Osmotic stress induces phosphorylation of histone H3 at threonine 3 in pericentromeric regions of *Arabidopsis thaliana*

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Histone phosphorylation plays key roles in stress-induced transcriptional reprogramming in metazoans but its function(s) in land plants has remained relatively unexplored. Here we report that an *Arabidopsis* mutant defective in *At3g03940* and *At5g18190*, encoding closely related Ser/Thr protein kinases, shows pleiotropic phenotypes including dwarfism and hypersensitivity to osmotic/salt stress. The double mutant has reduced global levels of phosphorylated histone H3 threonine 3 (H3T3ph), which are not enhanced, unlike the response in the wild type, by drought-like treatments. Genome-wide analyses revealed increased H3T3ph, slight enhancement in trimethylated histone H3 lysine 4 (H3K4me3), and a modest decrease in histone H3 occupancy in pericentromeric/knob regions of wild-type plants under osmotic stress. However, despite these changes in heterochromatin, transposons and repeats remained transcriptionally repressed. In contrast, this reorganization of heterochromatin was mostly absent in the double mutant, which exhibited lower H3T3ph levels in pericentromeric regions even under normal environmental conditions. Interestingly, within actively transcribed protein-coding genes, H3T3ph density was minimal in 5′ genic regions, coincidental with a peak of H3K4me3 accumulation. This pattern was not affected in the double mutant, implying the existence of additional H3T3 protein kinases in *Arabidopsis*. Our results suggest that At3g03940 and At5g18190 are involved in the phosphorylation of H3T3 in pericentromeric/knob regions and that this repressive epigenetic mark may be important for maintaining proper heterochromatic organization and, possibly, chromosome function(s).

epigenetics | heterochromatin | histone phosphorylation | abiotic stress | protein kinase

Plants, due to their sessile nature, adopt special strategies to cope with adverse environmental conditions, resulting in complex interactions among signaling pathways and developmental programs that impinge on chromatin organization and transcriptional activity (1–3). A growing body of evidence indicates that changes in DNA methylation and certain posttranslational histone modifications are involved in plant responses to environmental stresses, possibly by modulating the expression of stress responsive genes and/or chromosomal structures (1–6). Environmental cues, especially high temperature, can also transiently influence gene expression or heterochromatin organization via changes in histone variants and/or nucleosome occupancy, without obvious alterations in well-characterized epigenetic marks (2, 3, 7–10).

Histone phosphorylation can occur on serine, threonine, and/or tyrosine residues and is generally induced in response to extracellular signals, DNA damage, or entry into mitosis/meiosis (11–16). Phosphorylation of histone H3 is evolutionarily conserved at several sites including Ser 10 and 28 (H3S10ph and H3S28ph) and Thr 3 and 11 (H3T3ph and H3T11ph) (11–13, 15). In metazoans, a variety of environmental stimuli can trigger histone H3 phosphorylation, both globally and at specific genes in interphase cells (11, 14, 17–19). In plants, dynamic changes in histone H3 phosphorylation were also observed in cultured cells exposed to abiotic stress, such as high salt or low temperature, as well as to abscisic acid treatments (20, 21). Thus, phosphorylation of histone H3 and alterations in chromatin structure are emerging as critical factors in organismal responses to environmental cues, although in many cases the specific molecular mechanisms are not clearly understood.

Histone H3 phosphorylation has also been linked to chromosome structure and segregation based on the cell-cycle–dependent phosphorylation patterns of certain residues, concurrent with mitotic and/or meiotic chromosomal condensation (11–13, 21). The phosphorylation of histone H3 at Thr 3, mainly in mitotic cells, has been recently characterized in a number of organisms (12, 13, 15, 16, 21–23). In mammals, H3T3ph is more prevalent in the centromeric region of metaphase chromosomes and has been implicated in chromosome alignment and centromeric cohesion (12, 13, 16). Indeed, H3T3ph generated by the mitosis-activated Haspin kinase provides a binding site at inner centromeres for Survivin and the recruitment of Aurora B kinase, which is involved in accurate chromosome segregation (12, 13, 16, 24). In plants, however, homologs of Survivin and several subunits of the chromosomal passenger complex have not been found (21, 25) and cytogenetic analyses indicate that H3T3ph is distributed along the entire length

Significance

Histone phosphorylation and chromatin reorganization are emerging as critical factors in eukaryotic responses to environmental stimuli. Phosphorylation of histone H3 at Thr 3 is evolutionarily conserved but its role(s) in interphase cells has remained unexplored. In *Arabidopsis*, we found that H3T3ph increases in pericentromeric regions upon drought-like treatments and it might be required for maintaining proper heterochromatin/chromosome structure. In active genes the distribution of H3T3ph appears to be antagonistic to that of H3K4me3, suggesting that H3T3ph may have a repressive function. Interestingly, H3T3 phosphorylation depends on several protein kinase families, including some that are lineage specific. Understanding the dynamics of H3T3 phosphorylation and the kinases involved in its deposition may provide insights into epigenetic aspects of stress responses.

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of mitotic chromosomes rather than at centromeres (15, 21, 23). Moreover, characterization of *AtHaspin*, encoding the *Arabidopsis* Haspin homolog, suggested that the kinase is essential for embryo development and that H3T3ph might be involved in chromatin condensation (25, 26).

Interestingly, multiple kinase families have been implicated in H3T3 phosphorylation in eukaryotes and several appear to be lineage specific (11–13, 15). We have previously characterized MUT9, a green algae/land plant-specific Ser/Thr protein kinase capable of phosphorylating H3T3 in the alga *Chlamydomonas reinhardtii*, and found that H3T3ph functions as a repressive epigenetic mark (15, 27, 28). In land plants, however, there is scant information on the existence of H3T3 phosphorylation in interphase cells and on its possible involvement in responses to abiotic stress. We describe here the genome-wide distribution of H3T3ph in *Arabidopsis* and changes in this pattern associated with osmotic stress. The characterization of a mutant lacking protein kinases largely responsible for this modification in pericentromeric regions provided novel insights on the potential function(s) of H3T3ph in maintaining heterochromatin organization.

Results

At5g18190 and At3g03940, Homologs of *Chlamydomonas* MUT9, Are Required for *Arabidopsis* Development. We previously demonstrated that the MUT9 Ser/Thr protein kinase is necessary for heritable epigenetic silencing in the alga *Chlamydomonas* (15, 27, 28). To address the role(s) of related protein kinases in land plants, we have now extended our studies to *Arabidopsis*. This model plant encodes four homologs of MUT9 (*SI Appendix*, Fig. S1 *A* and *B*) but homozygous transfer DNA (T-DNA) insertion lines in individual *MUT9*-like kinase genes are largely indistinguishable in their phenotype from the wild type (Fig. 1 *A* and *B*). In contrast, a double mutant, generated by crossing T-DNA insertion lines of the closest paralogs *At5g18190* and *At3g03940* (*SI Appendix*, Fig. S1*A*), exhibited pleiotropic morphological abnormalities and developmental aberrations (Fig. 1*A* and *SI Appendix*, Fig. S2). *At5g18190* and *At3g03940* were named *MUT9-LIKE KINASE1* and *2* (*MLK1* and *2*), respectively; and the double mutant is hereafter abbreviated as *dm*. Mutant plants remained dwarf throughout their life cycle and displayed various defects in reproductive organs (*SI Appendix*, Fig. S2). RT-PCR analyses demonstrated depletion of the corresponding transcripts in each T-DNA insertion mutant and in the *dm* (Fig. 1*C*). To obtain direct evidence that the pleiotropic phenotypes were caused by loss of function of the protein kinase genes, the cDNA of *MLK1* driven by the cauliflower mosaic virus (CaMV) 35S promoter was introduced into the *dm*. Expression of *MLK1* was clearly detected in the transgenic plants, coincidental with successful rescue of the phenotypic defects (Fig. 1*D*). Thus, two highly similar Ser/Thr protein kinases, encoded by *MLK1* and *MLK2*, might function redundantly in important cellular and/or developmental processes in *Arabidopsis*.

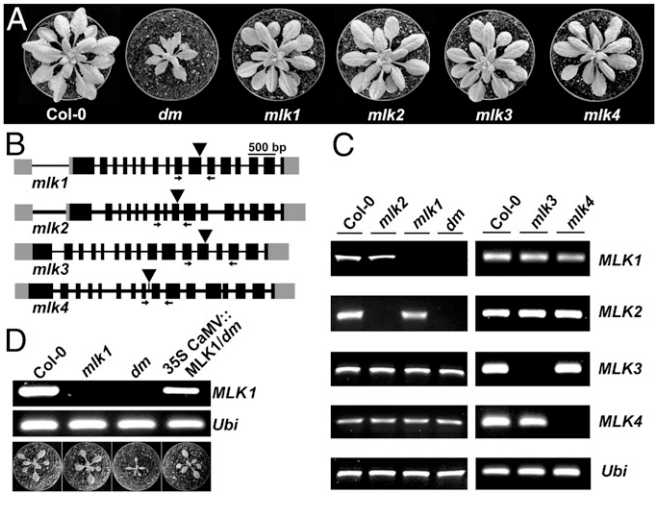

Fig. 1. Characterization of *Arabidopsis* mutants defective in MUT9-like kinases. (*A*) Three-week old plants of T-DNA insertion lines defective in individual *MUT9*-like kinase genes and of the *mlk1 mlk2* double mutant (*dm*). (*B*) Schematic representation of gene structures indicating coding sequences (black boxes) and untranslated regions (gray boxes). T-DNA insertion sites and the locations of RT-PCR primers are indicated by arrowheads and arrows, respectively. (*C*) RT-PCR analysis of transcript abundance in the T-DNA lines. *POLYUBIQUITIN 10* (*Ubi*) was used as an internal standard. (*D*) *MLK1* transcript levels and phenotypes of the indicated strains. A construct harboring the *MLK1* cDNA, driven by the 35S CAMV promoter, was introduced into the *dm* background to rescue the dwarf phenotype.

The *mlk1 mlk2* Double Mutant Is Hypersensitive to Salt/Osmotic Stresses. The *Chlamydomonas mut9* mutant is very sensitive to several abiotic stresses (27, 28). Thus, we examined the response of the *Arabidopsis dm* to drought mimicking conditions. When seedlings were exposed to 30% (wt/vol) PEG or 0.1 M NaCl, all genotypes were affected in their growth relative to seedlings on control plates (Fig. 2*A*). However, *dm* plants experienced greater growth reduction and some did not survive the treatments (Fig. 2*A*). Compared with the single mutants and the wild type, which displayed similar growth defects, the *dm* showed more severe root growth retardation (Fig. 2 *B* and *C*). Likewise, comparison of size-matched plants from the wild type and *dm* grown in soil demonstrated that the latter wilted faster during water deprivation (Fig. 2*D*). Moreover, after rewatering, 40% of the wild-type plants survived but only 10% of the *dm* did. These observations suggested that the MLK1 and MLK2 protein kinases might play a role(s) in the response of *Arabidopsis* to osmotic and salt stresses.

Osmotic Stress Stimulates Global Phosphorylation of Histone H3 at Threonine 3, Which Depends on Functional *MLK1* and *MLK2* Genes. Recombinant MLK1 protein was demonstrated to phosphorylate histone H3 Thr 3 in in vitro assays (*SI Appendix*, Fig. S3*A*). To examine whether the *Arabidopsis* MUT9-like kinases might phosphorylate H3T3 in vivo, as their *Chlamydomonas* homolog (28), immunoblot analyses were performed with a modification-specific antibody (*SI Appendix*, Fig. S3*B*). Interestingly, global levels of H3T3ph were substantially reduced in the *dm* (Fig. 2*E*), although not entirely lost, presumably because of the remaining activity of other kinases in the MUT9 subfamily (*SI Appendix*, Fig. S1*A*).

Given the role of certain posttranslational histone modifications in plant responses to environmental stresses (1–6) and the hypersensitivity of the *Arabidopsis dm* to PEG or NaCl treatments, we also examined whether H3T3ph levels change under drought mimicking conditions. We initially verified that exposure of *Arabidopsis* seedlings to 30% (wt/vol) PEG can mirror the stimulatory effect of drought on stress-responsive genes such as *RD29a*, *RD29b*, and *COR15a* (6, 29) (*SI Appendix*, Fig. S3*C*). Immunoblot analyses revealed that global H3T3ph levels increased in wild-type seedlings exposed to PEG for 4–5 h (Fig. 2*F*). In contrast, in the *dm*, phosphorylated H3T3 remained relatively constant and at lower steady-state levels during PEG treatments (Fig. 2*F*). In addition, we examined whether global changes in H3T3ph correlated with changes in methylation of Lys 4, the adjacent residue in the histone H3 tail. However, mono- or trimethylated histone H3 Lys 4 (H3K4me1 or H3K4me3) did not show conspicuous alterations in their status under PEG treatment, in either wild-type or *dm* seedlings (*SI Appendix*, Fig. S3*D*). These results indicated that increased H3T3ph global levels are part of the *Arabidopsis* response to osmotic stress and that this effect requires functional MLK1 and MLK2 kinases.

Genome-Wide Distribution of H3T3ph, H3K4me1, and H3K4me3. To gain insight into the role(s) of H3T3ph in *Arabidopsis*, its genome-wide distribution and that of H3K4me1 and H3K4me3 were examined, under different environmental conditions, by chromatin immunoprecipitation and deep sequencing (ChIP-Seq). For each treatment, two independent biological replicates were analyzed supporting a high degree of experimental reproducibility with

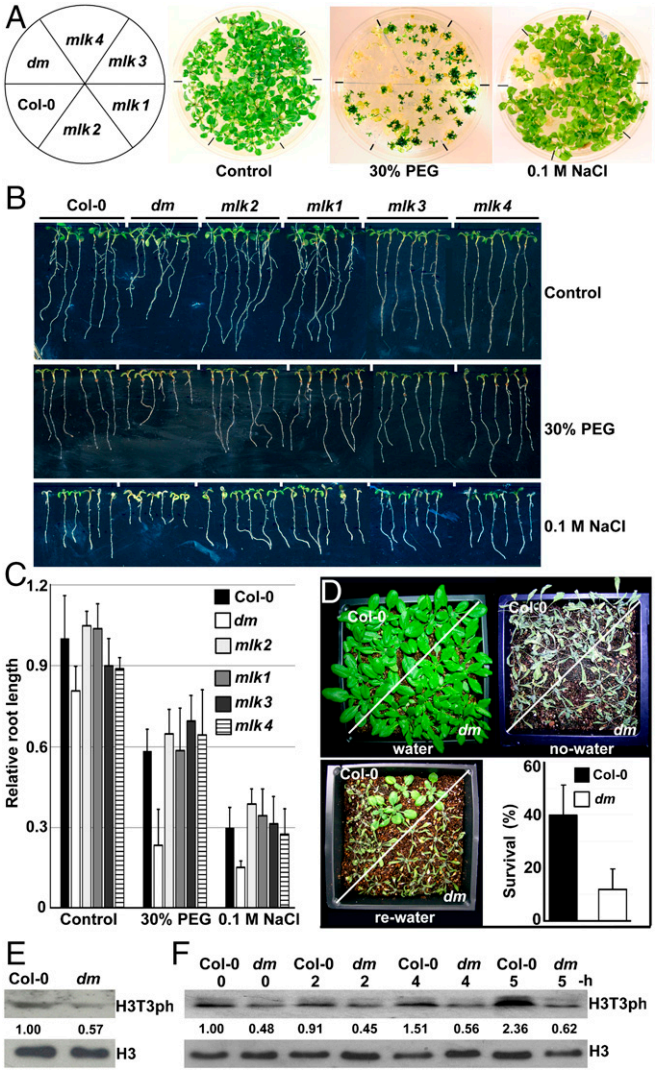

Fig. 2. Plant responses to osmotic/salt stress and H3T3 phosphorylation status. (*A*) Phenotypes of *Arabidopsis* lines subject to osmotic or salt stress. (*B*) Root elongation under the indicated treatments. (*C*) Relative root elongation. Values, normalized to those of Col-0 control, are means ± SD of three independent experiments. (*D*) Drought stress in soil-grown plants. Survival rate was calculated as percentage of plants regaining growth upon rewatering, following 7-d water withdrawal. Values indicate means ± SD of three independent experiments. (*E* and *F*) Immunoblot analyses of H3T3ph levels in plants grown under normal conditions (*E*) or subject to PEG osmotic stress for different periods (*F*). H3T3ph signal intensity normalized to that of histone H3 is indicated.

correlation coefficients between replicates ranging from 0.77 to 0.99 (*SI Appendix*, Table S1). The data for each histone posttranslational modification were normalized to histone H3 levels, to take into account changes in nucleosome occupancy as previously described (30).

On a chromosomal scale, the abundance of both H3K4me1 and H3K4me3 was greater in gene-rich euchromatic regions with relative depletion in transposon/repeat-rich pericentromeric/knob regions (*SI Appendix*, Fig. S4 *A–D*). These H3K4me1 and H3K4me3 distribution profiles were highly consistent with prior results from ChIP-chip experiments (31). Interestingly, in wild-type seedlings exposed to PEG for 5 h, we observed a slight increase in the relative levels of H3K4me3 associated with pericentromeric heterochromatin (*SI Appendix*, Fig. S4*D*). The overall patterns of H3K4me1 and H3K4me3 distribution in the *dm* were very similar to those in the wild type but without clearly discernible changes induced by PEG treatment (*SI Appendix*, Fig. S4 *E–H*).

The abundance of H3T3ph was fairly uniform along entire chromosomal lengths in control seedlings (Fig. 3*A*). Upon exposure to PEG, however, H3T3ph showed relative enrichment in pericentromeric/knob regions (Fig. 3 *B* and *C*), consistent with the global H3T3ph increase detected by immunoblotting (Fig. 2*F*). Intriguingly, this change was accompanied by a slight decrease in histone H3 abundance in pericentromeric/knob heterochromatin, presumably reflecting partial nucleosome depletion triggered by osmotic stress (Fig. 3*D*). These differences were deemed statistically significant by *t* test assays (*SI Appendix*, Table S2). Analysis of H3T3ph and H3K4me3 distribution within mostly pericentromeric transposable elements (TEs) and repeats also supported an overall increase in both epigenetic modifications, along entire element lengths, upon PEG treatment (*SI Appendix*, Figs. S4*I* and S5).

Unlike the wild type, the *dm* showed depletion of H3T3ph relative to histone H3 occupancy in pericentromeric/knob regions under normal conditions (*SI Appendix*, Fig. S6*A*), although the data were noisier, given the overall lower H3T3ph levels in the mutant background (Fig. 2*E*). Moreover, PEG treatment did not affect the H3T3ph profile (*SI Appendix*, Fig. S6 *B* and *C*) or the abundance of histone H3 (*SI Appendix*, Fig. S6*D*) in pericentromeric/knob regions of the *dm*. Consistent with these observations, relative H3T3ph levels within long TEs were somewhat reduced in the mutant (*SI Appendix*, Fig. S6*E*), under normal conditions, compared with those in the wild type (*SI Appendix*, Fig. S4*I*), and exposure to PEG did not change H3T3ph or H3K4me3 abundance (*SI Appendix*, Figs. S5 and S6*E*). In particular, the *dm* showed diminished H3T3 phosphorylation in transposons located in pericentromeric regions, whereas this defect was less pronounced in transposons located near euchromatic regions (Fig. 3*E* and *SI Appendix*, Fig. S7). These findings strongly suggested that the MLK1 and MLK2 kinases might be required for H3T3 phosphorylation in pericentromeric/knob regions and for certain chromatin changes associated with plant responses to osmotic stress.

The *mlk1 mlk2* Double Mutant Exhibits Somewhat Decondensed Chromocenters and Modest Activation of Certain Transposons/Repeats in a DNA Methylation-Independent Manner. Because H3T3 phosphorylation, dependent on MLK1 and MLK2 activities, seemed to be mainly linked to pericentromeric/knob regions, we examined the intracellular localization of MLK1 as well as the heterochromatin organization in nuclei. Notably, an MLK1-GFP fusion protein was detected predominantly associated with chromocenters (Fig. 4*A* and *SI Appendix*, Fig. S1*C*). These structures, consisting of compact heterochromatin containing inactivated transposable elements and tandemly repeated DNA, commonly appear as intensely DAPI-stained foci within nuclei (32). The wild type showed, as expected, compact and brightly stained chromocenters, whereas the *dm* exhibited broader DAPI-stained foci of lower intensity (Fig. 4*B*), suggesting partial decondensation of its pericentromeric/knob heterochromatin. Quantification of the fluorescence of stained nuclei (33) revealed that the *dm* had a lower relative heterochromatic fraction (RHF), about 70% of the wild type (Fig. 4*C*). In addition, when exposed to PEG, the RHF increased to a greater degree in the wild type relative to the *dm* (Fig. 4*C*), possibly as a result of chromocenter aggregation (Fig. 4*B*).

In agreement with a more relaxed heterochromatic structure, minor transcriptional activation of several endogenous loci, including the *TRANSCRIPTIONALLY SILENT INFORMATION* (*TSI*) element, centromeric 180-bp repeats, and the *ATHILA6A* and *Ta3LTR* transposons, was detected in the *dm* under normal conditions (Fig. 4*D*). This activation was slightly enhanced in certain elements by osmotic stress, whereas in the wild type these loci remained repressed (Fig. 4*D*). However, the very modest increase in expression of these repetitive sequences in the *dm* occurred without gross alterations in DNA methylation (*SI Appendix*, Fig. S8). In addition, the abundance of a centromere-specific histone H3 variant (CENH3), required for centromere function and chromosome segregation (34), was reduced in the mutant background (*SI Appendix*, Fig. S9*F*). Interestingly, the *dm* dwarfism and pleiotropic morphological and developmental

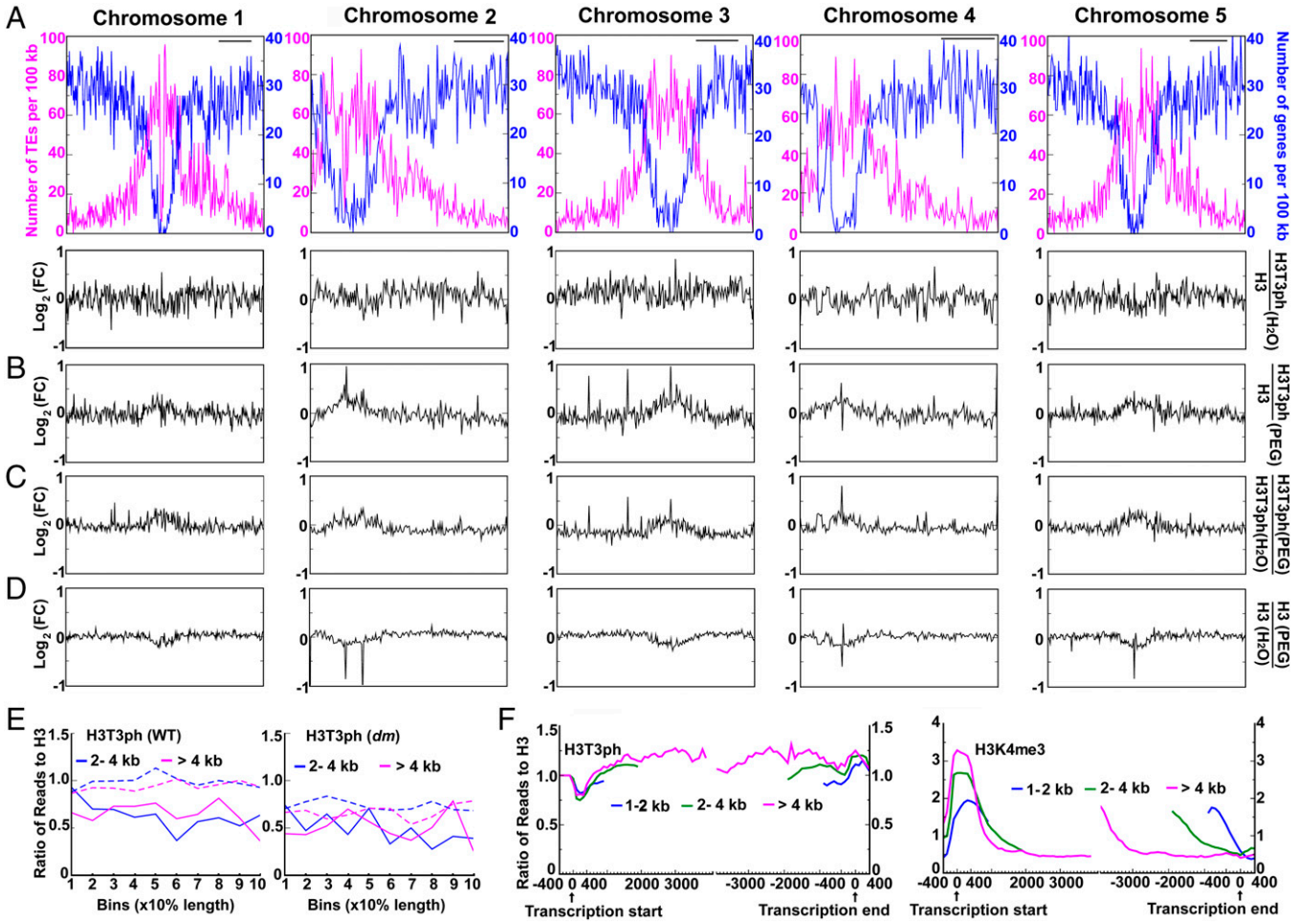

Fig. 3. Genome-wide distribution of H3T3ph in wild-type *Arabidopsis*. (*A*) Chromosomal distribution of genes (blue) and transposable elements (TEs) (pink). (Horizontal scale bar, 5 Mb.) (*Lower*) Chromosomal distribution under normal environmental conditions of H3T3ph normalized to histone H3. The binary logarithm of the fold change (FC), average of two independent experiments, is shown. (*B* and *C*) Chromosomal distribution of normalized H3T3ph in plants grown under the conditions indicated on the right axis. (*D*) Chromosomal distribution of histone H3 in PEG-treated plants relative to that in well-watered controls. (*E*) Distribution of H3T3ph within TEs of different lengths in wild-type and *dm* plants under normal environmental conditions. Solid and dashed lines represent TEs located in gene rich (i.e., chromosomal arms) or pericentromeric regions, respectively. (*F*) Distribution of H3T3ph and H3K4me3 in active protein-coding genes in wild-type plants grown under normal environmental conditions. Genes were aligned at the transcription start sites or the transcription end sites within each length group. The ratio of reads was determined at 100-bp intervals.

abnormalities might result partly from a defect in cell division, rather than in cell expansion, because the size of cells in stems was similar to that in the wild type (*SI Appendix*, Fig. S9 *A–E*). Thus, MLK1 and MLK2 might be involved in maintaining a condensed pericentromeric/knob heterochromatin and correct centromere organization, needed for proper chromosome function(s) and, to a lower degree, for preventing the expression of some transposable elements and repeats under osmotic stress.

H3T3 Phosphorylation in Actively Transcribed Protein-Coding Genes Does Not Depend on the *MLK1* and *MLK2* Genes. The association of H3T3ph with protein coding genes was examined within transcribed regions as well as in 400-bp upstream and downstream regions. The genic distribution of H3T3ph was determined as previously described for H3K4 methylation (6, 31, 35). Consistent with prior findings (6, 35), H3K4me3 levels peaked sharply after the transcription start site of active protein-coding genes and this pattern was particularly obvious in longer genes (Fig. 3*F* and *SI Appendix*, Fig. S10*A*). In contrast, H3T3ph levels were fairly uniform along the entire transcribed region of protein-coding genes but with a clear valley immediately downstream from the transcription start site (Fig. 3*F*).

Interestingly, the pattern and relative levels of H3T3ph associated with protein-coding genes were not affected in the *dm* (*SI Appendix*, Figs. S6*F* and S10*B*). Accordingly, relatively few protein-coding genes were differentially expressed in transcriptomic analyses comparing the *dm* and wild type, although about one-third of these genes corresponded to those annotated as implicated in responses to stress (*SI Appendix*, Fig. S11 and Dataset S1). PEG treatments did not affect H3T3ph profiles within protein-coding genes in either the wild-type or *dm* backgrounds (*SI Appendix*, Fig. S6 *G* and *H*). Thus, MLK1 and MLK2 do not appear to play a major role(s) in the gene-associated H3T3 phosphorylation, which must rely on additional protein kinases, possibly including the other MUT9-like paralogs. Indeed, our results, taken together, suggest that MLK1 and MLK2 catalyze predominantly H3T3 phosphorylation in pericentromeric/knob regions and that H3T3ph is a repressive epigenetic mark, possibly involved in heterochromatin/chromosome organization.

Discussion

The MUT9-like family of Ser/Thr protein kinases, which is specific to the green algae/land plant lineage, appears to play an important role(s) in *Arabidopsis*. The *dm* abnormal phenotypes were highly reminiscent of those of *Arabidopsis* RNAi lines with diminished

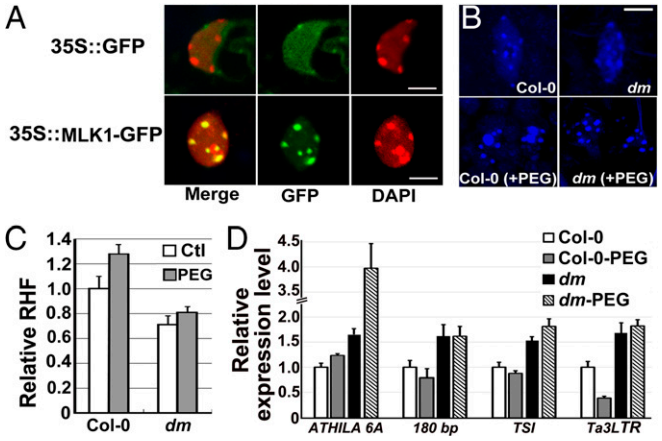

Fig. 4. Localization of the MLK1-GFP protein, heterochromatin organization, and transposon expression. (*A*) Subcellular localization of the MLK1-GFP fusion protein. Pseudocolored images of leaf cells are shown with nuclei indicated by DAPI staining. (Scale bar, 5 μm.) (*B*) Chromocenter organization of nuclei, stained with Hoechst 33342, from leaves of the wild type and *dm*. (Scale bar, 5 μm.) (*C*) The relative heterochromatin fraction (RHF) corresponds to the fluorescence intensity of all chromocenters relative to that of the entire nucleus (47). Fluorescence intensity of nuclei was determined with FociCounter (33). Values shown, normalized to those of Col-0 control, are means ± SD of three independent experiments (n = 50). (*D*) Transcript abundance of the indicated endogenous loci examined by real-time RT-qPCR. Values indicate normalized means ± SD of three independent experiments. *POLYUBIQUITIN 10* was used as the internal standard.

levels of CENH3 (34), and also partly overlapped those of *AtHaspin* RNAi lines (26) and some *CROWDED NUCLEI* mutants (36), a family of proteins involved in nuclear size control and heterochromatin organization. Given that MLK1 and MLK2 are required for proper H3T3 phosphorylation in pericentromeric regions and that this epigenetic mark has been implicated in chromosome segregation and/or condensation (11–13, 16, 21, 25, 26), these members of the MUT9 family might be primarily involved in heterochromatin organization and chromosome function(s) throughout the cell cycle.

Reduction of global H3T3ph levels in the *dm* supports the proposition that MLK1 and MLK2, like their *Chlamydomonas* homolog MUT9 (28), are involved in histone H3T3 phosphorylation in vivo. However, MLK1 and MLK2 depletion did not completely abolish H3T3ph, indicating the existence of other kinases capable of targeting histone H3 at Thr 3 in *Arabidopsis*. In agreement with this interpretation, genome-wide ChIP-Seq analyses revealed similar levels and distribution of H3T3ph associated with transcribed protein-coding genes in the wild-type and *dm* backgrounds. Additionally, this genic H3T3ph pattern was largely unaffected by osmotic stress. Interestingly, the distribution of H3T3ph was fairly uniform along protein-coding genes but with a distinct valley immediately after the transcription start site, coincidental with a well-characterized peak of H3K4me3 accumulation (6, 31, 35). An antagonistic relationship between the relative abundance of H3T3ph and H3K4me3 at several loci was previously observed in *Chlamydomonas* (28) and our findings in *Arabidopsis* also suggest an inverse correlation between H3T3ph and H3K4me3 in expressed genes. In mammals, a "phospho/methyl switch," where phosphorylation of a histone residue may affect the readout of a stable methylation mark in a neighboring residue, has been proposed to operate at the H3T3/K4 site (13, 14, 37, 38). However, H3K4 methylation by the mixed-lineage leukemia 1 protein in vitro is strongly reduced by H3T3ph (39). Conversely, the activity of Haspin and AtHaspin is inhibited by H3K4me3 (40, 41). Our observations in *Arabidopsis* are more consistent with antagonistic deposition of H3K4me3 and H3T3ph in expressed protein-coding genes and, by inference, that H3T3ph may function as a repressive epigenetic mark as demonstrated in *Chlamydomonas* (28).

Changes in DNA methylation, several posttranslational histone modifications, as well as a linker histone H1 variant have been implicated in plant responses to osmotic/salt stress (2, 3, 6, 10, 20, 21, 42–45), but in most cases the relationship among chromatin status, transcriptional responsiveness, and physiological outcomes is not clearly understood. Interestingly, in both metazoans and plants, histone phosphorylation appears to be involved in responses to osmotic/salt stress (3, 17, 19–21). In *Arabidopsis* the expression of most histone genes is down-regulated by dehydration conditions (43) and exposure of tobacco BY-2 cells to sucrose or NaCl resulted in induction of H3T3 phosphorylation (21). In the pericentromeric/knob regions of *Arabidopsis* wild-type seedlings subject to PEG treatment, we observed an increase in H3T3ph, slight enhancement in H3K4me3, and a modest decrease in histone H3 abundance, presumably reflecting nucleosome depletion. At the same time, the relative heterochromatic fraction in nuclei increased, possibly involving aggregation of chromocenters in contracted nuclei, whereas pericentromeric transposons and repeats remained largely repressed. In contrast, the *dm* showed no alteration in the examined chromatin features of the pericentromeric/knob regions, whereas several transposable elements and repeats displayed very modest transcriptional activation. Moreover, even under normal environmental conditions, the *dm* exhibited depletion of H3T3ph from pericentromeric/knob heterochromatin. These results, taken together with the prevalent localization of the MLK1-GFP fusion protein to chromocenters, strongly suggested that the MLK1 and MLK2 Ser/Thr protein kinases are mainly involved in the phosphorylation of H3T3 in pericentromeric/knob regions. H3T3ph and/or other events depending on MLK1 and MLK2 may be important for maintaining proper heterochromatin and centromere organization, required for normal chromosome segregation and cell division.

Our findings indicate, as previously reported (1–3, 7, 8, 42–44), that chromatin reorganization in plants is not restricted to developmental needs but is also a response to environmental stress. Among abiotic stress factors, prolonged exposure to heat causes nucleosome depletion and transient heterochromatin decondensation associated with transposon activation (1–3, 7, 8). Similarly, under osmotic stress *Arabidopsis* pericentromeric/knob heterochromatin appears to undergo partial nucleosome loss and a slight increase in H3K4me3, suggestive of transcriptional activation of the underlying DNA sequences. However, the heterochromatin fraction of the nuclei actually gets larger and transposons are not activated under these conditions. These changes are accompanied by increased H3T3ph levels in pericentromeric/knob regions and we speculate that this modification helps maintaining a condensed heterochromatic structure and proper chromosome organization. Moreover, this reorganization of heterochromatin was largely absent in the *dm*, indicating the need of functional MLK1 and MLK2 kinases for these processes. Numerous factors have already been implicated in epigenetic regulation in plants and the challenge ahead will be to define the precise function of H3T3ph, its relationship to other epigenetic mechanisms, and the multiple kinases responsible for this modification.

Materials and Methods

Plant Materials and Growth Conditions. Three T-DNA insertion SALK lines [*MLK3* (*At2g25760*): SALK-017102; *MLK2* (*At3g03940*): SALK-035080; and *MLK1* (*At5g18190*): SALK-002211] were obtained from the *Arabidopsis* Biological Resource Center. The remaining T-DNA insertion line [*MLK4* (*At3g13670*): GK-756G08] was obtained from GABI-Kat. Plants were grown under long-day conditions (16-h light/8-h dark). Specific growing conditions and plant treatments are described in *SI Appendix, SI Materials and Methods*.

Transgenic Constructs and Plant Transformation. The ORF of *MLK1* was amplified from cDNA (reverse transcribed from total RNA) with two sets of primers (*SI Appendix*, Table S3) using Phusion DNA Polymerase (NEB). One ORF was cloned into pSTBlue-1 (Novagen) and, after sequence verification, ligated into the BamHI and KpnI sites of pROK2 for complementation

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experiments. The other ORF was cloned into pENTR/D-TOPO (Life Technologies) and then transferred into pK7FWG2.0, to generate a GFP fusion, using Gateway LR Clonase II Enzyme Mix (Life Technologies). Transgenic *Arabidopsis* plants were generated by Agrobacterium-mediated transformation using the floral dip method.

Cytological Analyses. For the localization of the GFP fusion protein, leaves of transgenic plants were fixed in 4% paraformaldehyde and stained with Vectashield Mounting Medium containing DAPI (VECTOR Laboratories). Sequential fluorescence images were obtained on a Nikon 90 microscope using a Nikon A1 filter with NIS-Elements 4.20. For chromocenter analyses, samples were prepared as previously described (46). Images were captured with a confocal laser scanning microscope (Olympus).

Immunoblot Analyses. Nuclear proteins from plant leaves were isolated as reported (6) and separated by polyacrylamide gel electrophoresis. Histone posttranslational modifications were examined with specific antibodies against H3K4me1 (Abcam, ab8895), H3K4me3 (Abcam, ab8580), or H3T3ph (Upstate, 07–424). Antibody specificity was tested with a panel of peptides containing the modifications of interest (*SI Appendix*, Fig. S3*B*). Histone H3 was examined with a modification insensitive antibody (Abcam, ab1791). *Arabidopsis* CENH3 was analyzed with an antibody raised against its N-terminal domain (Agrisera, AS12 2226).

ChIP-Seq. After being transferred to control plates or to 30% (wt/vol) PEG plates for 5 h, plants were harvested and immediately cross-linked with 1% formaldehyde (6). Isolated chromatin was sheared by sonication and immunoprecipitated, as described (6), with the antibodies listed above. ChIP-Seq librar-ies were prepared according to Illumina's protocol and sequenced using a Genome Analyzer IIx (Illumina).

Computational Methods for Sequence Data Analysis. The primary analysis of the Illumina Genome Analyzer IIx output was performed as described (6). Analysis of the sequenced reads and their distribution along chromosomes and within genes and TEs was carried out as previously reported (31, 35). For detailed information see *SI Appendix, SI Materials and Methods*.

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