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Regulation of disease-responsive genes mediated by epigenetic factors: interaction of Arabidopsis–Pseudomonas

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SUMMARY

Genes in eukaryotic organisms function within the context of chromatin, and the mechanisms that modulate the structure of chromatin are defined as epigenetic. In Arabidopsis, pathogen infection induces the expression of at least one histone deacetylase, suggesting that histone acetylation/deacetylation has an important role in the pathogenic response in plants. How/whether histone methylation affects gene response to pathogen infection is unknown. To gain a better understanding of the epigenetic mechanisms regulating the interaction between *Pseudomonas syringae* and *Arabidopsis thaliana*, we analysed three different Arabidopsis *ash1-related* (*absent, small or homeotic discs 1*) mutants. We found that the loss of function of *ASHH2* and *ASHR1* resulted in faster hypersensitive responses (HRs) to both mutant (*hrpA*) and pathogenic (DC3000) strains of *P. syringae*, whereas control (Col-0) and *ashr3* mutants appeared to be more resistant to the infection after 2 days. Furthermore, we showed that, in the *ashr3* background, the *PR1* gene (*PATHOGENESIS-RELATED GENE 1*) displayed the highest expression levels on infection with DC3000, correlating with increased resistance against this pathogen. Our results show that, in both the *ashr1* and *ashh2* backgrounds, the histone H3 lysine 4 dimethylation (H3K4me2) levels decreased at the promoter region of *PR1* on infection with the DC3000 strain, suggesting that an epigenetically regulated *PR1* expression is involved in the plant defence. Our results suggest that histone methylation in response to pathogen infection may be a critical component in the signalling and defence processes occurring between plants and microbes.

INTRODUCTION

Plants protect themselves against biotic and abiotic stresses by developing a wide range of strategies known as 'defence' or 'stress' responses. Some of the most serious dangers to plants are infections by bacteria, viruses and pathogenic fungi. Cell wall reinforcement, phytoalexin production and the accumulation of antimicrobial proteins are among the defence mechanisms used by plants to restrict pathogen growth and, ultimately, to destroy it. The temporal and spatial regulation of these mechanisms influences the host–pathogen interaction, resulting in a phenotype that is susceptible or resistant to a specific organism (Wojtaszek, 1997).

The phenomenon known as systemic acquired resistance (SAR) represents an inducible defence mechanism that is activated in the distal organs of a plant in response to a local leaf infection with a pathogen (Vlot *et al*., 2008). The SAR communication requires a mobile 'systemic signal' and a medium for the translocation of this signal from the pest-colonized organ through the rest of the plant (Shah, 2009). For a long time, salicylic acid (SA) was thought to be the translocational signal associated with SAR. However, it has been demonstrated that SA is not the signalling molecule, although its accumulation is needed as an essential factor for the expression of multiple modes of plant disease resistance mechanisms (Vernooij *et al*., 1994; Wojtaszek, 1997).

Many disease- and defence-responsive mechanisms in plants appear to be under epigenetic control mediated by histone acetylation and/or methylation modifications (Alvarez-Venegas *et al*., 2007; van Lohuizen, 1999; Sharples and DePinho, 1999; Zhou *et al*., 2005). Studies in Arabidopsis have shown that pathogen infection induces the expression of at least one histone deacetylase (*HISTONE DEACETYLASE 19*, *HDA19*), suggesting that histone acetylation/deacetylation has an important role in the pathogenic response in plants (Zhou *et al*., 2005). *HDA19*, involved in several developmental processes, is induced by jasmonic acid (JA) and by 1-aminocyclopropane-1-carboxylic acid [ACC; an ethylene (ET) precursor]. Overexpression of the *HDA19* gene increases the expression of chitinase and glucanase genes, as well as resistance against the pathogenic fungus *Alternaria brassicicola* (Zhou *et al*., 2005).

Loss of function of the Arabidopsis homologue of trithorax-1 (ATX1) methyltransferase affects the expression of many pathogen- and disease resistance-encoding genes, including mildew-resistant factors, lectins, chaperones and heat shock proteins, as well as several members of the WRKY family of transcriptional regulators (Alvarez-Venegas *et al*., 2006). Among the WRKY transcription factors with altered expression, *WRKY70* presents a 7.2-fold down-regulation in *atx1* mutants, implicating ATX1 in the transcriptional activation of the gene, apparently by trimethylating the histone H3 lysine 4 (H3K4me3) of its nucleosomes. This

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mechanism is clearly distinct from the *Pseudomonas syringae* or SA-driven mechanisms (Alvarez-Venegas *et al*., 2007).

WRKY70 has been defined as a mediator in the cross-talk between the SA and JA signalling pathways, activating the SA- but repressing the JA-responsive genes (Li *et al*., 2004). Interestingly, the loss of ATX1 function significantly down-regulates the expression of the SA-responsive 'marker' gene *PR1* (*PATHOGENESIS-RELATED GENE 1*), whereas genes from the JA-responsive pathway [*THIONIN2.1* (*THI2.1*), *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*), *PLANT DEFENSIN1.2* (*PDF1.2*), *HEVEIN-LIKE PROTEIN* (*HEL*)] are up-regulated. These results reveal a novel mechanism for the regulation of disease-responsive genes mediated by the SET domain [for *S*u(var)3–9, *E*(z), *T*rithorax]-containing protein ATX1 with a histone H3 lysine 4 methyltransferase activity (Alvarez-Venegas *et al*., 2007).

In a recent study, Berr *et al*. (2010) have shown that the *ASHH2* (also named *EFS*, *SDG8*, *LAZ2* and *CCR1*) gene, a yeast SET2 and Drosophila ASH1 (absent, small or homeotic discs 1) homologue, plays a crucial role in plant defence against necrotrophic fungal pathogens through histone H3 lysine 36 trimethylation (H3K36me3)-mediated activation of a subset of genes within the JA/ET signalling defence pathway. The histone deacetylase HDA19 also regulates gene expression involved in JA/ET signalling in response to fungus invasion (Zhou *et al*., 2005). Furthermore, the ASHH2 methyltransferase (SDG8) is required for basal and R-protein-mediated pathogen resistance in Arabidopsis and to maintain a transcriptionally active state at *LAZ5* (an RPS4-like R-protein) by modifying its chromatin at H3K36 (Palma *et al*., 2010).

In this study, we investigated the susceptibility/resistance to pathogenic and nonpathogenic strains of *P. syringae* of several Arabidopsis lines mutated in three *ASH1* homologous genes. The results reveal specific chromatin modifications occurring between antagonistic defence signalling pathways depending on the nature of the bacterial elicitor contacting the plant.

RESULTS

Plants mutated in SET domain-encoding genes display reduced resistance to pathogens

The T-DNA insertion lines *ashh2* (SALK_014569), *ashr1* (SALK_127952C) and *ashr3* (SALK_128444) were analysed to establish whether mutations in these SET domain-containing proteins affected the susceptibility/resistance of Arabidopsis plants to pathogen attack (Figs 1 and 2; Experimental procedures). Segregation analysis of the kanamycin-resistant marker indicated that there was only one T-DNA insertion in each of the mutant lines (Fig. 1). Previously, it has been shown that the Arabidopsis *ashh2* T-DNA insertion line is a null mutant line and that *ASHH2* (*SDG8*) encodes an H3K36 methyltransferase (Dong *et al*., 2008). Whether ASHR1 and ASHR3 possess histone methyltransferase activity is unknown. As suggested by phylogenetic approaches, similar protein architecture and cladistic distributions, based on the relatedness of the SET domains among the proteins (Alvarez-Venegas and Avramova, 2002; Baumbusch *et al*., 2001; Springer *et al*., 2003), seem to correlate with functional similarity, offering a tool to pursue unknown gene functions. ASHH2 and ASHR3 have been classified as class II SET domain-containing proteins in plants, whereas ASHR1 belongs in class VI (or proteins with an interrupted SET domain) (Ng *et al*., 2007). Related proteins with interrupted SET domains (the SET-I region interrupted by a Zf-MYND domain)

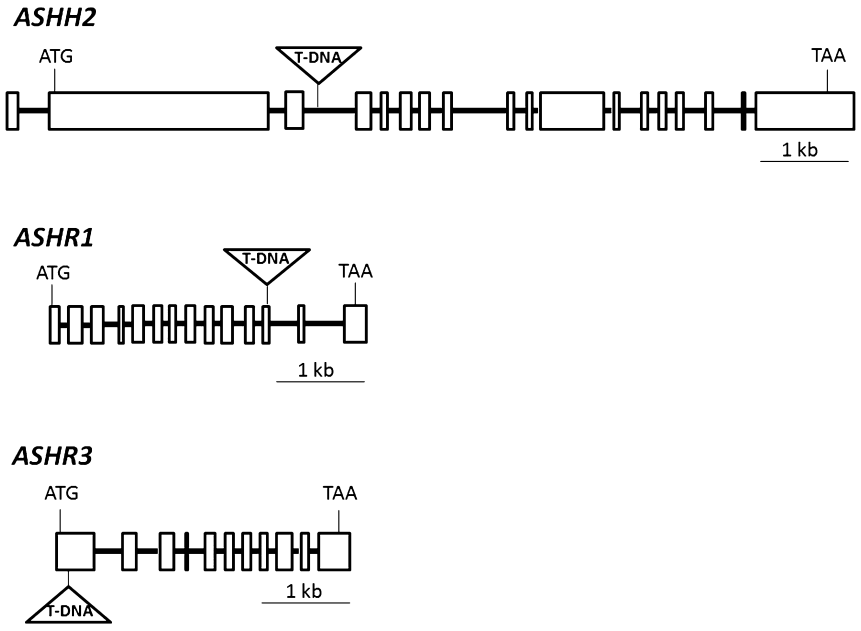

Fig. 1 Representation of the T-DNA insertions. Knockout mutations of ashh2, ashr1 and ashr3. T-DNA insertion sites are represented by triangles. Exons and introns are represented by boxes and lines, respectively.

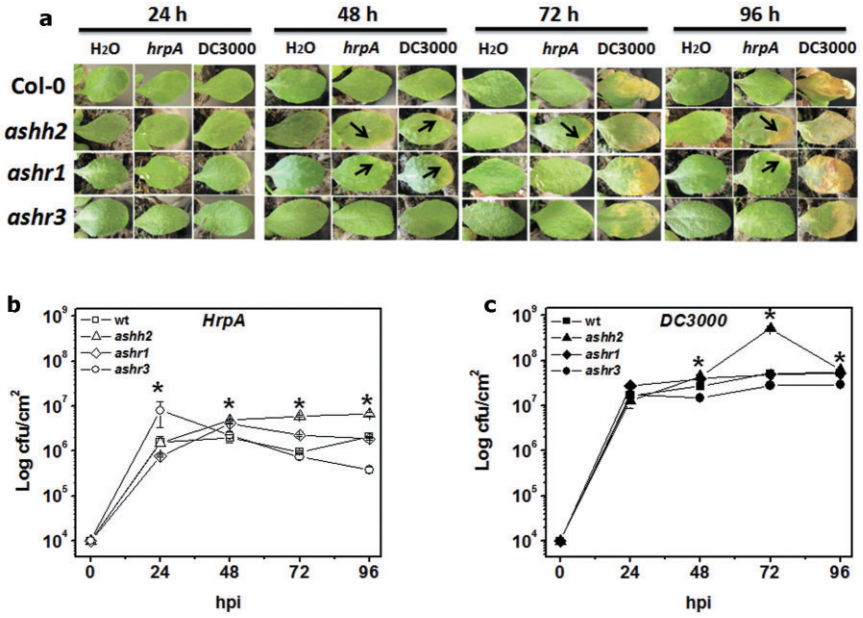

Fig. 2 Plant susceptibility to *Pseudomonas syringae*. (a) Wild-type (Col-0) and mutant plants (*ashh2*, *ashr1* and *ashr3*) after leaf infiltration with bacterial suspensions of wild-type DC3000 or *hrpA* mutant defective in type III secretion. Disease symptoms were tracked for a period of 4 days. The arrows indicate the hypersensitive response in the leaves. Growth of *P. syringae hrpA* (b) and DC3000 (c) strains in Col-0 (wt), *ashh2*, *ashr1* and *ashr3* mutant plants. Leaves were syringe infiltrated with a suspension (10^4 cfu/mL) of bacteria. Bacteria present in leaves were monitored at 24, 48, 72 and 96 h post-inoculation (hpi). Data bars represent the mean $\log_{10}$ cfu/cm^2 ± sample standard deviation for three biological replicates. Statistical comparisons were performed by analysis of variance (ANOVA) ($P < 0.05$).

are present in maize (SDG122, SDG123, SDG130 and SDG140) and rice (SDG716, SDG740, SDG739, SDG722 and SDG741), suggesting that this class of protein existed before the divergence of monocot and dicot plants, and is seemingly distantly related to class II SET domain-containing proteins (Ng *et al*., 2007), although their function has not yet been established in plants. Thus, cladistic analysis indicated that ASHH2 and ASHR3 are distantly related to ASHR1, a difference that could, in part, explain the 'opposite' effects described below.

Plants deficient in ASHH2, ASHR3 or ASHR1 function were inoculated with the pathogenic wild-type *P. syringae* DC3000 or with a *P. syringae hrpA* strain defective in the type III protein secretion system (see Experimental procedures). The presence of disease symptoms and bacterial growth (colony-forming units, cfu) *in planta* were examined. To ascertain that the chlorotic zones in the leaves were not caused by mechanical damage, the leaves of wild-type *A. thaliana* Col-0 were infiltrated with water (Fig. 2a). Although there were slight differences in the phenotypic appearance of wild-type and mutant plants at 24 h post-inoculation (hpi) with DC3000 and *hrpA*, the main signs of infection were observed at 48 hpi (Fig. 2b). Thus, the mutant *ashh2* and *ashr1* lines showed faster emergence of the hypersensitive response (HR) to both the *hrpA* and DC3000 strains, whereas the control (Col-0) and *ashr3* lines seemed to be more resistant to the infection. By 96 hpi, greater chlorotic areas were evident in *ashh2* and *ashr1* infected with the nonpathogenic strain *hrpA* compared with control (water-treated) plants and plants inoculated with the pathogenic strain DC3000. Interestingly, *ashr3* showed greater resistance, measured by the lack of visual HR to the pathogenic bacteria DC3000 at 96 hpi, than Col-0 or the *ashh2* and *ashr1* mutant lines.

To determine how fast bacteria grew before the first appearance of disease symptoms, we analysed cfu at 24, 48, 72 and 96 hpi (Fig. 2b,c). After 24 hpi, all plants (Col-0, as well as the mutants) displayed greater growth of DC3000 (Fig. 2c) than of *hrpA* (Fig. 2b). However, even when the first HR signs were not visible at 24 hpi, *hrpA* displayed greater growth in *ashr3* plants when compared with wild-type Col-0. This result suggests that *ASHR3* might be involved in the basal resistance against bacterial spread. In the case of *ashh2* and *ashr1* inoculated with *P. syringae hrpA*, the growth of the bacteria at 48 hpi was more than two-fold higher than that in either Col-0 or *ashr3* (Fig. 2b). However, greater necrotic effects were observed in the *ashh2* than in the *ashr1* background (Fig. 2a). For *ashh2* and *ashr1*, the cfu values for DC3000 infection were very similar, except at 72 hpi, when the cfu value in *ashh2* increased up to 10 times the level observed in the other backgrounds (Fig. 2c). The highest bacterial count for DC3000 (Fig. 2c; 5.18×10^8 cfu/cm^2) was observed at 72 hpi in *ashh2*. This is the only mutant line of the Arabidopsis ASH1 family with a known pleiotropic phenotype (Grini *et al*., 2009). By 96 hpi, the *ashh2* line demonstrated greater susceptibility to DC3000 and *hrpA* (Fig. 2a) in comparison with the other plants, even when the bacterial growth seemed to decrease (Fig. 2b,c).

Pseudomonas syringae infection increases repressive histone marks

The effect of bacterial infection on the expression of the putative histone methyltransferase genes was analysed by reverse transcription-polymerase chain reaction (RT-PCR) assays of Col-0 leaves inoculated with water, *hrpA* or DC3000 (Fig. 3a). Inoculation with the nonpathogenic bacteria increased slightly the expression levels of *ASHR1*, *ASHR3* and *ASHH2* compared with

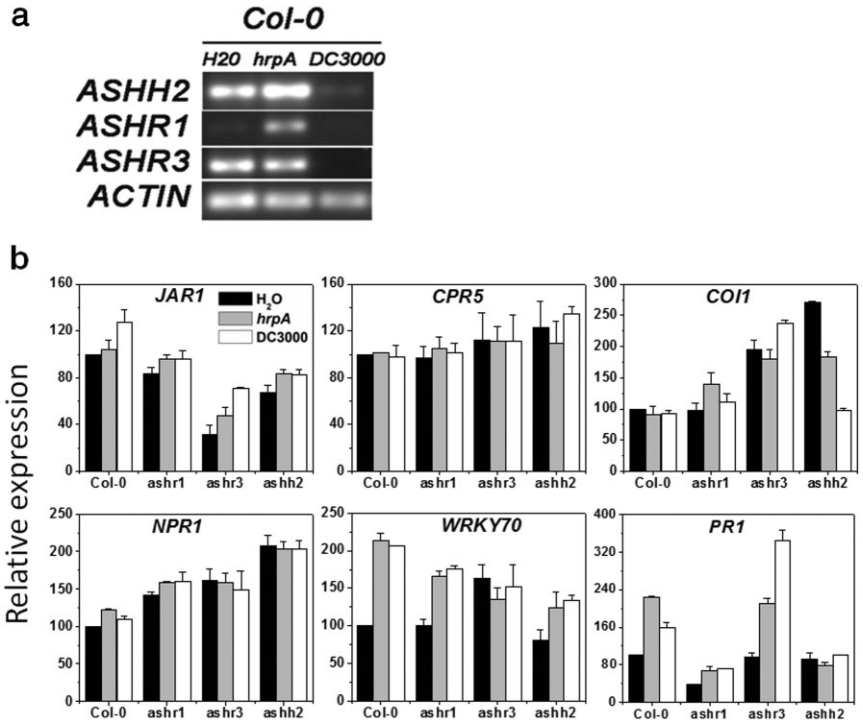

Fig. 3 Effects of *Pseudomomas syringae* on gene expression of defence-related genes in the wild-type and mutant backgrounds. (a) Expression of *ASHH2*, *ASHR1* and *ASHR3* genes on Col-0 plants after infiltration with water, with *P. syringae* DC3000 and with DC3000 *hrpA* strains. (b) Densitometric analysis of defence-related gene expression. Wild-type or mutant Arabidopsis plants (*ashr1*, *ashr3*, *ashh2*) after infiltration with water, and with the pathogenic *P. syringae* DC3000 and nonpathogenic *hrpA* strains. Leaf samples were collected 24 h post-inoculation (hpi) and were examined for the expression of the *JAR1*, *CPR5*, *COI1*, *NPR1*, *WRKY70* and *PR1* genes. ACTIN7 used as a control for each template preparation was amplified under exactly the same conditions as the tested genes. Each gene expression was conducted twice with three biological replicates.

leaves infiltrated with water only. However, the expression levels of all three genes were suppressed when plants were infected with the pathogenic DC3000. In the case of *ASHR3*, we did not detect expression of the gene at 24 hpi. This result might suggest that, as a response to the pathogen, there is a decrease in the expression of genes associated with the 'writing' of histone-activating marks (H3K4 methylation), leading to the formation of more heterochromatic structures as a defence mechanism against the pathogen. This is associated with the decrease in global H3K4me3 patterns and the increase in H3K9me2 seen in the *ashh2* line (a histone methyltransferase involved in H3K4me3, H3K36me2 and H3K36me3, and required for trimethylation of lysine 9 of histone H3; Cazzonelli *et al*., 2009; Dong *et al*., 2008; Grini *et al*., 2009; Xu *et al*., 2008), and with the increase in H3K27me3 patterns detected for all three *ashr1*, *ashr3* and *ashh2* mutant lines (Fig. 4).

To determine the possible effects of *P. syringae* on gene expression, we analysed the expression patterns of several genes involved in the defence response in the mutant and Col-0 plants (Fig. 3b): *JAR1* (*JASMONATE RESISTANT-1*, a locus essential for pathogen defence in the JA pathway), *CPR5* (*CONSTITUTIVE EXPRESSION OF PR GENES 5*, a gene that participates in signal transduction pathways involved in plant defence), *COI1* (*CORONATINE INSENSITIVE 1*, an F-box protein required for wound- and jasmonate-induced transcriptional regulation), *NPR1* (*NONEXPRESSER OF PR GENES-1*, a key regulator of the SA-mediated SAR pathway), *WRKY70* (a gene that functions as an activator of SA-dependent defence genes and a repressor of JA-regulated genes) and *PR1* (*PATHOGENESIS-RELATED GENE 1*, a molecular marker for the SAR response) (Fig. 3b).

All lines were leaf inoculated with *hrpA* and DC3000 and RT-PCR assays were performed at 24 hpi to avoid any interference from tissue damage. We determined that *JAR1* showed the lowest expression in the *ashr3* background, although the gene was still up-regulated in response to infection with DC3000 (Fig. 3b). This result indicates that JAR1 needs ASHR3 to reach the levels required for the response to pathogens (Fig. 5b). *JAR1* expression was also lower in the *ashr1* and *ashh2* mutant lines, although less strongly than in the *ashr3* line.

Unexpectedly, the transcriptional behaviour of another JA-regulated gene, *COI1*, showed a markedly different pattern. Under the treatments, *COI1* produced about two-fold higher transcript levels in *ashr3* and *ashh2* mutants, except when treated with DC3000. No major changes were observed in the *ashr1* line when compared with Col-0 (Fig. 5b). Elevated *COI1* transcript levels in *ashr3* and *ashh2* were associated with a decrease in H3K9me2 marks (see ChIP assay section). *CPR5* and *NPR1* genes followed similar expression patterns under the different treatments, displaying the highest expression in the *ashh2* background (Fig. 3b).

The *WRKY70* and *PR1* genes are positioned downstream in the defence signalling pathway and are considered to be key genes for SAR. The expression of these two genes was influenced by the putative histone methyltransferases in the presence of the pathogen. Thus, *WRKY70* was highly expressed in the presence of both

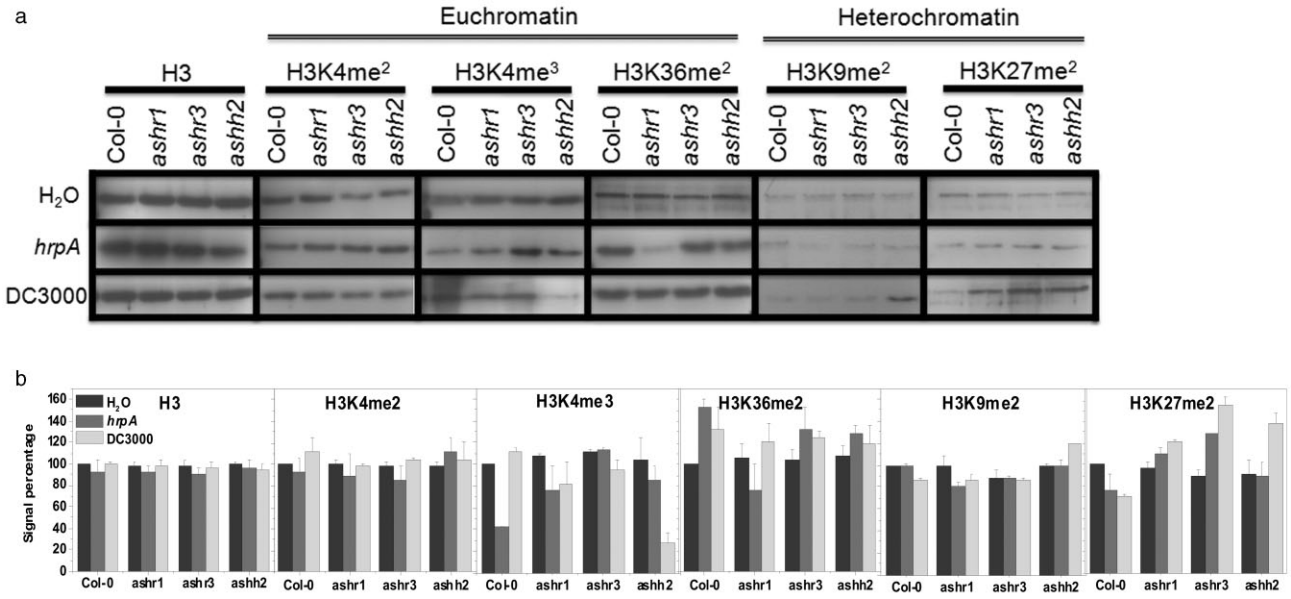

Fig. 4 Effects of *Pseudomonas syringae* on the global histone H3 methylation status in the wild-type and *ashh2*, *ashr1* and *ashr3* backgrounds. (a) Histone H3-tail methylation patterns in wild-type and mutant plants. Total histones extracted from 3-week-old wild-type and mutant plants at 24 hpi were probed with specific antibodies in Western blots. Subsequent to the hybridization, membranes were stripped off and reprobed with antibodies specific for nonmodified histone H3. (b) Densitometric analysis of the overall histone H3K4 methylation patterns. The levels of histone H3-tail methylation for the different treatments, defined as the ratio of meK/H3 to H3 intensity signals, were taken as 100%.

types of bacteria in Col-0, *ashr1* and *ashh2*. In *ashh2*, *WRKY70* displayed the lowest expression under all treatments. In *ashr3*, the mutant line that showed resistance to pathogen attack (Figs 2 and 3), *WRKY70* was highly expressed, even with water, in comparison with the other plants. This suggests a dysregulation of *WRKY70* as a result of the mutation (possibly through the lack of expression of a repressor regulating this gene). In the case of *PR1*, this gene was expressed with both bacteria in Col-0. However, in *ashr1* and *ashh2*, the mutant Arabidopsis lines that showed susceptibility to bacterial infection (Fig. 2), *PR1* showed no major changes in expression in comparison with Col-0. It could be that ASHR1 and ASHH2 are involved, to a certain extent, in the activation of *PR1* gene expression. Furthermore, it was observed that, in *ashr3*, *PR1* displayed the highest expression levels when infected with DC3000, which could be correlated with its resistance against this pathogen (Fig. 3b).

Formation of heterochromatin-like structures as a result of pathogen attack

To determine the possible effects of *P. syringae* on the global patterns of histone methylation in the different mutant lines, *ashh2*, *ashr1* and *ashr3* inoculated with both the wild-type DC3000 and the *hrpA* strains (Fig. 4) were analysed at 24 hpi (see Experimental procedures) by Western blots using antibodies against the H3K4 di- and trimethylated isoforms, as well as for the H3K9me2, H3K27me2 and H3K36me2 marks (Fig. 4a,b). We observed important changes in global methylation patterns in the different lines only when the plants were in contact with the bacteria. For instance, after inoculation with the pathogen DC3000, we detected, in *ashh2*, a decrease in the global H3K4me3 mark (~300%; densitometric analysis; Fig. 4b) and an increase in H3K9me2 (~30%; densitometric analysis; Fig. 4b) and H3K27me2 (~50%; densitometric analysis; Fig. 4b) marks, when compared with the wild-type Col-0 line. This might suggest the possible formation of heterochromatin-like structures as a result of biotic stress. Similarly, the *ashr1* and *ashr3* lines demonstrated an increase in the global H3K27me2 mark when exposed to the pathogen, but showed no reduction in activating marks such as H3K4me2, H3K4me3 and H3K36me2. These global histone methylation changes, together with a down-regulation in the expression of histone methyltransferases after inoculation with the bacterial pathogen (Fig. 3a), may lead to the formation of more heterochromatic structures as a defence mechanism to the pathogen. This observation deserves further analysis.

Histone methylation status on defence genes: chromatin immunoprecipitation (ChIP) assay

Infiltration with the bacterial pathogen *P. syringae* pv. *tomato* DC3000 induces the expression of genes, such as *WRKY70*, a mediator in the cross-talk between the SA and JA signalling pathways (Li *et al*., 2004, 2006). Here, we examined, by ChIP, the molecular events accompanying the epigenetically induced

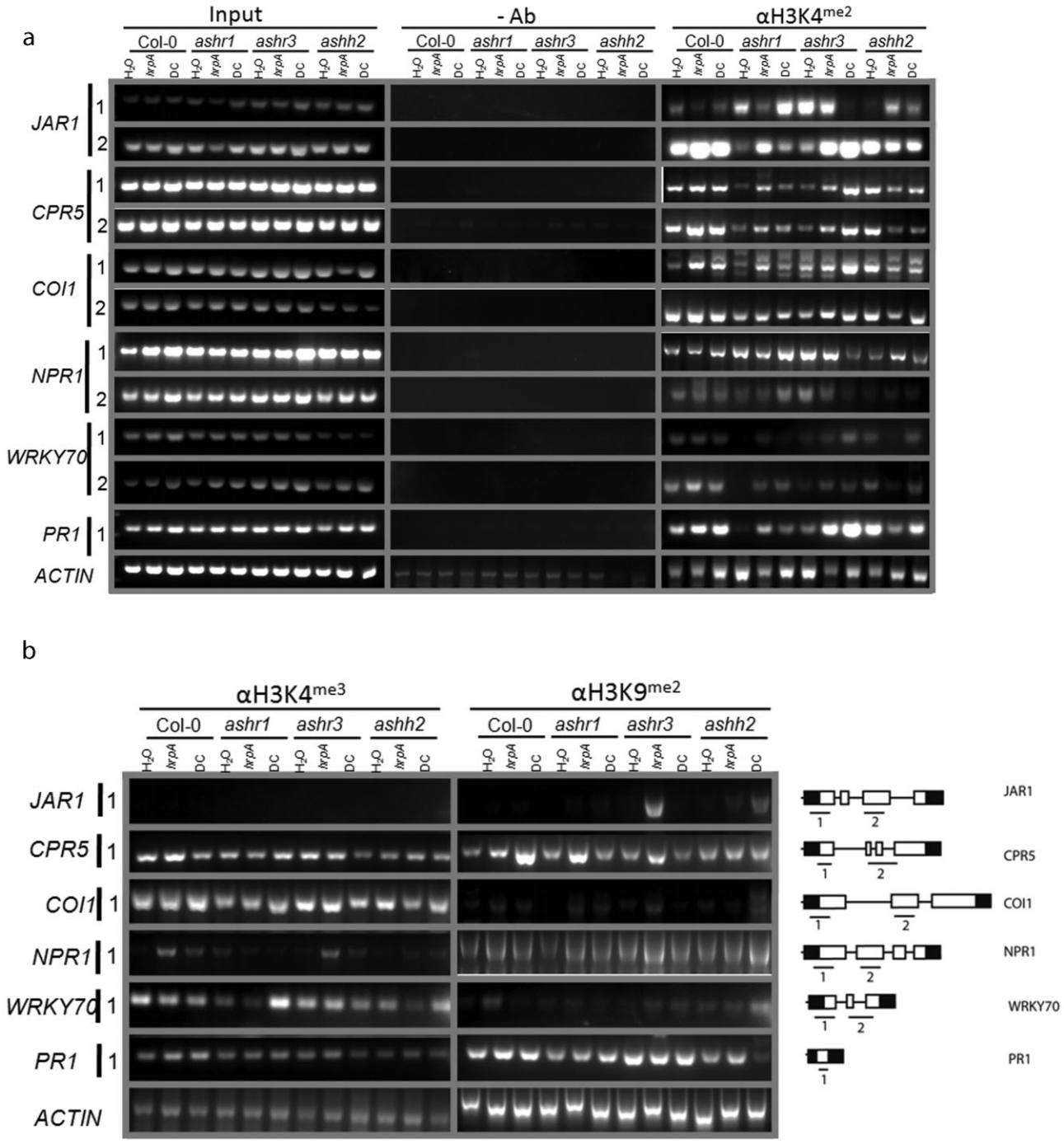

Fig. 5 Effects of *Pseudomonas syringae* on the histone H3 methylation patterns of wild-type and mutant backgrounds using chromatin immunoprecipitation. Samples were collected from wild-type and mutant Arabidopsis plants at 24 h post-inoculation with the pathogenic *P. syringae* DC3000 and nonpathogenic *hrpA* strains (water was used as a control), and were examined for histone H3-tail methylation patterns at the indicated genes (*JAR1*, *CPR5*, *COI1*, *NPR1*, *WRKY70* and *PR1*) in wild-type and *ashr1*, *ashr3* and *ashh2* mutants. Chromatin from noninduced and induced wild-type and mutant plants was analysed. (a) Input (input DNA), 15-fold diluted samples were used as templates for the input lanes. Negative controls (–Ab), no antibody samples treated in the same way as immunoprecipitated chromatins with H3K4me2. Amplified ACTIN7 with specific primers was used as a control for the quality of the samples. (b) Samples were immunoprecipitated with H3K4me3 and H3K9me2 antibodies. The schematic illustration (not drawn to scale) of the *JAR1*, *CPR5*, *COI1*, *NPR1*, *WRKY70* and *PR1* gene sequences illustrates the regions amplified by the primers (1, region 1; 2, region 2); the empty boxes show exons, the connecting lines are the introns and the black boxes are the 5′ and 3′ untranslated regions (UTRs). The black line below shows the regions amplified by the primers.

modification of *JAR1*, *CPR5*, *COI1*, *NPR1*, *WRKY70* and *PR1* in Arabidopsis (Fig. 5).

The normalization of histone H3 immunoprecipitated from infected (Col-0, *ashr1*, *ashr3*, *ashh2* with *hrpA* and DC3000) and control (Col-0, *ashr1*, *ashr3*, *ashh2* infiltrated with water) samples to the corresponding input fraction revealed major changes in H3K4me2 at regions 1 (promoter/first exon) and 2 (second exon), and H3K9me2 at region 1 (promoter/first exon) (Fig. 5). All studied genes have the H3K4me2 marks at both regions in the Col-0 background. However, there was an increase in this mark in *JAR1* and *CPR5* at region 2 (exons/coding), at *COI1* in region 1, and in the exon of *PR1* when the wild-type plant was infected with either bacterium. In the *ashr1* line, the H3K4me2 mark was increased at region 2 (exons/coding) in *CPR5*, *JAR1* and *WRKY70*, as well as in *PR1*. The H3K4me2 methylation levels in *PR1* and at region 1 of *WRKY70* were hardly detectable, but they increased after bacterial infection. An opposite effect was seen on *ashr3* for the *NPR1* and *JAR1* genes. Thereby, on infection with the DC3000 strain, the H3K4me2 levels decreased at the promoter region in these genes. Importantly, in *PR1*, this mark was highly accumulated only in the presence of the bacteria, which suggests that an expression of *PR1* as a result of epigenetic regulation is needed for plant defence. In the case of the susceptible line *ashh2*, a decrease in the levels of the H3K4me2 mark at the *CPR5* and *PR1* genes was observed when the plant was infected with either bacterium. Considering these results and those obtained with gene expression, it appears that *PR1* expression (Fig. 3b) is directly correlated with H3K4me2 levels.

Furthermore, we determined the levels of H3K9me2, a mark related to heterochromatin and repressive gene regions, at the promoters (region 1) of all analysed genes (Fig. 5b). We found that, in DC3000-inoculated Col-0 plants, there was an increase in H3K9me2 at region 1 of the *CPR5* gene, whereas region 1 of the *JAR1* gene in *ashh2* plants showed an increase in this mark when inoculated with DC3000. *CPR5* also showed an increase in H3K9me2 in *ashr1* and *ashr3* mutants on *hrpA* infection. The results on the *WRKY70* gene in DC3000-inoculated *ashh2* plants were also consistent with a more condensed chromatin conformation.

Likewise, the levels of H3K4me3, a mark related to active gene regions, were determined at region 1 of the analysed genes (Fig. 5b; see Experimental procedures). We found that, after bacterial inoculation, a major change was seen for the *NPR1* gene. After inoculation with either the wild-type DC3000 or *hrpA* strain, the H3K4me3 mark increased in the Col-0, *ashr3* and *ashh2* lines, independent of the strain used for infection. We did not see changes in the patterns of histone methylation on the other genes analysed (by ChIP) as a result of pathogen infection. This might suggest that the effect on gene expression is independent of/indirect from the methyltransferase, but directly related to the type of bacterium, i.e. pathogenic or nonpathogenic.

DISCUSSION

It is well known that plants are continuously exposed to pathogens throughout their lifetime. Much of what is known about how plants respond to pathogen attack has come from studies of *Arabidopsis thaliana*, rice or tobacco (Abad *et al*., 1996; De-la-Peña *et al*., 2008; Hugot *et al*., 1999; Legrand *et al*., 1987; Liu *et al*., 2007; van Loon *et al*., 2006). The first signalling pathways to be turned on when a microorganism interacts with a plant are those related to JA or SA (Feys and Parker, 2000; Kunkel and Brooks, 2002; Leon-Reyes *et al*., 2009; Métraux *et al*., 2002; Pozo *et al*., 2004). The signals for resistance to biotrophic pathogens occur mainly through the SA-dependent pathway, whereas defences against necrotrophs are mainly stimulated by the JA/ET pathway.

It has been shown that infiltration with the bacterial pathogen *P. syringae* pv. *tomato* DC3000 induces *WRKY70* expression (Li *et al*., 2004; 2006) and its regulation also occurs via the histone methyltransferase ATX1 (Alvarez-Venegas *et al*., 2007). Specifically, ATX1-dependent H3K4me3 at the promoter of *WRKY70* correlates with WRKY70 transcriptional up-regulation. More recently, Palma *et al*. (2010) have demonstrated that *ashh2* plants (also named *efs*, *sdg8*, *laz2* and *ccr1*) inoculated with *P. syringae* pv. *tomato* DC3000 present elevated bacterial growth 4 days after infection (nine-fold higher titres than in the wild-type), indicating that ASHH2 (SDG8) is required for full resistance to virulent pathogens. Furthermore, transcript levels of *RPM1* [a resistance (*R*) gene], as well as H3K36me3 levels at *RPM1* chromatin, are low or absent in 4-week-old *sdg8* (*ashh2* null mutant) plants compared with the wild-type, suggesting that ASHH2 (SDG8) targets a subset of *R* genes and other genes involved in general aspects of basal defence (Palma *et al*., 2010).

We have found that, when Col-0 is infected with DC3000, it provokes the down-regulation of the *ASHH2*, *ASHR1* and *ASHR3* genes (Fig. 3a), which suggests that, on pathogen attack, more heterochromatic-like structures may be formed. This is in agreement with our global histone methylation analysis. In addition, we have confirmed that, after infiltration with DC3000, *WRKY70* expression is stimulated in Col-0 and the activation is independent of any of the mutants analysed (Fig. 3b). Because the pathogen induces *WRKY70* expression in the absence of *ASHR1* and *ASHH2*, this suggests that in the DC3000 inoculated plants, ASHR1 and ASHH2 activate *WRKY70* by separate mechanisms. In particular, the expression of *WRKY70* in *ashr1* does not seem to be affected by the mutation and is only stimulated by DC3000. In contrast, ASHH2 is involved in the up-regulation of *WRKY70* transcription (*WRKY70* transcripts are diminished in the *ashh2* background), indicating that ASHH2 positively contributes to *WRKY70* transcription in the wild-type (similar to the effect seen by ATX1; Alvarez-Venegas *et al*., 2007). Furthermore, ASHR3 seems to indirectly regulate *WRKY70* expression, most probably through the

activation of a repressor, as suggested by the *WRKY70* up-regulation in the *ashr3* background, and its expression in this background independent of the treatment.

In contrast, studies using the *jar1*-1 mutant have shown that this mutation has no detectable impact on plant susceptibility to virulent *P. syringae* (Clarke *et al*., 2000; Kloek *et al*., 2001; Pieterse *et al*., 1998). This result is not surprising because *JAR1* encodes an enzyme involved in JA modification rather than a component of the jasmonate signalling pathway (Staswick and Tiryaki, 2004; Staswick *et al*., 2002). Thus, the up-regulation of *JAR1* seen in the Col-0, *ashr1*, *ashr3* and *ashh2* lines (Fig. 3b) suggests that, as a result of pathogen attack, *JAR1* is activated in order to catalyse the formation of modified forms of JA [e.g. JA amino acid (JAAA) conjugates and methyl jasmonate (MeJA)] that mediate different responses (Seo *et al*., 2001; Staswick and Tiryaki, 2004; Staswick *et al*., 2002).

Epigenetic mechanisms have been found to be important in the signalling pathways involved in plant defence (Hamon and Cossart, 2008; Stokes *et al*., 2002). Our results here support this idea. For example, we found that *P. syringae* pv. *tomato* DC3000 infection in *A. thaliana* provokes chromatin changes as a result of histone methylation changes, and that methylation patterns at different lysine residues in histone H3 contribute to the *in planta* resistance to the pathogen (Fig. 4). However, the growth difference between the *hrpA* mutant on Col-0 and the *ashr3* mutant was statistically significant and may suggest that ASHR3 is involved in basal resistance, similar to the earlier suggested role for ATX1(Alvarez-Venegas *et al*., 2007) (Figs 2 and 3).

The highest levels of global H3K9me2 marks and the lowest levels of global H3K4me3 marks on DC3000 infection were found in the *ashh2* line, whereas an increase in H3K27me2 marks was found in all three mutant lines (Fig. 4). Collectively, these results suggest that the formation of heterochromatic-like structures and/or gene down-regulation serves as a mechanism to cope with pathogen attack. This conclusion is in agreement with results showing that infection with necrotrophic fungal pathogens results in a reduction in the global H3K36me2 and H3K36me3 levels in the *sdg8* (*ashh2*) null mutant background (Berr *et al*., 2010). Thus, ASHH2 (SDG8) plays a crucial role in plant defence against necrotrophic fungal pathogens through H3K36me3-mediated activation of a subset of genes within the JA/ET signalling defence pathway (Berr *et al*., 2010).

In addition, we have shown that *ASHH2* (*SDG8*) expression in Col-0 plants is inhibited as a result of DC3000 infection, in agreement with a report that *sdg8* (*ashh2* null mutant) is significantly more susceptible at 3 days post-inoculation (dpi) to pathogen attack (Palma *et al*., 2010; Fig. 2). Thus, the loss of ASHH2 (SDG8) appears to enhance susceptibility towards *P. syringae* pv. *tomato* DC3000 (Palma *et al*., 2010), whereas our results show that *ASHH2* (*SDG8*) expression is inhibited as a result of DC3000 infection (Fig. 3a), suggesting an unknown role for effectors in the suppression of *ASHH2* (*SDG8*) gene expression. Although the *ashh2* line has the greatest susceptibility to DC3000 (Fig. 2a), compared with the other backgrounds, this HR might be increased as a result of an altered carotenoid composition displayed by the *ashh2* phenotype (Cazzonelli *et al*., 2009, 2010). Reduced lutein levels in leaves as a result of the down-regulation of *CAROTENOID ISOMERASE* (*CRTISO*) could partially affect the plant's susceptibility to the pathogen, as carotenoid pigments are critical for plant survival. Further research is needed in order to answer such questions.

We found that DC3000-infected *ashh2* affects the H3K4me patterns specifically in the genes related to the SA signalling pathway (Figs 4 and 5). As mentioned previously, ASHH2 (SDG8) is a multicatalytic enzyme with H3K36me2 and H3K36me3 activity (Grini *et al*., 2009), and H3K4me3 activity (Cazzonelli *et al*., 2009), and is required for the trimethylation of lysine 9 of histone H3 (Dong *et al*., 2008). Thus, there are no consistent data on whether the loss of ASHH2 influences H3K4 methylation, H3K36 methylation, or both. How ASHH2 modifies only a fraction of Arabidopsis histones, and its relationship with repressive epigenetic marks (H3K9me2 and H3K27me2), remains to be elucidated.

We hypothesize that, when a microorganism makes contact with a plant, it turns on different signalling pathways. If the microorganism is nonpathogenic, JA-related genes, such as *JAR1*, are induced. ASHR1 may be responsible for dimethylating *JAR1* nucleosomes, whereas ASHR3 seems to act indirectly on this gene, as the H3K4me2 levels remain high, although *JAR1* expression is low, following pathogen infection (Figs 3b and 5). When the interaction is pathogenic, two pathways turn on: the *NPR1* dependent of SA via *CPR5* and *COI1* JA-dependent. In this pathway, ASHR1 seems to be involved directly in the methylation of the *CPR5* nucleosomes, whereas ASHH2 acts indirectly in the nucleosome methylation of the *CPR5* gene. ASHH2 seems to methylate the nucleosome of *WRKY70* (small changes in gene expression, when compared with Col-0, associated with no histone modifications on pathogen infection) and mediates the methylation of an unknown repressor for *NPR1*. The most interesting hypothesis is that ASHR3 seems to activate the expression of a gene with repressive activity, because, in the *ashr3* mutant, the expression of *WRKY70* is high, but the H3K4me2/me3 marks are low, or unchanged.

There are several possible mechanisms of response to biotic stress via chromatin remodelling, histone modifications or DNA methylation, such as the establishment of new states of gene expression or epialleles, regulation of genome rearrangements, transposon activity and stress-mediated regulation of genes by small-RNA activation (Boyko and Kovalchuk, 2008). Clearly, all deserve further investigation in order to elucidate the epigenetic mechanisms in the plant–pathogen interaction. Direct evidence on how pathogen-induced epigenetic modifications may contribute to the transgeneration memory of stress has not been provided so

far, and the effect of bacterial pathogens on the epigenetic status in plants and the epigenetic control of plant immunity represents an emerging field. Our results provide data that can contribute to an understanding of the mechanisms by which changes in H3 methylation regulate the expression of specific genes.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Seeds from *Arabidopsis thaliana* wild-type Col-0 plants and *ashh2*, *ashr1* and *ashr3* mutant lines were surface sterilized, stratified for 48 h at 4 °C and grown on germination medium (Murashige and Skoog, 1962) supplemented with 2% sucrose at 24 °C under long-day (16 h light/8 h darkness) light cycles. After germination on germination medium, 7-day-old seedlings were transferred to 'Jiffy 7 peat pellets' (Novosel Enterprises, Oberlin, PA, USA) and placed in a growth chamber (Percyval AR-36, Percyval, Perry, IA, USA) at 24 °C under long-day (16 h light/8 h darkness) conditions.

Arabidopsis mutant lines and genotyping

Seeds from the different T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA), and were analysed after kanamycin selection of seeds grown in agar plates plus germination medium [2.25 g Murashige and Skoog salts (Sigma-Aldrich, St. Louis, MO, USA) and 10 g/L sucrose, pH 5.8] containing 50 mg/mL kanamycin.

T-DNA insertions were verified by PCR genotyping using T-DNA left border and gene-specific primers. Homozygotes of each line were identified by PCR from F2 generation plants. Segregation analysis of the kanamycin-resistant marker indicates that there is only one T-DNA insertion in each of the mutant lines. All PCRs were performed in 25-μL volumes: 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 2 min, and a final cycle of 72 °C for 5 min. Primers used for genotyping were those suggested by the Salk Institute Genomic Analysis Laboratory (SIGnAL) 'iSect Tool' (http://signal.salk.edu/tdnaprimers.2.html) and were generated with the default conditions. The T-DNA insertion lines analysed were *ashh2* (At1g77300, SALK_014569), *ashr1* (At2g17900, SALK_127952C) and *ashr3* (At4g30860, SALK_128444).

Plant infection

To perform pathogenicity assays and *in planta* bacterial growth assays, 21-day-old *Arabidopsis thaliana* wild-type Col-0 and mutant lines *ashh2*, *ashr1* and *ashr3* were leaf infiltrated (abaxial side) with 100 μL of a fresh bacterial suspension [optical density at 600 nm (OD_{600}) = 0.0005] in sterile water (control). The bacterial strains used in these assays were wild-type *P. syringae* pv. *tomato* DC3000 or a DC3000 *hrpA* mutant, also called A9 (Lee *et al*., 2005), which is defective in the type III secretion system. Symptoms were recorded for a period of 4 days after infection.

Bacterial growth *in planta* was followed for a period of 4 days by excising leaf discs measuring 0.4 cm^2, grinding in water and plating serially diluted suspensions on medium containing the appropriate antibiotic markers. Individual samples were taken from different plants. Each experiment was conducted with three biological replicates. Statistical comparisons were performed by one-way analysis of variance (ANOVA) ($P < 0.05$).

RT-PCR analysis

Total RNA was extracted from 0.3 g of inoculated leaf tissue (24 h after infection of 21-day-old plants) using the BRL Trizol reagent (Invitrogen, Carlsbad, CA, USA) and re-purified with the Qiagen RNeasy Mini Kit (Qiagen, Germantown, MD, USA), following the manufacturer's instructions. RT reactions were performed in a 20-μL volume containing 5 μg of total RNA and 200 units of the M-MLV Reverse Transcriptase (Invitrogen), following the manufacturer's conditions. Platinum Taq polymerase (Invitrogen) was used during PCR, and the conditions were as follows: 5 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and a final cycle of 72 °C for 5 min. Each RT-PCR was conducted twice with three biological replicates.

Gene expression

The following primers were used to determine the expression in wild-type Col-0 plants of the endogenous wild-type genes: *ASHH2* (AT1G77300): forward, 5′-AGACTAAAAGCACATCTCCCACG-3′; reverse, 5′-AAGGATTTTCCTTGTGGCAAGA-3′ (610 bp); *ASHR1* (AT2G17900): forward, 5′-CCTCTTCCACTGTCAGTGTGCC-3′; reverse, 5′-TGAACTCACCGCCTCTTTGG-3′ (522 bp); *ASHR3* (AT4G30860): forward, 5′-CGCTTATCACTCGCTTTGTGC-3′; reverse, 5′-CGGATCTTCCTTCACAACAGAATC-3′ (355 bp); Actin 2/7 (ATU27811): 5′-GGCCGTTCTTTCTCTCTATGCC-3′; 5′-GGCTGTCTCTAGCTCTTGCTCG-3′ (280 bp).

The primers used to determine gene expression after infection were as follows: *NPR1* (AT1G64280): forward, 5′-AGCACCTTTCAGAATCCTAGAAGAGC-3′; reverse, 5′-CTGCAGCAATAATACACAGGATGC-3′; *COI1* (AT2G39940): forward, 5′-CAGAAGCTAGAGATGAGAGGTTGTTGC-3′; reverse, 5′-TCCCAAAAACATCGACGACACAAC-3′; *JAR1* (AT2G46370): forward, 5′-AGATTGCTGCAACTGTTTAGACCGAG-3′; reverse, 5′-AGACAACAACGACGGAAATAAACAACT-3′; *WRKY70* (AT3G56400): forward, 5′-CATGGATTCCGAAGATCACAAGAG-3′; reverse, 5′-CCACTCTACATGGCCTAATTAATGATT-3′; *PR1* (AT2G14610): forward, 5′-CAATGGAGTTTGTGGTCACTACAC-3′; reverse, 5′-GATTCTCGTAATCTCAGCTCTTATTTG-3′; *CPR5* (AT5G64930): forward, 5′-CCTCGGTATATTTTGCGGTGTATCG-3′; reverse, 5′-CGATACAATTGTTTCAACAGCACCC-3′; Actin 2/7 (as control): forward, 5′-CGTTTCGCTTTCCTTAGTGTTAGCT-3′; reverse, 5′-AGCGAACGGATCTAGAGACACCTT-3′.

Histone isolation and Western blots

Histones from Arabidopsis were isolated from 1.5 g of leaf tissue from wild-type and mutant plants (24 hpi of 21-day-old plants) using sulphuric acid extraction of nuclei, followed by acetone precipitation, according to established protocols (Jackson *et al*., 2004). Ten micrograms of isolated histones per sample were used for Western blots. The proteins were transferred to nitrocellulose membrane (0.45 μm) by electrophoresis for 3 h at 250 mA. Membranes were blocked with 5% milk, 0.5% Tween in phosphate-buffered saline (PBS), and probed with various antibodies

(Millipore, Billerica, MA, USA) as follows: dimethyl-histone H3 [Lys-4] (cat. #07-030), trimethyl-histone H3 [Lys-4] (cat. #04-745), dimethyl-histone H3 [Lys-9] (cat. #07-441), dimethyl-histone H3 [Lys-27] (cat. #07-452) and anti-dimethyl-histone H3 [Lys-36] (cat. #07-274). Dimethylated (me2/H3) and trimethylated (me3/H3) levels were measured and compared in histones isolated from the different mutant lines and from wild-type plants. The amount of loaded histone H3 in each sample was determined from Western blots using antibodies specific to nonmethylated H3 (cat. #06-755). Signals from bands obtained with methylation-specific antibodies were normalized against the respective histone H3 amounts (measured as the signal intensities of Western blot bands obtained with anti-histone H3 antibodies). Data from four independent measurements consistently gave the same results.

Densitometric analysis

Band intensities were quantified using ImageQuant™ software (GE HealthCare, Pittsburgh, PA, USA). Intensities were normalized against the anti-histone H3 samples. Histone H3-tail methylation patterns in wild-type and mutants were immunoprecipitated with antibodies against specific H3-tail lysines at 24 hpi with the pathogenic *P. syringae* pv. *tomato* DC3000. Quantified band intensities plotted as a percentage of the input (taken as 100%) are shown on the adjacent histograms.

ChIP assay

ChIP assays were performed as described in detail elsewhere (Alvarez-Venegas and Avramova, 2005; Saleh *et al*., 2008). Calibration curves were constructed before immunoprecipitation experiments to determine the optimal amounts of chromatin to be used in each experiment and to ensure equivalent amounts of starting material. Serially diluted chromatin samples were used to define the point at which detectable bands would be amplified from the tested chromatin templates (immunoprecipitated with each of the three anti-meK antibodies), whereas controls (mock ChIP-ed chromatin templates) would be below the concentrations capable of amplifying visible bands. Antibodies were from Millipore (Billerica, MA, USA): anti-dimethyl histone H3 [Lys9] (cat. #07-441), anti-dimethyl histone H3 [Lys4] (cat. #07-030) and anti-trimethyl histone H3 [Lys4] (cat. #05-745). For all ChIP experiments, chromatin was isolated from rosette leaves of experimental and control plants. Each immunoprecipitation experiment was independently performed three times with separately isolated biological samples. All PCRs were performed in 25-μL volumes: 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 2 min, and finally 72 °C for 5 min. Band intensities were quantified using ImageQuant™. Intensities were normalized versus the input sample representing 15% of the DNA used as template. The ChIP primer sequences used were as follows (see map location in Fig. 5b): *COI1* (AT2G39940) promoter region plus first exon (or region 1): F1, 5′-CTCTGGTGGTTAT GTGTGGATCTGC-3′; R1, 5′-ACCTCAAGTTCGGGAATCGACG-3′; *COI1* exon (or region 2): F2, 5′-AAGGATGGTAAGTGGCTTCATGAGC-3′; R2, 5′-GGGCCGCGAATGGAAATAGTATT-3′; *NPR1* (AT1G64280) first exon (region 1): F1, 5′-CAATTCATCGGAACCTGTTGATGG-3′, R1, 5′-GCTAAA GCGCTCTTGAAGAAAGAGC-3′; *NPR1* exon (region 2): F2, 5′-AGTCTTG AAAAGTCATTGCCGG-3′; R2, 5′-GTATCAATTGTGGCTCCTTCCG-3′; *JAR1* (AT2G46370) first exon (region 1): F1, 5′-AAAAACTTTCCCTTTATGG ATGCTC-3′; R1, 5′-TTAAGGAAATGGCAGGAACAGG-3′; *JAR1* third exon (region 2): F2, 5′-GATGTCCATCAAGCCTTGTATTGC-3′; R2, 5′-GTCTC AGCTTTGGCACATACGG-3′; *WRKY70* (AT3G56400) first exon (region 1): F1, 5′-GTTATGAACCAACTCGTTGAAGGC-3′; R1, 5′-GCATCCTCTTTTACCC TTAACGG-3′; *WRKY70* plus third exon (region 2): F2, 5′-GGAGA TTCTTAATGCCAAATTCCCA-3′; R2, 5′-AACACCATGAGATCCTGAGAACC-3′; *PR1* (AT2G14610) first exon: F1, 5′-TTTAATCGTCTTTGTAGCTCTTGTA-3′; R1, 5′-CATTGCACGTGTTCGCAGCGTAGTT-3′; *CPR5* (AT5G64930) first exon (region 1): F1, 5′-GGAACCCCAAAATCAAATCACC-3′; R1, 5′-GAA CAACCCGTACCAGCAAAGC-3′; *CPR5* to fourth exon (region 2): F2, 5′-GGTTATTGCGAGAAAGAATGCC-3′; R2, 5′-GCGATCTAAATTACAACTCCC ACC-3′.

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