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Yong Ding
University of Nebraska Center for Biotechnology and Center for Plant Science Innovation and School of Biological Sciences, dingyong@ustc.edu.cn

Zoya Avramova
University of Nebraska School of Biological Sciences, zavramova2@unl.edu

Michael Fromm
University of Nebraska Center for Biotechnology and Center for Plant Science Innovation, mfromm2@unl.edu

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The Arabidopsis Trithorax-like Factor ATX1 Functions in Dehydration Stress Responses via ABA-Dependent and ABA-Independent Pathways

Yong Ding,[1,2] Zoya Avramova,[2] and Michael Fromm,[1]

1. University of Nebraska Center for Biotechnology and Center for Plant Science Innovation, 1901 Vine Street, Lincoln, Nebraska, USA
2. University of Nebraska School of Biological Sciences, 1901 Vine Street, Lincoln, Nebraska, USA

Corresponding authors – Z. Avramova, email zavramova2@unl.edu or M. Fromm, email mfromm@unlnotes.unl.edu

Abstract

Emerging evidence suggests that the molecular mechanisms driving the responses of plants to environmental stresses are associated with specific chromatin modifications. Here, we demonstrate that the Arabidopsis trithorax-like factor ATX1, which trimethylates histone H3 at lysine 4 (H3K4me3), is involved in dehydration stress signaling in both abscisic acid (ABA)-dependent and ABA-independent pathways. The loss of function of *ATX1* results in decreased germination rates, larger stomatal apertures, more rapid transpiration and decreased tolerance to dehydration stress in *atx1* plants. This deficiency is caused in part by reduced ABA biosynthesis in *atx1* plants resulting from decreased transcript levels from *NCED3*, which encodes a key enzyme controlling ABA production. Dehydration stress increased ATX1 binding to *NCED3*, and ATX1 was required for the increased levels of *NCED3* transcripts and nucleosomal H3K4me3 that occurred during dehydration stress. Mechanistically, ATX1 affected the quantity of RNA polymerase II bound to *NCED3*. By upregulating *NCED3* transcription and ABA production, ATX1 influenced ABA-regulated pathways and

genes. ATX1 also affected the expression of ABA-independent genes, implicating ATX1 in diverse dehydration stress-response mechanisms in Arabidopsis.

Keywords: NCED3, ATX1, abscisic acid, histone methylation, chromatin, Arabidopsis

Introduction

Plants respond to external stimuli by activating signaling pathways that rapidly alter physiological processes and expression patterns of genes involved in the response. The molecular mechanisms regulating gene expression are dependent in part on the structure of chromatin, which determines the accessibility of DNA to the transcriptional machinery. Chromatin-driven mechanisms, including noncoding RNAs, changes in histone variants, histone modifications, and DNA methylation are involved in the responses of plants to environmental cues (Chinnusamy and Zhu, 2009). Changes in DNA methylation and/or histone modifications associated with altered gene expression under various stresses have been reported in plants and in cell culture systems (Manzanero et al., 2002; Steward et al., 2002; Chua et al., 2003; Tsuji et al., 2006; Sokol et al., 2007). Chemical modifications of the histone amino-terminal tails may recruit specific transcription factors and/or induce nucleosome remodeling to facilitate transcription by RNA polymerase II (Pol II). The drought-induced H1-S linker histone in tomato affected stomatal conductance and transpiration, making antisense knock-down plants more sensitive to water deficit stress by an unknown mechanism (Scippa et al., 2004). Cold treatment of *Secale cereale* (rye), *Hordeum vulgare* (barley), and *Vicia faba* (field bean) caused changes in the distribution of histone H3 Ser10 phosphorylation (Manzanero et al., 2002). High salinity altered H3 Ser10 phosphorylation and histone H4 acetylation levels in *Nicotiana tabacum* (tobacco) and Arabidopsis culture cells (Sokol et al., 2007), and caused changes in nucleosome occupancy and in the levels of histone H3K4me3, H3K9ac, H3K14ac, H3K23ac, and H3K27ac modifications under stress at the RD29A, RD29B, RD20, and RAP2.4 genes in Arabidopsis (Kim et al., 2008). HOS15, a WD40-repeat protein, affects genomic histone acetylation levels and regulation of the cold inducibility of RD29A (Zhu et al., 2008).

The current knowledge of histone-modifying activities involved in dehydration stress-response mechanisms is limited. Abscisic acid (ABA)-induced stomatal closing is mediated by chromatin remodeling involving histone H4 deacetylation by activating deacetylases (Sridha and Wu, 2006; Zhu et al., 2008), whereas seed germination involves histone H2 monoubiquitination of several genes mediating ABA synthesis (Liu et al., 2007). Overexpression of the histone deacetylase AtHDAC2 in Arabidopsis reduced transpiration and affected ABA responses by an unknown mechanism (Sridha and Wu, 2006). ARABIDOPSIS HOMOLOG OF TRITHORAX1 (ATX1) trimethylates histone H3 at lysine 4 (H3K4me3) and regulates transcription from diverse classes of genes implicated in biotic and abiotic stress responses, including a subset of genes responding to water dehydration stress (Alvarez-Venegas et al., 2006b, 2007; Ding et al., 2009). The specific gene targets and biochemical mechanisms affected by these chromatin modifiers involved in the dehydration stress response remain to be established.

The plant hormone ABA is involved in seed development and dormancy as well as in responses to dehydration, high salinity (Yamaguchi-Shinozaki and Shinozaki, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007; Chinnusamy et al., 2008), and plant pathogen (Asselbergh et al., 2008) stresses. ABA is synthesized de novo in response to dehydration, and the limiting step of ABA biosynthesis is the cleavage of 9-*cis*-epoxycarotenoids by 9-*cis*-epoxycarotenoid dioxygenase (NCED3) (Qin and Zeevaart, 1999). Increased ABA levels activate signaling cascades that trigger responses such as stomatal closure and alteration of gene expression (Chinnusamy et al., 2008; Kim et al., 2008). Expression of *NCED3* is induced by dehydration stress, and overexpressed *NCED3* increases endogenous ABA levels, elevates transcription of ABA-inducible genes, reduces the transpiration rate from leaves, and improves dehydration tolerance in Arabidopsis. By contrast, reduced NCED3 expression in mutants or through antisense suppression causes dehydration-sensitive phenotypes (Iuchi et al., 2001; Wan and Li, 2006). Despite its importance in the dehydration stress response, the mechanisms regulating *NCED3* gene expression remain elusive.

Here we provide insights into a plausible mechanism by which ATX1 affects ABA-dependent responses. We demonstrate that ATX1 directly regulates *NCED3*, the rate-limiting enzyme in ABA biosynthesis, and that the levels of *NCED3* transcripts correlate with the level of H3K4me3 on its nucleosomes. The diminished levels of *NCED3* mRNA during dehydration stress result in lower ABA levels in *atx1* plants. Consequently, expression levels of ABA-dependent genes and ABA-dependent physiological responses are attenuated accounting for much of the dehydration sensitivity of *atx1* plants. Additionally, ATX1 is implicated in ABA-independent dehydration stress-signaling pathways in Arabidopsis.

Results

ATX1 loss of function lowers tolerance to dehydration stress

Three-day-old wild-type (WT) and *atx1* seedlings were tested for tolerance to osmotic or dehydration stress. Seedlings of *atx1* mutants developed more leaf chlorosis on media containing mannitol or polyethylene glycol (PEG) than WT seedlings after 12 days of exposure but had similar appearances on the control MS media (Fig. 1a). To verify that these phenotypes resulted from the loss of ATX1 activity, we performed experiments with *atx1* mutants transformed with an *ATX1* transgene. The *ATX1* transgene reversed the small plant and early flowering phenotype of *atx1*, indicating that the transgene functionally complements the atx1 mutation (Fig. S1a). Complemented *atx1* mutant plants grown on media containing mannitol or PEG were visibly indistinguishable from the wild type (Fig. 1b). In germination assays, *atx1* seeds displayed a lower percentage of germination than WT seeds in media containing mannitol, at each concentration of mannitol tested (Fig. 1c). Seedlings from two independently complemented lines grown in media containing mannitol displayed germination percentages similar to WT seeds at each concentration of mannitol tested (Fig. 1c).

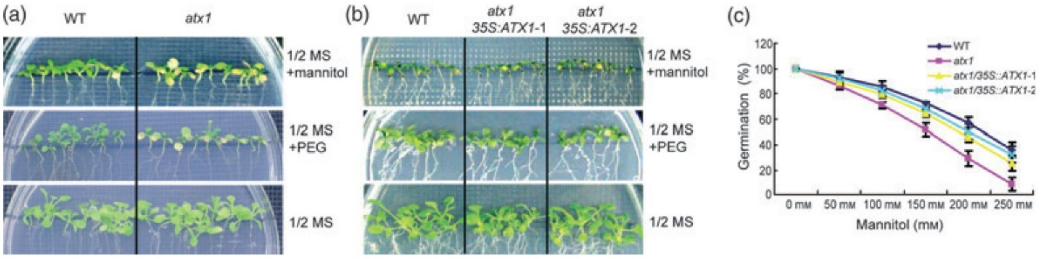

Figure 1. Response of *atx1* mutants to osmotic stress. Seedlings or seeds of the following genotypes were subjected to osmotic stress: wild type (WT), *atx1* or two independent isolates of an *ATX1* transgene transformed into the *atx1* mutant (*atx1/35S::ATX1*). (a) Three-day-old WT or *atx1* seedlings were transferred to half-strength MS medium, half-strength MS medium containing 200 mM mannitol or media previously infused with 30% polyethylene glycol (PEG), for 12 days. (b) As in A, except that seedlings of an *atx1* mutant complemented with an *ATX1* transgene in two independent lines (atx1/35S:ATX1-1, atx1/35S:ATX1-2) were compared with the WT. (c) Seeds of the indicated genotypes were germinated on half-strength MS medium or half-strength MS medium plus different concentrations of mannitol for 7 days, and the percentage of seeds that germinated is shown. Measurements are means ± SEMs (n = 4 independent experiments). Representative experiments in a and b are shown, and were performed at least three times.

Furthermore, soil-grown 14-day-old *atx1* plants were more sensitive to water withdrawal than WT plants (Fig. 2a). The number of WT plants surviving after 12 days of water withdrawal and 3 days of rewatering was 70%, whereas only 30% of the *atx1* plants recovered (Fig. 2a, right panel, and Fig. 2b). Plants subjected to soil water deficit for various times were used to determine their relative water content (RWC). This assay revealed that *atx1* plants had a lower RWC than WT plants after 9 or 12 days without water (Fig. 2c). As WT and *atx1* plants were grown within the same container, they were exposed to similar water potentials, suggesting that *atx1* plants were more sensitive to soil water deficits because of faster rates of transpiration. This hypothesis was tested directly by measuring the rate of water loss from detached leaves. Mutant *atx1* leaves lost water at a faster rate than the WT in this assay (Fig. 2d). To confirm that ATX1 was involved in this response, we measured the water content in *atx1* mutant plants complemented with an *ATX1* transgene. Analyses of the water-level profiles during dehydration revealed that the complemented *atx1* plants retained cellular water at higher levels than noncomplemented plants (Fig. 2d).

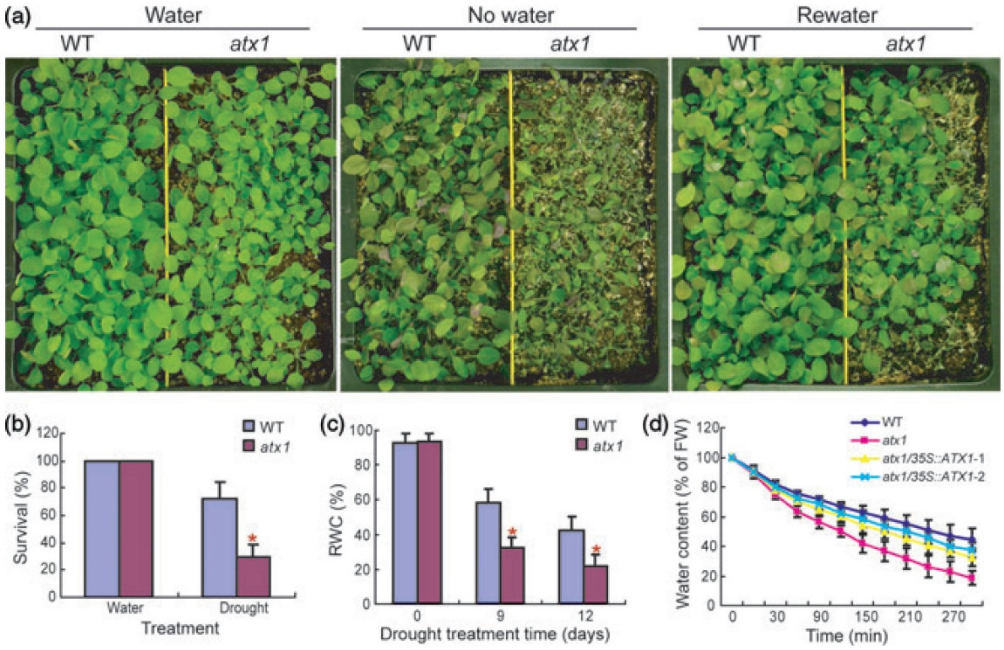

Figure 2. Response of *atx1* seedlings to dehydration stress. Wild-type (WT), *atx1* or *atx1/35S::ATX1* seedlings, or detached leaves, were subjected to dehydration stress. (a) Plants (14-days old) were grown with (Water) or without water (No water) for 9 days, or without water for 12 days followed by a 3-day watering recovery period (Rewater). (b) The percentages of WT or *atx1* plants surviving 12 days without water followed by 3 days of rewatering are shown. (c) Two-week old WT or *atx1* plants in soil were subjected to 9 or 12 days without watering, and the relative water content (RWC) was measured. (d) The volume of water lost from detached leaves of the indicated genotypes by air-drying was measured as the percentage change in the fresh weight (FW) of the leaves. A representative experiment is shown and was performed at least four times. Measurements are means ± SEMs ($n = 5$ for B; $n = 4$ for C and D; where n is the number of independent experiments); *$P \leq 0.05$.

In aggregate, *atx1* mutant plants displayed increased sensitivity to osmotic or dehydration stress, and this sensitivity could be explained, at least partly, by more rapid transpiration by *atx1* leaves. Rapid transpiration suggested a possible malfunction in the regulation of stomatal openings.

atx1 ***leaves have larger stomatal apertures and produce less ABA during dehydration stress***

Stomatal apertures were measured in watered or dehydration-stressed WT and *atx1* leaves. The *atx1* leaves had larger stomatal apertures than WT leaves in both the watered and nonwatered conditions (Fig. 3a,b). The *ATX1* transgene in *atx1* plants restored the smaller stomatal aperture during dehydration stress (Fig. S1b). Plants defective in ABA biosynthesis or perception are more sensitive to dehydration stress, in part, because of a more rapid transpiration resulting from a failure to reduce their stomatal apertures. Therefore, the sensitivity of the stomatal openings of WT and *atx1* leaves to ABA was measured

by immersing the leaf epidermis in buffer containing different concentrations of ABA. Stomata of *atx1* leaves showed similar percentage closings as WT stomata at the different ABA concentrations (Fig. 3c). This result demonstrated that the stomatal closure response in *atx1* leaves could be rescued by the addition of ABA, suggesting that ATX1 is not required for ABA signaling. To test a hypothesis that ABA production was impaired in *atx1* plants, ABA levels were measured in watered or dehydration-stressed plants. Control WT plants had higher levels of ABA under both conditions and showed a fourfold induction of ABA levels during dehydration stress. ABA levels in *atx1* plants were only 40% of those in WT plants, suggesting that ABA production was reduced in *atx1* plants (Fig. 3d).

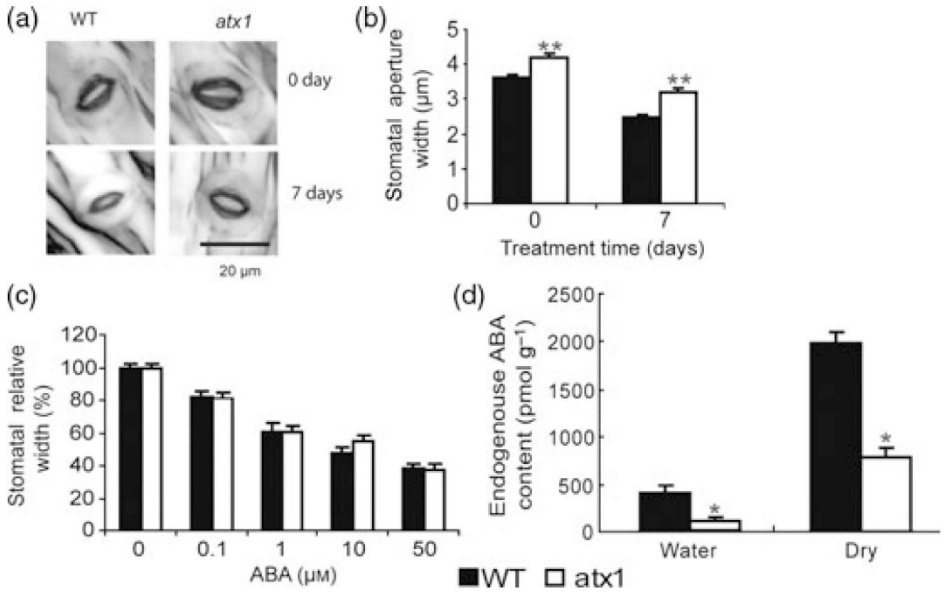

Figure 3. Stomatal openings and abscisic acid (ABA) levels in wild-type (WT) and *atx1* leaves. Stomatal openings were measured during watered, ABA-treated or soil water-deficit conditions, and ABA levels were measured in leaves from watered or dehydration-stressed plants. (a) Confocal microscope images of representative stomata of WT or *atx1* leaves from plants that were watered (0 day) or were not watered for 7 days (7 days). (b) The mean width of stomatal apertures for leaves treated as described in a are shown (means were derived from 60 stomatal measurements). (c) The widths of the stomatal openings for WT or *atx1* leaves immersed in various concentrations of ABA, relative to the stomatal opening in the control state (0 ABA), are shown. (d) Endogenous ABA levels were measured from watered or dehydration-stressed leaves in WT or *atx1* genotypes using an ELISA. Each bar represents a mean + SEM (n = 3 for b, c; n = 2 for d; where n is the number of independent experiments). *$P \leq 0.05$; **$P \leq 0.01$.

ATX1 affects the transcript levels of ABA biosynthetic genes

The expression levels of genes in the ABA biosynthetic pathway were examined as a possible explanation for the above phenotypes in atx1 mutants. The transcript levels of four ABA biosynthetic genes (*ABA1*, *ABA2*, *ABA3*, and *NCED3*) were measured in WT and *atx1*

backgrounds during noninduced (watered) and induced (ABA or dehydration stress) conditions. The results showed that mRNA levels of *ABA1*, functioning in the ABA and carotenoid biosynthesis required for late skotomorphogenic growth in Arabidopsis (Barrero et al., 2008), and of *ABA2*, a late expression gene with a fine-tuning function in ABA biosynthesis (Lin et al., 2007), were not strongly influenced by the ATX1 deficiency during either ABA treatment or dehydration stress (Fig. 4a). In contrast, *ABA3*, involved in the last step of ABA biosynthesis (Bittner et al., 2001), and NCED3, considered to be the rate-limiting step in ABA biosynthesis (Qin and Zeevaart, 1999), produced reduced levels of transcripts in *atx1* plants relative to WT plants under dehydration stress conditions (Fig. 4a). The downregulation of *ABA3* and *NCED3* in *atx1* cells presumably caused the reduction in endogenous ABA (Fig. 3d).

ATX1 directly binds NCED3 *and regulates its transcription*

The strong decreases in *ABA3* and *NCED3* transcript levels in *atx1* plants in response to dehydration raised the possibility that these genes were direct targets of ATX1. To answer this question, we used antibodies against ATX1 in a chromatin immunoprecipitation (ChIP) assay, with PCR primers to a region approximately 300 bp downstream of their transcription start sites. The ChIP-PCR signal at *ABA3* was similar to the IgG control, and was not affected by genotype or dehydration stress (Fig. S2). We conclude that ATX1 does not bind to *ABA3*. In contrast, although the ChIP-PCR *NCED3* signal from watered plants was very low in both WT and *atx1* plants, increased levels of ATX1 were bound to *NCED3* in WT plants relative to *atx1* plants during dehydration stress (Figs. S3a–c and 4b,c). As positive and negative controls, we analyzed *LIPID TRANSFER PROTEIN 7* (*LTP*, *AT2G 15050*), a direct target gene for ATX1 (Alvarez-Venegas and Avramova, 2005), and *ACTIN 7* (*ACT7*, *AT5G09810*), a nontarget gene. *LTP* bound more ATX1 in WT plants than in *atx1* plants (Fig. S3a), whereas *ACT7* did not bind ATX1, and the quantity of DNA recovered was not affected by genotype (Figs. S3a and 4c). We conclude that the quantity of ATX1 bound to *NCED3* increased significantly in WT plants subjected to dehydration stress (Fig. 4c).

Next, we asked whether ATX1 affected Pol-II levels at *NCED3*. An initial ChIP-PCR analysis of several regions of the *NCED3* gene in dehydration stress plants indicated that Pol-II levels were higher in WT plants than in *atx1* plants (Fig. S3b,c). Of the regions analyzed, the *NCED3* region about 300 bp downstream of the transcription start site had the highest Pol-II occupancy, and this region was used in the subsequent analysis. Pol-II occupancy was much higher on the *NCED3* gene after dehydration stress, and the increase was much larger in WT plants than in *atx1* plants (Figs. S3c and 4d). In contrast, the level of Pol II at ACT7 was not significantly affected by genotype or dehydration stress (Fig. 4d). The observed correlation between ATX1, Pol II and transcript levels for the *NCED3* gene supports a functional role for ATX1 in the increased rate of transcription of *NCED3* during dehydration stress.

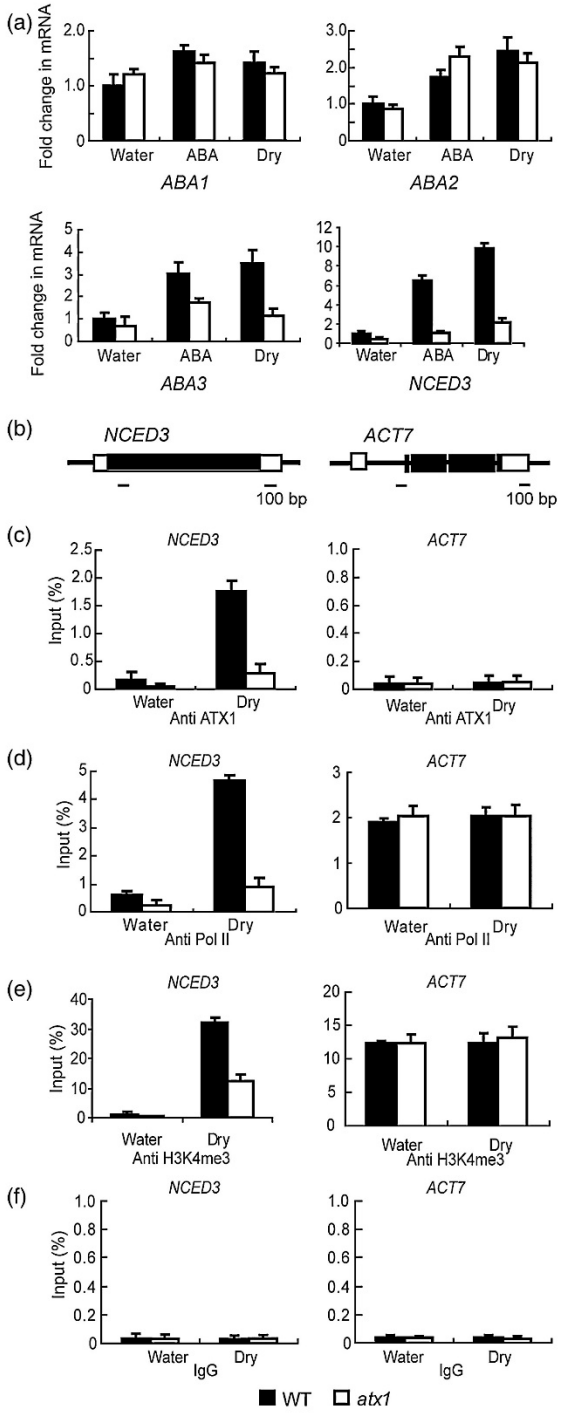

Figure 4. Transcript levels of abscisic acid (ABA) biosynthetic genes and chromatin changes at *NCED3*. Transcript levels were measured in leaves that were nonstressed (Water), ABA-treated for 1 h (ABA), or air-dried for 1 h (Dry) in wild-type (WT; black) or *atx1*

(white) genotypes. (a) The gene analyzed for its transcript levels is indicated below each panel. (b) A representation of the *NCED3* and *ACT7* genes, with the nontranscribed regions upstream (on the left side of panel) and downstream of the gene shown as lines; the noncoding transcribed regions (open boxes), coding regions (closed boxes), and introns (thin lines) are indicated. The regions of the genes analyzed by ChIP-PCR in panels c–e are indicated by the underlined areas near the 5′ end of the genes. (c–f) The panels show the results of ChIP-PCR with the antibody or serum used for ChIP indicated below each panel: (c) ATX1; (d) Pol II; (e) H3K4me3; (f) nonimmune IgG serum control (IgG). Representative experiments are shown and were performed three times in a and twice in c–f. Each bar represents a mean + SEM (n = 3).

Whether *NCED3* transcript levels, and the position of ATX1 on *NCED3*, correlated with H3K4 trimethylation levels and locations was determined next. The levels of H3K4me3 at *NCED3* were increased by dehydration stress, and this increase was much higher in a WT genotype than in an *atx1* genotype (Figs. S3c and 4e). This pattern is in agreement with *NCED3* transcript levels under these conditions (Fig. 4a). The locations of ATX1 and H3K4me3 on *NCED3* were similar, with peaks near the 5′ end of the gene (Fig. S3c). As controls we analyzed H3K4me3 levels at the *LTP* and *ACT7* genes. H3K4me3 levels at the ATX1-regulated *LTP* gene were reduced in *atx1* (Fig. S3d), whereas H3K4me3 levels at the nontarget gene *ACT7* were not affected by genotype or dehydration stress (Figs. S3d and 4e). These results demonstrated that ATX1 affected H3K4me3 levels at target genes but not at a nontarget gene.

Regulation of ABA-dependent and ABA-independent dehydration response genes by ATX1

Downregulation of *NCED3* in *atx1* plants implicated ATX1 in the ABA-dependent dehydration stress response pathway. To determine the extent of the involvement of ATX1 in the dehydration response, and whether its role was limited to the ABA-regulated pathway, we examined several dehydration-inducible genes displaying different sensitivity to exogenously applied ABA. Four ABA-dependent genes (*RD29A*, *RD29B*, *RD26*, and *ABF3*) were induced by dehydration stress as well as by treatment with ABA (Fig. 5a). During dehydration stress, transcript levels from these genes were reduced in *atx1* plants, indicating that ATX1 was required for WT levels of transcript production from these genes. The *ATX1* transgene in *atx1* plants significantly restored the dehydration inducibility of *NCED3*, *RD29A*, and *RD29B* (Fig. S4a), confirming that *atx1* was responsible for this phenotype. Much of the effects of the *atx1* mutation were mediated by ABA: treatment with exogenous ABA induced *RD29A* and *RD29B* transcripts in *atx1* plants to levels similar to those induced by ABA in WT plants, whereas those of *RD26* and ABF3 were partially restored (Fig. 5a). This result suggests that in the presence of ABA, the ABA inducibility of the first two of these genes was not compromised in the absence of functional ATX1, whereas *RD26* and ABF3 were partially dependent on ATX1.

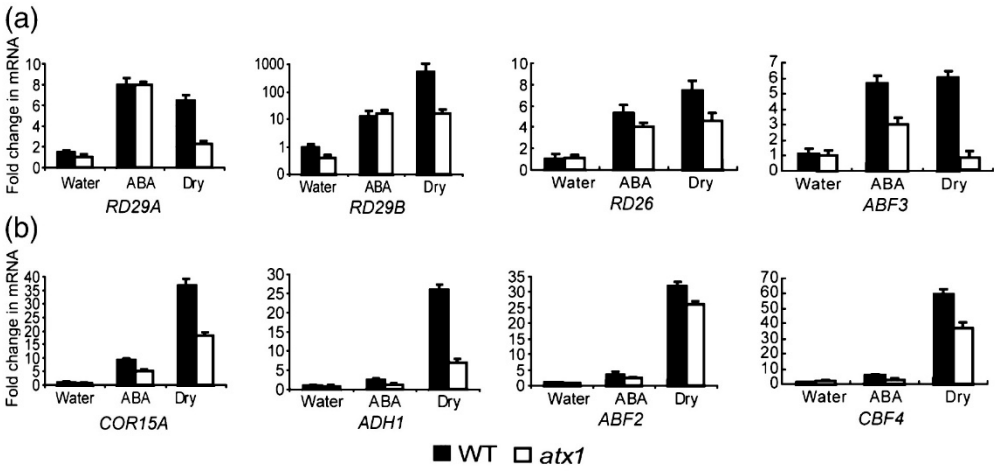

Figure 5. Transcript levels from abscisic acid (ABA)-dependent and -independent genes. Transcript levels were measured in leaves that were nonstressed (Water), ABA-treated for 1 h (ABA) or air-dried for 1 h (Dry) in wild-type (WT; black) or *atx1* (white) genotypes. The name of each gene analyzed is indicated below its respective panel. (a) Transcript levels from four ABA-dependent genes. (b) Transcript levels from four ABA-independent genes. Representative experiments are shown and were performed three times. Each bar represents a mean + SEM (n = 3).

As expected, the four ABA-independent genes (*COR15A*, *ADH1*, *ABF2*, and *CBF4*) only weakly responded to ABA relative to their induction during dehydration stress (Fig. 5b). Three of the genes in this group showed a strong ATX1-dependent induction upon dehydration stress, producing fewer transcripts in *atx1* than in WT plants, whereas ABF2 showed only a modest dependence on ATX1 (Fig. 5b).

The H3K4me3 levels from representative ABA-dependent and -independent genes (*RD29A*, *RD29B*, *COR15A*, *ADH1*, and *ABF2*) were measured by ChIP-PCR to determine how these levels varied in *ATX1* and *atx1* plants. We found H3K4me3 levels generally corresponded to transcript levels, and genes producing reduced levels of transcripts in *atx1* plants had reduced but measurable levels of H3K4me3 (Fig. S4b). These remaining H3K4me3 levels indicate that ATX1 is not the sole factor involved in activating and modifying the nucleosomes of these genes, adding to the complexity of the chromatin-based mechanisms involved in the response process.

Discussion

Here, we provide an example of the involvement of a histone methyltransferase, ATX1, in some of the physiological responses and altered gene transcription caused by osmotic or dehydration stress in Arabidopsis. Our results indicated that the loss of ATX1 function caused the lower germination of *atx1* seeds under increasing concentrations of mannitol, larger stomatal apertures during dehydration stress, increased water loss and increased sensitivity to dehydration stress. The reduced dehydration tolerance in *atx1* plants was

primarily caused by reduced levels of endogenous ABA during dehydration stress (Fig. 3d). The reduced ABA levels were consistent with reduced production of *ABA3* and *NCED3* mRNAs under dehydration stress in *atx1* mutants (Fig. 4a). The *ABA3* and *NCED3* genes encode key enzymes in the ABA biosynthetic pathway, and their expression levels affect the sensitivity of the plants to dehydration. In particular, transient expression of *NCED3* in guard cells caused stomatal closure in field bean (Melhorn et al., 2008), whereas suppression of *NCED3* transcription caused enhanced transpiration, and higher sensitivity to dehydration, in Arabidopsis (Qin and Zeevaart, 1999; Iuchi et al., 2001). Here, we demonstrated that the stomatal closure response in *atx1* leaves could be rescued by the addition of ABA (Fig. 3c), consistent with impaired production of ABA in *atx1* plants.

Upregulation of *NCED3* transcripts during dehydration stress was associated with increased ATX1 and Pol-II occupancy, as well as elevated levels of nucleosomal H3K4me3, at *NCED3* (Fig. 4c–e). These results illustrate a requirement for ATX1 for the production of WT levels of NCED3 transcripts. In contrast, ATX1 regulation of *ABA3* appeared indirect, as ATX1 did not bind to *ABA3* (Fig. S2). Moreover, ATX1 accumulated at *NCED3*, but not *ABA3* or *ACT7*, in response to dehydration stress, illustrating that ATX1 recruitment was both environmentally responsive and gene specific (Fig. 4c).

The ABA-dependent (*RD29A*, *RD29B*, *RD26*, and *ABF3*) as well as the ABA-independent (*COR15A*, *ABF2*, *CBF4*, and *ADH1*) dehydration-inducible genes displayed reduced transcript levels in *atx1* mutants during dehydration stress (Fig. 5). These results indicated that ATX1 was required for inducing transcript levels from both groups of genes during a dehydration stress response, despite their participation in different signaling pathways. Recently, we showed that ATX1 was also involved in a lipid-signaling pathway through its interaction with phosphatidylinositol-5-phosphate (PtdIns5P) (Alvarez-Venegas et al., 2006b). Osmotic or dehydration stress in yeast, animal, and plant cells increases the levels of phosphatidylinositol molecules, including PtdIns5P, to act as secondary messengers in stress signaling (Dove et al., 1997; Sbrissa et al., 2002; Pendaries et al., 2005; Munnik and Testerink, 2009). ATX1 can bind PtdIns5*P*, and the two coregulate a distinct subset of genes (Alvarez-Venegas et al., 2006b). Furthermore, production of PtdIns5*P* is dependent upon the activity of a myotubularin enzyme encoded by *AtMTM1*, and the identification of a set of genes with altered responses during dehydration stress in *atx1* or in *AtMTM1*-overexpressing plants (approximately 140 overlapping misexpressed genes; Ding et al., 2009) confirmed the involvement of ATX1 in this signaling pathway.

Collectively, our results demonstrate that ATX1 participates in distinct dehydration stress-responding pathways, including ABA-dependent, as well as ABA-independent (including lipid-signaling) pathways. ATX1 affects transcript levels of regulatory proteins, including genes encoding transcription factors that are involved in multiple and diverse signaling mechanisms. More than 20 transcription factors implicated in salinity, temperature, wound and dehydration stress responses were misregulated in *atx1* plants (Alvarez-Venegas et al., 2006a), such as the *ZAT10* gene encoding a transcription factor stimulating expression of reactive oxygen-defense transcripts, and enhancing the tolerance of plants to salinity, heat, and osmotic stress, (Mittler et al., 2006), or RD26, encoding a NAM transcription factor, mediating the regulation between ABA and JA signaling pathways during responses to drought or wound stresses (Fujita et al., 2004). ATX1 also regulates the

expression of genes that are encoding key regulators and hubs in the ABA signal transduction network, like *ABI1* and *HAB1* (Saez et al., 2006). These findings, together with earlier results showing that ATX1 was selectively recruited to *WRKY70* when the gene was induced by exposure to a pathogen (Alvarez-Venegas et al., 2007), implicate ATX1 in multiple aspects of abiotic, biotic, and wound stress responses, and in the regulation of developmental processes.

The involvement of ATX1 in so many diverse pathways might explain why some of the phenotypes of *atx1* seeds or plants are not the same as those of ABA-deficient mutants. The reduced germination of *atx1* seeds on mannitol is different than the phenotypes of seeds with low ABA levels, which are less sensitive to osmotic inhibition (Leon-Kloosterziel et al., 1996). Additionally, ABA-deficient plants are hypersensitive to exogenous ABA (Xiong et al., 2002), whereas *atx1* plants did not show this hypersensitivity. We presume that the atypical responses in *atx1*, relative to ABA-deficient seeds or plants, in seed germination and in ABA-induced stomatal closure are the result of pleiotropic effects of ATX1 within multiple pathways.

The trithorax group of proteins (TrxG), of which ATX1 is a member, often function in conjunction with polycomb (PcG) proteins, which generally oppose the functions of TrxG proteins (Schuettengruber et al., 2007). Two members of the PcG chromatin modifying complexes in Arabidopsis, FVE and MSI1, are known as epigenetic regulators of development (Hennig et al., 2003; Kohler et al., 2003; Ausin et al., 2004; Kim et al., 2004). Similar to the pleiotropic roles of ATX1, FVE, and MSI1 regulate genes involved in more than one environmental response pathway. FVE acts within the thermosensory pathway, displaying dual roles in regulating flowering time and the cold response (Kim et al., 2004), whereas MSI1 regulates a subset of ABA-responsive genes involved in the response to drought and salt stress (Alexandre et al., 2009). The emerging picture is that, in addition to regulating development, the plant TrxG/PcG complexes participate in a diverse set of biotic and/or abiotic stress response mechanisms.

Experimental Procedures

Plant growth conditions

Seeds of WS, *atx1* (Alvarez-Venegas et al., 2003) and *atx1* mutants complemented with *ATX1* (*35S::ATX1*) were sterilized and kept for 3 days at 4°C in the dark prior to germination in sterile media. For seedling assays, sterile seeds were germinated on half-strength MS medium for 3 days and then transferred to half-strength MS agar medium supplemented with mannitol or plates infused with 1.5 volumes of a 30% solution of polyethylene glycol (Verslues et al., 2006). Plates were incubated at 22°C with a 16-h light photoperiod. For soil dehydration assays, plants were grown at 22°C with a 12-h light photoperiod for 14 days, and were then grown under the same conditions with or without additional water for 12 days. Plants were then watered for 3 days and their recovery measured by color and leaf turgidity.

Transgenic *atx1* plants expressing a full-length cDNA *ATX1* sequence under the 35S promoter were generated for complementation analyses. Two independently transformed lines were used in the assays (*35S::ATX1-1*, *35S::ATX1-2*).

Stomata size assay and ABA treatments

Stomatal aperture size measurements were performed as previously described (Yamaguchi et al., 2007). Briefly, epidermal strips from 3-week-old plants were fixed in a solution (1% glutaraldehyde, 10 mM PIPES, pH 7.0, 5 mM $MgCl_2$ and 5 mM EGTA) for 1 h, and samples were then cleared by an overnight treatment with chloralhydrate. Sixty or more mature stomata of the fixed epidermal strips were examined in each experiment using a confocal microscope (Olympus, http://www.olympusamerica.com/).

For ABA treatments, stomata were opened by exposing the leaves to light and high humidity by floating the leaves on buffer [10 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES), pH 6.5, 50 mM KCl, 0.1 mM $CaCl_2$] for 1.5 h in a light chamber. Stomatal apertures were measured in the same buffer 1.5 h after the addition of ABA using a confocal microscope (Pei et al., 1997). Control experiments were performed in parallel without ABA. The widths of 60 stomata were measured using a micro-ruler in three independent experiments. The pairwise comparisons were calculated using the Student's *t*-test ($P < 0.05$).

Relative water content

Relative water content (RWC) was measured to follow the plant's water status. This value was calculated using the formula: RWC (%) = [(FW – DW)/(TW – DW)] × 100% (Hewlett and Kramer, 1962), where FW is fresh weight, DW is dry weight and TW is turgid weight. Leaves were detached from plants and immediately weighed to determine their FW. The same leaves were submerged in deionized water for 24 h, blotted dry, and weighed to determine their TW. Measurements of DW were taken after the leaves were oven dried (65°C) in brown paper bags for 24 h.

Reverse transcription and real-time PCR

Total RNA isolation and RT-PCR were performed as described previously (Ding et al., 2007), using gene-specific primers (Table S1). Real-time PCR analysis was performed using the cyclerIQ real-time PCR instrument and SYBR Green mixture (Bio-Rad, http://www.bio-rad.com). The relative expression of specific genes was quantitated using the $2^{-\Delta\Delta Ct}$ calculation according to the manufacturer's software (Bio-Rad; Livak and Schmittgen, 2001), where $\Delta\Delta C_t$ is the difference in the threshold cycles, and the reference housekeeping genes were ubiquitin (*AT4G05320*) for expression analyses or relative to input DNA for chromatin immunoprecipitation assays. The mean threshold cycle values for the genes of interest were calculated from three or four replicates.

ABA assay

Leaves were harvested from plants that were watered or air-dried for 1 h. The leaves were ground in liquid N_2 and homogenized in 90% (v/v) methanol containing 200 mg L^{-1} of diethydithiocarbamic acid sodium salt. The extracts were then incubated overnight in a covered, silanized borosilicate tube in darkness at 4°C, followed by a low-speed centrifugation. The methanolic supernatant was recovered and evaporated, and the residue was dissolved by methanolic Tris buffer (10% methanol, 50 mM Tris, pH 8.0, 1 mM $MgCl_2$ and 150 mM NaCl). An ELISA kit was used for the determination of ABA following the manufacturer's instructions (Agdia, http://www. agdia.com).

Chromatin immunoprecipitation assay

A 3-g sample of leaves were fixed with 1% formaldehyde using vacuum infiltration for 10 min, quenched in 0.125 M glycine, and the leaves were ground in a mortar and pestle in liquid nitrogen and filtered through miracloth. After centrifugation, the supernatant was pre-cleared with protein A magnetic beads (Invitrogen, http:// www.invitrogen.com), and specific antibodies or control IgG serum were added for overnight incubation at 4°C. The specific antibodies used were: anti-trimethyl-H3K4 (ab8580; Abcam, http://www. abcam.com), anti-Arabidopsis RNA Pol II (sc-33754; Santa Cruz Biotechnology, http://www.scbt.com) or anti-ATX1 (Alvarez-Venegas et al., 2006b). The antibody–protein–DNA complexes were isolated by binding to protein-A beads. The washed beads were heated at 65°C for 8 h with proteinase K to reverse the formaldehyde cross linking and remove proteins. The immunoprecipitated DNA was then isolated by extraction with phenol/chloroform. Purified DNA was analyzed by real-time PCR with gene-specific primers (Table S1).

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: *ABF2*, *AT1G45249*; *ACT7*, *AT5G09810*; *ADH1*, *AT1G77120*; *ATX1*, *AT2G-31650*; *COR15A*, *AT2G42540*; *NCED3*, *AT3G14440*; *RD29A*, *AT5G52310*; *RD29B*, *AT5G52300*; ubiquitin, *AT4G05320*; *ABF4*, *AT3G19290*; *CBF4*, *AT5G51990*; *ABA1*, *AT5G67030*; *ABA2*, *AT1G52340*; *ABA3*, *AT1G16540*; *RD26*, *AT4G27410*; *LTP*, *AT2G15050*.

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Supporting Information

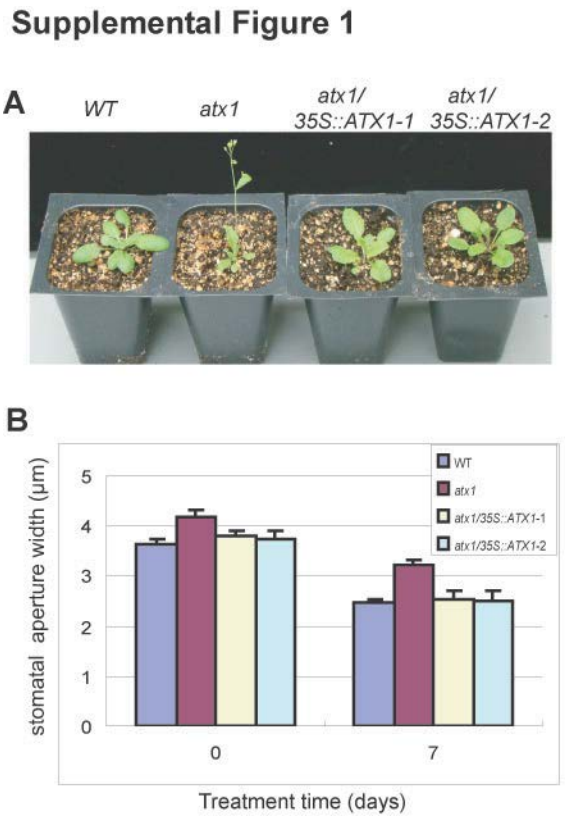

Figure S1. Complementation of *atx1* small-plant, early flowering and stomatal aperture phenotypes. WT, *atx1*, or *atx1* plants transformed with a *35S::ATX1* construct (*35S::ATX1-1* and *35S::ATX1-2*) were assayed for: (A) plant size and early flowering of 25 d old plants; (B) stomatal aperture widths before and after dehydration stress caused by withholding water from plants in soil (means were derived from 60 or more stomatal measurements). Each bar represents the mean + SEM (n = 3 independent experiments).

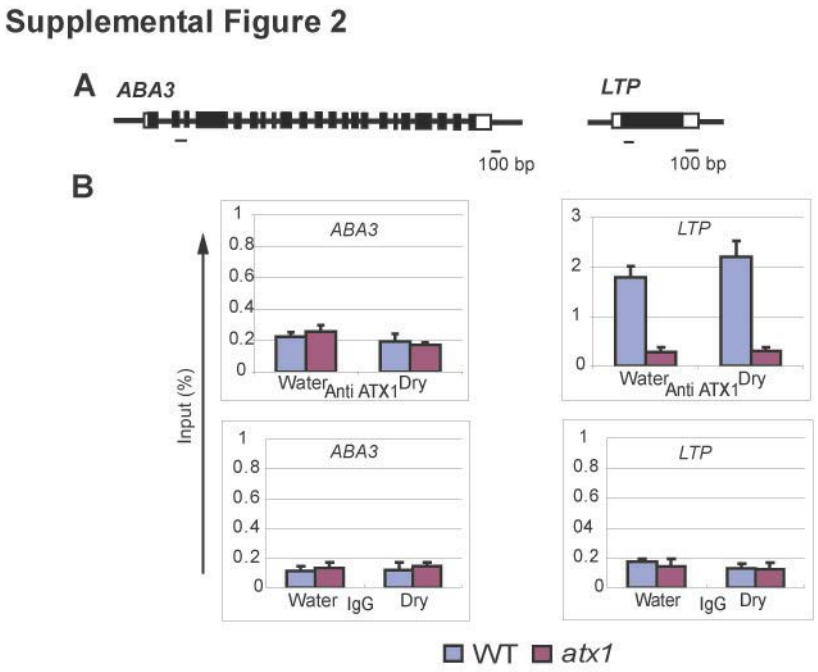

Figure S2. ChIP-PCR assays of ATX1 binding to *ABA3* in wild-type and *atx1* plants. (A) A diagram of *ABA3* and *LTP*, indicating the regions analyzed by ChIP-PCR. (B) The regions of *ABA3* and *LTP* indicated in panel A were analyzed by ChIP-PCR with antibody to ATX1 (Anti-ATX1) or with non-immune IgG (IgG).

Supplemental Figure 3

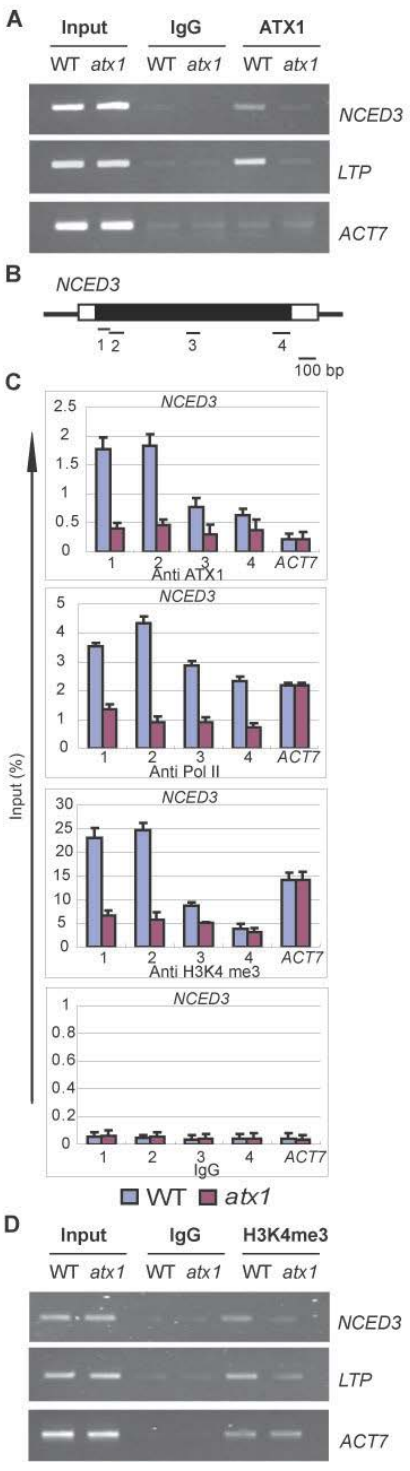

Figure S3. ChIP-PCR assays of ATX1, Pol II and H3K4me3 in wildtype and *atx1* plants. (A) Semi-quantitative PCR of the +300 bp regions of the *NCED3*, *LTP*, or *ACT7* genes was performed on input DNA (Input), or DNA from ChIP with nonimmune IgG serum (IgG), or with ATX1 antibody (ATX1) in WT or *atx1* dehydration-stressed plants. (B) A diagram of the *NCED3* gene indicating the regions analyzed by ChIP-PCR in panel C. The PCR primers used were: region 1 (5′-TCAAGTCGGAGCTTTGAGAA-3′, 5′-GGCTTCTTTCACGGCAAC-3′); region 2 (5′-ATGTTTCATCTGCGCTTCAC-3′, 5′-CGCTCTCTGGAACAAATTCA-3′); region 3 (5′-CTTTGATGGAATCGGGAAGT-3′, 5′-TTTGCTCCGGTGAATGAAC-3′); region 4 (5′-TAGCATGAGTCGGGCAATAC-3′, 5′-GGTTTGTTCAGGAACGTCAA-3′). (C) ChIP-PCR assays of the four regions of NCED3 shown in panel B or of the +300 bp region of ACT7 were performed with antibody to ATX1 (Anti ATX1), Pol II (Anti Pol II), H3K4me3 (Anti H3K4-me3), or with nonimmune IgG (IgG). (D) As in A except antibody to H3K4-me3 was used. Representative experiments are shown and were performed at least three times. Each bar represents the mean + SEM (n = 3).

Supplemental Figure 4

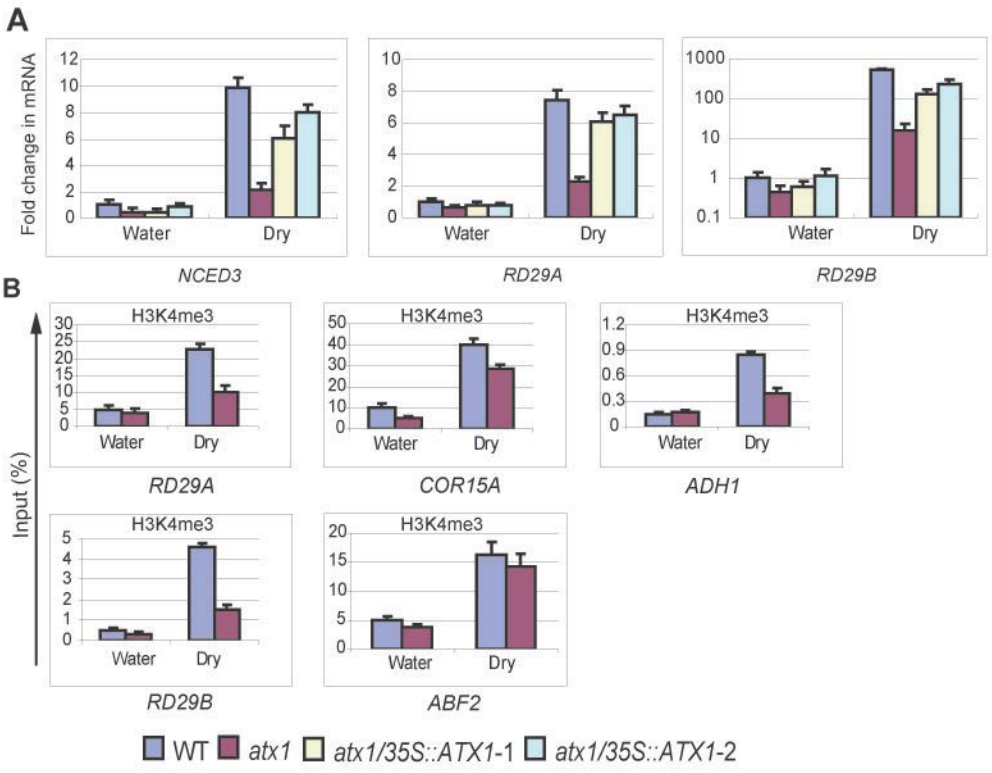

Figure S4. Change in mRNA levels in wild-type, *atx1*, or complemented plants, and H3K4me3 levels in selected genes before and after dehydration stress in wild-type and *atx1* plants. (A) WT, *atx1*, or *atx1* plants transformed with a *35S::ATX1* construct (*35S::ATX1-1* and *35S::ATX1-2*) were assayed for their transcript levels of the indicated genes before (Water) and after (Dry) dehydration stress. (B) H3K4me3 levels of selected ABA dependent (*RD29A*, *RD29B*) or independent genes (*COR15A*, *ADH1*, and *ABF2*) were measured by ChIP-PCR before and after dehydration stress in WT and *atx1* plants. Representative experiments are shown and were performed two times. Each bar represents the mean + SEM ($n = 3$).

Table S1. Primer sequences

RT-PCR primers

ABF2:	(5′- GGGTTGACTAGACAAGGTTCG -3′)
	(5′- ATCCATGTTCATTGACCCAA -3′)
ADH1:	(5′- AACACCGTTGTTTCCACGTA -3′)
	(5′- AACACCGTTGTTTCCACGTA -3′)
COR15A:	(5′- CAGATGGTGAGAAAGCGAAA -3′)
	(5′- CCCTACTTTGTGGCATCCTT -3′)
NCED3:	(5′- ATGTTTCATCTGCGCTTCAC -3′)
	(5′- CGCTCTCTGGAACAAATTCA -3′)
RD29A:	(5′- AGGAACCACCACTCAACACA -3′)
	(5′- ATCTTGCTCATGCTCATTGC -3′)
RD29B:	(5′- ACGAGCAAGACCCAGAAGTT -3′)
	(5′- AGGAACAATCTCCTCCGATG -3′)
Ubiquitin:	(5′- AGGATGGCAGAACTCTTGCT -3′)
	(5′- TCCCAGTCAACGTCTTAACG -3′)
ABF4:	(5′- TAGGAGGTGGTGGTCATCCT -3′)
	(5′- CCGGTCCACCTAGTGTGTT -3′)
CBF4:	(5′- CCGATCATAGATCTCCGGTT -3′)
	(5′- AAATCGGATGACGTGTCTCA -3′)
ABA1:	(5′- AACCCGGAGGAGTATCTGG -3′)
	(5′- AAACACCAATCCTCCGATTC -3′)
ABA2:	(5′- AAACAACGCAAACTCCGATT -3′)
	(5′- TCAATGCCACTTTACCCAAA -3′)
ABA3:	(5′- GGATCATGCTGGTTCTACTTTG -3′)
	(5′- TGTCGAGCATCCGCTATAAG -3′)
RD26:	(5′- AGTTCGATCCTTGGGATTTG -3′)
	(5′- ACCCGTTGCTTTCCAATAAC -3′).

ChIP-PCR primers

LTP:	(5′- ATCACAGCAAAGGCGGCTCTGAGCT -3′)
	(5′- TACGTGTTGCACTTGGTGTTGAACC -3′)
Actin7:	(5′- CGTTTCGCTTTCCTTAGTGTTAGCT -3′)
	(5′- AGCGAACGGATCTAGAGACTCACCTTG -3′)
NCED3:	(5′- ATGTTTCATCTGCGCTTCAC -3′)
	(5′- CGCTCTCTGGAACAAATTCA -3′).
RD29A:	(5′- AGGAACCACCACTCAACACA -3′)
	(5′- ATCTTGCTCATGCTCATTGC -3′)
RD29B:	(5′- ACGAGCAAGACCCAGAAGTT -3′)
	(5′- AGGAACAATCTCCTCCGATG -3′)
COR15A:	(5′- CAGATGGTGAGAAAGCGAAA -3′)
	(5′- CCCTACTTTGTGGCATCCTT -3′)
ABF2:	(5′- GGGTTGACTAGACAAGGTTCG -3′)
	(5′- ATCCATGTTCATTGACCCAA -3′)
ADH1:	(5′- AACACCGTTGTTTCCACGTA -3′)
	(5′- AACACCGTTGTTTCCACGTA -3′)