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# Methylation patterns of histone H3 Lys 4, Lys 9 and Lys 27 in transcriptionally active and inactive *Arabidopsis* genes and in *atx1* mutants

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## ABSTRACT

**Covalent modifications of histone-tail amino acid residues communicate information via a specific 'histone code'. Here, we report histone H3-tail lysine methylation profiles of several *Arabidopsis* genes in correlation with their transcriptional activity and the input of the epigenetic factor ARABIDOPSIS HOMOLOG OF TRITHORAX (ATX1) at *ATX1*-regulated loci. By chromatin immunoprecipitation (ChIP) assays, we compared modification patterns of a constitutively expressed housekeeping gene, of a tissue-specific gene, and among genes that differed in degrees of transcriptional activity. Our results suggest that the di-methylated isoform of histone H3-lysine4 ($m^2$K4/H3) provide a general mark for gene-related sequences distinguishing them from non-transcribed regions. Lys-4 (K4/H3), lys-9 (K9/H3) and lys-27 (K27/H3) nucleosome methylation patterns of plant genes may be gene-, tissue- or development-regulated. Absence of nucleosomes from the *LTP*-promotor was not sufficient to provoke robust transcription in mutant *atx1*-leaf chromatin, suggesting that the mechanism repositioning nucleosomes at transition to flowering functioned independently of ATX1.**

## INTRODUCTION

Histone-tail modifications provide 'tags' that are 'read' by factors recognizing the modified residue (1). The mechanism constitutes a 'code' that may instruct gene activity (1–3). Different biological systems have evolved specific ways of implementing and translating the code suggesting that the histone-tail 'language' is taxon (species) specific (4). For example, di-methylated K4/H3 ($m^2$K4/H3) modification is genome-wide in yeast (5) while in metazoa, $m^2$K4/H3 is concentrated around genes largely overlapping with tri-methylated ($m^3$K4/H3) modifications (6–9). Methylation at K4/H3 (mK4/H3) is linked with both active and non-active genes in *Saccharomyces cerevisiae*, dependent on whether two- or three-of the lysine-$NH_2$-groups are methylated (5,10,11). Eukaryotes may use specific 'dialects' of the epigenetic language (4) making deciphering a challenge.

Because of the totipotency of plant cells, plants have developed epigenetic mechanisms that are related, but not identical, with those of animals or yeast (4,12,13). Plants have a unique histone deacetylase (HDAC) family, a unique plant chromodomain DNA-methyltransferase (CMT) family (14), may have acetylated and methylated histone residues other than in known chromatins (4), and might 'read' information encoded in the modifications in a different way. For example, the *Arabidopsis* gene *LHP1*, although structurally similar to the animal *HP1* gene, does not function in heterochromatin (15). There is no homolog of *Polycomb* in the *Arabidopsis* genome and the K27/H3 modification is read in a plant-specific way: $m^2$K27/H3 needs to be present simultaneously with $m^2$K9/H3 in order to keep the *SUP* and *FWA* genes inactive (15). Methylated K9/H3 and K27/H3 were also found at the silenced *FLC* locus (16,17).

Less is known about histone-tail methylations associated with plant gene activation. Available data indicate the presence of $m^2$K4/H3 at one plant locus (17) and provide evidence that *Arabidopsis* euchromatin, in general, is enriched in $m^2$K4/H3, while heterochromatin is depleted in $m^2$K9/H3 but enriched in $m^2$K4/H3 (18,19). Whether the tri-methylated version of K4/H3 ($m^3$K4/H3) is associated with gene activation in plants, whether di- and tri-mK4/H3 co-localize at plant genes as in animals, or whether their distribution is analogous to that in yeast, is still unknown. A recent study reported interesting correlations between methylation and acetylation of several lysines of histone H3 isoforms (20). However, it is still unknown whether methylation of K4/H3 influences (cross-talks with) methylations at K9/H3 or K27/H3 at active or non-active gene loci.

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Earlier, we have shown that loss-of-ATX1 function had pleiotropic effects in *Arabidopsis* and that the mRNA levels of several flower homeotic genes were decreased in *atx1* mutant (21). Recombinant ATX1-SET-domain peptides displayed weak K4/H3 methyltransferase activity (21) suggesting that lowered expression might be due to loss-of-methylated K4/H3. The highly selective ATX1 effect upon some, but not all, homeotic genes (21) and microarray data (unpublished data) suggested that ATX1 was not involved in a genome-wide methylation of K4/H3. Here, we have determined that ATX1 is involved in the methylation of only a fraction of the overall histone H3-K4-residues. Nucleosomal methylation profiles were established at two ATX1-regulated loci: the ATX1- activated gene, *LTP*, and the ATX1-downregulated gene, *XET*. Their methylated profiles were compared with those of genes not regulated by ATX1, *SUP* and *ACT7*. In order to 'read' the H3-tail methylation code, we examined genes when expressed at low, moderate or high levels. To determine whether $m^2$H3/K4 and $m^3$H3/K4 co-localized at the start of transcription or throughout the gene sequence, we examined regions 5′-upstream (including the promotor) and downstream gene-coding (G)-regions; finally, we asked whether a gene transcribed in a tissue-specific manner would be similarly 'tagged' as an ubiquitously expressed housekeeping gene. To be able to correlate transcriptional activity with the methylation profile, we tested non-transcribed intergenic regions flanking a gene in activated and in silent states. We found that although di- and tri-methylated K4/H3 was often associated with transcriptionally active genes, mere presence of the tags did not necessarily define, or was not sufficient to induce, gene expression. Di-methylated K4/H3 was found on nucleosomes of all tested gene sequences, independent of whether the gene was active or not. It was also found in various combinations with $m^3$K4/H3, $m^2$K9/H3 and $m^2$K27/H3. Absence of $m^2$K4/H3 from intergenic regions suggested that this modification could be a general mark that distinguishes genes from non-transcribed sequences. Unlike patterns reported in animals or yeast (5–11), $m^3$K4/H3 always co-localized with $m^2$K4/H3 at both the 5′ end and downstream gene regions. We also found that the absent $m^3$K4/H3 did not necessarily correlate with low expression, that specific regulators (activators and repressors) affect transcription independently of H3-tail methylations, and that transcribing states might be labeled by different combinations of methylation tags at the promotor, in the gene-coding regions, or in both, in a gene-, tissue- and development-specific mode.

## MATERIALS AND METHODS

**ChIP Assays** were performed following the established protocols (22,23) with modifications. Four-week-old *Arabidopsis atx1* and wild-type plants were harvested after grown on soil. Two grams each of flowers, leaves and stems were immersed at room temperature in buffer A [0.4 M sucrose, 10 mM Tris–HCl (pH 8), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% formaldehyde] and kept under vacuum for 10 min. The cross-linking reaction was stopped by adding glycine to a final concentration of 100 mM, and by placing the tissue under vacuum for additional 5 min. The tissue was then washed with sterile de-ionized water, and frozen in liquid nitrogen. Frozen tissue was ground to a fine powder and re-suspended in 20 ml of cold nuclei isolation buffer [NIB; 15 mM PIPES (pH 6.8), 5 mM $MgCl_2$, 60 mM KCl, 0.25 M sucrose, 15 mM NaCl, 1 mM $CaCl_2$, 0.9% Triton X-100, 1 mM PMSF, 2 μg/ml pepstatin and 2 μg/ml aprotinin] as described (24). The slurry was filtered through four layers of cheesecloth and the filtrate was centrifuged at 10 000 *g* for 20 min on a Sorvall SA-600 rotor. The pellet (nuclei) was re-suspended in 3 ml lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 1 mM PMSF, 10 mM Na butyrate, 1 μg/ml aprotinin and 1 μg/ml pepstatin A) and the DNA was sheared by sonication to ∼1000 bp fragments. To pellet debris, the sample was centrifuged for 10 min at 13 000 r.p.m. (Sorvall SA-600 rotor), the supernatant (sonicated chromatin) was collected and used further.

Before immunoprecipitation experiments, calibration curves were constructed 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-methyl antibodies) while controls (mock ChIP-ed chromatin templates) would be below concentrations capable of amplifying visible bands. Titration assays were carried out for each immunoprecipitation experiment as follows: 50 μl, 100 μl, 200 μl and 300 μl aliquots of the supernatants adjusted to 500 μl with lysis buffer were pre-cleared with 60 μl salmon sperm (SS) DNA/Protein-A agarose (Upstate) for 50 min at 4°C and centrifuged. Supernatant was incubated with 60 μl SS DNA/Protein-A agarose without antibodies for 2 h at 4°C as negative control fractions (described below). An aliquot (50 μl) of sonicated chromatin diluted 10-fold in lysis buffer, contained the following amounts of DNA: 10.13 μg for *atx1* flowers, 6.6 μg for *atx1* leaves, 5.25 μg for *atx1* stems, 7.7 μg for wild-type flowers, 7.5 μg for wild-type leaves, and 7.2 μg for wild-type stems.

For the immunoprecipitation experiments, aliquoted samples were pre-cleared, as described above, and the supernatant fraction was incubated with 5 μl of the respective antibody (Upstate): anti-dimethyl-Histone H3 [Lys-9], product #07–441, anti-dimethyl-Histone H3 [Lys-4] #07–030, anti-trimethyl-Histone [Lys-4] #07–473, anti-dimethyl-Histone H3 [Lys-27] #07–322 and anti-Histone H3 #06–755. After overnight incubation with rotation at 4°C, 60 μl SS DNA/Protein-A agarose were added and incubation was continued for 2 h at 4°C. The agarose beads were then washed for 4 min at 4°C, with 1 ml of each of the following: low salt wash buffer, high salt wash buffer (Upstate), LNDET [0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris (pH 8)]; finally, beads were washed three times with 1 ml TE buffer [10 mM Tris–HCl (pH 8) and 1 mM EDTA]. Immuno-complexes were eluted from the beads after two washings with 250 μl of freshly prepared elution buffer (1% SDS and 0.1 M $NaHCO_3$) and were incubated at room temperature for 15 min with rotation. The eluates were combined with 20 μl of 5 M NaCl, and crosslinks were broken by incubation at 65°C for 5–6 h.

Residual protein was degraded by incubation with 20 µg of Prot K, incubated at 45°C for 1 h, followed by phenol extraction and ethanol precipitation. The DNA was re-suspended in 20 µl sterile distilled water.

Antibodies specific against ATX1 were raised in rabbits (CoCalico) and ChIP experiments were performed following a similar protocol.

An aliquot (50 µl) of the initial sonicated chromatin solution was treated to reverse the crosslinks and was used as a template for input samples. 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. Each immunoprecipitation experiment was independently performed at least three times. All PCR were done in 25 µl: 5 min at 95°C, followed by 35 cycles of 95°C for 30 s, 56°C for 30 sec, 72°C for 2 min and 72°C for 5 min. The following PCR primers were used: 5′-*LTP* F:cccaatatctccaatccataagtt, R:gagtagggtgatgaa-gagagttgt; *LTP* Gene F: atcacagcaaaggcggct-ctgagct, R: tacgtgttgcacttggtgttg-aacc; 5′-*XET* F: cccacataatcttcatgttgtgtt, R: ccacttcatcttcaagtc-a-tttgt; *XET* Gene F: gacaacattccggttaggcagtttc, R: cttcgacaa-cgtggcaaaatcctga; *Actin 2/7* F: cgtttcgctttccttagtgttagct, R: agc-gaacggatctagagactcacctt; *SUP* Gene F:ggccaccaagatcctacactt, R:agcatctttactggtgaaccttgt; 5′-*SUP* F:ttcacgaaacttaaaggt-gtaacat, R:agtaatgatttgtggtacatcagttt; *SUP* upstream region F:cgtacatttcaagcgtaattaaacc R:tttgtgttcttc-cgttgtgttag; *SUP* downstream region F:tccgctcacagcaggaac, R:tgggactt-ggtttctcttctttc.

### Nuclei isolation and MNase digestions

Nuclei were isolated according to established protocols (25). Three grams of fixed tissue were frozen in liquid Nitrogen, ground to a fine powder and re-suspended in 6 ml of Honda buffer [2.5% Ficoll 400, 5% dextran T40, 0.4 M sucrose, 25 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, +1 mM PMSF, 2 µg/ml pepstatin and 2 µg/ml aprotinin]. The homogenate was filtered through a 62 µm mesh and Triton X was added to a final concentration of 0.5%; the homogenate was incubated on ice for 15 min, centrifuged at 1500 *g* in an SS34 rotor for 5 min and washed with Honda buffer containing 0.1% Triton. The pellet was gently re-suspended in 1 ml of Honda buffer without Triton, transferred to a microcentrifuge tube and centrifuged at 100 *g* in a benchtop eppendorf microcentrifuge for 5 min. The supernatant, containing the nuclei was transferred to a new tube, centrifuged at 1800 *g* in a benchtop eppendorf microcentrifuge for 3 min to pellet the nuclei, and then washed with 800 µl of buffer N [0.34 mM sucrose, 15 mM HEPES (pH 7.5), 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine and 0.15 mM β-mercaptoethanol]. After centrifugation, the pellet was re-suspended in 400 µl of buffer N, adjusted to 3 mM $CaCl_2$ and 50 µl aliquots were incubated with 200 U/ml of microccocal nuclease (MNase/Worthington) for the desired time periods at 37°C. The reaction was stopped by addition of an equal volume of buffer S [90 mM HEPES (pH 7.9), 220 mM NaCl, 10 mM EDTA, 2% Triton X; 0.2% Na-deoxycholate, 0.2% SDS, 0.5 mM PMSF, 2 µg/ml pepstatin and 2 µg/ml aprotinin]. DNA was isolated and re-suspended in 20 µl $ddH_2O$. A 2 µl sample was used per PCR.

## RESULTS

### ATX1 is involved in the modification of only a fraction of *Arabidopsis* histones

Our results (Figure 1) suggested that ATX1 was not involved in overall methylation of histone H3-lysine4 (K4/H3). Di-($m^2$K4/H3) and tri-($m^3$K4/H3) methylated levels were measured and compared in histones isolated from *atx1* and from wild-type plants. The amount of loaded histone H3 in each sample was determined from western blots using antibodies specific against non-methylated H3. Signals from bands obtained with methylation specific antibodies were normalized against the respective histone H3 amounts (measured as signal-intensities of western-blot bands obtained with anti-histone H3-antibodies). This approach allowed us to evaluate even small variations in the methylation levels of overall K4/H3. Data from six independent measurements consistently indicated that histone H3 from the *atx1* mutants displayed lower K4 methylation (6–8% of $m^2$K4/H3 and ∼15% of less $m^3$K4/H3) than histone H3 from wild-type. Apparently, other methyltransferases contribute for the genome-wide K4/H3-modifications in *Arabidopsis*.

### Histone H3-tail methylation of the *LTP* gene up-regulated by ATX1

The *LTP* gene, a member of an antimicrobial peptide family (26), was highly expressed in wild-type vegetative tissues (leaves and stems), to a lesser degree in flowers, but was significantly downregulated in all tested *atx1* mutant chromatins. The results indicated that ATX1 positively controlled *LTP* expression (Figure 2a). To examine how repression by ATX1-loss-of function would influence *LTP* methylation profiles, we performed ChIP analyses with chromatins of three different tissues isolated from wild-type and from *atx1* plants.

In wild-type flower chromatin (moderately expressing *LTP*), we documented presence of all four methylated modifications ($m^2$K9/H3, $m^2$K27/H3, $m^2$K4/H3 and $m^3$K4/H3) (Figure 2c). In the highly expressing leaf and stem chromatins, the patterns were different: the nucleosomes from the (G)-region retained the $m^2$H3/K4 and $m^3$H3/K4-tags but the

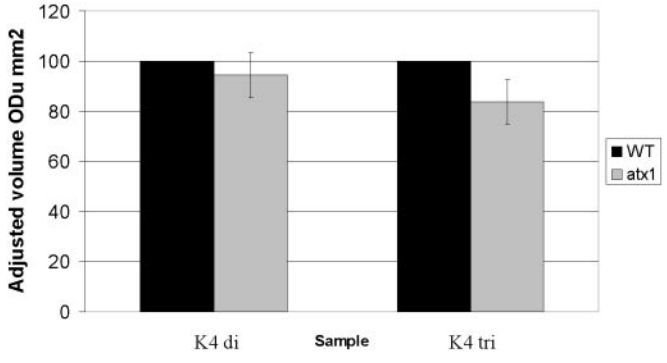


**Figure 1.** Overall histone H3-K4 methylation in wild-type and *atx1* mutants. Total histones extracted from 3-week old wild-type and *atx1* mutant plants were probed with antibodies specific for di- or tri-methylated K4/H3 in western-blots. Subsequent to the hybridization, membranes were stripped off and re-probed with antibodies specific for non-modified histone H3. The levels of histone H3-tail methylation of wild-type histones, defined as the ratio of mK/H3-to-H3 intensity signals, were taken as 100%.

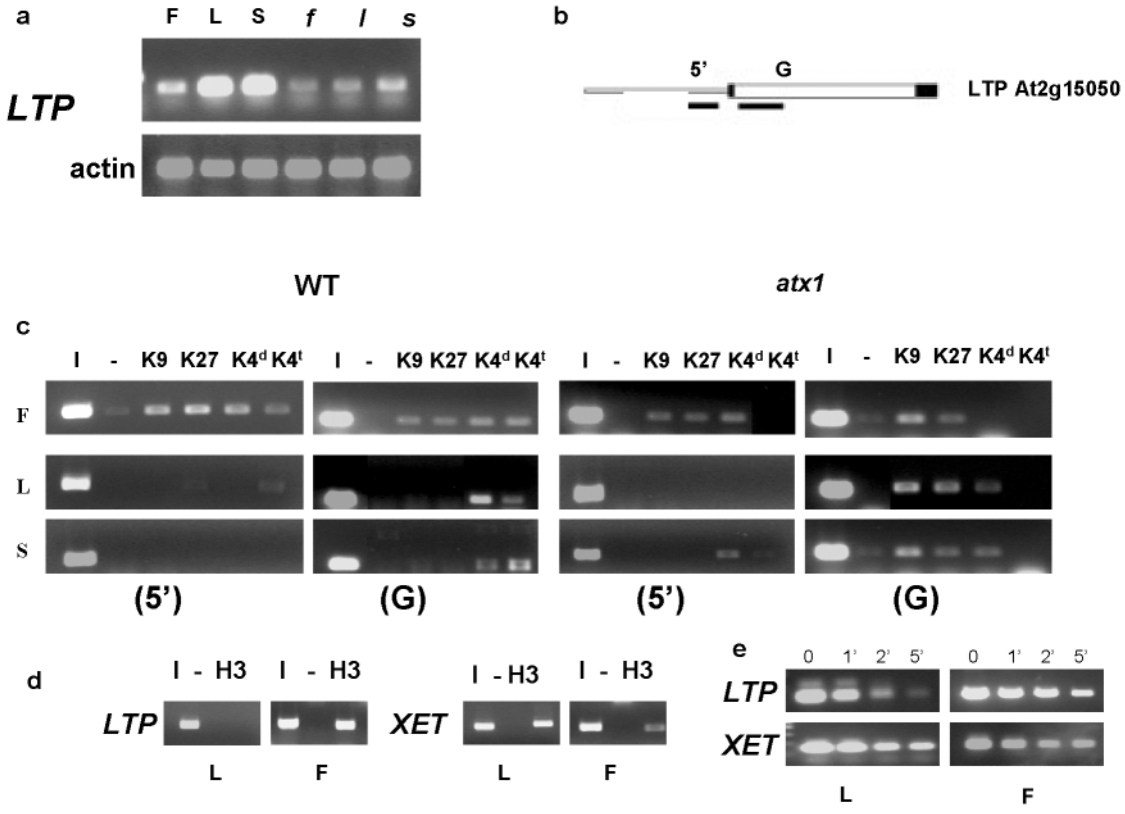


**Figure 2.** Tissue-specific expression of *LTP* and histone H3-tail methylation patterns in wild-type and in *atx1* mutants. (**a**) Expression of the *LTP* gene (At2g15050) in wild-type flowers (F), leaves (L) and stems (S) and in the respective *atx1* mutant tissues (*f*), (*l*) and (*s*). Actin was used as a loading control for each template. (**b**) *LTP* gene structure and location of specific primers used to PCR amplify tested gene regions. Empty boxes indicate exons, black boxes indicate non-translated regions; (5′)-contain upstream sequences and, presumably, the promotor; (G)-contains sequences within the coding region. (**c**) Chromatins isolated from wild-type and *atx1* tissues (F-flowers, L-leaf) immunoprecipitated with antibodies against specific H3-tail lysines. (I)-input DNA; 15-fold diluted samples were used as templates for the input (I) lanes. Negative controls (−), no antibody samples treated in the same way as immunoprecipitated chromatins; K9, K27, $K4^d$ and K4′- represent amplified bands from templates ChIP-ed with methylated histone H3- $m^2$K9/H3, $m^2$K27/H3, $m^2$K4/H3 and $m^3$K4/H3-antibodies, respectively (see Materials and Methods for dilutions, calibration and quantitation of chromatin used as template). (**d**) Leaf and flower chromatins, ChIP-ed with antibodies specific against unmodified histone H3 used as a general probe for nucleosome-associated DNA sequences. (**e**) Sensitivity to MNase digestions of the *LTP* and *XET* promotor regions in leaf (L) and flower (F) chromatins.

$m^2$K9/H3 and $m^2$K27/H3 bands were not present. Given the results from other chromatin experiments, the association of active genes with methylated K4/H3 (in di- and tri- isoforms) were not unexpected. However, the observation that moderately transcribed genes associated with multiple methylated lysines of the histone H3-tails is novel. Similar patterns were displayed by other genes (see below), suggesting that these might be general 'tags' for 'moderately'-expressing states in *Arabidopsis*.

In *atx1* mutants, lack of $m^3$H3/K4 on nucleosomes from both 5′ end and (G)- sequences, correlated with lowered *LTP* expression in *atx1* chromatins (Figure 2a and c; see also Figure 3d). Absence of $m^3$H3/K4 bands clearly implicated ATX1 in the methylation of *LTP* nucleosomes but presence of $m^2$H3/K4 on some *atx1* nucleosomes suggested that ATX1 was not involved in di-methylating K4/H3 at *LTP*.

### The *LTP*-promotor region in actively expressing leaf chromatin is depleted of nucleosomes

Most striking was the disappearance of all methylation signals from the 5′-*LTP* nucleosomes in highly expressing leaf and stem chromatins. This was an *LTP*-specific feature because another gene, *XET*, displayed clear methylation bands at its 5′-sequence (Figure 3c). Absence of all tested histone H3-modifications suggested that either the methylation tags were removed from *LTP*-promotor nucleosomes upon activation, or that the 5′-*LTP* region was depleted of nucleosomes.

To distinguish between the two possibilities, we performed ChIP with specific antibodies against unmodified histone H3 as a general probe for nucleosome-associated DNA. Using specific primers for the 5′-*LTP* region, we could not amplify the corresponding band from leaf chromatin but we did recover the promotor sequence from flower chromatin (Figure 2d). The results indicated that the 5′-*LTP* sequence was devoid of nucleosomes in leaf chromatin but that it was associated with nucleosomes in flower chromatin. Absence of nucleosomes from the leaf *LTP*-promotor was a gene-specific feature, because the 5′-region of a different gene, *XET*, was successfully recovered from the same leaf chromatin preparation (Figure 2d).

These conclusions were independently confirmed by micrococcal nuclease (MNase)-digestion experiments. Increased nuclease sensitivity at the 5′-*LTP* region displayed by leaf, but not flower chromatin (Figure 2e) correlated with the higher transcribed state of *LTP* in leaves than in flowers. The relative

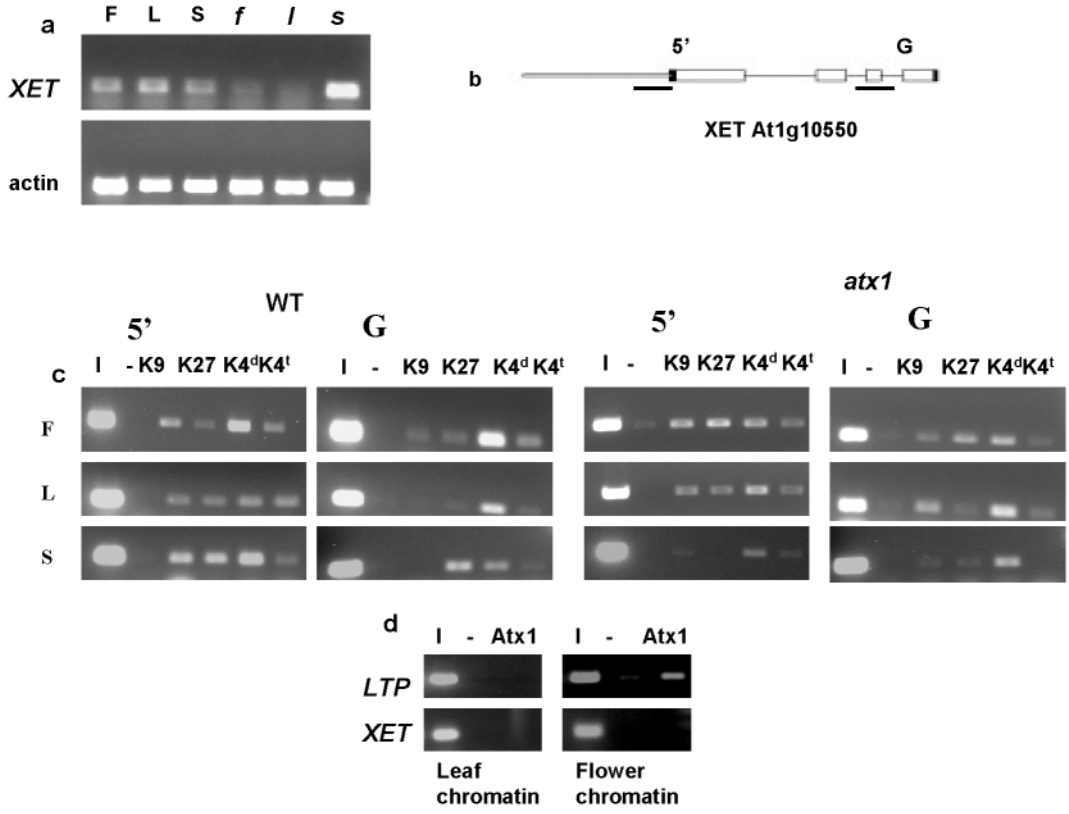


**Figure 3.** Tissue-specific expression of *XET* in wild-type tissues and in *atx1* mutants and methylation profiles at the histone H3-tails. (**a**) Expression of the *XET* gene (At1g10550), a putative xyloglucosyl transferase of the Glycosyl hydrolases family 16. (**b**) *LTP* gene structure and location of specific primers used to PCR amplify tested gene regions. (**c**) Chromatins isolated from wild-type and *atx1* tissues, immunoprecipitated with antibodies against specific H3-tail lysines. Annotations are as in Figure 2 (see also text for details); (**d**) ChIP assays for presence of 5′-*XET* and 5′-*LTP* nucleosomes from leaf, flower and stem chromatins with anti-ATX1 antibodies.

resistance of the *XET* promotor region to MN-digestion (Figure 2e) pointed to gene-specific differences in the structure of the two promotors. Collectively, the results reinforced the idea that chromatin structure of the 5′-*LTP* region was gene- and tissue-specific.

Interestingly, although *LTP* was poorly expressed in *atx1*-leaf and stem chromatins, the respective promotor regions were devoid of nucleosomes similar to the structures observed in the wild-type (Figure 2c, and data not shown). This suggested that removal of nucleosomes was not sufficient to provoke robust transcription and that the mechanism positioning the nucleosomes at the *LTP*-promotor was not ATX1-dependent.

## Histone H3-tail methylation profile of a gene downregulated by ATX1

Microarray analyses (unpublished data) revealed that about one-half of the 1600 genes with altered expression in *atx1* background were significantly up-regulated, suggesting that ATX1 was repressing these genes in the wild-type. It was a puzzling observation because trithorax factors and H3/K4 methylation, in general, are associated with activation (5–12). Thus, we were interested to find out how ATX1-related silencing would be reflected in the H3-methylation profiles. The *XET* gene, a putative xyloglucosyl transferase, was expressed at comparable levels in wild-type flowers, leaves and stems (Figure 3a). In *atx1* background, *XET* showed unexpectedly diverse patterns: almost silent in *atx1* leaves and flowers but overexpressed ~9.5-fold in the *atx1* stems. This result provided the opportunity to study methylation profiles at different degrees of *XET*-transcriptional activity and an involvement of ATX1 in its regulation.

The nucleosomes from the 5′-*XET* region in moderately expressing wild-type flower, leaf and stem chromatins carried all four H3-tail lysine methylations (Figure 3c), similar to the patterns observed with the moderately expressed *LTP* (Figure 2c). However, the methylation profiles of *XET* (G)-nucleosomes were not the same in leaf, flower and stem chromatins illustrating tissue-specific variations of lysine methylations 'encoded' at the same locus.

## A role of ATX1 in the control of *XET* and *LTP* genes

An unexpected result from the ChIP analyses was low, sometimes absent, $m^3$K4/H3 signal from nucleosomes in *XET*-expressing chromatins (wild-type leaves and stems). Particularly striking was the absence of $m^3$K4/H3 from (G)-nucleosomes in *atx1* stems overexpressing *XET* (Figure 3c). On the other hand, $m^3$K4/H3 bands were present in low-expressing *atx1* flower and leaf chromatins. The results suggested that an $m^3$H3/K4 tag was not a requirement, or a predictor, of *XET* activity and that a methylase different from ATX1 was engaged in methylating *XET*-nucleosomes.

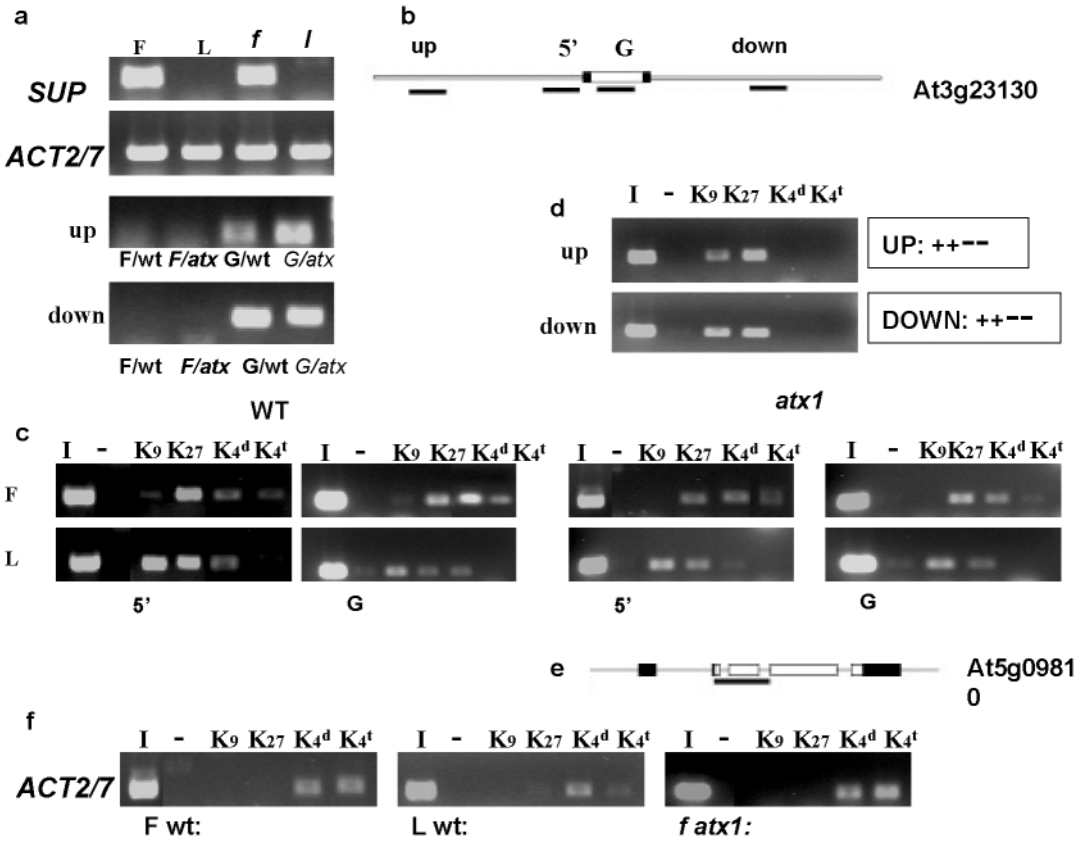


**Figure 4.** Tissue-specific expression and histone H3- methylation profiles of *SUP* and *ACT* genes as well as *SUP*-flanking intergenic sequences in wild-type and in *atx1* mutant tissues. (**a**) Expression of the *SUP* gene (At3g23130) and the *ACT* 2/7gene (At5g09810); The panels labeled up- and down- show absence of transcripts from the intergenic regions of wild-type (F/wt) and mutant (*F*/*atx*) flower chromatins; G/wild-type and *G*/*atx1* illustrate bands amplified with the same primers for the intergenic sequences using genomic DNA as template. (**b**) *SUP* gene structure and location of specific primers used to amplify tested regions by PCR. (**c**) Chromatins isolated from wild-type and *atx1* tissues, immunoprecipitated with antibodies against specific H3-tail lysines. Annotations are as in Figure 2 (see also text for details); (**d**) Nucleosomes at the non-transcribed flanking regions in flower chromatin. (**e**) Structure and location of specific primers used to amplify the *ACT* gene. (**f**) histone H3-methylation profiles of the constitutively expressed *ACT* gene.

Despite this result, however, repression of *XET* in *atx1* flowers and leaves, as well as its de-repression in *atx1* stems, clearly implicated ATX1 in the regulation of *XET*. We hypothesized that ATX1 did not interact directly with *XET*-nucleosomes but that it exercised its effect indirectly, i.e. through an activator (in flowers and leaves) or a repressor (in stems).

To test whether ATX1 targeted the 5′-*XET*-nucleosomes directly, we performed ChIP assays with anti-ATX1 specific antibodies. Neither leaf nor flower chromatins provided a template for the amplification of the 5′-*XET* region after immunoprecipitation with anti-ATX1 antibodies (Figure 3d). As a control, we used 5′-*LTP*-specific primers and the same chromatin preparations to amplify the *LTP*-promotor region. The expected *LTP* band was recovered from flower, but not from leaf chromatin (Figure 3d). The results were interpreted as ATX1 not being bound to 5′-*XET*-nucleosomes in either flower or leaf chromatins and as ATX1 being associated with 5′-*LTP* nucleosomes in flower, but not in leaf chromatin. The latter conclusion agreed with earlier results showing a lack of nucleosomes in the *LTP*-promotor region in leaves.

## Histone H3-tail methylation patterns of tissue-specific and of ubiquitously expressed genes not regulated by ATX1

To compare modification patterns of a gene activated in a tissue-specific manner with a ubiquitously expressed housekeeping gene, we investigated *SUPERMAN* (*SUP*) and *ACTIN7* (*ACT*) genes. The *SUP* gene is expressed in both wild-type and *atx1* flowers but not leaves; the *ACT* gene shows comparably high expression in leaves and flowers unchanged by ATX1-loss-of function (Figure 4a). Clearly, neither *SUP* nor *ACT* was controlled by ATX1. When transcriptionally silent, *SUP* carried $m^2$K9/H3 and $m^2$K27/H3 on both 5′ end and (G)-nucleosomes; upon activation in flower chromatin, $m^2$K9/H3 levels decreased while the methylation at K27/H3 did not change significantly. This result agreed with an earlier report that the simultaneous presence of both $m^2$K9/H3 and $m^2$K27/H3 was needed to keep the *SUP* gene silent (15). However, presence of $m^2$K4/H3, regardless of whether *SUP* was active or silent, was unusual. Tri-methylated K4/H3 was found in association only with the active *SUP* (Figure 4c). Because ATX1 did not regulate *SUP,* it is evident that a different methylase modified *SUP* histones. At the *SUP* locus,

$m^2$K4/H3 co-existed with $m^2$K9/H3 and $m^2$K27/H3 but $m^3$K4/H3 was found only with $m^2$K4/H3 and $m^2$K27/H3.

To correlate transcriptional activity with methylation profile, we analyzed the distribution of methylated residues on nucleosomes from non-transcribed intergenic regions flanking *SUP*. We wished to determine whether/how changes associated with transcriptional activation of *SUP* (i.e. disappearance of $m^2$K9/H3 and concomitant appearance of $m^3$K4/H3) would correlate with the methylation patterns of nucleosomes from adjacent upstream and downstream sequences. ChIP analysis of flower and leaf chromatins showed that nucleosomes from the flanking intergenic regions carried only $m^2$K9/H3 and $m^2$K27/H3 tags (Figure 4d) regardless of whether *SUP* was active or not. Notably methylations were absent at K4/H3 of intergenic nucleosomes suggesting that K4 methylation might label only histones from transcribed regions.

In the actively transcribed housekeeping gene, *ACT,* we detected methylations only at K4 of histone H3-tails, while neither K9/H3 nor K27/H3 were modified. Apparently, different combinations of methylation tags label active states of a tissue-specific gene and of a housekeeping gene. The patterns remained unchanged in *atx1* background supporting non-involvement of ATX1 in *ACT* regulation.

## DISCUSSION

ATX1 is involved in methylating histone H3- lysine 4 of only a fraction of *Arabidopsis* nucleosomes. ATX1-loss-of function affected ~15% of the overall tri- and >10% of the di-methylations suggesting that it is not responsible for overall K4/H3 methylations. Clearly, other plant methyltransferases are involved in genome-wide K4/H3 modification. The presence of multiple *Trithorax* and *Trithorax-related* genes in the *Arabidopsis* genome (27,28), as well as the highly selective ATX1-effects upon some, but not all, homeotic genes (21) are in agreement with this idea. Plant Trithorax factors may target specific nucleosomes, attesting further to the complexity of the plant code.

To reveal correlations between methylation profiles and the degree of transcriptional activity, we investigated genes in different expression states. These included a gene with tissue-specific expression activated by ATX1 (*LTP*) and a gene with comparable transcription levels in different wild-type tissues but altering expression in a tissue-specific mode in the absence of ATX1 (*XET*). Their methylation patterns were compared with patterns of genes not controlled by ATX1—a tissue-specific gene (*SUP*) and a housekeeping gene (*ACT7*).

A common feature displayed by the highly expressed genes, *ACT* and *LTP* (in leaves and stems) was that both carried only K4/H3 tags. Another actively transcribed gene, however, (*SUP* in flower chromatin), in addition to carrying the two K4-tags ($m^2$K4/H3 and $m^3$K4/H3) also carried $m^2$K27/H3. It was surprising that highly active states of gene expression did not always correlate with high levels of $m^3$K4/H3, as seen with overexpressed *XET* in *atx1*-stem chromatin and with the 5′-*LTP* in wild-type stems and leaves. Presence of $m^3$H3/K4 did not necessarily associate with augmented expression, as shown by *XET* in *atx1* flower and leaf chromatins. Thus, presence of an $m^3$H3/K4 tag was not a general requirement or predictor of gene activity in *Arabidopsis*.

Moderately and low-expressed genes could be labeled by all four modifications as well as by various combinations of 'silencing' ($m^2$K27/H3 or $m^2$K9/H3) and 'activating' ($m^2$K4/H3 or $m^3$K4/H3) tags (*LTP* in flowers, *XET* in wild-type flowers, leaves and stems). A combination of several lysine methylations might represent a general mode of 'tagging' plant genes when transcribed below their full potential. Inactive/low expression states usually lack $m^3$K4/H3 but carry $m^2$K4/H3 (i.e. *SUP* in leaves, *LTP* in *atx1* flowers and leaves). However, absence of $m^3$K4/H3 did not necessarily define low expression (*XET* in *atx1* stems) neither did presence of $m^2$K4/H3 and $m^3$K4/H3 cause activation of the silenced *XET* in *atx1*-leaf and flower chromatins.

A gene expressed in a tissue-specific manner and a constantly expressed (house keeping) gene displayed different methylation patterns: the active housekeeping gene carried only K4/H3-methylation, while various combinations of methylation were associated with the active and silent states of *SUP* (Figure 4c and f).

Presence of di-methylated K4/H3 in all tested gene loci and its absence from non-transcribed intergenic space, suggested that $m^2$K4/H3 could provide a general mark for gene-related sequences distinguishing them from non-transcribed regions. $m^2$K4/H3 was found in various combinations with other H3-tail lysine methylations, independent of whether pertinent genes were actively transcribed or not. In active genes, $m^2$K4/H3 usually co-localized with $m^3$K4/H3 at both the 5′- and the downstream regions. Thereby, the distribution of the K4/H3 methylations of the tested *Arabidopsis* genes was unlike the pattern reported in yeast (5,10,11) or the clustering found at transcription start sites of animal genes (6–9).

ATX1 may act as both an activator and a repressor of *Arabidopsis* genes. For a clue how this might be achieved at the molecular level, we compared the profiles of ATX1-activated (*LTP*) and repressed (*XET*) genes in *atx1* mutants. Absence of $m^3$K4/H3 labels and decreased transcription of *LTP* correlated with loss-of ATX1 function (Figure 2). In contrast, the methylation profiles associated with active and inactive states of *XET* in *atx1* mutants differed from those displayed by *LTP*. Histones from low-expressing *atx1* flower and leaf chromatins carried di- and tri-methylated K4/H3. The results suggested that a different methylase was involved at the *XET* locus and that the presence of 'activating' labels was not sufficient to stimulate gene expression.

The observation that overexpressed *XET* in *atx1*-stem chromatin displayed low levels of $m^3$K4/H3 was very unusual. It suggested that factors other than histone H3-methylases were dominant for the control of *XET*. However, the effect of ATX1-loss-of function upon *XET* expression (Figure 3a) clearly implicated ATX1 in its regulation. Most likely, this is achieved through an indirect mechanism, e.g. by activating a specific *XET*-repressor in wild-type stems. Results from ChIP experiments with anti-ATX1 antibodies also agreed that ATX1 might be indirectly involved in the control of *XET* (Figure 3d).

It is plausible that a stem-specific *XET*-repressor is related to factors influencing K9/H3 and K27/H3 methylations because these modifications were lower in *atx1*, than in wild-type, stems. However, it seems unlikely that low $m^2$K27/H3 and $m^2$K9/H3 levels were the main factors defining high *XET* expression: e.g. wild-type leaf and flower (G)-nucleosomes

also displayed low, or absent, $m^2$K27/H3 and $m^2$K9/H3 tags, yet *XET* expression was about a magnitude lower than in *atx1* stems. On the other hand, presence of $m^2$H3/K4 and $m^3$H3/K4 in *atx1* flower and leaf chromatins was not sufficient to drive-up expression to levels comparable with the wild-type. Clearly, H3-lysine methylations were not a primary cause for the low *XET* expression in *atx1*-leaf and flower chromatins, or for the high expression in *atx1* stems. Tissue-specific regulators may affect expression independent of H3-tail methylation profiles. A plausible possibility is that loss-of-ATX1 function has deactivated tissue-specific regulators of *XET* transcription, an activator in leaves and flowers and a repressor in stems.

ChIP assays with antibodies against non-modified histone H3 revealed that the 5′-*LTP* region was devoid of nucleosomes in actively transcribed leaf chromatin but that it was associated with nucleosomes in flower chromatin (Figure 2d). The absence of methylation bands and the increased nuclease sensitivity at the 5′-*LTP* region in highly expressing leaf and stem chromatins (Figure 2c and e) provided independent support to this conclusion. Lack of nucleosomes from the leaf *LTP*-promotor was a gene-specific feature (compare with the 5′-region of *XET*, Figure 2d and e). The nucleosomes were repositioned at the *LTP*-promotor with the transition to flowering concomitant with lowered transcriptional activity. The results indicated that the chromatin structure at the *LTP*-promotor was gene, tissue and development-stage-specific.

Interestingly, *LTP* was poorly expressed in *atx1*-leaf and stem chromatins although the respective 5′-regions were similarly devoid of nucleosomes as the wild-type (Figure 2c, and data not shown). This fact demonstrated that removal of nucleosomes was not sufficient to provoke robust transcription and that the nucleosome-positioning mechanism at the *LTP* locus functioned independently of ATX1. In contrast with the reported role of methylated K4/H3 for nucleosomes positioning in yeast (29), this result illustrated, once again, that plants might 'decode' and implement histone H3-tail methylation information in a plant-specific way.

Collectively, our results demonstrated that the methylation patterns of lysine residues 4, 9 and 27 of histone H3-tails could not serve as general indicators, or predictors, for the state of expression activity in *Arabidopsis*. Transcriptionally active genes may be labeled by different combinations of methylation tags at the promotor, in the gene-coding region, or in both. The resulting patterns are much more complex than currently acknowledged and might be developmentally regulated, gene- and tissue-specific. It remains to be established whether histone H3-tail lysine methylation modifications precede or trail established transcriptionally active states.

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