The Highly Similar Arabidopsis Homologs of Trithorax ATX1 and ATX2 Encode Proteins with Divergent Biochemical Functions

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Gene duplication followed by functional specialization is a potent force in the evolution of biological diversity. A comparative study of two highly conserved duplicated genes, ARABIDOPSIS TRITHORAX-LIKE PROTEIN1 (ATX1) and ATX2, revealed features of both partial redundancy and of functional divergence. Although structurally similar, their regulatory sequences have diverged, resulting in distinct temporal and spatial patterns of expression of the ATX1 and ATX2 genes. We found that ATX2 methylates only a limited fraction of nucleosomes and that ATX1 and ATX2 influence the expression of largely nonoverlapping gene sets. Even when coregulating shared targets, ATX1 and ATX2 may employ different mechanisms. Most remarkable is the divergence of their biochemical activities: both proteins methylate K4 of histone H3, but while ATX1 trimethylates it, ATX2 dimethylates it. ATX2 and ATX1 provide an example of separated K4 di from K4 trimethyltransferase activity.

INTRODUCTION

Gene duplication, followed by functional divergence of the resulting paralogous proteins, is a major force shaping molecular networks in living organisms (Ohno, 1970). Duplicated genes involved in signal transduction and transcription regulation might have been preferentially retained (Blanc and Wolfe, 2004). A duplicated transcription factor (TF) might lead to the origination of a nonoverlapping pathway to function in two different cell types, developmental stages, or environmental conditions. Because epigenetic regulators modulate expression of a large number of functionally linked genes (Alvarez-Venegas et al., 2007a), a duplicated gene encoding an epigenetic factor might contribute to the evolution of novel gene networks.

The highly conserved SET peptide [for Su(var)3-9, E(z), Trithorax], encoded by the Drosophila melanogaster Su(var)39-, E(z)-, and Trithorax-related genes, carries histone lysine methyltransferase (HKMT) activity with a preference for specific histone residue substrates (Rea et al., 2000). Although SET domain genes are ancient (Alvarez-Venegas et al., 2007b), they have proliferated in eukaryotes, particularly after the transition to multicellularity (Alvarez-Venegas and Avramova, 2002; Krauss et al., 2006). The genes from the Trithorax family encode factors that can modulate chromatin structure through their abilities to methylate the N-terminal lysine 4 of histone H3 (H3K4). Trithorax homologs have been found in both animals and plants, suggesting that common mechanisms of epigenetic regulation are derived from a shared ancestor. Subsequently, each lineage has evolved distinct subgroups of duplicated genes to meet lineage-specific needs.

According to current models, duplicated genes (paralogs) may have remained with redundant functions or may have acquired different fates: one copy might have been silenced to become nonfunctional or the two versions might have pared out the range of pleiotropic functions of the ancestral gene. The latter path may lead to separation of functions or subfunctionalization (Kondrashov et al., 2002). A general limitation of theoretical models is that it is unclear how closely biology follows. While it is logical to expect that structurally divergent paralogs might have evolved novel functions, it is impossible to predict the functions of duplicated genes with highly conserved coding sequences.

The Arabidopsis thaliana TRITHORAX family, ATX, contains five homologs segregating into two well-supported sister clades (Baumbusch et al., 2001; Alvarez-Venegas and Avramova, 2002), ATX1 and ATX2, originating from a segmental chromosomal duplication, belong in the same clade as sister paralogs.
Interesting questions regarding the fate of the ATX2 and ATX1 paralogs are whether they have retained redundant functions, whether one copy has lost its function, or whether the two have acquired divergent functions. The only member of the ATX family for which a biochemical function has been established is ATX1, which is involved in trimethylating histone H3-lysine 4 (H3K4me3) (Alvarez-Venegas et al., 2003). However, ATX1 is not responsible for the genome-wide methylation of histone H3K4: ~85% of the H3K4me3 signal is still present in the atx1 mutants, suggesting that other methyltransferases are involved as well (Alvarez-Venegas and Avramova, 2005).

The degree of H3K4 methylation (mono-, di-, or trimethylated K-NH2-groups) has important consequences for the transcriptional activity of pertinent genes in yeast and animal chromatinin (Bernstein et al., 2002; Milne et al., 2002; Nakamura et al., 2002; Santos-Rosa et al., 2002; Ng et al., 2003; van Dijk et al., 2005; Kouzarides, 2007). In Arabidopsis, dimethylated K4 (H3K4me2) was found at coding regions independent of whether the gene was active or not but was absent from intergenic regions, suggesting that H3K4me2 could be a general mark distinguishing potentially transcribed from nontranscribed sequences in the genome (Alvarez-Venegas and Avramova, 2005).

In yeast and animal systems, the same enzyme establishes both H3K4me2 and H3K4me3 marks: SET1 in Saccharomyces cerevisiae and the human trithorax homologs MLL1, MLL2, and hSset1 can produce mono-, di-, and trimethyl H3K4 marks (Bernstein et al., 2002; Santos-Rosa et al., 2002; Wysocka et al., 2005; Ruthenberg et al., 2007). However, the mammalian germ cell–specific factor Meisetz carries out K4 tri- but not mono- or dimethylation (Hayashi et al., 2005). Known histone H3K4 trimethyltransferases from Arabidopsis do not display dimethylating activity (Alvarez-Venegas and Avramova, 2005; Kim et al., 2005). Despite the broad distribution of the H3K4me2 in euchromatin (Jasencakova et al., 2003; Lippman et al., 2004) and its association with transcribed sequences (Alvarez-Venegas and Avramova, 2005), enzyme activity generating H3K4me2 marks in Arabidopsis has not been identified. Here, we report that the ATX2 encodes a putative H3K4 dimethyltransferase, providing an example of separated histone K4 dimethyltransferase and K4 trimethyltransferase activities in Arabidopsis.

Experimental design and data interpretation were performed in the context of a hypothesis that after duplication, the functions of ATX1 and ATX2 have diverged. Their possible functional divergence was assessed by (1) testing the ability of ATX1 and ATX2 to substitute for each other, as an indication of redundancy; (2) establishing patterns of expression of ATX1/2 in the plant; (3) identifying genes regulated by each ATX as an illustration of their specificity/redundancy, and (4) analysis of their biochemical functions.

RESULTS

Structural Relationship and Origin of the ATX1 and ATX2 Genes

The SET and the PHD (plant homeotic domain) domains are signature features of TRITHORAX family proteins of both animal and plant origin. In addition, two conserved peptides (FYR-C and FYR-N), together called DAST (for Domain Associated with SET in Trithorax; Alvarez-Venegas and Avramova, 2001), are located adjacent to each other in a subset of trithorax proteins. The presence or absence of DAST correlates with the division of Arabidopsis trithorax proteins in two subgroups (Baumbusch et al., 2001; Alvarez-Venegas and Avramova, 2002).

The ATX1 and ATX2 paralogs have originated from a segmental chromosomal duplication (Baumbusch et al., 2001; Figure 1A). The regions immediately surrounding ATX2 and ATX1 on chromosomes 1 and 2, respectively, are in reverse orientation, with collinearly positioned conserved paralogous genes interspersed with genes of no apparent similarity. All genes conserved between the two chromosomal regions have preserved their orientations with respect to the ATX gene, suggesting that the nonconserved genes are the products of insertions or deletions that have taken place since the duplication.

ATX1 and ATX2 are 65% identical and 75% similar at the amino acid level. The two proteins have similar architectural motifs (Figure 1B). To determine whether structural similarity translates into functional redundancy, we analyzed two atx2 lines (SALK_001880 and SALK_117262) with insertions in the promoter (atx2-1) and in the SET domain (atx2-2), respectively (Figure 2A). No transcripts could be amplified from the ATX2 gene sequences in atx2-1 plants, while atx2-2 mutant plants produced a truncated message with a disrupted SET domain sequence (see Supplemental Figure 1A online). However, neither of the homozygous atx2 mutant lines displayed obvious phenotypes. In contrast with the early bolting of atx1 under both long- and short-day conditions, mutant atx2 lines did not differ detectably from the wild type (Figure 2B). Upon maturation, atx2 plants were indistinguishable from the wild type (Figure 2C).

Differential Expression of ATX1 and ATX2 during Development

By being expressed in different temporal and/or spatial manners, redundant genes may acquire functional divergence (Pickett and Meeks-Wagner, 1995). To test this idea for the ATX1/ATX2 pair, we generated transgenic lines expressing the β-glucuronidase (GUS) coding region behind the ATX1 or ATX2 promoters (see Methods for the respective promoter regions used). Potential involvement of regulatory sequences located in introns could not be assessed by this approach. Five and seven independently transformed lines, for ATX1 and ATX2, respectively, each segregating for a single T-DNA insertion, were isolated and examined for GUS expression in various tissues in early development and later, at the flowering stage.
Striking differences were observed at the early stages of development: the absence of GUS expression driven by the ATX2 promoter in the tissues of young seedlings contrasted with the strong expression of GUS under the ATX1 promoter (Figure 3A). Later in development, the ATX2 promoter was activated as well, as illustrated by the blue staining of rosette leaves and roots (Figures 3B and 3C). The results suggested that ATX1 is expressed throughout development, while expression of ATX2 occurs later in life.

Apparently inactive in young roots, ProATX2:GUS expression was weakly stimulated in 2-week-old roots, although not in the vasculature (Figures 3A and 3B); ATX2 promoter activity appeared much stronger at 5 weeks as blue staining was seen in all root tissues. The tips of the lateral roots were not stained, which was in contrast with roots of the same aged plants expressing ProATX1:GUS. The expression of ATX1 in roots is under developmental in addition to tissue-specific regulation. In contrast with ProATX2, however, ProATX1:GUS is active in the vascular tissues of 6-d-old and 2-week-old roots. Later, the ATX1 promoter is turned on in adjacent tissues as well, suggesting that in older roots, the ATX1 and ATX2 expression patterns may overlap. Apparently, only ATX1 is expressed in dividing root tip cells at this stage.

In aerial tissues, the ATX1 promoter was active in rosette leaves, in growing inflorescence stems (in the upper younger regions), in cauline leaves (at hydathodes), and in the mature flowers (Figures 3C and 3D). P-ATX2 was weakly active in inflorescence nodes and at the base of the flowers, where ATX2-GUS expression overlapped with ATX1-GUS expression.

In flowers, except for common (but weak) staining at the tips of the stigma, ATX1 and ATX2 displayed distinct domains of expression (Figure 3D). The ATX1 promoter drives GUS expression in sepals, in the vasculature of petals, and in the filaments of the stamens. By contrast, ATX2-driven GUS expression was detected in pollen only. The tissue-specific staining within the same organ (stamens) illustrates spatial separation of ATX1/ATX2 expression.

According to available data on TF binding sites (see Methods), both promoters carry putative sites for regulation by light, UV radiation, pathogen attack, wounding, and abscisic acid (see Supplemental Figure 2 online). However, more putative TF binding sites were recognized upstream of ATX1 than of ATX2, suggesting that ATX1 is subjected to a broader array of regulatory signals than is ATX2.

**Genome-Wide Expression Analyses of atx2 Mutant Plants**

Collectively, the results so far imply that ATX1 and ATX2 encode both specific and redundant functions. Transcription profiling of genes with altered expression in atx1 and atx2 backgrounds offers a strategy to assess the functional divergence of the two paralogs at the genomic level.

Affymetrix gene chips (ATH1 Genome Arrays, with ~24,000 *Arabidopsis* genes) were used in whole-genome expression...
analysis of axt1 and axt2 homozygous mutant plants. RNA was isolated from two independent biological replicates of axt1, axt2-2, and from control wild-type plants, grown and handled under the same conditions; each experimental sample was analyzed versus each of the two wild-type control sets (see Methods). Expression patterns reflected whole-plant gene expression and not tissue-specific profiles.

We consistently detected ~60% of all expressed Arabidopsis genes in all samples tested under the reported experimental conditions (Alvarez-Venegas et al., 2006a, 2006b) (correlation coefficients: 0.93 for axt2, 0.97 for axt1, and 0.98 for the wild type). Performing multiple test corrections by the highly conservative Bonferroni method restricting false discovery rate (Benjamini and Hochberg, 1995), we identified 80 genes with altered expression in the axt2 background: 27 genes had increased, while 53 genes had decreased expression in axt2 mutant plants compared with the wild type; ~900 genes changed expression levels in axt1mutant plants. Derepressed and repressed genes resulting from ATX2 loss of function are listed in Supplemental Data Sets 1 and 2 online. Data from the hybridization experiments in the axt1 background are available elsewhere (Alvarez-Venegas et al., 2006a). Quantitative RT-PCR assays were consistent with the microarray data and confirmed the validity of the hybridization experiments (see below). Fewer genes altered transcription levels in axt2 than in axt1 backgrounds, suggesting a more limited role for ATX2 in Arabidopsis.

### Shared ATX1/ATX2 Targets

Overlap analysis provides a general strategy for distinguishing unique from redundant functions. Analysis of axt1- and axt2-affected genes in the four possible combinations is shown by the Venn diagram (Figure 4). Among the 80 ATX2-regulated genes, 34 genes (~42%) overlapped with the ATX1-regulated set. Therefore, 58% of the ATX2 targets were not shared with ATX1 and were ATX2 specific. Within the shared set, only eight genes were coregulated (one upregulated and seven downregulated). Interestingly, 26 shared genes changed expression in opposite directions, implying that ATX1 and ATX2 might have opposite effects upon the expression of these genes. To further elucidate these relationships at the molecular level, we tested a possible contribution by ATX2 to genome-wide H3K4 methylation and explored the roles of ATX1 and ATX2 in H3K4 nucleosomal modification at three selected loci.

### ATX2 and Genome-Wide Methylation of H3K4

Because ATX1 is not responsible for genome-wide methylation of H3K4 (Alvarez-Venegas and Avramova, 2005), we asked whether ATX2 contributes to the overall methylated profiles. Histones isolated from wild-type and from axt2-2 mutant plants were analyzed by protein gel blot assays using antibodies specifically recognizing the H3K4 di- or trimethylated isoforms. The axt2 line used in these analyses has a disrupted SET domain, allowing us to correlate H3K4 methylation patterns with the availability of the ATX2-SET peptide. To determine subtler variations in methylation levels, the amounts of loaded histone H3 in each sample were established with antibodies specific against nonmethylated H3; signal intensities of bands obtained with methylation-specific antibodies were normalized against the respective histone H3 amounts in 3-week-old mutant and wild-type plants.

In four independent measurements, the overall histone modification levels for either H3K4me2 or H3K4me3 varied between 2 and 6%, entirely within the standard error (see Supplemental...
Figure 3 online). We conclude that ATX2 is not involved in genome-wide histone H3K4 di- or trimethylation. However, this result could not resolve whether ATX2 does not possess an HKMT activity (consistent with a function lost after gene duplication), whether it modifies only a small fraction of Arabidopsis histones (consistent with the limited spatial and temporal patterns of expression of the ATX2 gene) or whether it is fully redundant (biochemically) with ATX1. To distinguish between these alternatives, we examined the methylation profiles at three shared loci.

NAP Gene Regulation by ATX1 and ATX2

The downregulated expression of At1g69490 in the atx1 and atx2 backgrounds (Figures 5A and 5B) suggested that in the wild type, this gene was activated by both ATX1 and ATX2. At1g69490 encodes a plant-specific TF from the NAC family (NAP, for NAC-LIKE ACTIVATED by AP3/PI) (Sablowski and Meyerowitz, 1998). At1g69490 showed reduced, but still significant, expression in both atx1 and atx2 mutant flowers, suggesting that ATX1 and ATX2 cooperate to maintain NAP’s wild-type expression levels. To reveal the molecular basis of this synergistic activity, as well as a possible role of ATX2 in the K4 methylation of NAP nucleosomes, we performed chromatin immunoprecipitation (ChIP) analyses with wild-type, atx1, and atx2 flower chromatins using specific antidimethylated and antitrimethylated H3K4 antibodies. NAP nucleosomes in wild-type flower chromatin show both H3K4me2 and H3K4me3 marks (Figure 5C). In atx1, the H3K4me2 band was present, but the H3K4me3 signal had disappeared, consistent with ATX1 being the sole H3K4 tri-methyltransferase acting at the NAP locus. On the other hand, the presence of the H3K4me2 signal indicated that ATX1 is not needed for dimethylating NAP nucleosomes. Surprisingly, the H3K4 methylation pattern reciprocally changed in the atx2 flower chromatin (Figure 5C). Disappearance of H3K4me2, but

Figure 3. Expression of ProATX1::GUS and ProATX2::GUS during Development.

(A) GUS expression under the control of the ATX1 or ATX2 promoter (left side and right side, respectively) in transgenic plants is shown. Six-day-old seedlings are shown in all panels, except the top right panel, which shows a 2-week-old plantlet. The ATX1 promoter is active in all tissues, particularly in the vasculature. In the roots, blue stain is seen only in differentiated vascular tissues. By contrast, the ATX2 promoter is apparently inactive in young seedlings (n = 16, five lines) even in two-week-old plantlets.

(B) Later in development, P-ATX1 and P-ATX2 show both complementary and overlapping expression patterns in roots. Two- and five-week-old roots are shown in the top two and bottom four panels, respectively. ProATX1::GUS expression (left panels) in two-week-old roots is seen in vascular tissues but not in adjacent tissues or in emerging lateral roots (arrow). In 5-week-old roots, broader domains of ProATX1 expression include nonvascular tissues and the tips of lateral roots. ProATX2 (right panels) is weakly active in 2-week-old transgenic roots, mainly in nonvascular tissues, but not in the dividing cells of bulging lateral roots (arrow). At the flowering stage, expression of ProATX2::GUS in roots overlaps with ProATX1::GUS, except at the tips.

(C) Tissues harvested from 5-week-old flowering plants. Both ATX1 and ATX2 promoters are active in rosette leaves, particularly in the vascular tissues. Only the top (younger) parts of primary inflorescence stems and patches of cauline leaf cells (arrows) show staining in plants carrying the ProATX1::GUS transgene. ProATX2::GUS showed more restricted expression domains and weaker expression in inflorescence stems and tissues.

(D) Domains of ProATX1::GUS and ProATX2::GUS expression in flowers. In addition to petals and sepal, ProATX1::GUS is expressed in stamens, particularly in the filaments; the P-ATX2-driven GUS expression is concentrated mainly in the pollen grains.
preservation of the H3K4me3 signal from atx2-NAP, nucleosomes implicated ATX2 in di- but not in tri-H3K4 methylation. The patterns of the housekeeping gene, ACTIN7, a control for the quality of the templates, was in full agreement with the reported data: the constitutively expressed housekeeping gene displayed both H3K4me2 and H3K4me3 bands (Figure 5D), confirming that missing signals from the NAP nucleosomes reflect specific ATX1 and ATX2 effects.

Thereby, ATX2 encodes a putative H3K4 dimethyltransferase separate from the ATX1-trimethylating activity. The disappearance of the H3K4me2 signal in atx2 correlates with lower expression from NAP despite the presence of H3K4me3. This observation is important because it suggests that both H3K4me2 and H3K4me3 marks are required for NAP expression at wild-type levels. The necessity to establish both marks on the NAP nucleosomes provides the basis for the synergistic interaction of ATX1 and ATX2 at the molecular level.

ATX1 and ATX2 Coregulate the XTH33 Gene by Different Mechanisms

The XTH33 gene (At1g10550), from the Arabidopsis xyloglucan endotransglucosylase/hydrolase (XTH) family, encodes a wall-modifying activity with a very tightly regulated expression in development (Becnel et al., 2006; Divol et al., 2007). Real-time PCR assays of several plant tissues confirmed that XTH33 was expressed in flowers (see Supplemental Figure 4 online). Comparing XTH33 expression in flowers of different backgrounds revealed a sharp decrease in transcript levels in atx1 and atx2 flowers (Figure 6A). H3K4me2 marks were present on wild-type and atx1 XTH33 nucleosomes, although the signal appeared to be slightly reduced in the latter. However, H3K4me2 signal was undetectable on XTH33 nucleosomes in the atx2 lines (Figure 6B), implicating ATX2 in this modification. Importantly, the loss of H3K4me2 marks from the atx2 XTH33 nucleosomes,

**Figure 4.** Venn Diagram of Genes with Altered Expression in atx2-2 and atx1.

Overlapping genes with significantly altered expression from the microarray hybridization experiments examined in the four possible combinations. The genes in the atx1 and atx2 fractions represent the sets obtained after P values were subjected to multiple test corrections by the conservative Bonferroni method (see Methods). Overlaps indicate shared targets.

Divergence of ATX1 and ATX2 Functions

**Figure 5.** Expression and H3K4 Methylation Patterns of NAP in Wild-Type, atx1, and atx2 Mutant Chromatin.

(A) NAP expression in wild-type, atx2, and atx1 mutant flowers. The expression of ACTIN7 is included as a loading control, and a schematic drawing of the NAP gene is shown below. Empty boxes represent introns, filled boxes are exons, and arrows indicate the locations of primers used for amplification in the RT-PCR (A) and ChIP assays (C).

(B) Relative NAP transcript levels (mean ± se) in wild-type, atx2, and atx1 mutant flower chromatin as determined by real-time PCR (n = 3).

(C) NAP amplified from ChIP assays of wild-type, atx1, and atx2 flower chromatin immunoprecipitated with antibodies distinguishing between di- and trimethylated H3K4 isoforms. I, input sample representing 10% of the template amount used for the immunoprecipitation. −, negative controls: samples treated in the same way as immunoprecipitated chromatin, except without added antibody.

(D) As in (C), except that K4 methylation patterns of the housekeeping gene ACTIN7 are shown as a control for the quality of the chromatin used as templates.
accompanied by the decreased transcript levels, suggests that dimethylated H3K4 residues are required for XTH33 expression. By contrast, H3K4me3 marks were absent from XTH33 nucleosomes, including the transcriptionally active locus in the wild-type flower chromatin (Figure 6C). This feature, specific for the XTH33 nucleosomes (confirmed by the presence of the ACTIN7 bands amplified from the same DNA templates), reinforced the conclusion that H3K4me3 tags are not indispensable for active transcription of Arabidopsis genes (Alvarez-Venegas and Avramova, 2005). Accordingly, the decrease of XTH33 transcripts in atx1 flowers might reflect the inactivation of a TF driving XTH33 expression in the absence of H3K4me3 and/or the subtle reduction in H3K4me2 marks. The different methylation patterns of NAP and XTH33 nucleosomes illustrate that ATX1 and ATX2 use distinct mechanisms even when activating shared genes.

Differential Roles of ATX1 and ATX2 at the WRKY70 Gene Locus

The At3g56400 gene, encoding a TF from the WRKY family (WRKY70), is involved in the regulation of disease response genes at the intersection of two signaling pathways (Li et al., 2004, 2006). We have demonstrated that ATX1 directly associates with WRKY70 nucleosomes, trimethylates H3K4 residues, and stimulates its transcription (Alvarez-Venegas et al., 2007a). Furthermore, we showed that H3K4me2 marks were present on all tested WRKY70 nucleosomes regardless of whether WRKY70 was actively transcribed (in wild-type leaves) or not (in wild-type flowers and in all tissues from the atx1 mutants). Here, we examined whether ATX2 was involved in establishing the K4 dimethylation marks on WRKY70. First, we checked the transcriptional activity of WRKY70 in the wild type and in atx2-2 tissues by real-time PCR (Figure 7A). WRKY70 is transcribed at basal levels in all tested tissues but is tissue-specifically activated in rosette leaves. In contrast with the sharp decrease in WRKY70 transcripts in atx1 leaves, WRKY70 expression was not downregulated in the atx2 background; it was even slightly augmented in the atx2 stems (Figure 7A). The differential in the WRKY70 transcript levels in atx1 and in atx2 rosette leaves (Figure 7B) illustrates the different effects upon WRKY70 transcription exercised by ATX1 and ATX2. By ChIP assays, we show that both di- and trimethylated H3K4 marks were present on wild-type WRKY70 leaf nucleosomes. The lower WRKY70 transcript levels, accompanied by the disappearance of the H3K4me3 signal in atx1 leaf chromatin (Figure 7C), were in full agreement with ATX1 being responsible for the trimethylating activity. However, the preserved H3K4me2 bands in atx2 leaf chromatin suggested that ATX2 was not involved in modifying WRKY70 nucleosomes. ACTIN-specific bands amplified from the same atx1 and atx2 leaf chromatin templates provided evidence for the specificity of the WRKY70 methylation patterns.

DISCUSSION

Divergence of ATX1 and ATX2 Promoter Functions

ATX1 and ATX2 originated as a result of a segmental duplication involving the tip of chromosome 1 and an internal section of chromosome 2 (Baumbusch et al., 2001; Figure 1A). At least two rounds of duplications might have occurred in Arabidopsis resulting in mosaic rearrangements and segmental duplications. Most duplication blocks are estimated to have occurred 20 to 40
million years ago, before the evolution of the genus *Brassica* but after the separation of Brassicaceae from other close eudicot families (Simillion et al., 2002; Blanc et al., 2003).

The absence of visible *atx2*-related phenotypes, as well as the inability of ATX2 to substitute for ATX1 in an *atx1* background, could indicate that ATX2 has become nonfunctional after the duplication, while the ancestral function has been retained by ATX1. However, it is important to emphasize that the phenotypes of *atx2* mutant plants have been scored under laboratory growth conditions, which cannot preclude a role for ATX2 in the survival and/or adaptation of the plant in the wild. Further cellular and molecular analyses confirmed the functional relevance of ATX2.

In agreement with models predicting that changes in cis-regulatory modules (promoters) of duplicated genes might lead to specific shifts in expression patterns between paralogs (Gu, 2003; He and Zhang, 2005; Duarte et al., 2006), we found more putative TF binding sites upstream of ATX1 than of ATX2 (see Supplemental Figure 2 online). All motifs from the ATX2 promoter region are also present in the ATX1 promoter region, indicating that similar regulation of the two genes is possible as well. In addition, unrecognized TF binding sites and/or specific combinations of known motifs in the ATX2 promoter may guide its specific expression as seen, for instance, in 2-week-old roots and in pollen (Figures 3B and 3D). Although it appears that the ATX2 promoter may have lost upstream regulatory sequences, we do not exclude the acquisition of novel unrecognized motifs or changes in the modular combinations of TF binding boxes. These could lead to expression pattern shifts between the paralogs and allow the divergence of their functions (Wendel, 2000; Hughes, 2002; Duarte et al., 2006).

Nonredundant Roles of ATX1 and ATX2 in Overall Gene Regulation

The lower number of misexpressed genes in *atx2* mutants compared with *atx1* (Alvarez-Venegas et al., 2006a) is in accordance with a more restricted role for ATX2 in *Arabidopsis*. Approximately 40% of ATX2-regulated genes overlap with ATX1-regulated genes. These results, together with the largely nonoverlapping patterns of ATX1 and ATX2 expression, suggest nonredundant functions. Within the shared targets, only eight genes were similarly influenced in the *atx1* and *atx2* backgrounds compatible with redundant functions. The expression of most of the overlapping genes was oppositely influenced in the *atx1* and *atx2* backgrounds, suggesting that ATX1 and ATX2 might participate in specific antagonistic complexes. Another possibility is that ATX1 and ATX2 regulate different TFs with opposite effects on the expression of shared targets.

To explore the mechanisms behind the apparently redundant or antagonistic effects at the molecular level, we examined the HKMT activity of ATX2. Recombinant Trithorax-related proteins of yeast, animal, or plant origin do not display robust methyltransferase activity (Czermin et al., 2002; Milne et al., 2002; Alvarez-Venegas et al., 2003), making it impractical to analyze enzyme activity by in vitro biochemical approaches. The lack of detectable ATX2 involvement in overall H3K4 methylation could indicate that ATX2 has no enzyme activity, that redundant enzymes compensate for ATX2 loss, or that ATX2 modifies only a limited nucleosomal fraction. Analyses of selected shared loci allowed us to reveal ATX2 enzyme activity and the divergence of ATX1 and ATX2 biochemical functions.
Different Roles and Molecular Mechanisms Used by ATX1 and ATX2 to Regulate Shared Targets

ATX1 and ATX2 upregulate the transcription of the NAP gene. Reciprocal changes of its nucleosomal patterns in atx1 and atx2 mutant chromatinis suggested a molecular basis for this synergy. The lack of H3K4me2 signals on atx2 NAP nucleosomes defines ATX2 as a putative H3K4 dimethyltransferase involved in their dimethylation (Figure 5C). Furthermore, although both ATX1 and ATX2 upregulate the XTH33 gene, they use different mechanisms. While ATX2 is implicated in the deposition of the H3K4me2 marks, ATX1 does not modify the XTH33 nucleosomes, suggesting that its effect is indirect (Figure 6B). Moreover, trimethylation of H3K4 is not a requirement for XTH33 transcription (Figures 6A and 6C).

ATX1 and ATX2 are differentially involved in the regulation of WRKY70 as well. In leaves, ATX2 does not influence WRKY70 transcription and does not participate in generating the WRKY70- H3K4me2 marks. The presence of H3K4me2 tags on WRKY70 nucleosomes from atx2 chromatin suggests that a different dimethyltransferase is involved. By contrast, ATX1 modifies WRKY70 nucleosomes and stimulates WRKY70 transcription (Figures 7B and 7C).

Collectively, the results demonstrate that ATX1 and ATX2 have evolved different target specificity and use different molecular and biochemical mechanisms when regulating shared targets. However, they do not allow us to exclude a potential ability of ATX2 to carry out trimethylation or of ATX1 to dimethylate nucleosomal K4 at other gene loci.

It is unclear what determines the di- and trimethylating activities of ATX2 and ATX1, respectively. The aromatic ring amino acid at position 4 in the conserved motif EfX(F/Y/W)/D/DY is important for the specificity of the SET domain peptide in establishing mono-, di-, or trimethylation of a single Lys residue (Zhang et al., 2003; Cheng et al., 2005; Collins et al., 2005; Thorstensen et al., 2006). Generally, proteins carrying W or F are involved in H3K9me3, while those carrying Y act as mono- and dimethyltransferases (Jackson et al., 2002, 2004; Cheng et al., 2005; Collins et al., 2005; Ebbs and Bender, 2006; Casas-Mollano et al., 2007). Interestingly, F/Y substitution may alter the substrate specificity, underscoring the significance of this amino acid in defining tri- versus dimethyltransferase activity (Cheng et al., 2005; Collins et al., 2005). It is important to emphasize, however, that this correlation has been demonstrated only for SET peptides of the SUVAR family. By contrast, all members of the Trithorax SET domain family have an invariant Y and still they can perform trimethylation (this report; Santos-Rosa et al., 2002; Wysocka et al., 2005; Ruthefenberg et al., 2007). Therefore, the presence of a Y in the active site of Trithorax-type SET peptides, apparently, is not restrictive to the trimethylation of K4. Furthermore, recombinant SUVAR SET domain peptides display robust HKMTase activity, while the trithorax SET domains require partners to be able to methylate H3K4 in vitro (Petrak et al., 2001; Milne et al., 2002; Nakamura et al., 2002). It seems that the steric requirements for the deposition of the substrate and the conformation of the product at the active site of the Trithorax SET domain are different from those governing the SUVAR SET domain.

In contrast with the actively studied roles of histone K4 di and trimethylation as chromatin marks, the significance of mono-methylated H3K4 has remained elusive. In Chlamydomonas, a separate activity is involved in generating K4me1, which tends to act as a silencing chromatin mark (van Dijk et al., 2005). However, animal- or plant-specific K4 monomethyltransferases have not been identified so far, despite the abundance of H3K4me1 marks in Arabidopsis chromatin (Zhang et al., 2007). It remains to be established whether K4me1 marks are labeling nucleosomes associated with coding sequences and whether they are reflective of gene transcription states. Preliminary results suggest that ATX1 and ATX2 are not responsible for genome-wide K4me1 modification. Whether they are involved in monomethylation could not be determined reliably due to the low K4me1 levels at the loci studied here. We shall need better tools than those currently available to provide conclusive answers to this question. Isolation and characterization of specific complexes assembled by ATX1 or ATX2, as well as structural analysis of the ATX SET domain peptides, will be critical steps toward overcoming the obstacles for direct biochemical assessment of Trithorax function.
respective genomic sequence. Primers for the ATX1 and ATX2 promoters used in this study were as follows: (ATX1) forward, 5’-cctccgccgtggagtttgagaatcc-3’; reverse, 5’-tcaacCGGTAAGGaaaatagccgagggagaaagc-3’; (ATX2) forward, 5’-ctaggACGTCCGctgcatcatactagttag-3’, reverse, 5’-acatGCCGCTTaaagagggagaaatag-3’. DNA sequences amplified from DNA template under PCR conditions as those described for the PCR genotyping were cloned in the pCambia1303 vector (Canberra), replacing the original 3S promoter with the desired experimental sequence. Constructs were verified by sequencing, and the plasmids were introduced in Arabidopsis plants by the dip infiltration method (Clough and Bent, 1998) to produce transgenic GUS-expressing lines. Six independently transformed lines were selected for each transformed construct and analyzed for GUS expression. Staining was done according to Silverstone et al. (1997). Color appeared after 4 h but was allowed to develop overnight. Samples were examined under a Nikon SMZ800 dissecting microscope equipped with an Optronics digital camera and MagnaFire 2.1 software. Single-copy insertion lines were used in the comparative experiments. For analyses of TF binding motifs, information available from the Ohio State University site was used (http://arabidopsis.med.ohio-state.edu/AtcisDB).

RNA Sample and Microarray Preparation

atx1, atx2-2, and wild-type control plants were grown and handled under identical conditions. Tissues were collected from whole, 4-week-old plants. For each experiment and its separate control, total RNA was isolated from two plants and frozen in liquid nitrogen using the TRIzol reagent following the manufacturer’s instructions (Invitrogen) and further purified using a Qiagen RNaseasy column. Fifteen micrograms of total RNA was used to synthesize cDNA using the Affymetrix One-Cycle cDNA synthesis kit according to the manufacturer’s instructions. All sample preparations followed the protocols of the Affymetrix GeneChip Expression Analysis Technical manual. Hybridizations were performed on Affymetrix Arabidopsis Genome ATH1-121501 arrays, stained with streptavidin-phycocyanin conjugate on an Affymetrix Fluidics Station 450. Images were obtained using the GeneChip 3000. More data and discussion of hybridization experiments in the atx1 background are available elsewhere (Alvarez-Venegas et al., 2006a).

Microarray Data Analysis

We used Affymetrix ATH1-121501 microarrays that cover 23,489 genes using 22,810 probe sets. Probes were mapped to genes using the 2006 Arabidopsis Information Resource gene annotation (ftp://ftp.arabidopsis.org/home/tair/Microarrays/Affymetrix/) that represents a major improvement over the original 2001 annotation in the Affymetrix GCOS software. Since correcting by the mismatched probes now is believed to increase the noise (Irizarry et al., 2005), we used only the perfectly matched probes. The quality of hybridization was assessed by MA plots, pairwise correlation, and RNA degradation. Statistical analyses were performed using the Bioconductor suite (Gentleman et al., 2004) implemented in the R programming language (Dalgaard, 2002). Raw intensity measurements were background corrected and quantile normalized (Irizarry et al., 2003b) using robust multiarray average analysis (Irizarry et al., 2003b) implemented in the affy package (Gautier et al., 2004). The background-corrected, normalized, and log2-transformed expression values were fitted to a linear model using the limma package (Smyth, 2004). Linear models were refined by an empirical Bayes method. The resulting P values were subjected to multiple test corrections. Since the number of downregulated genes exceeded the number of upregulated transcripts, a correction that may have biased the false discovery rate (Benjamini and Hochberg, 1995) calculations, multiple test corrections were performed by the more robust and conservative Bonferroni method.

Overall Histone H3-K4 Methylation in the Wild Type and atx1 and atx2 Mutants

Total histones extracted from 3-week-old wild-type and atx1 mutant plants were probed with antibodies specific for di- or trimethylated H3K4 protein gel blots exactly as described earlier (Alvarez-Venegas and Avramova, 2003). Membranes then were stripped and reprobed with antibodies specific for nonmodified histone H3. The levels of histone H3-tail methylation of wild-type histones, defined as the ratio of mH3-H3- to H3-intensity signals, were computed from four independent experiments.

RT-PCR

RT-PCR analysis was done exactly as described in Saleh et al. (2007) using the following primers: NAP, forward, 5’-ctagccaggaactcact-gt-3’, reverse, 5’-taacactcgtgctagatgtaggtgt-3’; ATX1 (band a; see Supplemental Figure 1 online), forward, 5’-GACTCGGCCGCTTGGTCACAG-3’, reverse, 5’-GCCCTGCTAGAAATGGAAAGC-3’; ATX2 (band b; see Supplemental Figure 1 online), forward, 5’-GGACCTGAGGCTCCTGTGC-3’, reverse, 5’-GACATTTGCAAAACACAGT-3’.

Real-Time PCR Analysis

Total RNA was isolated using the Invisorb Spin Plant RNA Mini kit (Invitek) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed on 500 ng of RNA using the M-MLV System for RT-PCR (Invitrogen). Real-time PCR reactions were performed in a final volume of 25 μL containing 12.5 μL of iQ SYBR Green PCR supermix (Bio-Rad), 1 μL of each primer (forward and reverse, 50 ng/μL), 5 μL of cDNA (1 ng/μL), and 6.5 μL sterile deionized water. The PCR products were amplified under the following conditions: 95°C for 3 min, 39 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 10 min using an iCycler iQ real-time PCR detection system (Bio-Rad), iQ 96 well PCR plates (Bio-Rad), and optical quality sealing tapes (Bio-Rad). The ACTIN7 gene sequence was used as an internal control. The primer sequences were as follows: NAP, forward, 5’-catgcaggaagctcactaggg-3’, reverse, 5’-tagcctcgctaggtagatgtg-3’; ATX1, forward, 5’-TGGTGGTCCTTCAACACAGGAA-3’, reverse, 5’-GACCTGAGGCTCCTGTGC-3’; WRKY70, forward, 5’-GACGAGCAAATGGTGGACACAT-3’, reverse, 5’-ATACCTCCTTCGAAAAACCTTCTGTG-3’, ACTIN7, forward, 5’-CTAGCAGGGTGATGCTCCTTCCTCAT-3’, reverse, 5’-CTGAAAGACTGTCCTGTGACTCT-3’.

ChIP Assays

A protocol described earlier (Alvarez-Venegas and Avramova, 2005) was used with some modifications: harvested tissues from examined plant samples were cross-linked (0.4 M sucrose, 10 mM Tris, pH 8.5, 1 mM EDTA, 1 mM PMSF, and 1% formaldehyde) under vacuum, followed by freezing and grinding in liquid nitrogen. After resuspension in buffer (15 mM PIPES, pH 6.8, 5 mM MgCl2, 60 mM KCl, 0.25 M sucrose, 15 mM NaCl, 1 mM CaCl2, 0.9% Triton X-100, 1 mM PMSF, 2 μg/mL pepstatin A, and 2 μg/mL aprotinin), the slurry was filtered (four layers of cheesecloth) and the filtrate centrifuged at 10,000 g for 20 min in a Sorvall SA-600 rotor. The nuclear pellet, resuspended in 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, 1 μg/mL aprotinin, 1 μg/mL pepstatin A), was sonicated to shear the DNA. After removing cell debris (centrifugation at 13,000 rpm in cold benchtop centrifuge for 10 min), the chromatin fraction was harvested and used for immunoprecipitation. Antitrimethyl Histone H3 Lys4 (Upstate) and antidimethyl Histone H3K4 (Upstate) were used. Negative control samples were treated in the same way, except without antibodies added. Each immunoprecipitation was performed at least three separate experiments. Calibration curves were built to determine optimal amounts of chromatin to be used in each experiment and to
ensure equivalent amounts of starting material (Alvarez-Venegas and Avramova, 2005). The PCR products were amplified under the following conditions: 95°C for 3 min, 38 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and 72°C for 10 min. The primer sequences used were as follows: WRKY70, forward, 5′-AGCAACTCCTCTCAACCCG-3′, reverse, 5′-CCATTGAGTAACTGCGCTGA-3′; XTH33, forward, 5′-TTGGTTTC-TTCACACAGCAGGAA-3′; reverse, 5′-GACCTACAGCAGCATGATTGTTT-3′; NAP, forward, 5′-ctctctcagctgctct-3′, reverse, 5′-ctggctctgcagcagctctg-3′, reverse, 5′-tgggagatccggacccgcaagatc-3′.

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At2g31650 (ATX1), At1g05830 (ATX2), At5g09810 (ACTIN7), At1g69490 (NAP), At3g56400 (WRKY70), and At1g10550 (XTH33).

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure 1. Expression of ATX2 in Different Genetic Backgrounds and Delayed Abscission Phenotypes in atx1 and atx2 Flowers.
Supplemental Figure 2. Transcription Factor Binding Motifs Recognized in the Promoter Regions of ATX1 and ATX2.
Supplemental Figure 3. Overall Histone H3-K4 Methylation in Wild-Type and in atx2-2 Plants.
Supplemental Figure 4. Real-time PCR Tissue-Specific Expression Analysis of XTH33.
Supplemental Table 1. atx2 Flowers Defective in Flower Organ Abscission.
Supplemental Data Set 1. Upregulated Genes in the atx2 Background.
Supplemental Data Set 2. Downregulated Genes in the atx2 Background.

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REFERENCES


Correction


Figure 7C (WRKY70 ChIP-PCR assay) has been corrected to clarify that the wild-type control sample for H3K4m3 was run on a separate gel from the atx1 and atx2 samples, indicated by the space (white line, bottom row). The samples for H3K4m2 (top row) were run together on the same gel. The legend and labeling on the figure have been amended slightly for clarity. The original conclusions of the manuscript are unaltered by these corrections.

![Figure 7C](image.png)

**Figure 7. Original:** Expression and H3K4 Methylation Patterns of WRKY70 in Wild-Type, atx1, and atx2 Chromatin.

*(C)* WRKY70 amplified from ChIP assays of wild-type, atx1, and atx2 flower chromatin immunoprecipitated with antibodies distinguishing between di- and trimethylated H3K4 isoforms. Labeling is as in Figure 5C. A schematic illustration of the WRKY70 gene including the regions amplified for the ChIP assay is shown at top of the panel.

![Figure 7C](image.png)

**Figure 7. Corrected:** Expression and H3K4 Methylation Patterns of WRKY70 in Wild-Type, atx1, and atx2 Chromatin.

*(C)* WRKY70 amplified from ChIP assays of wild-type, atx1, and atx2 flower chromatin immunoprecipitated with antibodies distinguishing between di- and trimethylated H3K4 isoforms. The control wild-type samples for H3K4m3, separated by a space (white line in the bottom panel), were resolved in a different gel from atx1 and atx2 samples. The blots were developed and processed in parallel. I, input sample representing 10% of the template amount used for the immunoprecipitation. −Ab and +Ab are immunoprecipitated chromatin without and with added antibody, respectively. A schematic illustration of the WRKY70 gene including the regions amplified for the ChIP assay is shown at top of the panel.

Editor’s note: the corrected figure and accompanying text were reviewed by members of The Plant Cell editorial board.
### The Highly Similar *Arabidopsis* Homologs of Trithorax ATX1 and ATX2 Encode Proteins with Divergent Biochemical Functions

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