

# The WD40 Domain Protein MSI1 Functions in a Histone Deacetylase Complex to Fine-Tune Abscisic Acid Signaling

Saher Mehdi,<sup>a,1</sup> Maria Derkacheva,<sup>a,b,1</sup> Margareta Ramström,<sup>c</sup> Lejon Kralemann,<sup>a</sup> Jonas Bergquist,<sup>c</sup> and Lars Hennig<sup>a,2</sup>

<sup>a</sup>Department of Plant Biology, Uppsala BioCenter, Swedish University of Agricultural Sciences and Linnean Center for Plant Biology, SE-75007 Uppsala, Sweden

<sup>b</sup>Department of Biology and Zurich-Basel Plant Science Center, ETH Zurich, CH-8092 Zurich, Switzerland

<sup>c</sup>Department of Chemistry, Analytical Chemistry and Science for Life Laboratory, Uppsala University, SE-75124 Uppsala, Sweden

ORCID IDs: 0000-0003-4602-9821 (S.M.); 0000-0001-8355-6546 (M.D.); 0000-0003-1471-7291 (M.R.); 0000-0002-8154-9315 (L.K.); 0000-0002-6645-1862 (L.H.)

**MSI1 belongs to a family of histone binding WD40-repeat proteins. *Arabidopsis thaliana* contains five genes encoding MSI1-like proteins, but their functions in diverse chromatin-associated complexes are poorly understood. Here, we show that MSI1 is part of a histone deacetylase complex. We copurified HISTONE DEACETYLASE19 (HDA19) with MSI1 and transcriptional regulatory SIN3-like proteins and provide evidence that MSI1 and HDA19 associate into the same complex in vivo. These data suggest that MSI1, HDA19, and HISTONE DEACETYLATION COMPLEX1 protein form a core complex that can integrate various SIN3-like proteins. We found that reduction of MSI1 or HDA19 causes upregulation of abscisic acid (ABA) receptor genes and hypersensitivity of ABA-responsive genes. The MSI1-HDA19 complex fine-tunes ABA signaling by binding to the chromatin of ABA receptor genes and by maintaining low levels of acetylation of histone H3 at lysine 9, thereby affecting the expression levels of ABA receptor genes. Reduced MSI1 or HDA19 levels led to increased tolerance to salt stress corresponding to the increased ABA sensitivity of gene expression. Together, our results reveal the presence of an MSI1-HDA19 complex that fine-tunes ABA signaling in *Arabidopsis*.**

## INTRODUCTION

Proteins related to budding yeast (*Saccharomyces cerevisiae*) MSI1 form a family of WD40 domain proteins found in all eukaryotes (Ruggieri et al., 1989; Hennig et al., 2005). MSI1-like proteins bind histones and can tether various protein complexes that act on histones or chromatin to their substrates (reviewed in Hennig et al., 2005). In *Arabidopsis thaliana*, this protein family contains the five members MSI1 to MSI5 (Ach et al., 1997; Kenzior and Folk, 1998; Hennig et al., 2003). *Arabidopsis* MSI4 and MSI5 associate with the histone deacetylases HDA5 and HDA6 and are involved in deacetylation and silencing of *FLOWERING LOCUS C* and endogenous small interfering RNA-directed DNA methylation target loci (Ausín et al., 2004; Kim et al., 2004; Gu et al., 2011; Luo et al., 2015). *Arabidopsis* MSI1 is a subunit of chromatin assembly factor CAF-1 and of various Polycomb-repressive complexes (Kaya et al., 2001; Köhler et al., 2003; De Lucia et al., 2008; Derkacheva et al., 2013). Recently, MSI1 was shown to also copurify with HISTONE DEACETYLASE19 (HDA19) (Derkacheva et al., 2013), but it is not known whether MSI1 functions in histone deacetylation.

HDA19 and HDA6 belong to the RPD3/HDA1 superfamily containing homologs of REDUCED POTASSIUM DEPENDENCY3 (RPD3) from budding yeast (reviewed in Hollender and Liu, 2008).

RPD3 is a core subunit of the SWI-INDEPENDENT3 (SIN3) complex that is conserved in yeast and animals (Carrozza et al., 2005; Keogh et al., 2005; Grzenda et al., 2009). In animals, the core SIN3 complex includes SIN3, an RPD3 homolog, the SIN3-associated proteins SAP18 and SAP30, and an MSI-like protein (Hassig et al., 1997; Zhang et al., 1997). *Arabidopsis* contains six homologs of SIN3: the SIN3-like proteins SNL1 to SNL6 (Bowen et al., 2010). HDA19 was shown to interact with SNL3 (Song et al., 2005) in yeast two-hybrid assays and with SNL1 in bimolecular fluorescence complementation assays (Wang et al., 2013). In yeast two-hybrid assays, *Arabidopsis* HDA6 and HDA19 both also interacted with a homolog of the animal SIN3 complex subunit SAP18 (Song and Galbraith, 2006; Hill et al., 2008). The yeast SIN3 complex also contains the subunit RXT3 (Carrozza et al., 2005), and the *Arabidopsis* RXT3-domain protein HISTONE DEACETYLATION COMPLEX1 (HDC1) was shown to interact with HDA19 and HDA6 in bimolecular fluorescence complementation assays (Perrella et al., 2013). These findings suggest that SIN3-related complexes are present in plants, but additional biochemical and molecular evidence is needed.

Histone deacetylases can be recruited to target genes by DNA binding transcriptional regulators and are often involved in a wide range of processes. However, the six SIN3-like, five MSI1-like, and 12 RPD3-like proteins in *Arabidopsis* may form a large number of SIN3-like complexes with potentially specialized functions, and more work is needed to assign physiological functions to distinct histone deacetylase complexes. Both HDA6 and HDA19 have been implicated in abscisic acid (ABA)-mediated responses to drought and high salinity (Chen et al., 2010; Chen and Wu, 2010; Luo et al., 2012b). Similarly, HDC1 was shown to affect responses

<sup>1</sup> These authors contributed equally to this work.

<sup>2</sup> Address correspondence to lars.hennig@slu.se.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Lars Hennig (lars.hennig@slu.se).

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**Table 1.** HDA19 Copurifies with Homologs of Subunits of the SIN3 Complex

Gene ID	Name	No. of Unique Peptides	IP1-IP2-IP3-IP4	Sequence Coverage (%)	IP1-IP2-IP3-IP4	Sequest Score	IP1-IP2-IP3-IP4
AT4G38130	HDA19	19-7-15-5		52-15.8-54.3-12.8		213-181-190-46	
AT5G58230	MSI1	5-3-5-4		22.6-7.8-17.2-14.4		16.7-18.3-22.7-34	
AT3G01320	SNL1	5-4-3-2		5.4-4.1-4.3-3		23.6-20.9-27.9-21	
AT5G15020	SNL2	20-10-14-8		19.6-9.3-14-8.6		97.8-54.7-121-67	
AT1G24190	SNL3	15-5-6-2		13.8-5.3-4.9-1.9		49-28.5-32.8-6.3	
AT1G70060	SNL4	4-2-0-1		4.8-2.6-0-1		20-19.6-0-2.6	
AT1G59890	SNL5	24-9-13-8		25.8-9.7-14.7-9.2		84.9-46.9-120-96	
AT1G10450	SNL6	11-14-8-5		11.9-13-9.2-5.2		42.5-101-105-29.3	
AT5G08450	HDC1	14-14-14-0		18.3-16.3-23.2-0		44.3-60-89-0	
AT3G20050	ATTCP-1	10-5-3-4		22-9.5-5.7-7.5		30.7-19.7-10.3-12.8	

HDA19 binding proteins were identified by immunoaffinity purification of HDA19:GFP from *Pro35S:HDA19:GFP* plants and mass spectrometry. The experiment was performed with four biological replicates. Shown are all proteins identified in the *Pro35S:HDA19:GFP* but not in a *Pro35S:GFP* line.

to abiotic stress and ABA (Perrella et al., 2013). Part of HDA19's function is mediated by its association with ERF transcription repressors to regulate gene expression in response to abiotic stresses (reviewed in Luo et al., 2012a). However, it is likely that HDA19 complexes can also be recruited by other DNA binding proteins. Interestingly, a role in abiotic stress responses has also been reported for MSI1, which appears to be independent of the well studied MSI1-containing CAF-1 and Polycomb-repressive complexes (Alexandre et al., 2009). In particular, reduction in MSI1 levels led to increased expression of many genes with ABA-responsive elements (ABREs) in their promoters (Alexandre et al., 2009). Because MSI1 copurified with HDA19 and SIN3-like proteins (Derkacheva et al., 2013) and both MSI1 and HDA19 have been implicated in abiotic stress responses, we hypothesized that MSI1 affects ABA responsive genes via functioning in a SIN3 histone deacetylase complex.

Here, we tested this hypothesis and provide several lines of experimental evidence in its support. First, biochemical evidence for a MSI1-HDA19 SIN3-like complex is shown: MSI1 and HDA19 can interact in yeast and exist in complex(es) of ~600 kD in plants. HDA19 interacting partners were purified *in vivo*, and MSI1 as well as a common set of proteins that interact with both MSI1 and HDA19 were identified. Reduction of MSI1 or HDA19 levels caused hypersensitivity of ABA-regulated genes. MSI1 and HDA19 bind to the chromatin of ABA receptor genes *PYL4*, *PYL5*, and *PYL6* where they reduce histone acetylation and transcription, suggesting a model in which the effects of MSI1 and HDA19 on ABA sensitivity of gene expression are caused by increased expression of ABA receptor genes. Finally, genetic double mutant analysis confirmed that *MSI1* and *HDA19* function together in ABA receptor gene expression and ABA sensitivity of gene expression.

## RESULTS

### MSI1 Copurifies with HDA19 and SNLs

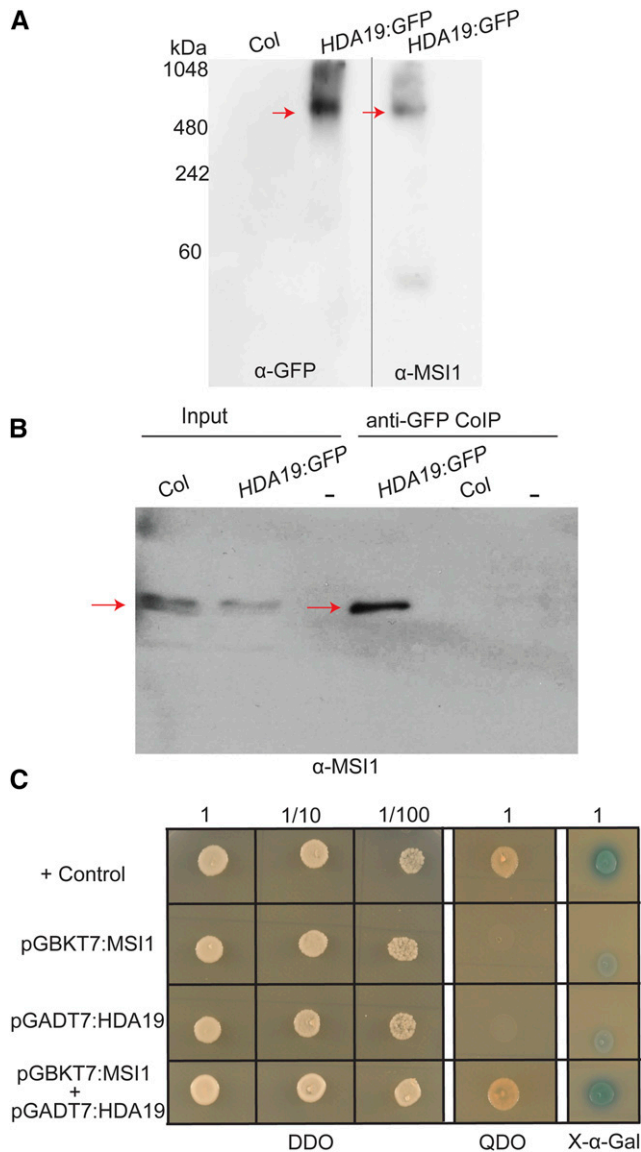
HDA19 is a candidate interaction partner of MSI1 (Derkacheva et al., 2013). To characterize HDA19 complexes *in vivo*, we used an Arabidopsis line that expressed GFP-tagged HDA19 (Fong et al., 2006) and immunoaffinity purified an HDA19:GFP complex

(see Methods for details). An Arabidopsis line expressing GFP (Derkacheva et al., 2013) was used as control. The purified fractions of four independent experiments were analyzed by high-resolution tandem mass spectrometry (MS/MS). Only proteins identified with at least two peptides in all four replicates but not in control samples were taken into account. HDA19 was found to copurify with all six SNL proteins (SNL1 to SNL6; Table 1), suggesting that HDA19 is a part of SIN3 complexes in plants. Biochemical identification of SNL1, SNL3, and HDC1 as interaction partners of HDA19 is in agreement with earlier reports based on yeast two-hybrid and bimolecular fluorescence complementation assays (Song et al., 2005; Perrella et al., 2013; Wang et al., 2013). In addition, the chaperonin ATTCP-1 (AT3G20050) was consistently found together with HDA19. This is consistent with a report that the mammalian TCP-1 Ring complex binds to the RPD3-like HDAC3 (Guenther et al., 2002). Finally, MSI1 but not MSI2-5 was found to copurify with HDA19 *in vivo* (Table 1). This finding, together with our previous results (Derkacheva et al., 2013), firmly establish that MSI1 acts together with HDA19 in Arabidopsis.

To further support that MSI1 and HDA19 are part of a common protein complex, we analyzed MSI1- and HDA19-containing complexes by native PAGE. Indeed, both HDA19 and MSI1 migrate as part of complexes of similar molecular weight (~600 kD; Figure 1A). Whereas HDA19 was mostly present in the high molecular weight fraction, for MSI1, signals of monomers were clearly detected as well. We also performed a coimmunoprecipitation assay using the *HDA19:GFP* line. Immunoblot analysis showed that MSI1 coprecipitated with HDA19, demonstrating that these proteins associate into the same complexes *in vivo* (Figure 1B). In addition, MSI1 and HDA19 also interacted in yeast two-hybrid assays, suggesting that the interaction is direct (Figure 1C).

Next, we tested whether *MSI1* and *HDA19* are coexpressed, which is required for a joint function. Using a compendium of transcript data for 79 developmental stages, organs, and tissues (Schmid et al., 2005), we observed a strong positive Pearson correlation of 0.95 for *MSI1* and *HDA19* expression (Supplemental Figure 1). In fact, *HDA19* was one of the genes most strongly correlated in expression with *MSI1*, and only 11 of the 21,500 probed genes had stronger correlation. This result demonstrates that *MSI1* and *HDA19* expression allows MSI1-HDA19 complex formation.

Common interaction partners of both MSI1 and HDA19 suggest that MSI1, HDA19, SNL2, SNL3, SNL4, and HDC1 could function



**Figure 1.** HDA19 Associates into the Same Complex with MSI1 in Vivo.

**(A)** MSI1 and HDA19 are components of the same histone deacetylase complex. HDA19:GFP and MSI1 were found to be part of 500- to 600-kD complexes (red arrows) detected by anti-GFP and anti-MSI1 using native PAGE. No anti-GFP signal was detected in wild-type Col. Specificity of the anti-MSI1 antibody was shown before (Hennig et al., 2003).

**(B)** MSI1 copurifies with HDA19. HDA19:GFP (red arrows) was precipitated from inflorescences of wild-type and *Pro35S:HDA19:GFP* plants. Precipitates were analyzed by immunoblotting using anti-MSI1 antibodies.

**(C)** MSI1 interacts with HDA19 in a yeast two-hybrid assay. Only yeast cotransformed with bait (pGBKT7:MSI1) and prey (pGADT7:HDA19) plasmids grow on selective medium (GDO, quadruple dropout, SD/-Ade/-His/-Leu/-Trp) and give a positive X-Gal signal (DDO, double dropout, SD/-Leu/-Trp without or with X-α-Gal).

in the same complex(es) in vivo. MSI1, HDA19, and HDC1 could form a core SIN3-like complex in vivo, while diverse associating SNLs could provide targeting specificity via binding to different transcription factors.

### Reduction of MSI1 and HDA19 Levels Causes Upregulation of ABA-Responsive Genes

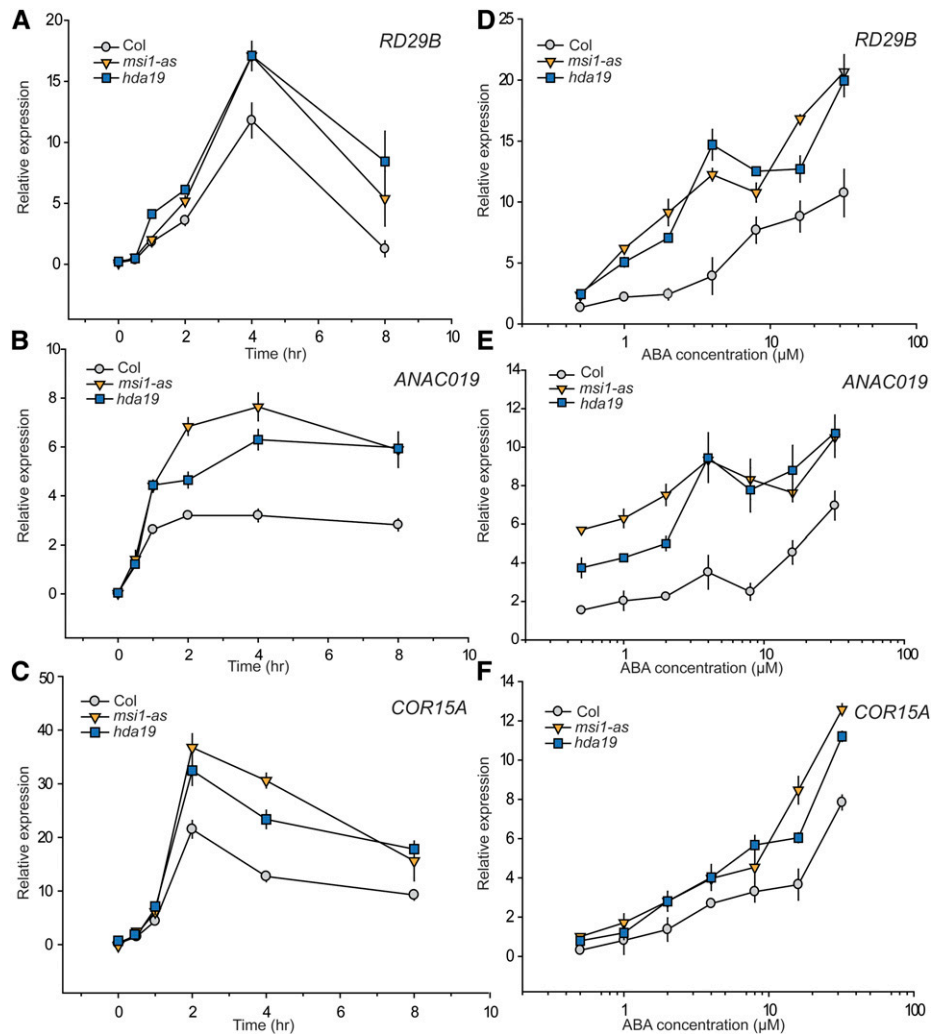
Various components of histone deacetylase complexes have been implicated in plant responses to biotic and abiotic stress (Chen and Wu, 2010; Perrella et al., 2013; Wang et al., 2013), and reduction of MSI1 levels can affect the expression of genes with ABREs in their promoters (Alexandre et al., 2009). Because ABA is a major mediator of responses to abiotic stress, we tested a potential role of MSI1 and HDA19 in activation of ABA-responsive genes using MSI1 antisense (*msi1-as*) and HDA19 RNAi (*hda19*) Arabidopsis lines. Ten-day-old seedlings of the wild type, *msi1-as*, and *hda19* were treated with 5  $\mu$ M ABA hydroponically for 8 h, and aliquots were harvested for RNA extraction every 2 h. Expression of three ABA-responsive genes was tested: *RD29B* (*RESPONSIVE TO DESSICATION29B*) (Msanne et al., 2011), *ANACO19* (*NAC DOMAIN CONTAINING PROTEIN19*) (Jensen et al., 2010), and *COR15A* (*COLD REGULATED15A*) (Baker et al., 1994; Steponkus et al., 1998), which all have ABREs in their promoter regions (Choi et al., 2000). After exposure to ABA, transcript levels of all three genes were considerably higher in *msi1-as* and *hda19* lines compared with the wild type at all time points (Figures 2A to 2C). Notably, the activation kinetics were not grossly changed but remained similar to that in wild-type plants.

To test whether the higher expression of stress-responsive genes in the *msi1-as* and *hda19* lines reflected increased sensitivity of gene expression to ABA, we treated 10-d-old seedlings of the wild type, *msi1-as*, and *hda19* with concentration between 0.5 and 32  $\mu$ M of ABA hydroponically for 4 h before harvesting material for RNA extraction. Consistent with the time course expression results, at most ABA concentrations, we observed higher expression of *RD29B*, *ANACO19*, and *COR15A* in *msi1-as* and *hda19* compared with the wild type (Figures 2D to 2F).

Together, these results demonstrate that several ABA-responsive genes respond much more strongly to ABA if MSI1 or HDA19 levels are decreased.

### *msi1-as* and *hda19* Plants Have Increased Transcript Levels of ABA Receptor Genes

A family of PYR/PYL receptor proteins mediates ABA signaling in plants (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009). ABA binding to PYR/PYLs increases their ability to bind and inhibit type 2C protein phosphatases (PP2Cs). In the absence of ABA, PP2Cs bind and inactivate SNF1-related kinases (SnRK2 kinases) (Umezawa et al., 2009). Inactivation of PP2Cs by PYR/PYLs leads to activation of SnRK2, which in turn causes activation of basic leucine-zipper transcription factors called ABFs/AREBs that bind ABREs and switch on stress response genes (Kobayashi et al., 2005; Furihata et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007). PYL4 interacts with PP2CA in an ABA-dependent manner (Lackman et al., 2011; Pizzio et al., 2013) and shows high expression levels in many tissues, and its inactivation is needed to generate ABA-insensitive mutants (Gonzalez-Guzman et al., 2012). This indicates that PYL4 is one of the key players in the ABA receptor family. Interestingly, *PYL4* acetylation and expression were previously reported to be affected by altered HDC1 levels (Perrella et al., 2013). We tested a potential role of the MSI1-HDA19 complex in ABA signaling at the level of receptor gene expression by measuring transcripts of *PYL4* and its two close homologs,



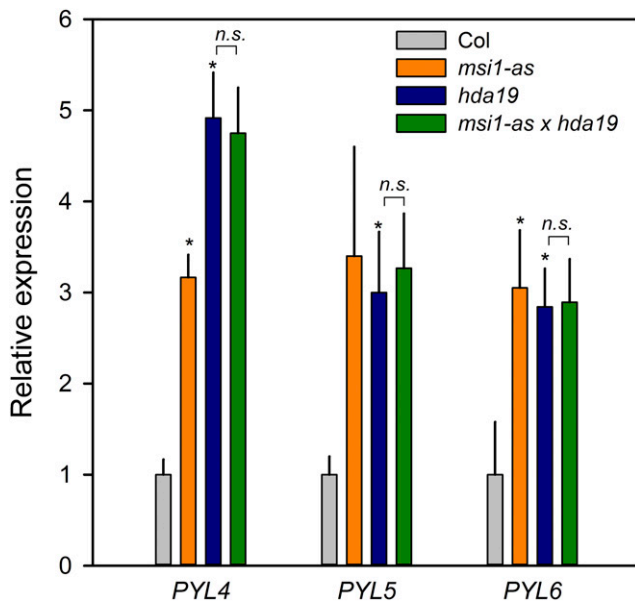
**Figure 2.** Both *msi1-as* and *hda19* Mutants Show Upregulation of ABA-Responsive Genes over Time in an ABA Dose-Dependent Manner.

(A) to (C) Expression kinetics of *RD29B*, *ANACO19*, and *COR15A*. Relative change in expression over 8 h of *RD29B*, *ANACO19*, and *COR15A* in the wild type (gray circles), *msi1-as* (orange triangles), and *hda19* (blue squares), respectively. Differences from the wild type are significant (two-sided *t* test,  $P \leq 0.05$ ) for all genes for time points at 3 h and longer and for *ANACO19* also at 1 h.

(D) to (F) ABA dose dependency of *RD29B*, *ANACO19*, and *COR15A* expression. Relative change in expression of *RD29B*, *ANACO19*, and *COR15A* in the wild type (gray circles), *msi1-as* (orange triangles), and *hda19* (blue squares), respectively, treated with different concentrations of ABA for 4 h. Differences from the wild type are significant ( $P \leq 0.05$ ) for *RD29B* above 0.5 μM ABA, for *ANACO19* at all concentrations, and for *COR15A* above 10 μM ABA. Expression levels are relative to *PP2A*. Graphs show the mean  $\pm$  SE of three biological replicates.

*PYL5* and *PYL6*, in *msi1-as*, *hda19*, and the wild type. Indeed, *PYL4*, *PYL5*, and *PYL6* transcript levels were increased in the lines with reduced levels of MSI1 and HDA19 (Figure 3). *PYL4*, *PYL5*, and *PYL6* are also examples of genes that are downregulated by ABA (Goda et al., 2008; Perrella et al., 2013). Notably, this repression by ABA was considerably stronger in *msi1-as* and *hda19* than in the wild type, demonstrating that not only ABA-induced but also ABA-repressed genes react more strongly to ABA in plants with reduced levels of MSI1 and HDA19 (Supplemental Figure 2). These results show that overexpression of *PYL4*, *PYL5*, and *PYL6* in *msi1-as* and *hda19* is alleviated by ABA and argue that although MSI1 and HDA19 affect the expression of *PYL4*, *PYL5*, and *PYL6*, they are not required for their ABA-dependent repression.

Increased expression of genes for ABA receptors is consistent with the increased ABA sensitivity of ABA-activated and ABA-repressed genes in plants with reduced MSI1 and HDA19 levels. To test whether MSI1 and HDA19 act in the same genetic pathway to affect *PYL4*, *PYL5*, and *PYL6* expression, we crossed the *msi1-as* and *hda19* lines and measured *PYL4*, *PYL5*, and *PYL6* expression (Figure 3). There was no further increase in *PYL* gene expression in the double mutant, suggesting that MSI1 and HDA19 function together in the same genetic pathway to affect *PYL* gene transcription, consistent with the protein-protein interaction data. Because ABA responses are sensitive to the dosage of *PYL* genes (Gonzalez-Guzman et al., 2012) and because overexpression of *PYL4* or *PYL5* increases ABA sensitivity



**Figure 3.** Increased Transcript Levels of ABA Receptors in *msi1-as* and *hda19*.

Relative expression of *PYL4*, *PYL5*, and *PYL6* in the wild type (gray), *msi1-as* (orange), *hda19* (blue), and *msi1-as hda19* (green). Expression levels are relative to *PP2A* and further normalized to the wild type. Graphs show the mean  $\pm$  SE of three biological replicates. Asterisks indicate values that are significantly larger ( $P \leq 0.05$ ) than in the wild type. *n.s.* indicates no significant difference between *msi1-as hda19* and *hda19*.

(Santiago et al., 2009; Pizzio et al., 2013), increased expression of *PYL4*, *PYL5*, and *PYL6* can explain the increased ABA sensitivity of gene expression in *msi1-as* and *hda19* plants.

### MSI1 Binds to Chromatin at the *PYL4*, *PYL5*, and *PYL6* Genes and Maintains Low H3K9 Acetylation

Next, we tested whether regulation of ABA receptor gene expression by MSI1 and HDA19 was direct. We performed a chromatin immunoprecipitation (ChIP) assay using a line expressing GFP-tagged MSI1 in an *msi1* mutant background (Alexandre et al., 2009) and the *HDA19:GFP* line. High MSI1 and HDA19 recovery was observed around the *PYL4*, *PYL5*, and *PYL6* transcriptional start sites (Figure 4). In contrast, only background signals were observed in regions more distant to the transcriptional start sites in a GFP control line and in IgG control reactions (Figure 4; Supplemental Figure 3). Thus, the effects of MSI1 and HDA19 on *PYL4*, *PYL5*, and *PYL6* expression involve direct binding to chromatin at the ABA receptor genes.

MSI1 and HDA19 function together in a complex in Arabidopsis. It is possible that altered expression of *PYL4*, *PYL5*, and *PYL6* in *msi1-as* and *hda19* is associated with increased histone acetylation. To test this hypothesis, we performed ChIP with antibodies against histone H3 acetylated at lysine 9 (H3K9ac) in wild-type, *msi1-as*, and *hda19* plants. In the wild type, levels of H3K9ac at *PYL4*, *PYL5*, and *PYL6* were not considerable higher than the level in an intergenic control region or IgG control values (Figure 5; Supplemental Figure 4). In contrast, in both *msi1-as* and *hda19*, H3K9ac levels were considerably increased around the transcriptional start site (Figure 5).

Thus, MSI1 and HDA19 are both needed to maintain low histone acetylation at *PYL4*, *PYL5*, and *PYL6*.

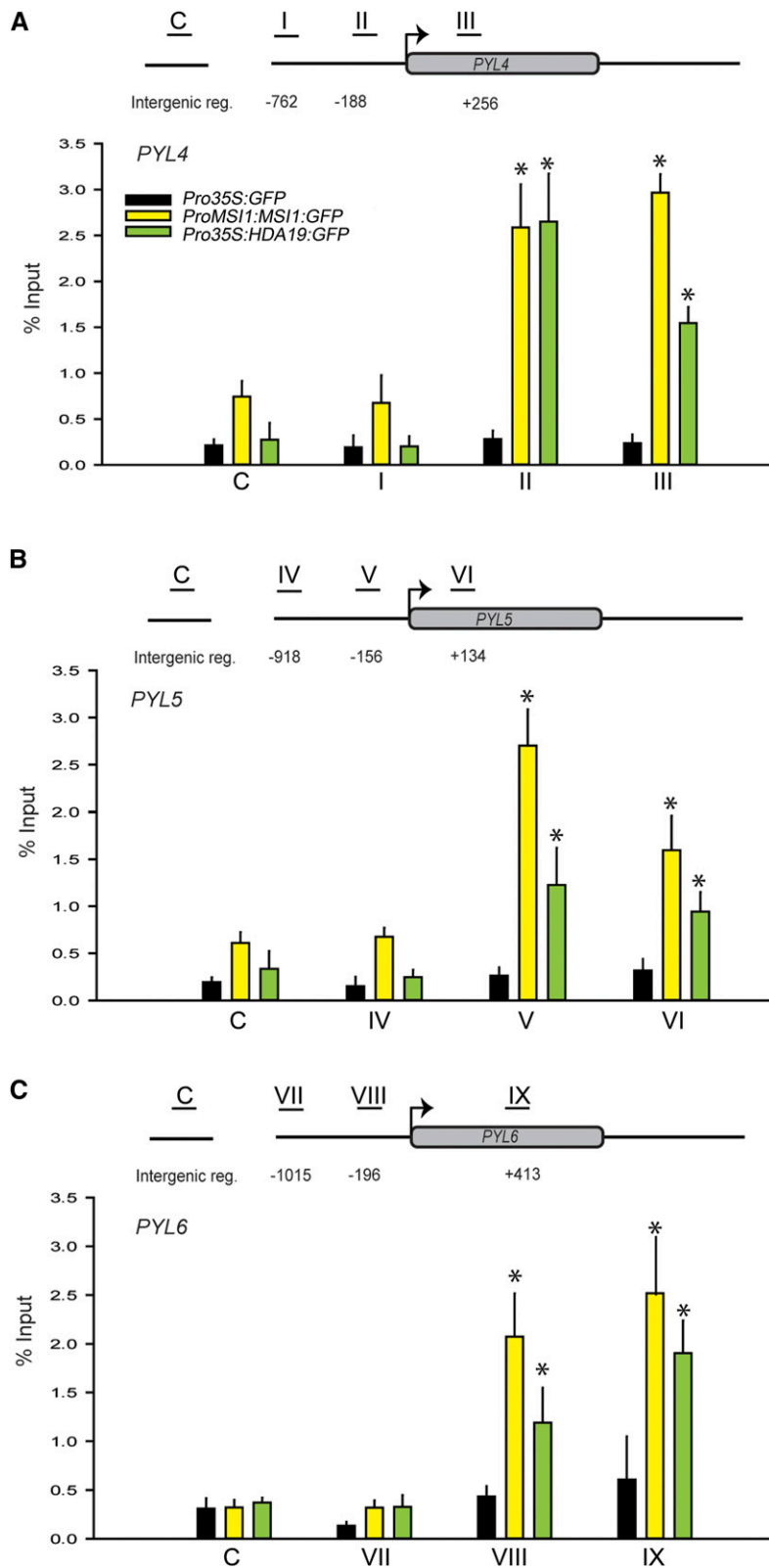
The notion that the MSI1-HDA19 complex is recruited to chromatin at *PYL4*, *PYL5*, and *PYL6* genes and represses their transcription implies that HDAC inhibitors should mirror the effects of reduced MSI1 or HDA19 dosage. To test this hypothesis, we measured the effect of the HDAC inhibitor trichostatin A (TSA) on *PYL4*, *PYL5*, and *PYL6* expression in wild-type, *msi1-as*, and *hda19* plants (Supplemental Figure 5). In the wild type, TSA indeed caused an increase in expression of the three genes similar to that observed in *hda19* plants. In *hda19* plants, however, TSA did not lead to a considerable further increase in *PYL4*, *PYL5*, and *PYL6* transcript levels, suggesting that HDA19 is the major HDAC involved in regulating *PYL4*, *PYL5*, and *PYL6* expression. The *msi1-as* plants were still responsive to TSA in this assay, probably because of the remaining MSI1 levels (~30% of wild-type levels) in this line (Exner et al., 2006). In contrast, TSA strongly increased *ANAC019*, *RD29B*, and *COR15A* transcript levels in unstressed wild-type, *msi1-as*, and *hda19* plants (Supplemental Figure 6), showing that TSA treatment has effects on *msi1-as* and *hda19* plants and suggesting that HDACs other than HDA19 maintain low *ANAC019*, *RD29B*, and *COR15A* expression in unstressed plants. Together, these results are consistent with the notion that HDAC activity mediates the effects of MSI1 and HDA19 on *PYL* gene expression.

### Loss of MSI1 or HDA19 Leads to Increased Tolerance to Salt Stress

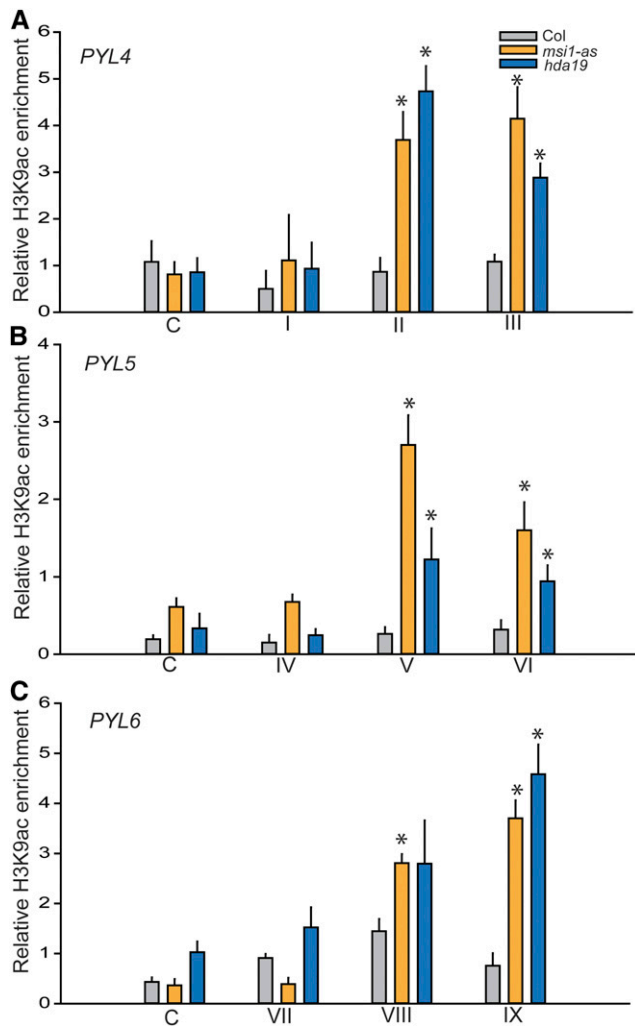
Because MSI1 and HDA19 dampen ABA-responsive gene expression in seedlings, it was possible that the gene expression response to abiotic stress was also altered in *msi1-as* and *hda19* plants. To test this hypothesis, we exposed wild-type, *msi1-as*, and *hda19* plants to salt and tested the expression of the salt-responsive gene *RD29B*. Consistent with the increased ABA sensitivity of gene expression, we detected increased salt sensitivity of gene expression as well (Supplemental Figure 7). Next, we tested whether the physiological response to salt was also altered in *msi1-as* and *hda19* plants. Seven-day-old seedlings were transferred onto medium containing 150 mM NaCl. This severe stress causes chlorophyll loss, which can be considerably delayed by ABA (Figure 6). Similarly, *msi1-as* and *hda19* plants tolerated 150 mM salt much longer than the wild type, as evident from the delayed chlorophyll loss (Figure 6). TSA pretreatment for 48 h delayed chlorophyll loss in the wild type considerably but had only a minor effect on *hda19*, consistent with the notion that HDAC activity, mainly contributed by HDA19, is required for normal chlorophyll loss upon salt exposure (Figure 6C). Notably, TSA affected chlorophyll loss in *msi1-as*, which is similar to its effects on *PYL4*, *PYL5*, and *PYL6* expression in this background. Together, the results indicate that the increased ABA sensitivity of gene expression in *msi1-as* and *hda19* plants was accompanied by increased tolerance to salt stress.

## DISCUSSION

HDAC protein complexes play key roles in the regulation of gene expression in diverse eukaryotes, such as budding yeast, *Drosophila melanogaster*, and mammals (Yang and Seto, 2008). In Arabidopsis, HDA6 and HDA19 are the most widely studied



**Figure 4.** MSI1 and HDA19 Bind to Chromatin at ABA Receptor Genes.



**Figure 5.** MSI1 and HDA19 Affect the H3K9 Acetylation Status at ABA Receptor Genes.

The graphs show increased H3K9ac levels in *msi1-as* (orange) and *hda19* (blue) compared with the wild type (gray) at the *PYL4*, *PYL5*, and *PYL6* locus, respectively. Fragment labels below the graphs are as in Figure 4. Anti-H3K9ac ChIP-qPCR was performed using 14-d-old plants. Values represent recovery expressed as relative enrichment of H3K9ac compared with H3 levels. Results of IgG control ChIP assays were plotted separately (Supplemental Figure 3). Shown are means  $\pm$  SE of three biological replicates. Asterisks indicate significant ( $P \leq 0.01$ ) difference from the wild type.

HDACs, and both are involved in a wide range of developmental processes and environmental responses (Tian and Chen, 2001; Tian et al., 2003; Zhou et al., 2005; Long et al., 2006; Chen and Wu, 2010; Jang et al., 2011). Given that HDA6 and HDA19 are closely

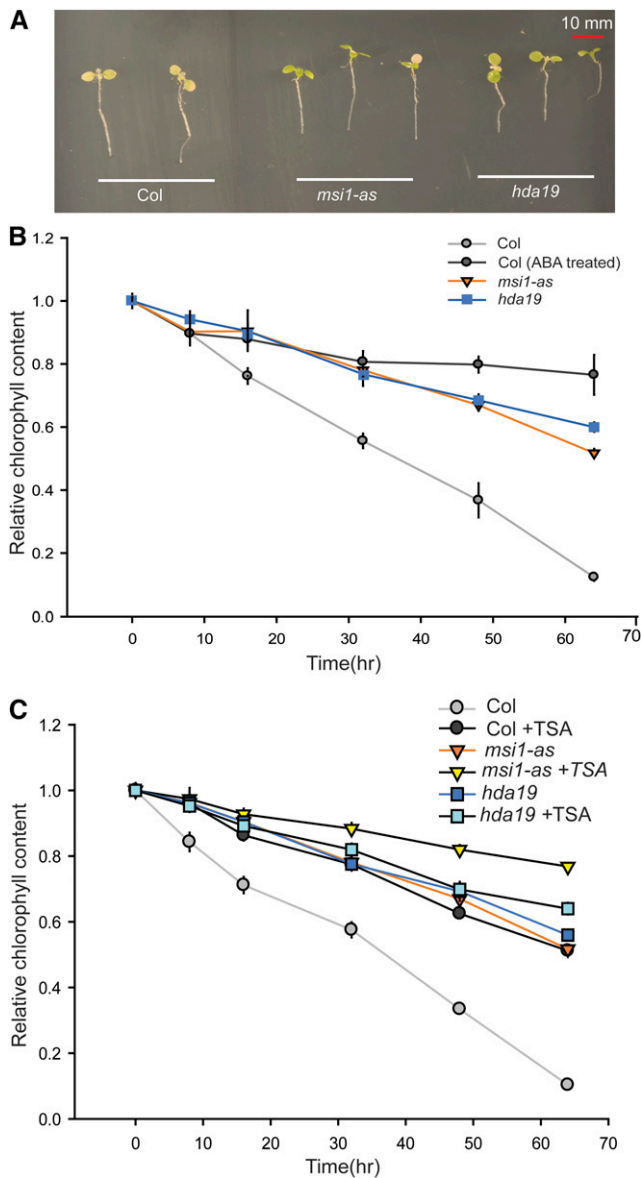
related, it is not surprising that they have partly overlapping functions. Both enzymes are involved in pathogen and jasmonic acid responses (Zhou et al., 2005; Wu et al., 2008) as well as in suppression of embryonic programs after germination (Tanaka et al., 2008), abiotic stress responses (Chen and Wu, 2010), promotion of flowering, and senescence (Wu et al., 2008; Yu et al., 2011). However, HDA6 also has specific functions in silencing of transposons, transgenes, and rRNA genes (Aufsatz et al., 2007; To et al., 2011; Liu et al., 2012). HDA6 forms complexes with MSI4 and its homolog MSI5 (Gu et al., 2011). These complexes are recruited to target genes where they lead to histone deacetylation and transcriptional gene silencing (Gu et al., 2011). In contrast to HDA6, HDA19 is presumably localized to euchromatin and is excluded from the nucleolus (Fong et al., 2006). We demonstrate that HDA19 and MSI1 form a complex and share common biological functions. Our data did not reveal any association of MSI1 with HDA6 or of MSI4/5 with HDA19, suggesting that these proteins form separate RPD3-like HDAC complexes in plants.

Arabidopsis MSI1 has been reported to be part of CAF-1 and several Polycomb-Repressive Complex 2 (PRC2)-like complexes such as the FERTILIZATION INDEPENDENT SEED, VERNALIZATION, and EMBRYONIC FLOWER complexes (Kaya et al., 2001; Köhler et al., 2003; De Lucia et al., 2008; Derkacheva et al., 2013). Because we found that MSI1 forms a complex with HDA19, it is possible that MSI1 links HDA19 to PRC2-like complexes or CAF-1. Notably, earlier work had suggested an association of RPD3 with PRC2 subunits in *Drosophila* (van der Vlag and Otte, 1999; Tie et al., 2001, 2003). In contrast, our work did not reveal any association of HDA19 with Arabidopsis PRC2 components. Similarly, a purification of the Arabidopsis VERNALIZATION PRC2 complex did not reveal an association with HDACs (De Lucia et al., 2008). This suggests that a potential association of the MSI1-HDA19 core complex with Arabidopsis PRC2 is not prevalent. Also, no other CAF-1 subunits or other known chromatin-related proteins were found to associate with HDA19, suggesting that the functions of the MSI1-HDA19 complex do not involve prevalent stable interactions with other chromatin-related protein complexes. Extending what has been known about MSI1 in PRC2 and CAF-1, we provide four key lines of evidence to support a role of MSI1 in a histone deacetylation complex to repress transcription. (1) MSI1 and HDA19 form a complex in vivo. (2) Reduction of MSI1 and HDA19 levels leads to increase transcript levels of the ABA receptor genes *PYL4*, *PYL5*, and *PYL6*. (3) Reduction of MSI1 and HDA19 levels leads to increased H3K9 acetylation levels at *PYL4*, *PYL5*, and *PYL6*. (4) MSI1 and HDA19 bind to the chromatin at *PYL4*, *PYL5*, and *PYL6*.

Loss of MSI1 is lethal and/or strong downregulation leads to severe developmental defects, making such lines inappropriate for the assays used here (Hennig et al., 2003; Köhler et al., 2003; Guitton et al., 2004). The report that MSI1 binds to the chromatin of

**Figure 4.** (continued).

ChIP was performed using 14-d-old *Pro35S::GFP*, *ProMSI1::MSI1::GFP*, and *Pro35S::HDA19::GFP* plants and anti-GFP antibodies. The graphs show MSI1 (yellow) and HDA19 (green) recovery at *PYL4*, *PYL5*, and *PYL6* compared with a *Pro35S::GFP* control (black). Schemes above the diagrams represent the recovery at *PYL4* (A), *PYL5* (B), and *PYL6* (C) loci. Black lines with Roman numerals represent the regions probed by PCR. Results of IgG control ChIP assays were plotted separately (Supplemental Figure 2). Values are recovery as percent of input; shown are mean  $\pm$  SE of three biological replicates. Asterisks indicate significant ( $P \leq 0.001$ ) difference from *Pro35S::GFP* controls.



**Figure 6.** *msi1-as* and *hda19* Have Increased Tolerance to Salt Stress.

**(A)** Performance of plants grown on NaCl. *msi1-as* and *hda19* plants remain green longer on 150 mM NaCl compared with the wild type.

**(B)** Change in chlorophyll content over time on 150 mM NaCl. *msi1-as* (orange), *hda19* (blue), and ABA-treated wild type (dark gray) have substantially more chlorophyll after 64 h than the wild type (gray). Differences from the wild type are significant (two-sided *t* test,  $P \leq 0.05$ ) for time points at 16 h and longer.

**(C)** Effect of pretreatment with TSA for 48 h on changes in chlorophyll content during exposure to salt (150 mM). Wild type treated with TSA (dark gray) show delayed loss of chlorophyll compared with the untreated wild type (gray). Differences from the wild type are significant ( $P \leq 0.05$ ) for time points at 8 h and longer. Shown are means  $\pm$  SE for three biological replicates.

the ABA-responsive gene *RD20*, which is upregulated even without exogenous ABA in adult plants of an Arabidopsis line with greatly reduced MSI1 levels and strongly altered development (Alexandre et al., 2009), is consistent with a role of MSI1 in gene

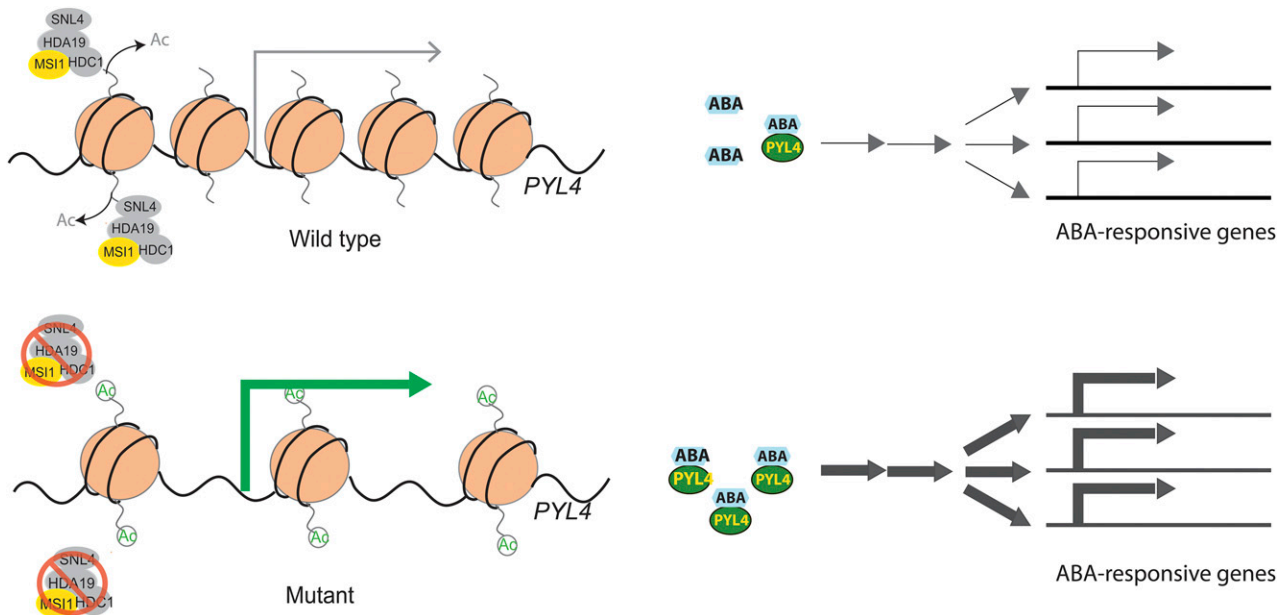
expression that goes beyond the effects of an MSI1-HDA19 complex on *PYL4*, *PYL5*, and *PYL6*, which was the focus of this study. To limit confounding effects of altered development, an antisense line of *MSI1* was used here, in which MSI1 levels were reduced to  $\sim 30\%$  of wild-type levels and which has only mild developmental alterations (Exner et al., 2006, 2008). However, the residual MSI1 levels in *msi1-as* may allow the formation of residual MSI1-HDA19 complexes that may still be sufficient to perform some functions. This could explain why *msi1-as* plants were responsive to TSA in some assays.

This study revealed a robust upregulation of *PYL4*, *PYL5*, and *PYL6* in plants with reduced MSI1 or HDA19 levels. An earlier transcriptome study had not identified these genes as upregulated in plants with reduced MSI1 levels, possibly due to the limited power of microarrays to robustly detect weakly expressed genes (Alexandre et al., 2009). Because inhibition of HDAC activity by TSA did not lead to activation of *PYL4*, *PYL5*, and *PYL6* beyond that observed in *hda19* plants, *PYL4*, *PYL5*, and *PYL6* expression appears to be mainly regulated by HDA19. In contrast, TSA treatment strongly activated *RD29B*, *COR15A*, and *ANAC019*, even in an *hda19* background. This is consistent with the notion that another HDAC complex can regulate these ABA-responsive genes. As MSI4 represses *COR15A* expression, binds to *COR15A* chromatin, and associates with HDA6 (Ausín et al., 2004; Kim et al., 2004; Gu et al., 2011; Jeon and Kim, 2011), it is possible that an MSI4-HDA6 complex directly regulates *COR15A*, while the MSI1-HDA19 complex indirectly regulates *COR15A* via affecting *PYL4*, *PYL5*, and *PYL6* expression.

In Arabidopsis, several components of potential RPD3-like HDAC complexes have been implicated to affect development. For example, SNL1 affects seed dormancy via changing the expression of ABA- and ethylene-responsive genes (Wang et al., 2013). Similarly, HDC1 affects plant growth via modulation of ABA-sensitive genes (Perrella et al., 2013). HDA19 and HSL1 may act together to repress the expression of seed maturation genes during germination (Zhou et al., 2013). Notably, many of the developmental roles that were described for HDA19 or other complex subunits may be explained by altered ABA signaling. We argue that at least some of these effects are mediated by repression of ABA receptor genes by an MSI1-HDA19 SIN3-like complex. First, our protein purification data establish that MSI1, HDA19, HDC1, and SNL1-6 can form a complex in vivo. Second, we found that MSI1 and HDA19 affect ABA sensitivity of gene expression via modulation of ABA receptor gene expression. These results are also consistent with earlier reports of the effects of MSI1 on the expression of ABA-responsive genes (Alexandre et al., 2009) and of ABA hypersensitivity of plants with reduced SNL3 levels (Song et al., 2005). Plants accumulate ABA in response to diverse abiotic stresses such as drought, cold, and salt; ABA then regulates the expression of many stress-responsive genes, promoting stress tolerance (reviewed in Nakashima and Yamaguchi-Shinozaki, 2013). Consistently, increased ABA sensitivity of gene expression in plants with reduced MSI1 or HDA19 levels was associated with increased tolerance to salt stress.

Together, we propose a model of how MSI1 functions as part of a histone deacetylase complex to fine-tune ABA-responsive gene expression (Figure 7). In wild-type plants, MSI1 together with HDA19 directly represses the expression of ABA receptor genes





**Figure 7.** Proposed Model of MSI1 Function in ABA Signaling.

In the wild type, a MSI1-HDA19 SIN3-like complex deacetylates chromatin at ABA receptor genes such as *PYL4* and thus maintains low transcription of the receptor genes (gray arrow). Increased expression of ABA receptor genes (green arrow) in *msi1* or *hda19* mutant plants causes increased expression of ABA-responsive genes in the presence of ABA (blue hexagons). Reduction of MSI1 or HDA19 levels has no strong effect on the expression of ABA-responsive genes in the absence of ABA.

such as *PYL4*, which causes dampening of ABA signaling. However, in the absence of MSI1 or HDA19, increased expression of ABA receptor genes leads to increased sensitivity of expression of ABA-responsive genes. However, ABA levels are high enough to efficiently activate ABA receptor-dependent activation of gene expression only upon stress or ABA treatment, while under control conditions, ABA-responsive genes have only small changes in basal expression. Because ABA represses *PYL4*, *PYL5*, and *PYL6*, their overexpression is alleviated by extended exposure to ABA, suggesting that the MSI1-HDA19 complex most strongly affects a subset of the initial responses to ABA in unstressed plants. Future work will focus on establishing how specificity in complex formation and target gene selection is established for the MSI1-HDA19 versus the MSI4/5-HDA6 complex.

## METHODS

### Plant Material

Seeds of *Arabidopsis thaliana* (accession Columbia) wild-type control and mutants were sterilized, stratified, and germinated on half-strength Murashige and Skoog medium and 0.8% agar at pH 5.7 in controlled growth rooms with 20 to 22°C and long-day conditions (16 h light/8 h dark; 110  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR full-spectrum white light supplied by Osram Lumilux cool white fluorescent tubes), unless stated otherwise. Ten-day-old seedlings were used for RNA extraction and ChIP experiments. For ABA and NaCl dose-response experiments, 10-d-old seedlings (five to eight) were transferred onto a 16-well hydroponic plate supplemented with either ABA, NaCl, or TSA at the concentration mentioned in the figures. Chlorophyll was assayed as described (Moran and Porath, 1980). The

*Pro35S::GFP*, *Pro35S::HDA19::GFP*, *ProMSI1::MSI1::GFP*, *HDA19 RNAi* (*hda19*), and *MSI1* antisense (*msi1-as*) plant lines were described before (Hennig et al., 2003; Zhou et al., 2005; Exner et al., 2006; Fong et al., 2006; Alexandre et al., 2009; Derkacheva et al., 2013). In the *HDA19 RNAi* lines, *HDA19* transcript levels are reduced to ~10% of the value in the wild type, and there is no considerable off-target effect on the *HDA19* homolog *HDA6* (Zhou et al., 2005; Supplemental Figure 8). Similarly, there are no considerable off-target effects on *MSI1* homologs in *msi1-as* lines (Hennig et al., 2003).

### Immunoprecipitation and Protein Immunoblot Analyses

For immunoprecipitation (IP) followed by mass spectrometry, 10 g of plant material was ground in a mortar with liquid nitrogen; for coimmunoprecipitation, 2 g of plant material was used. Soluble proteins were extracted in two volumes of extraction buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% IGEPAL, 1% Triton X-100, and protease inhibitors [Roche]) at 4°C for 30 min with gentle rocking. The centrifuged (4500g) supernatant was precleared with 30  $\mu\text{L}$  of prewashed protein A Sepharose beads (GE Healthcare) at 4°C for 20 min with gentle rocking. An input aliquot was taken from the precleared centrifuged (2000g) supernatant before the rest of the supernatant was subjected to IP with 50  $\mu\text{L}$  of bead-coupled antibodies at 4°C for 2 h with gentle rocking. The precipitate was washed six times with extraction buffer and eluted in 2 $\times$  Laemmli buffer. Anti-GFP Trap-A beads (*gta-10*; Chromotek) were used for IP. For protein immunoblots, proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride membrane (Roth) by semidry blotting in 25 mM Tris-HCl, pH 8.3, 150 mM glycine, and 10% methanol for 1 h at 15 V. Enhanced chemiluminescence detection was performed as recommended by the manufacturer (GE Healthcare). Mouse anti-MSI1 (Hennig et al., 2003) antibodies, 1:1000, were used for immunoblotting.

### MS/MS Analyses

After IP, the proteins were separated by 12% SDS-PAGE, lanes were cut into three sections, and in-gel digestion was performed (Shevchenko et al., 1996). The peptides from each tryptic digest were dissolved in 10  $\mu$ L of 0.1% formic acid (FA). Five microliters of each sample was injected on an EASY-nanoLC-system (Thermo Scientific). The enzymatic peptides were separated in reversed phase on a 10-cm-long C18-A2 column (i.d. 75  $\mu$ m; Thermo Scientific) using mobile phase A = 0.1% FA and B = 0.1% FA, 99.9% acetonitrile, and were then electrosprayed on-line to the mass spectrometer. Mass spectrometry measurements were performed on an LTQ Orbitrap Velos ETD mass spectrometer (Thermo Finnigan). MS/MS was performed, applying collision-induced dissociation in the LTQ mass spectrometer. MS/MS spectra were searched with Sequest (Eng et al., 1994) against the UNIPROT protein datABase, using Arabidopsis as a target organism. Carbamidomethylation of cysteine was set as fixed modification, and oxidation of methionine was set as a variable modification. The data were analyzed by Proteome Discoverer (Proteome Software). The cutoff for data analyses was set to a medium and high confidence for peptide identification. Proteins identified with at least two unique peptides in at least two replicates but never in control samples were taken into account.

### Native PAGE of Nuclear Proteins

Native protein was extracted from 1 g of 10-d-old wild-type and *HDA19:GFP* seedlings in 10 mL MEB extraction buffer (0.775 M hexylene glycol, 20 mM PIPES-KOH, pH 7.6, 10 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 60 mM KCl, 1  $\times$  Protease inhibitor, 0.5% Triton, and 5 mM  $\beta$ -mercaptoethanol) using a MACS dissociator (Miltenyi Biotec). The extract was filtered through two layers of Miracloth, and the filtrate was centrifuged at 1500g at 4°C for 10 min. The extracted nuclei were suspended in 500 mL of protein extraction buffer (65 mM Tris-HCl, pH 7.4, 10% glycerol, and 0.05% bromophenol blue). Proteins were precipitated with 4 volumes of ice-cold acetone and redissolved in 100  $\mu$ L extraction buffer. After centrifugation at 14,000g, 20  $\mu$ L of each sample was loaded onto precast 4 to 15% Mini-Protean TGX gels (Bio-Rad) and separated using Tris-glycine buffer (25 mM Tris and 190 mM glycine) at 70 V for 2 h. Transfer to polyvinylidene fluoride membranes was done in 25 mM Tris, 190 mM glycine, and 10% methanol at 30V overnight in a wet-blot transfer cell. Proteins were detected using mouse anti-MSI1 (Hennig et al., 2003) (1:3000) and anti-GFP (Clontech; 1:3000) antibodies.

### ChIP

For ChIP, 100 mg of 10-d-old seedlings was collected and cross-linked. Nuclei were extracted as described by Shu et al. (2013). For anti-MSI1:GFP ChIP, extracted nuclei were resuspended in 1 mL of PBS, pH 8.0, with 10 mM dimethyladipimidate dihydrochloride (Sigma-Aldrich) at 4°C for 2 h. To stop cross-linking, 50 mM of Tris-HCl, pH 7.5, was added followed by incubation with gentle rotation at 4°C for 20 min. Cross-linked nuclei were collected by centrifugation at 1500g for 10 min. Extracted nuclei were washed once with ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, and 1  $\times$  Complete EDTA-free protease inhibitor [Roche]) and resuspended in 100  $\mu$ L of lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, and 1  $\times$  Complete EDTA-free protease inhibitor). Chromatin was sheared by seven cycles of sonication of 30 s On/30 s Off using a Bioruptor (Diagenode) and diluted 10-fold with ChIP dilution buffer, followed by clearing by centrifugation (4500g, 5 min, 4°C). Twenty-five microliters of chromatin was used as input control and 250  $\mu$ L of chromatin was used in one immunoprecipitation reaction with 1  $\mu$ g of antibody and incubated overnight. Immunoprecipitated complexes were collected using 20  $\mu$ L of Dynabeads Protein A (Life Technologies) per reaction after incubation for 1.5 h at 4°C. Beads were washed two times for 5 min with 300  $\mu$ L of low salt wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 1  $\times$  Complete EDTA-free

protease inhibitor) and two times for 5 min with 300  $\mu$ L of high salt wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 1  $\times$  Complete EDTA-free protease inhibitor). Bound complexes were eluted using an IPure kit (Diagenode). Antibodies used were anti-GFP (Molecular Probes Invitrogen), IgG (Sigma-Aldrich), antihistone H3 (Millipore), and anti-H3K9ac (Millipore). DNA recovery after ChIP was quantified as percentage of input or relative to anti-H3 signal (relative abundance). qPCR with gene-specific primers (Supplemental Table 1) was performed using a CFX Connect system (Bio-Rad) and SsoAdvance Universal SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions.

### Yeast Two-Hybrid Assay

Constructs for yeast two-hybrid analysis were generated using the Matchmaker Gold Yeast Two-Hybrid System (Clontech) vectors pGBKT7 and pGADT7, which express protein fusions to the GAL4 DNA binding domain or transcriptional-activation domain, respectively. cDNA inserts encoding MSI1 and HDA19 were introduced in pGBKT7 and pGADT7 using the Gateway cloning system and Matchmaker Gold Yeast Two-Hybrid System as described in the user manuals. The analysis was performed in strain AH109 carrying HIS3 and MEL1 reporters for reconstituted GAL4 activity.

### RNA Isolation and RT-qPCR

RNA extraction and RT-qPCR were performed using an RNeasy Plant Mini Kit (Qiagen), followed by cDNA synthesis using a RevertAid first-strand cDNA synthesis kit (Thermo Scientific) on biological triplicates. qPCR with gene-specific primers (Supplemental Table 2) was performed using a CFX Connect system and SsoAdvance Universal SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Data were analyzed using a method described by Simon (2003).

### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: *MSI1* (AT5G58230), *HDA19* (AT4G38130), *HDC1* (AT5G08450), *SNL1* (AT3G01320), *SNL2* (AT5G15020), *SNL3* (AT1G24190), *SNL4* (AT1G70060), *SNL5* (AT1G59890), *SNL6* (AT1G10450), *ATTCP-1* (AT3G20050), *PYL4* (AT2G38310), *PYL5* (AT5G05440), *PYL6* (AT2G40330), *RD29B* (AT5G52300), *COR15A* (AT2G42540), and *ANAC019* (AT1G52890).

### Supplemental Data

**Supplemental Figure 1.** Expression of *MSI1* and *HDA19* is strongly correlated.

**Supplemental Figure 2.** Effect of *MSI1* and *HDA19* on the expression of the ABA-repressed genes *PYL4*, *PYL5*, and *PYL6*.

**Supplemental Figure 3.** IgG controls of anti-GFP ChIP.

**Supplemental Figure 4.** IgG controls of anti-H3K9ac ChIP.

**Supplemental Figure 5.** Effect of TSA on the expression of ABA receptor genes.

**Supplemental Figure 6.** Effect of TSA on the expression of ABA-responsive genes.

**Supplemental Figure 7.** Effect of salt on *RD29B* expression.

**Supplemental Figure 8.** Absence of off-target effect on *HDA6* in *HDA19 RNAi* lines.

**Supplemental Table 1.** Sequences of primers used for ChIP-qPCR.

**Supplemental Table 2.** Sequences of gene-specific primers used for RT-qPCR.

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## AUTHOR CONTRIBUTIONS

M.D., S.M., and L.H. designed the research. M.D., S.M., M.R., and L.K. performed research. M.D., M.R., J.B., and L.H. analyzed data. S.M., M.D., J.B., and L.H. wrote the article.

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