RESEARCH ARTICLE
Dual Role of the Histone Variant H2A.Z in Transcriptional Regulation of Stress-Response Genes

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Short title: Changes of H2A.Z levels in stress response
One-sentence summary: H2A.Z is removed from nucleosomes localized in genes upon transcriptional activation in response to drought stress conditions in Arabidopsis thaliana.

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ABSTRACT
The influence of the histone variant H2A.Z on transcription remains a long-standing conundrum. Here, by analyzing the actin-related protein6 mutant, which is impaired in H2A.Z deposition, and by H2A.Z profiling in stress conditions, we investigated the impact of this histone variant on gene expression in Arabidopsis thaliana. We demonstrate that the arp6 mutant exhibits anomalies in response to osmotic stress. Indeed, stress-responsive genes are overrepresented amongst those hyperactive in arp6. In wild-type plants, these genes exhibit high levels of H2A.Z in the gene body. Furthermore, we observed that in drought-responsive genes, levels of H2A.Z in the gene body correlate with transcript levels. H2A.Z occupancy, but not distribution, changes in parallel with transcriptional changes. In particular, we observed H2A.Z loss upon transcriptional activation and H2A.Z gain upon repression. This data suggest that H2A.Z has a repressive role in transcription and counteracts unwanted expression in non-inductive conditions. However, reduced activity of some genes in arp6 is associated with distinct behavior of H2A.Z at their +1 nucleosome, which exemplifies the requirement of this histone for transcription. Our data support a model where H2A.Z in gene bodies has a strong repressive effect on transcription, whereas in +1 nucleosomes it is important for maintaining the activity of some genes.

INTRODUCTION
The organization of chromatin has profound implications for the regulation of gene expression in diverse biological processes, including genome stability, recombination, developmental reprogramming and response to external stimuli (Feng et al., 2010; Soria et al., 2012; Zhu et al., 2013). The last of these processes is especially important for
terrestrial plants, which as sessile organisms are inevitably exposed to daily and seasonal environmental changes (Kim et al., 2015). Plants respond to environmental stimuli by activation of signaling pathways that rapidly modify transcription rate of responsive genes and trigger physiological reactions. Thousands of genes are involved in the response; therefore global genome regulation at the chromatin level is required to achieve the appropriate level of responsiveness (Zhu et al., 2011; Probst and Mittelsten Scheid, 2015). This may be achieved by nucleosome remodeling and repositioning via covalent modifications of histone proteins, especially trimethylation of histone H3 lysine 4 (H3K4me3) (van Dijk et al., 2010; Kim et al., 2008) and redistribution of histone variants. Recent studies also suggest an important role for the H2A.Z histone variant in this process (Coleman-Derr and Zilberman, 2012).

H2A.Z is a conserved variant of histone H2A that has been implicated in different processes such as transcriptional regulation, telomeric silencing, genome stability, cell cycle progression, DNA repair and recombination (Zlatanova and Thakar, 2008). The distribution of this histone variant is conserved among eukaryotes. H2A.Z is highly enriched at the transcription start site (TSS) of a large set of genes across cell types, consistent with a role in the regulation of transcription. Genome-wide studies in yeast have shown that H2A.Z enrichment at promoter-distal nucleosomes is required for initiation of transcription, whilst being inversely correlated with transcript levels (Millar et al., 2006; Zhang et al., 2005; Guillemette et al., 2005; Rhee et al., 2014). In Drosophila, the +1 nucleosome blocks RNA polymerase II transit, causing its increased stalling and backtracking at the promoters (Weber et al., 2014). Importantly, H2A.Z deposition at the +1 nucleosomes decreases RNA polymerase II (RNAPII) stalling, suggesting that its presence reduces the energy required for polymerase progression. H2A.Z levels negatively correlate with H3-H4 nucleosome turnover indicating that the nucleosomal H2A.Z facilitates RNAPII elongation without depletion of (H3-H4)_2 tetramers.

In Arabidopsis thaliana there are three functional genes encoding H2A.Z, HISTONE H2A 8 (HTA8), HTA9, and HTA11 (March-Díaz and Reyes, 2009). Both double and triple mutants are viable, although the last one shows severe developmental changes (Coleman-Derr and Zilberman, 2012). Deposition of H2A.Z into nucleosomes is carried out by the specific chromatin remodeling complex SWI2/SNF2-Related 1
(SWR1). It has been shown that eliminating genes encoding key subunits of this complex, *ACTIN-RELATED PROTEIN 6* (ARP6, Choi et al., 2005; Deal et al., 2005) or *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1* (PIE1, Noh and Amasino, 2003), results in inability to efficiently incorporate H2A.Z into nucleosomes and phenocopies the double mutant *hta9 hta11* (March-Díaz et al., 2008), but not the triple mutant *hta8 hta9 hta11* (Coleman-Derr and Zilberman, 2012). This may suggest that in the absence of a functional SWR1 complex, other mechanisms can occasionally incorporate H2A.Z into chromatin (Coleman-Derr and Zilberman, 2012; Hardy et al., 2009). However, as no other biological function of SWR1 has been identified, both *arp6* and *pie1* mutants have been widely used to study the effects of H2A.Z depletion from chromatin (Choi et al., 2013; Kumar and Wigge, 2010; Smith et al., 2010; Bieluszewski et al., 2015; Zilberman et al., 2008; Rosa et al., 2013).

Although the involvement of H2A.Z in the regulation of gene transcription has been extensively investigated, how H2A.Z influences gene expression in a physiological context-dependent manner has remained elusive. In this work we present data suggesting that H2A.Z may have different effects on gene expression depending on its nucleosomal location within the gene body. At the +1 nucleosomes, H2A.Z may be required for transcriptional activity, likely by reducing the energy required by RNAPII to overcome the first nucleosomal barrier. This is consistent with previous results in *Drosophila* (Weber et al., 2014; Rhee and Pugh, 2012). On the other hand, H2A.Z has negative influence on transcription in nucleosomes located across gene bodies. Upon drought stress, H2A.Z is removed from induced genes independently of its location in the gene.

**RESULTS**

**Plants deficient in nucleosomal H2A.Z show distorted germination rate in osmotic stress conditions**

The H2A.Z variant is significantly enriched across gene bodies of responsive genes suggesting a role in their transcriptional regulation (Coleman-Derr and Zilberman, 2012). To test this hypothesis, we sought to investigate whether H2A.Z-deficient mutants
present an abnormal response to osmotic stresses. Delayed germination was observed for hta9 hta11, arp6, and pie1 mutants under control conditions suggesting that nucleosomal H2A.Z-deficiency may affect seed germination (Fig. 1A, D; Supplemental Table 1). Interestingly, we observed that the effect was stronger in the pie1-5 mutant than in hta9 hta11 and arp6 (Fig. 1A, D). When NaCl-supplemented medium was used for germination (150 mM NaCl), the difference between arp6, pie1-5 and wild-type (wt) plants was enhanced significantly (Fig. 1B, E; see Supplemental Table 1 for statistical analysis). In the arp6 and wt comparison, we observed the highest difference at 96 hours after stratification. For pie1-5, the highest difference was observed 144 hours (radicle tip emergence) or 168 hours (green cotyledon emergence) after stratification (Fig. 1B, E). In these conditions the difference between pie1-5 and arp6 was also increased. Surprisingly, the differences were not significant for radicle tip emergence in the hta9 hta11 double mutant, which developed green cotyledons faster than wt during the first 96 hours after stratification (Fig. 1B, E; Supplemental Table 1). Next, we tested the effect of sorbitol-supplemented media on plant germination. We observed a similar delay effect of this treatment on seed germination rate, which was highest between 72 and 96 hours (radicle tips emergence) or 96 and 120 hours (green cotyledon emergence) after stratification for all three mutants (Fig. 1C, F; Supplemental Table 1). The double mutant hta9 hta11 exhibits a relatively mild phenotype (Fig. 1). It should be noted that other phenotypic differences observed between mutants and wt were also reported to be smaller in hta9 hta11 than in arp6 and pie1 (March-Díaz et al., 2007; Deal et al., 2005; Choi et al., 2005; March-Díaz and Reyes, 2009). This indicates that hta9 hta11 is less representative of H2A.Z deposition deficiency than the other two, which is not surprising as the third H2A.Z-encoding gene, HTA8, remains fully functional in this line (March-Díaz et al., 2008). Interestingly, in the case of the pie1-5 mutant, the plants did not reach the wt germination rate defined as the radicle tip emergence percentage after 10 days (Supplemental Table 1). The difference observed between arp6 and pie1-5 phenotypes may be due to potential other functions of the PIE1 protein, which has been previously suggested (Jarillo and Piñeiro, 2015; Coleman-Derr and Zilberman, 2012; Lu et al., 2009; Berriri et al., 2016). For this reason, the arp6 mutant
was used for further analyses. Collectively, our data indicate that H2A.Z deposition-deficient mutants show higher sensitivity to osmotic stress.

**Transcriptional changes in response to drought stress in wt and arp6 mutant**

To further investigate the impact of H2A.Z deficiency on the plant response to osmotic stress we pursued drought stress, as severe drought changes the transcriptional activity of a large number of genes. As we believed that H2A.Z has a direct effect on gene transcriptional regulation, this would provide us with an opportunity to test, on a large scale, whether H2A.Z changes its distribution along the responsive genes during their activation or repression. The *arp6* and wt plants did not show any obvious differences in sensitivity to drought; however significant changes were observed at the level of gene expression.

First, we compared the transcriptional changes in response to drought stress between *arp6* and wt plants. For clarity, genes that exhibit changes in transcript level in response to drought will be referred to as down- and upregulated, whereas genes that exhibit changes in transcript level in *arp6* relative to wt will be referred to as hypo- and hyperactive. To minimize any potential secondary effects due to the early flowering phenotype of the *arp6* mutant, we analyzed plants grown in short days (SD) for 30 days before the onset of drought conditions (*arp6* plants flower in SD photoperiod at 50 days from germination; Deal et al. 2005). Relative water content (RWC) was used as an indicator of stress progression and after 7 days without watering RWC of plants exposed to drought was ca. 55% for *arp6* and wt mutants and ca. 85% for both genotypes grown in control conditions (Supplemental Fig. 1A). In control conditions, we identified 1235 differentially expressed genes between *arp6* and wt using an estimated false discovery rate (FDR) of 5% and threshold log$_2$ (*arp6/wt*) > 0.5. Of these, 542 genes were hypoactive and 693 genes were hyperactive in *arp6* (Supplemental Data Set 1A, Supplemental Fig. 1B). A similar number of 1246 genes misregulated in *arp6* were detected in stress conditions: 610 genes were hypoactive and 636 were hyperactive in comparison to wt plants (Supplemental Data Set 1B Supplemental Fig. 1C).

Analysis of the gene ontology (GO) term for biological function revealed that both in control and stress conditions the genes hypoactive in *arp6* were significantly enriched
for DNA metabolic process ($P < 2.3E-04$ and $4.4E-05$ for control and stress, respectively). Moreover, Nitrogen compound metabolic process genes were enriched in stress conditions ($P < 7.8E-04$) and Response to DNA damage stimulus were enriched in control conditions ($P < 2.79E-05$) (Supplemental Data Set 2A and B). The enrichment for these classes is indicative of the role of H2A.Z in the double-strand break repair process, which has been previously reported (Van et al., 2015; Xu et al., 2012).

Analysis of genes hyperactive in arp6 when compared to wt under drought conditions (636 genes) did not reveal any specific GO classes. This is in accordance with previous reports of the global effect of H2A.Z on gene expression. The germination assay and the transcriptome analysis were performed in different experimental setups; therefore it is difficult to speculate whether the differences observed in germination upon osmotic stress result from distorted expression of single responsive genes. As we observed differences in control conditions between wt and arp6 mutant plants in both germination assays and the transcriptomic experiment, it is also possible that stress conditions additionally enhance distortion in seed germination already present in control conditions. By contrast, a similar comparison for control conditions (693 genes) showed overrepresentation of genes involved in Response to stimulus ($P < 2.12E-09$), particularly Response to chemical stimulus ($P < 1.02E-06$) (Supplemental Data Set 2C). This result suggests that ARP6 acts as a repressor of stress-responsive genes in control conditions.

In addition, we analyzed the expression of 27 genes selected from those identified by RNA-seq as hyperactive, hypoactive, or unaffected in our arp6 mutant (arp6suf3; Choi et al., 2005) in another arp6 allele (arp6-1; Deal et al., 2005) and in the pie1-5 mutant (Noh and Amasino, 2003). This analysis showed that all three mutant lines behave similarly when compared to wt plants (Supplemental Fig. 2). This confirms that the arp6 allele used in this work is representative of other mutants affecting SWR1C-dependent deposition of H2A.Z.

Construction of H2A.Z-tagged lines and analysis of gene expression in plants exposed to water deficit
To investigate H2A.Z distribution and occupancy in drought stress, we adapted the in vivo biotinylation system (Mito et al., 2005). We generated transgenic lines with Arabidopsis H2A.Z histone tagged with a peptide specifically recognized by BirA ligase (BLRP, biotin ligase recognition peptide) and expressing BirA. A line expressing BirA, transformed with an empty vector with BLRP sequence, was used as a control in further analyses. We purified biotinylated chromatin from rosette leaves following digestion with microccocal nuclease (MNase) to mostly mononucleosomes. Comparison of H2A.Z deposition in those lines with published data on H2A.Z distribution in FLOWERING LOCUS C (Deal et al., 2007) confirmed that our tagged H2A.Z histone is deposited at the same sites as the native protein (Supplementary Fig. 3A). We also performed a complementation test by crossing one of our lines with an hta9-1 hta11-1 mutant plant and observed restoration of wild-type flowering phenotype (Supplemental Fig. 3B, C). This shows that the tagged H2A.Z is functional and may at least partially complement the native protein.

The 4-week old H2A.Z-tagged plants were subjected to water deficit stress (see Materials and Methods). In this experiment, water content reached 45-55% in treated plants and ca. 85% in control plants (Supplemental Fig. 4). Rosette leaves were used to isolate chromatin for investigating nucleosome positioning (MNase-seq) and for affinity purification (ChAP) of H2A.Z bound DNA followed by high throughput sequencing (ChAP-seq; see below). To evaluate transcriptome changes in response to drought stress we used the same plant material for RNA isolation. Genome-wide transcriptional changes were analyzed by RNA-sequencing. This resulted in identification of 3344 genes, for which we observed at least two-fold change in transcription level when compared to control conditions. Of these, 2068 genes were downregulated, and 1276 genes were upregulated (Supplemental Data Set 3). As expected, the most overrepresented GO class for drought-induced genes was Response to stimulus ($P < 2.9E-24$), which includes Response to chemical stimulus ($P < 6.77E-23$), abiotic stimulus ($P < 1.09E-22$), stress ($P < 1.07E-21$) and endogenous stimulus ($P < 2.16E-10$) (Supplemental Data Set 4A). Among downregulated genes, the most overrepresented GO classes for Biological Processes included Photosynthesis ($P < 1.14E-16$) and a number of other photosynthesis-related GO classes, Metabolic process ($P < 3.2E-10$)
connected primarily to pigment synthesis, and starch metabolism, *Cell surface receptor-linked signaling pathway* \( (P < 2.75\text{E-09}) \), and *Response to abiotic stimulus* \( (P < 5.14\text{E-06}) \) (Supplemental Data Set 4B).

The level of H2A.Z enrichment in gene bodies correlates with the fold of transcriptional response to drought-stress

Previous work suggested that H2A.Z enrichment across gene bodies, rather than at the TSS, is correlated with higher measures of gene responsiveness (Coleman-Derr and Zilberman, 2012). However, the authors examined H2A.Z enrichment in control conditions, while applying measures of gene responsiveness from various published data gathered from different experimental conditions (e.g. different tissues). Therefore it was not possible to test this relationship in detail and propose the actual role of H2A.Z in control of gene responsiveness. Our H2A.Z-ChAP and RNA-seq data obtained from both control and stress conditions allow us to analyze this relationship more specifically. For the purpose of this work, we defined gene drought-responsiveness as transcription fold change between control and drought stress conditions.

First, genes up- and downregulated in drought conditions were divided into subclasses according to the change in their expression \( (\log_2 \text{ fold-change; from 1.5 to 3, } n=644, \text{ from 3 to 5, } n=358, \text{ and } >5, \ n=291 \text{ for upregulated, from -3 to -1.5, } n=896, \text{ from -5 to -3, } n=722, \text{ and } <-5 \ n=463 \text{ for downregulated}) \). We observed that levels of H2A.Z positively correlate with gene responsiveness for both up- and downregulated gene groups along gene bodies, but not for TSS (Fig. 2A-C; Supplemental Fig. 5). Furthermore, we applied a method of Zilberman et al. (2007): For each expressed gene in which H2A.Z was detected \( (n=16,332) \), we calculated 'H2A.Z enrichment scores' by summing all H2A.Z enrichment values. This was done independently for TSS (positioned 50 to 180 bp downstream from TSS), transcription termination site (TTS, positioned 360 to 230 bp upstream from TTS), and exon start site (ESS, positioned 40 to 170 bp downstream from ESS; mean H2A.Z enrichment was used when a gene consisted of more than one exon). We grouped all H2A.Z-containing genes into percentiles based on their responsiveness calculated as a change in expression level (absolute values of \( \log_2 \) fold-change in mRNA abundance between control and drought). Next, we calculated a
'total H2A.Z enrichment score' by summing scores for all of the genes in each percentile. This was further used to calculate Spearman's rank correlation coefficient. We observed that levels of H2A.Z correlate with the gene responsiveness in drought stress across gene bodies ($\rho = 0.852$ and $0.673$; $P = 2.29E-29$ and $1.68E-14$ for TTS and ESS, respectively) (Fig. 2D). On the other hand, we observed a weak negative correlation for TSS ($\rho = -0.539$, $P = 7.01E-9$) (Fig. 2D). This supports our hypothesis that H2A.Z enrichment significantly influences gene responsiveness via regulation of transcription elongation and termination, but not initiation. H2A.Z enrichment at TSS appears to play a different role in gene activation, likely by involvement in poising genes for transcription initiation (Zhang et al., 2005).

Importantly, when a 'H2A.Z enrichment score' vs. expression level in control conditions was calculated, weak negative correlations were observed (Spearman $\rho = -0.401$, -0.446 and -0.492, $P = 3.61E-5$, 3.29E-6 and 2.01E-7 for TSS, ESS and TTS, respectively) (Fig. 2E). Together, these data confirm that H2A.Z levels are relatively stable, permanent features of genes and have a repressive influence on gene transcription.

**Genes induced and repressed in stress differ in their chromatin structure**

To investigate potential differences between genes upregulated and downregulated in stress, we grouped all of the Arabidopsis genes based on our RNA-seq results from the water deficit experiment into three categories: 'Upregulated in drought' ($n=1276$), which have increased expression levels ($\log_2$ fold change $> 2$), 'Downregulated in drought' ($n=2068$), which have reduced expression levels ($\log_2$ fold change $< -2$), and 'All expressed' ($n=15,558$), which provide a control set. Having defined these three groups of genes, we examined their H2A.Z pattern in the data obtained in control conditions (normal watering). As expected, we observed more H2A.Z across the gene bodies of up- and downregulated genes in comparison to 'All expressed' genes (Fig. 3A). Surprisingly, we detected differences in H2A.Z level in the +1 nucleosome between the three groups of genes. The genes 'Upregulated in drought' have significantly lower H2A.Z occupancy in the +1 nucleosome than 'Downregulated in drought' ($P=7.98E-13$, Mann-Whitney Test) and 'All expressed' ($P=8.36E-12$, Mann-Whitney Test) (Fig. 3A). This is likely due
to the fact that the +1 nucleosome is more "fuzzy" in this gene group than in the other
two groups (Fig. 3C, D). Moreover, we observed broader nucleosome-depleted regions
(NDRs; a valley on plots on Fig. 3C and D directly before a +1 nucleosome peak) in
genes 'Upregulated in stress' (green lines) when compared to genes 'Downregulated in
drought' and 'All expressed' (red and blue lines, respectively, on Fig. 3C, D). This might
suggest that NDRs of genes induced in stress conditions have different chromatin
structure than corresponding regions in constitutively active and stress-repressed genes.

Transcriptional response of drought-induced genes is associated with reduction
of H2A.Z occupancy across their bodies

Our data collected from both control and stress conditions provided an opportunity to
investigate how H2A.Z levels change in relation to modification of gene transcription
activity. We answer this question using several approaches. First, we carried out
differential binding analysis to identify loci exhibiting gain or loss of H2A.Z during drought
stress. We identified 701 chromosomal regions that show a reduction in H2A.Z
occupancy upon stress, and 359 regions showing an increase in H2A.Z occupancy
(Supplemental Data Set 5A and 5B, respectively). Those regions corresponded to 512
and 329 genes, respectively, for which we performed GO classification. The GO
overrepresented classes for genes showing reduction of H2A.Z levels in drought
occupied well to classification of genes induced in drought stress (Supplemental
Data Set 6). GO analysis of genes corresponding to regions that increase H2A.Z levels
upon drought did not produce a significant category, which is likely due to the smaller
size of this group. This result suggests that H2A.Z is removed from nucleosomes during
transcriptional activation.

Next, we analyzed changes in H2A.Z profiles in response to stress for genes
upregulated and downregulated in drought, and compared them to 'All expressed'. When
the profile of H2A.Z enrichment in the 'All expressed' group was analyzed, no significant
difference between control and stress conditions was observed (Supplemental Figs 6A
and 7). Genes upregulated in stress showed a striking reduction in H2A.Z occupancy in
stress conditions at the TSS, especially at the +1 position (Fig. 3A, B, E, G)
Supplemental Figs 6B, D and 7). Genes downregulated presented a less evident
change in H2A.Z occupancy at the +1 nucleosome (Fig. 3A, B, E, G; Supplemental Figs 6C and 7). Across the gene body, genes 'Upregulated in stress' lost H2A.Z in drought conditions, whilst an opposite but less evident trend was observed for downregulated genes (Fig. 3A, B, E; Supplemental Figs 6B, D and 7). This was further confirmed via ChAP followed by quantitative PCR (ChAP-qPCR) on selected genes in an independent drought-stress experiment (Supplemental Fig. 8). These experiments provided evidence for a statistically significant reduction in H2A.Z occupancy in upregulated genes, however we failed to confirm that a significant gain of this histone variant occurs in repressed genes.

Finally, to statistically validate the relationship detected, we applied an approach based on H2A.Z enrichment scores. In this case we sorted all the genes according to their difference in expression, and for each percentile calculated the difference between the H2A.Z enrichment score in control and stress conditions. We observed a strong negative correlation for TSS, ESS, and TTS (Spearman $\rho = -0.944$, -0.937 and -0.907, $P = 6.96E-49$, 1.55E-46 and 1.16E-38 for TSS, ESS and TTS, respectively) (Fig. 3F). This indicates that changes in H2A.Z levels are similar for both TSS and gene bodies. When this analysis was repeated separately for genes upregulated and downregulated in stress, the correlation was significantly stronger for the first group (e.g. for ESS upregulated $\rho = -0.889$, $P = 1.30E-18$, downregulated $\rho = -0.631$, $P = 5.74E-6$). Again this confirms that H2A.Z gain in repressed genes is less evident when compared to the opposite effect in induced genes, yet still significant.

**Genes hyperactive in arp6 are enriched for H2A.Z across their bodies in wild-type plants**

The expression data for arp6, the mutant impaired in H2A.Z deposition, can be compared with the genome-wide profiling of H2A.Z in wt plants to examine how nucleosomal H2A.Z deficiency affects transcription. If the transcriptional changes observed in arp6 result directly from the absence of H2A.Z in nucleosomes occupying the misregulated genes, then in the presence of intact ARP6, the pattern of H2A.Z distribution or occupancy in these genes should be distinct from that of genes which are transcriptionally unaffected in arp6 mutant.
To test this hypothesis we performed a global analysis of H2A.Z enrichment across gene bodies in the BLRP-H2A.Z plants. Based on our RNA-seq data for *arp6* mutants, we grouped all the genes into 'Hyperactive in *arp6* (n=693) and 'Hypoactive in *arp6* (n=542), and compared their wild-type nucleosome profile with the profile of 'All expressed' genes (n=15,558). We did not observe differences in the H2A.Z nucleosomal occupancy between 'Hypoactive in *arp6* and 'All expressed' gene groups (Fig. 4). This suggests that most of the genes hypoactive in *arp6* are indirectly affected by H2A.Z levels, as many of them are likely linked to a negative regulation of DNA repair pathways (Supplemental Data Sets 2A and 2B). On the other hand, genes 'Hyperactive in *arp6* showed dramatically higher levels of H2A.Z across gene bodies in comparison to 'All expressed' genes (Fig. 4, Supplemental Fig. 9A). We concluded that in the majority of genes 'Hyperactive in *arp6*, H2A.Z has a direct effect on their expression by changing the property of underlying nucleosomes in the gene body, resulting in reduced transcriptional activity.

It is interesting to note that we did not observe significant differences in H2A.Z levels at the +1 nucleosome between the three gene groups (Fig. 4A), which indicates that the presence of this histone variant around TSS is not decisive for expression level regulation. We repeated this analysis for the set of data that was obtained for drought stress conditions and observed virtually identical profiles of H2A.Z enrichment (Supplemental Fig. 9B), providing further support for this interpretation.

**Drought-responsive genes with altered expression in *arp6* show different patterns of H2A.Z**

In order to take full advantage of our dataset, we reanalyzed the data focusing on the drought responsive genes. We investigated the transcriptional behavior of genes responsive to drought stress that change their expression profile in the *arp6* mutant. We identified 253 genes, which were both upregulated in drought in wt plants and showed altered expression in the *arp6* mutant (either in control conditions or in stress) when compared to wild type. When the genes were categorized by their expression profile in both control and stress conditions in *arp6*, the largest group consisted of genes hyperactive in both control and stress conditions (n=96 genes, 37.9%) followed by
genes hyperactive in control/ unaffected in stress ($n=64$ genes, 25.3%), and unaffected in control/ hyperactive in stress ($n=30$ genes, 11.9%) (Fig. 5A). For genes downregulated in wt in stress conditions that displayed altered expression in the $arp6$ mutant, the largest group was those unaffected in control/ hyperactive in stress ($n=80$ genes, 34.6%) followed by genes hyperactive in both control and stress conditions ($n=44$ genes, 19.0%) and hyperactive in control/ unaffected in stress ($n=32$ genes, 13.9%) (Fig. 5B). This shows that lack of ARP6 protein results primarily in gene hyperactivity irrespective of whether they are upregulated or downregulated in stress.

To examine whether the observed alterations in gene transcriptional activity are a direct result of changes in H2A.Z levels, we compared H2A.Z profiles of genes upregulated in drought and misregulated in $arp6$ ($n=204$) with all the genes upregulated in drought ($n=1844$, from hereafter referred to as the 'control group'). We divided them into two groups: 'Hyperactive in $arp6$ in control conditions' ($n=166$, 81.4%) and 'Hypoactive in $arp6$ in control conditions' ($n=38$, 18.6%). For these two gene groups, H2A.Z levels at TSS, gene body and TTS were calculated based on our ChAP-seq data (Fig. 5C). We observed that genes hypoactive in $arp6$ in control conditions had significantly lower H2A.Z levels in TSS in wt (Fig. 5C). Importantly, those genes do not lose H2A.Z at TSS during their transcriptional activation, as do other genes upregulated in drought (compare solid and dashed lines in Fig. 5C). We suggest that those genes require some H2A.Z at the +1 nucleosome to stay active, and an inability to efficiently incorporate H2A.Z at this location in the $arp6$ mutant results in their transcriptional hypoactivity. By contrast, genes hyperactive in $arp6$ in control conditions show significantly higher H2A.Z levels across their whole length indicating repressive effect on their transcription (Fig. 5C).

Very similar results were obtained for genes downregulated in drought in wt plants ($n=2226$; 'Control group'). Of them, 130 genes showed altered expression in the $arp6$ mutant in control conditions: 80 genes (61.5%) were hyperactive and 50 genes (38.5%) were hypoactive (Fig. 5D). The hypoactive group has significantly less H2A.Z in TSS under stress conditions (Fig. 5D). Moreover, this group of genes loses H2A.Z upon transcriptional repression, while other genes downregulated in drought behave the opposite (compare solid and dashed lines in Fig. 5D). This indicates that some genes...
'hypoactive in *arp6* require H2A.Z for their activation. Genes hyperactive in *arp6* in control conditions show significantly higher H2A.Z levels in gene body and TTS (Fig. 5D), which is consistent with the repressive effect of H2A.Z on their expression.

These results are in line with the repressive role of gene body H2A.Z in the regulation of gene expression and suggest a direct effect of H2A.Z levels on expression of many drought-responsive genes. At the same time, presence of H2A.Z in +1 nucleosomes in some genes may be important for maintenance of transcriptional activity, as was previously demonstrated (Deal et al., 2007; Kumar and Wigge, 2010).

The *arp6* mutant exhibits a dramatic reduction in H2A.Z occupancy within transcriptionally active genes

The previously presented interpretation of our results assumes that H2A.Z is largely absent from chromatin in the *arp6* mutant. This assumption is based on the fact that the SWR1 complex is considered as the only chromatin remodeler capable of H2A.Z deposition (Billon and Côté, 2011). However, this was not examined in plants, with the exception of three genes linked with flowering regulation (*FLC*, *MAF4* and *MAF5*; Deal et al., 2007; Zhang et al., 2015).

To investigate the extent to which lack of ARP6 protein affects H2A.Z deposition, we crossed the BLRP-H2A.Z line (5D) with the *arp6* mutant. The selected homozygous mutant plants carrying a tagged H2A.Z gene (called hereafter *arp6*-5D) were used to isolate chromatin (MNase-based protocol) and to perform ChAP. We selected 30 genes from groups 'Hyperactive in *arp6*', 'Hypoactive in *arp6*' and 'Unaffected in *arp6*', for which we carried out ChAP-qPCR experiments. Altogether, 78 genomic regions (amplicons) were analyzed, which corresponded to TSS nucleosomes, gene-body located nucleosomes or TTS nucleosomes. For 69 of them, the H2A.Z enrichment was significantly lower in the *arp6*-5D mutant than in the 5D line (Student’s *t*-test), which is wild-type for *ARP6* (Fig. 6A, Supplementary Fig. 10, Supplemental Table 2). The difference in H2A.Z enrichment between 5D and *arp6*-5D lines was significant between all three genic region types (i.e. TSS, *P* = 2.0E-8; gene body, *P* = 2.45E-9; TTS, *P* = 1.6E-4, Mann-Whitney Test), indicating that lack of *arp6* affects H2A.Z levels at different intergenic locations (Fig. 6A). We observed a dramatic 76.5% reduction in
H2A.Z enrichment in the *arp6*-5D when compared to the control 5D line, on average (Fig. 6A). RT-qPCR analysis revealed that *arp6*-5D plants exhibit 3.3 times higher expression of tagged *HTA11* than the 5D line, therefore the difference in H2A.Z occupancy between the two lines is likely due to lack of ARP6 protein and not lower levels of tagged H2A.Z. Altogether, these results indicate that the *arp6*-dependent pathway is a major determinant of H2A.Z deposition in Arabidopsis.

In addition, for several genomic regions with the highest and lowest H2A.Z enrichment in the *arp6*-5D line, we performed negative controls using the *arp6* line (without tagged H2A.Z). The differences between four regions with the highest, and three with the lowest, H2A.Z levels were significant in *arp6*-5D (*P* = 0.017; Mann-Whitney Test), but not between the same regions tested in the *arp6* line (*P* = 0.362; Mann-Whitney Test). Therefore, it is possible that some limited H2A.Z deposition may occur independently of the ARP6 protein.

Characterization of H2A.Z depletion in regards to the gene transcriptional profile in the *arp6* mutant indicates that all three gene groups, hyperactive, hypoactive and unaffected in *arp6*, exhibit significant reduction in H2A.Z levels in an *arp6* background (75.98%, *P* = 3.38E-08, 71.78%, *P* = 1.27E-08 and 82.53%, *P* = 2.18E-05, respectively; Mann-Whitney Test) (Fig. 6B, Supplemental Fig. 10). When H2A.Z enrichment in the 5D line was compared between regions in genes hyperactive and hypoactive in *arp6* mutant, the first group show significantly higher enrichment (*P* = 0.035; Mann-Whitney Test). However, when the same two groups of regions were compared in *arp6*-5D, no significant difference was observed (*P* = 0.438; Mann-Whitney Test). Moreover, Spearman rank correlation indicated that the level of H2A.Z depletion in the *arp6*-5D line is strongly correlated with H2A.Z level in the 5D line (ρ = 0.985, *P* = 1.20E-59, Fig. 6C). These results support our assumption that H2A.Z is largely absent from chromatin in the *arp6* mutant, and the residual levels of H2A.Z remain evenly distributed and statistically indifferent.

**DISCUSSION**

In this work, we studied the role of the histone variant H2A.Z in transcriptional regulation using two approaches: by analyzing its distribution and occupancy in response to
drought stress, and by investigating its distribution in genes deregulated in the "arp6" mutant. Although the repressive role of H2A.Z in transcription in *A. thaliana* was previously suggested (March-Díaz et al., 2008; Kumar and Wigge, 2010; Smith et al., 2010), our work is the first to test changes in occupancy of this histone variant in genes during the response to stress on a genome-wide scale. Here, we present solid evidence for H2A.Z depletion from the gene body upon transcriptional activation (Fig. 4 and Supplemental Figs 6-8). H2A.Z deposition in genes repressed during stress was less evident, yet still notable and statistically significant (Fig. 4, Supplemental Figs 6-8). Assuming that H2A.Z gain and loss accompanying transcriptional changes are active processes, this may suggest that they have different kinetics. This seems likely when one takes into account that H2A.Z deposition and eviction from nucleosomes depends on two different chromatin remodeling complexes, SWR1 and INO80 (Mizuguchi et al., 2004; Altaf et al., 2010; Luk et al., 2010; Yen et al., 2013; Watanabe et al., 2013; Zhang et al., 2015; Papamichos-Chronakis et al., 2011). An alternative explanation of the changes in H2A.Z levels observed in response to stress conditions would be an increased turnover of nucleosomes under transcriptional activation. It is known that passage of RNAPII causes a temporary loss of the H2A/H2B dimer (Kireeva et al., 2002; Workman, 2009). We recognize this explanation as less likely, because our H2A.Z enrichment is normalized to nucleosome occupancy as analyzed by MNase-seq.

We found that H2A.Z levels in gene bodies correlate well with gene responsiveness (Fig. 2). This is similar to observations made by Coleman-Derr and Zilberman (2012) and Latorre *et al.* (2015). We further analyzed this phenomenon and observed that the levels of H2A.Z in the chromatin of responsive genes remain high even if changes in H2A.Z enrichment are considered. Consistent with this, the distribution profile of H2A.Z along the gene remains unchanged, independently of whether the gene is induced or repressed during stress (Fig. 3A-E). This indicates that the nucleosomal H2A.Z pattern can be considered as a permanent property of genes. We hypothesize that high levels of H2A.Z are important to counteract unwanted transcription driven by strong promoters of responsive genes in non-inductive conditions. At the same time, a weak negative correlation between H2A.Z level and gene responsiveness was observed at TSS, likely reflecting a different non-repressive role of
H2A.Z in +1 nucleosomes on transcription (Fig. 2D), which was further investigated by
our analysis of the arp6 mutant (see below).

Interestingly, we observed that in genes upregulated in drought, the +1
nucleosome is more 'fuzzy' and is relatively poor in H2A.Z, which is opposite to the case
in drought repressed genes, where it is well positioned with high H2A.Z level (Fig. 3A,
B). This indicates that localization of H2A.Z at TSS is important for transcription initiation
in normal conditions, where it facilitates RNA polymerase transit through the +1
nucleosome (Weber et al., 2014). In stress conditions however, stress-induced genes
require another way to overcome the +1 nucleosome transcriptional barrier. We
hypothesize that this role is undertaken by longer nucleosome-depleted regions (NDRs),
which we found to be typical for stress-induced genes (Fig. 3C, D). The longer NDRs
may serve to bind specific transcription activators and enable the RNA polymerase to
pass the +1 nucleosome barrier even without high H2A.Z levels. Similar broader NDRs
for stress-induced genes were reported in fission yeast (Sansó et al., 2011; García et al.,
2014). Moreover, it has been shown that responsive genes are associated with highly
accessible chromatin in A. thaliana (Sullivan et al., 2014) and that TFs for stress-induced
genes are often permanently associated with NDRs enabling rapid transcriptional
activation of genes upon exposure to a stress signal (Liu et al., 2015; Zhang et al.,
2012).

It has been widely accepted that in pie1 and arp6 mutants which lack specific and
crucial subunits for the SWR1 complex, nucleosomal H2A.Z level is significantly reduced
(March-Díaz et al., 2008; Deal et al., 2007, 2005; Choi et al., 2005). Studies on pie1 and
arp6 mutants of A. thaliana demonstrated a uniform distribution of remaining H2A.Z
across gene bodies of three genes involved in flowering time regulation without the
prominent peaks observed in wild-type plants (Deal et al., 2005; Zhang et al., 2015). To
confirm that this is true also for other genes, including those misregulated in arp6, we
performed an extensive analysis of H2A.Z enrichment in 78 different genic regions. This
showed that the majority of tested regions significantly lose H2A.Z in the arp6
background and this is largely independent of their location within a gene (TSS, gene
body or TTS; Fig. 6A) or the transcriptional behavior of the gene in arp6 (hyperactive,
hypoactive or unaffected; Fig. 6B). It is important to emphasize that we did not find
evidence for a redistribution of H2A.Z, which could occur via a hypothetical *arp6*-independent H2A.Z deposition pathway (Jeronimo et al., 2015) (Fig. 6C, Supplemental Fig. 10) or passive H2A.Z incorporation during nucleosome reassembly (no correlation with expression rate was observed) (Hardy and Robert, 2010). This indicates that the SWR1 complex is the major factor controlling spatial distribution of H2A.Z in chromatin in plants. Therefore, we assume that the *arp6* mutant represents a chromatin state in which nucleosomal H2A.Z level is evenly decreased throughout the genome.

Wild type H2A.Z occupancy of genes that are hyperactive in *arp6* background is characterized by dramatically increased levels of the variant in gene bodies when compared to other genes (Fig. 4). As we observed no difference in H2A.Z level at the +1 nucleosome (Fig. 4A, E), it is tempting to speculate that the increase in the transcriptional activity in the *arp6* mutant is directly linked to H2A.Z deficiency in gene bodies. This indicates a different role of H2A.Z in the first nucleosome from the TSS than in nucleosomes located farther downstream. We found that the *arp6*-hyperactive genes belong primarily to stress-response related genes when GO classification is applied. This is consistent with studies that suggest a role of H2A.Z in transcription control of responsive genes in plants (March-Díaz et al., 2008; Coleman-Derr and Zilberman, 2012; Kumar and Wigge, 2010; Choi et al., 2016; Latorre et al., 2015). Moreover, our data obtained from the *arp6* mutant analysis strongly suggests that H2A.Z adopts a repressive role in transcription at the stage of elongation and termination, as its loss in gene bodies leads to an increase in their transcriptional activity, as estimated by RNA-seq. The molecular basis of this repressive function is not clear. H2A.Z may contribute to differential stability of nucleosomes (Ishibashi et al., 2009; Jin et al., 2009; Jin and Felsenfeld, 2007; Zhang et al., 2005) or specifically interact with elongation factors (Santisteban et al., 2011). However, in yeast H2A.Z stimulates transcription elongation by affecting the assembly of the RNAPII elongation complex (Santisteban et al., 2011), hence its repressive effect on transcription elongation in plants needs to be studied further.

In contrast to the conspicuous relationship described above, the pattern of H2A.Z distribution in *arp6*-hypoactive genes as measured by ChAP-seq in wild type plants is relatively similar to the pattern in 'All expressed' genes (Fig. 4). GO analysis indicates
that many genes from this group fall into a category that could be linked with the well-established role of H2A.Z and the SWR1 complex in DNA repair (Choi et al., 2013; Xu et al., 2012; Morillo-Huesca et al., 2010; Van et al., 2015; Horigome et al., 2014; Rosa et al., 2013). This may suggest existence of a feedback between the ARP6/SWR1 complex or nucleosomal H2A.Z and DNA repair pathways, which is currently unknown. On the other hand, the observation that genes hypoactive in *arp6* fall into this GO category may be coincidental, and the effect could be due to disturbed activity of transcription activators or a signaling pathway controlling their expression (Pecinka and Mittelsten Scheid, 2012; Mirouze and Paszkowski, 2011). Regardless of the causes, our study clearly demonstrates that in many cases reduced transcriptional activity of genes in the *arp6* mutant is not directly linked to changes in H2A.Z levels.

To investigate whether there are any genes in which transcriptional activation depends directly on H2A.Z, we focused on genes which are both drought-responsive and exhibit altered expression in *arp6*. When the pattern of H2A.Z occupancy was compared between genes hypoactive in *arp6* and all genes up- or down-regulated in drought, we observed lower H2A.Z levels in the +1 nucleosome for genes hypoactive in *arp6*, regardless of their transcriptional behavior in stress (Fig. 5C, D). In addition, these genes show no or opposite change in H2A.Z at TSS during their transcriptional activation when compared to genes in their control groups (compare solid and dashed lines in the middle panels of Fig. 5C and D). These results indicate that indeed, there are genes that require H2A.Z for activation, at least in the +1 nucleosome (Fig. 7). However, based on our experimental setups, where some residual H2A.Z levels could be observed, it is difficult to tell how universal this mechanism is.

The two opposite roles of H2A.Z in transcriptional regulation could be explained by the differences in the biochemical properties of H2A.Z-containing nucleosomes. This could result from posttranscriptional modifications of this histone variant, such as acetylation or ubiquitination. Although specific antibodies for investigation of these modifications have not yet been developed in plants, they have been studied in other organisms (Hu et al., 2013; Ku et al., 2012; Bruce et al., 2005; Valdes-Mora et al., 2012; Sarcinella et al., 2007; Millar et al., 2006). H2A.Z acetylation is generally considered as an important activating mark localized primarily in the +1 nucleosome (Hu et al., 2013;
Ku et al., 2012; Bruce et al., 2005; Valdes-Mora et al., 2012; Millar et al., 2006), whereas H2A.Z ubiquitination is usually linked with its repressive role (Ku et al., 2012; Sarcinella et al., 2007). Specific properties of promoter-proximal nucleosomes may also result from different histone variant composition, e.g. it was suggested that nucleosomes containing both H2A.Z and H3.3 histone variants are much less stable than nucleosomes of other composition (Jin and Felsenfeld, 2007).

Taken together, our findings suggest that in A. thaliana H2A.Z may have either a promoting or repressive effect on transcription, depending on the location of H2A.Z-containing nucleosomes within the gene (Fig. 7). Specifically, in +1 nucleosomes, H2A.Z is required for transcriptional activity. In nucleosomes located farther within gene bodies, it enhances the inhibitory effect of nucleosomes on transcriptional elongation and termination, thus acting as a repressive mark. This effect seems to be important for all the genes containing H2A.Z in their gene bodies. We show that the occupancy of H2A.Z across gene bodies decreases upon stress-dependent transcriptional activation, and these changes negatively correlate with changes in expression level. At the same time, levels of H2A.Z in gene bodies are positively correlated with gene responsiveness, suggesting that this histone variant may counteract unwanted transcription in non-inductive conditions.

MATERIALS AND METHODS
Germination assay
To ensure the same quality of wt (Col-0), arp6 (Choi et al., 2005), pie1-5 (SALK_096434) and hta9-1 hta11-1 (March-Díaz et al., 2008) seeds used for germination experiments, the seeds were harvested from the plants growing side-by-side in the same conditions. To remove potential differences in seed dormancy between the lines, harvested seeds were stored for at least 4 weeks before the germination assay. Seeds were surface-sterilized and sown on 1/2 Murashige and Skoog Media containing 0.8% (w/v) agar and 2.3 mM MES. For stress conditions, the media were additionally supplemented with 150 mM NaCl or 300 mM sorbitol. All three lines were sown on each plate. Eleven biological replicates were performed, each containing 17-18
seeds per line. After sowing, seeds were stratified for 3.5 days at 4°C and then transferred to a plant culture room (22°C, 16/8 h light/dark, 150 µmol m⁻² s⁻¹). Germination rates were scored daily as radicle tip emergence or green cotyledons emergence. Student’s t-test was used to assess statistical significance of observed differences.

**Construction of H2A.Z-tagged lines**

To obtain H2A.Z-tagged lines the *in vivo* biotinylation system (Mito et al., 2005) was adapted. The gene *HTA11* (AT3G54560) encoding *A. thaliana* H2A.Z histone was cloned under the control of its endogenous promoter and tagged at the 5'-end with biotin-ligase recognition peptide (BLRP). The *BLRP-HTA11* cassette was further cloned into the pMOA34 binary vector (Barrell and Conner, 2006), modified by addition of the *BirA* gene encoding prokaryotic biotin ligase under the control of the *Act2* gene promoter. *A. thaliana* plants (Col-0) were transformed as previously described (Zhang et al., 2006). After initial selection on hygromycin-enriched media, expression of tagged H2A.Z gene was analyzed in ~20 transformants by RT-qPCR and expression of the cassette was confirmed at the protein level by immunoblotting using streptavidin-HRP conjugate (Sigma-Aldrich). Several transgenic lines were tested along with lines transformed with empty vector containing the *Act2:BirA* cassette, which was further used as a mock.

**Complementation assay and analysis of H2A.Z enrichment in *arp6* background**

To test the effect of our tagged HTA.Z expression on early flowering phenotype observed in the *hta9-1 hta11-1* double mutant (March-Díaz et al., 2008), we performed a complementation assay. The *hta9-1 hta11-1* line was crossed with a 5D line expressing *BLRP-HTA11* and *BirA* cassettes and plants homozygous for both mutations were selected in the F₂ generation. The progeny of those plants were sowed in long-day conditions alongside wild-type plants (Col) and *hta9 hta11* plants, and flowering time was scored as the number of leaves at bolting. To measure H2A.Z enrichment in the *arp6* mutant, the *arp6* line was crossed with the 5D line expressing *BLRP-HTA11* and *BirA* cassettes. The plants homozygous for the *arp6* mutation carrying the tagged *HTA11* gene were selected in the F₂ generation and the leaf material for ChAP and RNA extraction was collected from long-day conditions. The amplicons for ChAP-qPCR were
selected from genes misregulated in \textit{arp6} (Supplemental Data Sets 1A and 1B) based on location of H2A.Z-containing nucleosomes as revealed by our ChAP-seq analysis. The full list of amplicons along with the primers used for qPCR is shown in Supplemental Table 3. For statistical analysis, Real Statistics Resource Pack was used (Zaiontz C., www.real-statistics.com).

\textbf{Plant growth conditions and drought stress}

\textit{Arabidopsis thaliana} seeds were sown in moistened peat pellets (Jiffy Products), stratified at 4°C for three days, and then transferred to a Sanyo growth chamber, where they were grown at 22°C and 70% humidity under long-day (16/8 h light/dark, 130 μmol m$^{-2}$ s$^{-1}$, fluorescent lamps). Drought stress was applied to 4-week old plants by withholding watering for 8 days. To eliminate a potential effect of early flowering of the \textit{arp6} mutant compared to \textit{wt} plants, for the \textit{arp6} RNA-seq experiment we used short day conditions (8/16 h light/dark). Drought stress was applied to 3-week old plants by withholding watering for 10 days. At least three biological replicates were performed per each experiment. The recovery test indicated that all the plants were able to survive these conditions.

\textbf{Determination of relative water content}

RWC is the percentage water content in tissue relative to the water content at full turgor (Nishiyama et al., 2011). To determine RWC, leaves were collected and immediately weighed to quantify fresh weight (FW). Turgid weight (TW) was obtained after overnight immersion in distilled water and dry weight (DW) after subsequent overnight drying at 50-60°C. RWC was calculated from the following formula: $\text{RWC} = \frac{(\text{FW} - \text{DW})}{(\text{TW} - \text{DW})}$.

\textbf{Chromatin affinity purification (ChAP)}

Approximately 4 g of leaves (2g for stressed plants) were fixed for 10 min under vacuum at room temperature in buffer containing 1% formaldehyde (0.4 M sucrose, 10 mM Tris-HCl pH 8.0, 1 mM PMSF, 1 mM EDTA, 1% formaldehyde). The reaction was quenched by adding glycine to a final concentration of 100 mM and applying vacuum for another 5 min. Next, plant material was washed five times in water and ground in liquid nitrogen to fine powder. Material was stored at -80°C. The powder was resuspended in ice-cold Honda buffer (25 mM Tris-HCl, pH 7.5, 0.44 M sucrose, 10 mM MgCl$_2$, 0.5% Triton X-
100, 10 mM β-mercaptoethanol, 2 mM spermine) and incubated for 30 min on ice with
shaking. Then, the homogenate was filtered through two layers of Miracloth, spun down,
and the pellet washed three times with Honda buffer (last time with no spermine). The
nuclei pellet was resuspended in ice-cold TNE buffer (10 mM Tris-Cl pH 8.0, 100 mM
NaCl, 1 mM EDTA) and after addition of CaCl₂ to a final concentration of 4 mM, MNase
(16 U/ml) digested for 20 min at 37°C. The reaction was terminated by addition of EGTA
to a final concentration of 25 mM. After centrifugation, the supernatant was stored in
liquid nitrogen until chromatin affinity purification. Chromatin was diluted 10-fold with
Dilution Buffer (16.7 mM Tris-Cl pH 8.0, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-
100, 1.1 mM PMSF and 1.1% Protease Inhibitor Cocktail, Sigma) and incubated with
Dynabeads M-280 Streptavidin (Life Technologies) overnight at 4°C with gentle
agitation. The slurry was washed with Wash buffer (20 mM Tris-Cl pH 8.0, 2 mM
EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1mM PMSF).
For ChAP-qPCR, DNA was extracted with 10% Chelex (Biorad) as described previously
(Wierzbicki et al., 2008). ChAP-qPCR was performed using Applied Biosystems 7900HT
Fast Real-Time PCR System and Maxima SYBR Green/ROX qPCR Master Mix
(Thermo Scientific). Three biological replicates were used for each experiment. Percent
of input was calculated as follows:

\[
\text{Percent of Input} = 100 \times 2^{\Delta Ct},
\]

\[
\text{where } \Delta Ct = Ct^{\text{Input}} - \log_2(\text{Input dilution factor}) - Ct^{\text{ChIP}}
\]

and all the results were normalized using the ACTIN7 gene. For high-throughput
sequencing, DNA was eluted and purified with the use of the IPure kit (Diagenode)
according to the manufacturer's instructions. For both ChAP-qPCR and ChAP-seq, DNA
from MNase-treated chromatin was used as input. DNA library preparation and
subsequent DNA sequencing on Illumina HiSeq2000 platform was performed by BGI
(Hong-Kong).

**ChAP-seq data analysis**

MNase-seq and ChAP-seq reads were mapped to the TAIR10 genome using Bowtie2
software (Langmead and Salzberg, 2013). H2A.Z enrichment was established by dividing
the ChAP-seq reads by MNase-seq (input) reads (after normalization to library size).
DANPOS2 (Chen et al., 2013) software was used for signal normalization, drawing
profiles and differential analysis of ChIP–Seq and MNase–Seq data. Differential binding of the H2AZ between stress and control samples was performed using the Dpeak algorithm, while the Dpos algorithm was used to analyze different types of nucleosome changes (occupancy change, fuzziness change and position shift), as described in DANPOS documentation. For all analyzes, the FDR threshold of 0.01 was used. HOMER package (Heinz et al., 2010) was used for the annotation of detected differential binding regions and nucleosomes, as well as for the analysis of GO terms, molecular pathways and protein domains overrepresented in affected sets of genes. Custom genome browser containing the processed experimental data was created using JBrowse software (Skinner et al., 2009).

**RNA isolation for RT-qPCR analysis and RNA-seq**

RNA for each biological repeat was extracted from 100 mg of leaves (from at least eight plants) with Trizol and rounds of phenol-chloroform and chloroform extractions followed by isopropanol precipitation. RNA was treated with DNase (Promega), then extracted with phenol-chloroform and precipitated with ethanol. Pure RNA water solution was sent to BGI Hongkong, where libraries and sequencing were performed using Illumina chemistry with single-end protocol. After quality inspection (FastQC, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimming (fastx, http://hannonlab.cshl.edu/fastx_toolkit/index.html) we obtained at least 12 million reads representing each sample. The sequences were mapped onto reference TAIR10 genome using TopHat2 aligner (Kim et al., 2013) with default parameters. The counts corresponding to each of the genes were scored using HTseq (Anders et al., 2015) tool using the ‘union’ option. Subsequently, the counts representing each gene were used in estimation of differentially expressed genes by DESeq2 (Love et al., 2014) from R Bioconductor package. The significant differences in expression were selected based on $p_{adj}$ value ($P$-value adjusted with the Benjamini-Hochberg procedure) lower than 0.05 unless otherwise indicated. For GO annotations, we used VirtualPlant 1.3 platform run with TAIR10 GO classification (Katari et al., 2010). Fischer exact test was used to estimate $p$-values for functional categories. For RT-qPCR analysis cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and
analyzed in triplicates on Applied Biosystems 7900HT Fast Real-Time PCR System using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific).

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers:

*AR*P6, AT3G33520; *HTA*11, AT3G54560. Sequence data are under accession numbers SAMN05504885–SAMN05504902 in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra). For additional accession numbers, please see Supplemental Table 2 and Supplemental Data Sets 1 and 3.

**Supplemental Data**

**Supplemental Fig. 1.** Comparison of the RWC for control plants and plants subjected to drought stress in wild-type and *arp6* plants used for RNA-seq and ChIP-seq analysis.

**Supplemental Fig. 2.** Different SWR1C mutants exhibit similar changes in transcription when compared to wild-type plants.

**Supplemental Fig. 3.** H2A.Z-tagged lines exhibit similar nucleosomal H2A.Z levels on the *FLC* gene and complement *hta9 hta11* mutations.

**Supplemental Fig. 4.** Comparison of the RWC for H2A.Z-tagged line 5D and lined K3F transformed with an empty vector in control and drought stress conditions.

**Supplemental Fig. 5.** H2A.Z levels in gene bodies correlates with gene responsiveness.

**Supplemental Fig. 6.** H2A.Z levels in control and stress conditions.

**Supplemental Fig. 7.** H2A.Z-ChAP signal in control conditions relative to TSS, ESS, TTS, and across the gene body.

**Supplemental Fig. 8.** ChAP-qPCR validation of changes in H2A.Z occupancy in drought stress at selected genes.

**Supplemental Fig. 9.** Genes hyperactive in *arp6* have significantly higher levels of H2A.Z across their bodies.

**Supplemental Fig. 10.** ARP6-dependent pathway is a major determinant of H2A.Z enrichment within Arabidopsis genes.
Supplemental Table 1. Germination assay in wt (Col), arp6, hta9 hta11 and pie1-5 plants in osmotic stress. *P*-values were calculated using Student’s *t*-test.

Supplemental Table 2. ChAP-qPCR analysis of H2A.Z enrichment for 5D and 5D-arp6 plants.

Supplemental Table 3. List of primers used for ChAP-qPCR and RT-qPCR analyses.

Supplemental Data Set 1. Genes that change their expression in arp6 mutant when compared to wild type plants in control and drought conditions.

Supplemental Data Set 2. Significantly enriched GO classes for genes hypoactive in arp6 for control and drought conditions, and genes hyperactive in arp6 in control conditions.

Supplemental Data Set 3. Genes that change their expression in drought stress conditions in wild type plants.

Supplemental Data Set 4. Significantly enriched GO classes for genes upregulated and downregulated in drought conditions.

Supplemental Data Set 5. Chromosomal regions that lost or gained H2A.Z levels in drought when compared to control conditions.

Supplemental Data Set 6. Significantly enriched GO classes for genes corresponding to regions, which lost H2A.Z levels in drought when compared to control conditions.

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Author Contributions

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Fig. 1. Nucleosome H2A.Z-depleted plants show delayed germination upon osmotic stress conditions. (A-C) Radicle tip emergence rate in wt, *hta9 hta11*, *arp6* and *pie1-5* on control media (A) and media containing 150 mM NaCl (B) and 300 mM sorbitol (C). (D-F) Green cotyledon emergence on control media (D), media containing 150 mM NaCl (E), and 300 mM sorbitol (F).
Fig. 2. H2A.Z levels in gene-bodies correlates with gene responsiveness. H2A.Z profiles for three gene classes divided according to their change of transcript level (log_{2} fold-change) are shown for genes induced (A) and repressed (B) in stress. (C) Heatmap visualization of H2A.Z enrichment (IP - input) for the gene bodies represented in panels (A-B). Genes were sorted (top to bottom) from highest to lowest H2A.Z enrichment. (D) Spearman rank correlation for H2A.Z level and expression change (absolute values from log_{2} fold change) for TSS, ESS and TTS. (E) Spearman rank correlation for H2A.Z level and expression level in control conditions for TSS, ESS and TTS.
Fig. 3. Distinct pattern of H2A.Z in constitutive, stress-induced and stress-repressed genes and its changes upon drought conditions. (A) H2A.Z enrichment in control conditions (normalized H2A.Z-ChAP reads minus normalized MNase-seq [input]) relative to TSS, TTS, and across gene body is shown for genes upregulated (green), and downregulated (red) in drought stress in comparison with 'All expressed genes' (blue). (B) As for (A) but in stress conditions. (C-D) Nucleosome distribution across TSS as determined by MNase-seq in control (C) and stress (D) conditions. (E) Heatmap visualization of H2A.Z enrichment (IP - input) for the TSS / TTS / gene bodies represented in panels (A, B) and MNase-seq (from panels C, D). Genes were sorted (top to bottom) from highest to lowest H2A.Z enrichment (for TSS, TTS and Gene body) or nucleosome density (for MNase TSS). For the class 'All expressed', 2000 genes were randomly selected to compare similar number of elements as for other classes. (F) The change in H2A.Z occupancy correlates with the change in expression level between control and drought conditions (Spearman rank correlation) for TSS, ESS and TTS. (G) Comparison of changes in H2A.Z enrichment between control (blue) and stress (green) conditions shown for all expressed, stress-induced and stress-repressed genes. Plots show the same data as for A, B and E.
Fig. 4. Genes hyperactive in *arp6* have significantly higher levels of H2A.Z across their gene bodies. H2A.Z enrichment (normalized H2A.Z-ChAP reads minus normalized MNase-seq [input]) relative to TSS (A), TTS (B), exon start site (ESS) (C) and across gene body (D) is shown for genes upregulated (green), and downregulated (red) in *arp6* in comparison with 'All expressed' genes (blue). (E) Heatmap visualization of H2A.Z enrichment (IP - input) for the TSS / TTS / ESS / gene bodies represented in panels (A-D). Genes were sorted (top to bottom) from highest to lowest H2A.Z enrichment. For the class 'All expressed', 1200 genes were randomly selected to compare similar number of elements as for 'Hyperactive in *arp6*' and 'Hypoactive in *arp6*' classes.
Fig. 5. Analysis of H2A.Z enrichment in drought responsive genes misregulated in arp6 mutant. (A,B) Classification of drought-upregulated (A) and drought-downregulated (B) genes according to their relative expression in arp6 mutant in control and stress conditions (control → stress). “Hpr”, “Unf”, “Hpo” are used as abbreviations for genes hyperactive, unaffected or hypoactive in arp6 relative to wt, respectively. Transcriptional profiles characteristic of three groups represented by the largest number of genes in each circle graph are shown schematically in the line graph on the right hand side of each circle graph. (C,D) H2A.Z enrichment in genes upregulated (C) and downregulated (D) in drought, divided according to the directionality of their misregulation in arp6 in control conditions (Hyper- or Hypoactive in arp6). The sizes of the groups are compared in the circle graphs on the left in (C) and (D) and the number of genes in each group is given in brackets. Note that each group covers three groups presented in (A) or (B), as this classification is based on control conditions only. The line plots show H2A.Z enrichment (mean values) at TSS, gene body (ESS) and TTS in control (red or blue solid lines) and drought conditions (red or blue dashed lines) as compared to the same for all genes up- (C) or down-regulated (D) in drought (gray lines). The significance of the difference in H2A.Z levels is indicated as stars for control (above the plots) and drought conditions (below the plots) (Mann-Whitney Test; *, P < 0.05; **, P < 0.01; ***, P < 0.001).
Fig. 7. The role of H2A.Z in the regulation of gene activity in Arabidopsis. Some genes require H2A.Z in TSS to maintain their transcriptional activity (left panel). These genes stay hypoactive in the arp6 mutant. As the arp6 mutant exhibits some residual evenly-distributed H2A.Z, it is difficult to speculate how general this mechanism is. On the other hand, H2A.Z across the whole gene length adopts a repressive role in transcription in most genes that change their expression under stress conditions (right panel). These genes stay hyperactive in the arp6 mutant. We hypothesize that the biological role of H2A.Z repressive influence on transcription is to counteract unwanted expression under non-inductive conditions. H2A.Z is partially removed from nucleosomes during transcriptional activation. The model assumes that in +1 nucleosomes H2A.Z may adopt both a promoting and repressive role, depending on additional factors such as nucleosome composition or histone modifications.

Fig. 6. ARP6-dependent pathway is a major determinant of H2A.Z enrichment within Arabidopsis genes. H2A.Z is depleted from the chromatin in an arp6 mutant background irrespective on the genic location (A) and in genes hyperactive, hypoactive and unaffected in arp6 (B). (C) Spearman Rank correlation showing the relationship between H2A.Z enrichment in wild-type (5D) and its depletion in arp6 background (arp6-5D).

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