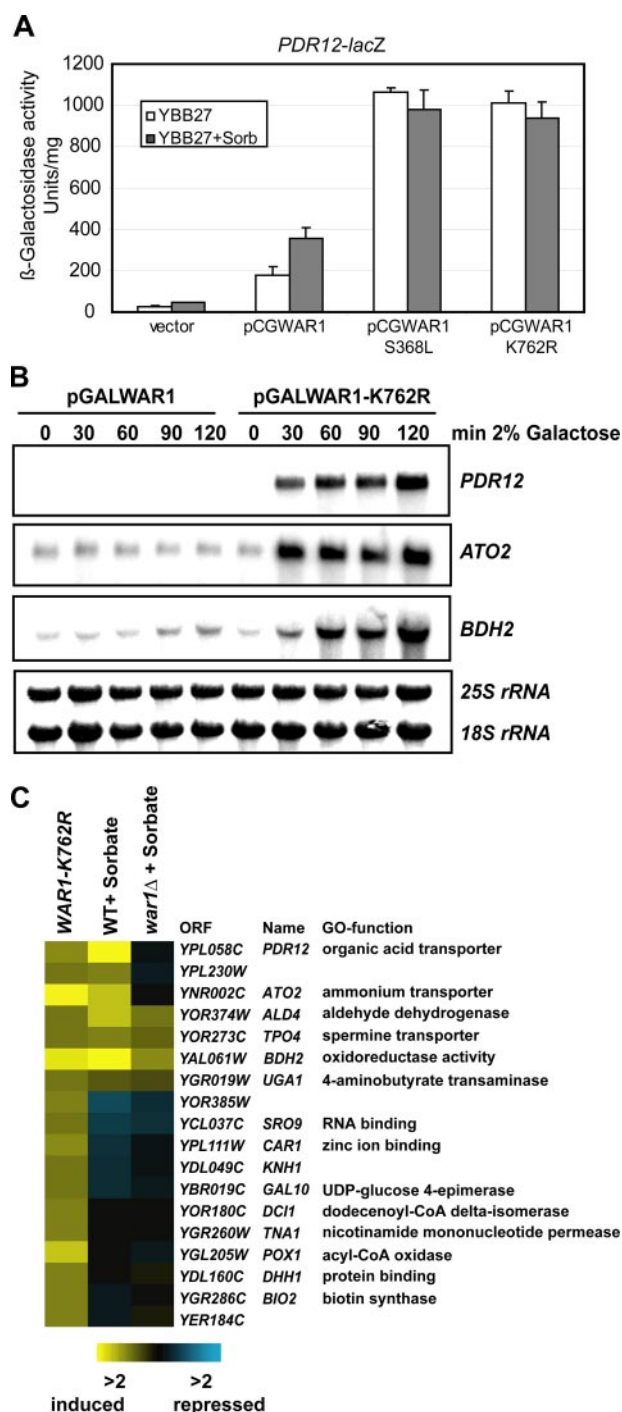


FIGURE 3. Increased transcriptional activity of War1 mutants in unstressed cells. A, strain YBB27 (*PDR12-promoter-LacZ*, *war1Δ*) transformed with pCGWAR1, pCGWAR1-S368L, pCGWAR1-K762R, or the empty control plasmid pRS315 was grown to an A_{600} of 1.2 to 1.5 and the culture divided; one-half was grown in the presence of 8 mM sorbate for 1 h. β -Galactosidase assays were carried out in triplicate in crude extracts. B, sorbate-stressed and constitutively active War1 share similar transcript profiles. Cultures of YAK120 transformed with either pGalWAR1 or pGalWAR-K762R were cultivated to the early exponential growth phase in YP containing raffinose. War1 expression was induced by adding 2% galactose, and mRNA was prepared from samples taken at the indicated time points. Northern blots were probed for *PDR12*, *ATO2*, and *BDH2* mRNAs, and methylene blue-stained rRNA served as a loading control. C, hierarchical clustering of induction values representing average inductions over three replicate profiles of each wild type (WT) and the isogenic *war1Δ* strain after 20 min of 8 mM sorbate treatment, as well as the *war1Δ* strain transformed with pGalWAR1-K762R in the absence of sorbate.

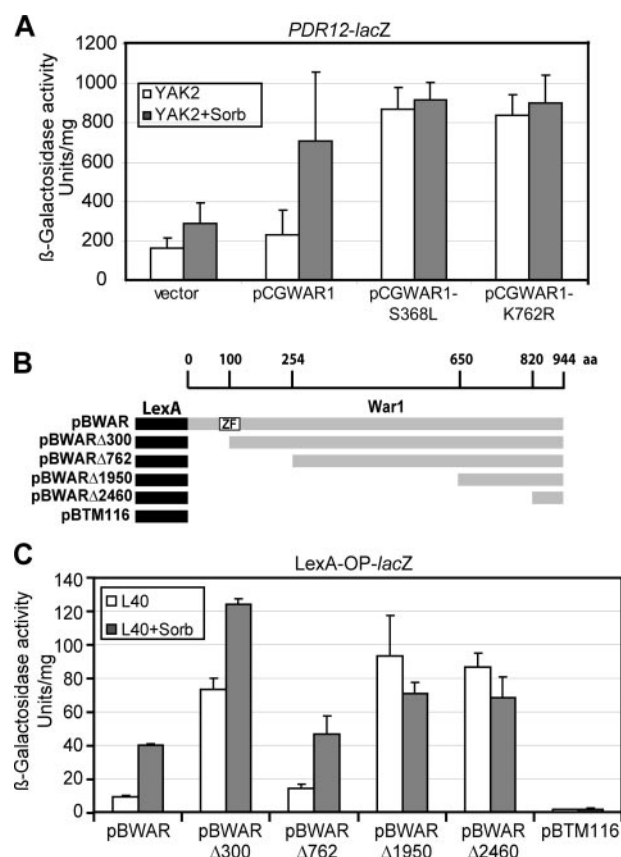


FIGURE 4. Mutations in and deletion of the central region cause hyperactivity. A, strain YAK2 (*PDR12-promoter-LacZ*) transformed with either pCGWAR1, pCGWAR1-S368L, pCGWAR1-K762R, or the empty control plasmid pRS315 was grown to A_{600} of 1.2–1.5 and the culture divided; one-half was grown in the presence of 8 mM sorbate for 1 h and the other remained as an untreated control. β -Galactosidase assays were carried out in triplicate in crude cell-free extracts. B, schematic depicting the LexA-War1 fusion and truncated constructs. C, L40 transformed with pBWAR, pBWARΔ300, pBWARΔ762, pBWARΔ1950, pBWARΔ2460 or the empty control plasmid pBTM116 was grown to an A_{600} of 1 to 1.3 and divided; one-half was grown in the presence of 10 mM sorbic acid for 40 min and the other remained as an untreated control. β -Galactosidase assays were carried out in triplicate in crude extracts.

The War1 Hyperactive Mutant Alleles Are Dominant—As many other related zinc finger transcription factors, War1 forms homodimers (7). We therefore tested whether gain-of-function alleles are dominant over the wild type War1 protein. Strain YAK2 (*P_{PDR12}-lacZ*) was transformed with plasmids encoding either wild type *WAR1* or the hyperactive alleles *WAR1-S368L* or *WAR1-K762R*. *PDR12* promoter activity was measured in unstressed conditions and in 8 mM sorbate-treated cells. YAK2 transformed with the vector control or the plasmid harboring wild type *WAR1* had low promoter activity in unstressed cells, which was, however, increased in response to stress (Fig. 4A). In cells expressing both hyperactive and wild type War1, the *PDR12* promoter is already up-regulated without addition of sorbate (Fig. 4A), showing that these gain-of-function alleles are dominant. The *PDR12* promoter *lacZ* activity in cells expressing the mutant allele was comparable in both wild type (Fig. 4A) and *war1Δ* backgrounds (Fig. 3A) and was essentially unchanged after sorbate treatment.

Partial Deletion of War1 Internal Regions Leads to Constitutive Activity—Most of the mutants identified are located in the central region between the DNA-binding domain and the acti-

vation domain of War1. We therefore tested the function of this region for weak acid-regulated activation of War1. We constructed plasmids fusing the LexA DNA-binding domain with truncated War1 derivatives, lacking parts of the N terminus. The plasmids were derivatives of pBTM116 and named pBWΔ300, pBWΔ762, pBWΔ1950, and pBWΔ2460, corresponding to the number of base pairs lacking from the 3'-end of *WAR1* (Fig. 4B). Fusion protein expression was equal in stressed and unstressed situations as confirmed by immunoblotting. Only pBWΔ2460 expressed very low levels of LexA fusion protein in both situations (data not shown). Transcription factor activity of War1 truncations fused to LexA was measured by β -galactosidase assays in the yeast reporter strain L40 (*lexA-OP-lacZ*) under stressed and unstressed conditions. pBWAR1 expressing full-length War1 fused to LexA was used as the wild type control and the empty vector pBTM116 as a negative control. The LexA-War1 fusion lacking 100 amino acids of the N terminus (pBWΔ300) showed elevated levels of activity in unstressed cells when compared with wild type. Sorbate stress still further increased its activity (Fig. 4C). Strain L40 harboring pBWΔ762 behaved exactly as the strain harboring the wild type plasmid. In contrast, the two War1-LexA fusions lacking 650 residues or more from the N terminus showed weak acid-independent high activities (Fig. 4C, pBWΔ1950 and pBWΔ2460). The region deleted in these hyperactive truncation mutants precisely corresponds to the region harboring the mutations causing War1 hyperactivity (Fig. 1A). Taken together, these results indicate that the War1 DNA-binding domain is dispensable for stress sensing or signaling, because replacement of the zinc finger with the LexA DNA-binding domain keeps the protein responsive to sorbate. In contrast, the internal domain plays a major role in regulating War1 activity.

War1 Promoter Binding Is Enhanced in Sorbate-stressed Cells—Gain-of-function mutants isolated during the genetic screen strongly induce *PDR12* expression in the absence of sorbate. One possible explanation for this might be enhanced binding of War1 variants to the *PDR12* promoter. To address this question, we used ChIP to follow the association of wild type War1, the hyperactive mutant War1-K762R, and the inactive mutant War1-R764Δ (21) to the *PDR12* promoter before and after weak acid stress. War1 variant proteins cross-linked to chromatin were immunoprecipitated with polyclonal War1 antibodies. PCR detected the relevant region of the *PDR12* promoter, as well as a fragment of the *CLB2* promoter as a control. Binding sites of oligonucleotides used for amplification of the *PDR12* promoter fragment are illustrated in Fig. 5A. Gel bands were quantified, and the ratios of the *PDR12*/*CLB2* signals are shown. Previous data obtained with *in vivo* dimethyl sulfate footprinting indicated constitutive binding of War1 to the *PDR12* promoter (7). Confirming these data, we observed weak binding of wild type War1 to the *PDR12* promoter in the absence of weak acid stress when compared with the *war1Δ* strain. However, addition of sorbate strongly increased War1 DNA binding (Fig. 5, B and C). Interestingly, the gain-of-function variant War1-K762R showed already increased promoter association in the unstressed state. Sorbate treatment had only a marginal effect on promoter association of War1-K762R (Fig. 5B). Association of the loss-of-function mutant War1-R764Δ

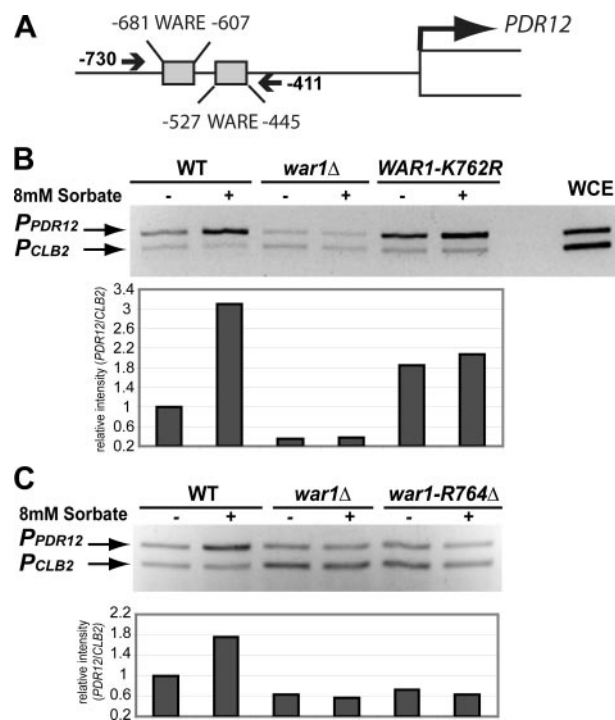


FIGURE 5. Binding of wild type and mutant War1 variants to the *PDR12* promoter. A, schematic illustration of the region in the *PDR12* promoter amplified after ChIP. Small black arrows indicate primer-binding sites. B, ChIP experiments with strains expressing either wild type (WT) or the constitutively active War1 variant (War1-K762R), including the *war1Δ* control, were performed. Protein-DNA complexes from sorbate-treated and untreated cultures were immunoprecipitated using polyclonal anti-War1 antibodies, and DNA fragments were amplified by PCR using primers specific for the *PDR12* promoter and *CLB2* as internal control. PCR fragments were analyzed by gel electrophoresis. *P_{PDR12}* PCR fragments were quantified and normalized to *P_{CLB2}* and expressed relative to the untreated wild type level. WCE, whole cell extract. C, signals from ChIP experiments with strains expressing either wild type or the loss-of-function mutant War1-R764Δ as well as the negative control (*war1Δ*) are shown.

was undetectable in stressed or unstressed cells (Fig. 5C). The state of chromatin association of War1 variants reflected and verified their gain- or loss-of-function phenotypes. These results demonstrate that weak acid stress changes the War1 recruitment to the *PDR12* promoter, suggesting a higher affinity of activated War1 for target promoters in the presence of weak acid.

FRET Analysis of War1—In a simple model, one could speculate that weak acids directly or indirectly trigger conformational changes of War1, thereby “unmasking” the War1 activation domain and release from inhibition. As a way to investigate conformational changes *in vivo*, we therefore exploited fluorescence resonance energy transfer (FRET) occurring between CFP and YFP molecules attached to the N and C termini of the same War1 molecule. Because of relatively low expression of genomic War1, we were forced to use the *ADH1* promoter to increase War1 levels and obtain measurable fluorescence signals. We generated a plasmid-based construct (pACWY) encoding a CFP-War1-YFP (C-War1-Y) variant. In addition to C-War1-Y, War1 derivatives tagged at the C terminus with either a single CFP or YFP were constructed. These variants were expressed from plasmids with *LEU2* (pAWC) and *URA3* (pAWY) markers, respectively, allowing for simultaneous expression in yeast. Despite selecting for both markers, many

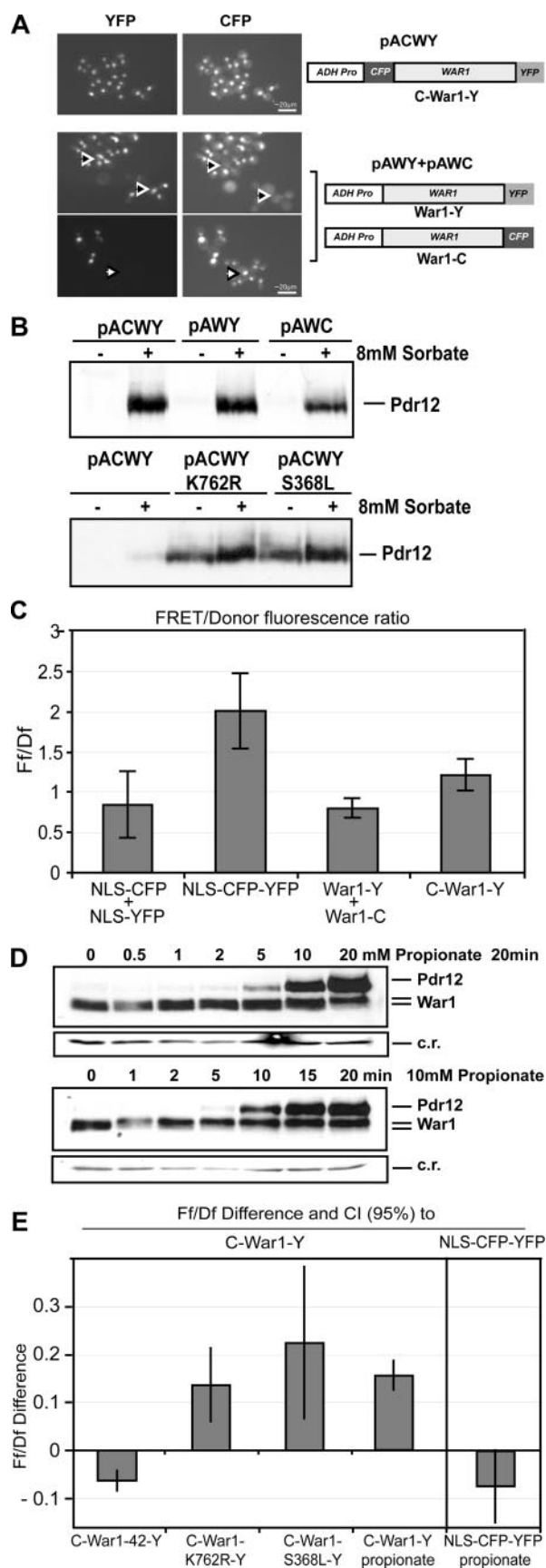


FIGURE 6. FRET analysis of CFP- and YFP-tagged War1. *A*, strain YAK120 harboring pACWY as well as YAK120 harboring pAWY and pAWC were grown to the early exponential growth phase. Localization of fluorescence signals

TABLE 3

FRET setup

The following abbreviations are used: Ff, FRET fluorescence; Df, donor fluorescence; *n*, number of cells quantified.

Construct	FRET/donor (Ff/Df)	Variance	S.D.	<i>n</i>	<i>p</i> value
NLS-CFP + NLS-YFP	0.841	0.162	0.402	81	3.201E-129
NLS-CFP-YFP	2.013	0.198	0.445	183	
War1-Y + War1-C	0.796	0.011	0.103	41	8.759E-25
C-War1-Y	1.215	0.032	0.178	64	

cells displayed different fluorescence intensities in the YFP and CFP channels (Fig. 6A). Notably, War1 function was not compromised by these tags, as regulatory properties remained unchanged when compared with the wild type protein (Fig. 6B). Sorbate-treated cells still drove increased Pdr12 expression. Moreover, like their untagged forms, the CFP/YFP double-tagged derivatives of constitutive active War1 alleles led to elevated Pdr12 levels (Fig. 6A, lower panel), whereas the double-tagged control C-War1-42-Y allele remained inactive (data not shown). Furthermore, cells expressing single- or double-tagged War1 variants displayed growth properties under sorbate and propionate stress similar to the wild type strain (data not shown).

We next tested our FRET set up using appropriate control plasmids (27). To calibrate our system, we performed measurements with covalently linked CFP and YFP proteins expressed from the plasmid pNLS-CFP-YFP to visualize the maximum FRET under these conditions. Importantly, we also expressed both chromophores from two separate plasmids pNLS-CFP and pNLS-YFP as individual proteins (27) to measure FRET generated by stochastic and random association. All molecules were targeted to the nucleus using an SV40-derived nuclear localization signal. Fluorescence intensity over the nuclei of captured pictures was measured, and the relation of the FRET to the CFP channel was calculated (Table 3). In the case of C-War1-Y and NLS-CFP-YFP, both chromophores were pres-

was recorded in living cells with YFP and CFP filter sets. In the upper panel, cells expressing C-War1-Y are shown. Cells expressing either both War1-Y and War1-C or only one of the two tagged variants (War1-Y: filled black arrows; War1-C: filled white arrows) are shown in the lower two panels. Schematics illustrate the different WAR1 CFP and YFP fusion constructs used. *B*, strain YAK120 (*war1Δ*) transformed with the indicated plasmids was grown to the early exponential growth phase; the cultures were split, and one-half was stressed with 8 mM sorbate for 40 min. Cell extracts equivalent to 0.5 A_{600} were separated by 7% SDS-PAGE followed by immunodetection of Pdr12 using polyclonal antisera. *C*, FRET versus donor ratio (Ff/Df) of C-War1-Y and NLS-CFP-YFP. W303-1A cells transformed with FRET control plasmids expressing NLS-CFP-YFP or both NLS-CFP and NLS-YFP, as well as YAK120 expressing C-War1-Y or both War1-C and War1-Y, were analyzed as described under "Materials and Methods." Data are listed in Table 3 and derived from supplemental Table 5, and the standard deviations are indicated. *D*, propionate induces Pdr12 expression and activates War1. Cultures of W303-1A were grown to an A_{600} of 1 before adding the indicated amounts of propionate for 20 min (upper panel). Alternatively, 10 mM propionate was added to the culture, and samples were taken at the indicated time points (lower panel). Cell extracts equivalent to 0.5 A_{600} were separated on a 10% SDS-PAGE. Immunoblotting was carried out using specific polyclonal antibodies detecting War1 and Pdr12. A cross-reaction (c.r.) to War1-antibodies served as a loading control. The mobility shift of War1 to a slower migrating form is indicated. *E*, variance analysis of FRET in C-War1-Y and mutant alleles. The mean differences between wild type C-War1-Y and mutant alleles are represented with bars; confidence intervals (95%) of the differences are indicated. For propionate treatment, the relation of Ff/Df values of NLS-CFP-YFP and C-War1-Y between treated and untreated cells are shown. Data are listed in Table 4 and are derived from supplemental Table 5.

TABLE 4

FRET of War1 variants compared with wild type War1

Construct compared with C-War1-Y	Difference of Ff/Df to C-War1-Y	Confidence interval (95%)
C-WAR1-42-Y	-0.063	0.021
C-War1-K762R-Y	0.137	0.076
C-War1-S368L-Y	0.225	0.158
C-War1-Y 10 mM propionate	0.156	0.030
Propionate control	Difference to NLS-CFP-YFP untreated	0.075
NLS-CFP-YFP 10 mM propionate		

ent in equimolar amounts, because they are included in the same molecule. Hence, these constructs did not require bleed-through corrections for data analysis (31). We therefore calculated the ratio of the FRET (Ff) fluorescence signal *versus* the donor CFP (Df) fluorescence (Ff/Df or FRET ratio). For the separately tagged constructs, we selected cells showing nuclear staining corresponding to roughly equimolar amounts of NLS-CFP and NLS-YFP for analysis. The results shown in Fig. 6C demonstrate that FRET signals were readily detectable with our setup.

In a second set of measurements, we tested the chromophore interaction when fused to War1. Cells displaying nuclear staining for roughly equimolar amounts of War1-C and War1-Y were selected for microscopic analysis. Fluorescence of cells expressing C-War1-Y or NLS-CFP-YFP had some stochastic variation of the fluorescence signal intensity but always showed similar levels in both CFP and YFP channels. Calculating the ratio of the FRET channel to the donor channel (CFP) for a large number of nuclei in several independent experiments showed a significant relative increase of the Ff to Df ratio of the C-War1-Y harboring both chromophores, when compared with all other separated control constructs (Fig. 6C and Table 3). These results demonstrate a close vicinity of C- and N-terminal ends of War1.

FRET Analysis of War1 and Mutant War1 Variants—We next asked if the FRET signal obtained with C-War1-Y remains constant or is correlated to the War1 function or activation status. We therefore constructed double-tagged derivatives of the inactive (C-War1-42-Y) or hyperactive alleles (C-War1-K762R-Y and C-War1-S368L-Y). Both inactive and active derivatives were expressed at similar levels and localized to the nucleus. A slightly enhanced cytoplasmic staining was noticed in the inactive (War1-42) allele (data not shown). We then performed a number of independent experiments to determine the FRET (Ff) fluorescence signal *versus* the donor CFP (Df) fluorescence (Ff/Df or FRET ratio) as listed in supplemental Table 5. Variance analysis of the obtained Ff/Df ratios indicated a characteristic difference to the wild type construct over all series. As shown in Fig. 6E and Table 4, we detected a reduction of FRET signals in the inactive mutant relative to wild type with a nonoverlapping weighted confidence interval. By contrast, hyperactive mutants behaved in an opposite manner. We tested two mutant alleles, S368L and K762R, and in both cases obtained increased constitutive FRET to donor (Ff/Df) signals when compared with wild type War1.

We next analyzed FRET signals of C-War1-Y under weak acid stress. Control experiments with the NLS-CFP-YFP fusion

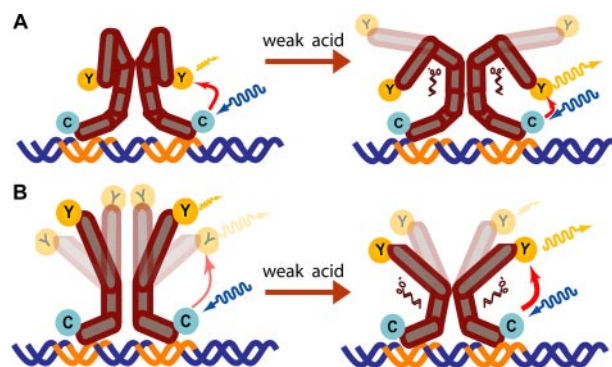


FIGURE 7. Models for conformational changes of War1 in the presence of weak acids. A, weak acids promote release of an autoinhibitory domain located at the extreme C terminus, which normally interacts with a central domain. This results in increased flexibility and higher probability of FRET occurrence. B, weak acid binding to War1 favors a conformational switch bringing the C and N termini in closer proximity. To comply with the accompanying increased DNA affinity, both models suggest enhanced homodimerization, thus increasing binding of the complex to the WARE, and perhaps improving recruitment of the mediator complex. For simplicity, the models do not consider interactions with other possible factors and/or post-translational modifications.

construct indicated a reduction of signal (Ff/Df) in cells exposed to 10 mM propionate, and a slight increase under sorbic acid stress (data not shown). The shorter C₂ chain of propionate might cause fewer side effects on protein function when compared with the C₆ chain sorbic acid. Propionic acid caused activation of War1 with the same kinetics as sorbate but required a slightly higher concentration (Fig. 6D). Under 10 mM propionic acid stress, the double-tagged War1 showed an increase of the Ff/Df signal to a level comparable with the hyperactive alleles, indicating conformational changes occurring during or triggered by War1 activation.

War1 Initiates Stress Adaptation after an Activation-triggered Conformational Change—In summary, our data provide compelling evidence for potential models describing the function of War1 in stress adaptation (Fig. 7). In model A, War1 is locked in an inactive conformation through the interaction of the extreme C terminus with the central region, yet it is still loosely associated with the *PDR12* promoter DNA. Weak acid stress opens this conformation, releasing the activation domain and allowing for interaction with accessory factors, as well as increasing the affinity for the *cis*-acting recognition motif. In model B, interaction with weak acid anions forces War1 from an open flexible conformation toward a closed and activated state, bringing the N and the C terminus in close vicinity. In both models conformational changes also enhance homodimerization, increasing War1 promoter affinity. Moreover, we suppose that these changes in conformation are tightly linked to phosphorylation events, although the precise role of phosphorylation in the process of War1 regulation or activation remains elusive.

DISCUSSION

Weak acid-triggered induction of the plasma membrane ABC transporter Pdr12 is extremely rapid, leading to Pdr12 levels similar to the H⁺-ATPase, the most abundant plasma membrane protein (1, 4). This seems especially important in case of sudden exposure to weak organic acid stress. Remark-

ably, it takes only 10–15 min before newly synthesized Pdr12 is detectable, including transcription of the corresponding 5-kb gene and translation (Fig. 6D). This rapid response is exclusively demanding the weak acid response regulator War1 (7). In this study, we provide new insights into the mechanisms of War1 regulation and its role in stress adaptation. We identified conserved regions of the transcription factor required for regulated activity, and we show that stress activation of War1 is causing dynamic conformational changes.

Regulation of War1 Activity by Weak Acid Stress—Zinc cluster transcription factors are a class of proteins exclusively found in fungi (9). They often control small and specific regulons representing a particular task in the cell metabolism. Only few members, like the drug resistance regulators Pdr1 and Pdr3, seem to play more than one distinct role (23, 32). War1 is another highly specific member of this class, even conserved beyond the yeast *S. cerevisiae*. Short chain organic acids (C_2 to C_7) are main triggers of War1 activation, leading to protection through Pdr12 induction. War1 is constitutively nuclear and appears always bound to its target WARE sites in stressed and unstressed cells (7). The main War1 regulon includes only about four genes, with only one, *PDR12*, shown to be necessary and sufficient for protection during weak acid stress.

The regulation mechanisms of this class of transcription factors are largely devoid of classical signal transduction cascades. The idea that many zinc cluster factors are directly responding to small molecules, metabolites, or drugs has been substantiated by the analysis of several other yeast transcription factors (19, 11). Such a mode of action can explain the rapid response seen with weak acid-induced War1. Likewise, activation of the Put3 zinc cluster factor upon binding of an inducing compound has been demonstrated *in vitro* (12). The induction of the proline utilization pathway is required for growth on proline as sole nitrogen source. Put3 activates transcription of the two genes coding for the enzymes Put1 and Put2 only in the presence of proline and the absence of other preferred nitrogen sources (33, 34). Similar to War1, Put3 is constitutively bound to the upstream regulatory sequence of its target genes (34) and is differentially phosphorylated depending on the quality of nitrogen sources (35). The Put3 transcription factor undergoes conformational changes from an inactive to an active state, triggered by the binding of the inducer as suggested by protease protection assays *in vitro* (36). Several regulators of metabolism and biosynthetic pathways appear to follow this scheme. Leu3 requires the presence of the intermediate of leucine biosynthesis α -isopropyl malate (37); Ppr1 requires dihydroorotic acid for pyrimidine biosynthesis (38), and Lys-14 requires α -amino adipate semialdehyde (39). Interestingly, Gal4 appears as one exception, because it requires two accessory factors for regulation, with one of them required for binding galactose (40). Regarding War1, a physical interaction with an inducer has not yet been demonstrated. Moreover, genetic evidence for similar upstream regulators is not available (21). Hence, based on the similarity to the above-mentioned family members, a direct regulatory mechanism exerted by weak organic acids seems plausible. The use of reconstituted *in vitro* transcription systems as established for Put3 (12) will clarify this question.

Conserved Internal Domains Have Inhibitory Functions—Here we explored the activation mechanism of War1 employing several approaches. We identified seven distinct mutations in *WAR1*, each of them leading to constitutive hyper-activity of the regulator triggering extremely high Pdr12 levels. Notably, a loss-of-function (R764 Δ) and two gain-of-function mutations (K762N and F763M) were identified earlier in the same region (21). One highly interesting allele is truncated, encoding a translational stop instead of the conserved tryptophan residue 936. Truncation of Put3 and Pdr1/Pdr3 also leads to constitutively active forms (41, 42). A similar effect was achieved by introducing a hemagglutinin tag at the very C terminus of Put3 (36), and a tandem affinity purification tag in the Pdr3 regulator.⁴ The other six residue exchanges localize to regions highly conserved between War1-like factors from other fungi, located between the so-called coiled-coil and the C-terminal activation domain. However, in our hands, constitutive *WAR1* mutant alleles do not confer higher levels of weak acid resistance. A possible beneficial effect of constitutive high Pdr12 levels is probably compensated by an efficient induction mechanism.

The region bridging the zinc finger and the activation domain in War1 clearly exerts regulatory functions. The so-called MHR is found in a large subset of the family but not War1 (43). Notably, mutations within this putative MHR cause hyperactivity of Leu3 and Pdr1/Pdr3 (16, 42, 44). Likewise, our data demonstrate that loss of the central region leads to constitutive War1 activity. MHR deletions also activate the zinc finger transcription activators Leu3 and Hap1 (18, 45). Furthermore, fusions of the Put3 activation domain to a heterologous DNA-binding domain cause hyperactivity, suggesting the existence of an internal inhibitory domain in Put3 (46). Our genome-wide microarray analysis suggests that the War1-K762R mutant allele has a very similar DNA-binding specificity when compared with the sorbate-induced wild type (22). Therefore, the War1 regulation is likely to be independent from the DNA-binding domain. Our data indicate that the central region and the extreme C terminus of War1 are essential for regulated function in weak acid stress adaptation.

The Role of Phosphorylation in War1 Regulation—In extracts from weak acid-stressed cells War1 undergoes a characteristic mobility shift in SDS-polyacrylamide gels reflecting phosphorylation changes (7). Furthermore, mass spectrometric analysis of activated *versus* normal War1 demonstrates a massive bulk phosphorylation of War1 after stress exposure. These data also show that phosphorylation of several residues of War1 is responsible for the appearance of slower migrating isoforms.⁵ Nonfunctional *WAR1* alleles are also not further phosphorylated in response to stress, strongly linking War1 activity to the phosphorylation status (21).

The cause and effect relationship between phosphorylation and transcription factor activation has remained an open question and thus has been under discussion for several yeast zinc cluster proteins (20). The War1 kinase(s) have not been identified, despite intense efforts through several biochemical and

⁴ Y. Mamnun, unpublished data.

⁵ I. E. Frohner, C. Gregori, C. Anrather, C. Schüller, G. Ammerer, and K. Kuchler, manuscript in preparation.

systematic genetic approaches. This implies either a marked functional redundancy or an essential function of the kinase(s) involved. In any case, our data clearly demonstrate that conformational changes of War1 must occur during the activation, whereas inactive mutants might lack the ability to undergo conformation changes upon stress.

War1 Transcriptional Activity Is Linked to Conformational Changes—Conformation changes and possible intra-molecular interactions of domains in zinc cluster regulators have been reported using two techniques. Protease digestion assays indicated different accessibility of domains in Put3 (36). Two-hybrid assays between separated parts of Leu3 suggested interactions between central and C-terminal regions (14, 15). Our attempts using similar two-hybrid approaches with War1 failed.⁶

In this work, however, we report dramatic changes in the chromatin association of War1 in stressed cells using ChIP assays. This result is further supported by increased constitutive binding of dominant mutants, whereas an inactive War1 variant displays only weak promoter association. The increase in promoter occupancy observed after stress is likely to result from conformational changes, potentially influencing the War1 on/off rate to promoter DNA or the affinity through stabilizing homodimerization. Alternatively, activated War1 could recognize binding sites otherwise not detected by regular footprinting approaches.

To gain further support for possible conformation changes, we used a special FRET setup with a double-tagged, functional War1 carrying CFP and YFP at the N and C terminus, respectively. For detection sensitivity reasons, we had to increase gene dosage of War1 for the FRET approach. We feel that this is acceptable, because simple overexpression of wild type War1 fails to cause elevated weak acid resistance, suggesting that only the activated state is functionally relevant. FRET approaches were successfully used to show interactions of Gal4 with Gal80 and subunits of the SAGA complex (47). Moreover, FRET-based sensors have been used to measure dynamic intramolecular changes in response to biochemical signals (reviewed in Ref. 48). Our FRET analysis suggests that stress exposure rapidly triggers conformational changes in War1. Furthermore, inactive and hyperactive War1 mutant alleles seem locked in one conformation only, active or inactive, as they show constitutive changes in the FRET and ChIP assays. Strikingly, these changes also suggest that hyperactive War1 alleles mimic a stress conformation, whereas inactive alleles resemble the unstressed state, which may be considered a “sensing” mode. Furthermore, the identification of a hyperactive allele lacking the last nine residues is consistent with the idea of long range interactions between distant regions or domains of War1. The stress adaptation process is rapid and efficient, leading to the rapid appearance of Pdr12 at the cell surface. Hence, we would speculate that activated War1 may also cause an enhanced recruitment of the mediator complex. Indeed, drug-induced interaction of Pdr1 and Pdr3 with the mediator complex subunit Gal11 was recently demonstrated (11). Additional experi-

ments will clarify whether the sensing conformation of War1 requires a direct binding of weak acids for conversion into the activated state. Finally, long term attempts to obtain high resolution structural information on War1 will reveal the nature of activated *versus* sensing conformations and thus the molecular mechanism of War1 function.

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