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HDA19 is required for the repression of salicylic acid biosynthesis and salicylic acid-mediated defense responses in Arabidopsis

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SUMMARY

To cope with a lifetime of exposure to a variety of pathogens, plants have developed exquisite and refined defense mechanisms that vary depending on the type of attacking pathogen. Defense-associated transcriptional reprogramming is a central part of plant defense mechanisms. Chromatin modification has recently been shown to be another layer of regulation for plant defense mechanisms. Here, we show that the RPD3/HDA1-class histone deacetylase HDA19 is involved in the repression of salicylic acid (SA)-mediated defense responses in Arabidopsis. Loss of HDA19 activity increased SA content and increased the expression of a group of genes required for accumulation of SA as well as *pathogenesis related* (*PR*) genes, resulting in enhanced resistance to *Pseudomonas syringae*. We found that HDA19 directly associates with and deacetylates histones at the *PR1* and *PR2* promoters. Thus, our study shows that HDA19, by modifying chromatin to a repressive state, ensures low basal expression of defense genes, such as *PR1*, under unchallenged conditions, as well as their proper induction without overstimulation during defense responses to pathogen attacks. Thus, the role of HDA19 might be critical in preventing unnecessary activation and self-destructive overstimulation of defense responses, allowing successful growth and development.

Keywords: HDA19, histone acetylation, chromatin, salicylic acid, pathogenesis, PR1, *Arabidopsis thaliana*.

INTRODUCTION

Plants are at risk of attack from hazardous microbial or fungal pathogens. Upon pathogen infection, plants activate various defense systems, depending on the infection strategies and lifestyles of the pathogen, that lead to multiple physiological reactions such as programmed cell death (called the hypersensitive reaction), the biosynthesis of signaling molecules, cell wall synthesis, and the degradation of bacterial cell walls. Such defense responses to pathogen attacks are associated with the expression of a wide variety of defense-related genes (Dangl and Jones, 2001; Durrant and Dong, 2004; Eulgem, 2005).

Chromatin modification has recently been described as another layer of regulation for transcriptional reprogramming during the activation of defense systems. SPLAYED, an ATP-dependent chromatin remodeler, and HISTONE MONOUBIQUITINATION 1, a RING-finger E3 ligase, were reported to be required for defense against necrotrophic fungal pathogens (Walley *et al.*, 2008; Dhawan *et al.*, 2009). Arabidopsis Trithorax 1, a histone methyltransferase, directly regulates the transcription of WRKY70, a positive regulator of salicylic acid (SA)-mediated defense signaling (Alvarez-Venegas *et al.*, 2007; Saleh *et al.*, 2008). In addition, it was also reported that components of the Arabidopsis SWR1-like complex, which replaces the histone H2A with the histone variant H2AZ, are required for the repression of SA-dependent defense genes (March-Díaz *et al.*, 2008).

HDA19, an Arabidopsis histone deacetylase (HDAC) in the RPD3/HDA1 superfamily, has been implicated in defense

systems. It was first reported to be involved in the ethylene (ET)/jasmonic acid (JA) signaling pathways of defense responses based on observations that *HDA19* overexpression increased the expression of several ET/JA-regulated genes and resistance to a fungal pathogen, *Alternaria*, whereas *HDA19* knock-down plants had the opposite phenotypes (Zhou *et al.*, 2005). In addition, Kim *et al.* (2008) recently proposed that HDA19 positively regulates SA-mediated basal defense and the expression of *Pathogenesis Related 1* (*PR1*) by opposing the transcriptional activator activities of WRKY38 and WRKY62 by physical interactions with them. However, as the role of HDA19 in the absence of WRKY38 and WRKY62 was not studied, it is uncertain whether HDA19 has WRKY38- and WRKY68-independent roles in defense responses. On the other hand, Tian *et al.* (2005) reported that the basal expression of the SA-induced *PR* genes *PR1* and *PR5* is upregulated in *hda19* mutants under pathogen-free conditions, reflecting a negative role of HDA19 in defense responses.

PR1 and *PR2* are well-known markers of the SA-mediated defense systems, including basal defense, resistance (R) gene-mediated defense, and systemic acquired resistance (SAR) against biotrophic pathogens (Ward *et al.*, 1991; Van Loon, 1997; Rairdan and Delaney, 2002). Transcription of *PR1* and *PR2*, which requires the coactivator NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1; Cao *et al.*, 1994; Delaney *et al.*, 1995; Cao *et al.*, 1997; Shah *et al.*, 1997), is induced in response to infection by several biotrophic pathogens, the presence of pathogen-derived molecules, and the presence of SA or SA analogs. Interestingly, several recent publications have shown that the state of histone acetylation is tightly associated with the expression of *PR1*. The SA-induced activation of *PR1* accompanies an increase in the level of acetylated histones at the *PR1* locus in Arabidopsis (Mosher *et al.*, 2006; Koornneef *et al.*, 2008) and tobacco (Butterbrodt *et al.*, 2006). In addition, loss of *SUPPRESSOR OF NPR1* (*SNI1*), which increases the basal expression of *PR1*, also causes hyper-acetylation of histones within *PR1* chromatin (Mosher *et al.*, 2006). These studies suggest that histone acetylation is possibly involved in the transcriptional regulation of *PR1* and perhaps other *PR* genes. Thus, the identification of histone acetyltransferases (HATs) or HDACs that control histone acetylation at *PR1* will be essential to prove this possibility and will shed light on the comprehensive regulatory mechanisms of *PR1* transcription.

In this study, we report that HDA19 plays a negative role in basal defense mediated by the SA-dependent signaling pathway. Loss of *HDA19* causes increased expression of defense genes, specifically through the SA-dependent pathway, and it promotes resistance to the virulent *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000). HDA19 prevents unnecessary accumulation of SA and subsequent activation of SA-mediated defense responses in the absence of pathogen attack by repressing the expression of genes required for SA accumulation. In addition, HDA19 associates directly with the promoters of *PR1* and *PR2* and deacetylates histones. Thus, HDA19 forms a repressive chromatin environment that ensures low basal expression of defense genes under unchallenged conditions as well as proper induction of *PR* genes without harmful overstimulation during defense responses to pathogen attack.

RESULTS AND DISCUSSION

Loss of *HDA19* causes increased expression of *PR1* and *PR2*

Our preliminary transcriptome analysis using the T-DNA insertion mutant *hda19-3* (Figure 1a) revealed that several defense-related genes are upregulated by the mutation. This observation prompted us to hypothesize that HDA19 might play a role in plant immunity and to examine the mRNA levels of *PR1* and *PR2*, well-known marker genes for the SA-mediated defense pathway, and *PLANT DEFENSIN 1.2* (*PDF1.2*) and *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*), marker genes for the ET/JA-mediated and JA-mediated defense pathways, respectively, in wild-type (wt) and *hda19*

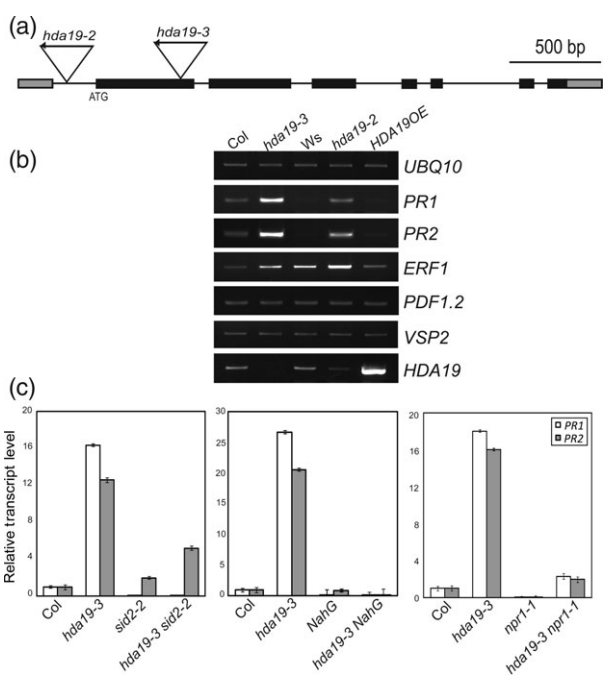

Figure 1. Repression of *PR* genes by HDA19.
(a) Schematic of the genomic structure of *HDA19*. The T-DNA insertion sites in the *hda19-2* and *hda19-3* mutants are indicated. Black and gray boxes represent exons and untranslated regions, respectively. Solid lines indicate introns.
(b) Expression of defense genes in *hda19* mutants. Plants grown in short days (SD) for 28 days (d) were used for RT-PCR analyses (b and c). *Ubiquitin 10* (*UBQ10*) was included as an expression control.
(c) Quantitative RT-PCR analyses of *PR1* and *PR2* expression in *hda19 sid2*, *hda19 NahG*, and *hda19 npr1*. The wild type (Col) levels were set to 1 after normalization by *UBQ10*. The means ± SE of three technical replicates are shown.

mutant plants. As shown in Figure 1(b), loss of *HDA19* increased the mRNA levels of *PR1* and *PR2* in both *hda19-2* (Figure 1a) and *hda19-3* compared with the corresponding wt plants. However, the mRNA levels of *PDF1.2* and *VSP2* were not affected by the *hda19* mutations, although the mRNA level of *ETHYLENE RESPONSE FACTOR 1* (*ERF1*), a positive regulator of *PDF1.2* (Solano *et al.*, 1998), was marginally increased in *hda19* (Figure 1b). Furthermore, the overexpression of Myc-tagged HDA19 (HDA19OE) in *hda19-2* complemented the morphological phenotypes of the mutant (Figures S1a and S2a) and restored wt mRNA levels of *PR1* and *PR2* (Figures 1b and S2b). These results suggest that in a pathogen-free environment, HDA19 specifically represses the expression of genes regulated by the SA-mediated defense pathway but not by the ET/JA-mediated pathways.

In a previous study (Zhou *et al.*, 2005), an overexpression of *HDA19* in wt caused morphological deformities. In another study (Kim *et al.*, 2008), similar morphological deformities were observed only in the T_1 generation of *HDA19* overexpressing plants but not in the next generation of homozygous transgenic plants. However, such deformities were not seen in our seven independent T_2 *HDA19OE* lines in which we could observe *HDA19* mRNA (Figure S2) and protein (Figure S9c) overexpression. Because our *HDA19OE* plants were generated in the *hda19-2* mutants of the Wassilewskija (Ws) ecotype, while the *HDA19* overexpressing plants of the previous studies were of wt Col background, we also generated *HDA19OE* transgenic lines in wt Columbia (Col) and grew them in the same conditions as Kim *et al.* (2008)(12 h light:12 h dark photoperiod at 22°C). Forty-one of 50 T_1 lines displayed morphology comparable to wt Col whereas the remaining nine T_1 lines were somewhat similar to the *hda19-3* mutants (Figure S2c). We then analyzed the expression level of *HDA19* in six representative lines that showed wt morphology and two representative lines that had *hda19*-like morphology. The *HDA19* mRNA level was greatly increased in the former six lines compared with wt; however, the level was lower in the latter two lines than in wt, probably due to co-suppression (Figure S2d). Thus, our results clearly demonstrate that *HDA19* overexpression causes no morphological abnormalities irrespective of the Arabidopsis ecotype and the presence of endogenous HDA19 activity.

To further clarify the roles of HDA19 in the SA-mediated defense pathway, we made double mutants of *hda19-3* and several important components of the SA-mediated defense pathway and examined the mRNA levels of *PR* genes. Mutation of *ISOCHORISMATE SYNTHASE 1* (*ICS1*) or *SA-INDUCTION DEFICIENT 2* (*SID2*) is known to impair the biosynthesis of SA through the isochorismate pathway in Arabidopsis (Dewdney *et al.*, 2000; Wildermuth *et al.*, 2001). The increased mRNA levels of *PR1* and *PR2* in *hda19-3* were completely and largely abolished, respectively, by the *sid2-2* mutation (Figure 1c), suggesting that SA is required for the upregulation of *PR1* and *PR2* in *hda19*. Similarly, the constitutive expression of SA-degrading salicylate hydroxylase (NahG; Gaffney *et al.*, 1993; Delaney *et al.*, 1994) also eliminated the upregulation of *PR1* and *PR2* mRNAs in the *hda19* mutant (Figure 1c). In line with these findings, the loss of *NPR1*, an essential coactivator for the induction of *PR* genes by SA during the development of SAR (Cao *et al.*, 1994, 1997; Delaney *et al.*, 1995; Shah *et al.*, 1997), abolished the expression of *PR1* and *PR2* in *hda19-3* (Figure 1c). Taken together, these results indicate that *PR1* and *PR2* are de-repressed by the loss of *HDA19* in an SA- and NPR1-dependent manner.

hda19 mutants show increased resistance to *Pst* DC3000

Because *PR1* is a good molecular marker for disease resistance against biotrophic pathogens, we examined whether the mutations in *HDA19* increase resistance to the virulent pathogen *Pst* DC3000. As shown in Figure 2(a), the *hda19-3* and *hda19-2* leaves infiltrated by pathogens showed fewer severe disease symptoms than the corresponding wt leaves. Consistently, the growth of *Pst* DC3000 was suppressed approximately 10-fold in *hda19-3* compared with wt at 3 days post-infection (dpi; Figure 2b). A similar result was also obtained from *hda19-2* (Figure 2b), and the enhanced resistance of *hda19-2* to *Pst* DC3000 was eliminated by the overexpression of *HDA19* in *hda19-2* (Figure 2a,b), further demonstrating the role of HDA19 in disease resistance. It has been noted that the constitutive resistance phenotype of a number of defense mutants in the Col ecotype is influenced by SNC1, a Col-specific TIR-NBS-LRR protein (Stokes *et al.*, 2002; Yang and Hua, 2004; Yang *et al.*, 2006; Kim *et al.*, 2010). However, the enhanced bacterial resistance of *hda19-2* mutants in the Ws ecotype, which lacks functional SNC1, clearly showed that the role of HDA19 in disease resistance is not solely SNC1-dependent. Similarly to the expression patterns of *PR1* and *PR2* (Figure 1c), the resistance of *hda19-3* to *Pst* DC3000 was suppressed by the mutations in *SID2/ICS1* and *NPR1* and by the overexpression of *NahG* (Figure S3), indicating that SA and NPR1 are essential for the disease resistance conferred by the *hda19* mutations. In summary, the data above indicate that HDA19 acts as a negative regulator of the SA-dependent basal defense to *Pst* DC3000.

We then monitored the induction of *PR1* and *PR2* during the defense response against *Pst* DC3000. *PR1* and *PR2* mRNAs increased more rapidly in *hda19-3* and *hda19-2* than in the corresponding wt (Figures 2c and S4). Reverse transcription followed by quantitative real-time PCR (RT-qPCR) analyses of *PR1* mRNA levels showed that *PR1* is induced at higher rates in *hda19* mutants than in wt. The *PR1* mRNA levels remained higher in *hda19* mutants than in wt for up to 72 h post-infection (hpi). On the other hand, the

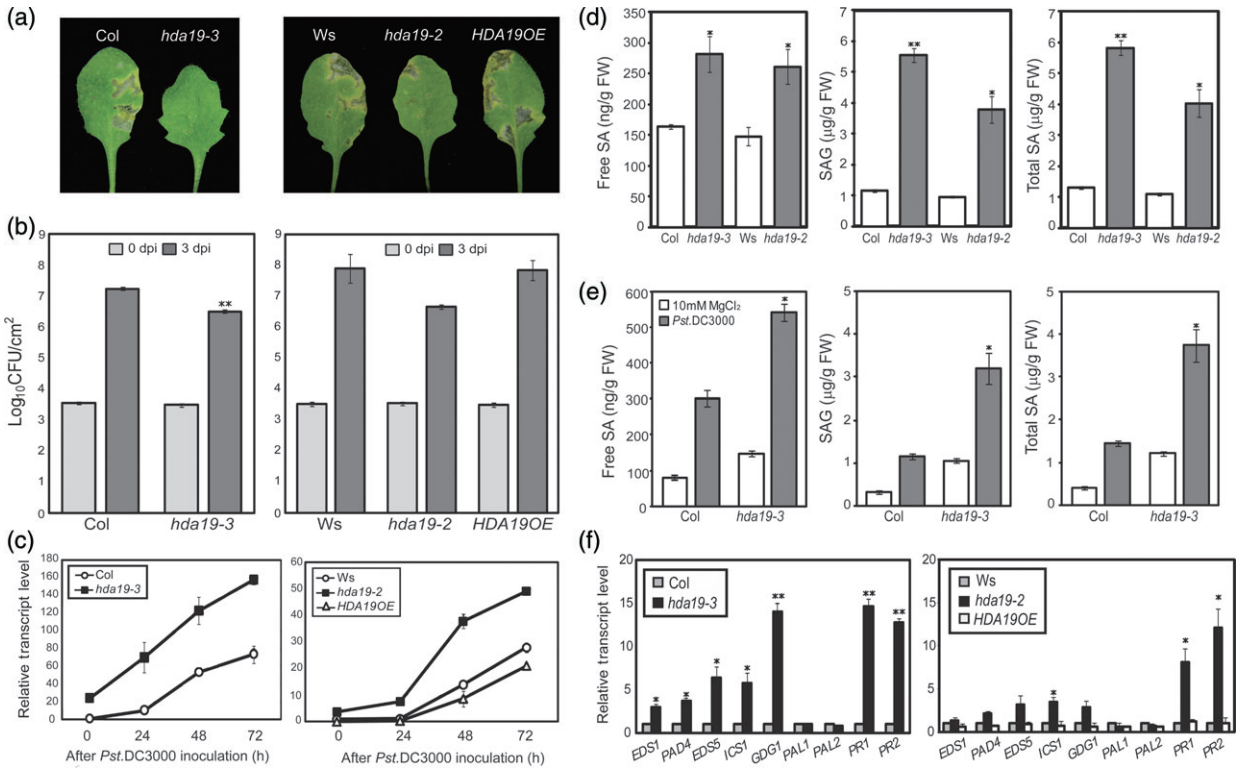

Figure 2. Resistance of *hda19* mutants to *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) and the repression of salicylic acid (SA) biosynthetic genes by HDA19.

(a) Representative leaves at 5 days post-infection (dpi).

(b) Bacterial cell growth at 0 or 3 dpi is shown as the means ± SE of colony-forming units (CFU) from six replicates for Col and *hda19-3* or four replicates for Ws and *hda19-2*. For each replicate, three leaves from three different plants were used.

(c) The quantitative RT-PCR (RT-qPCR) analyses of *PR1* expression after infection. The wild type (wt) levels at 0 h post-infection (hpi) were set to 1. The values are means ± SD of two independent biological replicates. Error bars for some data points are obscured by the symbols.

(d) Contents of free SA or SAG (SA in glucose-conjugated form) in wt and *hda19*.

(e) Contents of free SA or SAG in wt and *hda19-3* at 24 hpi. Ten millimolar $MgCl_2$ was used as a pathogen-free control. For (d) and (e), plants grown in SD for 28 days were used for SA extraction. Averages were obtained from three independent experiments, and error bars represent the SE. FW, fresh weight.

(f) Expression of SA biosynthetic genes, *PR1*, and *PR2* in *hda19* as studied by RT-qPCR. The wt (Col or Ws) levels were set to 1 after normalization by *UBQ10*. The means ± SE of three biological replicates are shown. Asterisks indicate statistically significant differences compared with wt (*$P < 0.05$, **$P < 0.005$ in a Student's *t*-test).

overexpression of *HDA19* resulted in slower *PR1* induction than in wt, such that the *PR1* mRNA levels remained lower in the overexpressor for up to 72 hpi (Figures 2c and S4b). These results indicate that HDA19 prevents the overstimulation of defense responses during pathogen infection as well as their activation prior to pathogen attack.

Our observations that *hda19* mutations increase pathogen resistance and pathogen-induced *PR1* expression disagree with the observations of Kim *et al.* (2008). In their study, one of the *hda19* alleles we used, *hda19-3*, showed reduced resistance to the same strain of pathogen as the one we used, and the induction of *PR1* after the pathogen attack was also attenuated in the mutants. The differences between the two studies are that 10 times more pathogen was used to infiltrate leaves in our study and that the plants were grown under short day (SD; 8-h light/16-h dark) and day-neutral (12-h light/12-h dark) conditions in our and their study, respectively. However, these differences are probably not attributable to such opposing effects, because when we performed the pathogen resistance test under conditions comparable to those of Kim *et al.* (2008), the growth of *Pst* DC3000 was also suppressed approximately 10-fold in *hda19-3* compared with wt in three biologically independent experiments (Figure S5). We did not observe the increase in *HDA19* mRNA levels after pathogen infection nor the degradation of *HDA19* transcript in *sid2* or *npr1* mutants (Figure S6) that were reported by Kim *et al.* (2008). However, importantly, our study demonstrates increased levels of free and conjugated forms of SA in *hda19* mutants compared with wt, as mentioned below (Figure 2d,e), which is consistent with the increased resistance to the pathogen (Figures 2a,b, S3, and S5) and increased expression of *PR* genes (Figures 1b,c, 2c, and S4) before and after pathogen challenge in the mutants.

Salicylic acid content and the expression of SA biosynthetic genes are increased in *hda19* mutants

Genetic analyses using *hda19 npr1*, *hda19 sid2*, and *hda19 NahG* overexpressing double mutant plants (Figures 1c and S3) suggested that the *hda19* mutation causes induction of the *PR* gene, possibly through altered SA levels. To test this possibility, we measured the endogenous levels of SA and its glucose-conjugated form (SAG) in wt and *hda19* plants. As shown in Figure 2(d), both SA and SAG levels were 1.72–1.77-fold and 3.97–4.82-fold higher, respectively, in the mutants. We also measured SA and SAG levels after infection with *Pst* DC3000. Levels of SA and SAG were increased 24 h after pathogen infection in both wt and *hda19-3* and were 1.8- and 2.8-fold higher, respectively, in *hda19-3* than in wt (Figure 2e), indicating that *hda19* mutants contain higher SA and SAG levels than wt in infected state as well as before pathogen infection. It has been proposed that SA is synthesized in plants through two different pathways: the phenylalanine ammonia lyase (PAL) pathway (Klessig *et al.*, 1998) and the isochorismate pathway, via the activity of isochorismate synthase (ICS) (Delaney *et al.*, 1994). Thus, we examined the mRNA levels of *PAL1*, *PAL2*, and *ICS1* in wt and *hda19* plants to test whether the *hda19* mutations increase the accumulation of SA and SAG by influencing the expression of these genes. Interestingly, *ICS1* was upregulated in the *hda19* mutants (Figure 2f), but *PAL1* and *PAL2* were not, suggesting that SA and SAG are highly accumulated in *hda19* via the ICS pathway.

In addition, the mRNA levels of *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*; Falk *et al.*, 1999; Feys *et al.*, 2001), *PHYTOALEXINE DEFICIENT 4* (*PAD4*; Zhou *et al.*, 1998; Feys *et al.*, 2001), *ENHANCED DISEASE SUSCEPTIBILITY 5* (*EDS5*; Nawrath *et al.*, 2002), and *GH3-LIKE DEFENSE GENE* (*GDG1*; Jagadeeswaran *et al.*, 2007; Lee *et al.*, 2007; Nobuta *et al.*, 2007), which encode essential components for SA accumulation, were also elevated in the *hda19* mutants (Figure 2f). Therefore, it was possible that the high levels of SA in *hda19* plants might have resulted from increased expression of these genes in the mutants. However, as the expression of *EDS1*, *PAD4*, *EDS5*, *GDG1*, and *ICS1* is also known to be induced by SA (reviewed in Shah, 2003; Jagadeeswaran *et al.*, 2007), it was also possible that the increased expression of these genes might be caused by the increased SA content in *hda19*. To resolve this issue, we analyzed the mRNA levels of *EDS1*, *PAD4*, *EDS5*, and *GDG1* in *sid2-2* single mutants and *hda19-3 sid2-2* double mutants in which the biosynthesis of SA is severely impaired. Among the genes tested, the mRNA levels of *GDG1* and *EDS5* were higher in *hda19-3 sid2-2* than in *sid2-2*, although the mRNA levels of *EDS1* and *PAD4* were not significantly different between the two genotypes (Figure S7).

On the basis of these results it appears that the loss of *HDA19* leads to elevated SA levels, primarily by increasing the expression of some of the genes, such as *GDG1* and *EDS5*, required for accumulation of SA. The elevated levels of SA in *hda19* might in turn increase the expression of SA-inducible genes such as *EDS1*, *PAD4*, and *ICS1* that are also required for SA accumulation, leading to signal amplification through positive feedback. However, a more accurate 'cause and effect' relationship in this hypothesis is yet to be established through genetic interaction studies between *HDA19* and genes such as *GDG1* and *EDS5*. Because SA is a key signaling molecule in local defenses and SAR, the maintenance of a low level of SA in the absence of pathogen attack should be important to prevent unnecessary activation of energetically costly defense responses. HDA19 has a role in this regard by directly or indirectly repressing the expression of the subset of genes involved in the accumulation of SA.

hda19 mutations cause hyper-acetylation of histones at *PR* loci

Previous reports on the tight association of histone acetylation with transcriptional activity at the *PR1* locus (Butterbrodt *et al.*, 2006; Mosher *et al.*, 2006; Koornneef *et al.*, 2008) raised the possibility that HDA19 might affect the transcription of *PR1* through histone deacetylation in addition to regulating endogenous SA levels. Chromatin immunoprecipitation (ChIP) assays with antibodies against acetylated histone H3 (H3Ac) and acetylated histone H3 lysine 9 (H3K9Ac) showed that the levels of H3Ac and H3K9Ac in the *PR1* and *PR2* promoter regions are higher in *hda19-3* than in wt (Figure 3a,b). Treatment with 2,6-dichloroisonicotinic acid (INA), a functional analog of SA, also increased the levels of H3Ac and H3K9Ac in the *PR1* and *PR2* promoter regions (Figure 3a,b). These results, along with previous reports on the SA-mediated increase of H3Ac at *PR1* (Mosher, 2006; Koornneef *et al.*, 2008), imply a role for HAT(s) in SA- or SA-analog-mediated *PR1* induction. Unlike H3K9Ac levels, H3K27Ac and H4K5Ac levels were not significantly altered by either the *hda19-3* mutation or INA treatment (Figure S8a). Similarly to the *PR1* and *PR2* loci, H3Ac and H3K9Ac levels at the *EDS5* and *GDG1* loci were also increased in the *hda19-3* mutant (Figure 3a,b). However, H3Ac and H3K9Ac levels at the *EDS1*, *PAD4*, and *ICS1* loci were not affected by the *hda19-3* mutation (Figure S8b,c), suggesting that the increased expression of *EDS1* and *PAD4* in *hda19* might be independent of the histone deacetylase activity of HDA19. Thus, only a portion of the genes upregulated in *hda19* might be repressed by histone deacetylation within their chromatin.

To address whether the increased H3Ac at *PR1* is a consequence or a cause of the active transcription in *hda19*, we compared H3Ac levels at *PR1* in wt, *hda19-3*, *sid2-2*, and *hda19-3 sid2-2*. The SA level of *hda19-3 sid2-2* was not determined and compared with the level of *sid2-2*. However, *PR1* expression has been demonstrated to be fully dependent on SID2/ICS1 activity in *hda19-3* (Figures 1c and S7) as well as

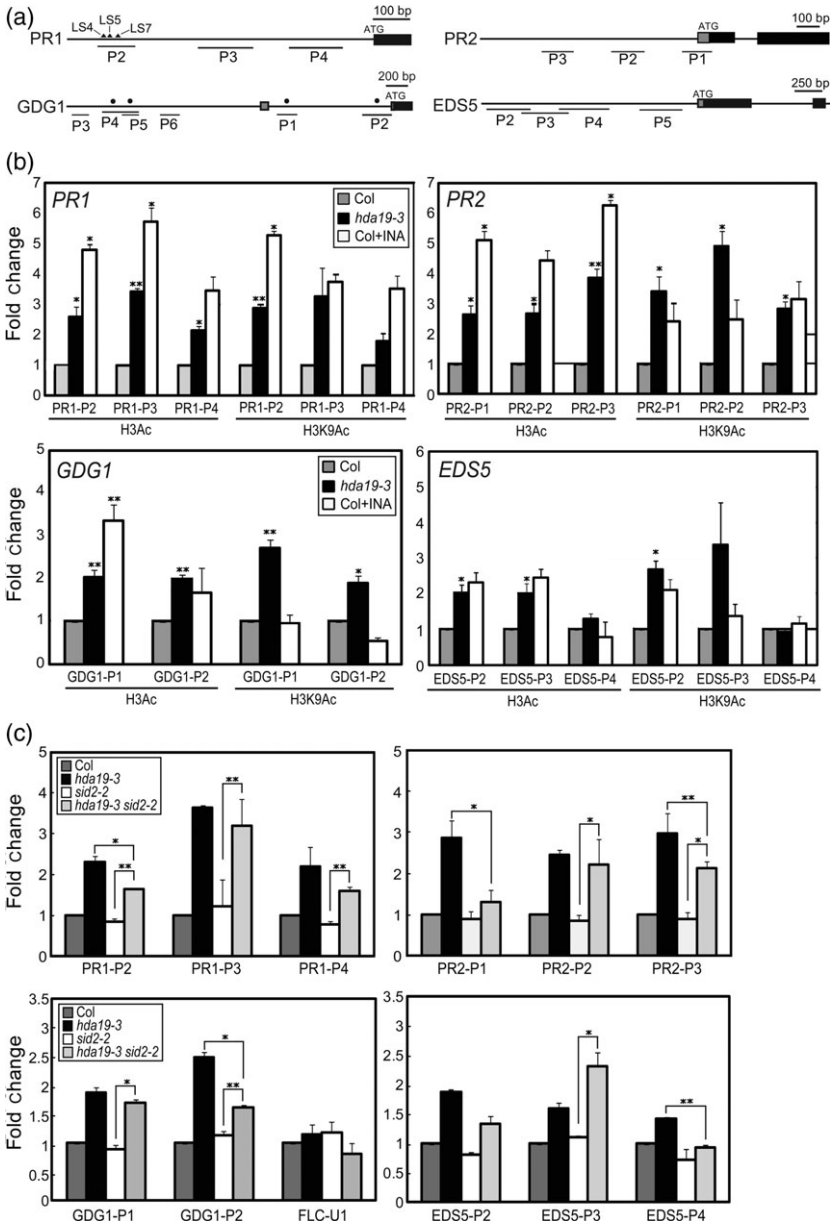

Figure 3. Increased acetylation of histone H3 at the *PR1*, *PR2*, *GDG1*, and *EDS5* loci by *hda19* mutation and 2,6-dichloroisonicotinic acid (INA) treatment.

(a) Schematics of the *PR1*, *PR2*, *GDG1*, and *EDS5* loci showing regions amplified by the primers used for chromatin immunoprecipitation (ChIP) assays. Black and gray boxes represent exons and 5′ untranslated regions, respectively. Solid lines indicate 5′ upstream promoters or introns. The *cis*-acting elements, LS5, LS6, and LS8 in the *PR1* promoter (Lebel *et al.*, 1998) and the putative W-boxes in the *GDG1* promoter (Jagadeeswaran *et al.*, 2007) are represented as closed triangles and circles, respectively, above each promoter.

(b) Quantitative PCR analyses of the ChIP assays with antibodies against acetylated histone H3 (H3Ac) or acetylated histone H3 lysine 9 (H3K9Ac). Shown are means ± SE of at least three biological experiments performed in triplicate. Plants of each genotype were grown in short-day conditions (8-h light/16-h dark) for 35 days and harvested for the ChIP assay (b and c). For INA treatment, Col plants were sprayed with 300 μM INA 24 h before harvest. The wt Col levels were set to 1 after normalization by input and the internal control *UBQ10* (b and c).

(c) Quantitative PCR analyses of the ChIP assays with antibody against H3Ac. Shown are means ± SE of three biological experiments performed in triplicate. Asterisks indicate statistically significant differences between the two comparisons marked by brackets (**P* < 0.05, ***P* < 0.01 in a Student's *t*-test).

in wt (Nawrath and Métraux, 1999; Wildermuth *et al.*, 2001). Therefore, *sid2-2* single and *hda19-3 sid2-2* double mutants would possess similar H3Ac levels at *PR1* if the H3Ac levels were affected by the transcriptional status of *PR1*. Although the H3Ac levels in the *PR1* regions were slightly decreased by the *sid2-2* mutation, the levels were significantly higher in *hda19-3 sid2-2* than in *sid2-2* (Figure 3c). Likewise, H3Ac levels in the *PR2*, *EDS5*, and *GDG1* regions were also significantly higher in *hda19-3 sid2-2* than in *sid2-2* (Figure 3c). These results clearly show that the increased H3Ac at the *PR1* locus in *hda19* is not merely the consequence of active transcription, and suggest that the increased H3Ac at *PR1* and other loci, such as *PR2*, *EDS5*, and *GDG1*, might cause active transcription of these genes in *hda19*.

The results in Figures 1(c), 3(c), and S7 also indicate that the increased H3Ac itself is not sufficient for the activation of *PR* transcription. Accordingly, SA-dependent transcriptional activators, including NPR1, might eventually be required to achieve full transcriptional activity of *PR1*. Accumulation of SA either localizes NPR1 in the nucleus (Mou *et al.*, 2003) or allows NPR1 to form an enhanceosome, resulting in the activation of target genes such as *PR1* and *PR2* (Rochon *et al.*, 2006; Boyle *et al.*, 2009). Therefore, although the repression imposed by the activity of HDA19 is relieved, the lack of functional NPR1 activity due to either the absence of SA or the mutation in *NPR1* would result in the failure of *PR* activation. Interestingly, it was reported that SA-induced histone acetylation was not detected in *npr1* mutants

(Koornneef *et al.*, 2008). Our ChIP data show that histone acetylations induced by *hda19* and SA are likely to be additive, at least in part (Figure 3c). Thus, SA-activated NPR1 might recruit HAT activity to *PR* promoters and/or exclude HDAC activity from them.

The higher basal levels of *PR1* mRNA and increased histone acetylation at the *PR1* locus observed in *hda19* are also seen in *sni1* mutants (Mosher *et al.*, 2006), suggesting that HDA19 and SNI1 might work together in *PR1* repression. However, there are several differences between *hda19* and *sni1*: the endogenous SA level is elevated in *hda19* (Figure 2d,e) but not in *sni1* (Li *et al.*, 1999); the loss of NPR1 almost completely blocks the SA-mediated induction of *PR1* in *hda19* (Figure 1c) but only partially blocks it in *sni1* (Li *et al.*, 1999; Durrant *et al.*, 2007); the pathogen susceptibility of *npr1* mutants is consistently relieved by mutations in *SNI1* (Durrant *et al.*, 2007) but only very slightly relieved by mutations in *HDA19* (Figure S3). These discrepancies suggest that the NPR1-independent pathway (Shah *et al.*, 1999) might be relieved or activated by *sni1* mutations but not by *hda19* mutations, i.e. HDA19 but not SNI1 is specific to the NPR1-dependent pathway.

PR1 and *PR2* promoters are direct targets of HDA19

To address whether HDA19 plays a direct role in the transcriptional regulation of *PR1* and *PR2*, ChIP assays were performed using *hda19* plants carrying either the *HDA19::FLAG* or the *HDA19::HA* construct. These constructs contain either the FLAG-tagged or hemagglutinin (HA)-tagged full-length *HDA19* genomic fragment, including the promoter, and both fully rescued the defective expression pattern of *PR* genes as well as the morphological defects of *hda19* (Figure S9). Chromatin immunoprecipitation assays followed by PCR analyses showed that both the HDA19::FLAG and the HDA19::HA proteins can associate with the PR1-P2, PR1-P3, PR2-P1, and PR2-P2 regions (Figure 4a). However, despite the increased H3Ac within the PR2-P3 region in *hda19-3* (Figure 3a,b), association of HDA19::FLAG or HDA19::HA was not detected in this region. Enrichment of HDA19::FLAG and HDA19::HA in the *PR1* and *PR2* promoters was not significantly decreased after infection by *Pst* DC3000 (Figure S10), suggesting that HDA19 binds constitutively to the *PR1* and *PR2* promoters. These results are consistent with the observation that the induction fold of *PR1* after pathogen infection or INA treatment is higher in *hda19* than in wt (Figures 2c, 5a, and S4), implying that HDA19 plays a role in attenuating *PR1* transcriptional activity in an induced state as well as at the basal level.

The association of HDA19::FLAG and HDA19::HA proteins was also detected in *GDG1* promoter regions containing W-boxes (GDG1-P4 and GDG1-P5) but not in other *GDG1* promoter regions (Figures 3a and 4b). HDA19::FLAG and HDA19::HA proteins did not show any association with the promoter region (PAD4-P2) of *PAD4* (Figure 4a), in which the histone acetylation level was not increased by *hda19* (Figure S8b,c). Surprisingly, there was no detectable enrichment of HDA19::FLAG or HDA19::HA in the regions spanning the *EDS5* promoters (Figure 4c), where H3Ac and H3K9Ac levels were increased by *hda19* (Figure 3a,b). These results suggest that the increased histone acetylation in *EDS5* might not be directly caused by the loss of *HDA19*. One possibility is that *hda19* mutations might downregulate the expression/activity of different histone deacetylases or upregulate the expression/activity of histone acetyltransferases, affecting histone acetylation levels at the *EDS5* locus. Recently, the SIRT family histone deacetylase AtSRT2 was reported to be involved in the repression of *PAD4*, *EDS5*, and *ICS1* (Wang *et al.*, 2010), although whether this effect is direct and due to altered histone acetylation levels is not known. Thus, it would be of future interest to test whether HDA19 represses *EDS5* through AtSRT2. However, we cannot completely exclude the possibility that the epitope tags attached to HDA19 are not efficiently detected by monoclonal antibodies during ChIP, as they could be hidden by other components of the HDA19-containing protein complex working on these promoters. In fact, an immunoblot (IB) analysis after IP showed that the IP efficiency was largely decreased when plant tissues were cross-linked prior to protein extraction, as in the ChIP procedure, compared with when plant tissues were not cross-linked (Figure S9d).

Because HDA19 does not have a DNA-binding motif, it would require a DNA-binding transcriptional repressor to be recruited to target loci. It is known that the *PR1* promoter contains negative *cis*-elements (Lebel *et al.*, 1998). Although the presence of a TGA-box and a W-box (*cis*-elements for TGA and WRKY family transcription factors, respectively) in these negative *cis*-elements suggests that TGA and WRKY family transcription factors might be DNA-binding *PR1* transcriptional repressors, few of these family members have been proven to actually bind to the negative elements. It is known that TGA2 and TGA5 act as transcriptional repressors of *PR1* under uninfected conditions but turn into transcriptional activators upon pathogen infection (Zhang *et al.*, 2003; Rochon *et al.*, 2006; Kesarwani *et al.*, 2007; Boyle *et al.*, 2009). However, HDA19 is not likely to be recruited by TGAs because histone acetylation levels at *PR1* in *tga2 tga5* double mutants were not increased compared with those in wt (Figure S11a), although the mRNA level of *PR1* was significantly increased in the double mutants (Figure S11b). Identification of the HDA19-recruiting factor(s) and study of the biochemical relationships among transcriptional repressors will shed light on the mechanism responsible for negative regulation of *PR1* and other *PRs*.

Modulation of *PR1* promoter responsiveness to INA by HDA19

To substantiate the role of HDA19 and histone acetylation in *PR* gene expression, a time course induction of *PR1* mRNA in

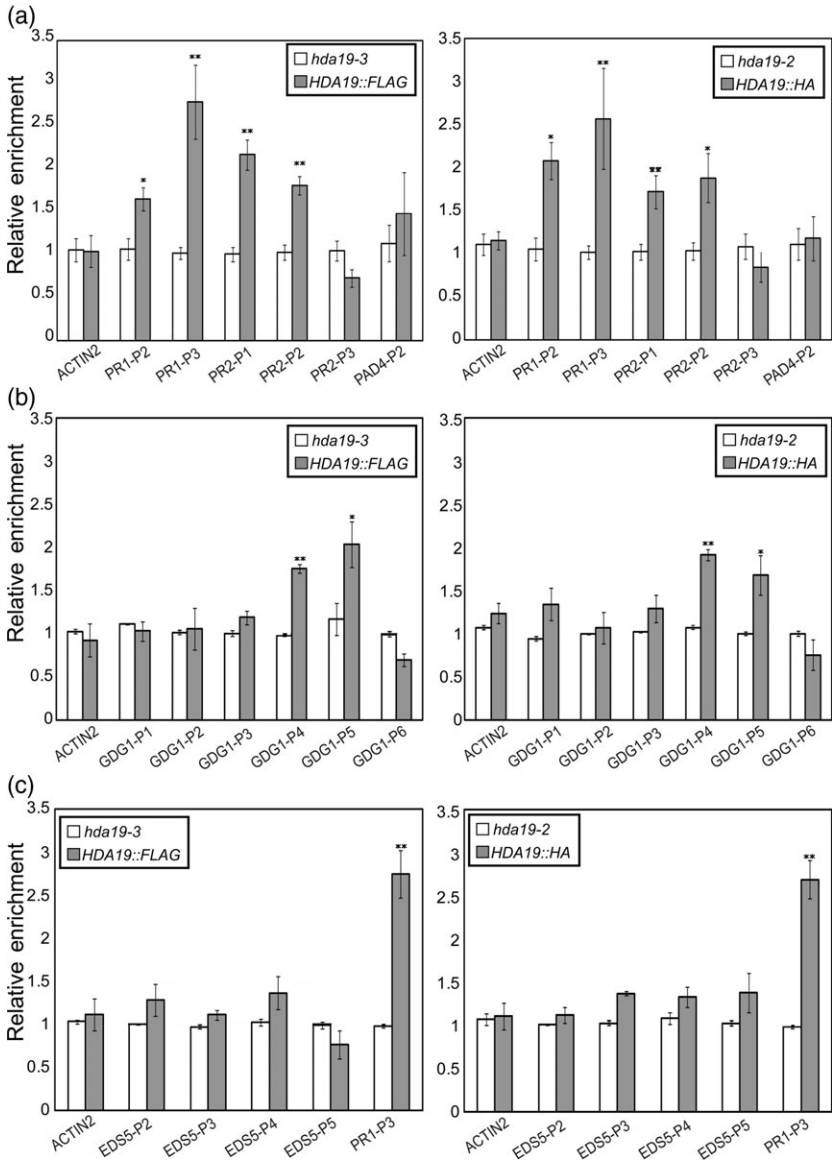

Figure 4. Direct association of HDA19 with *PR1*, *PR2*, and *GDG1* chromatin.
(a) Chromatin immunoprecipitation–quantitative PCR (ChIP-qPCR) for *PR1* and *PR2* chromatin.
(b) Chromatin immunoprecipitation–qPCR for *GDG1* chromatin.
(c) Chromatin immunoprecipitation–qPCR for *EDS5* chromatin. Regions tested with the ChIP assay are indicated in Figure 3(a). Plants of each genotype were grown in short-day conditions (8-h light/16-h dark) for 35 days and harvested for the ChIP assay using antibody against FLAG or hemagglutinin (HA). The amount of immunoprecipitated chromatin was normalized to the corresponding input and compared with untagged plants. Shown are the means ± SE of three biological experiments performed in triplicate. Asterisks indicate statistically significant differences compared with untagged plants (*$P < 0.06$, **$P < 0.01$ in a Student's *t*-test).

wt, *hda19-2*, and *HDA19OE* lines in response to INA was monitored (Figure 5a). Induction of *PR1* mRNA by INA was detected at 3 h post-treatment (hpt) in all genotypes tested. The *PR1* mRNA level increased most rapidly in *hda19-2* but most slowly in *HDA19OE*. These results suggest that *hda19* mutations potentiate INA-induced expression of *PR1*. To avoid confusion from the higher basal expression of *PR1* in *hda19*, *PR1* induction upon INA treatment was also monitored in the absence of SID2 activity (Figure 5b). In the absence of INA, *PR1* mRNA was not detected in *sid2-2* and *sid2-2 hda19-3*, as has already been shown in Figure 1(c). Upon treatment with either 50 or 300 μM of INA, *PR1* mRNA was induced at higher levels in *sid2-2 hda19-3* than in *sid2-2*. In summary, the results above indicate that the *hda19* mutation increases the responsiveness of the *PR1* promoter to INA, and this phenomenon might be caused by increased histone acetylation, which raises the accessibility of the *PR1* promoter to transcription factors in the SA-mediated defense signaling pathway.

The observation that the increased histone acetylation *per se* in *hda19* without SA could not activate the transcription of *PR1* and *PR2* (Figures 1c, 3c, and S7) but rather potentiated the induction of *PR1* and *PR2* in response to INA (Figure 5), suggests that histone acetylation might be a component of the molecular mechanism of priming for defense. Thus, it might be postulated that SA-mediated histone acetylation at target loci caused by the first pathogen infection or SAR primes the target loci to be more responsive to subsequent infection. Such transcriptional memory has been reported in innate immunity in animals. For example, when a macrophage is first exposed to lipopolysaccharide, the chromatins of a class of Toll-like receptor-induced genes are modified

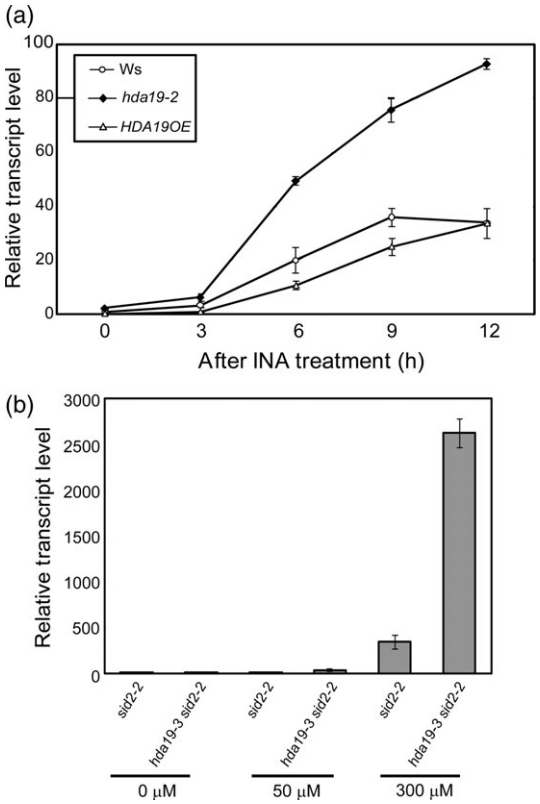

Figure 5. Enhanced responsiveness of the *PR1* promoter to 2,6-dichloroisonicotinic acid (INA) in the *hda19* mutant.
(a) Reverse transcriptase-quantitative PCR (RT-qPCR) analysis of *PR1* expression upon INA treatment. Plants of each genotype were grown in short-day conditions (8-h light/16-h dark) for 28 days, treated with 300 μM INA, and harvested at the indicated time for RNA extraction. The wild-type (wt) level at 0 h was set to 1 after normalization by *UBQ10*. The means ± SE of three independent biological replicates are shown.
(b) *sid2* single or *hda19 sid2* double mutant plants grown as in (a) were treated with the indicated concentration of INA for 24 h before harvest and RNA extraction. *PR1* expression was measured by RT-qPCR. The *sid2* level at 0 μM was set to 1 after normalization by *UBQ10*, and the means ± SE of three independent biological replicates are shown.

and, as a result, the induction of these genes by a second lipopolysaccharide occurs with faster kinetics and larger amplitude (Foster *et al.*, 2007). Interestingly, it was recently reported that histone modifications associated with transcription activation are set in several *WRKY* gene promoters by the priming stimulus before gene activation in Arabidopsis, suggesting a role for histone modification as a memory system for the subsequent stress stimulus (Jaskiewicz *et al.*, 2011).

It has been reported that the overexpression of some individual defense-related genes produces disease resistance which is usually slight and limited to specific pathogens in specific plant species (Van Loon *et al.*, 2006), suggesting that multiple PR proteins are required to promote effective defense responses (Durrant and Dong, 2004; Van Loon *et al.*, 2006). Moreover, individual overexpression of *PR1* to *PR5* in Arabidopsis had no effect on resistance against *Pst* DC3000 (Seo *et al.*, 2008). Therefore, although our work focuses on the role of HDA19 in the transcription of *PR1* and *PR2*, there might be other defense-related genes of which transcriptions are regulated in the same manner as *PR1* and *PR2*. The activation and maintenance of defense systems is a costly process. Therefore, a regulatory system for the accurate control of such processes is required to prevent unnecessary activation and self-destructive overstimulation of defense responses and to ensure successful growth and development. Our study shows that HDA19 is a critical component in such a regulatory system.

EXPERIMENTAL PROCEDURES

Plant materials, growth conditions, and INA treatment

The *hda19-3* mutant (Kim *et al.*, 2008) in the Col background was isolated from the SALK collection (http://signal.salk.edu/; *hda19-3*, SALK_139445) and the *hda19-2* mutant (Long *et al.*, 2006) in the Ws background was provided by Jeff Long (Salk Institute, La Jolla, CA, USA). The *npr1-1*, *sid2-2*, and *NahG* overexpressing plants were in the Col background and have been described previously (Gaffney *et al.*, 1993; Cao *et al.*, 1997; Wildermuth *et al.*, 2001). The *hda19-3 npr1-1*, *hda19-3 sid2-2*, *hda19-3 NahG* double mutants were generated by genetic crosses, and their genotypes were confirmed by PCR genotyping (Table S1). To detect the *npr1-1* allele, which contains a point mutation, PCR amplification using the NPR1-F and NPR1-R primers (Table S1) was performed, followed by restriction digestion with *Nla*III, as previously described (Kesarwani *et al.*, 2007). For *sid2-2*, which contains a deletion and/or rearrangement in the nineth exon, PCR was performed using the genotyping sid2F and genotyping sid2R primers (Table S1), which amplify only the wt allele. The *NahG* transgene was detected by PCR using the NahG-F and NahG-R primers (Table S3). All plants were grown at 22°C under approximately 100 μE m^{-2} sec^{-1} cool white fluorescent light with an 8-h light/16-h dark photoperiod (SD). For INA treatment, 5-week-old plants were sprayed with water or INA [Sigma, http://www.sigmaaldrich.com/)] dissolved in water.

Pathogen inoculation and measurement of bacterial growth

Pst DC3000 was grown at 28°C in King's B medium supplemented with 100 mg L^{-1} rifampicin (Whalen *et al.*, 1991). Pathogen inoculation was performed as previously described (Katagiri *et al.*, 2002). Briefly, leaves of 5-week-old plants were pressure-infiltrated with 10 μl of *Pst* DC3000 suspension at OD_{600} = 0.001. Three inoculated leaves were harvested at 3 dpi and homogenized in sterile H_2O. Leaf extracts were serially diluted and plated on King's B medium, followed by incubation at 28°C for 2 days before counting the colony-forming units.

Constructs and plant transformation

To generate the *HDA19OE* construct, *HDA19* cDNA was obtained from Col RNA by RT-PCR using the HDA19F–*Nco*I and HDA19R–*Sma*I primers (Table S2) and cloned into pGBKT7 (Invitrogen, http://www.invitrogen.com/). After *Xba*I/*Sma*I digestion, the resulting 1×*Myc::HDA19* fragment was cloned into the 35S-pPZP221-RbcS vector, which had been modified from pPZP221 (Hajdukiewicz *et al.*, 1994) to contain the Cauliflower Mosaic Virus 35S promoter and the Arabidopsis Rubisco small subunit (RbcS) terminator. For the construction of *HDA19::FLAG* and *HDA19::HA,* a genomic *HDA19* fragment including the 1.5-kb promoter region upstream of the start

codon was PCR amplified using the Gateway HDA19F and Gateway HDA19R primers (Table S2). The *HDA19* genomic DNA was cloned first into the pENTR/SD/D-TOPO entry vector (Invitrogen) and then integrated into either the pEarlyGate 301 or the pEarlyGate 302 destination vector (Earley *et al.*, 2006) via recombination. The final constructs were introduced into the *hda19-3* or *hda19-2* mutants by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* strain C58C1, and transformants were selected on MS media supplemented with 1% sucrose and 25 μg ml^{-1} glufosinate ammonium (Sigma).

Quantification of SA and SAG

Extraction and quantification of endogenous SA and SAG were performed using leaf tissues from 4-week-old plants as previously described (Enyei *et al.*, 1992). Leaf tissues (0.5 g fresh weight) were frozen in liquid nitrogen, ground to a fine powder, and extracted sequentially with 90 and 100% methanol. After vacuum drying of the pooled methanol extracts, the residue was resuspended in 5 mM sodium acetate buffer (pH 5.5) containing 80 units of β-glucosidase (Sigma) per gram of plant tissue (fresh weight). Following enzymatic hydrolysis (90 min at 37°C), the reaction was stopped by the addition of 10% trichloroacetic acid (TCA). The solution was then partitioned using ethyl acetate:cyclopentane:isopropanol (100:99:1, v/v/v). Salicylic acid was quantified by measuring the fluorescence (excitation 301 nm, emission 412 nm) after separation through a C_{18} reverse-phase HPLC column (Waters Corp., http://www.waters.com/). The HPLC column was maintained at 40°C and equilibrated with 0.5% glacial acetic acid:methanol (75:25, v/v) at a flow rate of 1.5 ml min^{-1}. Three minutes after injection, a methanol gradient (25–60%) was applied over 7 min, after which the methanol concentration was returned to 25%. All data were corrected based on the recovery rate of spiked samples.

RNA extraction and RT-qPCR analyses

Total RNA was isolated from leaves of 4–5-week-old plants using TRI Reagent (Molecular Research Center, http://www.mrcgene.com/) according to the manufacturer's instructions. Reverse transcription was performed with Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (Fermentas, http://www.fermentas.com/) using 4 μg of total RNA followed by either semi-quantitative PCR with i-Taq DNA polymerase (iNtRON Biotechnology, http://eng.intronbio.com/) or real-time quantitative PCR (qPCR) analyses of first-strand DNA. Primers used for RT-PCR or RT-qPCR are listed in Tables S3 and S4. Quantitative PCR was performed in 96-well blocks with an Applied Biosystems 7300 real-time PCR system (http://www.appliedbiosystems.com/) using the SYBR Green I master mix (Bio-Rad, http://www.bio-rad.com/) in a volume of 20 μl. The reactions were performed in triplicate for each run and at least two biological replicates were included. Absolute quantification was performed using standard curves generated by amplification of dilution series of plasmid DNA containing individual genes. The transcript levels of each gene in different samples were normalized to the internal control *UBQ10* mRNA and presented as a fold change relative to the transcript level of corresponding gene in wt without any treatment.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation was performed as described by Han *et al.* (2007) using 5-week-old plants grown in SD. The antibodies used for ChIP were anti-acetyl-H3 (Millipore 06-599, http://www.millipore.com/), anti-acetyl-H3K9 (Millipore 07-352), anti-acetyl-H3K27 (Millipore 07-360), anti-acetyl-H4K5 (Millipore 07-327), anti-FLAG (Sigma F1804), and anti-HA (Abcam ab9110, http://www.abcam.com/). The amount of immunoprecipitated chromatin for each locus was determined by semi-quantitative PCR or qPCR with primer pairs in Table S5. To determine the relative amount of each amplified product in the samples, the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) was used. For histone acetylation ChIP, the amount of immunoprecipitated DNA was normalized to the respective input, and the fold enrichment was obtained by comparing the normalized value of each fragment with that of either *ACTIN2* or *UBQ10*. The fold enrichments in all regions for wt were set to 1 in the graphs. For binding ChIP, the enrichment was defined as the change in the normalized Ct value relative to the control untagged plants. Fold enrichment was presented as mean of nine measurements from three biological repeats performed in triplicate.

Accession numbers

The sequences of genes cited in this article can be obtained from the TAIR database (http://www.arabidopsis.org) with the following accession numbers: *HDA19* (At4g38130), *PR1* (At2g14610), *PR2* (At3g57260), *NPR1* (At1g64280), *EDS1* (At3g48090), *PAD4* (At3g52430), *EDS5* (At4g39030), *ICS1/SID2* (At1g74710), *GDG1* (At5g13320), *PAL1* (At2g37040), *PAL2* (At3g54260), *ERF1* (At3g23240), *PDF1.2* (At5g44420), *VSP2* (At5g24770), *FLC* (At5g10140), *SPLAYED* (At2g28290), *HISTONE MONOUBIQUITINATION 1* (At2g44950), *ATX1* (At1g66240), *WRKY70* (At3g56400), *WRKY38* (At5g22570), *WRKY62* (At5g01900), *SNI1* (At4g18470), and *SRT2* (At5g09230).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Morphological phenotypes of *hda19* single or *hda19 sid2*, *hda19 NahG*, and *hda19 npr1* double mutant plants.

Figure S2. Morphological phenotypes of transgenic plants overexpressing *Myc::HDA19*.

Figure S3. Suppression of the *hda19*-induced resistance to *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) by *sid2*, *NahG*, and *npr1*.

Figure S4. Pathogen-induced mRNA expression of *PR1* and *PR2*.

Figure S5. Resistance of *hda19* mutants grown under day-neutral condition to *Pst* DC3000.

Figure S6. Expression of *HDA19* after pathogen infection in wt, *npr1*, or *sid2* mutants.

Figure S7. SID2-independent increase of *EDS5* and *GDG1* mRNA levels in the *hda19* mutant.

Figure S8. Effect of *hda19* mutation or 2,6-dichloroisonicotinic acid (INA) on histone acetylation at the *PR1*, *PR2*, *EDS1*, *PAD4*, and *SID2/ICS1* loci.

Figure S9. Complementation of *hda19* mutant phenotypes by *HDA19::FLAG* or *HDA19::HA*.

Figure S10. Binding of HDA19 to *PR1* and *PR2* chromatin 48 h after infiltration of *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) suspension (+) or water (–).

Figure S11. Effect of *tga* mutations on histone acetylation at the *PR1* and *PR2* loci.

Table S1. Oligonucleotides used for genotyping.
Table S2. Oligonucleotides used for *HDA19OE*, *HDA19::FLAG*, and *HDA19::HA* constructs.
Table S3. Oligonucleotides used for RT-PCR analysis.
Table S4. Oligonucleotides used for quantitative RT-PCR analyses.
Table S5. Oligonucleotides used for chromatin immunoprecipitation (ChIP) assays.
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