Diurnal changes in the histone H3 signature H3K9ac|H3K27ac|H3S28p are associated with diurnal gene expression in Arabidopsis

Journal Article

Author(s):
Baerenfaller, Katja; Shu, Huan; Hirsch-Hoffmann, Matthias; Fütterer, Johannes; Opitz, Lennart; Rehrauer, Hubert; Hennig, Lars; Gruissem, Wilhelm

Publication date:
2016-11

Permanent link:
https://doi.org/10.3929/ethz-b-000121422

Originally published in:
Plant, Cell & Environment 39(11), https://doi.org/10.1111/pce.12811

Plant, Cell & Environment

Plant, Cell and Environment (2016) **39**, 2557–2569 doi: 10.1111/pce.12811

Original Article

Diurnal changes in the histone H3 signature H3K9ac|H3K27ac|H3S28p are associated with diurnal gene expression in Arabidopsis

Katja Baerenfaller[1], Huan Shu[1,4], Matthias Hirsch-Hoffmann[1], Johannes Fütterer[1], Lennart Opitz[2], Hubert Rehrauer[2], Lars Hennig[3] & Wilhelm Gruissem[1]

[1]Department of Biology, ETH Zurich, Zurich 8092, Switzerland, [2]Functional Genomics Center Zurich, ETH Zurich/University of Zurich, Zurich 8057, Switzerland, [3]Department of Plant Biology, Swedish University of Agricultural Sciences and Linnean Center for Plant Biology, Uppsala SE-75007, Sweden and [4]Program of Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, USA

ABSTRACT

Post-translational chromatin modifications are an important regulatory mechanism in light signalling and circadian clock function. The regulation of diurnal transcript level changes requires fine-tuning of the expression of generally active genes depending on the prevailing environmental conditions. We investigated the association of histone modifications H3K4me3, H3K9ac, H3K9me2, H3S10p, H3K27ac, H3K27me3 and H3S28p with diurnal changes in transcript expression using chromatin immunoprecipitations followed by sequencing (ChIP-Seq) in fully expanded leaves 6 of *Arabidopsis thaliana* grown in short-day optimal and water-deficit conditions. We identified a differential H3K9ac, H3K27ac and H3S28p signature between end-of-day and end-of-night that is correlated with changes in diurnal transcript levels. Genes with this signature have particular over-represented promoter elements and encode proteins that are significantly enriched for transcription factors, circadian clock and starch catabolic process. Additional activating modifications were prevalent in optimally watered (H3S10p) and in water-deficit (H3K4me3) plants. The data suggest a mechanism for diurnal transcript level regulation in which reduced binding of repressive transcription factors facilitates activating H3K9ac, H3K27ac and H3S28p chromatin modifications. The presence of activating chromatin modification patterns on genes only at times of the day when their expression is required can explain why some genes are differentially inducible during the diurnal cycle.

Key-words: *Arabidopsis thaliana*; chromatin immunoprecipitation; chromatin modifications; diurnal; epigenetics; histone; leaf.

Correspondence: K. Baerenfaller and W. Gruissem. e-mail: kbaerenfaller@ethz.ch; wgruissem@ethz.ch

INTRODUCTION

Gene expression in eukaryotes is regulated at different levels, including chromatin organization that modulates the accessibility of DNA to transcriptional regulatory proteins such as transcription activators and repressors (Kouzarides 2007; Pfluger & Wagner 2007). Chromatin activity and function are determined by various modifications, including DNA methylation, histone modifications, as well as exchange of histone variants (Pfluger & Wagner 2007; Barneche *et al.* 2014; Seo & Mas 2014; Shu *et al.* 2014). Histones H2A, H2B, H3 and H4 are post-translationally modified mainly in their amino-terminal sequences and H2A also in its carboxy-terminal sequence by acetylation, phosphorylation, methylation, sumoylation or ubiquitylation. These modifications can either directly modulate chromatin compaction and accessibility or change the interaction with other proteins (Kouzarides 2007; Ruthenburg *et al.* 2007). Establishment and maintenance of histone modifications in plants are related but not identical to those in fungi or animals (Pfluger & Wagner 2007; Zhou 2009; Liu *et al.* 2010). Acetylation of histone amino-terminal tails facilitates transcriptional activation either by neutralizing the lysine positive charge and thereby relaxing the chromatin structure, or by forming a binding site for bromodomain-containing proteins (Barneche *et al.* 2014; Lee *et al.* 2010). Histone H3 acetylation and deacetylation were implicated in the regulation of light-responsive genes (Chua *et al.* 2003; Bertrand *et al.* 2005; Benhamed *et al.* 2006; Charron *et al.* 2009) and have therefore been proposed as a switch between permissive and repressive states of chromatin for gene expression (Shahbazian & Grunstein 2007). Histone methylation can have both activating and repressive functions in gene expression and the distinct mono-methylation, di-methylation or tri-methylation modifications often have different biological roles (Zhou 2009; Liu *et al.* 2010). For example, histone H3K4 methylation activates transcription by recruitment of chromatin modifying proteins, while histone H3K27 methylation represses transcription by promoting a compact chromatin structure (Bernstein *et al.* 2006). In *Arabidopsis*, histone lysine methylation occurs mainly at K4, K9, K27 and K36 of histone H3. Histone H3K9 is

predominantly mono-methylated or di-methylated, and together with H3K27 tri-methylation, these methylations are associated with silenced regions, whereas H3K4 and H3K36 methylation are associated with active genes (Berger 2007; Liu *et al.* 2010). In yeast and mammals, histone phosphorylation occurs in response to DNA damage and during meiotic and mitotic chromatin remodelling. Histone H3 phosphorylation also controls H3 acetylation and methylation and was therefore implicated in the regulation of gene expression (Rossetto *et al.* 2012). Plant histones are phosphorylated at multiple sites as well, including H3S10 and H3S28, which correlates with chromosome segregation and metaphase/anaphase transitions (Kurihara *et al.* 2006; Houben *et al.* 2007; Bigeard *et al.* 2014). In addition, histone H3 phosphorylation in differentiated mesophyll cells is mostly associated with rRNA genes (Granot *et al.* 2009). However, phosphorylation of histone H3 has so far not been implicated in regulating transcription in plants.

Histone modifications have been proposed to constitute a code that determines the transcription state of a gene, but the temporal order and enzymatic mechanism by which they are established can also affect the level of transcriptional activity (Karlić *et al.* 2010; Lee *et al.* 2010). For example, interactions have been reported between phosphorylation at H3S10 and methylation at H3K9 or acetylation at H3K9 and H3K14 (Demidov *et al.* 2009; Fry *et al.* 2004; Li *et al.* 2005), or between phosphorylation at H3S28 and acetylation at H3K27 (Lau & Cheung 2011).

In plants, light-regulated gene expression has been investigated mainly in the context of light signalling and the transition to photomorphogenesis (Barneche *et al.* 2014). Light signals are integrated by transcription factors that bind to light-responsive elements (LREs) in the promoter regions of genes, but transcriptional regulation also depends on changes in histone modifications. These play an important role in light-driven processes and light signalling, for transient adaptations to changing light conditions, and to control circadian gene expression (Barneche *et al.* 2014; Bertrand *et al.* 2005; Chua *et al.* 2003; Li *et al.* 2012; Schroeder *et al.* 2002; Seo & Mas 2014; Van Zanten *et al.* 2012). The rhythmic expression of several circadian clock genes including *LHY*, *CCA1*, *TOC1*, *PRR9*, *PRR7* and *LUX* correlates with oscillating acetylation at H3K9, H3K14 and H3K56, as well as tri-methylation at H3K4 (Perales & Más 2007; Farinas & Mas 2011; Hemmes *et al.* 2012; Malapeira *et al.* 2012; Song & Noh 2012). Histone acetylation and tri-methylation at H3K4 seem to be active marks that promote the rhythmic activation of clock genes, and thus, the precise temporal and combinatorial accumulation of histone modifications may regulate the shape of the circadian waveforms of core clock gene transcription (Malapeira *et al.* 2012; Seo & Mas 2014). The circadian clock is functionally connected to light signalling because light entrains the clock, and the clock modulates the expression of many light-regulated genes. It was therefore not unexpected that the control of many light-driven and circadian-driven processes such as growth, flowering and metabolism also involves chromatin-based mechanisms (Barneche *et al.* 2014).

We previously reported that transcript levels in *Arabidopsis* leaf 6 strongly oscillate between the end of the day (EOD) and end of the night (EON) (Baerenfaller *et al.* 2012). These diurnal transcript level changes were dependent on the growth stage and experimental conditions because fewer transcripts fluctuated in fully expanded leaves compared with earlier development stages and also in water deficit compared with optimal water conditions (Baerenfaller *et al.* 2012). We therefore hypothesized that temporal changes in chromatin modifications and between experimental conditions could cause differences in diurnal transcript level fluctuations. To test this hypothesis, we performed a chromatin immunoprecipitation (ChIP) experiment using antibodies against seven different histone H3 modifications. We now show that diurnal transcript oscillations correlate with previously undetected diurnal changes in H3K9 and H3K27 acetylation and H3S28 phosphorylation. Genes marked by these modifications encode core clock and other proteins. We therefore argue that their diurnal transcript level changes depend on the dynamic reversible acetylation and phosphorylation of their corresponding genomic regions, while their general active state is controlled by a more static activating H3K4 tri-methylation and the absence of repressive H3K9 di-methylation and H3K27 tri-methylation.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana accession Col-4 (N933) plants were grown in a growth chamber equipped with the PHENOPSIS automaton (Granier *et al.* 2006) as described previously (Baerenfaller *et al.* 2012). For the optimal water condition experiment, the soil water content was adjusted to 0.40 g water g^{-1} dry soil, and for the water deficit experiment, plants were grown in mild water deficit conditions with soil water content adjusted to 0.24 g water g^{-1} dry soil. Leaves 6 at fully expanded stage were harvested during the last hour before the light is on and before the light is off, and each sample was prepared by bulking material from numerous plants. The frozen plant material was sent to the MPI in Golm, where it was ground and aliquoted using a cryogenic grinder (German Patent No. 08146.0025U1).

Chromatin immunoprecipitation-quantitative PCR and chromatin immunoprecipitation-sequencing

Native ChIP was performed as described (Shu *et al.* 2014) with minor modifications. One hundred milligrams of cryogenic grinded plant material was treated in 8 mL of nuclei extraction buffer (1.0 M hexylene glycol, 20 mM PIPES-KOH pH 7.6, 10 mM $MgCl_2$, 0.1 mM EGTA, 20 mM sodium butyrate, 60 mM KCl, 0.5% Triton 100, 5 mM β-mercaptoethanol, supplemented with complete EDTA-free protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche, Basel, Switzerland)) for 15 min at 4 °C on a slow rocker. The homogenate was filtered through 50 μm CellTrics nylon-mesh filters (Partec, Georlitz, Germany), and a nuclei pellet was collected by centrifugation for 10 min at 1500 × g at 4 °C. Isolated nuclei were washed once in MNase buffer (50 mM

Tris–HCl pH 8, 10 mM sodium butyrate, 5 mM $CaCl_2$, complete EDTA-free protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche)), treated with 2 μL of RNase A, 30 μg/μL (Sigma-Aldrich, St. Louis, MO, USA) and used for Micrococcal Nuclease (New England BioLabs, NEB, Beverly, MA, USA) digestion for 1 min (final concentration 8 gel units/μL) in 200 μL MNase buffer at 37 °C. The reaction was stopped with 10 mM EGTA. After centrifugation, the supernatant was collected as phase 1 chromatin preparation. The pellet was treated in 200 μL buffer S2 (1 mM Tris–HCl pH 8, 0.2 mM EDTA, complete EDTA-free protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche)) for 30 min at 4 °C. After centrifugation, the supernatant was collected as phase 2 chromatin preparation. The two phases of chromatin preparations were combined and diluted with 600 μL Dilution Buffer (25 mM Tris-HCl pH 8, 220 mM NaCl, 20 mM sodium butyrate, 5 mM EDTA, complete EDTA-free protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche)) to adjust the Na^+ concentration to 150 mM. The majority of the chromatin was of mononucleosome size (data not shown). The Triton-X 100 concentration in the mononucleosomal chromatin was adjusted to 0.1% followed by pre-clearing using non-immune rabbit IgG and 100 μL Dynabeads Protein A (Invitrogen, Carlsbad, CA, USA). Fifty microliters of pre-cleared mononucleosomal chromatin were kept as input control, and 100 μL were used for each immunoprecipitation with respective antibodies (Table S1). Antibody-recognized chromatin was collected with Dynabeads Protein A (Invitrogen). After washing, beads were resuspended in TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA), and DNA was extracted using phenol-chloroform extraction and ethanol/salt precipitation. ChIP was performed in biological duplicates. DNA was amplified using the Ovation Ultralow IL Multiplex Kit (NuGEN, San Carlos, CA, USA) according to manufacturer's instructions with 15 amplification cycles. Amplification fidelity was confirmed by quantitative PCR using DNA samples before and after amplification for five selected genomic loci (data not shown). Sequencing of stranded, paired-end 100 bp reads was carried out on an Illumina HiSeq 2000 platform using v3 chemistry (Illumina, San Diego, CA, USA).

Sequencing data processing

For processing of the sequencing data, we followed the workflow depicted in Figure S1. The sequencing data were loaded into the CLC Genomics Server (version 5.0.2) (CLC bio, Aarhus, Denmark) and mapped to the *Arabidopsis* genome using the following parameters: auto-detect paired distances = Yes; collect un-mapped reads = No; colour space alignment = Yes; colour error cost = 3; deletion cost = 3; global alignment = No; insertion cost = 3; length fraction = 0.9; masking mode = NO_MASKING; mismatch cost = 2; non-specific match handling = IGNORE; Output mode = CLUSTER; similarity fraction = 0.9. The mapped sequencing data have been deposited to the European Nucleotide Archive with accession number PRJEB9989 (available at http://www.ebi.ac.uk/ena/data/view/PRJEB9989).

The sequencing data exported from CLC were subsequently binned with binsize = 200 bp starting at position 1 of each chromosome by counting the fragment reads in the individual bins using the Set::IntervalTree perl module (Booth 2010), generating lists with a bincount for each bin. Assessing the distribution of the bincounts, we realized that some genomic regions comprised bins with extremely high bincounts as compared with the general distribution of the bincount data. As these high bincounts could potentially compromise data normalization, we needed to identify these regions using the criteria: 1) as the extremely high bincounts are outside of the general distribution of the data, the bincounts were sorted and the gap in the distribution was identified by determining where the bincount was larger than the previously ranked bincount plus the average bincount of the sample (Figure S2) and 2) as the reason for these extremely high bincounts most probably lies in the repetitive nature of the genomic sequence, it is by definition not sample dependent, and we therefore only considered regions when they had extremely high bincounts in at least three quarter of all samples. After visual inspection and curation of these regions, they were masked by setting the bincounts in the corresponding bins to 0 (Table S2). After masking, the bincounts in each sample were normalized by scaling the average bincount to a value of 1000.

For the correlation analyses between transcript expression and chromatin modification levels, the average bincounts for regions comprising the transcription start site (TSS), gene body and transcription termination site (TTS) were determined and normalized to histone H3 to account for altered nucleosome density.

Identification of genes with differentially modified chromatin regions between two samples

For the identification of differentially modified chromatin regions, we applied the general principle of the ChIP norm method (Nair *et al.* 2012) with some alterations.

1) Identification of significant bins. For the removal of the stochastic noise, we initially assumed that the noise follows an amplified binomial distribution (ABD) as suggested in Nair *et al.* (2012). After binning the data in different binsizes, we calculated the theoretical ABD, and based on this, we determined the minimum bincount cut-offs for achieving a false discovery rate (FDR) < 5%. We found that the calculated bincount cut-offs would only leave few significant bins and that the theoretical ABD is very different compared with the distribution of the unspecific IgG antibody background data. From this, we concluded that the ABD only poorly represents the stochastic noise in our data. We therefore applied the FDR calculation to determine the bincount cut-off on the IgG background data instead. After filtering at FDR < 5%, we found that the number of remaining significant bins in general only depended on the binsize but not the antibody or the sample. However, for some samples with poorly performing IgG data, the number of remaining significant bins was particularly low, and filtering at FDR < 5% based on the IgG data would lead to skewed analyses for these samples. For the removal of stochastic noise, we

therefore decided to calculate the average bincount cut-off for achieving an FDR < 5% only including the well performing IgG data. Based on this, we defined that for binsize = 200, the lowest 5.5% of bincounts of each sample correspond to stochastic noise. If the bincounts for a given bin were in the stochastic noise in sample 1 and in sample 2, the bincounts for that bin were set to 0 in both samples.

2) Identification of enriched bins. After removal of the noise, the bincounts of the samples and the input DNA control were again normalized by scaling. To identify differentially modified chromatin regions between two samples, only those regions that are enriched with respect to the input DNA control should be compared. If this step is omitted, the results will mainly comprise chromatin regions with very low bincounts. For the identification of the enriched bins, we therefore required a minimum sample/input DNA ratio of 2. Because we found that a poorly performing input DNA sample led to skewed results, we took the average input DNA bincounts from all samples to determine the sample/input DNA ratio.

3) Identification of differentially enriched bins. For the identification of a differentially enriched bin between samples 1 and 2, we required that the bin was defined to be enriched in at least one of the samples and that the bincount ratio between sample 1 and sample 2 was >1.5 or <1/1.5 (if the bincount was 0 in one of the samples, it was set to 0.1 before calculating the ratio). All the bins that passed these criteria were given out together with the information on their genome localization based on the gene annotation and the previously applied definition of the TSS, gene body and TTS regions. For the identification of genes containing differentially modified chromatin regions between two samples, we required that a gene contained at least two differentially enriched bins comparing sample 1 with sample 2. For the genes that fulfilled this criterion, the bincounts for all enriched bins in sample 1 and sample 2 were written out and a Wilcoxon rank sum test on the bincounts of the differentially enriched bins was performed to calculate the *P*-value for the statistical significance of the difference in the bincounts. Genes with a *P*-value < 0.05 were considered to contain differentially modified chromatin between samples 1 and 2 (Supporting Information 2). Analyses were carried out using perl and R (R Core Team 2015).

Tiling array transcript data

Previously reported AGRONOMICS1 microarray transcript data of leaf number 6 at developmental stage 4 in three biological replicates in short-day optimal water (SOW) and short-day mild water deficit (SWD) conditions were taken (Baerenfaller *et al.* 2012). After exclusion of probe sets for plastid and mitochondrion encoded transcripts and of probe sets matching to more than one gene, 30 442 probe sets remained. To identify genes that are differentially expressed between SOW and SWD and between EOD and EON in each experimental condition, the following tests were performed: 1) paired *t*-test between all six SOW and six SWD samples, 2) *t*-test between the three SOW EON and SOW EOD samples, 3) *t*-test between the three SWD EON and SWD EOD samples. The *P*-values were corrected for multiple testing with the Benjamini–Hochberg method (Benjamini & Hochberg 1995). Transcripts with a *P*-value < 0.05 and a fold-change >log2 (1.5) between the respective mean expression values were considered to change significantly. This gave 2105 transcripts with significantly different expression levels between SOW EOD and EON (1333 higher at EOD, 772 higher at EON), 374 transcripts with significantly different levels between SWD EOD and EON (211 higher at EOD, 163 higher at EON) and 294 transcripts with significantly different levels between SOW and SWD (170 higher in SOW, 124 higher in SWD) (Supporting Information 3).

Gene ontology categorization

Gene ontology (GO) categorization was performed using the Ontologizer software (http://compbio.charite.de/ontologizer) (Bauer *et al.* 2008) in combination with the *Arabidopsis* annotation file (download on 6 May 2014) considering aspect biological process (BP). Annotations with GO evidence codes inferred from electronic annotation (IEA) or inferred from reviewed computational analysis (RCA) were excluded from analyses. Over-representation analysis was carried out using the Topology–Elim method, and after correction of the *P*-values with Bonferroni, GO terms with *P*-values < 0.01 were considered to be enriched.

Promoter element analyses

The promoter region was defined as 1000 bp upstream of the annotated TSS or until the TTS of the preceding gene. The TSS corresponds to the genome region spanned by the bin containing the TSS plus the two preceding bins, while the TTS comprises the genome region spanned by the bin containing the TTS plus the two following bins. Over-representation and under-representation was assessed separately for the genes with higher modification levels at EOD or EON using Fisher's exact test and by comparing the number of identified genes with the number that would be expected by chance.

Co-expression cluster analysis

Co-expression clusters provide an alternative tool to classify gene groups, in this case in relation to the general expression pattern. The ATTED-II co-expression database (Version 5.0) (Obayashi *et al.* 2014) was used to assemble for every gene a co-expression cluster containing all genes with a mutual rank (MR) value <100. To extract a smaller number of these clusters that represent the majority of all genes with minimal overlap between the clusters, the complete gene list was iteratively matched against all clusters and each time the cluster containing the largest number of genes from the complete list was extracted and the genes present in this cluster removed from the query list. This was repeated until no new clusters containing more than eight genes were retrieved. The resulting collection of 384 clusters covers 19′734 (94.7%) of the 20′836 genes in the complete list, with 18′645 (89.5%) genes that are present in

maximum five clusters and no gene that is present in more than 12 clusters. For each cluster, the over-representation of the genes with the respective differential chromatin modifications higher at EON or EOD was determined with a Fisher's exact test. The clusters were sorted according to the presence of the H3K9ac, H3K27ac or H3S28p differential modification at EON or EOD, respectively, in the replicate intersect of SOW (Supporting Information 2).

RESULTS

Experimental set-up

We investigated a set of diagnostic histone H3 modifications in fully expanded *Arabidopsis* leaf number 6 in which all cell division has ceased to identify differential chromatin modifications that correlate with diurnal expression level changes and with changes between two different experimental conditions. For this, we collected fully expanded leaves 6 of plants grown under SOW and SWD (60% soil water content) conditions at EON and EOD as previously reported (Baerenfaller *et al.* 2012). Chromatin was prepared from leaf powder and immunoprecipitated using antibodies against the H3 modifications K4 tri-methylation (H3K4me3), K9 acetylation (H3K9ac), K9 di-methylation (H3K9me2), K27 acetylation (H3K27ac), K27 tri-methylation (H3K27me3), S10 phosphorylation (H3S10p) and S28 phosphorylation (H3S28p), as well as against histone H3 (histone) and using an unspecific antibody (IgG) as background control (Fig. 1(a)). The captured DNA fragments from the ChIPs and input DNA (input) were subjected to deep sequencing (ChIP-Seq) generating paired end sequence data.

Binning chromatin immunoprecipitation-sequencing reads reveals activating and repressive H3 modifications

For data processing and evaluation, we adopted the workflow outlined in Figure S1. Sequence reads were mapped to the *Arabidopsis* genome using the CLC Genomics Workbench and their number per sample after mapping is summarized in Table S3. Mapped reads were sorted into bins of 200 bp, and fragment reads in the individual bins were counted, generating lists with bincounts per bin. A few regions of the genome contained bins with extremely high bincounts outside of the general distribution of the bincount data that could compromise data normalization. These regions were therefore masked by setting the bincounts of the corresponding bins to zero, followed by normalization of the bincount data by scaling (Methods). Clustering of the bincounts per bin for the seven antibodies and eight samples (two biological replicates) after normalization separates the activating from the repressive chromatin modifications (Figure S3), demonstrating that the main difference in the data is between the activating or repressive functions of the individual chromatin modifications.

Histone modifications are correlated with transcript levels

To correlate the bincounts of the different chromatin modifications with transcript expression values, we first defined the antibody over histone and histone over input ratios for the regions comprising the TSS, gene body and TTS of each gene. The value for the TSS region is the average of the bincounts in the bin comprising the start site and the two proximal bins (promoter region); the value for the TTS region is the average of the bincounts in the bin comprising the termination site and the two distal bins; and the gene body is the average of the bincounts of all bins in between. Because the allocation of bins starts with the TSS region followed by the TTS region and the body, 1069 very short genes had no bins specific for their body. These genes comprise 78 pseudogenes, 217 genes for non-coding RNAs and 774 genes encoding short proteins.

Correlation of chromatin modifications in the regions comprising the TSS, body and TTS with transcript expression data from the same leaf samples plus a third biological replicate reported previously (Baerenfaller *et al.* 2012) revealed that H3K4me3, H3K9ac, H3K27ac and H3S10p are positively correlated with expression in all samples with highest correlation in the gene body, while the correlation for H3S28p is highest in the TTS region. In contrast, H3K9me2 and H3K27me3 are negatively correlated with expression with the most negative correlation in the gene body. Histone density is negatively correlated with transcript expression in the TSS and TTS regions (Fig. 1(b)). The negative correlation of histone density and transcript expression is consistent with the binding of transcription factors to regulatory DNA regions, which causes displacement of nucleosomes and chromatin remodelling and results in DNase I hypersensitivity (ENCODE Project Consortium 2012; Neph *et al.* 2012; Thurman *et al.* 2012).

H3K9ac, H3K27ac and H3S28p modifications in genes with diurnal transcript oscillations vary between EOD and EON

We next asked if genes with significantly different transcript levels between EOD and EON (2105 for SOW and 374 for SWD) have histone modifications that can separate the EOD and EON samples in a principal component analysis. The separation of the EOD and EON samples in the first two principal components for a given histone modification indicates that the difference between modification levels at EOD and EON is a major source of variation in the corresponding data. The results show that the variation in H3K9ac, H3K27ac and H3S28p modifications separate the EOD and EON samples both in SOW and SWD, but more pronounced in SWD (Figures S4, S5). The EOD and EON samples could not be separated when all genes were taken into account or only genes with significantly different expression levels between SOW and SWD. Genes with transcript expression changes between EOD and EON therefore have varying H3K9ac, H3K27ac and H3S28p modifications in the EOD and EON samples.

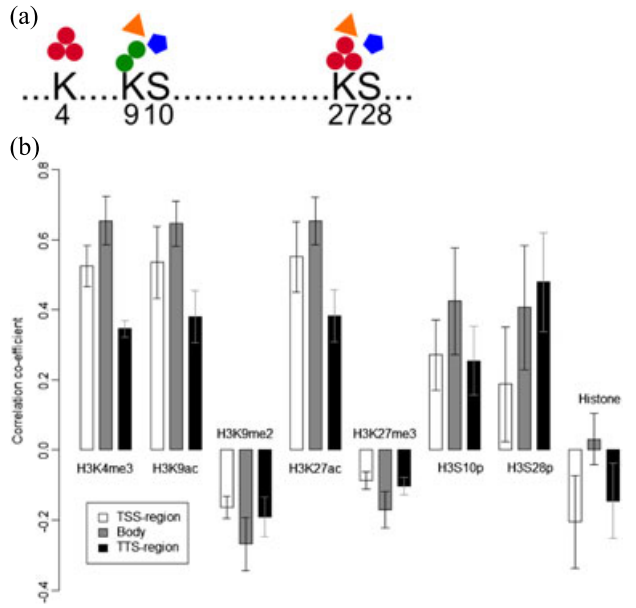

Figure 1. Histone H3 modifications included in this study and their correlation with transcript expression. (a) Modifications of histone H3 included in this study are tri-methylation of K4 (H3K4me3) and K27 (H327me3) (red circles), di-methylation of K9 (H3K9me2) (green circles), acetylation of K9 (H3K9ac) and K27 (H3K27ac) (orange triangle) and phosphorylation at S10 (H3S10p) and S28 (H3S28p) (blue pentagon). (b) Spearman correlation between transcript expression and chromatin modifications expressed as antibody over histone ratios in the regions containing the TSS, TTS or gene body. For histone, the transcript expression values were correlated with the histone over input ratios to detect regions of nucleosome depletion. The indicated values are the mean correlation coefficients over all eight samples and their standard deviations.

The H3K9ac|H3K27ac|H3S28p signature is indicative for genes with differential EOD and EON chromatin modifications

To identify genes with differentially modified chromatin between EOD and EON, we removed stochastic noise from the bincount data to include only significant bins in the analysis. The filtered data were re-normalized, and a data/input threshold was used to retain only enriched bins. We then searched for differentially enriched bins between two different samples and identified genes comprising at least two differentially enriched bins. Using a *P*-value cut-off of 0.05, we included only genes with a significant difference between enriched bins (Methods). When comparing the identified genes with differential EOD and EON chromatin modifications in the SOW and SWD biological replicates, we found the highest replicate consistency for H3K9ac, H3K27ac and H3S28p (Tables S4, S5). Inconsistencies between the biological replicates for the other modifications are likely a result of the complex Chip-Seq protocol that causes higher variability than any true differences in these modifications between the EOD and EON samples, which were all harvested on the same day. Thus, differences in the H3K9ac, H3K27ac and H3S28p modifications are most indicative for chromatin changes between EOD and EON.

We next investigated the extent of co-occurrence of two different modifications in specific genes and found that differential modifications of H3K9ac&H3K27ac co-occurred most often in the SOW and SWD samples, followed by H3K27ac&H3S28p and H3K9ac&H3S28p (Tables S6, S7). Because H3K9ac, H3K27ac and H3S28p alone or in combination differ most between EOD and EON, we defined genes to have a H3K9ac|H3K27ac|H3S28p signature when they had differential chromatin modifications between EOD and EON in H3K9ac, H3K27ac or H3S28p. GO categorization of the genes with the H3K9ac|H3K27ac|H3S28p signature revealed the enrichment of various response pathways and the GO terms *circadian rhythm* and *starch catabolic process* (Table S8). Of the 84 genes assigned to GO category *circadian rhythm,* 43 had a differential chromatin modification between EOD and EON and 36 had the H3K9ac|H3K27ac|H3S28p signature (Table S9). Differential modifications of H3K9ac, H3K27ac or H3S28p in H3K9ac|H3K27ac|H3S28p signature genes were either all higher at EOD or all higher at EON, but never intermixed, which confirms the co-occurrence of these modifications on specific genes at specific times of the day. The 12 genes with higher levels of all three chromatin modifications (H3K9ac& H3K27ac&H3S28p) at EON include the key components of the morning loop *circadian clock associated 1* (*CCA1*, *AT2G46830)* and *late elongated hypocotyl 1* (*LHY*, *AT1G01060*), as well as *night light-inducible and clock-regulated 3* (*LNK3*, *AT3G12320*) and *reveille 1* (*RVE1*, *AT5G17300*). Genes with higher levels of H3K9ac&H3K27ac& H3S28p at EOD were the evening loop components *timing of CAB expression 1* (*TOC1*, *AT5G61380*), *pseudo-response regulator 5* (*PRR5*, *AT5G24470*), *Constans-like 9* (*COL9*, *AT3G0707650*), *glycine-rich protein 7* (*GRP7*, *AT2G21660*), *flavin-binding, kelch repeat, F box 1* (*FKF1*, *AT1G68050*) and *Gigantea* (*GI*, *AT1G22770*). The strong diurnal transcript oscillation of clock genes between EOD and EON therefore involves reversible acetylation and phosphorylation of histone H3.

Of the 21 genes assigned to GO category *starch catabolic process,* 12 have differential chromatin modifications between EOD and EON. Of these, 10 have the H3K9ac|H3K27ac|H3S28p signature with higher modification levels at EOD, and only *beta-amylase 1* (*BAM1*, *AT3G23920*) has higher modification levels at EON (Table S10). The four genes with higher levels of all three chromatin modifications (H3K9ac&H3K27ac&H3S28p) at EOD encode pyruvate phosphate dikinase (GWD1, SEX1, AT1G10760), phosphoglucan, water dikinase (GWD3, PWD, AT5G26570) and dual-specificity protein phosphatase 4 (DSP4, SEX4, AT3G52180), which are key enzymes of starch breakdown involved in transient glucan phosphorylation, as well as disproportionating enzyme 2 (DPE2, AT2G40840), which metabolizes the cytosolic maltose that is produced in starch breakdown (Streb & Zeeman 2012). The H3K9ac|H3K27ac| H3S28p signature is therefore also associated with the diurnal expression of genes that encode starch breakdown proteins.

Differential chromatin modification versus differential expression

When we compared the genes that are differentially expressed with those that have differential chromatin modifications

between EOD and EON, we again found that many genes in the overlap have differential modifications of H3K9ac, H3K27ac or H3S28p. The average expression level of the genes that are differentially expressed between EOD and EON was generally higher for those with the H3K9ac|H3K27ac|H3S28p signature than for those without. However, the distributions were overlapping, and genes in almost the full range of expression levels can have the H3K9ac|H3K27ac|H3S28p signature (Figure S6). Furthermore, almost all of the genes that contain a differential chromatin modification and are also differentially expressed have the H3K9ac|H3K27ac|H3S28p signature (89.2% for SOW and 97.3% for SWD in the replicate intersect; Table S11). The differential H3K9ac|H3K27ac|H3S28p modification signature between EOD and EON also corresponds with differential transcript expression levels between EOD and EON in SOW and SWD, except for two genes in SOW (Fig. 2). Higher levels of modifications in the H3K9ac|H3K27ac|H3S28p signature at EOD are therefore indicative of higher transcript expression at EOD and vice versa. In support of this conclusion, the fold-change in transcript expression between EON and EOD for all genes with a differential H3K9ac|H3K27ac|H3S28p modification signature and higher levels at EON was positive, while it was negative for the genes with higher modification levels of the H3K9ac|H3K27ac|H3S28p signature at EOD. This was not the case for the genes with differential chromatin modifications between EOD and EON that did not have a differential modification of the H3K9ac|H3K27ac|H3S28p signature (Fig. 3, Figure S7).

The H3K9ac|H3K27ac|H3S28p signature is associated with co-expressed genes that have diurnal fluctuation of expression

To obtain an unbiased presentation of gene activities based on general co-expression, we used the ATTED-II database (Obayashi *et al.* 2014) to group *Arabidopsis* genes in a basic set of 384 co-expression clusters with maximum gene coverage and minimal overlap. Genes with differential chromatin marks are over-represented in specific co-expression clusters, and these clusters clearly discriminate between genes showing increased H3K9ac, H3K27ac and H3S28p modification levels at EON or EOD, with the three modifications co-occurring in the respective clusters (Fig. 4, Figure S8). Genes in the top ranking clusters have a diurnal fluctuation of expression in previously reported short-day diurnal expression profiles (Endo *et al.* 2014). Genes in EON clusters have expression maxima at EON and/or the early light period and low expression at EOD and in the early dark period. Genes in EOD clusters show the reciprocal pattern with peak expression at EOD and in the early dark period (Figure S9). Diurnal expression therefore contributes to the co-expression pattern of the selected gene clusters.

H3S10p and H3K4me3 are condition-dependent co-occurring modifications that correlate with increased diurnal transcript level changes

When comparing the SOW and SWD experiments, we found that in SWD, fewer genes were differentially expressed and had differential chromatin modifications between EON and EOD (Fig. 2). The genes with differential modifications in the H3K9ac|H3K27ac|H3S28p signature in both conditions had higher modification levels at either EON or EOD in both experiments (Figure S10). Thus, the general principle of diurnal changes in the H3K9ac|H3K27ac|H3S28p chromatin modification signature is the same in both conditions. However, analysis of the co-occurrence of differential chromatin modifications between EON and EOD on specific genes revealed that in SOW, the pairwise co-occurrences with the second highest values were between H3S10p and H3K9ac, H3K27ac or H3S28p (Table S6), while in SWD, they were between H3K4me3 and H3K9ac, H3K27ac or H3S28p (Table S7). The additional H3S10p and H3K4me3 marks were found both in the genes that showed differential chromatin modifications in the overlap between the SOW and SWD experiment and in those that were specific for one or the other condition (Figure S10). The fold-change in transcript expression between EON and EOD was higher for genes that had the H3S10p mark in addition to the H3K9ac|H3K27ac|H3S28p signature in SOW and the H3K4me3 modification in addition to H3K9ac|H3K27ac|H3S28p in SWD (Fig. 3). This suggests that the additional H3S10p and H3K4me3 modifications are activating and that they depend on the prevailing experimental condition.

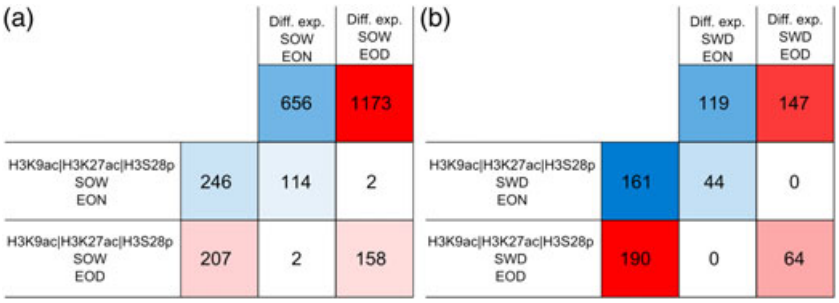

Figure 2. Overlap between differential H3K9ac|H3K27ac|H3S28p modification and differential expression at end of the day (EOD) and end of the night (EON). Diagram of the overlap between genes with the differential H3K9ac|H3K27ac|H3S28p modification pattern in the replicate intersect and higher modification levels either at EON (blue) or EOD (red) and genes that are differentially expressed and have higher expression levels either at EON (blue) or EOD (red) in the (a) short-day optimal water (SOW) and the (b) short-day water deficit (SWD) experiment.

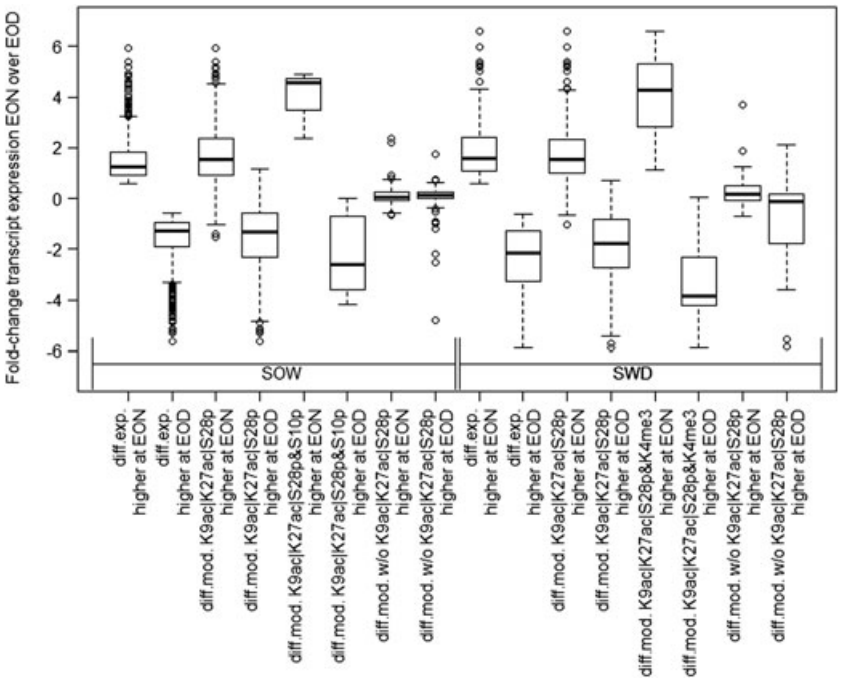

Figure 3. Association between modifications in the H3K9ac|H3K27ac|H3S28p signature and transcript expression at end of the day (EOD) and end of the night (EON). Fold-change between the log-transformed transcript expression values at EON and EOD for the short-day optimal water (SOW) and the short-day water deficit (SWD) experiment for 1) the genes with differential expression values between EON and EOD and higher expression levels at EON or EOD, 2) for the genes in the replicate intersect with differential chromatin modifications between EON and EOD displaying the H3K9ac|H3K27ac|H3S28p pattern and higher modification levels at EON or EOD, 3) for the genes in the replicate intersect with differential chromatin modifications between EON and EOD displaying the H3K9ac|H3K27ac|H3S28p pattern and differential modification of H3S10p for SOW and of H3K4me3 in SWD and higher modification levels at EON or EOD and 4) for the genes in the replicate intersect with differential chromatin modifications between EON and EOD not displaying the H3K9ac|H3K27ac|H3S28p pattern and higher modification levels at EON or EOD.

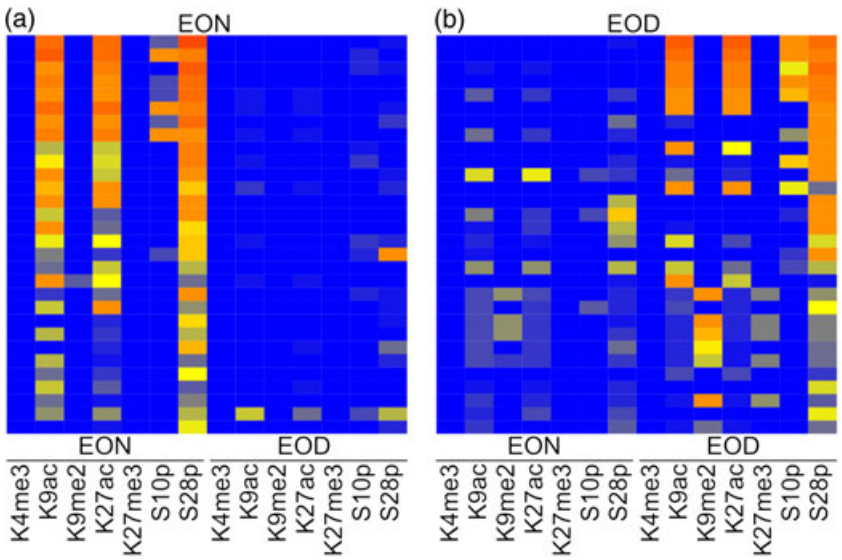

Figure 4. Correlation of the H3K9ac|H3K27ac|H3S28p signature with gene co-expression. Heatmap displaying the over-representation of the genes with the respective differential modifications of histone H3 higher at end of the night (EON) or end of the day (EOD) in the individual clusters. Displayed are the –log10 of the *P*-values from Fisher's exact test (0–2: blue, 2–4: yellow, 4–100: red). Clusters were sorted according to the presence of the H3K9ac, H3K27ac or H3S28p differential modification at (a) EON or (b) EOD, respectively, and the top 30 clusters are displayed.

The genes that have the differential H3K9ac|H3K27ac|H3S28p&H3S10p chromatin modification in SOW and H3K9ac|H3K27ac|H3S28p&H3K4me3 in SWD and that have higher modification levels at EON are *LHY* and *early-responsive to dehydration stress (ERD) family protein* (*AT4G15430*), while those with higher modification levels at

EOD are *PRR5*, *alpha-glucan phosphorylase 2* (*PHS2*, *AT3G46970*), *B-S glucosidase 44* (*BGLU44*, *AT3G18080*) and *thiamin C* (*THIC*, *AT2G29630*). The list of genes with H3K9ac|H3K27ac|H3S28p&H3S10p in SOW or H3K9ac|H3K27ac|H3S28p&H3K4me3 in SWD includes the clock components *CCA1*, *reveille 1* (*RVE1*, *AT5G17300*), *cycling DOF factor 2* (*CDF2*), *GI*, *FKF1*, *GRP7*, as well as *short vegetative phase* (*SVP*, *AT2G22540*) (Table S12). This suggests that strict diurnal transcript expression control of clock and metabolism genes is tuned with additional activating modifications.

Histone H3 acetylation and phosphorylation are distributed differently across genes

For the genes with differential H3K9ac, H3K27ac and H3S28p modifications between EOD and EON and in addition H3S10p for SOW and H3K4me3 for SWD, we investigated where in the gene the significantly enriched bins are located. In both experiments, we found that for most genes, H3K9ac-enriched and H3K27ac-enriched bins mapped mainly to the gene body and TSS region. In contrast, H3S28p-enriched bins are mainly located in the gene body and TTS region (Fig. 5). Correspondingly, the gene regions containing most of the enriched bins in the respective modifications primarily separated the EOD and EON samples in the principal component analysis, while the other regions often showed a separation of the biological replicates (Figures S4, S5). The genes with differential H3S10p in the SOW experiment have the enriched bins mostly in the gene body (Fig. 5(a)), while the differential H3K4me3-enriched bins in SWD are similarly distributed as the H3K9ac and H3K27ac-enriched bins (Fig. 5(b)). Thus, although the acetylated and phosphorylated amino acids in H3 are directly next to each other, they are enriched in different gene regions. It therefore seems unlikely that H3K9ac, H3S10p, H3K27ac and H3S28p co-occur on individual H3 proteins.

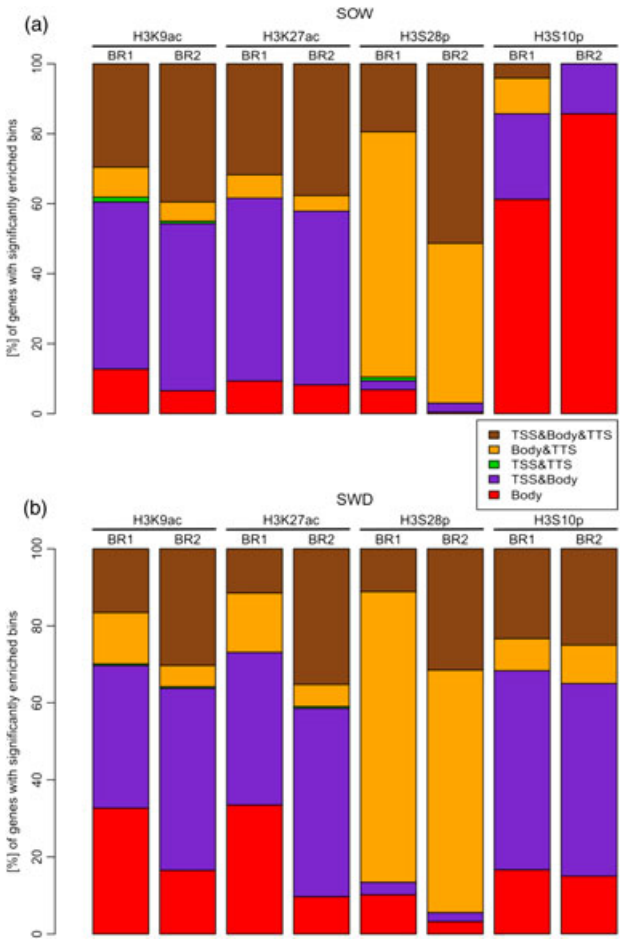

Figure 5. Localization information for the significantly enriched bins. For the significantly enriched bins of the specified antibody, the localization on the genes with differential chromatin modification between end of the day and end of the night in the replicate intersect were assessed. Considered were the regions comprising the TSS, the gene body, the TTS and combinations thereof. There were no significantly enriched bins located solely on the TSS or the TTS regions. TSS, transcription start site; TTS, transcription termination site.

Diurnally controlled transcription factors are associated with the H3K9ac|H3K27ac|H3S28p signature

Considering the diurnal dynamics in the H3K9ac|H3K27ac|H3S28p signature, histone H3 acetylation and phosphorylation must be both reversible within 8 hours and specific for individual genes. This can be achieved by available chromatin modifying complexes together with proteins that tether them to chromatin at the right time of the day. Transcription factors are ideally suited for specific tethering and in fact, genes assigned to GO category sequence-specific DNA binding transcription factor activity are significantly over-represented in genes with the H3K9ac|H3K27ac|H3S28p signature in SOW and SWD with higher modification levels at EON (Table S13). The repressive transcription factors CCA1, LHY and RVE1 belong to this group, and their increased modification and expression level at EON corresponds well with the concomitant repression of their target genes. Genes with higher EOD H3K9ac|H3K27ac|H3S28p modification levels also have a significant over-representation of the evening element (EE) and the canonical CCA1/RVE1 element (Franco-Zorrilla *et al.* 2014), especially in the TSS and promoter regions, as well as in the TTS region. In addition, the non-canonical RVE_a element and G-box are over-represented in the TSS and promoter regions (Table S14). Direct binding of the EE by CCA1, for example, represses TOC1 expression at dawn, and this repression is relieved when CCA1 is degraded during the day (Harmer *et al.* 2000; Alabadí *et al.* 2001). Relief of repression of genes with promoter elements such as EE, CCA1/RVE1 or RVE_a therefore coincides with increased modifications in the H3K9ac|H3K27ac|H3S28p signature. Together, the activity of genes with diurnally oscillating transcript levels thus might not only depend on the combinatorial presence or absence of circadian control transcription factors but also on prevailing histone H3 modifications.

Genes with higher EOD H3K9ac|H3K27ac|H3S28p modification levels include fewer transcription factors, and these transcription factors are not associated with the over-represented

promoter elements found in genes with higher H3K9ac|H3K27ac|H3S28p modification levels at EON. In the genes with higher EON modification levels, over-represented promoter elements include the G-box, the strong G-box-like ABA-responsive element (S_G_ABRE) (Choi *et al.* 2000), the hormone-up-at-dawn element (Michael *et al.* 2008) and the PIF3 element (Franco-Zorrilla *et al.* 2014) (Table S14). These elements are recognized by phytochrome interacting factors (PIFs), which are transcription factors that are phosphorylated and degraded after light activation of phytochromes (Chen & Chory 2011). PIF4 (AT2G43010) has the H3K9ac|H3K27ac|H3S28p signature with higher modification levels at EOD in SOW and SWD as well as the additional H3K4me3 modification in SWD (Tables S12, S13). PIF4 ChIP-Seq identified 4363 PIF4 target genes (Oh *et al.* 2012) that are strongly over-represented among H3K9ac|H3K27ac|H3S28p signature genes (*P*-value = 2.31E-34 for SOW and *P*-value = 3.75E-20 for SWD in a Fisher's exact test, Figure S11). The transcription factor LONG HYPOCOTYL 5 (HY5, AT5G11260) promotes photomorphogenesis downstream of photoreceptors by binding to the G-box in the promoter of target genes (Lee *et al.* 2007), but in contrast to PIFs, HY5 is degraded in the dark (Osterlund *et al.* 2000). Although HY5 is differentially expressed between EOD and EON in SOW with higher mRNA levels at EON, HY5 does not have differential chromatin modifications between EOD and EON. HY5 target genes are also strongly over-represented among the H3K9ac|H3K27ac|H3S28p signature genes (*P*-value = 8.75E-103 for SOW and *P*-value = 9.51E-33 for SWD in a Fisher's exact test). Together, the abundance levels of circadian clock and photoreceptor-controlled transcription factors most likely regulate diurnal expression level changes of their target genes also by timing enzymes that modify the histone H3 H3K9ac|H3K27ac|H3S28p signature.

DISCUSSION

We have established that in the mature *Arabidopsis* leaf 6 grown in SOW, diurnal changes in transcript expression between EON and EOD correlate with changes in a novel histone H3 H3K9ac|H3K27ac|H3S28p signature of their genes. The transcript level fluctuations may contribute to the time-dependent optimization of the cellular energy status (Baerenfaller *et al.* 2012). Accordingly, in long days, fewer genes have significant diurnal transcript changes because in this condition, plants do not need to economize their energy budget (Baerenfaller *et al.* 2015). In contrast, the smaller numbers of genes with significant diurnal transcript fluctuations in SWD plants are likely a consequence of the restricted water regime (Baerenfaller *et al.* 2012). The similar changes of histone H3 H3K9ac, H3K27ac and H3S28p modifications in genes with fluctuating transcript levels in both conditions suggest that the H3K9ac|H3K27ac|H3S28p signature is part of a general mechanism of diurnal transcript level regulation. Moreover, we identified additional condition-dependent activating H3 modifications, namely H3S10p in SOW and H3K4me3 in SWD. Because H3K4me3 is regarded as a strongly activating modification by itself (Bernstein *et al.* 2006; Berger 2007; Liu *et al.* 2010), it appears that under non-optimal growth conditions, the genes that have high diurnal transcript level changes require an additional activation.

Genes with significantly different transcript levels between the morning and evening are by definition active genes, and correspondingly, their expression levels are positively correlated with H3K4me3 and H3K27ac and negatively with H3K9me2 and H3K27me3 modifications. The presence and absence of these modifications therefore mark the general 'activeness' of these genes, but they are not part of a mechanism that regulates their diurnal transcript level fluctuations. We found that diurnal gene expression changes between EOD and EON are correlated with the absence or presence of the novel activating H3K9ac|H3K27ac|H3S28p signature.

Histone modifications modulate various biological processes including the circadian rhythm (Barneche *et al.* 2014; Seo & Mas 2014). The histone acetylation pattern at the TOC1 promoter follows a circadian oscillation that is associated with the rhythmic expression of the gene (Perales & Más 2007). In the morning, TOC1 transcript expression is repressed through binding of the partly redundant transcription factors CCA1 and LHY to the EE promoter element (Harmer *et al.* 2000; Alabadí *et al.* 2001). Transcription factor binding in the TOC1 promoter antagonizes H3 acetylation, most likely by blocking histone acetyltransferase (HAT) accessibility (Stratmann & Más 2008). As CCA1 levels and binding decrease during the day, repression of TOC1 transcription is relieved. TOC1 itself functions as a general repressor of oscillator gene expression through rhythmic association with the promoters of the oscillator genes. In the evening and at night, when TOC1 levels are high, it prevents the activation of morning-expressed genes (Huang *et al.* 2012). The declining phase of TOC1 expression is associated with histone deacetylase (HDAC) activities, resulting in histone H3 hypoacetylation (Perales & Más 2007; Farinas & Mas 2011). Regulation of circadian expression by oscillating histone marks is not exclusive to TOC1 but has also been shown for the core clock genes CCA1, LHY, PRR9, PRR7 and LUX (Hemmes *et al.* 2012; Malapeira *et al.* 2012; Song & Noh 2012; Seo & Mas 2014). We demonstrate that this type of gene expression regulation is not limited to core clock genes but has a broader role in the diurnal regulation of gene expression.

For transcription factors that mainly act as repressors, the following model therefore emerges for the diurnal control of transcript expression. At EON, transcription factors of the morning loop are highly expressed both at transcript and protein levels. The proteins bind to their recognition sequences in the promoters of genes, such as the EE or the canonical CCA1/RVE1 element, which might prevent H3 modification with activating chromatin marks. Consequently, the activity of target genes at EON is suppressed because of the absence or removal of activating chromatin marks, perhaps via a more condensed and less accessible chromatin structure. During the day, the abundance of the repressive transcription factors decreases and hence also their promoter binding. This could either increase the placement of activating modifications or decrease their removal in H3 of their target genes to result in higher EOD transcript levels. Increased expression at

transcript and protein level of evening transcription factors such as TOC1 and PIF4 will lead to increased binding to their promoter recognition sequences, which either suppresses or activates the expression of their target genes. Because mainly genes with higher H3K9ac|H3K27ac|H3S28p modification levels at EOD have promoter elements bound by repressive transcription factors such as CCA1, LHY and RVE1, this mode of regulation seems to be more prevalent for genes with higher transcript expression at EOD.

The association of the H3K9ac|H3K27ac|H3S28p signature with diurnal transcript level changes between EOD and EON is consistent with the over-representation of these H3 modifications in co-expression clusters that contain genes with diurnal expression patterns. The presence of the activating H3K9ac|H3K27ac|H3S28p signature only at times of the day when the corresponding genes are transcribed can also explain why certain genes are regulated differently between day and night. For example, about 70% of the light-inducible genes respond more strongly to a light pulse in the middle of the subjective day than during the subjective night. The light-inducible and clock-regulated (LNK) gene family seems to have a key role in the process. LNK genes are morning phased, more strongly induced by light in the middle of the night, and expression of LNK1 and LNK2 is repressed by TOC1/PRR proteins in the evening (Rugnone *et al.* 2013). Here, we found that of the four LNK genes LNK2 (AT3G54500), LNK3 (AT3G12320) and LNK4 (AT5G06980) have the H3K9ac|H3K27ac|H3S28p signature with higher modification levels at EON. The presence of the activating pattern only during the night can therefore explain why they are especially light-inducible during the night.

The chromatin modification complex(es) responsible for placing and removing the H3K9ac|H3K27ac|H3S28p signature must minimally consist of one or several HATs, HDACs, kinases and phosphatases. The specificities of HATs and HDACs are regulated by interactions with sequence-specific DNA-binding or chromatin regulatory proteins (Marmorstein & Roth 2001; Carrozza *et al.* 2003). However, HATs can also have global acetylating activity independent of recruitment by DNA-binding transcription activators (Imoberdorf *et al.* 2006). The *Arabidopsis* genome encodes 12 HATs and 18 HDACs that are responsible for the deposition and the removal of acetylation marks, respectively. This makes it difficult to rigorously establish which HATs and HDACs are involved in the dynamic regulation of the H3K9ac|H3K27ac|H3S28p signature. A well-characterized HAT required for light-regulated gene expression and growth is general control non-repressible 5 (GCN5, AT3G54610) (Benhamed *et al.* 2006). GCN5 recruitment to promoters is mediated either directly by transcription factor binding to promoters or indirectly through interaction of GCN5-containing complexes with promoter-binding transcription factors, or the bromomodomains of GCN5 can bind directly to acetylated histone tails (Benhamed *et al.* 2008). HDACs were classified in three subfamilies, of which the HD2 (histone deacetylase) family is plant-specific and comprises four proteins (Wu *et al.* 2000, 2003; Dangl *et al.* 2001; Pandey *et al.* 2002). Their transcript levels are higher at EOD in SOW and SWD, with significant changes between EOD and EON for HD2A (AT3G44750), HD2B (AT5G22650) and HD2D (AT2G27840). The only other HDAC with a significant transcript expression change between EOD and EON is HDA2 (AT5G26040), which is also more highly expressed at EOD. This suggests that plant-specific HDACs in particular remove the activating acetylation marks in the evening. All three Aurora kinases in *Arabidopsis* can phosphorylate H3S10, and treatment with the Aurora kinase inhibitor hesparadin inhibits histone H3 phosphorylation (Weimer *et al.* 2016). For example, Aurora1 is responsible for the cell cycle-dependent phosphorylation of H3S10 (Demidov *et al.* 2009). Aurora3 phosphorylates both H3S10 and H3S28, and these phosphorylations are associated with chromosome segregation and metaphase/anaphase transition (Kurihara *et al.* 2006). However, in the Aurora1 and Aurora2 double T-DNA insertion mutant, histone H3 phosphorylation was not impaired (Van Damme *et al.* 2011). Furthermore, plant Aurora kinases are considered mitotic kinases because their expression levels are highest during mitosis in actively dividing cells (Weimer *et al.* 2016). It therefore seems rather unlikely that they also have a role outside of the cell cycle in the regulation of gene expression. In mammalian cells, H3S10 and H3S28 are phosphorylated in response to EGF treatment by the kinases RSK2, MSK1, MSK2 and MLTK-α (Rossetto *et al.* 2012). Human MSK1 is a direct transcription activator. Phosphorylation of H3S28 correlates with transcription initiation and can also induce a methyl-acetylation switch in the adjacent K27 residue (Lau & Cheung 2011). The *Arabidopsis* MSK homologues are the S6 kinases S6K1 and S6K2, which are part of the TOR signalling pathway (Mahfouz *et al.* 2006). Further research is required to establish if these kinases phosphorylate histone H3 and if this phosphorylation regulates gene expression.

TOC1 diurnal transcript levels correlate with circadian rhythms in the binding of chromatin remodelling factors such as SSRP1 and SPt16 of the facilitates chromatin transcription (FACT) complex that functions as transcriptional co-activators (Perales & Más 2007). Co-activation by FACT may occur via nucleosome destabilization, which facilitates RNA polymerase II passage, although FACT is absent in terminally differentiated cells (Duroux *et al.* 2004). In fully expanded leaves, other factors may therefore be needed to facilitate transcription. The activity of chromatin modification complexes may also be regulated by post-translational modifications. For example, the HDACs HD2A and HD1 (HDA19, AT4G38130) as well as GCN5 can be phosphorylated (Bigeard *et al.* 2014). GCN5 also interacts specifically with a phosphatase 2C protein (AtPP2C-6-6) and can be phosphorylated by a nuclear protein kinase, potentially SNF1 (Servet *et al.* 2008). Although we have identified concerted H3K9ac|H3K27ac|H3S28p modifications as a novel chromatin signature for diurnally regulated genes, the mechanism for the timely placement and removal of the H3K9ac|H3K27ac|H3S28p signature and the composition of the responsible chromatin modification complexes are currently not known and will need to be elucidated in future research.

ACKNOWLEDGMENTS

This work was supported by ETH Zurich and in part by the AGRON-OMICS integrated project funded in the European Framework Programme 6 (LSHG-CT-2006-037704) awarded to W.G.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR'S CONTRIBUTIONS

W.G. designed the research, H.S. performed the experiment, L. O. and H.R processed the data, K.B., M.H-H., J.F. and W.G. analysed and interpreted the data, K.B. wrote the manuscript, J.F., L.H. and W.G. edited the manuscript and all authors approved the final version.

REFERENCES

Alabadí D., Oyama T., Yanovsky M.J., Harmon F.G., Más P. & Kay S.A. (2001) Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. *Science* **293**, 880–883.

Baerenfaller K., Massonnet C., Hennig L., Russenberger D., Sulpice R., Walsh S., … Gruissem W. (2015) A long photoperiod relaxes energy management in Arabidopsis leaf six. *Current Plant Biology* **2**, 34–45.

Baerenfaller K., Massonnet C., Walsh S., Baginsky S., Bühlmann P., Hennig L., … Gruissem W. (2012) Systems-based analysis of Arabidopsis leaf growth reveals adaptation to water deficit. *Molecular Systems Biology* **8**, 606.

Barneche F., Malapeira J. & Mas P. (2014) The impact of chromatin dynamics on plant light responses and circadian clock function. *Journal of Experimental Botany* 2895–2913.

Bauer S., Grossmann S., Vingron M. & Robinson P.N. (2008) Ontologizer 2.0 – a multifunctional tool for GO term enrichment analysis and data exploration. *Bioinformatics* **24**, 1650–1651.

Benhamed M., Bertrand C., Servet C. & Zhou D.-X. (2006) Arabidopsis GCN5, HD1, and TAF1/HAF2 interact to regulate histone acetylation required for light-responsive gene expression. *The Plant Cell* **18**, 2893–2903.

Benhamed M., Martin-Magniette M.L., Taconnat L., Bitton F., Servet C., De Clercq R., … Hilson P. (2008) Genome-scale Arabidopsis promoter array identifies targets of the histone acetyltransferase GCN5. *The Plant Journal* **56**, 493–504.

Benjamini Y. & Hochberg Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B* **57**, 289–300.

Berger S.L. (2007) The complex language of chromatin regulation during transcription. *Nature* **447**, 407–412.

Bernstein B.E., Mikkelsen T.S., Xie X., Kamal M., Huebert D.J., Cuff J., … Lander E.S. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**, 315–326.

Bertrand C., Benhamed M., Li Y.-F., Ayadi M., Lemonnier G., Renou J.-P., … Zhou D.-X. (2005) Arabidopsis HAF2 gene encoding TATA-binding protein (TBP)-associated factor TAF1, is required to integrate light signals to regulate gene expression and growth. *The Journal of Biological Chemistry* **280**, 1465–1473.

Bigeard J., Rayapuram N., Bonhomme L., Hirt H. & Pflieger D. (2014) Proteomic and phosphoproteomic analyses of chromatin-associated proteins from Arabidopsis thaliana. *Proteomics* **14**, 2141–2155.

Booth B. (2010) Set::IntervalTree-0.01. Retrieved from http://search.cpan.org

Carrozza M.J., Utley R.T., Workman J.L. & Côté J. (2003) The diverse functions of histone acetyltransferase complexes. *Trends in Genetics* **19**, 321–329.

Charron J.-B.F., He H., Elling A.A. & Deng X.W. (2009) Dynamic landscapes of four histone modifications during deetiolation in Arabidopsis. *The Plant Cell* **21**, 3732–3748.

Chen M. & Chory J. (2011) Phytochrome signaling mechanisms and the control of plant development. *Trends in Cell Biology* **21**, 664–671.

Choi H., Hong J., Ha J., Kang J. & Kim S.Y. (2000) ABFs, a family of ABA-responsive element binding factors. *The Journal of Biological Chemistry* **275**, 1723–1730.

Chua Y.L., Watson L.A. & Gray J.C. (2003) The transcriptional enhancer of the pea plastocyanin gene associates with the nuclear matrix and regulates gene expression through histone acetylation. *The Plant Cell* **15**, 1468–1479.

Dangl M., Brosch G., Haas H., Loidl P. & Lusser A. (2001) Comparative analysis of HD2 type histone deacetylases in higher plants. *Planta* **213**, 280–285.

Demidov D., Hesse S., Tewes A., Rutten T., Fuchs J., Ashtiyani R.K., … Houben A. (2009) Aurora1 phosphorylation activity on histone H3 and its cross-talk with other post-translational histone modifications in Arabidopsis. *The Plant Journal : For Cell and Molecular Biology* **59**, 221–230.

Duroux M., Houben A., Růzicka K., Friml J. & Grasser K.D. (2004) The chromatin remodelling complex FACT associates with actively transcribed regions of the Arabidopsis genome. *The Plant Journal : For Cell and Molecular Biology* **40**, 660–671.

ENCODE Project Consortium (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**, 57–74.

Endo M., Shimizu H., Nohales M.A., Araki T. & Kay S.A. (2014) Tissue-specific clocks in Arabidopsis show asymmetric coupling. *Nature* **515**, 419–422.

Farinas B. & Mas P. (2011) Functional implication of the MYB transcription factor RVE8/LCL5 in the circadian control of histone acetylation. *The Plant Journal* **66**, 318–329.

Franco-Zorrilla J.M., López-Vidriero I., Carrasco J.L., Godoy M., Vera P. & Solano R. (2014) DNA-binding specificities of plant transcription factors and their potential to define target genes. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 2367–2372.

Fry C.J., Shogren-Knaak M.A. & Peterson C.L. (2004) Histone H3 amino-terminal tail phosphorylation and acetylation: synergistic or independent transcriptional regulatory marks? *Cold Spring Harbor Symposia on Quantitative Biology* **69**, 219–226.

Granier C., Aguirrezabal L., Chenu K., Cookson S.J., Dauzat M., Hamard P., … Tardieu F. (2006) PHENOPSIS, an automated platform for reproducible phenotyping of plant responses to soil water deficit in Arabidopsis thaliana permitted the identification of an accession with low sensitivity to soil water deficit. *New Phytol* **169**, 623–635.

Granot G., Sikron-Persi N., Li Y. & Grafi G. (2009) Phosphorylated H3S10 occurs in distinct regions of the nucleolus in differentiated leaf cells. *Biochimica et Biophysica Acta – Gene Regulatory Mechanisms* **1789**, 220–224.

Harmer S.L., Hogenesch J.B., Straume M., Chang H.S., Han B., Zhu T., … Kay S. A. (2000) Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. *Science* **290**, 2110–2113.

Hemmes H., Henriques R., Jang I.C., Kim S. & Chua N.H. (2012) Circadian clock regulates dynamic chromatin modifications associated with arabidopsis CCA1/LHY and TOC1 transcriptional rhythms. *Plant and Cell Physiology* **53**, 2016–2029.

Houben A., Demidov D., Caperta A.D., Karimi R., Agueci F. & Vlasenko L. (2007) Phosphorylation of histone H3 in plants – a dynamic affair. *Biochimica et Biophysica Acta – Gene Structure and Expression* 308–315.

Huang W., Perez-Garcia P., Pokhilko A., Millar A.J., Antoshechkin I., Riechmann J.L. & Mas P. (2012) Mapping the core of the Arabidopsis circadian clock defines the network structure of the oscillator. *Science* 75–79.

Imoberdorf R.M., Topalidou I. & Strubin M. (2006) A role for gcn5-mediated global histone acetylation in transcriptional regulation. *Molecular and Cellular Biology* **26**, 1610–1616.

Karlić R., Chung H.-R., Lasserre J., Vlahovicek K. & Vingron M. (2010) Histone modification levels are predictive for gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 2926–2931.

Kouzarides T. (2007) Chromatin modifications and their function. *Cell* **128**, 693–705.

Kurihara D., Matsunaga S., Kawabe A., Fujimoto S., Noda M., Uchiyama S. & Fukui K. (2006) Aurora kinase is required for chromosome segregation in tobacco BY-2 cells. *The Plant Journal : For Cell and Molecular Biology* **48**, 572–580.

Lau P.N.I. & Cheung P. (2011) Histone code pathway involving H3 S28 phosphorylation and K27 acetylation activates transcription and antagonizes polycomb silencing. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 2801–2806.

Lee J., He K., Stolc V., Lee H., Figueroa P., Gao Y., … Deng X.W. (2007) Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. *The Plant Cell* **19**, 731–749.

Lee J.-S., Smith E. & Shilatifard A. (2010) The language of histone crosstalk. *Cell* **142**, 682–685.

Li J., Terzaghi W. & Deng X.W. (2012) Genomic basis for light control of plant development. *Protein & Cell* **3**, 106–116.

Li Y., Butenko Y. & Grafi G. (2005) Histone deacetylation is required for progression through mitosis in tobacco cells. *The Plant Journal : For Cell and Molecular Biology* **41**, 346–352.
Liu C., Lu F., Cui X. & Cao X. (2010) Histone methylation in higher plants. *Annual Review of Plant Biology* **61**, 395–420.
Mahfouz M.M., Kim S., Delauney A.J. & Verma D.P.S. (2006) Arabidopsis target of rapamycin interacts with raptor, which regulates the activity of S6 kinase in response to osmotic stress signals. *The Plant Cell* **18**, 477–490.
Malapeira J., Khaitova L.C. & Mas P. (2012) Ordered changes in histone modifications at the core of the Arabidopsis circadian clock. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 21540–21545.
Marmorstein R. & Roth S.Y. (2001) Histone acetyltransferases: function, structure, and catalysis. *Current Opinion in Genetics and Development* **11**, 155–161.
Michael T.P., Breton G., Hazen S.P., Priest H., Mockler T.C., Kay S.A. & Chory J. (2008) A morning–specific phytohormone gene expression program underlying rhythmic plant growth. *PLoS Biology* **6**, 1887–1898.
Nair N.U., Das S.A., Bucher P. & Moret B.M.E. (2012) Chipnorm: a statistical method for normalizing and identifying differential regions in histone modification chip-seq libraries. *PLoS ONE* **7**, e39573.
Neph S., Vierstra J. & Stergachis A. (2012) An expansive human regulatory lexicon encoded in transcription factor footprints. *Nature* **489**, 83–90.
Obayashi T., Okamura Y., Ito S., Tadaka S., Aoki Y., Shirota M. & Kinoshita K. (2014) ATTED-II in 2014: evaluation of gene coexpression in agriculturally important plants. *Plant and Cell Physiology* **55**, e6.
Oh E., Zhu J.-Y. & Wang Z.-Y. (2012) Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. *Nature Cell Biology* 802–809.
Osterlund M.T., Hardtke C.S., Wei N. & Deng X.W. (2000) Targeted destabilization of HY5 during light-regulated development of Arabidopsis. *Nature* **405**, 462–466.
Pandey R., Müller A., Napoli C.A., Selinger D.A., Pikaard C.S., Richards E.J., … Jorgensen R.A. (2002) Analysis of histone acetyltransferase and histone deacetylase families of Arabidopsis thaliana suggests functional diversification of chromatin modification among multicellular eukaryotes. *Nucleic Acids Research* **153**, 5036–5055.
Perales M. & Más P. (2007) A functional link between rhythmic changes in chromatin structure and the Arabidopsis biological clock. *The Plant Cell* **19**, 2111–2123.
Pfluger J. & Wagner D. (2007) Histone modifications and dynamic regulation of genome accessibility in plants. *Current Opinion in Plant Biology* **10**, 645–652.
R Core Team. (2015) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria Retrieved from http://www.r-project.org.
Rossetto D., Avvakumov N. & Côté J. (2012) Histone phosphorylation: a chromatin modification involved in diverse nuclear events. *Epigenetics* **7**, 1098–1108.
Rugnone M.L., Faig A., Sanchez S.E., Schlaen R.G., Hernando C.E., Danelle K., … Más P. (2013) LNK genes integrate light and clock signaling networks at the core of the Arabidopsis oscillator. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 12120–12125.
Ruthenburg A.J., Li H., Patel D.J. & Allis C.D. (2007) Multivalent engagement of chromatin modifications by linked binding modules. *Nature Reviews. Molecular Cell Biology* **8**, 983–994.
Schroeder D.F., Gahrtz M., Maxwell B.B., Cook R.K., Kan J.M., Alonso J.M., … Chory J. (2002) De-etiolated 1 and damaged DNA binding protein 1 interact to regulate arabidopsis photomorphogenesis. *Current Biology* **12**, 1462–1472.
Seo P.J. & Mas P. (2014) Multiple layers of posttranslational regulation refine circadian clock activity in Arabidopsis. *The Plant Cell* **26**, 79–87.
Servet C., Benhamed M., Latrasse D., Kim W., Delarue M. & Zhou D.X. (2008) Characterization of a phosphatase 2C protein as an interacting partner of the histone acetyltransferase GCN5 in Arabidopsis. *Biochimica et Biophysica Acta – Gene Regulatory Mechanisms* **1779**, 376–382.
Shahbazian M.D. & Grunstein M. (2007) Functions of site-specific histone acetylation and deacetylation. *Annual Review of Biochemistry* **76**, 75–100.
Shu H., Nakamura M., Siretskiy A., Borghi L., Moraes I., Wildhaber T., … Hennig L. (2014) Arabidopsis replacement histone variant H3.3 occupies promoters of regulated genes. *Genome Biology* **15**, R62.
Song H.R. & Noh Y.S. (2012) Rhythmic oscillation of histone acetylation and methylation at the arabidopsis central clock loci. *Molecules and Cells* **34**, 279–287.
Stratmann T. & Más P. (2008) Chromatin, photoperiod and the Arabidopsis circadian clock: a question of time. *Seminars in Cell and Developmental Biology* **19**, 554–559.
Streb S. & Zeeman S.C. (2012) Starch metabolism in Arabidopsis. *Arabidopsis Book* **10**, e0160.
Thurman R., Rynes E. & Humbert R. (2012) The accessible chromatin landscape of the human genome. *Nature* **489**, 75–82.
Van Damme D., De Rybel B., Gudesblat G., Demidov D., Grunewald W., De Smet I., … Russinova E. (2011) *Th* Arabidopsis α Aurora kinases function in formative cell division plane orientation *e*. *The Plant Cell* **23**, 4013–4024.
Van Zanten M., Tessadori F., Peeters A.J.M. & Fransz P. (2012) Shedding light on large-scale chromatin reorganization in Arabidopsis thaliana. *Molecular Plant* **5**, 583–590.
Weimer A.K., Demidov D., Lermontova I., Beeckman T. & Van Damme D. (2016) Aurora kinases throughout plant development. *Trends in Plant Science* **21**, 69–79.
Wu K., Tian L., Malik K., Brown D. & Miki B. (2000) Functional analysis of HD2 histone deacetylase homologues in Arabidopsis thaliana. *The Plant Journal* **22**, 19–27.
Wu K., Tian L., Zhou C., Brown D. & Miki B. (2003) Repression of gene expression by Arabidopsis HD2 histone deacetylases. *The Plant Journal* **34**, 241–247.
Zhou D.X. (2009) Regulatory mechanism of histone epigenetic modifications in plants. *Epigenetics* **4**, 15–18.

Received 26 April 2016; received in revised form 26 July 2016; accepted for publication 27 July 2016

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supporting Information 1: PDF document with Tables S1-S14 and Figures S1-S11 with descriptions in the corresponding table headers and figure legends.

Supporting Information 2: Table with genes with differentially modified chromatin between EOD and EON in the replicate union and intersect in the SOW and SWD experiment.

Supporting Information 3: Table with genes with significantly differential expression between EOD and EON in the SOW and the SWD experiment and between SOW and SWD.