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Research Paper

Epigenetic Control of a Transcription Factor at the Cross Section of Two Antagonistic Pathways

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ABSTRACT

The expression of the *Arabidopsis* gene *WRKY70* is known to be antagonistically regulated by the salicylic acid (SA) and jasmonic acid (JA) signaling pathways. The gene encodes a transcription factor functioning at the crossroad of the two pathways. Here we show that the *Arabidopsis* homolog of Trithorax, ATX1, activates the expression of the *WRKY70* gene and is involved in establishing the trimethylation pattern of histone H3 tail lysine 4 (H3K4me3) residues of its nucleosomes. Chromatin immunoprecipitation (ChIP) analyses with antiATX1 specific antibodies demonstrated that *WRKY70* is a primary target for the ATX1 histone methylase activity, while the SA-responsive gene, *PR1,* and the JA-responsive gene, *THI2.1*, are secondary targets. The unexpected finding that *PR1* and *THI2.1* nucleosomes carryH3K4me3-marks unrelated to their transcription states suggests that the defense-response genes *PR1* and *THI2.1* keep their nucleosomes in 'actively' modified state, perhaps, in preparation for quick-changes of transcription when needed by the cell. Based on the experimental data, we propose a model that could explain the ability of a single epigenetic factor to orchestrate expression of a large number of genes, particularly in cases involving response reactions.

INTRODUCTION

Eukaryotic genes function within the context of chromatin. Mechanisms that modulate the structure of chromatin, leading to activation or repression of pertinent genes, are defined as epigenetic. A paradigm for epigenetic control is the function of the Polycomb/Trithorax-Group (PcG/TrxG) complexes regulating animal and plant homeotic genes and developmental processes.[1-4] The molecular nature of epigenetic mechanisms has been a longstanding enigma until the recent discovery that the SET domain [SuVar(3-9)-E(z)-Trithorax] conserved in both PcG and TrxG components has histone methyltransferase activity.[5] Covalent modifications of histone-tail amino acid residues are though to constitute a 'code' that controls gene activity.[6] Certain lysines at histone tails can be acetylated, methylated, ubiquitinated, or poly-ADP-ribosylated, creating recognition sites for cellular complexes with activating or repressing roles.[7,8] SET domain peptides of the Trithorax- family can methylate lysine 4 of histone H3 (H3/K4), a modification associated with transcriptional activation. Activity depends on whether two- or three-of the lysine-NH_2-groups are methylated, adding a new layer of complexity to the 'code'.[9-13] Plants may have developed epigenetic mechanisms that are related, but not identical, with those of animals or yeasts.[14-16]

Disruption of the gene *ARABIDOPSIS HOMOLOG OF TRITHORAX, ATX1*, causes pleiotropic phenotypes including homeotic stem growth, root, and leaf defects.[4] ATX1 carries the highly conserved SET domain of the Trithorax family[17] and lowered expression of its target genes was, most likely, due to loss of methylated H3/K4.[4] Downregulated genes in the *atx1* background often display lowered levels of H3K4me3 but ATX1 is not responsible for the genome-wide H3/K4 methylation modifying only a fraction of the nucleosomes, instead.[18] Approximately 1,600 genes robustly changed transcription in the *atx1* background.[19] The large number of misexpressed genes raised the question of how ATX1 could synchronize control over such large target numbers. In addition, ATX1 both activates and represses expression raising further questions of how this is achieved and what chromatin modifications reflect this dual role. One plausible scenario is that some genes, i.e., genes encoding transcription factors (TF) are primary targets of epigenetic modifiers, while downstream genes (or entire networks) controlled by the TFs are secondary targets. Thereby, some of the *atx1*- misexpressed genes are simply reflecting altered expression of

pertinent TF (see model in Fig. 6). In such a context, epigenetic regulation may be viewed as a secondary level of control superimposed on the primary level represented by TF. Deactivation of a repressor or upregulation of an activator, then, could provoke a rapid increase of transcription of all genes within the network without the need to modify (prepare) each gene individually. Such *modus operandi* has clear advantages in providing flexibility and rapid gene responses when required by the cell.

To test this hypothesis, we searched the *atx1* microarray expression profiling data (Table 1 in Ref. 19) for ATX1-regulated genes that could serve as a model. Among the genes with altered expression in the *atx1* background, we noticed significant representation of genes encoding disease-resistance, pathogenesis and defense-related proteins including members of the TIR-NBS-LRR classes of disease resistance proteins (>30 genes) and more than 100 genes implicated in various defense-responses: mildew-resistant factors, lectins, major latex proteins, LEA, and Bet v I allergen family proteins; over 20 genes encoding chaperones and heat shock proteins, as well as six members of the WRKY family of transcriptional regulators,[20] were misexpressed in the *atx1* background. Apparently, many disease and defense responsive mechanisms in *Arabidopsis* appear to be under epigenetic control, analogous to roles played by animal PcG/Trx complexes in the regulation of senescence, disease and cancer.[21,22]

Downregulation of *WRKY70* (7.2-fold in *atx1* mutants) implicated ATX1 in the transcriptional activation of the gene. In the meantime, WRKY70 was defined as a mediator in the cross talk between the Salicylic acid (SA) and Jasmonic acid (JA)-signaling pathways activating the SA- but repressing the JA-responsive genes.[23,24] Interestingly, loss-of ATX1 function significantly downregulated the expression of the SA- responsive 'marker' gene, *PR1*, while genes from the JA-response pathway (*THI2.1, VSP2, PDF1.2, HEL*) were upregulated. Thereby, the relationships between *WRKY70* and downstream components of the two signaling pathways were reversely mirrored in the ATX1-loss-of-function background. These findings suggested an ATX1-driven *WRKY70*- activating mechanism and provided an attractive opportunity to test our model (Fig. 6). Positioning of *WRKY70* at the nod of convergence of the two pathways implied that lower transcript levels resulting from the ATX1-loss of function could resonate along the two pathways, oppositely affecting the expression of genes downstream of *WRKY70*. The expression of the *WRKY70* gene is stimulated by the SA- and repressed by the JA-pathways;[23,24] misexpression of *WRKY70* in the *atx1* background[19] suggested an additional epigenetic route for its control. Here, we analyzed the methylation patterns at lysine 4 residues of histone H3 tails of *WRKY70*, *PR1* and *THI.2.1* nucleosomes in correlation with their actively transcribed and repressed states. We were interested in defining the molecular basis of the ATX1-driven mechanisms that activated *WRKY70* and *PR1,* but repressed the *THI.2.1,* genes. The model proposed for the genome-wide function of ATX1 accounts for most of our experimental data.

MATERIALS AND METHODS

Plant material, inoculations with *P. Syringae* and assessments of effects. To perform pathogenicity assays and in planta bacterial growth assays, *Arabidopsis thaliana* Col-0 was dip-inoculated into bacterial suspensions at 1 X 10^7 cells/ml in 10 mM $MgCl_2$ with 0.02% silwet L-77 (Lehle Seeds, Round Rock, TX) and sampled as described.[25] The bacterial strains used in these assays were wild type *P. syringae* pv. tomato DC3000 or a DC3000 *hrcC* mutant, which is defective in type III secretion. Symptoms were recorded 4 days after infection. Bacterial growth in planta was tracked for a period of 4 days by excising leaf disks measuring 0.4 cm^2, grinding in water, and plating serially-diluted suspensions on media containing the appropriate antibiotic markers. Individual samples were taken from different plants. Measurements were taken from experiments repeated six times over a nine-month period. For inoculations of *Arabidopsis* plants expressing the *uidA* gene under the control of the *ATX1* promotor, DC3000 and the *hrcC* mutant strain were infiltrated into leaves at 1 X 10^5 cells/ml using a needless syringe. β-glucuronidase (GUS) activity was assessed after infiltrated leaves were histochemically stained for 24 h with 5-bromo-4chloro-3-indolyl (X-gluc) as a substrate.[26] Seeds from *Arabidopsis* Col 0 wild type and from the *atx1*-mutant line were grown at 24°C under 14 hr light/10 hr darkness. The *atx1* mutant line carried a *Ti-i*nsertion deleting the PHD-SET domains.[4] For SA and meJA treatments, aqueous solutions of 1 mM SA or 0.1 mM meJA were sprayed directly on the leaves until imminent run off. Leaves were harvested at the indicated time points after treatment.

Generation of ATX1 Promotor-GUS Constructs. For cloning the promotor region, we have included sequences lying between the transcription start sites of *ATX1* and the end of the upstream neighbor gene. The designed promotor included the 5'-untranslated regions of *ATX1*. Primers begin with a six-nonsense nucleotide sequence, followed by the restriction sequence used in the cloning step, followed then by the respective genomic sequence. Primers for the *ATX1* promotors used in this study was:

ATX1, F: 5'- cgatgcGGATCCtctccgtggagtttgagaatcc-3'

R: 5'-tcagacCCATGGggagattattcggagggagaaagc-3';

Amplified DNA sequences were cloned in the pCambia1303 vector (Canberra, Australia) after substituting the original 35S promotor with the ATX1-promotor sequence. Constructs were verified by sequencing and the plasmids were introduced in *Arabidopsis* plants by the dip-infiltration method to produce transgenic GUS-expressing lines. Six independently transformed lines were selected and analyzed for GUS-expression.

RT-PCR analysis. Total RNA was extracted from 0.3 g of tissue by using the BRL Trizol reagent and repurified with the Qiagen RNeasy Mini Kit, following the manufacturer's instructions. Fifteen micrograms of total RNA was used to synthesize cDNA using Affymetrix One-Cycle cDNA Synthesis Kit Affymetrix). All sample preparations followed prescribed protocols (Affymentrix Genechip Expression Analysis Technical manual). RT reactions were performed in a 20 μl volume containing 2.5 μg of total RNA and 200 units of the M-MLV Reverse Transcriptase from Invitrogen, following the manufacturer conditions. The TaKaRa Ex-Taq polymerase was used during the PCR reaction, and the conditions were as follow: 95°C for 30", 60°C for 30" and 72°C for 1' for 34 cycles.

The following PCR primers were used:

ATX1,	F: tgtatctgaaggcacacaggcttc	R:gatgatatgccacgcgacaagaag;
WRKY70,	F: ggagattcttaatgccaataaccca	R:aacaccatgagatcctgagaacca;
PR1	F: tttaatcgtctttgtagctcttgta,	R:cattgcacgtgttcgcagcgtagtt;
THI2.1	F: gagtctggtcatggcacaagttcaa,	R:ccaggtgggactacatagctcttgg;
LTP_{WAX9}	F: atcacagcaaaggcggctctgagct,	R:tacgtgttgcacttggtgttgaacc;
Actin 2/7	F: cgtttcgctttccttagtgttagct,	R:agcgaacggatctagagacacctt;

ChIP assays. ChIP assays were performed as described in details elsewhere.[18] It is important to note that 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 when detectable bands would be amplified from tested chromatin templates (immunoprecipitated with each of the four anti meK antibodies) while controls (mock ChIP-ed chromatin templates) would be below concentrations capable of amplifying visible bands. Titration assays with a series of diluted mock treated samples were performed with each set of primers used in the study to ensure that comparable amounts were used as templates in the PCR. Antibodies used in this study were from Upstate: anti-dimethyl-Histone H3 [Lys9], product number (07-441), anti-dimethyl-Histone H3 [Lys4] (07-030), anti-trimethyl-Histone [Lys4] (07-473), anti-dimethyl-Histone H3 [Lys27] (07-322), and anti-Histone H3 (06-755). Antibodies specific against ATX1 were raised in rabbits (CoCalico) and purified by affinity-column chromatography; specificity of antiATX1 antibodies has been reported in Ref. 19. The gene LTP (*At2g15050*), shown earlier to be a direct target of ATX1,[18] was used as a positive control for the association of ATX1 with nucleosomes of tested genes. ChIP experiments with the antiATX1 antibody were performed following a protocol similar to the one used for the histone modifications. For all ChIP experiments in this study chromatin was isolated from rosette-leaves of experimental and control plants. Each immunoprecipitation experiment was independently performed three to five times with separately isolated biological samples. All PCR reactions were done in 25 μl: 5min at 95°C, followed by 35 cycles of 95°C 30 sec, 56°C 30 sec, 72°C 2 min, and 72°C 5 min. Band intensities were quantified using ImageQuant™. Intensities were normalized versus the input sample representing 15% of the DNA used as template. Results were statistically analyzed by the Two- Sample t-test.

RESULTS

Correlation between ATX1 activity and the methylation profile of *WRKY70* nucleosomes. Infiltration with the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 induces *WRKY70* expression[23,24] and microarray hybridizations suggested that *WRKY70* transcription is also upregulated by ATX1.[19] Here, we examined the molecular events accompanying the epigenetically induced activation of *Arabidopsis* genes. First, we confirmed that, in our hands, DC3000 did stimulate *WRKY70* expression and that ATX1 was involved in upregulating *WRKY70* transcription (Fig. 1A): *WRKY70* transcripts were noticeably diminished in the *atx1* background. After infiltration with DC3000, *WRKY70* expression increased and higher transcript levels were still visible 48 hours later, similar to the profile of the wild type; however, transcript levels were always below the wild type when measured at the same time points. Thereby, ATX1 positively contributed to *WRKY70* transcription in the wild type. Because the pathogen induced *WRKY70* expression in the absence of ATX1 it suggested that, most likely, DC3000 and ATX1 activate *WRKY70* by separate mechanisms.

The Trithorax family members activate gene expression by generating epigenetic tags on pertinent nucleosomes that positively influence transcription.[8-13,18] To establish correlations between the ATX1-dependent activation and H3-lysine methylation profiles of *WRKY70*-nucleosomes (under induced and non-induced transcription states) we performed chromatin immunoprecipitation (ChIP) assays with antibodies specific against several histone H3-lysine methylated isoforms: di-methylated K9 (H3K9me2), di-methylated K27 (H3K9me2), di-methylated K4 (H3K4me2) and tri-methylated K4 (H3K4me3). Non-induced (basal) levels of *WRKY70* transcription were accompanied by low H3K9me2 and H3K4me3 and prominent H3K27me2 and H3K4me2 signals (Fig. 1B). Twenty-four hours after inoculation with DC3000 (coincident with enhanced transcription of *WRKY70*) intensities of H3K4me2 and H3K4me3 bands increased, while K27/H3 bands decreased. Analyses of quantified intensities indicated that K27/H3-decrease and H3K4me3 and

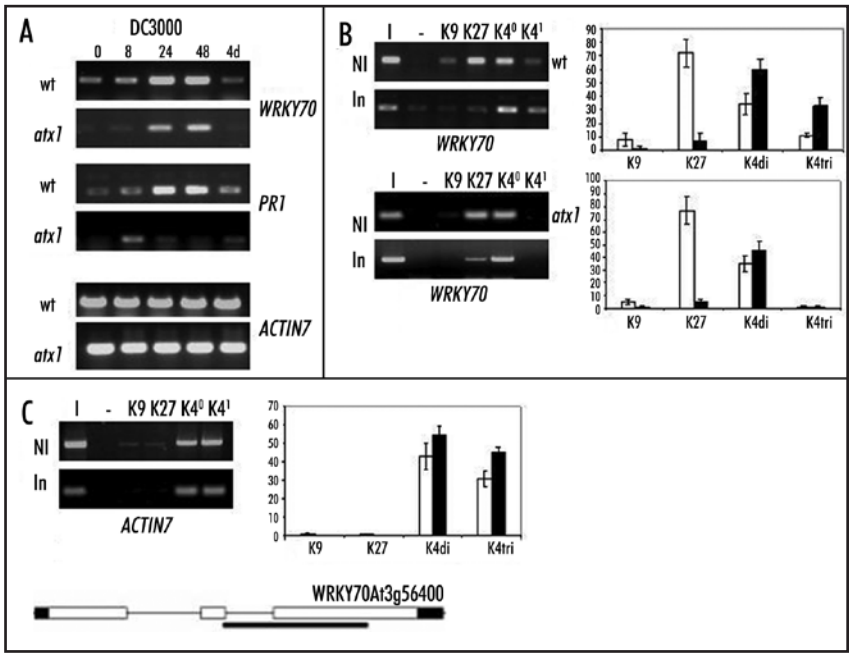

Figure 1. Effects of *P. syringae* upon the expression and the histone H3-methylation patterns of *WRKY70* in the wild type and in *atx1* backgrounds. (A) Wild type or *atx1*-mutant *Arabidopsis* plants after infiltration with the pathogenic *P. syringae DC3000* strain. Samples collected at the indicated post-inoculation time points were examined for the expression of the *WRKY70* and *PR1* genes. *ACTIN7* used as a control for each template preparation was amplified under exactly the same conditions as tested genes. (B) Histone H3-tail methylation patterns of the *WRKY70* gene, in wild type and in *atx1* mutants. Chromatins from non-induced (NI) and from induced (In) wild type and *atx1* plants. Samples were immunoprecipitated with antibodies against specific H3-tail lysines 24 hours post inoculation with the pathogenic *P.syringae* DC3000 when gene expression was at highest levels. (I)-input DNA; fifteen fold diluted samples were used as templates for the input lanes. Negative controls (-), no antibody samples treated in the same way as immunoprecipitated chromatins; K9, K27, $K4^{d,}$ and $K4^{t,}$- represent amplified bands from templates ChIP-ed with methylated histone H3-H3K9me2, H3K27me2, H3K4me2, and H3K4me3 antibodies, respectively. Quantified band intensities plotted as percentage of the input (taken as 100%) are shown on the adjacent histograms. Empty columns are for samples before induction; black columns are for signals obtained post-induction. Each column is from three independent measurements. Absence of H3K4me3 signals from the *WRKY70* gene in *atx1* chromatin (under non-induced and induced conditions) implicate ATX1 in this modification and are not result of poor chromatin templates, as evidenced in (C); (C) *atx1* chromatin templates, as described in (B), amplified with *ACTIN7* specific primers as a control for the quality of the samples. Empty and black columns in the histogram are as indicated above; (D) schematic illustration (not drawn to scale) of the WRKY70 gene sequence: the empty boxes show exons, the connecting lines are the introns and the black boxes are the 5′ and 3′ UTR. The black line below shows the region amplified by the primers.

H3K4me2 increases were statistically significant ($p<0.003$, $p<0.02$, and $p<0.01$, respectively), while changes in H3K9me2 and were insignificant ($p>0.1$). Thereby, the changes in the H3K27me2 and H3K4me3 methylation profiles of the *WRKY70* nucleosomes were consistent with patterns known to accompany transcriptional activation.[13,27] However, H3K9me2 modification appeared not to be involved in regulating *WRKY70* expression.

To determine a role of ATX1 in the *WRKY70* nucleosomal profile, *atx1* plants were inoculated with DC3000 to induce *WRKY70* transcription and its chromatin environment was analyzed by ChIP. The methylation patterns of chromatins isolated from non-treated *atx1* plants and 24 hours later were compared with the patterns of wild type nucleosomes. The main difference was the absence of H3K4me3 from the *atx1- WRKY70* nucleosomes (Fig. 1B), while changes in H3K4me2 levels in wild type and *atx1* chromatins were statistically not significant ($p>0.5$ for both the induced and non-induced states). Absence of H3K4me3 signal from *atx1* chromatin implicated ATX1 in tri-methylating the *WRKY70* nucleosomal H3/K4-residues. Bands amplified from the same chromatin templates with specific primers for the *ACTIN7* gene provided evidence for the quality of *atx1* chromatin templates and underscored the involvement of ATX1 in modifying *WRKY70* but not *ACTIN7* nucleosomes (Fig. 1C). Consistent with previous data, the housekeeping gene carried only H3K4me2 and H3K4me3-tags.[18]

Next, we examined whether the expression of the *ATX1* gene, itself, was sensitive to *P. syringae,* or to the SA and/or JA-triggered mechanisms.

Effects of *P. Syringae,* SA and JA Upon the Expression of *ATX1*. Possible effects of *P. syringae,* of the SA, and the JA-induced mechanisms upon *ATX1* expression were tested by RT-PCR assays and, also, in transgenic plants expressing the GUS-reporter under the control of the ATX1 promotor.

No significant changes in *ATX1* transcription were detected (Fig. 2A) when plants were treated with either the pathogenic DC3000 or with the *hrcC* mutant strain lacking the ability to inject bacterial effector proteins into plant cells[28] (except for an apparent increase 48 hours after inoculation with *hrcC,* see Discussion). Transgenic plants expressing the *ATX1*-promotor driven reporter protein, GUS, were inoculated with DC3000, with the *hrcC* strain, or with water (mock- treatment). For each series, leaves were collected from the same plant to avoid differences in individual plant's GUS-expression (Fig. 2B). There were no significant differences in GUS expression among all samples when compared at the same time points. The virulent DC3000 strain did not alter visibly the staining suggesting that the *ATX1* gene was not under the influence of innate immunity pathways induced by DC3000. An apparent decrease in GUS expression was observed in all leaf samples collected four days post-inoculation. Most likely, this effect was connected with leaf-age than with a response to the pathogen. We note also that *ATX1* was not wound-induced, as torn and punctured leaf tissues did not elevate GUS expression (Fig. 2B).

Direct application of either SA or JA did not noticeably change *ATX1* gene expression as well (Fig. 2C) suggesting that *ATX1* expression was not influenced detectably by *P. syringae,* by the SA or JA-responsive pathways.

ATX1 and the SA-and JA-Responsive Genes. Downregulation of the SA-responsive gene *PR1* and upregulation of several JA-responsive genes in the *atx1* background[19] suggested that, in addition to the known regulatory pathways, the genes were also epigenetically regulated. To test this idea, the *PR1* (*AT2G14610*) and *THI2.1* (*AT1G72260*) genes activated by the SA and JA-pathways, respectively, were selected as examples for further examination. Consistent with available results[23,29,30] the expression of *PR1* in the wild type was upped after infiltration with *P. syringae* DC3000 or after direct application of SA (Figs. 1A and 3A). In the *atx1* mutant background, non-induced *PR1* transcript levels were lower than in the wild type and remained lower after inoculation with DC3000 (Fig. 1A) or after treatment with SA (Fig. 3A). The results, consistent with ATX1 activating *PR1*, suggested that, most likely, the mechanism would be an ATX1-driven modification of *PR1* nucleosomes. Contrary to the expectation, however, ChIP experiments with chromatins isolated from non-treated and from SA-treated wild type and *atx1* plants revealed that ATX1 was not involved in *PR1* nucleosomal modification (Fig. 3B). Presence of H3K4me3 marks on *PR1* nucleosomes isolated from *atx1* chromatin implicated a methyltransferase different from ATX1. Thereby, ATX1 does not methylate *PR1* nucleosomes despite the demonstrated effect of ATX1 upon *PR1* expression. One possibility, then, is that ATX1 controls *PR1* indirectly (see below). Finding of H3K4me3-marks on non-activated *PR1*-nucleosomes and subsequent results showing that H3K4me3 levels did not change significantly upon gene induction ($p>0.1$) (Figs. 3B, C), suggested that *PR1* nucleosomes carried the H3K4me3-tags in the non-induced state. We propose that the 'activating' tags are in place providing 'readiness' for a quick change of *PR1* expression when needed by the cell.

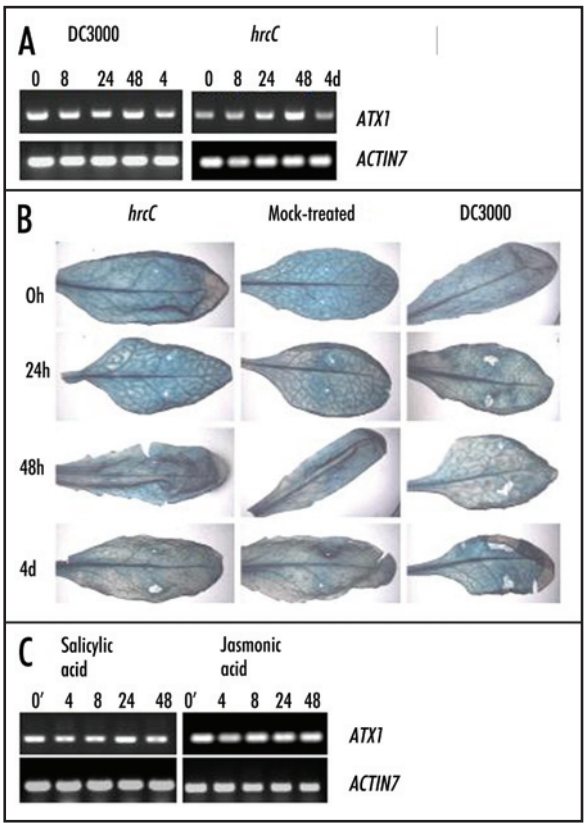

Figure 2. Expression of the *ATX1* gene. (A) *ATX1* gene expression levels after *DC3000* and *hrcC*-inoculations; (B) Transgenic plants expressing GUS under the control of ATX1 promotor collected and stained at the indicated time intervals. For each of the shown treatments, leaves were harvested from the same plant. (C) Levels of *ATX1* gene expression after SA-and JA-treatments.

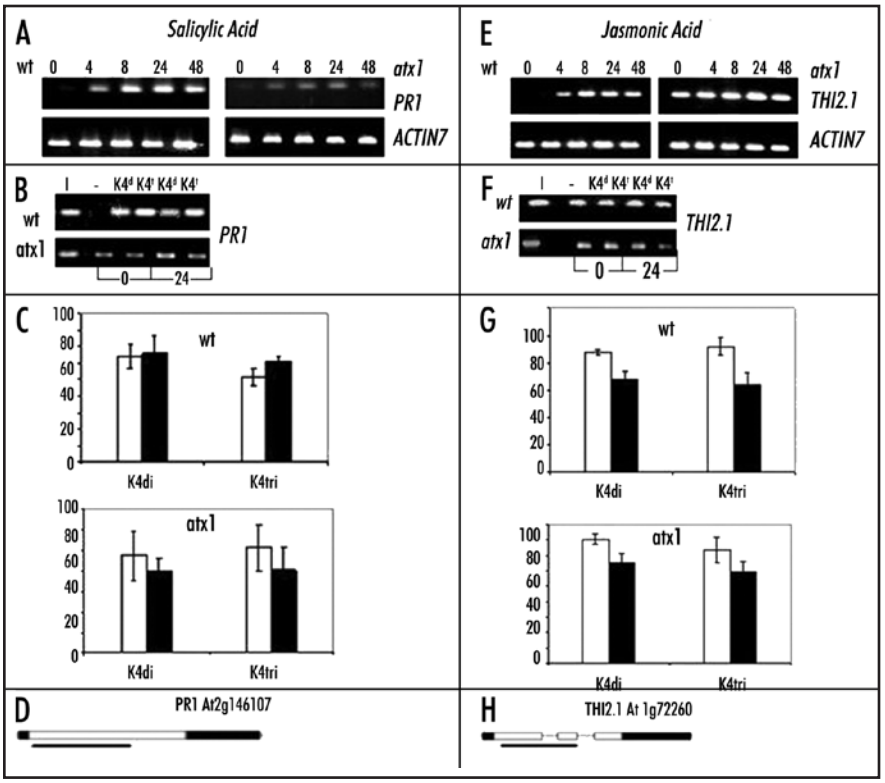

Figure 3. Expression and H3/K4 methylation patterns of the *PR1* and *THI2.1* genes activated by the SA- and JA-responsive pathways. (A) Transcription levels of the *PR1* gene in wild type and *atx1* backgrounds before and after spraying with SA. Leaves for analyses were collected at the times shown on top of lanes. Time points are shown as hours post infection. (B) Di- and tri-K4 methylation of *PR1* nucleosomes from wild type and *atx1* chromatins; ChIP assays with anti-H3K4me2 and antiH3K4me3 antibodies before induction (zero time point) and 24 h post treatment are shown in the bracketed lanes, as indicated. (C) Band intensities plotted as percent of the input sample; (D) and (H) are schematic drawings (not drawn to scale) of the *PR1* and *THI2.1* genes. Empty boxes show exons, the connecting lines are the introns and the black boxes are the 5′ and 3′ UTR. The black lines below show the regions amplified by the primers specific for the respective genes; (E) Transcription levels of the *THI2.1* gene in wild type and *atx1* backgrounds before and after spraying with meJA; (F) H3K4me2 andH3K4me3 K4 patterns of *THI2.1* nucleosomes from wild type and from *atx1* chromatins. (G) Band intensities of the K4methylation levels plotted as percent of the input sample. Empty columns represent samples before induction; black columns are for signals obtained post-induction. Each column is from three independent measurements.

In contrast with the activating roles of ATX1 in the *WRKY70* and the *PR1* expression, several JA-responsive genes are repressed by ATX1: the *THI2.1* gene is expressed in non-stimulated *atx1* plants at levels comparable with those achieved upon meJA-stimulation, suggesting that *THI2.1* is ATX1-repressed in the wild type (Fig. 3E). It was impossible to predict how H3/K4 methylation patterns would correlate with the *THI2.1* induced and non-induced expression states and how/if ATX1 contributed to this repression. To answer these questions, we examined the *THI2.1* H3/K4-methylation profiles in wild type and in *atx1* chromatins under non-induced and under meJA-induced conditions. ChIP analyses with leaf-chromatins from wild type plants before, and after stimulation with meJA, revealed nucleosomal patterns unexpectedly similar to those displayed by *PR1*: di- and tri-methylated H3/K4 nucleosomes were present in both low- and high-expressing *THI2.1*, indicating that the 'activating' tags were in place before induction of transcription (Figs. 3F and G). Furthermore, presence of H3K4me2 and H3K4me3 bands from the *atx1*- chromatin, indicated that a methylase different from ATX1 was modifying the *THI2.1* nucleosomes (Figs. 3F and G) suggesting that ATX1 controls *THI2.1* indirectly.

Primary and Secondary Targets of ATX1. To determine whether any of the three ATX1-controlled genes was a primary target for the histone modifying activity of ATX1, we performed ChIP assays with chromatin isolated from wild type non-induced plants and immunoprecipitated with specific anti-ATX1 antibodies. Specific primers for *WRKY70, PR1,* and *THI2.1* were used to amplify gene sequences associated with ATX1. We could not amplify *PR1* and *THI2.1* corresponding bands but we did recover the *WRKY70* specific band from the same DNA template (Fig. 4). The results were interpreted as ATX1 being bound with *WRKY70* nucleosomes but not with *PR1* and *THI2.1* nucleosomes in *Arabidopsis* chromatin. The experiments suggest that *WRKY70* is a primary target, while *PR1* and *THI2.1* are secondary targets for the histone methylating activity of ATX1.

Resistance to *P. Syringae* in Atx1 Mutants. Lastly, we examined whether downregulation of *WRKY70* and *PR1* in the *atx1* background influenced the plants' susceptibility to the pathogenic *P. syringae* DC3000. Wild type *A. thaliana* Col-0 and *atx1* plants inoculated with DC3000 and the DC3000 *hrcC* strain defective in type III protein secretion system were examined in parallel for production of disease symptoms and for bacterial multiplication *in planta* (Figs. 5A, B). There were no substantial differences in the phenotypic appearances of wild type and *atx1* mutant plants two and four-days post-inoculation. However, wild type DC3000 and the DC3000 *hrcC* defective in type III secretion displayed slightly better growth in *atx1* plants compared to wild type Col-0 (Fig. 5B). The growth difference between the *hrcC* mutant defective in type III secretion on Col-0 and the *atx1* mutant was statistically significant and may suggest that *atx1* is involved in basal resistance. The *hrcC* mutant strain defective in the type III protein secre-

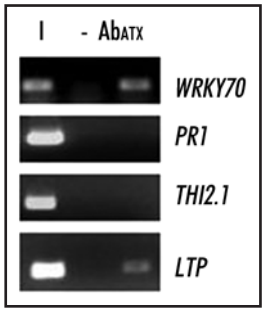

Figure 4. Primary and secondary targets of ATX1. ChIP assays with antiATX1-antibodies to establish primary and secondary targets of ATX1. Amplification with *WRKY70* and *PR1* specific gene-primers were performed on chromatin isolated 24 hours post-induction with *P. syringae* DC3000. Wild type chromatin before inoculation was used as a template for the *THI2.1* nucleosomes. Recovery of *WRKY70* gene-specific bands from the antiATX1-antibody precipitated fraction support direct association of *WRKY70* nucleosomes with ATX1. Absent *PR1, THI2.1* bands indicate lack of ATX1-bound nucleosomes. The *LTP* gene sequences were used as a positive control.

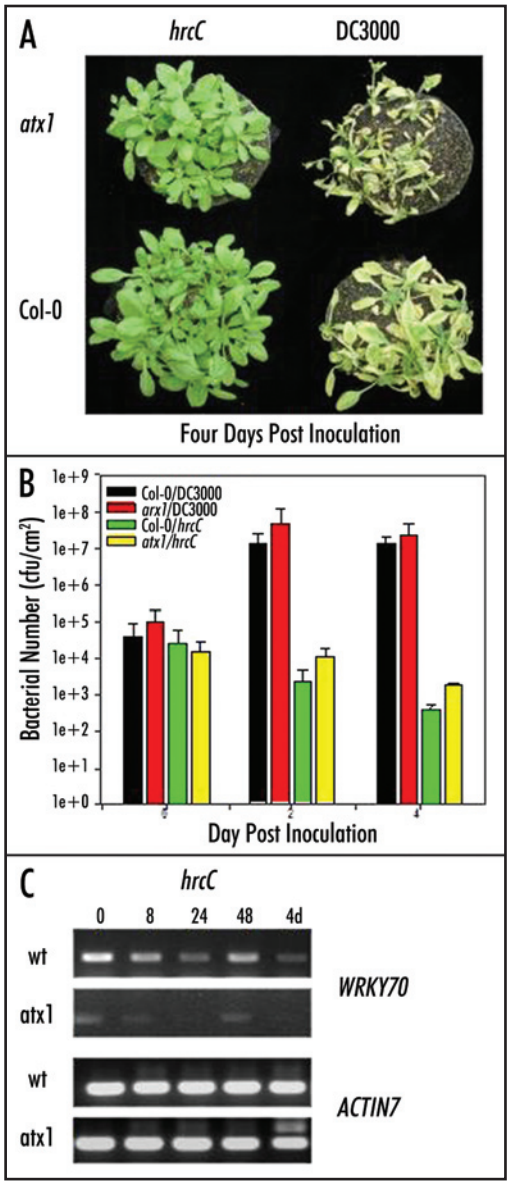

Figure 5. *atx1* plants do not display higher susceptibility to *P. syringae*. (A) Wild type and *atx1* mutant plants four days after being dip-inoculated into suspensions of wild type DC3000 or DC3000 *hrcC* mutant defective in type III secretion; (B) Bacterial growth *in planta* was tracked for a period of 4 days. DC3000 grew similarly in both wild type and *atx1* plants, whereas the *hrcC* strain grew slightly better in *atx1* plants than it did in wild type. (C) Expression of the *WRKY70* gene in wild type and *atx1* backgrounds upon treatment with the DC3000 *hrcC* strain.

tion system did not stimulate *WRKY70* expression in either the wild type or in the *atx1* backgrounds (Fig. 5C), indicating that *WRKY70* activation depended on the delivery of bacterial effector proteins in the cell. Lowered levels of *WRKY70* transcripts did not significantly compromise the *Arabidopsis* response to DC3000, consistent with results showing that loss of *WRKY70* function in *wrky70*-1 did not alter the resistance to DC3000.[24]

DISCUSSION

Approximately 12% of the active *Arabidopsis* genes were affected by the loss of ATX1 function: about equal numbers of genes decreased or increased expression in the *atx1* background (Supplementary Tables in Ref. 19). Thereby, wild type ATX1 functions both as activating and as repressing factor in *Arabidopsis*. The capacity of a single epigenetic factor to regulate expression of a large number of diverse classes of genes, in addition to the high specificity towards the targets, poses logistic problems. For instance, it is unclear how coordinated changes in transcript levels are achieved, how the same epigenetic factor accomplishes both activating and repressive functions, and what methylation patterns accompany transitions to highly transcribed states upon induction. Establishing the H3K4me3 patterns of ATX1- regulated defense genes allowed us to propose a plausible model that might provide a conceptual framework for understanding how an epigenetic factor might achieve regulation of multitudes of diverse genes even when participating in antagonistic pathways.

A model for epigenetic regulation of networks of genes. The central idea is that ATX1 acts as a 'master-regulator' selectively targeting transcription factors (activators or repressors). The latter, then, convey the effects upon genes, or entire downstream networks, under their control. Thereby, TFs are preferable primary targets of the ATX1 methyltransferase activity but genes other than TF might be primary targets as well, as shown for the *LTP* gene (Fig. 4). However, altered expression of TFs would be critical for passing on and multiplying the ATX1 effects along chains of downstream genes; secondary targets will simply be reflecting altered transcript levels of pertinent transcription factors. Another idea supported by the experimental results is that regulation by TFs is superior to the methylation profiles of the target genes in determining active or silenced states: presence of 'activating' or 'silencing' tags is not sufficient to initiate/repress transcription on its own (this study; 18). The novel observations that H3K4me3 tags were present on *PR1* and *THI2.1* nucleosomes before induction of transcription and that no significant changes in H3K4me3 levels accompanied their activation post induction (Figs. 3B and C; 3F and G) suggested that genes involved in response mechanisms have their nucleosomes in an actively modified 'ready' state. Low transcription levels, then, are maintained through a non-activated activator or a repressor. Altered expression of these factors (primary epigenetic targets) could increase transcription from downstream targets without the need to individually modify each gene. ATX1 is critical to maintain and to adjust expression levels of the primary target, which, in turn is responsible for the expression of downstream genes.

Such a model illustrates a mechanism that can produce rapid and coordinated changes in expression of entire gene networks. Epigenetic regulation may be viewed as superimposed on primary regulatory systems (achieved by TFs) for providing flexibility and rapid-responses to events by modulating transcriptional intensities of primary targets.

ATX1 controls transcription of the TF *WRKY70*, the SA-responsive gene *PR1* and the JA-responsive gene *THI2.1*. ATX1 is involved in maintaining higher levels of *WRKY70* expression, most likely by methylating of its nucleosomes. This mechanism is clearly distinct from the *P. syringae* and SA-driven mechanisms.

Genetic evidence has suggested that the SA and JA response pathways act antagonistically.[31,32] The transcriptional factor WRKY70 was positioned at the convergence nod of the SA- and JA-signaling pathways activating the SA-responsive *PR1* gene and repressing the JA-inducible genes.[23,24] As revealed by microarrays,[19] similar relationships between the expressions of *WRKY70, PR1* and the JA-responsive genes (*THI2.1, VSP2, PDF1.2, HEL*) were displayed also in a network regulated by ATX1 (see model in

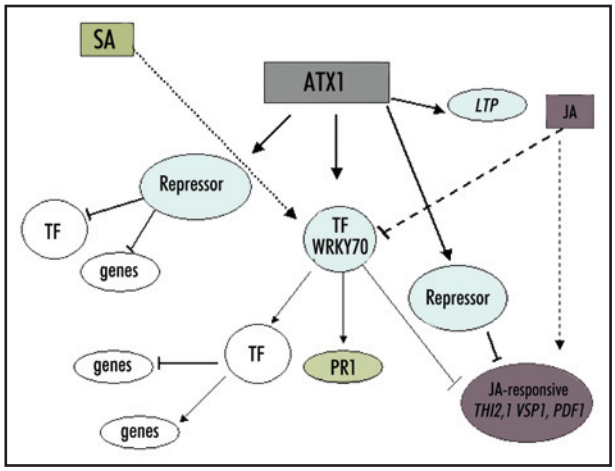

Figure 6. A model for the control of diverse classes of genes by ATX1. ATX1 is targeting directly transcriptional regulators (activators and repressors) by modifying their nucleosomes; genes other than TF could also be primary targets, i.e., the *LTP* gene. Altered transcript levels from these regulators influence expression of downstream genes or chains of genes; ATX1 directly activates *WRKY70* in a pathway independent from the SA-triggered activation. *PR1* expression is controlled by more than one pathway[32,33] but the ATX1-driven activation of *PR1* may be achieved via epigenetically activated *WRKY70*. Another indirect route of *PR1* control could be by an ATX1-mediated interference with *PR1* message stability (not shown). Arrows indicate activation; bars indicate repression. Broken lines show independent SA-and JA-acid controlled pathways. A putative repressor of JA-activated genes is kept active by ATX1: its deactivation in the *atx1* backgrounds leads to the derepression of the JA-responsive genes. *WRKY70* represses JA-responsive genes but does not seem to be involved in *THI2.1* repression.[23,24] Circles with TF indicate activators.

Fig. 6). These findings are important because they confirm the validity of our microarray data and provide biological context for their interpretation. None of the reported genes functioning upstream of *WRKY70* in the SA- and the JA-signaling pathways[23,29-33] were found among the misexpressed gene fraction in *atx1* plants. RT-PCR assays with specific primers for the *NPR1, TGA2, ERF, EDS3, EDS8, PAD3, PAD4,* and *COI2* genes failed to indicate significant changes in expression for any one of these genes in the *atx1* background (results not shown). Together with data from the microarray hybridizations, these results suggested that ATX1 did not influence expression of SA-and JA- responsive genes upstream of *WRKY70*. We propose that ATX1 exercises its control of the SA- responsive (*PR1*) and of the JA-responsive (*THI2.1*) genes at the level of *WRKY70*. More than one pathway is capable of activating *WRKY70* transcription[23,32,33] and our results define ATX1 as a novel positive regulator of *WRKY70*. Lower *WRKY70* transcripts resulting from ATX1 loss of function, then, would have consequences for the expression of the SA-and JA- responsive genes downstream of *WRKY70*. Because *PR1* and *THI2.1* are not directly targeted by ATX1 (Fig. 4), their regulation is mediated by a TF (activator or repressor, respectively). Despite its ability to repress JA-responsive genes, WRKY70 does not seem to be the repressor for *THI2.1*[23,24] suggesting that ATX1 controls *THI2.1 via* a different factor, while WRKY70 is a plausible activator for *PR1*.[23] Another possibility is that an ATX1-controlled factor could be involved in the posttranscriptional stability of the *PR1* mRNA as an alternative mechanism for the ATX1-mediated activation of *PR1* expression.

Molecular basis of the ATX1-mediated control of *WRKY70*, *PR1* and *THI2.1* genes. The ability to experimentally induce expression of these genes allowed us to analyze possible correlations between methylated H3/K4 patterns, gene expression states, and the involvement of ATX1 in this process. Apparently, ATX1 is involved in tri-, but not in di-, methylation of H3/K4 of *WRKY70* nucleosomes. Loss of H3K4me3, associated with the downregulation of *WRKY70* in *atx1* plants, illustrates a positive correlation between H3K4me3 and *WRKY70* expression. We note also the negative correlation between the activity state of *WRKY70* and the presence of H3K27me2. Consistent with its role as a 'repressive' mark, H3K27me2 levels drop significantly upon transcriptional induction of *WRKY70*; by contrast, H3K9me2 is low (Fig. 1B). Thus, the H3K27me2 and H3K9me2 patterns at the silent *WRKY70* locus provide an example different from the Clarke Kent epialleles of *SUP*, which require simultaneously present H3K27me2 and H3K9me2 to maintain *SUP* silent.[16] These results add to the evidence that the 'epigenetic crosstalk' at plant loci might be gene-specific. Furthermore, in the context of current ideas about the crosstalk between the activating and repressing epigenetic modifications, we note that absence of H3K4me3 (in the *atx1* background) did not result in increased H3K27me2 or H3K9me2 levels nor was low *WRKY70* transcription in the *atx1* background accompanied by higher levels of either of these silencing marks. Thereby, absent H3K4me3 marks did not trigger more methylation of K27 suggesting that the factors di-methylating K27 at the *WRKY70* locus were not correlated with the presence of the H3K4me3 marks for their activity; H3K9me2 is not involved in the epigenetic decoration of *WRKY70* nucleosomes.

ChIP assays with antiATX1-specific antibodies showed that ATX1 was bound to *WRKY70* nucleosomes defining it as a 'primary' target. Despite the role of ATX1 in the expression of *PR1,* and *THI2.1*, it does not modify *PR1,* and *THI2.1* nucleosomes (Figs. 3A–C; 3E–G). There are several methyltransferases in *Arabidopsis* that could play this role.[17,34] ATX1 does not associate directly with *PR1* and *THI2.1* nucleosomes (Fig. 4) consistent with the idea that, most likely, the ATX1-control is mediated by a TF.

Epigenetic regulation of disease-responses in plants. The chromatin-remodeling factor (DDM1) and DNA methylation are involved in plant pathogen interactions controlled by the *BAL* locus.[35] Histone deacetylases (HDA) were implicated in maize responses to pathogenic fungi and in JA, ethylene, and *Alternaria brassicicola* induced responses in *Arabidopsis*.[36-38] Histone deacetylase activity of HDA19 correlated with *PR* gene expression and with altered pathogen resistance to *A. brassicicola* in *hda19* mutants.[39] HDA19-associated deacetylation was both genome-wide and gene specific. However, lack of ChIP-based analyses in these reported studies, did not allow correlating induced gene expressions with covalent changes in chromatin patterns. Consistent with our earlier study,[18] we show that such correlations are not unequivocal: nonexpressed response genes might carry 'activating' tags in preparation for future transcription. Most likely, their expression is initiated by activation of an activator or deactivation of a repressor.

Induction of *WRKY70* transcription was dependent exclusively upon the pathogen-induced mechanism because exposure to the DC3000 *hrcC* mutant did not trigger upregulation of *WRKY70* in either wild type or in *atx1* backgrounds (Fig. 5C). Interestingly, since the *hrcC* mutant is defective in the delivery of bacterial type III effectors into plant cells via the type III system, it suggests that type III effectors induced *WRKY70*. This is not related to the induction of defenses by resistance protein surveillance because DC3000 is virulent on *Arabidopsis*. Lowered expression from *WRKY70* in *atx1* mutants showed only a slightly increased bacterial growth than wild type controls (Figs. 4A and B) in agreement with published results.[24] The apparent increase in ATX1 transcript levels 48 hours post inocu-

lation with *hrcC* (pointed out to us by the anonymous Reviewer) as well as the slightly better growth of this strain in the *atx1* plants (shown in Figs. 4A and B) might reflect an involvement of ATX1 in basal resistance and merit further studies.

Collectively, our results revealed a novel mechanism for the regulation of the disease-responsive *PR1* and *THI2.1* genes mediated by an epigenetically regulated TF. WRKY70, a direct target for the histone methyltransferase activity of ATX1 is, most likely, the TF activating *PR1*. The repressor of *THI2.1* is not identified; it will be interesting to establish whether it would be a direct target of ATX1. Other methylases modify H3/K4 residues on the *PR1* and *THI2.1* nucleosomes but presence of H3K4me3-tags does not indicate an actively transcribed gene: defense-response genes might carry these modifications in readiness for rapid change of transcription upon need.

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