NEW INSIGHTS INTO MECHANISMS OF EPIGENETIC MODIFIERS IN PLANT GROWTH AND DEVELOPMENT

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NEW INSIGHTS INTO MECHANISMS OF EPIGENETIC MODIFIERS IN PLANT GROWTH AND DEVELOPMENT

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Editorial: New Insights Into Mechanisms of Epigenetic Modifiers in Plant Growth and Development

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Editorial on the Research Topic

New Insights Into Mechanisms of Epigenetic Modifiers in Plant Growth and Development

In eukaryotic cells, chromatin, a highly dynamic nucleoprotein complex, plays a critical role in controlling gene expression notably by regulating the interaction between transcription factors and regulatory elements. The structure of the chromatin is determined by epigenetic mechanisms, including DNA methylation, histone modifications, and chromatin remodeling. A growing body of evidence indicates that epigenetic regulations are involved in plant adaptation to environmental stresses, and in plant development, including flowering control, fruit and root development, as well as seed maturation and germination. Furthermore, epigenetic mechanisms have the potential to stabilize cell identity and maintain tissue organization. Hence, epigenetic diversity is now emerging as a new source of phenotypic variation to improve adaptation to changing environment and ensure yield and quality of crops. The 14 articles published in this Research Topic highlight recent progresses, opinions, and reviews to advance our knowledge in the role of the epigenome on controlling plant development, plant response to environmental stresses, and plant evolution. For instance, gene duplication and chromatin remodeling contribute to increase the morphological and cellular complexity of plants during their evolution according to Hajheidari et al.

Chromatin modifications, including DNA methylation and histone modifications, are critical in regulating gene transcription, and thus may reprogram cell differentiation and development (Inácio et al.; Zhang et al.; Hajheidari et al.). For instance, Inácio et al. immunolocalized various epigenetic marks and correlated epigenomic changes with transcriptional regulation when studying cork formation and quality in cork oak, a genuinely forest-specific process. Furthermore, changes in the acetylation levels of the lysine 9 of the histone H3 (H3K9) and lysine 5 of the histone H4 (H4K5) were found associated with the heat stress-dependent inhibition of lateral root formation in maize

(Zhang et al.). Interestingly, whereas a global increase in histone acetylation was observed in response to heat stress, H3K9 and H4K5 acetylation decreased significantly in the promoter region of the haem oxygenase-1 (*ZmHO-1*) and giberellic acid–stimula ted like-1 (*ZmGSL-1*) genes, two inhibitors of lateral root forma tion (Zhang et al.).

Plant cells have the capability to dedifferentiate in totipotent cells, a prerequisite to asexual embryogenesis. Recent papers support a role of histone deacetylation and DNA methylation in cellular reprogramming leading to callus formation and asexual embryogenesis through the regulation of key developmental genes such as Wuschel (Pasternak and Dudits). In addition to somatic embryogenesis, the epigenome also controls the juvenile-to-adult developmental transition notably by modula ting the expression of regulatory genes. Indeed, in Arabidopsis plants, this transition is regulated by miR156/157 and its ta rget-squamosa promoter binding protein-like gene (Xu et al.). Other epigenetic changes controlling the juvenile-to-adult developmental transition include DNA methylation, and histone modification (Xu et al.). Ultimately, these chemical cha nges lead to a remodeling of the chromatin. The SWI/SNF chromatin remodeling complexes play a central role in this biological process by controlling phytohormone biosynthesis, the establishment and maintenance of meristems, organ development, and floral transition (Ojolo et al.; Maury et al.). Supporting the central role of chromatin remodeling and histone modifications in controlling development of plant, Kang et al. studied the role of the chromatin-remodeling factor inositol auxotrophy 80 and the histone chaperones nap1-related protein 1 and 2 in modulating auxin fluxes and the activity of the inflorescence and root apical meristems. Another interesting study highlights the impact of the epigenome in controlling tra nscriptional initiation. The single-stranded DNA-binding protein whirly1 promoted the acetylation of H3K9 and repressed the trimethylation of H3K4 to enhance the recruitment of the RNA polymerase II on the wrky53 promoter (Huang et al.).

Epigenetic alterations also control the response of plants to environmental stresses including light perception and various abiotic stresses (e.g. salinity, drought, UV-B radiation, temperature, and heavy metal toxicity). As described by Lee et al. the circadian regulation of two proteins of the Sin3-histone deacetylase complex, encoded by SAP30 function-related 1 (*AFR1*) and *AFR2* genes, is critical for the proper regulation of Arabidopsis circadian rhythm. These two proteins directly bind to the circadaian clock associated 1 (*CCA1*) and pseudo-response regulator 9 (*PRR9*) promoters in order to locally deacetylate the histone H3 and negatively affect their expression. This is just a first level of the epigenetic regulation of the Arabidopsis circadia n clock. Indeed, Hung et al. described a more complex tra nscriptional regulation of the circadian clock: the recruitment of the lysine-specific demethylase 1 (LSD1)-like 1/2 (LDL1/2) and histone deacetylase 6 (HDA6) proteins by circadian clock associated 1 (CCA1)/late elongated hypocotyl (LHY) is needed to repress the expression of timing of cab expression 1 (TOC1). Acting as a negative feedback regulatory loop, TOC1 also intera cts with LDL1/2 and HDA6 proteins to repress the expression of *CCA1/LHY*. A broader picture of the role of the epigenome on the plant circadian clock is provided in the Du et al. review paper.

Environmental stresses also induce the formation of stress-responding agents such as nitric oxide. In soybean, Sun et al. revealed that the *de novo* deposition of trimethylated histone H3 lysine 27 residue in the promoter and coding sequence of plant genes is needed to repress their transcription in response to salt stress. Mechanistically, Ageeva-Kieferle et al. described in their review the role of nitric oxide as inhibitors of histone deacetylase through the S-nitrosation of selected cysteine residues. Nitric oxide also regulates the epigenome by controlling the expression of genes encoding DNA and histone methyltransferases and demethylases. Taken together, nitric oxide is a chemical agent controlling plant gene activity in response to environmental stresses notably by regulating the activity of various histone acetyltransferases, deacetylases, methyltransferases and demethylases, and DNA methyl transferases and demethylases.

CONCLUDING REMARKS

This special topic clearly highlights the central role of the epigenome in the regulation of gene expression that influences many plant biological processes such as plant development and plant response to environmental stresses. A deeper analysis of the chromatin remodeling and transcription related mechanisms will be needed to better understand the epigenetic regulation of gene expression. Single cell -omic technologies such as single cell RNA-seq and ATAC-seq will enable further discoveries by capturing the transcriptome and epigenome for each cell composing a complex organ. While single cell RNA-seq was recently applied on Arabidopsis root protoplasts, there is a need to develop plant single cell ATAC-seq technology to gain a more complete picture of the plant cell epigenome.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Epigenetic Regulation of Juvenile-to-Adult Transition in Plants

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Epigenetic regulation is referred to as changes in gene function that do not involve changes in the DNA sequence, it is usually accomplished by DNA methylation, histone modifications (repressive marks such as H3K9me, H3K27me, H2Aub, or active marks such as H3K4me, H3K36me, H3Ac), and chromatin remodeling (nucleosome composition, occupancy, and location). In plants, the shoot apex produces different lateral organs during development to give rise to distinguishable phases of a juvenile, an adult and a reproductive phase after embryogenesis. The juvenile-to-adult transition is a key developmental event in plant life cycle, and it is regulated by a decrease in the expression of a conserved microRNA-miR156/157, and a corresponding increase in the expression of its target genes encoding a set of plant specific SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) proteins. Recent work has revealed that the miR156/157-SPL pathway is the master regulator of juvenile-to-adult transition in plants, and genes in this pathway are subjected to epigenetic regulation, such as DNA methylation, histone modifications, and chromatin remodeling. In this review, we summarized the recent progress in understanding the epigenetic regulation of the miR156/157-SPL pathway during juvenile-to-adult transition and bring forward some perspectives of future research in this field.

Keywords: epigenetic regulation, miR156, SPL, juvenile-to-adult transition, plants

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INTRODUCTION

Unlike mammals, in which organ formation is completed during embryonic development, plants produce new organs from self-sustaining stem cell populations known as meristems in different developmental processes. In plants, post-embryonic development can be divided into a juvenile vegetative phase, an adult vegetative phase and a reproductive phase, and each developmental phase is marked by changes in a series of distinct phase-specific traits (Poethig, 1990; Kerstetter and Poethig, 1998). The transition from the juvenile vegetative phase to the adult vegetative phase was referred to as the juvenile-to-adult transition or vegetative phase change.

In *Arabidopsis*, the juvenile-to-adult transition is characterized by the formation of leaf abaxial trichomes, an increase in leaf length/width ratio and serration, and a decrease in cell size (Telfer et al., 1997; Tsukaya et al., 2000; Usami et al., 2009). Genetic and molecular analyses demonstrated that the conserved miRNA-miR156/157 and its target genes-*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes act sequentially with miR172, another miRNA that targets a class of *AP2*-like transcription factors (TFs), to regulate juvenile-to-adult transition in plants (Wu and Poethig, 2006; Wu et al., 2009; He et al., 2018). miR156/157 is highly expressed in juvenile phase and its abundance declines gradually, while its target *SPL* genes increases during shoot development.

miR156/157 negatively regulates *SPL* gene expression through transcript cleavage or translational inhibition. *SPLs* were also responsive to photoperiodic induction and exhibited an miR156/157-independent expression pattern (Schmid et al., 2003; Jung et al., 2012). Therefore, the outcome of SPL levels fine-tuned by both miR156/157 and exogeneous cues orchestrates the timing of juvenile-to-adult transition (Huijser and Schmid, 2011; Poethig, 2013).

The Arabidopsis genome encodes eight miR156 genes (*MIR156A*∼*H*) and four miR157 genes (*MIR156A*∼*D*), and those genes function redundantly. The *mir156a mir156c* double mutant exhibited a similar phenotype to the *35S::MIMICRY156* transgenic plants with significantly reduced levels of miR156, which indicates that *MIR156A* and *MIR156C* are the two main loci contributing to the level of miR156 and have dominant roles in vegetative phase change within the miR156 family in *Arabidopsis* (Yang L. et al., 2013; Yu et al., 2013). miR157 functions redundantly with miR156, but has a much smaller effect on shoot morphology and *SPL* gene expression than miR156 (He et al., 2018). miR156/157 targets 10 out of 16 different *SPL* genes in *Arabidopsis*. Based on the amino acid sequence of the SBP domain, the miR156/157-targeted *SPL* genes can be classified into five clades, *SPL3/SPL4/SPL5*, *SPL9/SPL15*, *SPL2/SPL10/SPL11*, *SPL6*, and *SPL13A/B* (Xie et al., 2006; Riese et al., 2007; Preston and Hileman, 2013). Genetic and functional analysis of the role of *SPL* genes in vegetative phase change indicated that *SPL2/SPL9/SPL10/SPL11/SPL13/SPL15*, but not *SPL3/4/5/6*, contribute to the juvenile-to-adult transition with *SPL9/SPL13/SPL15* being more important for juvenile-to-adult transition than *SPL2/SPL10/SPL11* (Xu et al., 2016a).

As the master regulator of the juvenile-to-adult transition, miR156/157-SPL pathway has been shown to be subjected to transcriptional and post-transcriptional regulation. Those include the transcriptional regulation of *pri-MIR156/157* and *SPLs* genes, the regulation of miR156/157 biogenesis, and post-transcriptional regulation of *SPL* genes (**Figure 1**). Here, we review our current understanding of epigenetic regulation of the miR156/157-SPL pathway and the roles of corresponding players in juvenile-to-adult transition in plants.

DNA METHYLATION

DNA methylation [5-Methylcytosine (5mC)] is a hallmark of epigenetic gene silencing in both plants and mammals (Feng et al., 2010; Law and Jacobsen, 2010). DNA methylation is found at CG or non-CG sites including CHH and CHG (H represents A, T, or C) in plants in contrast to CG sites only in mammals (Henderson and Jacobsen, 2007; Cokus et al., 2008). In plants, CG methylation is carried out by DNA METHYLTRANSFERASE 1 (MET1), whereas DOMAINS-REARRANGED METHYLTRANSFERASEs (DRM) and CHROMOMETHYLASE 3 (CMT3) are responsible for the non-CG methylation (Law and Jacobsen, 2010).

The first indication of DNA methylation plays a role in phases of shoot development comes from the work done by Brink. In the 1950s, Brink noticed the similarity between phase change in plants and changes in cell states in non-plant organisms, he proposed that phases of shoot development might be regulated by reversible changes in chromatin based on his research on paramutation in maize (Brink, 1962). Subsequent work on *Spm* transposable elements (Banks and Fedoroff, 1989) and the Robertson's Mutator (*Mu*) element (Martienssen et al., 1990) suggest that DNA methylation may be the underlying mechanism for maintaining phases of shoot development in plants. Recent work in peach also demonstrated that levels of nuclear DNA methylation was higher in adult meristems than that in juvenile and juvenile-like meristems (Bitonti et al., 2002), and an increase in DNA methylation during development seems widespread in plants (Fraga et al., 2002; Ruiz-García et al., 2005). In *Arabidopsis*, the triple DNA methyltransferase mutant *drm1 drm2 cmt3* exhibited a developmental retardation phenotype (Cao and Jacobsen, 2002), indicating that DNA methylation is important for normal growth and development in plants. However, genome-wide DNA methylation analysis of 5-week-old Columbia wild type, *met1* and *drm1 drm2 cmt3* triple mutant (Zhang et al., 2006), and 25-day-old Columbia wild type (Zilberman et al., 2007) indicated that only the coding sequence *of* the *SPL10* gene contains non-CG methylation. These results suggest that genes upstream or downstream of the miR156/157-SPL pathway, instead of miR156/157 or *SPL* genes, might be regulated by DNA methylation. Therefore, phenotypic characterization of vegetative phase change phenotype of mutants of DNA methyltransferases (MET1, DRM, and DNMT2) or demethylation enzymes (ROS1, DME, DML2, and DML3), as well as bisulfite sequencing of *MIR156/157* and *SPLs* loci, will facilitate to uncover the role of DNA methylation in regulation of miR156/157-SPL pathway and juvenile-to-adult transition in plants.

HISTONE MODIFICATION

Histone modification at specific lysine sites functions as transcription repressive marks such as H3K9me, H3K27me, H2Aub, etc., or active marks such as H3K4me, H3K36me, H3Ac, etc., this modification is catalyzed by Polycomb group (PcG) protein complexes and Trithorax group (TrxG) protein complexes, respectively (Pien and Grossniklaus, 2007; Köhler and Hennig, 2010; Grossniklaus and Paro, 2014; Kingston and Tamkun, 2014). PcG complexes are repressors of gene transcription, and function in multi-subunit complexes, such as Polycomb Repressor Complex 1 (PRC1) or Polycomb Repressor Complex 2 (PRC2) (Grossniklaus and Paro, 2014).

PRC2 AND H3K27me3 MODIFICATION

PRC2 is a highly conserved and well-characterized PcG complex, and it represses target gene expression by trimethylating histone H3 at lysine 27 (H3K27me3) through the E(z) SET domain (Köhler and Hennig, 2010; Grossniklaus and Paro, 2014). In the

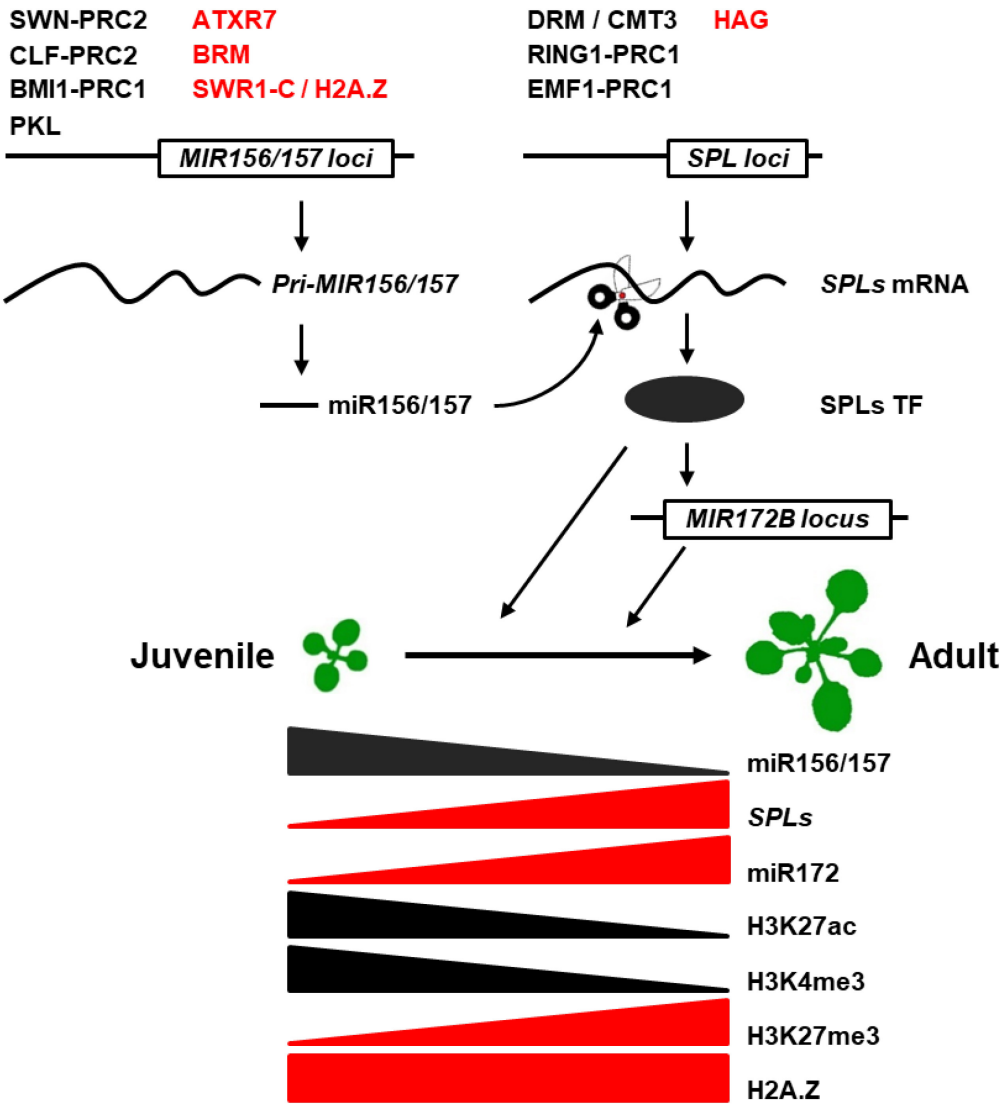

FIGURE 1 | Epigenetic regulation of juvenile-to-adult transition in plants. Active and repressive epigenetic regulators were marked in red and black at *MIR156/157* and *SPL* loci, respectively. Triangle indicates gradual increase or decrease in the epigenetic modification levels of *MIR156* loci.

Arabidopsis genome, three paralogous genes *MEDEA* (*MEA*), *SWINGER* (*SWN*), and *CURLY LEAF* (*CLF*) are orthologs of the *Drosophila E(z)* gene, which function as a histone methyltransferase subunit in the PRC2 complex. *MEA* appears to function in embryogenesis specifically, and *CLF* and *SWN* are broadly expressed and partially redundant in vegetative and reproductive development (Zheng and Chen, 2011; Bemer and Grossniklaus, 2012; Xu et al., 2016b).

Whole genome analysis in *Arabidopsis* uncovered 1000s of gene loci carrying the H3K27me3 mark catalyzed by the PRC2 complex, indicating that H3K27me3 is a major epigenetic silencing mechanism in plants (Zhang et al., 2007; Lafos et al., 2011). Among them, most *MIR156/157* loci, especially the dominant loci (*MIR156A*, *MIR156C,* and *MIR157A*), also carry H3K27me3 mark. However, except for *SPL4* and *SPL6* which play no obvious roles in juvenile-to-adult transition, miR156/157-targeted *SPL* genes are largely devoid of the H3K27me3 mark. These results imply that the PRC2 complex promotes *SPL* gene transcription indirectly by repressing the transcription of *MIR156/157* loci (Lafos et al., 2011).

During juvenile-to-adult transition in *Arabidopsis*, the decrease in the transcription of *MIR156A* and *MIR156C* loci is associated with an increase in the binding of the PRC2 complex to these two loci, causing an increase in the H3K27me3 mark in their promoter and transcribed regions as well as a decrease in the H3K27ac mark in the region immediately after transcription start sites (TSS) (Xu et al., 2016b,c). Loss-of-function mutant of *SWN*, but not the loss-of-function mutant of *CLF*, exhibited an obvious delayed juvenile-to-adult transition phenotype (Xu et al., 2016b,c). H3K27me3 was completely lost in *clf swn* double

mutant and it eventually dedifferentiated into a callus-like tissue, making it impossible to determine the phenotype of juvenile-to-adult transition (Xu et al., 2016b). Therefore, the question of if *SWN* and *CLF* functions redundantly in vegetative phase change remains unknown. However, the H3K27me3 mark at *MIR156A/MIR156C* loci was significantly reduced in *clf* mutants, but that in *swn* mutant remains controversial, which indicates that *SWN* and *CLF* may function redundantly to repress *MIR156A/MIR156C* by catalyzing H3K27me3 (Xu et al., 2016b,c).

PRC1 AND HISTONE UBIQUITINATION

PRC1 is thought to recognize the H3K27me3 mark to confer stable transcriptional repression (Lund and van Lohuizen, 2004). PRC1 is more dissimilar between *Arabidopsis* and animals, but it has related functions. In *Arabidopsis*, the function of PRC1 can be histone 2A mono-ubiquitination (H2Aub) dependent or independent. H2Aub dependent group requires the E3 ubiquitin ligase activity of Arabidopsis B lymphoma Moloney murine leukemia virus insertion region1 homolog 1A (AtBMI1A)/B/C or AtRING1A/B, while H2Aub independent group requires the activity of the EMBRYONIC FLOWER 1 (EMF1) (Yang C. et al., 2013; Calonje, 2014). BMI1-PRC1 and RING1-PRC1 are required for the repression of seed maturation program after germination, whereas EMF1-PRC1 is required for floral repression (Moon et al., 2003; Calonje et al., 2008; Chen et al., 2010).

PRC1 has been shown to be involved in juvenile-to-adult transition in *Arabidopsis*. BMI1-PRC1 maintains the repression of miR156 and accelerates juvenile-to-adult transition (Picó et al., 2015). The levels of *MIR156A* and *MIR156C* were upregulated in *atbmi1a/b* mutant and the juvenile phase was prolonged with the H2Aub and H3K27me3 marks being decreased in the TSS region of *MIR156A* and *MIR156C* (Picó et al., 2015).

RING1-PRC1 and EMF1-PRC1 function to repress *SPLs* to delay juvenile-to-adult transition (Li et al., 2017). In *ring 1a ring 1b* double mutant, the H2Aub mark was obviously decreased in the promoter and coding region of *SPL3*, *SPL9* and *SPL10*, causing upregulation of these genes to accelerate the appearance of adult traits (Li et al., 2017). Therefore, PRC1 variants function in vegetative phase change mainly by targeting different *MIR156/157* loci or *SPL* genes in the miR156/157-SPL pathway, and they have opposing roles in this process. However, how PRC1 variants recognize distinct targets still remains unclear, and more work is required to explore the mechanism of how PRC1 works.

ATXR7 AND H3K4me3 MODIFICATION

The Arabidopsis genome encodes three H3K4 methyltransferase, namely ARABIDOPSIS TRITHORAX1 (ATX1), ATX2, and ATXR7 (Avramova, 2009). ATX1 and ATX7 are members of the Trithorax family, and ATXR7 is the only member of the SET1 subfamily in *Arabidopsis* (Tamada et al., 2009). *atxr7-1*, but not *atx1-1*, *atx2-1*, or *atx1 atx2* double mutant, exhibits a precocious juvenile-to-adult transition phenotype. Chromatin immunoprecipitation (ChIP) analyses indicated that ATXR7 binds to a region adjacent to the TSS of *MIR156A* and deposits the H3K4me3 mark to activate *MIR156A* transcription (Xu et al., 2018).

HAG1 AND HISTONE ACETYLATION

Histone acetylation is generally considered as an active epigenetic mark, which is a balanced process regulated by histone acetyltransferases (HAG1) and histone deacetylases (HDA1, HAD6). Spt–Ada–Gcn5–acetyltransferase-like histone acetyltransferase complex (SAGA-like complex) is conserved in mammals, plants, files and yeast, and General Control Non-repressed 5 (GCN5) functions as the catalytic component for this complex (Turner, 2000).

In *Arabidopsis*, loss-of-function mutants in *HAG1* (the Arabidopsis homolog of *GCN5*), *hag1-6* and *hag1-7*, exhibited a significantly delayed juvenile-to-adult transition phenotype (Kim et al., 2015). In *hag1-6* mutant, transcripts of *MIR156* loci and mature miR156 remained stable; however, those of *SPL3*, *SPL4*, *SPL5*, *SPL9*, *SPL11*, *SPL13*, *SPL15,* and *SPL8* were greatly reduced, suggesting that the regulation of *SPLs* by HAG1 is independent of miR156. ChIP results showed HAG1 was bound to the promoters and transcribed regions of *SPL3* and *SPL9* directly, leading to histone acetylation at the H3K9, H3K14, and H3K27 sites in these genes (Kim et al., 2015). HAG1-mediated H3 acetylation (H3Ac) of *SPL9* is also responsive to light signals, which indicates that HAG1-mediated H3Ac of *SPL9* might function as a sensor of environmental conditions to modulate the developmental process in plants (Kim et al., 2015).

CHROMATIN REMODELING

Chromatin remodeling includes changes in nucleosome composition, nucleosome occupancy, nucleosome location, and the accessibility of the DNA to other transcriptional regulators.

SWR1-C AND H2A.Z HISTONE VARIANT

ATP-dependent SWR1 chromatin remodeling complex (SWR1-C) functions in exchanging the histone H2A-H2B dimer with the H2A.Z-H2B dimer, and then produces nucleosome variant (Mizuguchi et al., 2004; Luk et al., 2010). In *Arabidopsis*, mutations in the SWR1-C subunit coding genes (*ARP6*, *SEF*, and *PIE1*) and H2A.Z coding genes (*HTA8*, *HTA9*, and *HTA11*) exhibited a similar pleiotropic phenotype, which indicates that the primary function of SWR1-C is to deposit H2A.Z (Mizuguchi et al., 2004; Wu et al., 2005). However, the mechanism of H2A.Z modification by SWR1-C to regulate different target gene expression is distinguishable in that H2A.Z can change the nucleosome occupancy to destabilize nucleosomes or to increase nucleosome stability and/or to function with H3K4me3 mark together (Martin-Trillo et al., 2006; Kumar and Wigge, 2010; Choi et al., 2013).

In *arp6* and *hat9/hat11* mutants, *MIR156A/MIR156C* transcripts were reduced and juvenile-to-adult transition was accelerated (Choi et al., 2016; Xu et al., 2018). ChIP with H2A.Z antibody showed that H2A.Z was enriched at the first 500 nucleotides after TSS of *MIR156A/MIR156C*, and the level of H2A.Z was significantly reduced in *arp6* mutant. However, H2A.Z level does not change significantly during juvenile-to-adult transition, suggesting that H2A.Z and SWR1-C contribute to maintaining the expression of *MIR156A/MIR156C* early in shoot development, but do not regulate the timing of juvenile-to-adult transition (Xu et al., 2018). *MIR156A* transcript was reduced in *arp6* mutant due to higher nucleosome occupancy in its promoter region (Choi et al., 2016); however, it was suggested that H2A.Z increases the expression of *MIR156A/MIR156C* by promoting the deposition of H3K4me3 rather than by decreasing nucleosome occupancy in the *MIR156A* promoter region (Xu et al., 2018).

ATP-DEPENDENT CHROMATIN REMODELING PROTEIN

BRAHMA (BRM) is the ATPase subunit of the most widely studied SWI2/SNF2 chromatin remodeling protein complex. It uses the energy derived from ATP hydrolysis to change the histone octamer-DNA interaction (Saha et al., 2006; Clapier and Cairns, 2009). BRM regulates *MIR156A* transcription by directly binding to the promoter region and maintaining low occupancy of the −2 and +1 nucleosomes proximal to the TSS. *brm* mutants exhibit an accelerated juvenile-to-adult transition phenotype by reducing the transcription of *MIR156A* (Xu et al., 2016c). BRM also antagonizes the function of SWN in the PRC2 complex to remove H3K27me3 repressive mark in *MIR156A* (Xu et al., 2016c).

PICKLE (PKL) is a CHD3 ATP-dependent nucleosome remodeling protein, which is physically associated with the nucleosome remodeling and deacetylation complex (Perruc et al., 2007; Zhang et al., 2008; Ho et al., 2013). PKL is bound to the TSS adjacent region of *MIR156A/MIR156C* to promote the juvenile-to-adult transition by repressing the transcription of *MIR156A/MIR156C*. In *pkl* mutants, *MIR156A/MIR156C* transcripts were elevated due to the reduction in nucleosome occupancy at the +1 position, an increase in the H3K27ac mark, and a corresponding decrease in the H3K27me3 mark in the promoter and transcribed region (Xu et al., 2016b).

PERSPECTIVE

Although the miR156/157-SPL pathway has been shown to be the master regulator of juvenile-to-adult transition in plants, yet little is known about the upstream regulator of this pathway, especially for miR157. Recent studies have revealed that DNA methylation, histone modification, chromatin remodeling play important roles in regulating the expression of some components in the miR156/157-SPL pathway. However, there are still some critical questions remain to be solved as illustrated in **Figure 2**.

HOW ARE EPIGENETIC REGULATORS RECRUITED TO THE *MIR156/157* AND/OR *SPLs* LOCI?

MIR156/157 and/or *SPL* loci are subjected to epigenetic regulation to modulate juvenile-to-adult transition in plants. However, these epigenetic regulators, by their own, have no DNA binding specificity. Therefore, a central question is how these epigenetic regulators are recruited to their target genes.

PRC2-mediated H3K27me3 is a conserved epigenetic modification between plants and the animal kingdom (Mozgova and Hennig, 2015; Xiao and Wagner, 2015). Recent genomic study in *Arabidopsis* showed that PRC2 components bind to specific DNA motifs called Polycomb response elements (PREs) by interacting with specific TFs (Xiao et al., 2017). Interestingly, six top enriched motifs (CTCC, CCG, G-box, GA repeat, AC-rich, and Telobox motifs) out of 170 computationally defined PREs were present at the *MIR156A* locus (Xiao et al., 2017). The GA repeat and Telobox motifs were present adjacent to the TSS region of *MIR156A* and *MIR156C* loci together, these motifs are the potential binding sites for class I BPC and C1-2iD TFs, respectively. This information will be helpful to identity TFs through which the PRC2 complex interacts to be recruited to the *MIR156A/MIR156C* loci during juvenile-to-adult transition.

EPIGENETIC MODIFICATION OF *MIR156/157* AND/OR *SPL* LOCI BY STRESS?

Plants are sessile organisms and they are forced to adapt to the changing environment. The miR156/157-SPL pathway functions as the master regulator of juvenile-to-adult transition and flowering (Wang et al., 2009; Wu et al., 2009). Therefore, plants evolved a precise mechanism to adapt to the environment by shortening or prolonging the juvenile phase or changing the flowering time. Under salt or drought stress conditions, miR156 was induced to maintain plants in the juvenile phase for a relatively longer time; when they were returned to favorable conditions, miR156 was suppressed to accelerate the developmental transition (Cui et al., 2014). Under UV-B radiation conditions, the PRC2-mediated H3K27me3 modification in the *MIR156A/MIR156C* loci was decreased, and the corresponding up-regulation of miR156 delayed juvenile-to-adult transition (Dotto et al., 2018). Other studies also indicate that the expression of miR156 is responsive to ambient temperature (Stief et al., 2014), phosphate starvation (Hsieh et al., 2009), CO_2 treatment (May et al., 2013), suggesting a tight interaction between juvenile-to-adult transition and environment through the miR156/157-SPL pathway.

Epigenetic modification is a reversible mark, which can be removed or deposited to target genes to affect their expression in response to changing environment. It will be of great interest

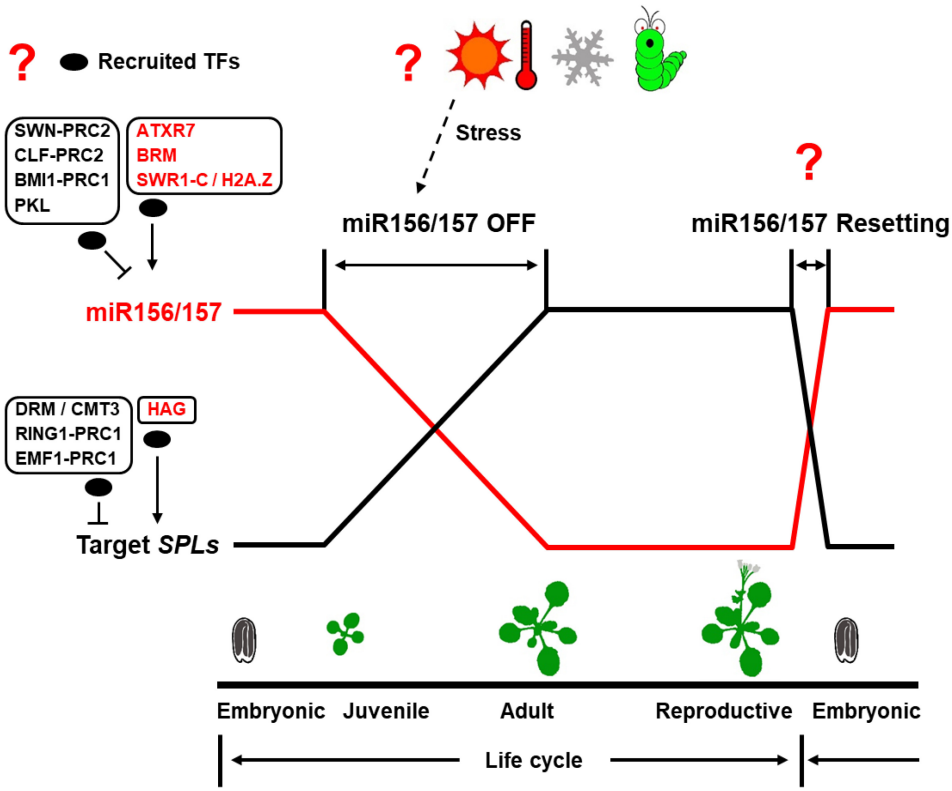

FIGURE 2 | Epigenetic regulation of the miR156/157-SPL pathway in plant lifecycle. In plant lifecycle, the transcription of genes in the miR156/157-SPL pathway exhibits a fixed temporal expression pattern. The major unknown parts in epigenetic regulation of the miR156/157-SPL pathway are shown in question mark. Oval represents recruited transcription factors (TFs).

to learn how epigenetic modification patterns of *MIR156/157* and/or *SPL* loci change in response to external cues, especially to environment stresses, as well as how this changing environment affects the juvenile-to-adult transition.

REVERSIBLE EPIGENETIC REGULATION OF miR156/157 RESETTING?

During juvenile-to-adult transition, miR156/157 transcription was reduced or silenced gradually to ensure the plant to enter the adult phase and flower. This is achieved by disposing of active epigenetic marks such as H3K4me3, H3K27ac and depositing some repressive epigenetic marks such as H3K27me3 to miR156 loci. Interestingly, this silencing process needs to be reset to an active state in each generation as miR156/157 is de-repressed again to be highly expressed in the pro-embryo stage (Nodine and Bartel, 2010) after flowering.

A similar example of Off-Resetting pattern in plant lifecycle is the regulation of *FLOWERING LOCUS C* (*FLC*). *FLC* is silenced by depositing H3K27me3 mark under winter cold treatment, and the silenced state was maintained in the mature pollen grains and the egg cells (De Lucia et al., 2008; Sheldon et al., 2008). In pro-embryo stage, *FLC* is activated by depositing active epigenetic marks such as H3K4me3, H3K36me3, and disposing of repressive marks such as H3K27me3. LEAFY COTYLEDON1 (LEC1), a seed-specific pioneer TF (Tao et al., 2017), and EARLY FLOWERING 6 (ELF6), a H3K27me3 demethylase (Crevillén et al., 2014), were shown to play critical roles in *FLC* re-activation.

As for *MIR156/157*, it is still unknown where and when the *de novo* re-activation occurs. Moreover, whether the resetting of miR156/157 depends on a reversible epigenetic regulation still remains elusive. Further study of when, where and how miR156/157 Off-Reset pattern is initiated during plant life cycle will be an important future task.

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YX and LZ wrote the article, and GW revised it.

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REVIEW
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Regulation of Plant Growth and Development: A Review From a Chromatin Remodeling Perspective

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In eukaryotes, genetic material is packaged into a dynamic but stable nucleoprotein structure called chromatin. Post-translational modification of chromatin domains affects the expression of underlying genes and subsequently the identity of cells by conveying epigenetic information from mother to daughter cells. SWI/SNF chromatin remodelers are ATP-dependent complexes that modulate core histone protein polypeptides, incorporate variant histone species and modify nucleotides in DNA strands within the nucleosome. The present review discusses the SWI/SNF chromatin remodeler family, its classification and recent advancements. We also address the involvement of SWI/SNF remodelers in regulating vital plant growth and development processes such as meristem establishment and maintenance, cell differentiation, organ initiation, flower morphogenesis and flowering time regulation. Moreover, the role of chromatin remodelers in key phytohormone signaling pathways is also reviewed. The information provided in this review may prompt further debate and investigations aimed at understanding plant-specific epigenetic regulation mediated by chromatin remodeling under continuously varying plant growth conditions and global climate change.

Keywords: chromatin remodeling, SWI/SNF complexes, histones, gene regulation, meristem, hormone signaling, plant development, flowering

INTRODUCTION

The packaging of genetic material into nucleosomes is a distinctive evolutionary feature of eukaryotic cells. Nucleosomes are repetitive units consisting of 147 bp DNA strands tightly wrapped around a central octamer comprising one histone heterotetramer (H3/H4) and two histone heterodimers (H2A/H2B). Histones are highly conserved proteins found in all eukaryotes as core nucleosome units (Suzuki and Bird, 2008). Together, the histone octamer and DNA resemble "beads" on a string and are referred to as chromatin (Luger et al., 1997). This packaging ensures that long DNA strands are tightly condensed by supercoiling to precisely fit

into the nucleus. Such compaction of nucleosomes in chromatin hinders DNA accessibility to important regulatory proteins essential for various nuclear processes. However, cells have evolved in response to this impediment through chromatin remodeling (Groth et al., 2007).

Gene regulatory mechanisms in eukaryotic cells (such as transcription, DNA repair and replication) act upon chromatin structure as a substrate. These activities induce cellular changes and regulate gene expression in a number of biological processes, including genome stability, recombination, developmental reprogramming and response to extracellular signals (Feng et al., 2010; Soria et al., 2012; Zhu et al., 2013). The regulatory precision of these fundamental genome processes relies on the high fidelity of chromatin remodeling mechanisms that permit temporal access to – or blocking of – vital DNA sequences, such as gene promoters. Histone modifications, nucleosome remodeling and DNA methylation have been shown to regulate chromatin structure and gene expression (Henikoff and Shilatifard, 2011).

There are two main players regulating chromatin dynamics: (1) chromatin remodelers that alter DNA-histone interactions by energy harnessed through ATP hydrolysis and (2) nucleosome-modifying enzymes that modulate DNA and histone residues by specifically adding or removing covalent modifications (Jerzmanowski, 2007). Organisms rely on gene expression regulation to achieve normal cell differentiation, organogenesis, growth and development. Moreover, gene expression regulation is temporally and spatially coordinated via crosstalk between chromatin remodeling complexes (CRCs) and the transcription machinery (Deem et al., 2012). In plants, such precise control of gene expression mediated by chromatin modifications in response to endogenous and environmental stimuli is fundamental for proper development and reproductive success (Sarnowski et al., 2005; Bezhani et al., 2007; Han et al., 2012; Archacki et al., 2013; Efroni et al., 2013; Sarnowska et al., 2013; Qin et al., 2014; Vercruyssen et al., 2014).

Plant development can be divided into the embryonic and postembryonic phases. The embryonic development phase includes the establishment of the seedling apical-basal axis, the shoot apical meristem (SAM) and the root apical meristem (RAM). The subsequent establishment of the leaf, stem and flower meristems occurs in the postembryonic phase. In this review, we discuss recent investigations underpinning the involvement of SWI/SNF chromatin remodeling ATPases in regulating key plant growth and development processes, including meristem establishment and maintenance, cell differentiation, organ initiation, flower morphogenesis and flowering time regulation. We also discuss the role of chromatin remodelers in the plant response to key phytohormone signals. This overview may be useful for framing the current knowledge gaps, thereby stimulating further debate and research aimed at a comprehensive understanding of epigenetic regulatory mechanisms in plant development. This in turn may guide molecular-based plant improvement techniques for desirable agronomic traits.

CLASSIFICATION OF EUKARYOTIC CHROMATIN REMODELING COMPLEXES

Chromatin remodeling complexes are evolutionarily conserved multi-unit protein complexes that regulate chromatin structure by altering nucleosome composition and interactions (Narlikar et al., 2002). A common feature of all purified CRCs is that they harbor an ATPase/helicase of the SWITCHING DEFECTIVE2/SUCROSE NON-FERMENTING2 (SWI2/SNF2) family as their catalytic core. The SWI/SNF family is part of superfamily2 (SF2), a large family of helicases and translocases, and is named after the first identified CRC (Peterson et al., 1994). SWI/SNF CRCs utilize the energy derived from ATP hydrolysis to control accessibility to vital DNA sequences by influencing nucleosome structure and position and determining the kind of variant histone subspecies to be incorporated (Eisen et al., 1995; Clapier and Cairns, 2009).

A number of *Arabidopsis* SWI/SNF complex subunits have been identified based on sequence similarity with metazoan subunits. These include four SWI2/SNF2 ATPases (BRAHMA [BRM], SPLAYED [SYD], MINU1/CHR12 and MINU2/CHR23) (Knizewski et al., 2008; Han et al., 2015); four SWI3 proteins (SWI3A to SWI3D); two SWI/SNF ASSOCIATED PROTEINS 73 (SWP73A/CHC2 and SWP73B/CHC1, also called BRAHMA ASSOCIATED FACTOR 60 (BAF60) in humans); two ACTIN RELATED PROTEINS belonging to SWI/SNF complexes (ARP4 and ARP7); and the BUSHY (BSH) protein (Meagher et al., 2005; Jerzmanowski, 2007; Kwon and Wagner, 2007; Sang et al., 2012). Eukaryotic SWI2/SNF2 family chromatin remodelers can be categorized into four classes/subfamilies based on phylogenetic analysis, and all four of these subfamilies are represented in plants (Farrona et al., 2008).

SWI/SNF Subfamily Remodelers

The SWI/SNF subfamily of remodelers initially purified from *Saccharomyces cerevisiae* is probably the most comprehensively studied. SWI/SNF subfamily CRCs have a well-established role in gene expression regulation. The SWI/SNF subfamily remodelers contain 8–14 subunits, and the catalytic ATPases of most SWI/SNF subfamily remodelers are composed of a helicase-SANT domain, a post-HSA domain and a C-terminal bromodomain. While the fungal SWI/SNF subfamily ATPases consist of a pair of actin-related proteins (ARPs) (Cairns et al., 1998), higher orthologs of SWI/SNF subfamily complexes contain a dimer of an ARP and actin (Lessard et al., 2007). The mode of action of SWI/SNF complexes has been characterized as sliding and/or ejecting nucleosomes at many target loci, but they reportedly have no vital role in the assembly of eukaryotic chromatin structure (Clapier and Cairns, 2009). It was recently reported that the *Arabidopsis* SWI/SNF complex expedites its role in activating and repressing target gene expression by binding to both promoters and terminators and that it regulates the expression of both promoter-centered genes and non-coding RNAs (Archacki et al., 2017).

Imitation Switch (ISWI) Subfamily Remodelers

The ISWI subfamily remodelers have 2–4 subunits. Eukaryotic ISWI remodeler complexes typically have 1 or 2 distinct catalytic subunits and specialized attendant proteins that give rise to various domains. These domains include plant homeodomains, bromodomains, DNA-binding histone fold motifs and additional DNA-binding motifs (Clapier and Cairns, 2009). ISWI complexes were initially purified from *Drosophila melanogaster*. They include the nucleosome remodeling factor (dNURF), chromatin accessibility complex (dCHRAC) and ATP-utilizing chromatin assembly and remodeling factor (dACF) (Corona and Tamkun, 2004). SANT-like ISWI ATPases contain a SANT domain adjacent to a SLIDE domain in their C-terminus, which acts as a binding site for unmodified histone tails and DNA. The diversity resulting from attendant subunits is exhibited in the modes of action of the ISWI family complexes. Both ACF and CHRAC have been shown to promote chromatin assembly and transcription repression through the optimization of nucleosome spacing, whereas NURF assists RNA polymerase II (RNAPII) activation by randomizing nucleosome spacing (Corona and Tamkun, 2004).

Chromodomain Helicase DNA-Binding (CHD) Subfamily Remodelers

The CHD subfamily remodelers were first purified from *Xenopus laevis* and comprise 1–10 subunits. The N-terminus of their catalytic subunit consists of two chromodomains arranged tandemly, with the composition varying from monomeric in lower eukaryotes to multimeric in vertebrates (Marfella and Imbalzano, 2007). CHD subfamily remodelers vary in their structure, composition and function, due in part to the diversity of their chromodomains. One of the members of the CHD subfamily in vertebrates, the nucleosome remodeling and deacetylase (Mi-2/NuRD) complex, has repressive roles due to its inclusion of histone deacetylases (HDAC1/2) and methyl CpG-binding domain (MBD) proteins. In contrast, other CHD subfamily remodelers have been reported to promote transcription by either ejecting or sliding nucleosomes along the DNA double strand (Denslow and Wade, 2007).

Inositol Requiring 80 (INO80) Subfamily Remodelers

The INO80 subfamily is the most recently characterized chromatin remodeler subfamily. Many INO80 subunit homologs have been identified in yeast, fruit fly, mammals and plants, making it the most conserved subfamily. The presence of a split ATPase subunit distinguishes INO80 complexes from the SWI/SNF, CHD and ISWI subfamilies (Morrison and Shen, 2009). In *S. cerevisiae*, the INO80 subfamily is represented by two complexes, INO80 and Swi2/snf2-related 1 (SWR1); in mammals, it is represented by three complexes, INO80, Snf2-related CBP activator protein (SRCAP) and p400. *D. melanogaster* has INO80 and p400 complexes (Morrison and Shen, 2009; Bao and Shen, 2011). *S. cerevisiae* INO80 and SWR1 ATPases are approximately 1.2–1.5 MDa in mass and are reported to contain 15 and 14 subunits, respectively (Shen et al., 2000; Krogan et al., 2003; Mizuguchi et al., 2004). In general, the INO80 subfamily has a relatively conserved composition of individual complexes and a high degree of homology in the ATPase subunit. Studies of the *S. cerevisiae* SWR1 complex indicate the importance of a spacer (split) region in the ATPase subunit, as deletion of this region leads to dissociation of a number of subunits from the complex, including the RuvB-like Rvb1 and Rvb2 subunits (Wu et al., 2005). The INO80 subfamily is responsible for various functions in eukaryotic cells including transcriptional activation and DNA double-strand break (DSB) repair (Ebbert et al., 1999).

INVOLVEMENT OF SWI/SNF ATPases IN MERISTEM ESTABLISHMENT AND MAINTENANCE

Plant development is characterized by the presence of stem cells with the capacity to self-renew and transform into tissue-specific founder cells. The stem cells of the RAM and SAM have been widely studied in plants and are found at the root and shoot tips, respectively (Shen and Xu, 2009). Stem cell homeostasis and identity in both plants and mammals are associated with the activity of SWI/SNF chromatin remodeling ATPases (Ori et al., 2000; Kwon et al., 2005; Ho et al., 2009; Aichinger et al., 2011). Perturbations in the Polycomb group (PcG) repressive complex, histone acetylation or chromatin assembly may lead to improper RAM or SAM development (Phelps-Durr et al., 2005; Schubert et al., 2006; Barrero et al., 2007; Xu and Shen, 2008; Kornet and Scheres, 2009). Individual *Arabidopsis* SWI/SNF subfamily chromatin remodelers (BRM, SYD, CHR12 and CHR23) (Flaus et al., 2006) are also involved in regulating numerous plant developmental processes (Farrona et al., 2004; Hurtado et al., 2006; Kwon et al., 2006; Bezhani et al., 2007; Tang et al., 2008; Wu et al., 2012).

SYD promotes SAM maintenance by binding to the promoter region of its target gene *WUSCHEL* (*WUS*) and activating *WUS* expression in the organizing center (OC) region of the SAM (**Figure 1**). Consistent with this function, *Arabidopsis syd* mutant plants exhibit reduced *WUS* transcript levels accompanied by abnormal SAM development (Kwon et al., 2005).

The CHR12 and CHR23 ATPases have redundant roles in plant development processes including meristem initiation and maintenance. Plants with strong double mutant *chr12chr23* combinations show embryonic lethality, endosperm defects and failure to initiate stem cell populations in both roots and shoots, whereas plants with weak double mutant *chr12chr23* combinations are small viable plants with noticeable defects in RAM and SAM maintenance (Sang et al., 2012).

A study by Wu et al. (2015) demonstrated a role of chromatin in local developmental progressions in the root and shoot. In the SAM, cells with high auxin levels initiate proteolysis of Aux/IAA leading to the dissociation of the co-repressor complex TOPLESS-HISTONE DEACETYLASE 19

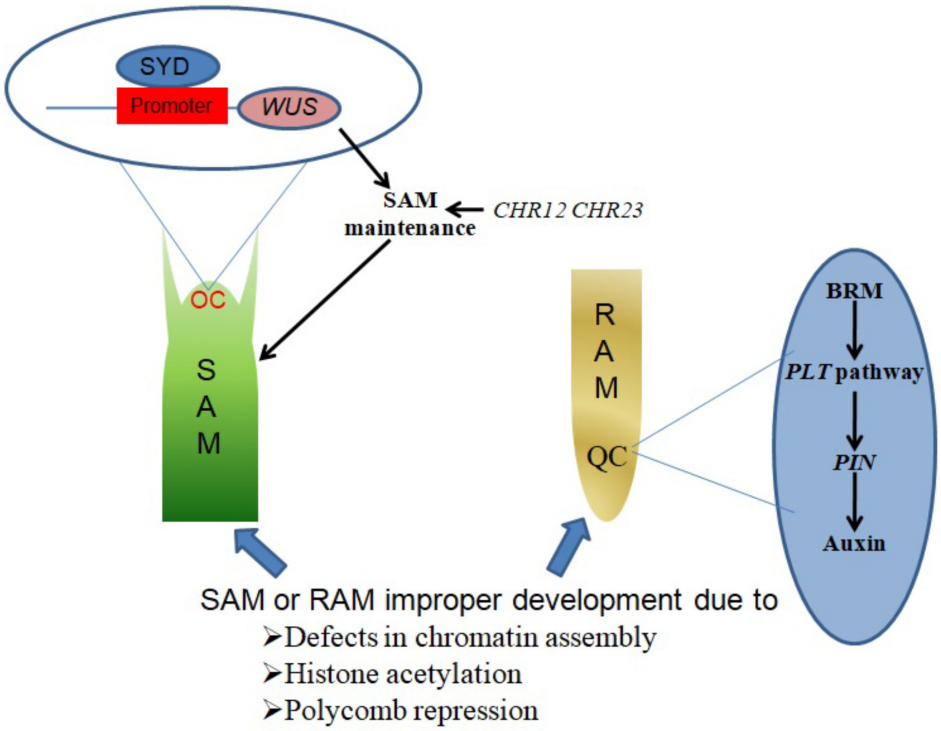

FIGURE 1 | Chromatin remodelers in shoot and root apical meristem establishment and maintenance. SYD binds to the promoter region of *WUS* in the OC region of the SAM, activating *WUS* expression to promote SAM maintenance. CHR12 and CHR23 have redundant roles in plant development regulation, as single mutations in either produce no obvious defects but strong double mutations lead to impaired SAM maintenance. BRM acts in the *PLT* pathway to regulate the expression of *PIN* genes, which in turn influence auxin distribution in the QC region to ensure RAM maintenance.

(TPL-HDA19). This enables the recruitment of SYD and BRM to direct the acquisition of flower primordium founder cell fate (Wu et al., 2015). In the RAM, a small group of mitotically inactive cells known as the quiescent center (QC) maintains the root stem cells. BRM was found to act in the *PLETHORA* (*PLT*) pathway to maintain the root stem cell niche by altering the expression of *PIN-FORMED* (*PIN*) genes responsible for auxin distribution in the RAM, as illustrated in **Figure 1**. *brm* mutants exhibit faulty root stem cell niche maintenance, reduced meristem activity and retarded root growth (Yang et al., 2015).

To attain continuous plant growth, cells derived from pluripotent stem cells must undergo asymmetric cell division and expansion. This allows for the generation of lateral organs and the maintenance of stem cell populations for additional growth (Jarillo et al., 2009). The role of BRM in ensuring asymmetric cell division and cell fate determination in both plant and animal stem cells was discussed extensively in a recent review (Pillitteri et al., 2016).

Our current knowledge of mechanisms involving chromatin-mediated regulation of plant stem cell initiation and maintenance remains limited to the few *Arabidopsis* SWI/SNF complexes discussed above. However, emerging evidence suggests complex interconnections among many players including key hormone signaling pathways. This underscores the ability of plants to activate or arrest lateral organ founder cell formation in response to both internal developmental signals and external biotic and abiotic stress signals. These attributes may eventually be harnessed or targeted for crop improvement applications.

CHROMATIN REMODELING IN CELL DIFFERENTIATION, ORGAN INITIATION AND DEVELOPMENT

Stem cells in the SAM initiate organ founder cells that later differentiate into aboveground organ-specific tissues such as leaves and stems (Sinha, 1999; Laux, 2003). Plant roots are formed from a reservoir of undifferentiated cells in the RAM called the root stem cells. Mature plant organs usually maintain relatively undifferentiated cells as a fallback for hormonal stimuli or mechanical injury, helping to generate new tissues and organs through cellular reprogramming (Ikeuchi et al., 2015). Cellular differentiation results from global changes in gene expression patterns and these changes involve many transcription regulators and are epigenetically mediated by chromatin remodeling (Bruex et al., 2012; Taylor-Teeples et al., 2015).

ANGUSTIFOLIA3 (AN3)/GRF-INTERACTING FACTOR1 (GIF1), a member of the GIF family of transcriptional coactivators, plays a key role in *Arabidopsis* shoot development (Kim and Kende, 2004), cotyledon identity establishment during

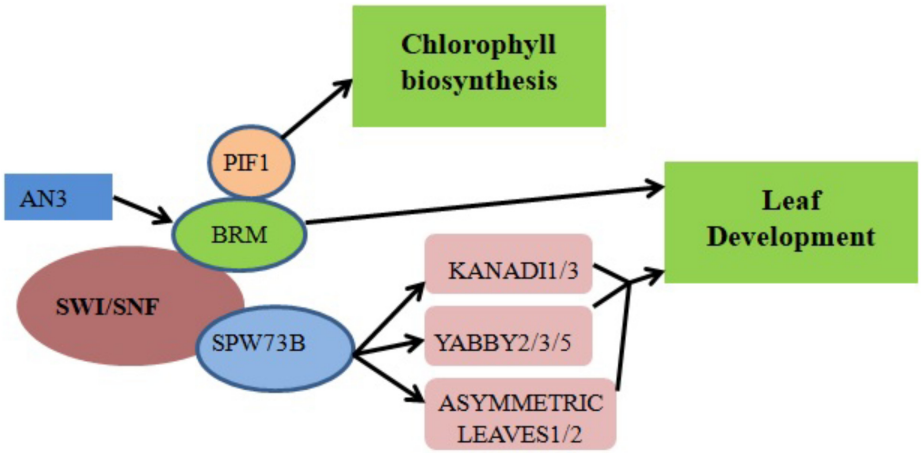

FIGURE 2 | Chromatin remodelers in cell differentiation and organ development regulation. The recruitment of BRM by the transcriptional coactivator AN3 promotes the transcription of genes responsible for leaf development. Direct binding of SWP73B to the promoter regions of *KANADI1/3*, *YABBY2/3/5* and *ASYMMETRIC LEAVES1/2* also controls leaf development. Physical interaction between PIF1 and BRM regulates chlorophyll biosynthesis by affecting the expression levels of associated enzymes.

embryogenesis (Kanei et al., 2012) and leaf size increase resulting from increased cell number (Horiguchi et al., 2005; Lee et al., 2009). Transcriptional coactivators often work together with DNA-binding transcription factors to promote transcription either by recruiting chromatin remodelers or stimulating general complex formation around RNA polymerase II (Pol II). Genetic interaction between AN3 and the *Arabidopsis* SWI/SNF chromatin remodeling complex BRM (**Figure 2**) suggests that AN3 recruits SWI/SNF complexes to promote cell division during leaf development (Vercruyssen et al., 2014). BRM is recruited to its target gene loci via association with the plant-specific H3K27 demethylase RELATIVE OF EARLY FLOWERING 6 (REF6). Through its zinc-finger (ZnF) domains, REF6 recognizes genomic loci harboring a CTCTGYTY motif (Li et al., 2016). A forward genetic screen in *Arabidopsis* indicated that BRM and *SWINGER* (*SWN*) (a key component of Polycomb Group Repressive Complex 2 in plants) antagonistically control vegetative phase change through the temporal expression of miR156 at the nucleosome level. Specifically, the accelerated vegetative phase change of *brm* mutants was accompanied by reduced miR156 expression and increased levels of H3K27me3 at the *MIR156A* locus (Xu et al., 2016).

SWP73 subunits of *Arabidopsis* SWI/SNF CRCs function in various plant development pathways including the regulation of leaf and flower development (Vercruyssen et al., 2014; Sacharowski et al., 2015). For example, SWP73B directly binds to the promoters of the *KANADI1/3*, *YABBY2/3/5* and *ASYMMETRIC LEAVES1/2* genes involved in leaf development (**Figure 2**) (Sacharowski et al., 2015). RNA interference (RNAi) silencing of *SWP73B* leads to dwarfism in *Arabidopsis*, evidencing its role in plant development (Crane and Gelvin, 2007).

A study of the chromatin remodeler protein ZmCHB101, which is the core subunit of maize SWI3, revealed its key roles in normal maize growth and development. ZmCHB101 controls the transcriptional reprogramming of a set of genes involved in gene expression regulation, photosynthesis, metabolic regulation and stress response. The RNAi maize lines generated in the study exhibited improper tassel and cob development and abaxially curling leaves caused by increased bulliform cell numbers (Yu et al., 2016).

Chlorophyll biosynthesis is a critical mark of the transition from heterotrophic to autotrophic growth in plants. Physical interaction between BRM and the transcription factor PHYTOCHROME-INTERACTING FACTOR 1 (PIF1) was shown to regulate chlorophyll biosynthesis in *Arabidopsis* (**Figure 2**). When exposed to light, dark-grown *brm* plants exhibit higher greening rates, reduced protochlorophyllide accumulation and lower levels of reactive oxygen species (ROS) compared to wild-type plants; there is also increased expression of *NADPH:protochlorophyllide oxidoreductase A* (*PORA*), *PORB* and *PORC*, enzymes which accelerate a key step in chlorophyll biosynthesis (Zhang et al., 2017).

The importance of chromatin remodelers for proper plant growth progression and development is exemplified by their involvement in asymmetric cell differentiation and in initiating organ founder cells. Proper plant growth also involves precise developmental phase transitions, and the antagonism between SWI/SNF ATPases and PcG repressive complex components is crucial for these shifts.

CHROMATIN REMODELER-MEDIATED FLOWERING AND FLOWER MORPHOGENESIS

The precise regulation of the transition from vegetative growth to flowering is paramount for plant reproductive success. This process is characterized by the transition of the vegetative

SAM into an inflorescence meristem (IM) and the initiation of floral meristems (FMs) (Sablowski, 2007; Kaufmann et al., 2010). While SAM maintenance ensures indeterminate plant growth, the determinate nature of FMs determines reproductive success, seed development and the yield of agricultural crops (Liu et al., 2011). Complex regulatory networks of transcription factors and chromatin remodelers guide flowering time and flower development while integrating both internal and external signals (Wils and Kaufmann, 2017). These networks comprise photoperiod, vernalization and thermo-sensory pathways for sensing long days, cold winter and ambient temperature, respectively, together with pathways responsive to internal factors, such as the age pathway and the gibberellins (GA) signaling pathway (He, 2012).

In *Arabidopsis*, a repressor complex that consists of the two MADS box transcription factors *MADS AFFECTING FLOWERING 4/5* (*MAF4/5*), *FLOWERING LOCUS C* (*FLC*) and *SHORT VEGETATIVE PHASE* (*SVP*) serves as a negative regulator of flowering time. The components directly repress the expression of the floral pathway integrators *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Lee et al., 2007; Li et al., 2008; He, 2012). *FRIGIDA* (*FRI*) promotes higher *FLC* levels that inhibit flowering (Michaels and Amasino, 2001) through the recruitment of multiple active chromatin modifications at the *FLC* locus (Choi et al., 2011). Vernalization overrides the *FRI*-mediated activation of *FLC* expression and thereby enables flowering (Kim et al., 2009; Crevilleńn and Dean, 2011).

The core subunit components of the *Arabidopsis* SWR1 chromatin remodeling complex (including *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1* (*PIE1*), *ACTIN RELATED PROTEIN 6* (*ARP6*) and *SWR1 COMPLEX 6* (*SWC6*)/*SERRATED LEAVES AND EARLY FLOWERING* (*SEF*)) have been shown to play important roles in regulating the proper growth and development of most plant organs. Importantly, SWR1 controls plant development by generating a balance between microRNAs and target mRNAs at the transcriptional level (Choi et al., 2016). It was recently demonstrated that SWR1 regulates gene expression by establishing lowly accessible and highly accessible nucleosome structure at the first nucleosome upstream and downstream of the transcription start site (TSS), respectively (Dai et al., 2017). SWR1 subunit loss-of-function mutants exhibit pleiotropic phenotypes including early flowering, serrated leaves, frequent absence of inflorescence internodes, bushy growth and flowers with altered organ number and size (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006; March-Diaz et al., 2007; Qin et al., 2014). H2A.Z deposition around the *FLC* TSS by SWR1 is necessary for *FRI*-meditated activation of *FLC* (**Figure 3**). One line of evidence is that functional disruption of SWR1 prevents H2A.Z deposition at *FLC* chromatin, suppressing its expression and leading to early flowering (Choi et al., 2007; Deal et al., 2007). Additionally, *PIE1* mutations were earlier shown to substantially reduce *FLC* transcript levels, with a concomitant conversion of the winter-annual habit to the rapid flowering summer-annual habit (Noh and Amasino, 2003). A recent study revealed an *Arabidopsis* homolog of the yeast SANT domain protein Swc4/Eaf2 as a novel SWR1-C subunit. SWC4 is a DNA-binding protein that contributes to the recruitment SWR1-C through specific recognition of AT-rich DNA segments in chromatin regions of target genes to deposit H2A.Z. Further, knock-out and knockdown studies showed that *SWC4* is essential for both embryo viability and the control of post-embryonic processes, including flowering time, by repressing transcription of a number of genes including the floral integrator *FT* and key transcription factors (Gomez-Zambrano et al., 2018).

The *At*INO80 complex is required for somatic homologous recombination (HR) and expression regulation of *FLC* and *MAF4/5* by facilitating the enrichment of H2A.Z at their ends. Independent *Atino80-5* and *Atino80-6* mutant alleles display similar pleiotropic phenotypes of small plant size, reduced organ size and late flowering. These observations provide a link between plant responses to environmental and developmental signals and epigenetic mechanisms involved in plant chromatin stability (Zhang et al., 2015).

The SWP73B (BAF60) subunit of the *Arabidopsis* SWI/SNF complex has also been implicated in flowering time control through its involvement in chromatin loop formation at the *FLC* locus (Jegu et al., 2014). SWP73A has been shown to be confined to flowering time modulation under short day conditions, with functional overlap between SWP73A and SWP73B during embryogenesis (Sacharowski et al., 2015).

SVP, another key flowering repressor, is highly expressed during the vegetative phase to promote growth (Hartmann et al., 2000) but is down-regulated during the floral transition by the autonomous and GA signaling pathways (Li et al., 2008). *FT* and *SOC1* are subsequently activated to promote flowering (**Figure 3**). One study proposed that BRM controls flowering time in *Arabidopsis* by directly activating *SVP* expression (Li et al., 2015). Genome-wide analysis of H3K27me3, a histone mark associated with gene repression, in *brm* mutant seedlings revealed increased H3K27me3 deposition at several genes including *SVP*, indicating an antagonism between BRM and PcG repressor proteins (Li et al., 2015).

Another key flowering time regulator in *Arabidopsis* activated by *CONSTANS* (*CO*) under long days to induce flowering is the *FT*-encoded florigen protein (FT) (Corbesier et al., 2007). FT moves from the phloem to the SAM and forms a complex with the bZIP transcription factor FD to activate the expression of the FM identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*). This activation leads to the formation of the floral primordium, which sequentially generates three types of lateral floral organs (sepals, petals and stamens) (Zik and Irish, 2003; Abe et al., 2005; Wigge et al., 2005). *LFY* recruits SYD and BRM ATPases to induce the expression of the floral homeotic regulator *AGAMOUS* (*AG*) by removing H3K27me3 marks on its second intron (**Figure 3**) (Wu et al., 2012). The SWR1 complex is also involved in *FT* expression regulation, with functional disruption of SWR1 leading to temperature-insensitive *FT* activation and early flowering. An ambient temperature increase from 17 to 27°C causes eviction of the

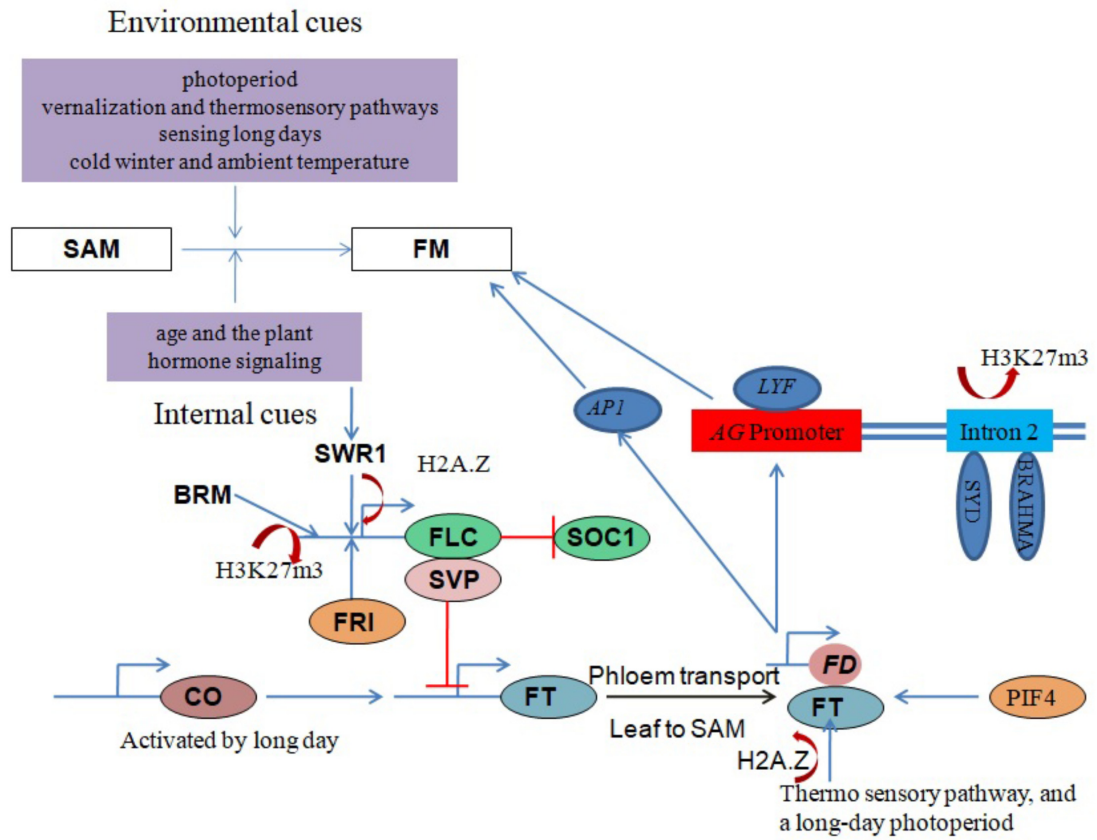

FIGURE 3 | Chromatin modulation in flowering regulation. Red lines with perpendicular bars denote repression, blue arrows indicate activation, and colored ovals represent proteins. Floral transition in the SAM involves IM and FM specification and is regulated by a balance between environmental and internal factors. SYD, BRM and SWR1 chromatin remodeling complexes coordinate with other factors to influence floral development by regulating the expression of critical genes, such as *AG*, *FT*, *FLC* and *SVP*, in response to both external and internal stimuli.

H2A.Z nucleosomes, which enables *FT* transcription by Pol II (Kumar and Wigge, 2010).

Studies of the CHD3 chromatin remodeler *PICKLE* (*PKL*) have highlighted its role in plant reproductive development by promoting crosstalk between the sporophytic and gametophytic generations. Loss of *PKL* in the maternal sporophyte leads to improper development of the *Arabidopsis* female gametophyte, integument and pollen tube, accompanied by delayed ovule and embryo development (Carter et al., 2016).

It was recently shown that during megasporogenesis, somatic cells cooperatively use SWR1 to restrict female reproductive founder cell specification to a single cell in the ovule primordia by incorporating H2A.Z at a particular *WRKY28* nucleosome and promoting expression of the gene (Zhao et al., 2018). The WRKY28 transcription factor is exclusively expressed in hypodermal somatic cells surrounding the megasporocyte and represses those cells from differentiating into functional megasporocytes (Zhao et al., 2018). Other separate findings demonstrated that both BRM and SWR1 are involved in determining inflorescence architecture in response to developmental cues (Zhao et al., 2015; Cai et al., 2017).

The above findings reveal mechanisms involving the recruitment of SWI/SNF ATPases to specific flower development regulatory pathways at an appropriate time. They also emphasize the centrality of SWI/SNF complexes in shaping plant growth and ensuring the continuity of plant viability through generations by precise spatiotemporal control of the relevant developmental processes. Nevertheless, these conclusions about important regulatory mechanisms are generally based on findings from model plants such as *Arabidopsis*. Thus, the understanding of such regulatory mechanisms in agriculturally important crop species remains inadequate and will require further investigation.

CHROMATIN REMODELERS IN PHYTOHORMONE SIGNALING PATHWAYS

Plant hormones are critical for the proper regulation of plant growth and development processes including seed germination, vegetative and reproductive growth and abiotic stress response. The biosynthesis and degradation of plant hormones are also

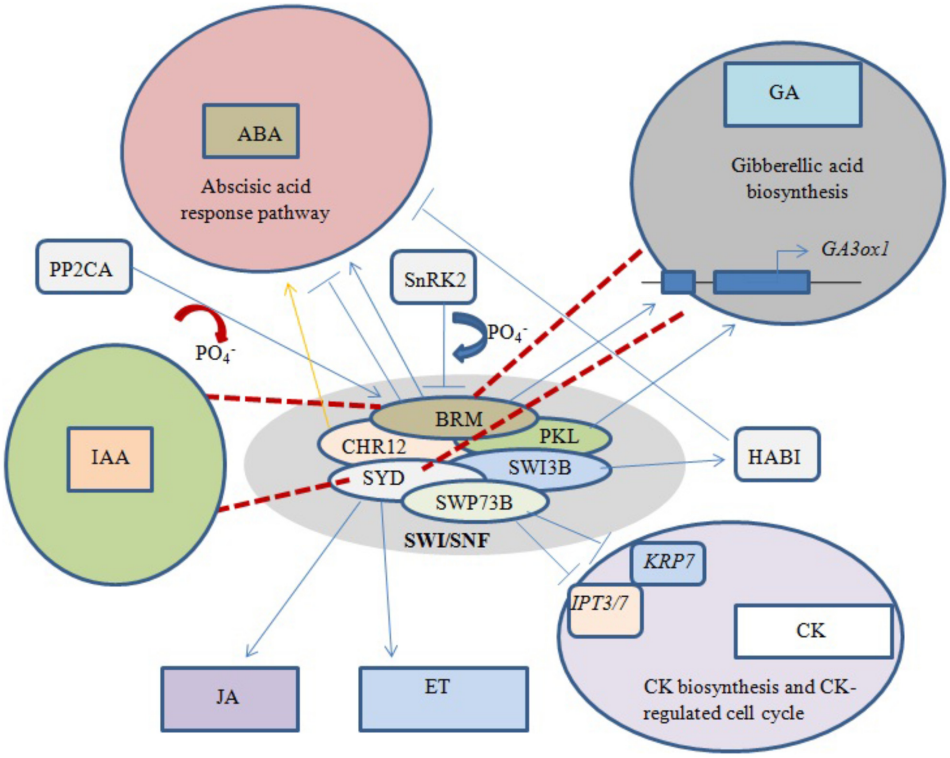

FIGURE 4 | Phytohormone signaling response under chromatin remodeling. Blue arrows represent activation/interaction, blue lines with perpendicular bars denote inhibition, red dashed lines indicate the mutated state of an ATPase, and the yellow arrow represents overexpression. Both BRM and PKL positively regulate GA biosynthesis by activating underlying genes. Phosphorylation of BRM and overexpression of CHR12 both activate the ABA response pathway, while dephosphorylation of BRM and interaction of SWI3B with HABI repress the ABA response. SWP73B negatively regulates CK biosynthesis and CK-mediated cell cycle changes. Mutations in BRM and SYD inhibit GA and IAA biosynthesis, while normal SYD positively regulates JA- and ET-dependent gene expression.

tightly controlled through the transcriptional regulation of target genes. SWI/SNF ATPases are involved in plant hormone responses through physical interaction with hormone signaling pathway components and transcriptional regulators of genes involved in hormone biosynthesis and perception (Sarnowska et al., 2016).

Abscisic acid (ABA) is an important phytohormone that promotes seed dormancy and arrests growth in post-germination embryos under water stress conditions. In the absence of stress stimuli, BRM was initially implicated in repressing the activity of the ABA pathway, with adult *brm* mutants exhibiting increased drought tolerance (Han et al., 2012). It was later revealed that important ABA signaling pathway components physically interact with BRM, leading to post-translational phosphorylation/dephosphorylation of BRM. For example, phosphorylation of BRM by SnRK2 kinases leads to its inhibition, while PP2CA-mediated dephosphorylation restores the ability of BRM to repress the ABA response (**Figure 4**). Moreover, the phosphomimetic BRM mutant shows hypersensitivity to ABA (Peirats-Llobet et al., 2016).

BRM has also been shown to play a direct role in the positive regulation of GA biosynthesis by binding to chromatin near the *GA3ox*1 promoter, thereby activating the gene (**Figure 4**). *Arabidopsis brm* null mutants exhibit a significant decrease in active GA levels (Archacki et al., 2013). Furthermore, transcriptional profiling revealed that most genes involved in the GA and auxin signaling pathways are affected in both *syd* and *brm* null mutants (Bezhani et al., 2007). Previous studies showed that plants over-expressing CHR12 had enhanced growth arrest when exposed to drought or heat stress (Mlynarova et al., 2007). In addition, SYD was linked to ethylene (ET)- and jasmonic acid (JA)-dependent gene regulation (Walley et al., 2008), and the SWI3B subunit was found to interact with a negative regulator of ABA signaling, the PP2C-type phosphatase HAB1 (Saez et al., 2008).

During development, plants transition from the embryonic stage to the seedling stage. Previous studies have characterized the joint effects of PKL and GA in repressing embryonic traits during plant development transitions (Zhang et al., 2008; Zhang et al., 2012). Specifically, *PKL* expression represses the embryonic state by controlling a significant number of GA-responsive genes. *pkl* seedlings exhibit a semi-dwarf phenotype similar to that of GA-response mutants and are able to express embryonic traits under GA-biosynthesis inhibition. In contrast, treatment of *pkl* mutants with GA greatly decreases their characteristic pickle-root phenotype (Henderson et al., 2004). Both PKL and BRM

CRCs have been proposed to act as positive regulators of the GA pathway possibly through distinct mechanisms. Unlike *brm* mutants, *pkl* mutant plants have an increased abundance of active GA (Archacki et al., 2013). Recently, PKL was found to positively regulate most GA-mediated developmental processes including promoting vegetative growth (hypocotyl, leaf, and inflorescence stem elongation) and phase transitions (i.e., the juvenile-to-adult and vegetative-to-reproductive transitions) (Park et al., 2017).

Excess cytokinin (CK) production inhibits primary root elongation and weakens lateral root development (Kuderova et al., 2008). SWP73B is one of the accessory subunits of *Arabidopsi*s SWI/SNF complexes shown to positively regulate root development and cell cycle progression in the root meristem by suppressing CK biosynthesis (**Figure 4**). SWP73B negatively regulates the CK biosynthesis genes *ADENOSINE PHOSPHATE-ISOPENTENYLTRANSFERASE* (*IPT3*) and (*IPT7*) and the CK-regulated cell cycle inhibitor *KIP-RELATED PROTEIN7* (*KRP7*) by hindering the deposition of active histone marks on their gene bodies (Jegu et al., 2015).

In aggregate, these findings indicate that both phytohormone biosynthesis and degradation in response to extracellular and intracellular cues partly depend on SWI/SNF chromatin remodeling ATPases. The findings also suggest the role of SWI/SNF ATPases as a hub in plant perception and response to phytohormone signaling as well as hormone crosstalk through direct physical interactions with hormone signaling pathway components. The outcomes of these interactions influence the plant response to abiotic stress, enabling plant adaptability under changing climates.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Chromatin remodeling has been extensively studied in the context of various regulatory and developmental processes in a number of eukaryotic organisms, including humans, mice, yeast, fruit fly and *Arabidopsis*. Phylogenetic analysis suggests that the chromatin remodeling machinery and their modes of operation are evolutionarily conserved across eukaryotes. However, SWI/SNF subunit loss-of-function studies have uncovered varied organism-specific phenotypes. These phenotypes also depend on the strength of a given mutation, leading to phenotypic differences among single and double mutants of SWI/SNF subunit genes. The functional redundancy of some SWI/SNF complex subunits also necessitates further in-depth analyses of their interaction patterns in different plant development pathways.

As discussed above, a number of plant regulatory and developmental transitions are controlled by chromatin modifiers through complex pathways in response to both endogenous and environmental factors. These precisely orchestrated mechanisms are heritable, underscoring their importance for plant survival in changing habitats. Moreover, the dynamics of these epigenetic controls go hand in hand with changes in plant growth conditions. Positive attributes that could potentially be integrated into plant breeding schemes are of interest to many plant scientists and breeders insofar as they may yield varieties that can withstand adverse weather conditions, early flowering or various biotic stresses and maximize nutrient utilization for improved yield. High-throughput technologies together with the vast genomic data presently available in public repositories should facilitate comparative and functional studies aimed at plant improvement. Future studies in other species of interest (e.g., rice and other crops) will likely build upon the findings on ATP-dependent CRCs in *Arabidopsis* and more recently in maize. Plant-specific molecular and genetic approaches should also characterize regulatory differences between developmental stages and under different growth conditions for a more comprehensive understanding of the involvement of SWI/SNF chromatin modifiers in plant growth and development.

GENERAL OUTLOOK

The following research questions and topics are of particular interest in terms of how CRCs may be involved in various processes either directly or indirectly related to plant development.

(1) Involvement of SWI/SNF ATPases in flowering regulation in the temperate legume model plant *Medicago truncatula*, whose flowering is prompted by winter cold and long-day photoperiods despite the absence of an *FLC* gene.
(2) The role played by chromatin remodelers in oxidative stress remediation processes resulting from various metabolic activities in plant cells other than DNA double-strand break repair.
(3) Besides their known function in the phosphate starvation response, there is a need to ascertain other roles of SWI/SNF remodelers in mediating the uptake of essential micro- and macronutrients from the soil in conjunction with nutrient transporter gene families and rhizosphere microbial activities.
(4) The mechanism by which the chromatin remodeling complex SWR1 mediates chromatin structural dynamics by replacing the core H2A-H2B histone dimer with the H2A.Z-H2B dimer in octamers is well characterized. However, the molecular mechanisms underlying the reverse process remain elusive.
(5) The specific roles of accessory subunits of *Arabidopsis* SWI/SNF CRCs in regulating plant development are only beginning to be understood. The latest example is SWP73 involvement in the cell cycle, leaf development, flowering time and embryogenesis. Much remains to be uncovered in this regard.

AUTHOR CONTRIBUTIONS

SO and SC conceived the idea, wrote the manuscript, and drafted the figures. SP designed and organized figures and revised the

manuscript. WL, MY, MA, and HZ revised the manuscript and organized the figures. YQ conceived the idea and revised and organized the manuscript.

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frontiers in Plant Science

ORIGINAL RESEARCH
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Cork Oak Young and Traumatic Periderms Show PCD Typical Chromatin Patterns but Different Chromatin-Modifying Genes Expression

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Plants are subjected to adverse conditions being outer protective tissues fundamental to their survival. Tree stems are enveloped by a periderm made of cork cells, resulting from the activity of the meristem phellogen. DNA methylation and histone modifications have important roles in the regulation of plant cell differentiation. However, studies on its involvement in cork differentiation are scarce despite periderm importance. Cork oak periderm development was used as a model to study the formation and differentiation of secondary protective tissues, and their behavior after traumatic wounding (traumatic periderm). Nuclei structural changes, dynamics of DNA methylation, and posttranslational histone modifications were assessed in young and traumatic periderms, after cork harvesting. Lenticular phellogen producing atypical non-suberized cells that disaggregate and form pores was also studied, due to high impact for cork industrial uses. Immunolocalization of active and repressive marks, transcription analysis of the corresponding genes, and correlations between gene expression and cork porosity were investigated. During young periderm development, a reduction in nuclei area along with high levels of DNA methylation occurred throughout epidermis disruption. As cork cells became more differentiated, whole nuclei progressive chromatin condensation with accumulation in the nuclear periphery and increasing DNA methylation was observed. Lenticular cells nuclei were highly fragmented with faint 5-mC labeling. Phellogen nuclei were less methylated than in cork cells, and in lenticular phellogen were even lower. No significant differences were detected in H3K4me3 and H3K18ac signals between cork cells layers, although an increase in H3K4me3 signals was found from the phellogen to cork cells. Distinct gene expression patterns in young and traumatic periderms suggest that cork differentiation might be under specific silencing regulatory pathways. Significant correlations were found between *QsMET1*, *QsMET2*, and *QsSUVH4* gene expression and cork porosity. This work evidences that DNA methylation and histone modifications play a role in cork differentiation and epidermis induced tension-stress. It also provides the first insights into chromatin dynamics during cork and lenticular cells differentiation pointing to a distinct type of remodeling associated with cell death.

Keywords: CORK, phellogen, lenticels, lenticular phellogen, 5-mC, H3K4Me3, H3K9me2, H3K18Ac

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INTRODUCTION

Plants are exposed to adverse environmental conditions like desiccation, freezing, heat injury, mechanical traumas, and disease. Enveloping protective tissues are thus fundamental to plants survival. Epidermis, the protecting tissue in primary tissues, is replaced by the periderm during stems and roots secondary growth (Evert, 2006). An exceptionally thick periderm is found in the cork oak (*Quercus suber* L.), the commercial cork, which due to several valuable properties, like imperviousness to liquids and insulation, is used for a wide number of important industrial applications (Pereira, 2007). Cork is the result of phellogen (cork cambium) meristematic activity followed by a particular differentiation process, involving cork cells expansion, cell walls suberization and deposition of waxes, ending with cell death and complete emptiness of the cells (Natividade, 1950; Pereira, 2007). In cork oak stems, the phellogen arises in the first year of growth in the subepidermal cell layer (Graça and Pereira, 2004) and continuously produces cork cells throughout the tree's lifespan accumulating a thick periderm very rapidly. Cork is allowed to be firstly harvested when the stem perimeter reaches the legal size (Oliveira and Costa, 2012). The separation of cork is obtained by the physical rupture of phellogen cells, leading to its death. A new traumatic phellogen is formed after cork extraction by a process of meristematic activation within the exposed non-conducting phloem (Fortes et al., 2004). After nine years of renewed growth, cork is thick enough to be stripped off again from the tree. This process is thereafter cyclically repeated allowing the sustainable exploration of cork-oak trees for more than 200 years. The cork produced by traumatic phellogens (*amadia* cork) has the best characteristics for industrial transformation, as opposed to the first cork divided by the original phellogen. However, even this cork can have widely variable characteristics, presumably due to both environmental and genetic factors, expressed as its industrial "quality." Cork quality is defined by the cork tissue thickness and homogeneity (Silva et al., 2005). The cumulative yearly layers of cork cells are locally crossed at certain points by lenticular channels, named cork pores. These channels are formed by the activity of particular regions of the phellogen, the lenticular phellogen, and are thought to permit gas diffusion between the inward living tissues, and the external environment. Cork porosity, meaning the number, dimension, and distribution of lenticular channels is widely variable in corks from different trees (Graça and Pereira, 2004). Corks with high levels of porosity strongly depreciate its industrial and economic value.

DNA methylation, post translational modifications of histones (HPTMs) and RNA-directed DNA methylation (RdDM) are hallmarks in modifying the functional state of chromatin, and together with nucleosome remodeling can alter the nuclear architecture during plant cell differentiation [reviewed in (Pikaard and Scheid, 2014; Ikeuchi et al., 2015; Takatsuka and Umeda, 2015; Latrasse et al., 2016)]. Plant genomes are methylated in CG, CHG, and CHH contexts which requires the activity of specific DNA methyltransferases (DNMTs) DNA METHYLTRANSFERASE 1 (MET1) maintains CG methylation; CHROMOMETHYLASE 3 (CMT3) maintains non-CG methylation in a self-reinforcing loop between histone H3K9 methylation and DNA methylation by requiring KRYPTONITE (KYP/SUVH4), SUVH5, and SUVH6, a H3K9 methyltransferases; and DOMAINS REARRANGED METHYLTRANSFERASES 1 (DRM1) and 2 (DRM2) responsible for CHH *de novo* methylation through the RdDM pathway [reviewed in (Pikaard and Scheid, 2014)]. CG methylation can result in gene silencing when found in promoter regions or be correlated with moderately high transcription when present within gene body (Zhang et al., 2006; Lister et al., 2008). Non-CG methylation is associated with the transcriptional silencing of transposable elements (TEs) (Cokus et al., 2008). Histone PTMs are also important components of chromatin-level control of gene activity contributing to define distinct chromatin states that modulate the access of transcription machinery to DNA [reviewed in (Kouzarides, 2007)]. Among these modifications, the dimethylation of histone H3 at lysine 9 (H3K9me2) is a highly conserved repressive mark found in heterochromatic regions with a particular role in the silencing of TEs and other repetitive DNA (Bernatavichute et al., 2008). Different HPTMs may be associated with transcriptionally active chromatin such as the trimethylation of histone H3 at lysine 4 (H3K4me3) which is a hallmark of transcription initiation (Roudier et al., 2011; Sequeira-Mendes et al., 2014) specifically accomplished by ATXR3 (Berr et al., 2010; Guo et al., 2010) and ATX3 (Chen et al., 2017). Another modification related to active chromatin is the acetylation of histone H3 at lysine 18 (H3K18ac) mainly found in regions surrounding the transcription start site and associated with transcription enhancers (Wang et al., 2008).

The regulation of specific plant developmental processes involving DNA methylation and HPTMs is well known [reviewed in (Pikaard and Scheid, 2014; Ikeuchi et al., 2015; Takatsuka and Umeda, 2015; Latrasse et al., 2016)]. Few studies were conducted in cork oak such as in pollen nuclei (Ribeiro et al., 2009), revealing an unexpected pattern of marks associated either with gene silencing or activation. Also, DNA methylation levels were correlated with tissue maturity during embryogenesis (Rodríguez-Sanz et al., 2014; Pérez et al., 2015), and with differences in cork cellular characteristics (cork quality traits) which could be directly related to original (Inácio et al., 2017) or traumatic phellogen activity (Ramos et al., 2013; Inácio et al., 2017). Notwithstanding the crucial role played by the cork as a protective tissue and its highly valued product, studies on the role of these modifications in cork formation and differentiation are still scarce.

In this work, we used cork oak periderm as a model to seek for the first insights into the formation and differentiation of secondary protective tissues at the chromatin level. We studied the chromatin organization and nuclei structural changes during cork cells differentiation together with the dynamics of DNA methylation and HPTMs in young and traumatic periderms, formed after cork extraction wounding. The relative expression of the corresponding chromatin-modifying genes was compared through qRT-PCR in both periderms. In addition, relationships between gene expression and the most relevant cork quality traits were investigated.

MATERIALS AND METHODS

Sampling

Young periderms were collected from sprigs harvested in randomly chosen cork oak adult trees located in Tapada da Ajuda field, Lisbon: herbaceous 'just burst' sprigs (hereafter referred as herbaceous); around one-year-old sprigs slightly lignified (hereafter referred as one-year old); and three-year-old sprigs heavily lignified (hereafter referred as three-year old). This was performed either by harvesting whole sprigs or by peeling off the periderm tissues.

Traumatic periderms in the form of cork planks were extracted from selected cork-oak trees during the harvesting season. The planks were harvested at breast height (at 1.30 m height from soil) from two adult trees located at Tapada da Ajuda field, Lisbon and seven adult trees located at a cork oak stand (*montado*) in Herdade dos Leitões, Montargil, Portugal. Trees were selected based on their cork quality parameters previously characterized (Inácio et al., 2017). All tissues were harvested during the period of more intense phellogen activity between July and September 2016. Samples of phellogen with contiguous differentiating tissue were collected by scraping the inner surface of cork planks and stored in liquid nitrogen until further use. Small pieces were also cut from the inner surface of the same cork planks and fixed for histologic and cytogenomic analysis. Neighboring cork planks from the same trees were collected for quality traits assessment [described elsewhere (Inácio et al., 2017)].

Fixation and Sectioning

Immediately after collection, detached periderms were fixed in 4% paraformaldehyde in 1× PBS (phosphate buffered saline: 137 mM NaCl; 0.27 mM KCl; 1 mM phosphate buffer, pH 7.4) under vacuum followed by overnight incubation in fresh fixative at 4°C. Periderms were then dehydrated with a graded ethanol series (50, 70, 85, 95, and 100%), cleared with histoclear (VWR Chemicals), and embedded in paraffin (VWR Chemicals). Tissue sections of 7 μm were made using a microtome and mounted in slides previously coated with poly-L-lysine (1 mg/mL, Sigma-Aldrich, Spain).

To preserve the 3D structure of the nuclei, thick sections of whole sprigs with one and three years old were made using the technique described by Conde et al. (2012).

Traumatic periderm and herbaceous sprigs were fixed in FAA (formaldehyde 37%, acetic acid, ethanol 50%, 1:1:18) under vacuum followed by overnight incubation in fresh fixative at 4°C, and dehydrated with a graded ethanol series (70, 96, and 100%) before embedding in glycol methacrylate – GMA (resin-based product – Technovit® 7100) following the manufacturer's instructions. Semi-thin sections (2 μm thick) were made using glass knifes on a piramitome LKB Bromma 11800.

Anatomy and Histology Studies

To identify the structures present in the herbaceous and one-year-old sprigs, these were stained with toluidine blue O (1%), and observed on a Leitz Biomed microscope (Leica Microsystems, Germany) under bright field and photographed with an AxioVision color camera (Carl Zeiss, Germany).

The traumatic periderms were stained with a combination of berberine 0.1% and crystal violet 0.5% to identify the cell walls composition. Preparations were observed in an Axio Imager.Z1 epifluorescence microscope (Carl Zeiss, Germany), and images were acquired with an AxioVision HRm camera (Carl Zeiss, Germany) using the Zeiss filter set 49 (445/50 nm).

The detection of autofluorescence in herbaceous, one-year-old sprigs and traumatic periderms observed with an Axio Imager.Z1 epifluorescence microscope (Carl Zeiss, Germany) using the Zeiss filter sets 49 (445/50 nm), 44 (530/50 nm), and 14 (590 nm) allowed the identification of tissues in the immunodetection experiments.

Immunolocalization of 5-Methylcytosine and Posttranslational Histone Modifications

Immunodetections of 5-methylcytosine and HPTMs in one and three-year-old sprigs were performed according to (Ribeiro et al., 2009) with some modifications. The antibodies used were chosen since they have been extensively tested in several studies in both plants and animals (Bianco-Miotto et al., 2010; Carvalho et al., 2010; She and Baroux, 2015; Groth et al., 2016) and their specificity has been verified (Nettersheim et al., 2013). Briefly, cell walls were partially digested with 2% cellulose (Sigma-Aldrich, Spain) in 1x PBS and hydrolysable tannins digested with 2 U/ml of tannase (Thermo Fisher Scientific, Waltham, MA, United States). The antigens were further retrieved using the microwave technique at full power for 8 min (Nic-Can et al., 2013). After cooling down, the sections were incubated with 5% BSA (bovine serum albumin, Sigma-Aldrich, Spain) to block non-specific binding, before the incubation with primary antibodies: anti-5mC (1:100 dilution, Abcam AB10805, Cambridge, United Kingdom), anti-H3K9me2 (1:5 dilution, Abcam AB1220, Cambridge, United Kingdom), anti-H3K4me3 (1:50 dilution, Abcam AB8580, Cambridge, United Kingdom), and anti-H3K18ac (1:100 dilution, Abcam AB1191, Cambridge, United Kingdom). Goat polyclonal secondary antibody to mouse or rabbit IgG – H&L conjugated to Alexa Fluor® 488 (Abcam AB150113, AB150077, Cambridge, United Kingdom) were added in a 1:100 dilution accordingly. The slides were mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, United Kingdom). Thick sections were examined on a Leica SP5 confocal coupled to a Leica DMI6000 (Leica, Germany) using a 63× 1.4 NA Oil immersion objective and HyD detectors in Standard Mode. Laser lines 405 and 488 nm where then used to excite DAPI and Alexa Fluor® 488 fluorochromes with spectral detection adjusted for each. Z-stacks with 0.5 μm were acquired to allow for the identification of entire nuclei.

Immunolocalization of 5-methylcytosine (5-mC) on semi-thin sections of herbaceous sprigs and traumatic periderms was carried out also according to (Ribeiro et al., 2009) but with a prior permeabilization with 1× PBS containing 0.5% Triton X-100. Sections were incubated with mouse monoclonal to 5-mC

(Abcam AB10805, Cambridge, United Kingdom), followed by incubation with anti-mouse Cy™3-labeled secondary antibody (Sigma-Aldrich C2181, Spain). The slides were mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, United Kingdom) and examined with Zeiss Axio Imager.Z1 epifluorescence microscope (Carl Zeiss, Germany) with a 63× 1.25 NA Oil immersion objective. Images were acquired with AxioVision HRm camera (Carl Zeiss, Germany) using Zeiss filter sets 49 (445/50 nm) and 14 (590 nm).

Fluorescence Intensity Quantification

To achieve precise and reliable comparisons between signals observed at distinct cell differentiation stages, confocal analysis was performed using the same laser excitation and sample emission capture settings (El-Tantawy et al., 2014). The same procedure was applied to epifluorescence analysis on herbaceous sprigs and traumatic periderms preparations. Measurements of the fluorescent signal intensity and nuclei area were performed on the different tissues and/or cork cell layers using Fiji (Schindelin et al., 2019). Projections of maximum fluorescence images from confocal Z-series were obtained and used to quantify fluorescence (Conde et al., 2012). The contour of each nucleus was manually outlined, and the fluorescence intensity was measured (sum of the fluorescence on each pixel within the outlined area). Fluorescence intensity in a non-labeled region was used to normalize all quantifications. The nucleus area was used to normalize the fluorescence intensity to avoid an artificial positive correlation between fluorescence intensity and nuclei size. The ratio fluorescence intensity/nucleus area reflects the amount of DNA methylation or histone modifications. Semi-thin sections of GMA (2 μm) and paraffin (7 μm) led to the segmentation of each nucleus, thus, for statistical analysis, these were grouped in classes according to its area (<15[, [15;25[, [>25 μm^2). Data are presented in standard boxplots as minimum, first quartile (bottom of box), median, third quartile (top of box), and maximum with the actual spread of individual observations represented by jittered dots. Differences in the ratio fluorescent intensity/nucleus area and in the nuclei area between tissues and/or cork cell layers were tested through Student's *t*-test and one-way ANOVA followed by Tukey's multiple comparison test, at a 5% significance level. For non-normally distributed data, Kruskal-Wallis non-parametric and Dunn's Multiple Correction *post hoc* tests were used. All statistical tests were performed using GraphPad Prism V5.0 software (GraphPad©, San Diego, CA, United States).

RNA Extraction

Total RNA was extracted from detached periderms and traumatic periderms with the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, Spain) according to manufacturer's instructions except for some minor modifications: the isolation buffer was supplemented with one volume of Plant RNA Isolation Aid (Thermo Fisher Scientific, Waltham, MA, United States) per unit mass of fresh tissue. Total RNA integrity was assessed by 1% (w/v) agarose gel electrophoresis. mRNA was isolated from total RNA using the Dynabeads mRNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, United States) following the manufacturer's directions. cDNA was synthesized from 45 ng of mRNA using oligo$(dT)_{18}$ in a 20 μL-reaction volume using RevertAid H Minus Reverse transcriptase (Thermo Scientific, Waltham, MA, United States) according to the manufacturer's protocol. cDNA was stored at −20°C until further use.

Putative *Quercus suber* Histone Methyltransferases Characterization

The cork oak *Qs*DNMTs (*QsCMT3*, *QsDRM2*, *QsMET1*, and *QsMET2*), and *QsSWC4* evaluated by qRT-PCR in this study were previously characterized (Ramos et al., 2011).

The putative cork oak sequences homologous of characterized histone methyltransferases (HMTs – *QsSUVH4*, *QsATXR3*, and *QsATX3*) were obtained by performing a BLAST at cork oak database[1] (Pereira-leal et al., 2014), using the Arabidopsis protein sequences as a query. Complete sequences were retrieved from the cork oak genome version 1.0 (Ramos et al., 2018), except for *QsATXR3*. Protein structure was analyzed using NCBI-CD[2] (Marchler-Bauer et al., 2016) and SMART searches[3] (Letunic and Bork, 2017). The potential cork oak *Qs*HMTs orthologous proteins in other angiosperms (**Supplementary Table 1**) were obtained by performing a BLAST at the NCBI database[4] (Altschul et al., 1997). All sequences were aligned with MUSCLE[5] (Edgar, 2004) and the alignment was trimmed with GBLOCKS[6] (Dereeper et al., 2008). *Qs*HMTs were used to perform a phylogenetic analysis with orthologous sequences. Phylogeny analysis was obtained with MEGA 7 software (Kumar et al., 2016), using the maximum likelihood method and a bootstrap of 1000.

Primer Selection and qRT-PCR Analysis

Seven target genes – *QsCMT3*, *QsDRM2*, *QsMET1*, *QsMET2*, *QsSWC4*, *QsSUVH4*, *QsATXR3*, and *QsATX3* – and four housekeeping genes – *ACT* (actin), *CACs* (clathrin adaptor complexes medium subunit family protein), *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), *EF-1α* (elongation factor 1-alfa) – were evaluated in this study. Primers were designed using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, United States) except for *QsDRM2* gene and housekeeping genes that was chosen from a previous gene expression studies in cork oak (Marum et al., 2012; Ramos et al., 2013). qRT-PCR experiments were performed in all tissues except for *QsSUVH4* which were only performed in three-year-old sprigs and traumatic periderms. Gene description, NCBI nucleotide and protein sequences accession numbers, primer sequences, and amplicon size are described in **Supplementary Table 2**.

The real-time qPCR was performed in 96 well white reaction plates (Bio-Rad, Hercules, CA, United States), using an IQ5 Real Time PCR (Bio-Rad, Hercules, CA, United States) with at least

[1]http://corkoakdb.org

[2]https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

[3]http://smart.embl.de

[4]https://blast.ncbi.nlm.nih.gov/Blast.cgi

[5]https://www.ebi.ac.uk/Tools/msa/muscle/

[6]http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=gblocks

six individuals and three technical replicates. All cDNA samples were diluted 20-fold and were amplified in triplicate in two independent PCR runs. The reaction mixture was composed of 1 μL diluted cDNA, 0.5 μM of each gene-specific primer and 5 μL master mix (SsoFast EvaGreen Supermix, Bio-Rad, Hercules, CA, United States). The following program was applied: initial polymerase activation, 95°C, 3 min; then 40 cycles at 94°C for 10 s (denaturation), 61°C (except for *ATXR3* which was 55°C) for 20 s (annealing), 72°C 15 s (extension), followed by a melting curve analysis to confirm the correct amplification of target gene fragments and the lack of primer dimmers. No template controls were also included in triplicate for each primer pair. Amplification efficiencies of all genes were estimated with the LinRegPCR quantitative PCR data analysis program (Ruijter et al., 2009) using the raw fluorescence data as input. According to NormFinder algorithm (Andersen et al., 2004) the *GAPDH* and *ACT* genes were chosen as the most stable ones to be used as references. The target genes relative expression ratio was calculated based on amplification efficiencies and expressed in comparison to the geometric mean of reference genes according to (Pfaffl, 2001).

Statistical analysis was performed by clustering data from each group of tissues (young periderms – one and three-years-old – and traumatic periderms) and evaluating statistical differences between them as well as between genes, through Student's *t*-tests and one-way ANOVA followed by Tukey's multiple comparison tests, at a 5% significance level.

Also, relationships between DNMTs, HMTs, and *QsSCW4* relative gene expression and cork quality traits previously assessed (Inácio et al., 2017) were analyzed by Pearson's correlations using 'rcorr' function from R environment. The studied traits were porosity coefficient, and pores area, length, and roundness (**Supplementary Table 3**), described in detail elsewhere (Inácio et al., 2017).

RESULTS

Cork Cells Keep Their Nuclei Up to the Last Phases of the Differentiating Process

In the herbaceous sprigs immediately after burst, only primary growth was detected. From the outside to the inside, epidermis with pluricellular trichomes, cortical parenchyma, primary phloem and xylem, and pith were identified. Periclinal divisions were detected in outermost cortex cell layer establishing the precursor of the phellogen (**Figure 1A**). Different tissues from the outermost to the innermost region of the one-year-old sprigs were clearly recognized: residues of the epidermis with trichomes, few cork cell layers, phellogen, phelloderm, cortex (**Figure 1B**), secondary and primary phloem, secondary and primary xylem, and pith. In the detached periderms the protection tissue is well individualized but the phellogen cell layer was not present since it was torn during the removal. In three-year-old sprigs, due to the increase in thickness of the stem, resulting from the underlying phellogen activity, the epidermis was no longer detectable, and increasing cork cell layers were detected (**Figure 1C**). Cork cell layers exhibited intense autofluorescence when excited with ultraviolet light, indicating the deposition of suberin in their cell walls, which favored the identification of the phellogen, a cell layer without fluorescence right below cork in whole sprigs.

The traumatic periderms, cork planks identical to commercial cork, had the thickness resulting from the nine years growth cycle. In the inner surface of cork planks, the tear zone, i.e., the region where the cork planks were detached from the tree, the cells were disrupted with several contiguous layers of differentiating cork cells already showing suberized walls from the early stages of the differentiation process (**Figure 1D**). Cork living cells showing entire nuclei (DAPI positive) with cytoplasmic content were observed in three-year-old sprigs and traumatic periderms several layers beyond the phellogen and the tear zone (**Figure 1E**). It was impossible to count the total number of living cork cell layers in traumatic periderms, since these were heavily corrugated. The cork cell layers will be referred hereafter as c1, c2, c3, c4, and c5 from phellogen to the outermost layers according to its age, being c1 the cork cell layer most recently formed and c5 the latter differentiation stages.

Chromatin Condenses, and Nuclei Area Decreases as Differentiation Proceeds in Cork Cells

Alterations in nuclei structure were noticed in differentiating cork cells in both young periderms (one and three-year-old sprigs) and traumatic periderms. Decreases in nuclei area from the phellogen to the cork layers at later stages of differentiation were detected ranging from 2.1-fold in one-year-old sprigs ($p < 0.001$, Tukey's multiple comparison test, **Figure 2A**) to 4.2-fold in traumatic periderm samples ($p < 0.001$, Tukey's multiple comparison test, **Figure 2B**). These changes were corroborated by the higher nuclei number with less than 15 μm^2 found in cork cells at later differentiating stages, particularly in traumatic periderm where no nuclei higher than 15 μm^2 were found (**Supplementary Figure 1**). The reduction in area was accompanied by drastic changes at the chromatin level. Chromatin progressively condensed as cork cells became more differentiated, and the condensed chromatin preferentially localized at the nuclear periphery.

Nuclei area in epidermis also suffered changes in morphology by decreasing its area from entire to disrupted stage (from herbaceous to one-year-old sprigs) (**Figure 2C**, $p < 0.001$, Tukey's multiple comparison test), as denoted by the absence of nuclei with areas higher than 25 μm^2 in the latter (**Figure 2D**).

Nuclear Fragmentation Is Present in Lenticels of Older Periderms

In younger periderms from one-year-old sprigs, small areas with intense meristematic activity denoted the lenticular phellogen. This meristem produces huge number of cells known as filling tissue forming the lenticels. The high number of cell layers produced by the lenticular phellogen propels the epidermis upwards causing its fracture (**Figure 3A**). In three-year-old

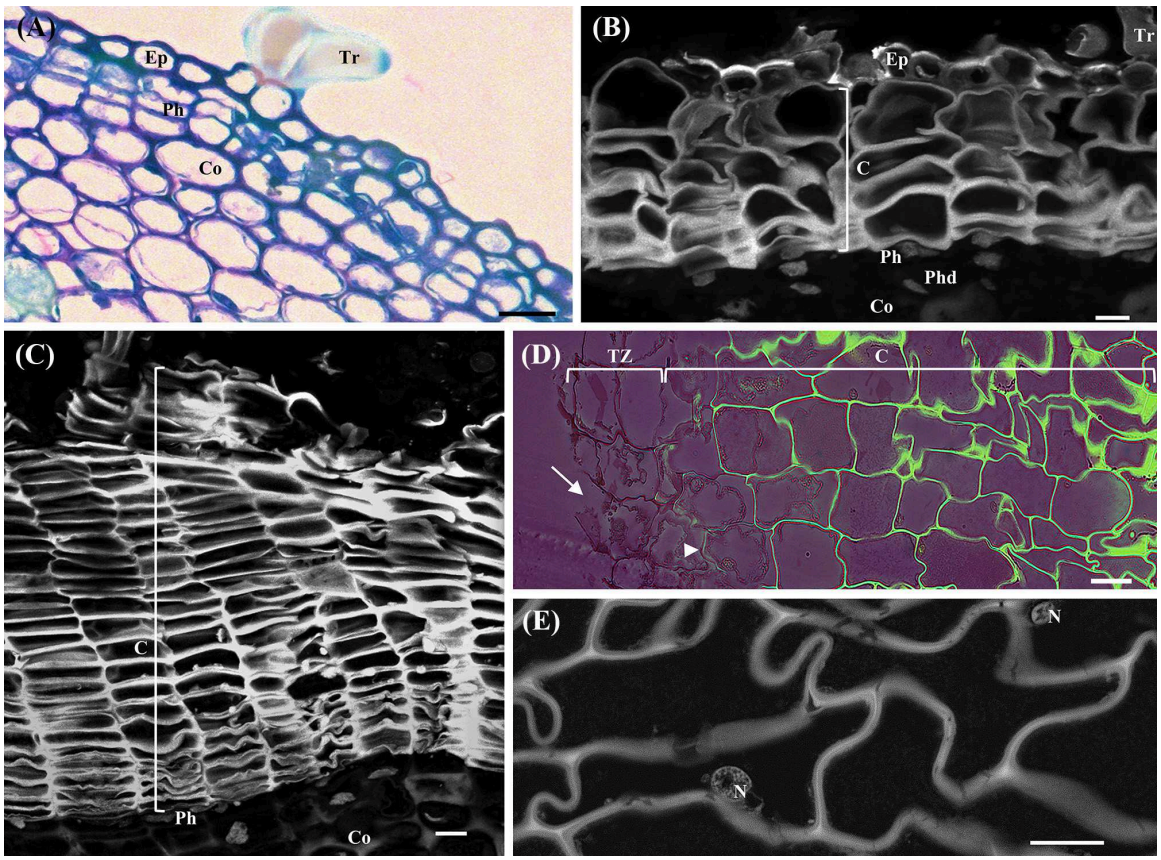

FIGURE 1 | Anatomical analysis of cross-sections of young sprigs (herbaceous, and one and three-year-old sprigs) and traumatic periderms. **(A)** Toluidine Blue O staining of the herbaceous sprigs showing the epidermis with pluricellular trichomes, cortical parenchyma, and the first periclinal divisions originating the precursor of the phellogen. **(B)** Autofluorescence detection under UV light of cork cells and residues of the epidermis with trichomes in cross-sections of one-year-old sprigs. Right below the cork cells is the phellogen, and the underlying tissues phelloderm and cortical parenchyma. **(C)** Autofluorescence detection under UV light of several cork cell layers resulting from the phellogen activity in cross-sections of three-year-old sprigs. **(D)** Berberine/crystal violet staining of traumatic periderms observed under UV light. The cells from the tear zone presented disrupted cellulosic reddish walls (arrows). The contiguous layer of cork cells already shows suberized walls at early stages of differentiation (arrowhead), and highly suberized walls at later stages of differentiation (intense green). **(E)** Cells at later stages of cork differentiation showing entire nuclei. DNA was counterstained with DAPI. Bar = 10 μm. Ep, epidermis; Tr, pluricellular trichomes; Co, cortex; Ph, phellogen; C, cork cells; Phd, Phelloderm; Tz, tear zone; N, nucleus.

sprigs, the lenticels were larger due to continuous lenticular phellogen activity during sprig development (**Figure 3B**). The wall of the cells produced by lenticular phellogen emitted much less autofluorescence than cork cells, indicative of a low suberin content, and a distinct cell wall composition. Contrastingly to cork cells, nuclei from older lenticels (from three-year-old sprigs) became misshapen (**Figure 3C**), and highly fragmented as cells differentiate, with portions of chromatin protruding from a central less condensed chromatin mass to the outermost regions of the lenticels (**Figure 3D**).

DNA Methylation and Posttranslational Histone Modifications Are Highly Dynamic During Cork Cells Differentiation

To assess the nuclear distribution of well-known markers of different chromatin functional states, immunolocalization of 5-mC and H3K9m2 (repressive marks), and H3K4me3 and H3K19ac (active marks) were performed. These studies revealed differences in the intensity and distribution patterns at distinct tissues and differentiation stages.

In very young herbaceous sprigs the nuclei showed discrete 5-mC signals distributed in small spots all over the chromatin both in cortex and epidermis (**Figure 4A**) with similar intensity level ($p > 0.05$, Unpaired *t*-test with Welch's correction, **Figure 4B**). In one-year-old sprigs, 5-mC signals were dispersed throughout the nucleus in all cork cell layers (**Figure 4C**) and significant higher intensity levels were detected in all cork cells when compared with phellogen, except for c2 ($p < 0.01$ for Ph vs. c1, $p < 0.05$ for Ph vs. c2, Dunn's multiple comparison test, **Figure 4D**). In fact, a 2.5-fold increase from phellogen to the more differentiated cork cell layer (c3) was noticed. Simultaneously, high levels of DNA methylation were found in epidermal nuclei in cells that remained alive (**Figure 4C**, arrow). In three-year-old sprigs,

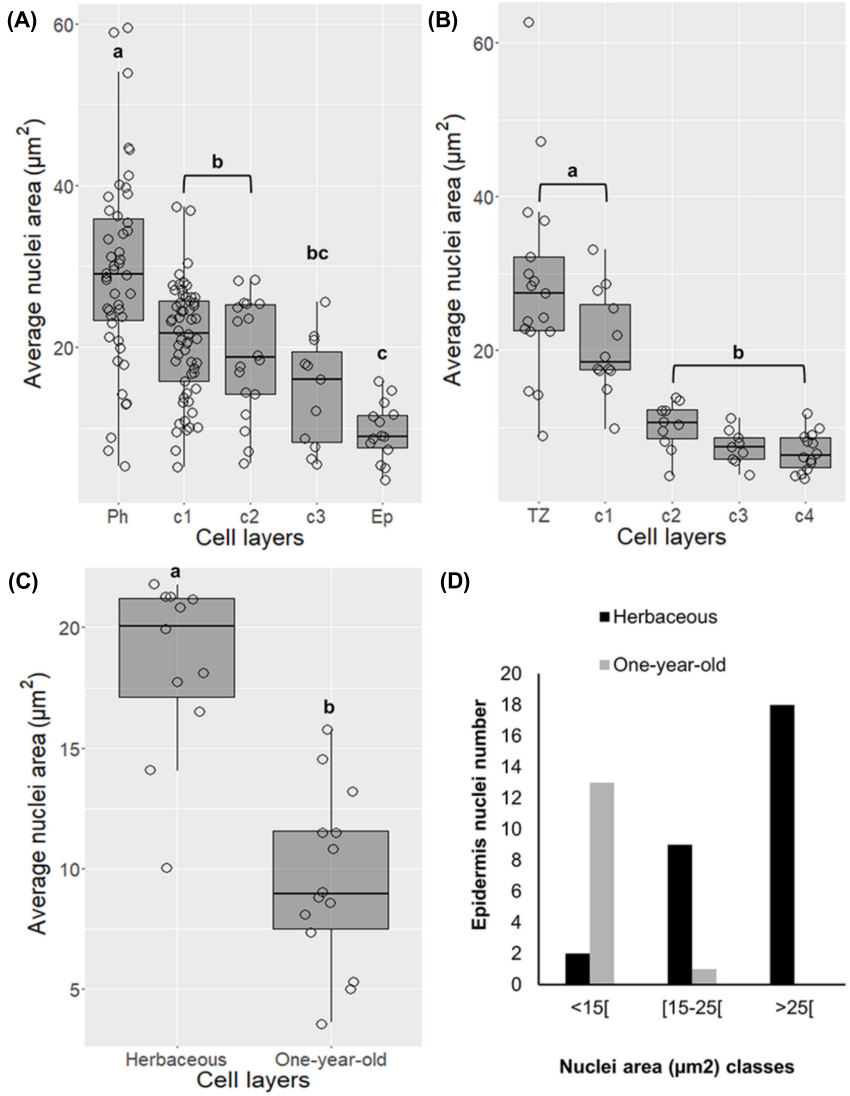

FIGURE 2 | Changes in nuclei area in differentiating cork cells in young periderms from one and three-year-old sprigs **(A)**, in traumatic periderms **(B)**, and in epidermis from herbaceous (entire epidermis) to one-year-old sprigs (disrupted epidermis) **(C)**. **(D)** Epidermis from one-year-old sprigs shows no nuclei with areas higher than 25 μm^2. Boxplots represent minimum nuclei area, first quartile (bottom of box), median, third quartile (top of box), and maximum. The distribution of every individual measurement is represented by jittered dots.

5-mC signals were dispersed throughout the nucleus in cork cells, however, the highest intensity was observed in nuclear periphery (**Figure 4E**). There was a significant increment in 5-mC intensity from c2 to c5 at the latter stages of differentiation ($p < 0.05$, Dunn's multiple comparison test, **Figure 4F**). In traumatic periderm, the tear zone, which locates at the immediate vicinity of the phellogen, showed faint 5-mC fluorescence signals distributed as very small spots all over the chromatin that became more intense at nuclear periphery in the more differentiated cork cells (**Figure 4G**). In nuclei with areas less than 15 μm^2, a remarkable 6.4-fold increase in the 5-mC level from the tear zone to cork cells at later differentiation stages (c4) was detected ($p < 0.01$, Dunn's multiple comparison test, **Figure 4H**).

The immunodetection of 5-mC in lenticels exposed differences in fluorescence signal between lenticel filling cells compared to the underlying lenticular phellogen, and neighboring phellogen and cork cells (**Figure 5A**). Although 5-mC signals were distributed throughout the nuclei within lenticels (**Figure 5B**), the quantification of changes in 5-mC levels along lenticular cell layers was unfeasible since nuclei were highly fragmented. Nevertheless, the less intensity in 5-mC signals in lenticular filling tissue compared with the surrounding tissues was quite evident (**Figure 5A**). Furthermore, lenticular phellogen exhibited significantly lower levels of 5-mC than the contiguous cork-forming phellogen ($p < 0.0001$, Unpaired t-test with Welch's correction, **Figure 5C**).

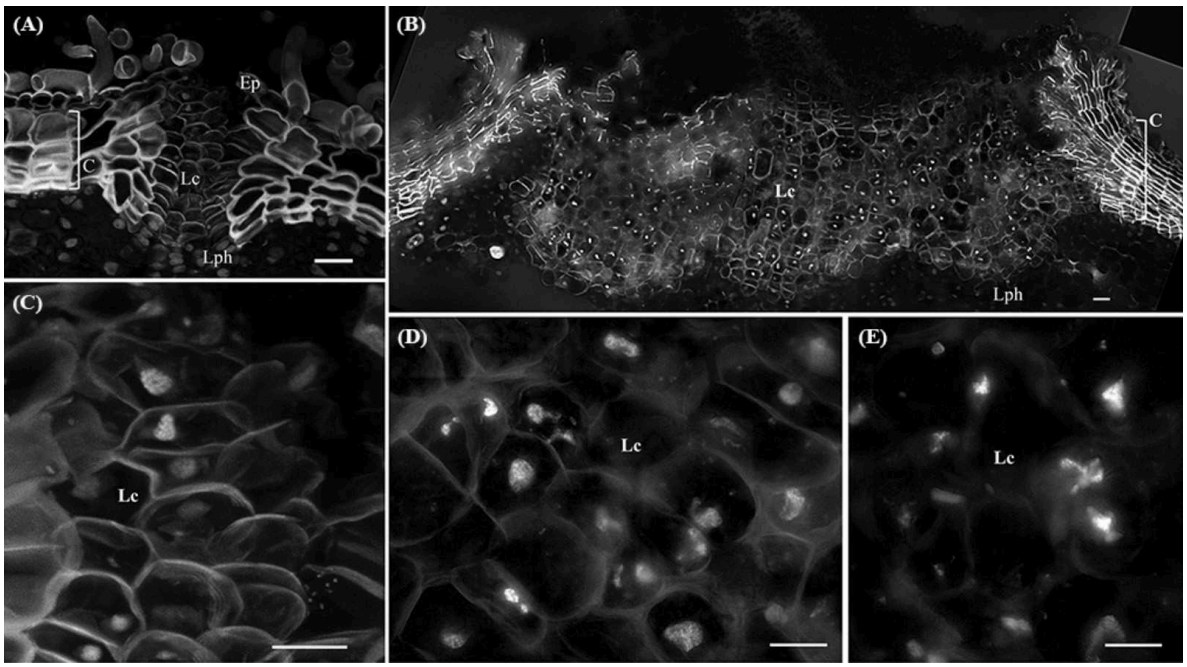

FIGURE 3 | Lenticels found in one-year-old sprigs **(A)** and in three-year-old sprigs **(B)**. The lenticels show a higher number of cell layers above the lenticular phellogen that propel the epidermis upwards causing it to fracture [arrowhead in **(A)**]. In three-year-old sprigs **(B)**, the lenticular phellogen localized in much larger areas due to anticlinal divisions and continuous activity during sprig development. The walls of the lenticular cells emit less autofluorescence than cork cells when excited with UV light, indicative of distinct cell wall composition. **(C)** Nuclei from younger lenticels are whole and round, while from older lenticels are misshapen and fragmented at the innermost regions of the lenticels **(D)**. **(E)** At the outermost regions of the lenticels nuclei are highly fragmented as cells are differentiating with portions of chromatin protruding from a central less condensed chromatin mass. DNA was counterstained with DAPI. Bars in **(A,B)** = 20 μm and in **(C–E)** = 10 μm. Ep, epidermis; Lph, lenticular phellogen; C, cork cells; Lc, lenticular cells.

Regarding the dimethylation of histone H3 at lysine 9 (H3K9me2) in one-year-old sprigs, two condensed chromatin knobs in the periphery of the nucleolus could be observed (**Figure 6**). The H3K9me2 signals were not quantified due to the reduced number of labeled nuclei.

The trimethylation of histone H3 at lysine 4 (H3K4me3) signals were distributed throughout the nuclei both in phellogen and differentiating cork cell layers in one and three-year-old sprigs (**Figures 7A,B**). In one-year-old sprigs, a 2.4 and 2.7-fold enrichment in the level of H3K4me3 was found from the phellogen to the two contiguous cork cell layers, respectively ($p < 0.001$, Dunn's multiple comparison test, **Figure 7C**), while in three-year-old an average of 2.6-fold increase was noticed from phellogen to each of the differentiating cork cell layers ($p < 0.001$, Dunn's multiple comparison test), with no differences between them (**Figure 7D**).

The nuclear distribution pattern of acetylation of histone H3 at lysine 18 (H3K18ac) revealed nuclei equally and thoroughly labeled in all cork cell layers in one-year-old sprigs (**Figure 8A**) and in all cork cell layers and in phellogen in three-year old sprigs (**Figure 8B**), respectively. No significant differences were detected in the intensity levels ($p > 0.05$ for both sprigs, Tukey's multiple comparison test, **Figures 8C,D**).

Quercus suber HMTs Proteins Showed All Expected Domains

The *Qs*DNMTs and *QsSWC4* studied in this work were previously characterized (Ramos et al., 2013). The three *Qs*HMTs analyzed revealed that *QsSUVH4* encodes a putative complete protein comprising all four domains found in SUVH4 proteins: SRA-YDG, Pre-SET, SET, and post-SET from N to C terminal; *QsATXR3* encodes a putative partial protein lacking the N-terminal, but with the SET domain of ATXR3 proteins detected at the C terminal; *QsATX3* encodes a putative complete protein, containing all domains described for ATX3 proteins: PWWP, plant homeodomains (PHD) finger, SET, and post-SET from N to C terminal (**Supplementary Figure 2**). All domains seemed conserved in the angiosperms used in this study (see **Supplementary Table 1** for list of angiosperms). Each *Qs*HMT grouped with their orthologous sequences establishing three well individualized groups (**Supplementary Figure 3**), revealing a high degree of conservation.

HMTs Differential Gene Expression Was Found Between Young and Traumatic Periderms

The relative expression of several *Qs*DNMTs, *Qs*HMTs, and *QsSWC4* was compared in young and traumatic periderms through qRT-PCR.

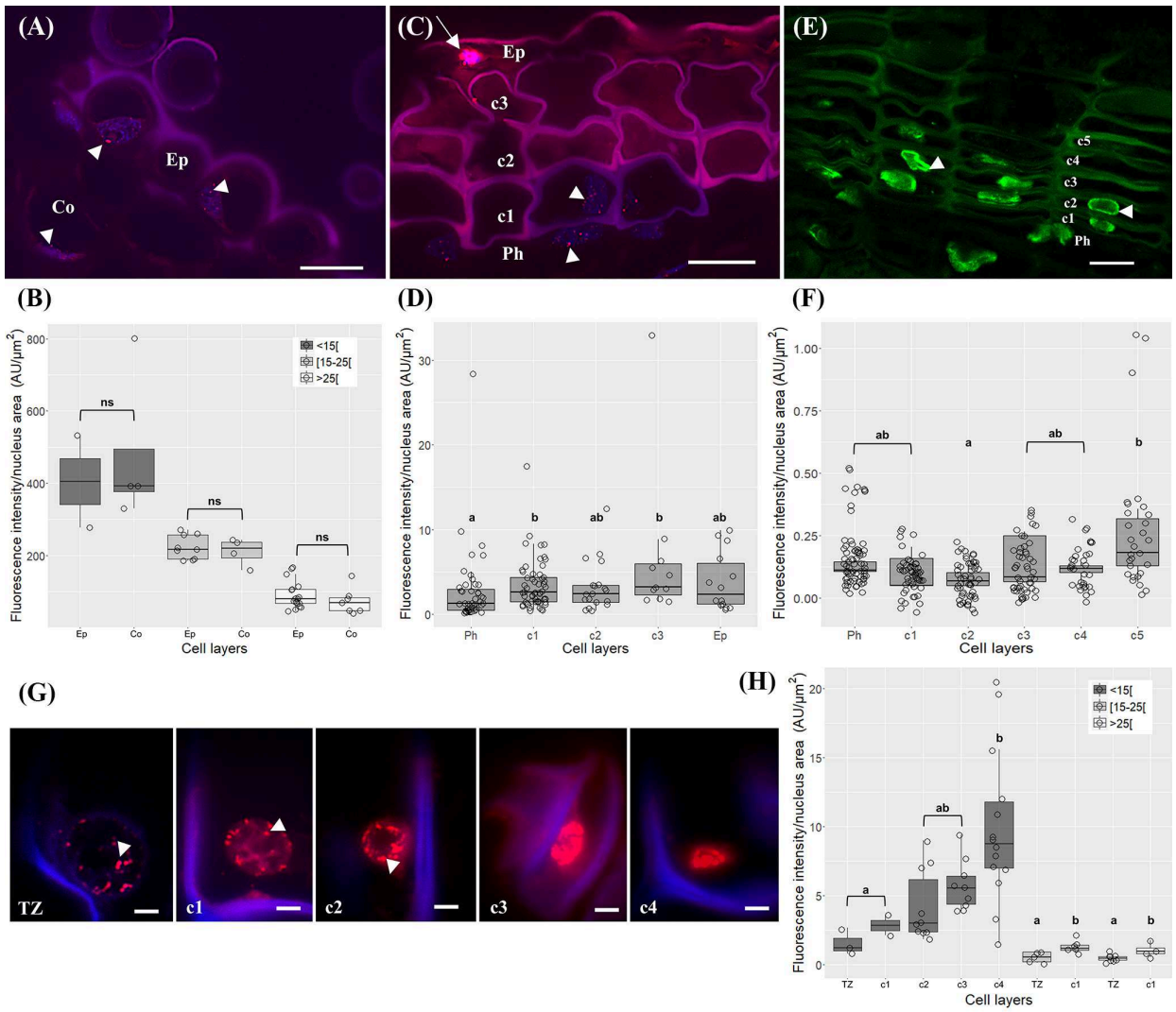

FIGURE 4 | Immunolocalization of 5-methylcytosine (5-mC) in periderms from young sprigs and traumatic periderms. **(A)** Herbaceous sprigs nuclei show discrete 5-mC signals distributed in small spots all over the chromatin in cortex and epidermis, with similar intensity level in all the nuclei area classes [<15[, [15;25[, and [<25[μm^2 **(B)**. **(C)** Nuclei of one-year old sprigs show 5-mC signals dispersed throughout the nucleus in all cork cell layers c1, c2, c3, c4; an epidermal nucleus shows high levels of DNA methylation (arrow); **(D)** all cork cells show significant higher intensity levels of 5-mC when compared with phellogen, except for c2 and a 2.5-fold increase is detected from the phellogen to the older cork cell layer (c3). **(E)** Nuclei of three-year-old sprigs show highest intensity of 5-mC signals at the nuclear periphery with a significant increase from c2 to c5 **(F)**. **(G)** Nuclei from traumatic periderms show increase and change in distribution: the nuclei from the tear zone and the most recently formed cork cell layer (c1) display slight dispersed 5-mC signals, that become stronger at the nuclear periphery in older cork cell layers (c2, c3, and c4). **(H)** A significant and pronounced increase in the 5-mC levels is noticed from the tear zone to the older cork living cell layer (c4) in nuclei with areas less than 15 μm^2. Boxplots represent minimum fluorescence intensity/nucleus area, first quartile (bottom of box), median, third quartile (top of box), and maximum. The distribution of every individual measurement is represented by jittered dots. Arrowheads indicate 5-mC signals. Similar small letters indicate no significant differences. DNA was counterstained with DAPI. Bars in (**A,C,E**) = 10 µm and in **(G)** = 2 µm. Ph, phellogen; Ep, epidermis; Co, cortex; Tz, tear zone.

A high accumulation of transcripts was found for *QsDRM2*, *QsMET1*, *QsATXR3,* and *QsATX3* genes in both young and traumatic periderms. Although variability was found between individuals, *Qs*DNMTs, *Qs*HMTs, and *QsSWC4* showed a tendency to be expressed at lower levels in traumatic than in young periderms, except for *QsSUVH4* and *QsATXR3* (**Figure 9**). *QsSUVH4* was amongst the most expressed genes in traumatic periderms, but significantly down-regulated in the young ones (p = 0.03, Mann Whitney test). In the latter, *QsSUVH4* was significantly down-regulated when compared with *QsCMT3* (p = 0.03, Unpaired t-test). The expression of *QsMET1* was positively and significantly correlated with porosity coefficient ($r \approx 0.95$, p = 0.003, 'rcorr' R function), while *QsMET2* and *QsSUVH4* expressions showed negative and significant correlations with porosity-related traits (pore length and roundness), one of the most relevant defects found in cork of ($r \approx 0.78$, p = 0.04, 'rcorr' R function; **Supplementary Table 4**).

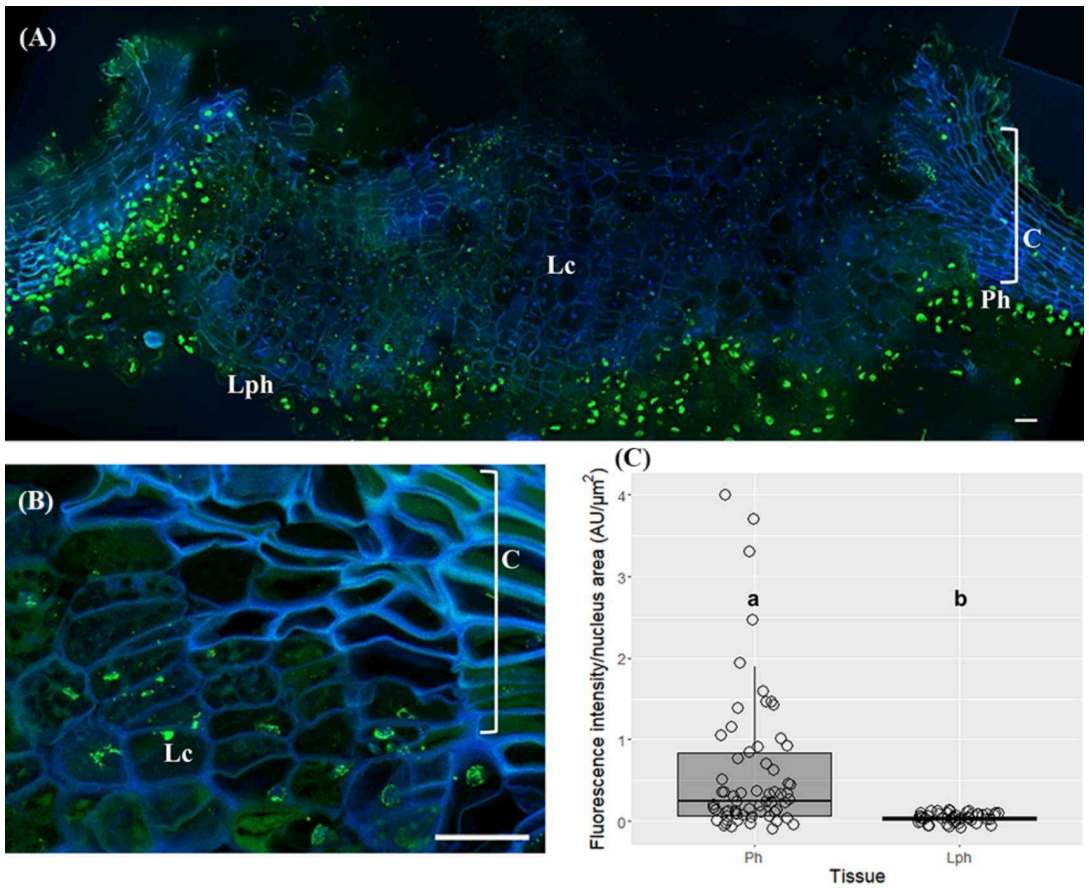

FIGURE 5 | Immunolocalization of 5-methylcytosine (5-mC) in lenticels from three-year-old sprigs. Less intense 5-mC signals in lenticular cells compared with the contiguous tissues like lenticular phellogen, phellogen, cork cells, and cortex **(A)**. **(B)** Fragmented nuclei of lenticular cells showing 5-mC signals. **(C)** Lenticular phellogen exhibit significant lower levels of 5-mC than the contiguous 'cork' phellogen; different small letters indicate significant differences. DNA was counterstained with DAPI. Bars = 10 μm. Lph, lenticular phellogen; Ph, phellogen; Lc, lenticular cells; C, cork cells; Co, cortex.

DISCUSSION

In this work, we used for the first time cork oak periderm as a model to study the formation and differentiation of secondary plant protective tissues and their behavior after traumatic wounding. Periderms are composed of three different types of tissues: the phellogen, a single cell layer secondary meristem with high cell cycling activity; the cork, a multi-cell layer tissue interrupted locally by lenticular channels; and the phelloderm, a single cell layer composed of living parenchymatous cells (Evert, 2006). An integrated approach that combines the immunodetection of several chromatin-modifying marks, and the expression of genes required for the imposition of these modifications enabled a detailed view of the dynamics of active and repressive chromatin marks in nuclei of periderm cells. Also, the establishment of correlations between gene expression and one of the most relevant cork quality traits was achieved. Although periderm formation and development has been extensively studied at the chemical and molecular level [(Miguel et al., 2015; Wunderling et al., 2018) and reviewed in (Franke et al., 2012; Graça et al., 2015; Vishwanath et al., 2015)], our results at the chromatin level brings novelty and adds significant value to the comprehension of its ontogeny.

Drastic Changes in Chromatin Structure Occurs During Cork Cells Differentiation

The periderm formation in cork oak involves several processes, including cork cells expansion, cell wall suberization (Graça and Pereira, 2004; Pereira, 2007), and likely programmed cell death (PCD) as a terminal differentiation step, resulting in several layers of dead cork cells with empty lumens, with a key function in insulation and protection. During cork cells differentiation, a repressive nuclear compartment was defined by a high level of DNA methylation at the nuclear periphery. Methylation at the nuclear periphery is usually associated with transposable elements (TEs) and repressed genes location (Bi et al., 2017). This suggests a striking reallocation of chromatin within these nuclei in oak meristematic cells since TEs are known to have an interspersed arrangement in potential gene-rich regions, accompanied by a dispersed 5-mC pattern all over the nuclei

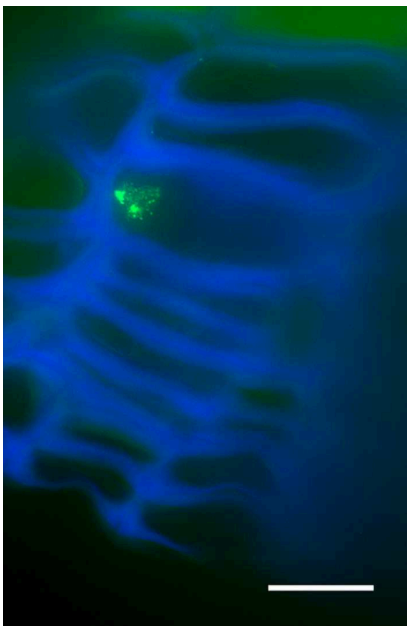

FIGURE 6 | Immunolocalization of histone H3 at lysine 9 (H3K9me2) in cross-sections of one-year-old sprigs shows two condensed chromatin knobs in the periphery of the nucleolus. Bar = 10 μm.

(Alves et al., 2012). Moreover, different types of nuclei can present different chromatin organizations, as detected by several epigenetic marks patterns in cork oak pollen nuclei (Ribeiro et al., 2009), corroborating a strong chromatin remodeling associated with development and differentiation processes in cork oak. Similar to cork cells differentiation are the changes in nuclear morphology and chromatin organization found amongst the most typical structural features in cells undergoing PCD [reviewed in (Van Hautegem et al., 2014; Latrasse et al., 2016)]. These alterations accompanied by increased levels and a change in the distribution of DNA methylation in differentiating cork cells has also been observed in tapetum cells PCD correlating with an up-regulation of MET1 (Solís et al., 2014), responsible for CpG methylation maintenance in cycling cells (Huang et al., 2010). Indeed, *QsMET1* was amongst the genes with higher levels of relative expression in the cork tissue contradicting, however, previous results in corks with different qualities (Ramos et al., 2013) where was argued that *QsMET2* might be substituting *QsMET1* function to maintain the CpG methylation during phellogen activity. In our work, although at lower levels than *QsMET1* this gene was amongst the genes with the highest expression. DNMT2 or MET2 are known to have weak or no DNA methyltransferase activity although still present in all eukaryotes (Ponger and Li, 2005). For these reasons it has been suggested that, at least in some species, it may have alternative roles (Vieira et al., 2017). In addition, it has been shown that AtDNMT2 interacts with AtHD2s, a unique plant-specific type of histone deacetylase family (Song et al., 2010). Moreover, increase in HD2 expression has been detected during fruit senescence (Kuang et al., 2011), indicating a possible role of DNMT2 in plant cell death programs through the association with histone deacetylases. Therefore, the preferential expression in tissues undergoing PCD, and the other functions might contribute to its high expression. Another important process in meristematic derivative cells is the *de novo* methylation mainly accomplished by DRM1 and DMR2 through the RdDM pathway [reviewed in (Pikaard and Scheid, 2014)]. In differentiating cork cells where DNA methylation is increasing it was not surprising to find the highest levels of gene expression for *QsDRM2*, according to previous results (Ramos et al., 2013). Different methyltransferases act together with chromatin remodeling complexes in an intricate interplay to modify chromatin structure and regulate transcription. CMT3 is required for CHG methylation maintenance, preferring hemimethylated CHG sites (Du et al., 2012), having an active role in TEs silencing (Tompa et al., 2002; Kato et al., 2003; Lippman et al., 2003). Indeed, in traumatic periderms, which are composed of phellogen and contiguous differentiating cork cells, around 20% of methylated CCG *loci* were found (Inácio et al., 2017), although a global DNA methylation view is compromised since the methylation in all other contexts could not be assessed. Considering the emergence of the repressive chromatin domain in these nuclei, and CMT3 function in silencing TEs, its expression in a tissue with intense meristematic activity and in its derivatives, is not surprising.

Epigenetic modifications are read by several protein complexes. DMAP1 is the human homologous of the Arabidopsis SWC4, which is a subunit of the chromatin remodeling complex SWR1C, and also a component of the NuA4 histone acetyltransferase complex, both involved in transcriptional regulation (Bieluszewski et al., 2015) by interacting with the promoters of target genes (Mozgová et al., 2015). SWC4 which has nucleosome acetyltransferase H4 activity (Doyon et al., 2004; Bieluszewski et al., 2015), is involved in several cellular processes such as, the regulation of mitotic cell cycle progression (Shin et al., 2010), DNA repair (Lee et al., 2010), and has been considered essential for plant development (Mozgová et al., 2015). The human DMAP1 is also a co-regulator that stimulates global maintenance of DNA methylation by co-working with MET1 (Rountree et al., 2000) at sites of double strand break repair (Negishi et al., 2009; Shin et al., 2010). The diverse functions of the DMAP1/SWC4 protein, as well as its involvement in two chromatin remodeling complexes associated with gene expression, should account for the expression detected in cork tissues during its differentiation, as already seen in traumatic corks (Ramos et al., 2013).

Like in cork cells, epidermis nuclei decrease in area whereas DNA methylation level increases from herbaceous to one-year-old sprigs, until this cell layer gradually disappear. During the enlargement of stems and roots due to secondary growth, the one-layered epidermis is replaced by the periderm, which thereafter assures the protective role (Evert, 2006). This replacement is caused by internal mechanical pressure driving to epidermis rupture. Thus, epidermis of woody species is a short-lived tissue that undergoes PCD likely as a response to mechanical stress signals, as the changes in chromatin structure observed are

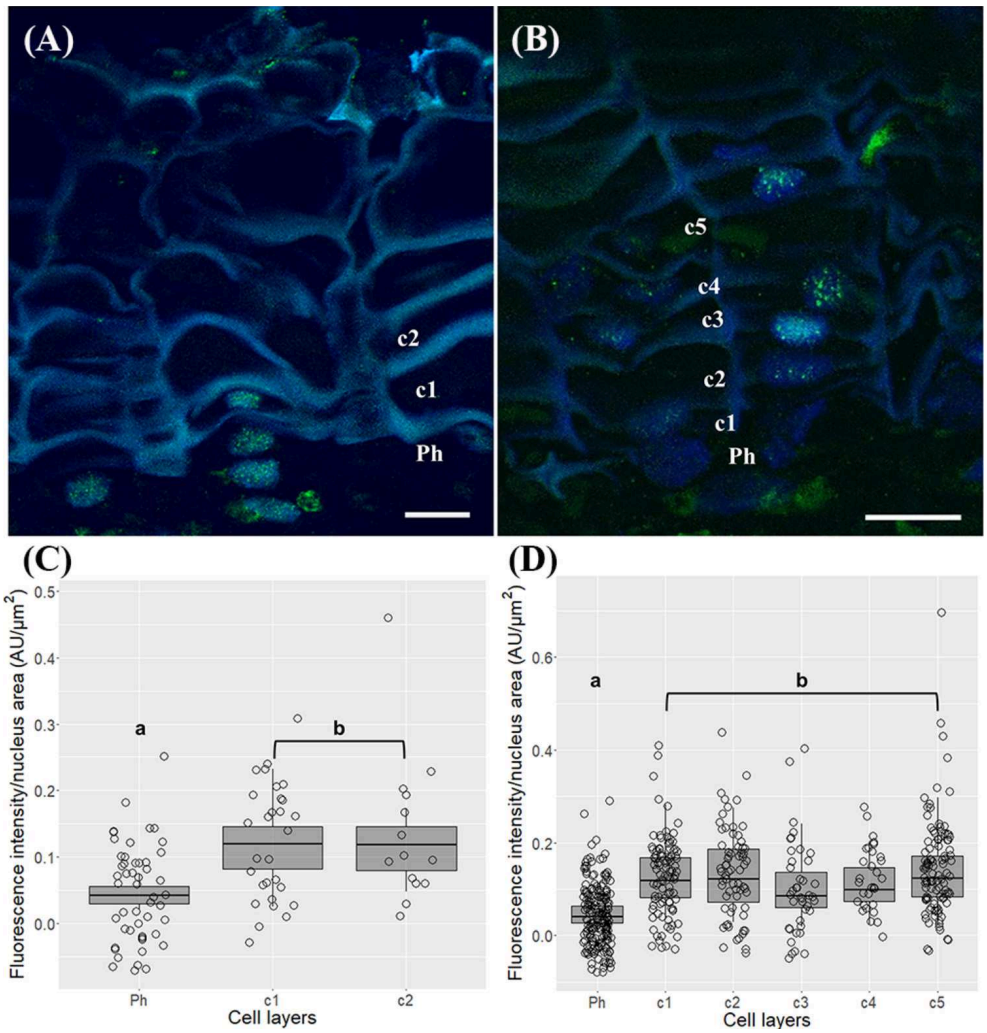

FIGURE 7 | Immunolocalization of the trimethylation of histone H3 at lysine 4 (H3K4me3) in periderms from young sprigs. **(A)** The nuclei from one-year old sprigs show H3K4me3 signals dispersed throughout the nucleus both in phellogen and cork cell layers c1, c2, c3, and c4 with an enrichment from the phellogen to the two contiguous cork cell layers (c1 and c2) with no differences between them **(B)**. Three-year old sprigs nuclei show H3K4me3 signals dispersed throughout the nucleus both in phellogen and cork cell layers c1, c2, c3, and c4 **(C)** with a significant increase from the phellogen to each of the cork cell layers (c1, c2, c3, c4, and c5) and no differences between them **(D)**; similar small letters indicate no significant differences. DNA was counterstained with DAPI. Bar = 10 µm. Ph, phellogen. Boxplots represent minimum fluorescence intensity/nucleus area, first quartile (bottom of box), median, third quartile (top of box), and maximum. The distribution of every individual measurement is represented by jittered dots.

typical features of this type of death. Indeed, mechanical stress-induced PCD has also been observed in lateral root cap cells in Arabidopsis driven by the expansion of underlying tissues (Fendrych et al., 2014) or in endosperm breakdown mediated by both endosperm softening and embryo growth (Fourquin et al., 2016). Mechanical stress-activated gene expression has been seen in Arabidopsis (Lee et al., 2005) and poplar (Coutand et al., 2009) in response to mechanic stimuli. Remarkably, columella cells which are subject to tensile stresses when root grows through soil particles, are the most highly methylated cells due to the hypermethylation of TEs (Kawakatsu et al., 2016). Thus, our results point to the modulation of the response to mechanical signals during epidermis replacement through DNA methylation.

In the present work, an enrichment in H3K4me3, a mark associated with gene activation (Roudier et al., 2011; Sequeira-Mendes et al., 2014), was found from the phellogen to all differentiating cork cell layers in young and traumatic periderms, accompanying the PCD process. In a similar process, senescent Arabidopsis leaves showed a strong correlation between up-regulated senescent-associated genes marked with H3K4me3 and gene expression (Hinderhofer and Zentgraf, 2001; Brusslan et al., 2015). Also, in developing secondary xylem of *Eucalyptus grandis*, cell wall-related genes with vital roles in wood formation were found to be H3K4me3-enriched (Hussey et al., 2017). Considering that periderm formation involves cork cells expansion, cell wall suberization and deposition of waxes (Graça and Pereira, 2004; Pereira, 2007), we may speculate that

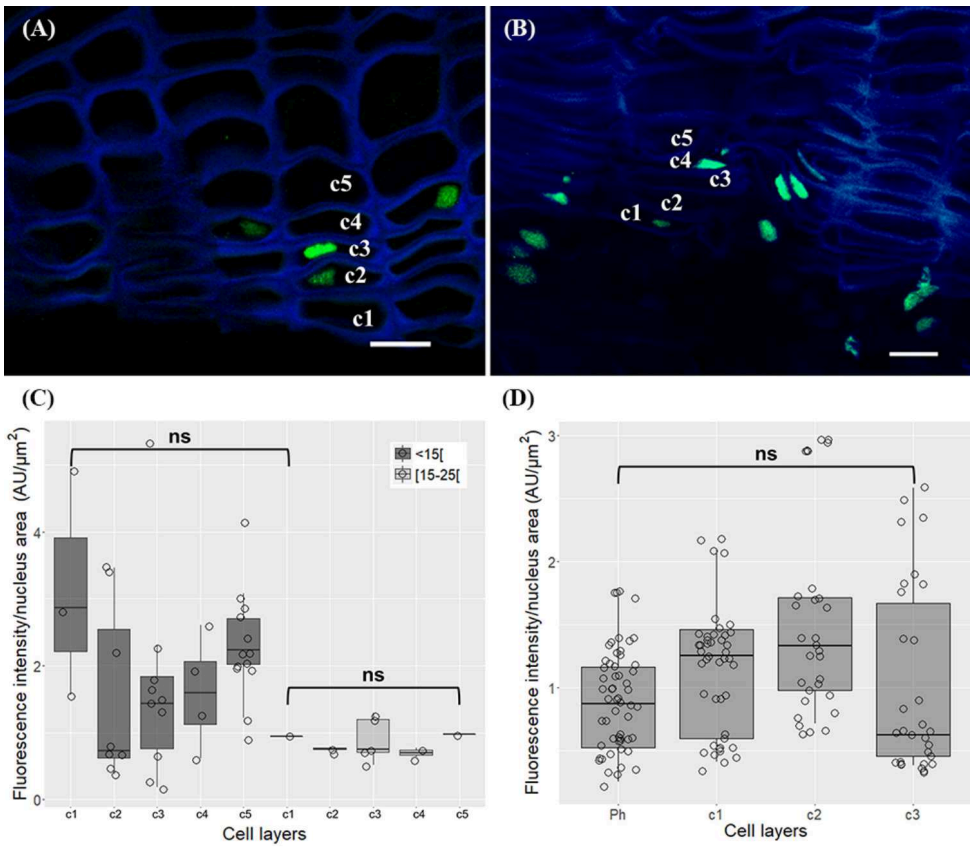

FIGURE 8 | Immunolocalization of the acetylation of histone H3 at lysine 18 (H3K18ac) in in periderms from young sprigs. Nuclei from all cork cell layers (c1, c2, c3, c4, and c5) show dispersed H3K18ac signals in one **(A)** and three-year-old sprigs **(C)**, with no significant differences detected **(B,D)**. DNA was counterstained with DAPI. Bar = 10 µm. Ph, phellogen. Boxplots represent minimum fluorescence intensity/nucleus area, first quartile (bottom of box), median, third quartile (top of box), and maximum. The distribution of every individual measurement is represented by jittered dots.

genes involved in suberin and waxes synthesis and deposition, as well as cell death-associated genes are up-regulated in cork cells (Soler et al., 2007; Teixeira et al., 2017; Boher et al., 2018) through H3K4me3 modification. Tri-methylation of histone H3 at lysine 4 is imposed by *ATXR3* and *ATX3* (Berr et al., 2010; Guo et al., 2010; Chen et al., 2017), which level of expression relates well to the amount of H3K4me3 in young and traumatic periderms.

Another important mark associated with transcription is the acetylation of histones. The levels of acetylation of histone H3 at lysine 18 were high, and similar in phellogen and all differentiating cork cell layers. H3K18ac is mainly located in the region surrounding the TSS and associated with enhancers (Wang et al., 2008), pointing to an increase of this mark in highly active cells. H3K18 acetyltransferase has been suggested to be required for the demethylation of a subset of ROS1 (repressor of silencing 1) targets such as the 35S rDNA arrays, and many TEs (Gong et al., 2002; Agius et al., 2006), since these repeats are enriched in this mark (Zhao et al., 2014; Tang et al., 2016). Although the role of H3K18ac in plant cell differentiation is largely unknown we may hypothesize that as cork cells differentiation involve extensive chromatin remodeling, and specific gene expression activation, H3K18ac might be needed to regulate repetitive sequences reorganization through ROS1.

Cork Differentiation in Young and Traumatic Periderms May Be Under Differential Silencing Pathways

The phellogen in young periderms is formed from cortical cells of primary origin right below epidermis shortly after burst of herbaceous sprigs (Graça and Pereira, 2004), while traumatic periderms are formed through a process of dedifferentiation and meristematic activation of the non-conducting phloem living cells (Evert, 2006). Phellogens with different ages and origins showed distinct DNA methylation profiles apparently linked to aging or/and to traumatic chromatin remodeling 'memories' during dedifferentiation (Inácio et al., 2017). High gene expression variability was found between individuals what could be related to their different cork qualities, since DNA methylation polymorphisms were previously found to be associated with distinct phenotypes (Inácio et al., 2017).

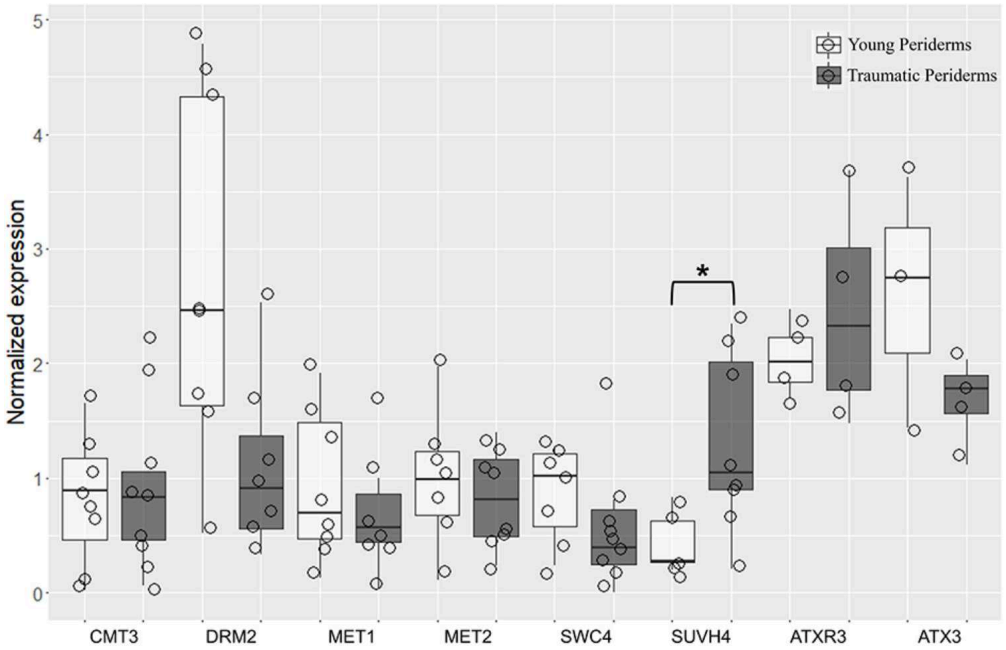

FIGURE 9 | Quantitative real-time PCR (qRT-PCR) evaluation of *QsCMT3*, *QsDRM2*, *QsMET1*, *QsMET2*, *QsSWC4*, *QsSUVH4*, *QsATXR3*, and *QsATX3* mRNA transcripts at different stages of periderm development: young (one and three-year-old sprigs) and traumatic periderms. Results are expressed as means ± standard deviation of at least six individuals and three technical replicates. Transcript levels were normalized to *actin* and *GAPDH*. Asterisks indicate the significance of the difference between young and traumatic periderms. Boxplots represent minimum relative expression, first quartile (bottom of box), median, third quartile (top of box), and maximum. The relative expressive of every individual is represented by jittered dots.

Although a close relationship between DNA and histone methylation is evidenced by a self-reinforcing loop between CMT3 and SUVH4 in controlling CHG DNA methylation through H3K9 methylation mark (Cao et al., 2000; Jackson et al., 2004; Huang et al., 2010; Du et al., 2015), *QsCMT3* and *QsSUVH4* expression did not followed the same tendency in young periderms. In fact, *QsSUVH4* expression was detected at significantly lower levels in comparison with *QsCMT3* while in traumatic corks similar levels were detected. *QsSUVH4* putatively contained all canonical domains identified for methylation of H3 at lysine 9 (Rea et al., 2000), including the SET domain and both pre-SET and post-SET domains required for methyltransferase activity (Baumbusch et al., 2001), and the SRA-YDG domain, reader of DNA methylation indispensable for both the interaction with histones and chromatin binding (Citterio et al., 2004). In addition, the phylogenetic analysis with orthologous SUVH4 proteins reinforced the belief of similar functions. All these facts presume the presence of this mark in cork oak nuclei, and although Vičić et al., 2013 were not able to detect H3K9me2 in oak's cycling cells nuclei, we clearly detected them in cork oak periderms, as previously in vegetative and generative pollen nuclei (Ribeiro et al., 2009). The distinct patterns observed in cork nuclei with two condensed chromatin knobs in the periphery of the nucleolus is in accordance with the enrichment of this mark in condensed and silent rDNA heterochromatic domains (Lawrence et al., 2004), which are preferentially located in nucleoli periphery (Leitch et al., 1992; Silva et al., 2008), and to the role of this mark in the silencing of TEs and other repetitive DNA elements (Bernatavichute et al., 2008). Recent studies on *cmt3* and *suvh4* mutants suggest that these enzymes strongly regulate CHH methylation through a different pathway (Stroud et al., 2013). Also, Zheng et al. (2012) proposed a novel role for SUVH4 in the control of Arabidopsis seed dormancy in a pathway not involving CMT3. The distinct expression patterns of *QsSUVH4* in periderms with different origins, together with different DNA methylation profiles found in traumatic periderms (Inácio et al., 2017), emphasizes the presence of these two silencing pathways in cork oak.

Cork and Lenticels Show Distinct Chromatin Remodeling Features

The homogeneity of the cork tissue is sometimes interrupted by discontinuities called lenticels that start to develop below stomata in the first periderm through the enhanced activity of the lenticular phellogen (Graça and Pereira, 2004). Lenticels are composed of a loosely filling tissue wherein the cells disaggregate giving rise to the lenticular channels or pores that radially cross the cork, providing a pathway for direct gas exchange between the exterior and the inner tissues (Lendzian, 2006). Although these cells also undergo a PCD program, the process is clearly different from the one suffered by cork cells, since developed lenticels showed highly fragmented nuclei with portions of chromatin protruding from a central less condensed

chromatin mass. Very similar chromatin organization and nuclear events were observed during protophloem sieve elements cell death program (Eleftheriou, 1986), and interestingly, in human cells (Dini et al., 1996) pointing to a common pathway in plant and animal kingdoms. Nuclear fragmentation has also been reported in several plant processes such as the ones associated with endosperm degradation during seed development (Wojciechowska and Olszewska, 2003), petal senescence (Yamada et al., 2006), abiotic stress (Ning et al., 2002), post-phloem transport in developing caryopsis (Kladnik et al., 2004), and hybrid lethality (Ueno et al., 2016). Although the features observed in this work are insufficient to comprehend the process underlying lenticular cells disaggregation it is clear that in cork oak periderms there are two types of chromatin remodeling processes associated with cell death: in cork tissue and in lenticels.

The nuclear morphology alterations seen in filling cells nuclei were accompanied by less intense 5-mC signals when compared with the surrounding tissues. This might suggest that other pathways such as the ones involving RNA-mediated gene silencing and/or Polycomb Group (PcG) proteins complexes [reviewed in (Derkacheva and Hennig, 2013; Pikaard and Scheid, 2014; Perera and Goodrich, 2016)] could contribute more than DNA methylation pathways to stably repress genes during lenticel filling cells death. To further test this hypothesis, components of these pathways should be studied in lenticels.

Moreover, the expression of *QsMET1* in traumatic periderms was positively and significantly correlated with porosity coefficient. Since lenticular channels (pores) are composed of cells with low suberin content it is tempting to speculate that this gene might be involved in the silencing of suberin-related genes in these cells. This is in accordance with previous work where this gene was also up-regulated in corks with high porosity (Ramos et al., 2013). Interestingly, differences in DNA methylation were also detected at the phellogen level. Lenticular phellogen showed significant lower levels of 5-mC when compared with cork-forming phellogen, what can be clearly related with the intense meristematic activity of the former when compared with the latter. On the other hand, a negative and significant correlation was found between *QsMET2* expression and pore length, which is supposedly related to regular lenticular phellogen activity throughout the years. *QsMET2* might be acting in cell-cycle genes silencing in lenticular phellogen through its possible interaction with the plant-specific histone deacetylase HD2s (Song et al., 2010). *QsSUVH4* expression and pore roundness showed a negative correlation. A more rounded pore apparently results from an irregular lenticular phellogen activity of all its cells along the years. Therefore, *QsSUVH4* might control genes responsible for its characteristic feature, i.e., higher cell division rates compared with cork-forming phellogen. These results strengthens the previous hypothesis that DNA methylation is likely involved in phellogen cells fate – whether it originates cork cells or lenticel filling cells – and is associated with differences in porosity (Inácio et al., 2017).

CONCLUSION

This is, to our knowledge, the first study focusing on the chromatin changes associated with periderm development and wound-periderm formation in trees. Particularly, it offers a comprehensive overview of DNA methylation and HPTMs distribution at the cell level and in distinct cell types. Distinct types of nuclear restructuring processes associated with cell death were also evidenced for the first time during cork and lenticular cells differentiation. Furthermore, this work strengthens the association of DNA methylation with phellogen cells fate and distinct cork quality and suggests that different silencing pathways might contribute to cork differentiation in young and traumatic periderms.

Regardless the limitations of this work in understanding the functional role of these marks in PCD programs, it puts forward a novel view and important breakthroughs into developmental processes in woody species.

To decipher the involvement of chromatin organization and the functional impact in cork differentiation and PCD, multidisciplinary approaches using reverse genetics, deep methylomes, and transcriptomes analyses must be conducted.

Taken together, our findings provide new insights into the dynamics of active and repressive chromatin marks in phellogen activity, cork differentiation and lenticel patterns that determine its industrial quality.

AUTHOR CONTRIBUTIONS

VI performed the laboratory work, data analysis, and worked on the original draft of the manuscript. MM contributed to the qRT-PCR experiments. JG conceived the tree sampling, analyzed the data, and critically reviewed the manuscript. LM-C conceived and designed the experiments, analyzed the data, and critically reviewed the manuscript.

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violet image. We also thank FCT (Portuguese Foundation for Science and Technology) research units: LEAF (Linking Landscape, Environment, Agriculture and Food – UID/AGR/04129/2013) and CEF (Forest Research Center – UID/AGR/00239/2013).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01194/full#supplementary-material

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The Role of Promoter-Associated Histone Acetylation of *Haem Oxygenase-1* (*HO-1*) and *Giberellic Acid-Stimulated Like-1* (*GSL-1*) Genes in Heat-Induced Lateral Root Primordium Inhibition in Maize

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In plants, lateral roots play a crucial role in the uptake of water and nutrients. Several genes such as *Zea mays Haem Oxygenase-1* (*ZmHO-1*) and *Giberellic Acid-Stimulated Like-1* (*ZmGSL-1*) have been found to be involved in lateral root development. In the present investigation, we observed that heat treatment might be involved in the inhibition of lateral root primordium (LRP) formation in maize, accompanied by an increase in global acetylation levels of histone 3 lysine residue 9 (H3K9) and histone 4 lysine residue 5 (H4K5), suggesting that histone modification was related to LRP inhibition. However, Trichostatin A (TSA), an inhibitor of histone deacetylases (HDACs), apparently did not inhibit the LRP formation, revealing that global hyperacetylation might not be the determining factor in the LRP inhibition induced by heat stress. Furthermore, expression of genes related to lateral root development in maize, *ZmHO-1* and *ZmGSL-1*, was down-regulated and the acetylation levels in the promoter region of these two genes were decreased under heat stress, suggesting that promoter-associated histone acetylation might be associated with the expression of *ZmHO-1* and *ZmGSL-1* genes which were found to be involved in the heat-induced LRP inhibition in maize.

Keywords: *Zea mays*, heat stress, epigenetics, histone acetylation, HDACs, H3K9, H4K5, lateral root primordium inhibition

INTRODUCTION

Plant roots play a crucial role in communication at the root-soil interface (i.e., water and nutrition uptake), and anchorage (Villordon et al., 2014). Root system architecture (RSA) is regulated by an endogenous genetic program, including cultivars or inbred lines (Song et al., 2016) and external factors, including biotic and abiotic environment (Gifford et al., 2013; Dondoni and Marra, 2014; Zhang et al., 2014; Yu et al., 2015). A typical plant root is divided into several functional zones along the root's longitudinal axis, including the root cap, meristem zone, elongation zone, mature zone, and lateral root zone (Wilson et al., 2015).

Maize (*Zea mays*), an agronomically important cereal crop, plays an important role in food, animal feed, and biofuel production worldwide and its roots indicate spatio-temporal complexity and have distinct genetic control (Smith and De Smet, 2012; Rogers and Benfey, 2015; Tai et al., 2016; Song et al., 2016). Morphology of maize roots consists of embryonic root system: an embryonic primary root forms 2 or 3 days after germination and a variable number of seminal roots arise a week after germination and extensive post-embryonic shoot-borne root system: crown roots arise from consecutive underground nodes of the stem which initiates ∼10 days after germination, and brace roots arise from consecutive aboveground nodes of the stem which initiates ∼6 weeks after germination (Hochholdinger et al., 2004; Zhang et al., 2014; Tai et al., 2016). Lateral root formation can be characterized into two major phases: pericycle activation (stimulation and dedifferentiation of pericycle cells) which proliferates to form a LRP and meristem establishment which occurs via cell expansion and activation of the lateral root meristem (Malamy and Benfey, 1997; De Smet, 2012; Zhang et al., 2014). Lateral roots arise from embryonic primary and seminal roots and post-embryonic shoot-borne roots, constituting an extensive underground branching network which increases the interacting surface (Malamy, 2005; Hochholdinger and Zimmermann, 2008; Rogers and Benfey, 2015).

It has been reported that maize lateral roots arise via the division of pericycle and endodermis cell (Fahn, 1990; Yu et al., 2016), whereas *Arabidopsis* lateral roots arise completely from pericycle cells (Dubrovsky et al., 2000; Beeckman et al., 2001). Lateral root formation is affected by various intrinsic and extrinsic factors, such as phytohormones and environmental stimuli (Marhavý et al., 2016; Yu et al., 2016). Substantial evidence has revealed that lateral root initiation is regulated by alteration of cyclins and cyclin-dependent kinases (CDKs) induced by auxin in pericycle cells (Malamy, 2005; Yu et al., 2015). Several related genes have been found to be associated with the lateral root initiation and formation in plants. Members of the plant-specific *Gibberellic Acid-stimulated Arabidopsis* (*GASA*) gene family play a significant roles in diverse biological processes, such as seed germination (Rubinovich and Weiss, 2010; Zhong et al., 2015) and flower induction (Roxrud et al., 2007). *GAST1* (*Gibberellic Acid Stimulated Transcript 1*) is the first member of the *GASA* gene family and has been identified in tomato which has a similar function in lateral root formation to *Root System Induced1* (*RSI1*) (Shi et al., 1992). The *GASA* gene family consists of 14 genes; evidence on *GASA4* has demonstrated that it expressed significantly in meristematic tissue namely, primary and lateral roots (Aubert et al., 1998). Several studies have suggested that *GASA*-like genes mainly regulated cell division and elongation (Ben-Nissan et al., 2004). *Gibberellic Acid Induced Petunia 1* (*GIP1*) identified from petunia (*Petunia hybrida*) has been suggested to be closely related to stem elongation. While *GIP4* and *GIP5* have shown similar expression changes during cell division regulation as *GASA4* in *Arabidopsis* (Aubert et al., 1998; Ben-Nissan et al., 2004). Ten members of *GASA*-like family have been identified in maize (Zimmermann et al., 2010). The *Z. mays Gibberellic Acid- Stimulated Like* (*ZmGSL*) family encodes small proteins of 75 to 128 amino acids which contain 12 perfectly conserved cysteines (Zimmermann et al., 2010). The result from lateral root mutants *lrt1* and *rum1* has indicated that *ZmGSL2*, *ZmGSL4*, *ZmGSL6,* and *ZmGSL9* contributed to lateral root formation and development (Zimmermann et al., 2010). Specifically, *ZmGSL2* represented a maize-specific checkpoint of lateral root formation in primary roots, while *ZmGSL4*, *ZmGSL6,* and *ZmGSL9* is likely to contribute to the initial events of lateral root formation. Recently, it was found that *Haem oxygenase* (*HO*) gene also acts as an important regulator in the lateral root formation (Han et al., 2012). HO catabolizes haem into three products: carbon monoxide (CO), biliverdin (BV) and free iron (Hsu et al., 2013). Three HO proteins, HO-1, HO-2, and HO-3, have been identified in mammals (Maines, 2004). However, several forms of *HO-1*-like genes have been isolated and identified from *Arabidopsis* (*AtHO-1*), Tomato (*LeHO-1*) and rice (*OsHO-1*) (Han et al., 2012; Meng and Liao, 2016; Mahawar and Shekhawat, 2018). The expression of *HO-1* can be induced by multiple stimuli, such as, reactive oxygen species (ROS) (Chen et al., 2009), salinity or heavy metal stress (Han et al., 2008; Xie et al., 2008), and ultraviolet radiation (Yannarelli et al., 2006). Hence, it is well established that the expression of *HO-1* is an antioxidant mechanism against oxidative damage subject to diverse stress (Shekhawat and Verma, 2010). In maize, ZmHO-1 has a conserved HO signature sequence and shares highest homology with rice OsHO-1 protein. It is established that up-regulation of *ZmHO-1* is closely associated with maize lateral root formation by modulating cell cycle regulatory genes and enhancing CO production (Han et al., 2012).

Histone post-translational modification can occur at the N-terminal tail of core histones (H2A, H2B, H3, H4) through acetylation, methylation, phosphorylation, and ubiquitination (Jenuwein and Allis, 2001). Previous studies have shown that histone acetylation are involved in plant resistance response to abiotic stimuli (Hu et al., 2011; Zhao et al., 2014). Here, we illustrated that heat stress might led to the inhibition of LRP formation in maize. To establish the role of epigenetic mechanisms in regulating LRP formation in maize, we have studied the role of TSA, an inhibitor of HDACs. Our findings revealed that TSA did not inhibit lateral root formation, suggesting that global hyperacetylation might not be the determining factor regulating inhibition of lateral root formation in maize. Furthermore, we have analyzed the role of lateral root initiation/development genes, such as *ZmHO-1* and *ZmGSL-1* genes under heat stress. Our research corroborates that *ZmHO-1* and *ZmGSL-1* genes showed down-regulation and acetylation of the promoter regions of these two genes was decreased under heat stress, indicating that promoter-associated histone acetylation of *HO-1* and *GSL-1* genes might be involved in the heat-induced LRP inhibition in maize.

RESULTS

Heat Stress Inhibits Lateral Root Formation

In an attempt to investigate the genetic mechanism of the plant response to heat stress, our results suggested that heat stress led

to LRP inhibition in maturation zone of maize seedlings. The seedlings of maize hybrid line *Huayu 5* were treated for 1–6 days at 45°C. No LRP initiation was observed at the individual level in the heat-treated seedlings, whereas several lateral roots appeared in maturation zone of the control roots, showing that heat stress inhibits the LRP formation (**Figure 1A**). To verify the inhibition effects of heat stress on lateral root formation, we performed paraffin section with maturation zones in control and heat treated roots at the individual and tissue level. Our work substantiates that lateral roots were initiated from vascular bundle in control and formation of lateral roots might be hardly observed in heat stressed seedlings (**Figure 1B**). Therefore, our results have shown that heat stress might significantly inhibit the initiation of LRP formation from vascular bundle.

Genomic Histone Hyperacetylation Accompanied the Inhibition Process

To analyze whether heat stress affects histone acetylation in the seedling roots, we have performed western blot analysis with anti-H3K9ac, anti-H4K5ac and anti-H3. H3K9ac and H4K5ac are the two important euchromatic marks positively regulating gene transcription. We have determined that genomic acetylation levels of H3K9 and H4K5 were both significantly increased under heat stress, compared with the untreated control roots (**Figures 2A–C**). Furthermore, we have carried out immunostaining assay to detect histone acetylation at the cellular level. The immunostaining results were consistent with previous results and the distribution of H3K9ac and H4K5ac were found to be unaltered under heat stress as compared with the control (**Figures 2D,E**). Besides, nucleus size was larger under heat stress than that of the control, and possibly linked to the genomic histone hyperacetylation (**Figures 2D,E**).

Histone acetylation is regulated by *histone acetyltransferases* (*HATs*) and *HDACs* (Kouzarides, 2007). To analyze the expression levels of *HATs* and *HDACs*, two *HAT* genes *GENERAL CONTROL NON-DEREPRESSIBLE 5* (*GCN5*) and *HAT-B* representing two types of *HATs* (*HAT-A* and *HAT-B*), and two HDAC genes (*HDAC101* and *HDAC106*) were investigated. The expression of *GCN5* consistently showed an increased level under heat stress and it was nearly four-fold higher after 6 days of treatment than that in the control group (**Figure 3A**). The expression of *HAT-B* was increased after 1 and 3 days of heat treatments, however it was decreased after 6 days of heat treatment (**Figure 3B**). In brief, the expression of *HATs* were increased under heat treatment. The expression of *HDAC101* exhibited no distinct changes after 1 and 3 days of treatments while it declined after 6 days of treatment (**Figure 3C**). The expression of *HDAC106* revealed a relatively decreased level after heat treatment and it was over 80% lower than that in the control after 6 days of treatment (**Figure 3D**). Overall, the expression of *HDACs* displayed a lower level after heat treatment. The expression changes in *HATs* and *HDACs* genes were consistent with the genomic histone hyperacetylation under heat stress.

Genomic Histone Hyperacetylation Perhaps Not Be the Key Regulator of LRP Inhibition

An increase in histone hyperacetylation under heat treatment led us to investigate the role of histone hyperacetylation in lateral root formation under heat stress. To examine the hyperacetylation level of the genome under heat stress, TSA, an inhibitor of HDAC, was used to treat maize seedlings; both analyses have clearly demonstrated the inhibitory effects. The western blotting results revealed that TSA had clearly enhanced the H3K9ac and H4K5ac levels (**Figure 4A**) and the immunostaining of nuclei further displayed similar results (**Figure 4B**). Notably, TSA might not be involved in the inhibition of LRP formation at both individual and tissue levels (**Figures 4C,D**), suggesting that genomic hyperacetylation

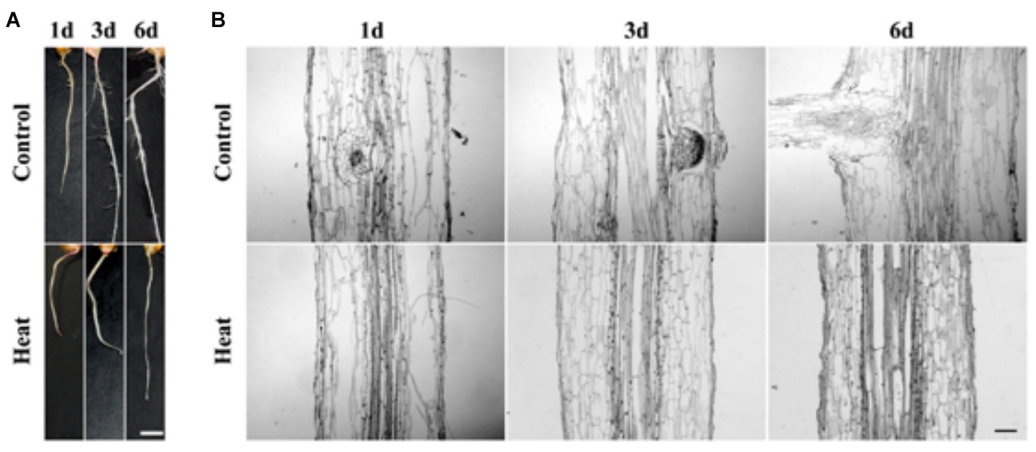

FIGURE 1 | (A) Phenotype characteristics of maize plant at 1, 3, and 6 days under heat treatment and control condition. The lateral root primordium (LRP) initiation was inhibited in maize seedling under heat treatment for 1 day hence no lateral root was observed. Bar = 5 mm. **(B)** Paraffin section of the LRP zone. Analysis of paraffin tissue section showed that the LRP initiation was inhibited under heat stress. As compared to the untreated controlled root LRP initiation was not observed under heat treatment incubation at 45°C, suggesting that LRP initiation was inhibited. Bar = 10 μm.

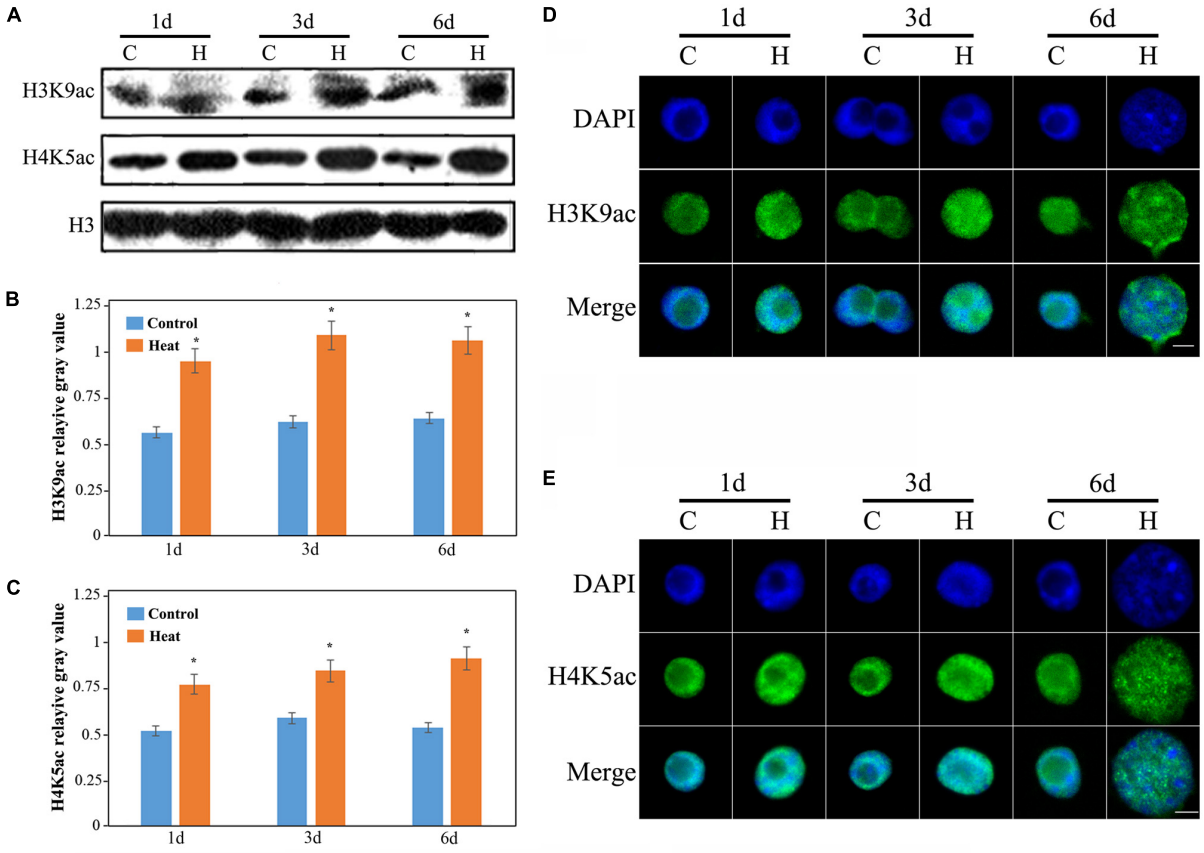

FIGURE 2 | Histone acetylation showed an increasing trend in the heat-induced inhibition of lateral root initiation in maize seedlings. **(A)** The levels of H3K9ac and H4K9ac in the roots of maize seedlings increased significantly under heat treatment. All the western blot assay were repeated three times, and histone H3 was used as the standard internal reference. **(B)** The mean gray value of H3K9ac bands. **(C)** The mean gray value of H4K5ac bands. **(D)** Immunological staining indicated an increase in the acetylation level of H3K9. **(E)** Immunologic staining showed an increase in the acetylation level of H4K5. The level of histone acetylation in the lateral root development of maize seedling was increased, and the nucleus was decondensed. Five hundred nuclei were observed in each sample. The Bar = 10 μm. Asterisk (*) indicated that the gene expression level of the heat treatment group which was found to be significantly different from that of the control group (*t*-test, $p < 0.01$).

perhaps not be the key regulator involved in the inhibition of LRP.

Heat Stress Inhibits Expression of *HO-1* Gene and *GSL* Gene Family

Due to the inhibition of LRP formation under heat stress, so we detected the expression of the related genes involved in lateral root formation. *ZmHO-1* gene was reported to play a role in determining lateral root development and several members of the *GSL* gene family are found to be involved in GA3-regulated lateral root formation. qRT-PCR analysis revealed that the expression of the *HO-1* gene after heat treatment was 70% lower than that under control conditions (**Figure 5A**). Besides, gene expression of *GSL-1* under heat stress was 80% lower than that under control at 1 day and decreasing trend was observed with the increasing days of treatment (**Figure 5B**). We further analyzed the expression patterns of other *GSL* gene family members and our work substantiates that *GSL-4* and *GSL-9* gene expression was similar with *GSL-1* gene expression under heat treatment whereas *GSL-2* and *GSL-6* gene expression was increased after heat treatment (**Supplementary Figure S1**).

Regulation of *HO-1* and *GSL-1* Gene Expression and Their Association With H3K9ac and H4K5ac on the Promoter Region

Histone acetylation on the promoter region plays a crucial role in the regulation of gene expression, including H3K9ac and H4K5ac. As, heat stress inhibited the LRP formation therefore, we have focused our study on the specific genes which displayed negative regulation under heat treatment. As a result, we selected two genes, *HO-1* and *GSL-1* to detect histone acetylation levels on the promoter regions; both genes were found to be significantly decreased under heat stress. Furthermore, ChIP assay with antibodies for H3K9ac and H4K5ac were performed (**Supplementary Figure S2**). H3K9ac and H4K5ac were both significantly decreased on three sets of *HO-1* gene under heat

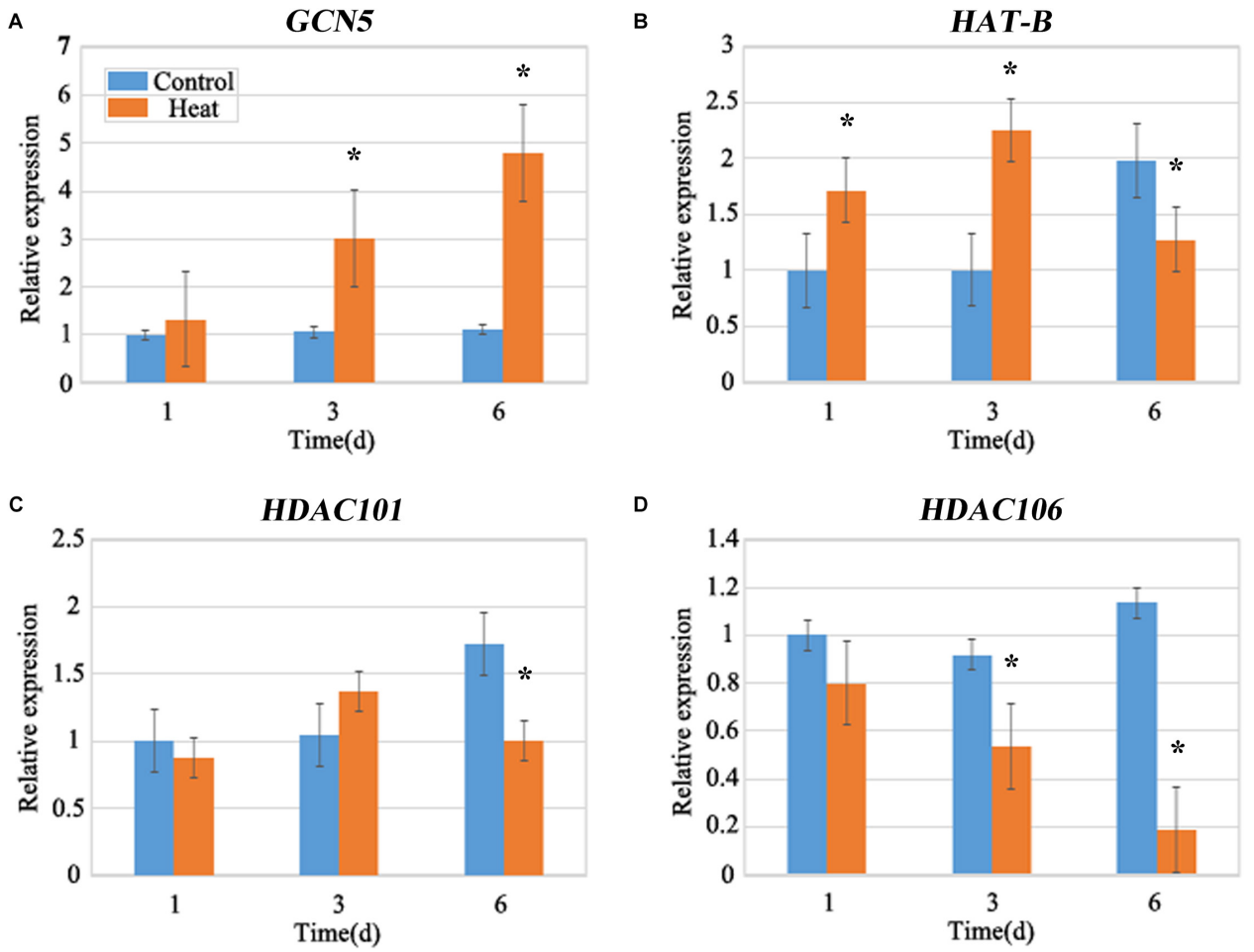

FIGURE 3 | Quantitative Fluorescence PCR analysis. The expression levels of the maize *histone acetyltransferase* (*HATs*), *GCN5* **(A)**, *HAT-B* **(B)**, and *histone deacetylase* (*HDACs*), *HDAC101* **(C)** and *HDAC106* **(D)** under heat stress. The expression of *HATs*, *GCN5* and *HAT-B* were up-regulated, and the expression of *HDACs*, *HDAC101* and *HDAC106* showed a decreasing trend under heat treatment for 1–6 days. The gene expression level of 1 day in the control group was set as 1. Actin is a standardized internal reference y. Each experiment was repeated three times. Asterisk (*) indicated that the gene expression level of the heat treatment group which was found to be significantly different from that of the control group (*t*-test, $p < 0.01$).

treatment as compared to control (**Figure 6A**); most of the differences were more than 50% and the big gap value has been consistent with the previous transcription results as shown by qRT-PCR (**Figure 6A**). H3K9ac was decreased on three sets of *GSL-1* gene under heat treatment, whereas H4K5ac level was fluctuating under heat stress (**Figure 6B**); H4K5ac was found to be slightly present on the set B after 3 days of treatment, whereas it increased significantly on the set C after 6 days of treatment (**Figure 6B**).

Heat Stress Inhibition of LRP Released in Recovery Group as Accompanied by Release of *HO-1* and *GSL-1* Gene Expression

In order to investigate whether the inhibition of LRP formation occurred under heat stress might be recovered and to elucidate the function of histone acetylation on promoter regions of *HO-1* and *GSL-1* genes, plants after 3 days of treatment were transferred to control conditions for 3 days. The results indicated that lateral roots appeared in the maturation zone of seedling roots after 3 days of recovery (**Figure 7A**). The paraffin section examination at tissue levels further supports the results obtained at the individual level (**Figure 7A**); the expression of *HO-1* and *GSL-1* genes were clearly enhanced as compared with their respective groups under heat stress (**Figures 7B–E**).

Histone Deacetylation Induces Inaccessibility of the Promoter Region of *ZmHO-1* and *ZmGSL-1* to Micrococcal Nuclease

As, histone acetylation/deacetylation usually alters chromatin conformation to regulate gene expression therefore, we analyzed the effect of TSA on local region chromatin accessibility to micrococcal nuclease (MNase) during heat treatment.

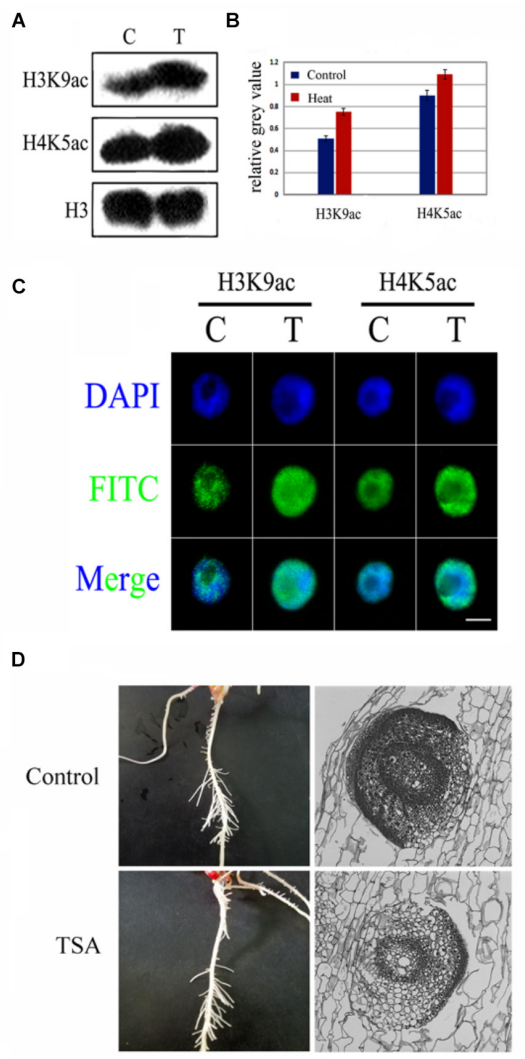

FIGURE 4 | TSA, an epigenetic inhibitor did not inhibit LRP initiation development in maize seedlings. **(A,B)** Western blot analysis showed that H3K9ac and H4K9ac levels in the maize seedlings was significantly increased after TSA treatment. **(C)** The nucleus was decondensed after TSA treatment. **(D)** Phenotype characteristics of maize plants under TSA treatment and control. TSA treatment did not inhibit the LRP initiation in maize, and the paraffin sections of the LRP in roots maize seedling at 3 days under TSA treatment. Western blot experiment is repeated three times. Five hundred nuclei were examined in each sample. Bar = 10 μm.

Condensed chromatin regions are more inaccessible to MNase digestion as compared to the decondensed chromatin regions, so real-time PCR (CHART-PCR) was used to analyze MNase digestion and chromatin accessibility. By CHART-PCR analysis we investigated the chromatin packaging of the specific region. In heat treated group, set A, B and C were less accessible to MNase (**Figure 8**) as compared to control group. Thus, HDACs might induce the chromatin decondensation across the promoter region during heat treatment, which apparently alter the expression of *ZmHO-1* and *ZmGSL-1*.

MATERIALS AND METHODS

Plant Materials and Heat Stress Treatment

Seeds of maize (*Zea mays*) hybrid line *Huayu 5* were germinated and seedlings were cultured in water for 3 days under untreated controlled environmental conditions (14 h light/120 mmol·m^{-2}·s^{-1}/25°C and 8 h dark/20°C, 70% relative humidity) (Yong et al., 2012). Subsequently, plants under heat treatment were incubated at 45°C with the similar photoperiod and humidity. Roots from different treatments were collected and used for different assays. The concentration of TSA used in the present investigation was 10 μM (Wang et al., 2015). In the TSA treated group, the maize seedlings were transferred to the solution of 10 μM TSA and the solution was supplemented with 10 μM TSA per day.

Fixation and Sectioning

Root samples of the control and under high temperature group for 1, 3, and 6 days were selected and immersed in FAA solution [glacial acetic acid, formalin (37%) and ethanol (70%); 1:2:17 (v/v)] for approximately 24 h at room temperature. Leica TP-1050 tissue processor was used to process fixed root tissues for critical drying and wax infiltration. Rotary microtome was used to cut chilled wax embedded root samples to obtain 8 μm tissue section and then flattened on the surface of a water bath at 42°C. The tissue sections were transferred onto slides by lifting the slides beneath the wax sections. Afterward, the wax sections were melted on a hot plate (∼60°C to be affixed upon the slides). The slides were sequentially processed through de-waxing, staining and mounting after oven-drying at 38°C. The prepared slides were examined using the Olympus BX-60 light microscope (Olympus, Tokyo, Japan) and photographed with the CCD monochrome camera Sensys 1401E.

Antibodies

The antibodies used in western blotting, immunostaining and ChIP assays were as follows: anti-H3 (ab1791) and anti-H4K5ac (ab1997) were obtained from Abcam; anti-H3K9ac (07-352) and fluorescein-conjugated goat anti-rabbit IgG (16-237) were obtained from Millipore and AP-conjugated goat anti-rabbit IgG (A4187) was obtained from Sigma.

Western Blot Analysis

Proteins were extracted from the maize seedlings, by grinding the roots in liquid nitrogen and re-suspending the powder in the protein extraction buffer [100 mM Tris-HCl pH 7.4, 5 mM EDTA, 50 mM NaCl and 1 mM PMSF] was performed as previously described (Zhao et al., 2014). Western blot detection was carried out as previously described (Zhang et al., 2011). The mean gray value of the signals of H3K9ac and H4K5ac was measured with ImageJ 1.48 software. Abundance index was calculated as H3K9ac or H4K5Ac band intensity/H3 band intensity. Histone H3 was used as a loading control. All assays were repeated three times.

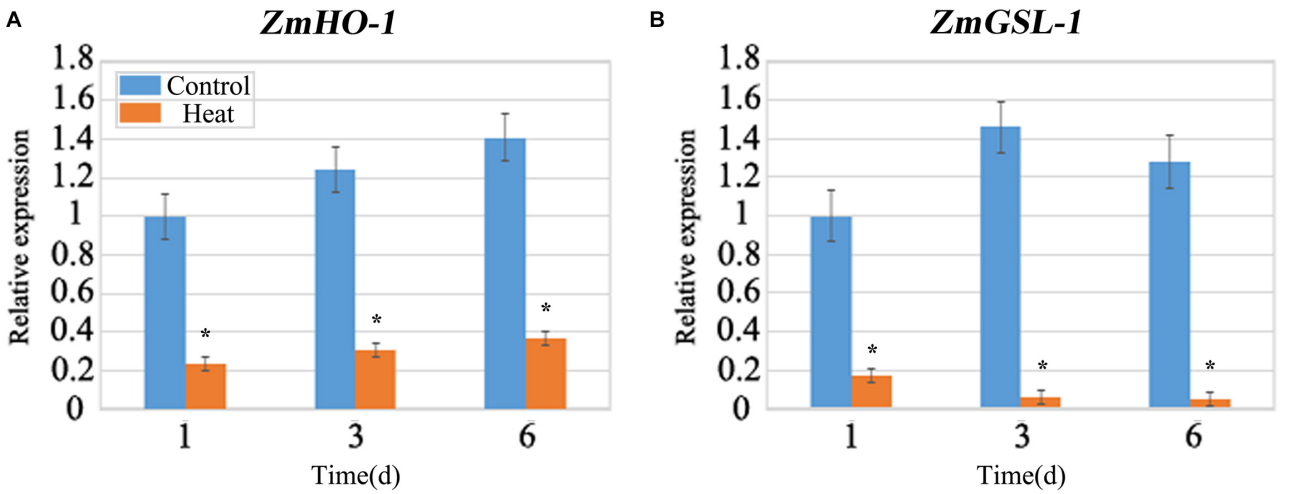

FIGURE 5 | qRT-PCR analysis of the expression levels of *ZmHO-1* and *ZmGSL-1* genes at 1, 3, and 6 days under heat stress. The transcription level of *ZmHO-1* **(A)** and *ZmGSL-1* **(B)** were significantly inhibited under heat stress. The relative expression level was evaluated from three biological replications. Asterisk (*) indicated that the expression level of the heat treatment group was significantly different from that of the control group (*t*-test, $p < 0.01$).

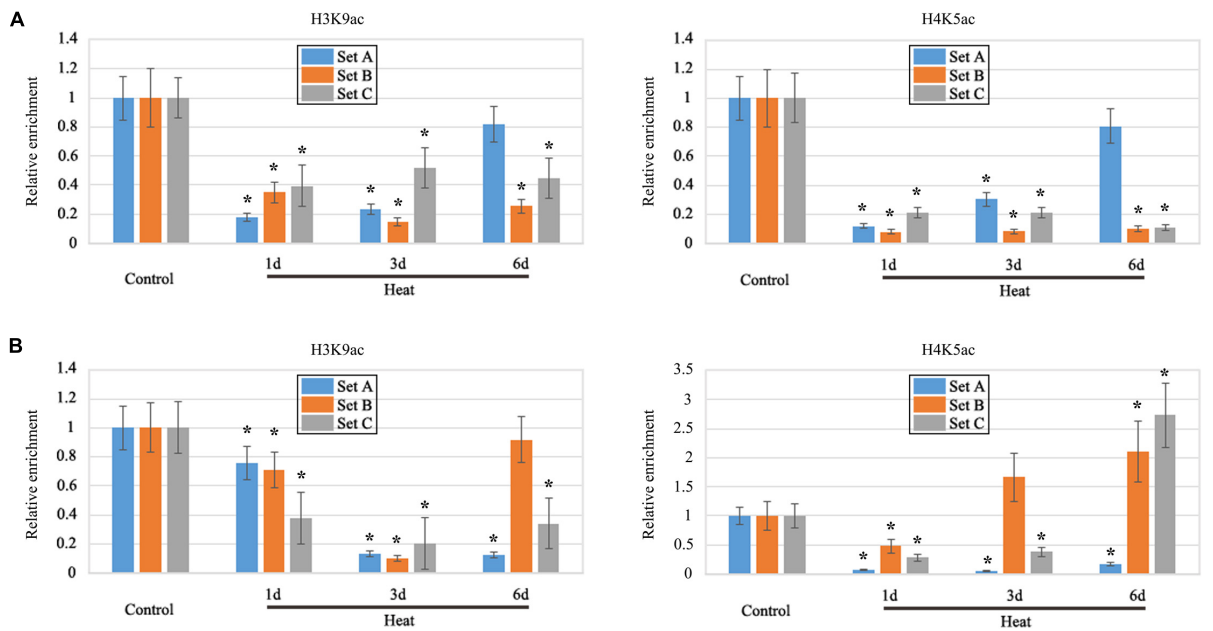

FIGURE 6 | ChIP analysis. It revealed that the modification of H3K9ac and H4K5ac in the promoter region of *ZmHO-1* and *ZmGSL-1* genes was significantly altered under the heat stress. **(A)** The level of H3K9ac and H4K5ac in the upstream region of *ZmHO-1* gene was significantly decreased under heat treatment. **(B)** The level of H3K9ac in the upstream region of *ZmGSL-1* gene was significantly decreased and H4K5ac was found to be slightly present on the set B after 3 days of treatment, whereas it increased significantly on the set C after 6 days of treatment. The acetylation level of the control group was set to 1.0 at each time point. The relative abundance of histone modification was repeated three times. Asterisk (*) indicated that the relative increase of H3K9ac or H4K5ac under heat treatment group is significantly different from that of the control group (*t*-test, $p < 0.01$).

Immunostaining Analysis

Nucleus preparation and immunostaining were performed as previously described (Fei et al., 2010). Briefly, isolated nuclei ($n = 500$) were spread on a slide and incubated with the primary antibody at 4°C overnight followed by an incubation at 37°C for 2 h with the secondary antibody. All slides were examined under an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan), after counterstained with 0.2 μg/ml DAPI (Sigma), mounted with Vectashield (Vector labs, Burlingame, CA, United States). Images captured with the CCD monochrome camera Sensys 1401E were pseudo-colored using the METAMORPH® 4.6.3 software (Universal Imaging

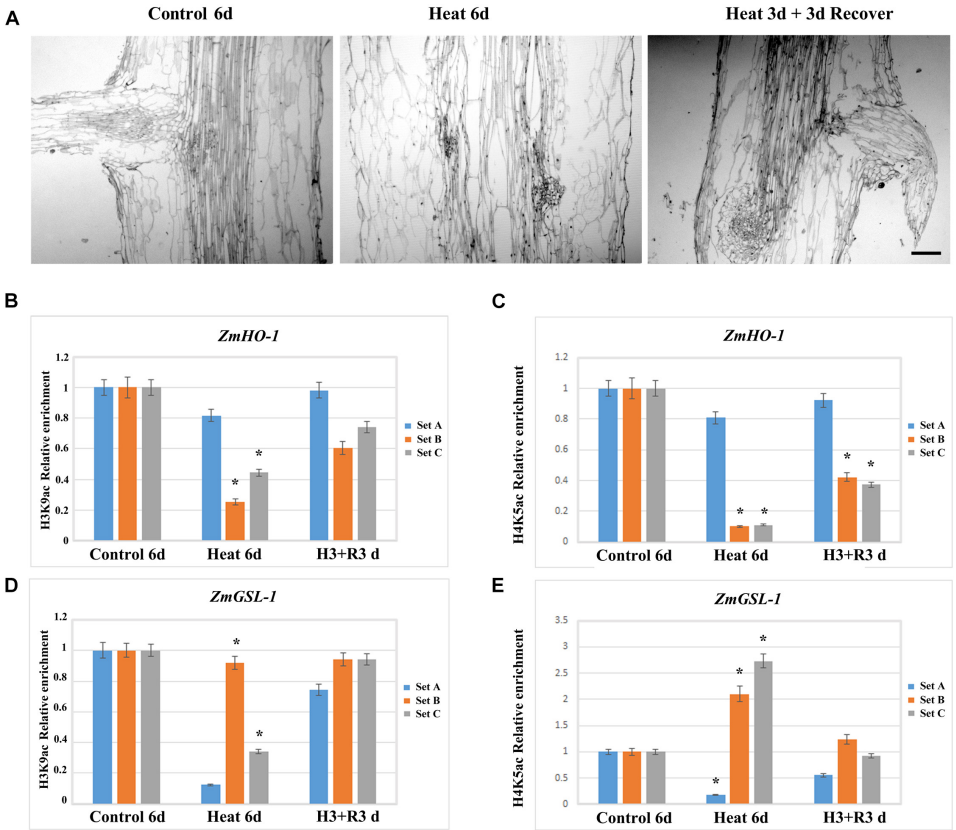

FIGURE 7 | Maize seedlings under heat treatment for 3 days were transferred to the control temperature condition for recovery, and the lateral root growth initiation was restored, while the level of epigenetic modification of *ZmHO-1* and *ZmGSL-1* gene promoter was restored to normal level. **(A)** Root section of maize seedlings at 6 days under control temperature condition and 6 days under heat treatment. **(B)** The alterations in H3K9ac in the upstream region of *ZmHO-1* gene promoter under heat treatment group and restored group. **(C)** The alterations of H4K5ac in the upstream region of *ZmHO-1* gene promoter in heat treatment group and restored group. **(D)** Changes in H3K9ac in the upstream region of *ZmGSL-1* gene promoter under heat treatment group and restored group. **(E)** Alterations in H4K5ac upstream of *ZmGSL-1* gene promoter under heat treatment group and restored group. All experiments were repeated three times. Asterisk (*) indicated that the relative increase in H3K9ac or H4K5ac under heat treatment is significantly different from that of the control group (*t*-test, $p < 0.01$).

Corp, Downingtown, PA, United States). Microscope settings and camera detector exposure time were kept constant for each respective channel (fluorescein or DAPI) but were optimized for individual experiments. For both control and treatment groups, three independent immunostaining experiments were performed with each antibody.

Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was isolated from maize roots using the RNAprep pure Plant Kit (Qiagen, Mannheim, Germany) following the supplier's instructions. To remove residual DNA contamination, 1 mg of total RNA was treated with 50 units of *DNase I* (Fermentas, Burlington, ON, Canada) at 37°C for 30 min. The purified RNA was reverse-transcribed to cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada).

qRT-PCR was performed using SYBR® Green Real-time PCR Master Mix (Toyobo, Tokyo Japan) in a StepOne Plus real-time PCR system (Applied Biosystems) with the following cycling conditions: 94°C for 2 min, followed by 40 amplification cycles at 94°C for 5 s, 56°C for 15 s and 72°C for 20 s. Fluorescence data were acquired at the 72°C step and during the melting curve program. The *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene (GenBank accession number: X07156.1) was selected as a reference gene in this study. Template-free and SYBR Green mix-free samples were amplified for each gene as negative controls. Triplicate PCR reactions for each of the three independently-purified RNA samples were carried out. Quantitative PCR primers were designed using the Primer Premier 5 software to amplify fragments of approximately 200 bp (**Table 1**).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed with anti-H3K9ac and anti-H4K5ac as previously described (Haring et al., 2007). The immunoprecipitated DNA was subjected to real-time PCR analysis with six primer sets, designated as A–C (**Table 2**) for

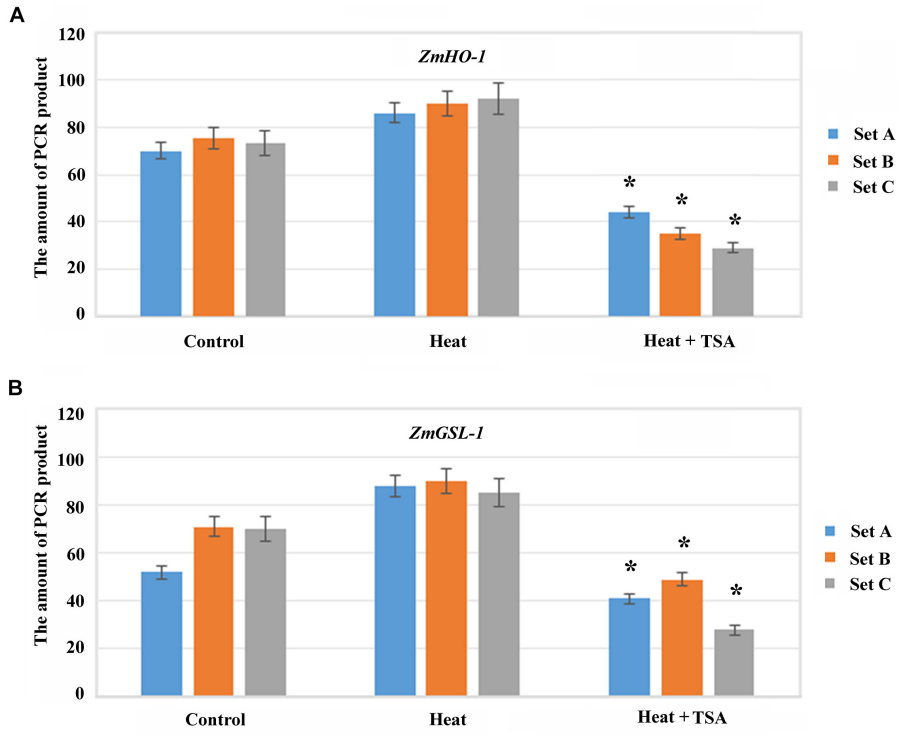

FIGURE 8 | Histone acetylation induced chromatin accessibility of the promoter region in *ZmHO-1* and *ZmGSL-1*. **(A,B)** CHART-PCR assay was performed with nuclei digested by MNase to measure chromatin accessibility of the specific promoter region of *ZmHO-1* and *ZmGSL-1*. The PCR products was converted from the Ct values according to the standard curve. All experiments were repeated three times. Asterisk (*) indicated that the relative PCR product in *ZmHO-1* and *ZmGSL-1* of the TSA treatment group was significantly different from that of the control group (*t*-test, $p < 0.01$).

TABLE 1 | Primers used for qRT-PCR.

RT-PCR	Sequence (5′-3′)	Efficiency %
ZmHO-1	ACACTGTTGGCTGATCCAGT	96
	AAACGTATCTGGGGGAGGGA	
ZmGSL-1	CTAATTTGCTGCGCGGCAATG	98
	CACTTGCGGCAGAAGAAGAG	
Actin	GATGATGCGCCAAGAGCTG	102
	CCTCATCACCTACGTAGGCAT	

the *ZmHO-1* and *GSL-1* gene promoter regions following the above-mentioned procedure.

Chromatin Accessibility Real-Time PCR (CHART-PCR)

CHART-PCR assay was performed to analyze the conformational change of chromatin as previously described (Xu et al., 2016). The seedlings treated with heat or heat-TSA for 1 day. Nuclei were extracted and digested using 5 U MNase for 5 min at 37°C (Hu et al., 2011). Subsequently, DNA was prepared using a Plant genomic DNA kit (Qiagen, Mannheim, Germany) and quantified using the Gene Quant calculator (Amersham Pharmacia Biotec, Piscataway, NJ, United States). 100 mg of genomic DNA from heat or heat-TSA treated samples was used for SYBR Green real-time PCR analysis. The primers used in chromatin accessibility by real-time PCR were similar to those used in the ChIP assays (**Table 1**). MNase accessibility is characterized to be inversely proportional to the amount of amplified PCR product.

DISCUSSION

In the present study, we have investigated the possible relationship between histone acetylation at the gene promoter regions and LRP inhibition in maize seedling under heat stress. The results provided a new insights into a possible epigenetic regulation of the heat-induced LRP inhibition.

Histone modification plays a vital role in plant response to abiotic stresses to modulate epigenetically the growth and development by remodeling the chromatin structure and activating or repressing gene transcription

TABLE 2 | Primers used for ChIP-PCR and CHART-PCR.

ChIP-PCR	Sequence (5′-3′)	Efficiency %
ZmHO-1 Set A	CCATACTCGAGCTGCTCA	101
	AGAGGGACATTCAGGGA	
ZmHO-1 Set B	GATAGTTCCGATGAAGAG	98
	AGTCATCTTCCTCAGACA	
ZmHO-1 Set C	GGACGGCTGAAGTTTCTCTG	97
	GCTTGCATAAGGGCGATAAG	
ZmGSL-1 Set A	CAGCTGACCTGATGGAGACT	104
	TTGGCATCTGCAACAGACGC	
ZmGSL-1 Set B	ACACTGTTGGCTGATCCAGT	96
	AAACGTATCTGGGGGAGGGA	
ZmGSL-1 Set C	CTAATTTGCTGCGCGGCAATG	98
	CACTTGCGGCAGAAGAAGAG	
ZmActin	GATGATGCGCCAAGAGCTG	102
	CCTCATCACCTACGTAGGCAT	

(Geiman and Robertson, 2002). Maize roots under heat treatment had shown distinct morphological and histological features of inhibited LRP formation. The global acetylation level of histone H3K9 and H4K5 increased significantly under heat treatment, which suggested the potential role of heat stress in histone modification. The increased expression of *GCN5* and *HAT-B* and the decreased expression of *HDAC101* and *HDAC106* genes might contribute to the histone acetylation changes in maize seedlings exposed to heat stress. The increased level of histone acetylation consistently leads to an open access of chromatin (Bannister and Kouzarides, 2011). In the present investigation, the increase in global histone acetylation of H3K9ac and H4K5ac accompanied by chromatin decondensation, indicated high accessibility of the whole genome allowing transcription factor recruitment in the process of LRP inhibition after heat treatment and our result were consistent with the histone acetylation and deacetylation study conducted on yeast (Kurdistani and Grunstein, 2003). Furthermore, we

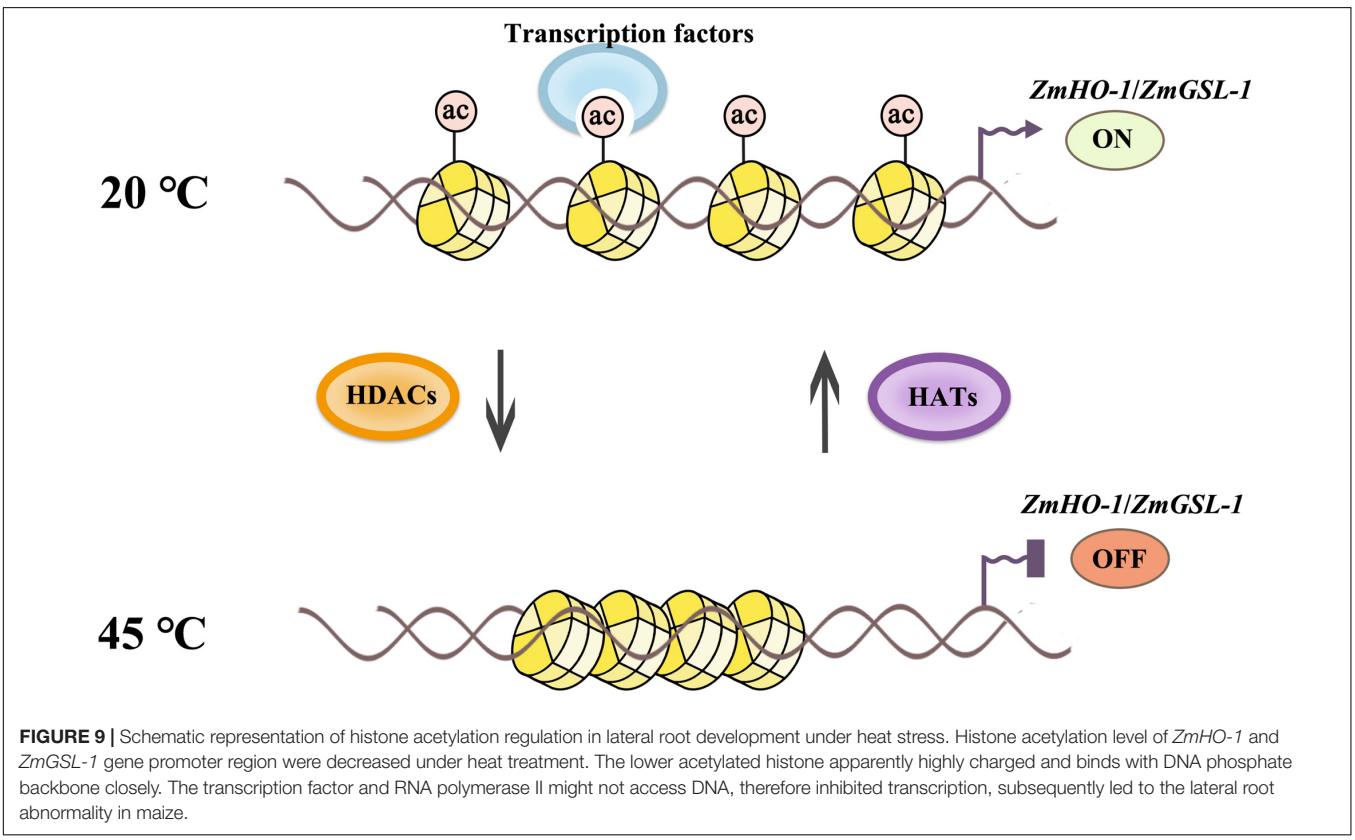

FIGURE 9 | Schematic representation of histone acetylation regulation in lateral root development under heat stress. Histone acetylation level of *ZmHO-1* and *ZmGSL-1* gene promoter region were decreased under heat treatment. The lower acetylated histone apparently highly charged and binds with DNA phosphate backbone closely. The transcription factor and RNA polymerase II might not access DNA, therefore inhibited transcription, subsequently led to the lateral root abnormality in maize.

investigated the role of histone hyperacetylation in the process of LRP inhibition. TSA, a HDACs inhibitor, was used to treat maize seedlings; our results had shown similar histone hyperacetylation level under heat stress (Wang et al., 2015). The western blotting analysis had indicated that TSA led to enhanced levels of H3K9ac and H4K5ac and the immunostaining of nuclei also suggested similar results in control condition roots as compared to heat stress. As the LRP formation was not inhibited by TSA, further suggesting that global hyperacetylation might not be the determining factor in the inhibition process of lateral root as induced under heat stress.

ZmHO-1 gene was reported to play a crucial role in lateral root development and several members of the GSL gene family were found to be crucial in GA3-regulated lateral root formation. *HO-1* and *GSL-1* genes were down-regulated under heat treatment as compared to control condition. After 3 days of recovery period, the expression of *HO-1* and *GSL-1* genes were increased as compared with the heat stress and LRP initiated in the maturation zone, exhibiting the release of LRP inhibition. Moreover, the expression level of other *GSL* gene family members were also detected using qRT-PCR. *GSL-4* and *GSL-9* gene expression were similar with *GSL-1* under heat stress. However, *GSL-2* and *GSL-6* gene expression levels were increased under heat stress (**Supplementary Figure S1**). Therefore, our result suggested that *ZmHO-1* and *ZmGSL-1* seems to be involved in the heat induced LRP inhibition in maize.

The GSL gene family *ZmGSL2* is only active in primary roots, while *ZmGSL6* is detected in secondary root. *ZmGSL4* is strongly expressed in wild-type and *lrt1* mutant primary roots but only transcribed at low levels in primary roots of *rum1*. However, *ZmGSL9* is strongly expressed in wild-type primary roots and only weakly transcribed in *lrt1* and *rum1*. In this study, expression level of *GSLs* under heat treatment is different, which might be attributed to their different function in lateral root development.

Histone modifications of chromatin on the promoters can reveal repository information about developmental and environmental cues. Histone acetylation/deacetylation at the promoter regions of some genes is usually involved in the alteration of the local chromatin conformation that regulates gene expression, and our findings were found to be consistent with the previous results. For example, suberoylanilide hydroxamic acid activates the transcription of the *p21WAF1* gene by increasing the levels of histone acetylation in the promoter region (Richon et al., 2000). *Arabidopsis HOS15* represses expression of *RD29A* through RD29A promoter-associated histone deacetylation (Zhu et al., 2008). *Arabidopsis AtHD2A*, *AtHD2B,* and *AtHD2C* were shown to repress transcription when targeted to the promoter of genes (Wu et al., 2003). In this study, ChIP assay with anti-*H3K9ac* and anti-*H4K5ac* indicated that histone acetylation levels on the promoter regions of *ZmHO-1* and *ZmGSL-1* genes were significantly decreased during heat stress, except for *H4K5ac* level of promoter area of *GSL-1* which was slightly accumulated in the set B after 3 days of heat treatment and certainly increased in the set C after 6 days of treatment. Besides, CHATR-PCR data suggested that the chromatin accessibility of the promoter regions of *ZmHO-1* and *ZmGSL-1* was decreased under heat treatment and increased under heat-TSA treatment, indicating that heat stress inhibits the expression of *ZmHO-1* and *ZmGSL-1* through chromatin alteration in specific sites that regulate expression of these genes (**Figure 9**). Additionally, our results has been consistent with the previously published report on inhibition of *sodCp* genes in response to abscisic acid through deacetylation of histones in the promoter regions in maize (Hou et al., 2015).

In the present investigation, we have suggested the role of promoter associated histone acetylation of *Haem Oxygenase-1* (*HO-1*) and *Giberellic Acid-Stimulated Like-1* (*GSL-1*) genes in heat induced LRP inhibition in maize, further experiments were required to establish the direct role of *ZmHO-1* and *ZmGSL-1* genes expression in the inhibition of LRP formation in maize under high temperature using promoter modified plants. In conclusion, the maize lateral root formation was found to be suppressed under heat treatment and an epigenetic control of expression of the lateral root formation related genes was observed in response to heat stress.

AUTHOR CONTRIBUTIONS

LL, SH, and HZ conceived and designed the experiments. HZ, MY, and XZ performed the experiments. LL and HZ analyzed the data. HZ contributed reagents, materials, and analysis tools. LL, HZ, and MG wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01520/full#supplementary-material

FIGURE S1 | qRT-PCR detection of *GSL* gene family expression levels under heat stress **(A)** *GSL-2* and **(C)** *GSL-6* gene expression was increased under heat treatment **(B)** *GSL-4* and **(D)** *GSL-9* gene expression was similar with *GSL-1* gene expression in heat stress. The gene expression level in the control group for 1 day was set to 1. Actin is used as a standardized internal reference. Experiments were repeated three times. Asterisk (*) indicated that the gene expression level of the heat treatment group was significantly different from that of the control group (*t*-test, $p < 0.01$).

FIGURE S2 | (A,B) Design of the detected fragment pattern in the promoter region of *ZmHO-1* and *ZmGSL-1* genes (Set A, B, C).

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WHIRLY1 Occupancy Affects Histone Lysine Modification and *WRKY53* Transcription in *Arabidopsis* Developmental Manner

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Single-stranded DNA-binding proteins (SSBs) are assumed to involve in DNA replication, DNA repairmen, and gene transcription. Here, we provide the direct evidence on the functionality of an *Arabidopsis* SSB, WHIRLY1, by using loss- or gain-of-function lines. We show that WHIRLY1 binding to the promoter of *WRKY53* represses the enrichment of H3K4me3, but enhances the enrichment of H3K9ac at the region contained WHIRLY1-binding sequences and TATA box or the translation start region of *WRKY53*, coincided with a recruitment of RNAPII. *In vitro* ChIP assays confirm that WHIRLY1 inhibits H3K4me3 enrichment at the preinitiation complex formation stage, while promotes H3K9ac enrichment and RNAPII recruitment at the elongation stage, consequently affecting the transcription of *WRKY53*. These results further explore the molecular actions underlying SSB-mediated gene transcription through epigenetic regulation in plant senescence.

Keywords: ssDNA binding protein, transcription, histone modification, leaf senescence, *Arabidopsis*

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INTRODUCTION

Plant senescence is the last stage of plant development. It is a controlled process that plants response to internal factors (age and hormone) and external factors (abiotic stress and biotic stress), in which plants remobilize nutrients from source leaves to developing tissues. In the past decades, molecular components underlying the onset of senescence have been studied, however, the age-dependent mechanisms that control the onset of senescence remains opening.

WRKY is a major transcription factor (TF) family of plants, in which many of them are the central players in gene regulation during leaf senescence. WRKY6 was found to activate several senescence-associated genes during leaf senescence (Robatzek and Somssich, 2002). Mutation and overexpression of WRKY6 retarded and accelerated both developmentally and dark-induced senescence (Robatzek and Somssich, 2002; Zhang et al., 2018). Similarly, overexpression or knockout of *WRKY22* also showed accelerated and delayed senescence phenotype in dark condition (Zhou et al., 2011). WRKY53 and WRKY70 were reported to be positive and negative regulators of senescence, respectively. Loss of WRKY53 delayed the leaf senescence (Miao et al., 2004), while *wrky70* mutant showed aggravated senescent phenotype during development and dark treatment (Ulker et al., 2007). WRKY54 can co-operate with WRKY70 to repress leaf senescence

(Besseau et al., 2012). Additionally, WRKY57 function as a repressor in JA-induced leaf senescence (Jiang et al., 2014) and WRKY45 positively regulate age-triggered leaf senescence *via* gibberellin (GA)-mediated signaling pathway (Chen et al., 2017). Mutual regulation exists between WRKYs and WRKYs can be regulated at different levels (Phukan et al., 2016).

WRKY53, a well-known regulator to plant early leaf senescence, is a convergence node tightly regulated by various process (Zentgraf et al., 2010). WRKY53 binds to more than 60 target genes directly (Miao et al., 2004). It has been reported that WRKY53 protein is regulated by MEKK1 kinase, epithiospecifying senescence regulator (Miao and Zentgraf, 2007; Miao et al., 2007), and UPL5 in the protein level (Miao and Zentgraf, 2010). Moreover, the expression of *WRKY53* gene is activated by GATA4 (Zentgraf et al., 2010), activation domain protein (AD protein) (Miao et al., 2008), and REVOLUTA protein (Xie et al., 2014) in the transcriptional level in leaf senescence, while it is repressed by ssDNA binding protein WHIRLY1 (Miao et al., 2013). The histone modification at *WRKY53* locus seemed to associate with SUVH2 histone methylase (Ay et al., 2009). Current research showed WRKY53 can interact with histone deacetylase 9 (HDA9) affecting downstream gene expression in leaf senescence (Chen et al., 2016). The AT-rich motif in WHIRLY1 binding domain of *WRKY53* has been shown to relate to chromatin structure and epigenetic mark modification (Lim et al., 2007). However, up to now, there is no further evidence about the transcriptional regulation of *WRKY53* by single stranded DNA binding WHIRLY1 protein with chromatin modification.

Single-stranded DNA binding proteins are ubiquitous in organisms and essential in recognition and processing of ssDNA during various cellular processes (Dickey et al., 2013). They bind to ssDNA with high sequence specificity or independent of sequence, stabilizing the ssDNA intermediates during several cellular processes, such as DNA replication, recombination, and repair as well as telomere maintenance (Dickey et al., 2013; Ribeiro et al., 2016; Hedglin and Benkovic, 2017; Huang S.H. et al., 2017). SSB proteins is also given a role in the regulation of gene expression (MacDonald et al., 1995; Liu et al., 2000; Kim et al., 2005; Choi et al., 2009; Miao et al., 2013) and regulation of the activity of many other DNA metabolic proteins (Lohman and Ferrari, 1994; Roy et al., 2007; Shereda et al., 2008; Roy et al., 2009). As a single-stranded DNA binding protein, WHIRLY domains are four structural topologies that have been structurally characterized with ssDNA (Dickey et al., 2013). WHIRLY domains are approximately 180-amino-acid-long domains characterized by two roughly parallel four-stranded β sheets with interspersed helical elements (Desveaux et al., 2005; Cappadocia et al., 2013).

As a member of WHIRLY family, WHIRLY1 has been proven to be a plastid nucleoid-associated protein affecting DNA replication (Krupinska et al., 2014b) and have a function in repair of organelle DNA (Etminan et al., 2010; Maréchal and Zou, 2014) and in maintenance of plastid genome stability (Lepage et al., 2013; Zampini et al., 2015). WHIRLY1 has been likewise implicated in telomere maintenance through binding to four copies of the telomere repeat (Yoo et al., 2007). However, WHIRLY1 protein first has been reported to bind to the inverted repeat sequence of the elicitor response element (ERE) on the promoter of *PR-10a* gene in potato, acting as a transcription activator (Desveaux et al., 2000). Then other studies have shown WHIRLY1 together with WHIRLY3 can bind to the AT-rich region of kinesin gene promoter to activate kinesin gene expression in *Arabidopsis* (Xiong et al., 2009). Recently, WHIRLY1 has been reported to bind to the GTCAAT motif of *S40* promoter in barley by Nanoelectrospray Mass Spectroscopy (Krupinska et al., 2014a). In our previous study, we have found that WHIRLY1 can bind to combination motif of GTNNNAAT and AT-rich motif of downstream target genes, such as *WRKY53*, *WRKY33*, *SPO11*, and *PR1* by *in vivo* chromatin immunoprecipitation sequencing (ChIP-seq). It has been verified that WHIRLY1 bind to the promoter of *WRKY53* to repress expression of *WRKY53* and *WRKY33* in *Arabidopsis* leaf senescence (Miao et al., 2013; Ren et al., 2017). Nevertheless, how WHIRLY1 as a single-stranded protein mediate the transcription of downstream genes in a fine-tune case remains elusive.

In this study, we first address WHIRLY1 occupancy on *WRKY53* promoter, enrichment of several histone modifications and recruitment of RNAPII at promoter and translation start regions of *WRKY53*, as well as gene expression of *WRKY53* in chronological during leaf aging. Biochemical and genetically evidences demonstrate that WHIRLY1 occupancy at the *WRKY53* promoter affects not only the enrichment of H3K4me3 and H3K9ac but also RNAPII recruitment at WHIRLY1 bind region and translation start region of *WRKY53 in vivo* and *in vitro*, which influences the transcription of *WRKY53* in the development manner. This study provides an implication for exploration of SSB-mediated transcription in epigenetically modification level in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All the plants used were *Arabidopsis thaliana (L.) Heynold ecotype Columbia 0* background, WHIRLY1 T-DNA insertion line *Salk_023713* (*why1*) were provided by the European Arabidopsis Stock Centre, while WHIRLY1 overexpression mutants (*oepnWHY1* and *oenWHY1*), WHIRLY1 complementary line (*PWHY1*) which harbors its own promoter, and WHIRLY1 CDS plus HA target were constructed at previous research (Miao et al., 2013). Seeds were germinated on wet filter paper after 48 h of vernalization. Then they were transplanted in pots in vermiculite in a climatic chamber with a 13-h light (100 μE/h) /11-h dark photoperiod, 22°C/18°C day–night temperature regime, and 60% relative humidity. Rosette leaves were labeled with colored threads after emergence, as described previously (Hinderhofer and Zentgraf, 2001). 5–8th rosette leaves from *PWHY1* mutants at 6th week were collected for treatment with 3-Deazaneplanocin A (Dzenp) to detect the influence of WHY1 binding affinity and enrichment of H3K4me3, H3K9ac.

Measurements of Chlorophyll Fluorescence and Chlorophyll Content

Chlorophyll fluorescence of leaf 5 from different developmental plants was measured using a Pocket PEA Chlorophyll Fluorimeter (Hansatech) after 15-minimum dark incubation. The average Fv/Fm value of leaf 5 from at least 12 individual plants was calculated. Chlorophyll concentrations of leaf 5 from 12 different developmental plants were measured by Dualex 4. Three points of each leaf were detected.

mRNA Preparation and qRT-PCR Analysis

Total RNA from 5th to 8th rosette leaves was isolated according to the manufacturer's protocol of TransZol UP (TRANSGEN), and was then treated with RNase-free DNase I (EN0521, Thermo Scientific). First-strand cDNA was generated from 1 μg portion of total RNA using RevertAid First-Strand cDNA Synthesis Kit (K1622, Thermo Fisher), following the instruction. PCR was performed to analyze the expression of genes. To determine the relative expression rate, data were normalized to the expression level of wild-type or of 5-week-old plants (which were set to 1) after normalized to the internal control of *GAPC2*. Additionally, three technical replicates of three biological replicates and the determination of a melting curve of the amplified PCR products were carried out.

In vivo ChIP Assay

The ChIP assay was performed with a modified method (Gendrel et al., 2005). About 0.75 g leaves (fifth to eighth leaves of WT and WHIRLY1 mutants during different developmental stages and whole rosettes of darkness-treated plants) were used. The detailed procedure is shown in **Supplementary Material**. The immunoprecipitated DNA was isolated by Universal DNA purification kit (TIANGEN, DP214-03). Purified DNA was analyzed by real-time PCR with specific primers (see **Supplementary Tables**). Relative histone modification levels in WT during leaf development were therefore normalized to the input, while the enrichment in WHIRLY1 mutants was normalized to WT again, referring to $\Delta\Delta$Ct method[1].

Recombinant WHY1 Protein Preparation

The recombinant WHY1 protein was expressed in *E. coli* as described by Miao et al. (2013).

Chromatin Assembly and Transcription Assays

Chromatin was assembled and transcription assays were performed as described by Active & Motif Chromatin Assembly Manuel[2]. pG5ML with promoter of *WRKY53* including mutated WRKY53II [mutant (GTNNNGGT) m1 or mutant (CTNNNNAAAT) m2 WHIRLY1 binding motif] (Miao et al., 2013) or mutated TATA-box or wild-type fragment was used as DNA template. The detail showed in **Supplementary Material**.

In vitro ChIP

In vitro ChIP assays were performed as described (Lauberth et al., 2013) (the detail in **Supplementary Material**). The levels of H3K4me3, H3K9ac are given relative to the total H3 levels. *In vitro* ChIP to detect the effect of WHIRLY1 on transcription stage was performed in the presence or absence of 0.01% sarkosyl, which inhibits PIC assembly but does not affect elongation by pre-formed complexes (Cai and Luse, 1987; Hawley and Roeder, 1987) or 800 nM B2 RNA, which inhibits transcription prior to PIC formation (Espinoza et al., 2004).

RESULTS

Enrichment of H3K4me2, H3K4me3, and H3K9ac Were Altered at the Promoter Region of *WRKY53* at Senescence Initiation Stage

In previous work, we performed ChIP-Seq and WHIRLY1 was found binding on the promoter of *WRKY53* as a repressor in the developmental manner (Miao et al., 2013). Leaves of 5–8 from plants grown under 80 $\mu molm^{-2}s^{-1}$ radiation were chosen to analyze the relationship of WHIRLY1 and downstream gene expression at the fine-turn level (**Supplementary Figure S1**). The expression of *RBCS*, a gene encoding the small subunit of ribulose 1,5-bisphosphate carboxylase, was downregulated at the 6th week while the expression of *SAG12* gene encoding a cysteine protease which expressed only in senescent tissues, was drastically induced at 8th week in current case (**Supplementary Figures S1A–C**). Therefore, we inferred that 5th to 7th week was the initiation and early stage of leaf senescence. WHIRLY1 was phosphorylated by calcineurin B-like-interacting protein kinase14 (CIPK14) and accumulated steady in the nucleus after 5W (Ren et al., 2017) (**Supplementary Figure S1E**). Although occupancy of WHIRLY1 on the promoter of *WRKY53* was conversed with the accumulation of nuclear form WHIRLY1, the expression level of *WRKY53* strikingly increased from 5th week to 6th week while the occupancy of WHIRLY1 on the promoter of *WRKY53* declined (**Supplementary Figures S1D,E**). Taken together, these results demonstrate that WHIRLY1 chronologically repressed *WRKY53* transcription and expression *via* binding to its promoter at senescence initiation stage from 5th week to 7th week.

To investigate whether WHIRLY1 binding on the *WRKY53* promoter affect the enrichment of selected histone methylation at senescence initiation stage, chromatin immunoprecipitation experiments were carried out with antibodies against H3K4me2, H3K4me3 (associated with transcriptional active), and H3K27me2 (associated with transcriptional inactive) (**Supplementary Figure S2**) using 5- to 8-week-old wild-type plants. The ChIP assay showed that enrichments of H3K4me2 and H3K4me3 at WRKY53P region which was downstream of WHIRLY1 binding domain at the promoter of *WRKY53* and at WRKY53II region which contained WHIRLY1 binding domain

[1]https://www.qiagen.com/us/resources/resourcedetail?id=34e05db7-689c-4abc-bde6-6488d097394f&lang=en

[2]www.activemotif.com

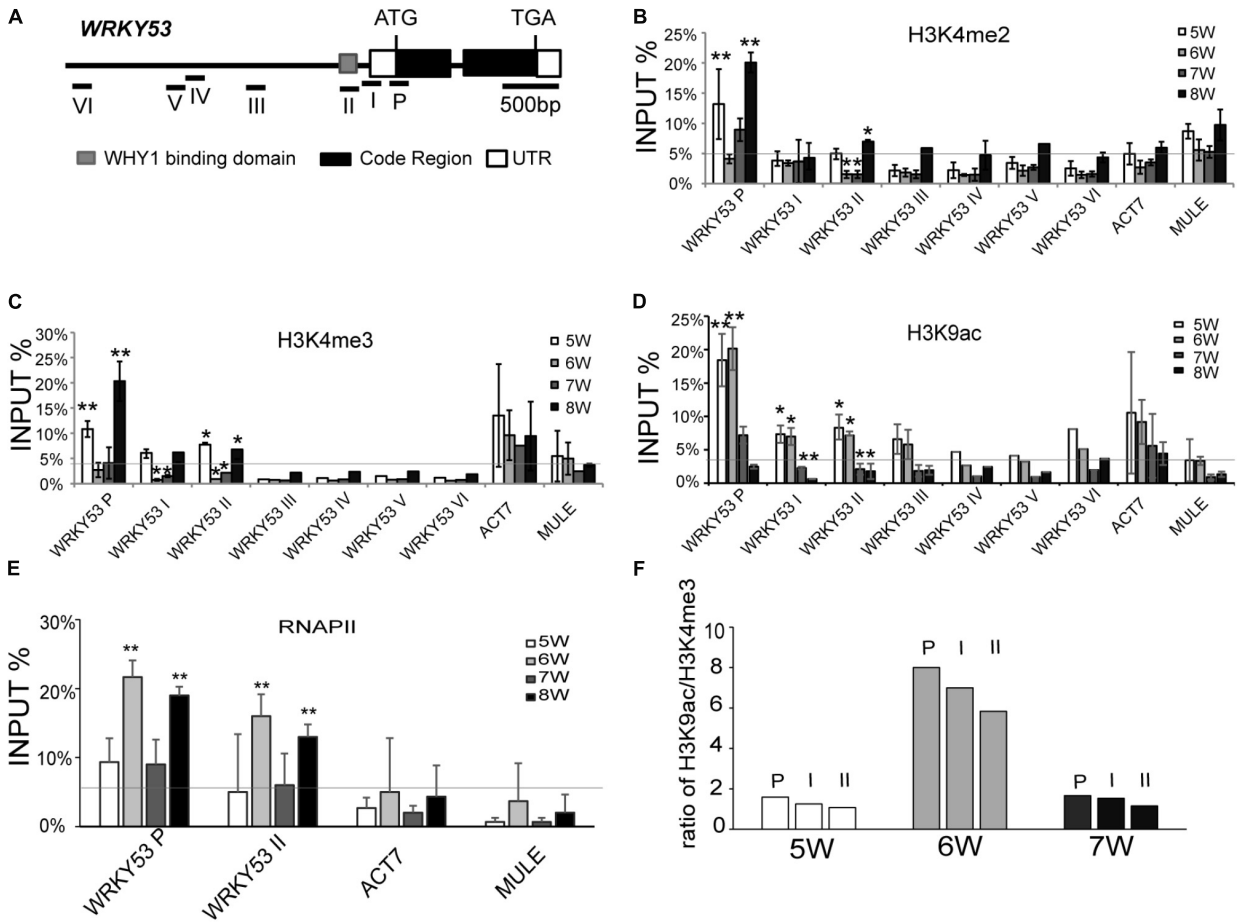

FIGURE 1 | Histone modification and RNAPII occupancy at the promoter regions and translation start region of *WRKY53*. **(A)** Schematic diagram of the genomic structure of the *WRKY53* gene. The lines with number represent qPCR amplicons in different regions of *WRKY53* gene. Gray box, black box, and blank box represent WHIRLY1 binding domain (gray), exon (black), and untranslated regions (blank). **(B–E)** ChIP analyses of changes in H3K4me2 **(B)**, H3K4me3 **(C)**, H3K9ac **(D)** levels, and RNAPII **(E)** occupancy at different regions of *WRKY53* from 5th to 8th week. The antibodies used for ChIP were optimized (**Supplementary Figure S2**). The quantitative PCR was performed with primers (I–VI) around the predicted WHIRLY1 binding sites on the promoter of *WRKY53* and primer (P) covered the 5′ untranslated region and translational start region of *WRKY53* (**Figure 2**) (Ay et al., 2009). *ACT7* (*Actin-related gene 7, AT5G09810*) and *MULE* (*Mutator-like transposable element, AT2G15810*) were analyzed as controls. The relative level was normalized to INPUT DNA. Three biological replicates and three technique replicates were used to analyze. Error bar show the SD ($n = 3 \times 3$). Asterisk indicates significant differences ($^*P < 0.05$ and $^{**}P < 0.01$) based on Student's *t*-test. **(F)** Alteration of ratio of H3K9ac/H3K4me2-3 at different regions of *WRKY53* from 5th to 7th week compared to occupancy of WHIRLY1 and transcripts level of *WRKY53*.

with TATA-box (**Figure 1A**), were at high level in 5-week-old plants, then significantly declined from 5th to 6th, and started to increase from 6th to 8th week, and significantly climbed up to 20% high level at 8th week (**Figures 1B,C** and **Supplementary Figure S3**). The enrichment of H3K4me2, H3K4me3 at the residual detected regions of *WRKY53* promoter were at low level compared to that of WRKY53P. H3K27me2 was the lowest at all the detected regions of *WRKY53* gene (**Supplementary Figure S4A**). Although the enrichment of WHIRLY1 at *WRKY53* promoter decreased gradually, still maintained at 40% level at 7th week (**Supplementary Figure S1F**), and *WRKY53* expression level increased rapidly from 5th to 6th week then declined at 7th week (**Supplementary Figure S1D**). Taken together, these results suggest that the occupancy of WHIRLY1 protein at *WRKY53* promoter may be associated with the enrichment of H3K4me2 and H3K4me3 at *WRKY53*. However, WHIRLY1 occupancy did not repress *WRKY53* expression *via* directly correlated with H3K4me2 and H3K4me3.

We also detected the pattern of H3K9ac, H4ac, and RNAPII occupancy at *WRKY53* in wild-type plants during leaf aging. The H3K9ac at promoter and P regions of *WRKY53* maintained high level at 5th and 6th week but decreased sharply from 7th week (**Figure 1D** and **Supplementary Figure S5**). H3K9ac has been reported to positively correlate with the recruitment of RNAPII (Zhang et al., 2017). Expectedly, the recruitment of RNAPII at WRKY53P and WRKY53II regions significantly increased at 6th week, which is consistent with the transcription of *WRKY53* (**Supplementary Figure S6** and **Figure 1E**). The enrichment of H4ac at promoter and P region of *WRKY53* showed a constant low level during leaf senescence in our

study (**Supplementary Figure S4B**). Taken together, these results indicate that histone modifications at the promoter and translational start region of *WRKY53* are dynamic from 5th to 8th week and H3K9ac seems more closely related to the transcription of *WRKY53*.

A combinatorial interplay between posttranslational modifications on the same histone was proposed based on the patterns of H3 methylation and acetylation at promoters of specific target genes (Taverna et al., 2007). We calculated the ratio of H3K9ac and H3K4me2-3 at the *WRKY53* promoter during the initiation period of plant senescence from 5th to 7th week. The results showed the ratio pattern of H3K9ac/H3K4me2-3 enrichment was chronologically associated with the occupancy of WHIRLY1 on the *WRKY53* promoter (**Supplementary Figure S1F**) and the transcription level of *WRKY53* **Supplementary Figures S1D,E** and **Figure 1F**) during the initiation period of plant senescence, which suggests that H3K9ac/H3K4me2-3 synergistically control *WRKY53* transcription in plant developmental manner.

Loss of WHIRLY1 Enhances the H3K4me3 Enrichment at *WRKY53*

To further investigate whether the binding of WHIRLY1 affects the enrichment of H3K4me2 and H3K4me3 at promoter and P regions of *WRKY53*, we detected the pattern of H3K4me2, H3K4me3, and RNAPII recruitment at WRKY53II and WRKY53P regions of *WRKY53* in *WHIRLY1* knockout line (*why1*), overexpression nucleus-located WHIRLY1 line (*oenWHY1*), *WHIRLY1* complementary line (*PWHY1*) as well as WT plants (Miao et al., 2013). The ChIP–qPCR results showed that the enrichments of H3K4me2 and H2K4me3 at WRKY53P region of *WRKY53* as well as H3K4me2 at WRKY53II region of *WRKY53* increased significantly at 6th week in *why1* mutant compared to WT plants (**Figure 2A** and **Supplementary Figure S3**), but RNAPII recruitment did not change (**Supplementary Figure S6**). Interestingly, overexpression nuclear isoform WHIRLY1 (Miao et al., 2013) did not have effect on H3K4me2, H3K4me3, and RNAPII recruitment at WRKY53P region, but the enrichment of H3K4me2 at WRKY53II region which contained the WHIRLY1 binding domain decreased (**Figures 2A,B**). The results suggest that loss of WHIRLY1 enhances the H3K4me2 and H3K4me3 enrichment at the 5′ untranslated region and translational start region of *WRKY53* (WRKY53P), while the occupancy of WHIRLY1 seems to inhibit the enrichment of H3K4me2 and H3K4me3 at its binding site on *WRKY53* at senescence initiation stage.

H3K4me3 at promoters and 5′-end regions have been reported to significantly correlate with active gene expression (Santos-Rosa et al., 2002; Zhang et al., 2009; Songa et al., 2014), and the H3K9 acetylation has been supposed to serve as a template for the gain of H3K4me3 marks during leaf senescence (Brusslan et al., 2015). We next investigate whether the enrichment of H3K4me3 affects the WHIRLY1 occupancy and H3K9ac enrichment at *WRKY53*. We treated 6-week-old *PWHY1* plants with different concentrations(0, 1, 5, and 10 μM) of 3-Deazaneplanocin A (DZnep), which is a *S*-adenosyl homocysteine hydrolase inhibitor and has been reported to affect H3K4me3 enrichment at the promoters of 3% genes in *zebrafish* (Ostrup et al., 2014). The results showed that DZnep deduce the enrichment of H3K4me3 at WRKY53II region of *WRKY53* in a dose-dependent manner (**Figure 2D**). However, DZnep had no effect on the occupancy of WHIRLY1 at *WRKY53* promoter (**Figure 2C**). Interestingly, DZnep treatment resulted in increase of H3K9ac at WRKY53II region of *WRKY53* (**Figure 2E**). Therefore, H3K4me3 marks at *WRKY53* promoter did not directly affect the occupancy of WHIRLY1 at *WRKY53* promoter, but H3K4me3 marks correlate with H3K9ac.

WHIRLY1 Enhances H3K9 Deacetylation and Represses RNAPII Recruitment at *WRKY53* During Early Leaf Senescence

To determine whether WHIRLY1 occupancy affects H3K9ac level and RNAPII recruitment at *WRKY53*, we performed ChIP experiment in *why1*, *oenWHY1*, *PWHY1*, and WT plants with anti-H3K9ac and anti-RNAPII antibody (**Supplementary Figure S2**). As showed in **Figure 3** and **Supplementary Figure S5**, the loss of WHIRLY1 led to an increase in H3K9ac at WRKY53II region of *WRKY53* at 6-week-old and 7-week-old wild-type plants, and enhanced the H3K9ac enrichment at WRKY53P region of *WRKY53* at 7-week-old wild-type plants. H3K9ac enrichment at WRKY53P region of *WRKY53* was inhibited at 6-week-old and 7-week-old *oenWHY1* mutant, while H3K9ac enrichment at WRKY53II region of *WRKY53* was only inhibited at 7-week-old *oenWHY1* mutant (**Figure 3A**). Interestingly, significant enrichment of RNAPII at WRKY53II and WRKY53P regions of *WRKY53* were also detected in the 7-week-old *why1* mutants. Overexpression of *WHIRLY1* reduced the RNAPII recruitment at WRKY53II and WRKY53P regions of *WRKY53* at 7-week-old *PWHY1* (**Figure 3B**). As shown in the **Figures 3C,D**, higher transcript level of *WRKY53* and *SAG12* in the *why1* mutant and lower transcript level of *WRKY53* and *SAG12* in the *oenWHY1* were detected, showing high proportion of yellow leaves and less chlorophyll content in the *why*, reversely, less proportion of yellow leaves and high chlorophyll content in the *oenWHY1* line. These results demonstrated that WHIRLY1 binding on *WRKY53* accelerated the deacetylation of H3K9ac and repressed RNAPII recruitment at promoter and translational start region of *WRKY53* to determine *WRKY53* transcript level and senescence-related parameter in a developmental manner.

WHIRLY1 Protein Occupied on the *WRKY53* Promoter Impacts H3K4me3, H3K9ac, and *WRKY53* Transcription Initiation *in vitro* at the Preinitiation Conformation Stage

To confirm occupancy of WHIRLY1 protein at the *WRKY53* promoter directly impacts H3K4 methylation, H3K9ac and *WRKY53* transcription initiation, we recruited a cell-free transcription system (**Figure 4A**) (An and Roeder, 2004). Chromatin was assembled by recombinant factors using Hela

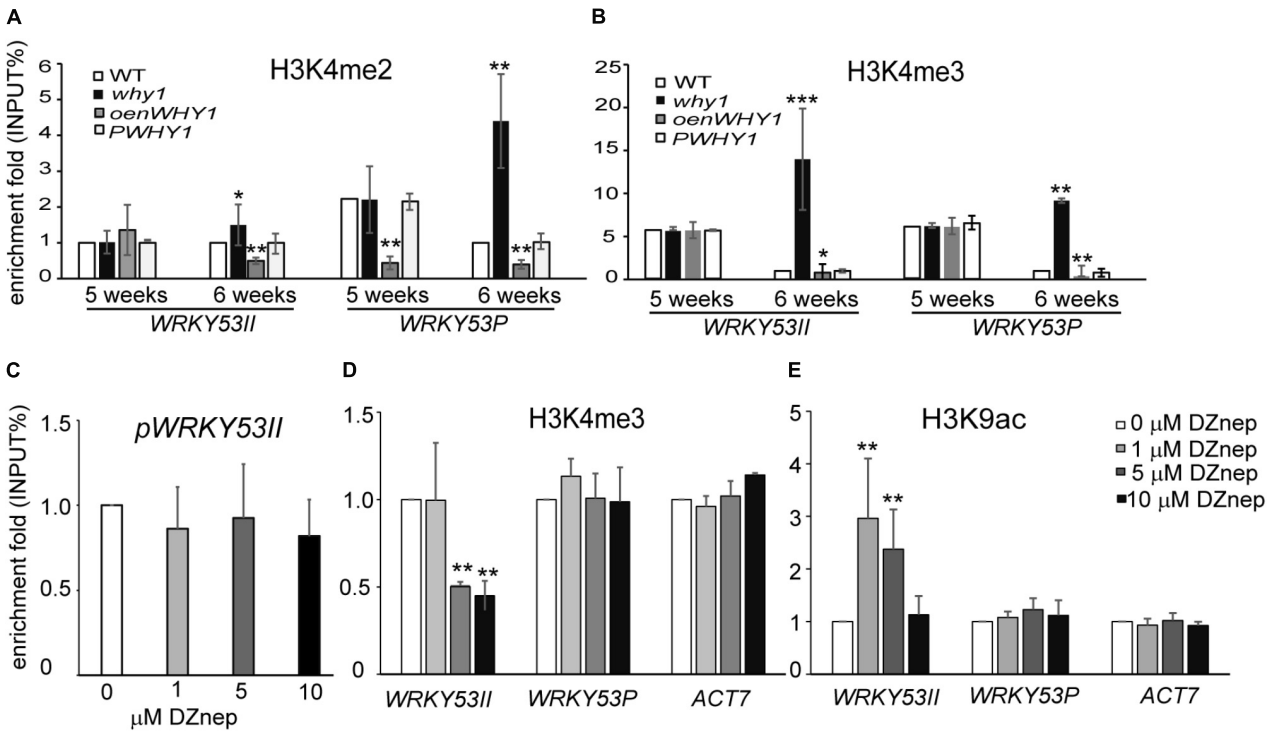

FIGURE 2 | WHIRLY1 regulates the H3K4me3 enrichment at *WRKY53* at senescence initiation stage. **(A,B)** ChIP experiments were performed to assess H3K4me2 **(A)** and H3K4me3 **(B)** levels at WHIRLY1 binding region (WRKY53II) and translation start region (WRKY53P) of *WRKY53* using rosette leaves of *why1*, *oenWHY1*, wild-type (WT), and *PWHY1* plants. *why1, whirly1*; *oenWHY1*, *oenWHIRLY1*; and *PWHY1*, *Pwhirly1:WHIRLY1.* The relative level (input %) was normalized to that of 5-week-old WT. Three biological replicates and three technique replicates were used to analyze. Error bar shows the SD ($n = 3 \times 3$). Asterisk indicate significant differences ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$) based on Student's *t*-test. **(C–E)** The binding affinity of WHIRLY1 at promoter of *WRKY53* (pWRKY53II) **(C)**, H3K4me3 **(D)**, and H3K9ac **(E)** levels at WRKY53II and WRKY53P regions of *WRKY53* using 6-week-old rosette leaves of *PWHY1* plants after 24 h induction with 1, 5, and 10 μM 3-Deazaneplanocin A, dimethylsulfoxide (Dznep) was used as a control in treatment. *Actin7* was used as an inner control for ChIP–qPCR. ChIP–qPCR were carried out with antibody against HA, H3K4me3, and H3K9ac. Three biological replicates and three technique replicates were used to analyze. Asterisk indicate significant differences ($^*P < 0.05$ and $^{**}P < 0.01$) based on Student's *t*-test.

core histone and pG_5ML template with the promoter of *WRKY53* including mutated WRKY53II or mutated TATA-box or wild-type fragment (**Figure 4B**). Micrococcal nuclease digestion showed that all templates are chromatinized equivalently (**Supplementary Figure S7**). *In vitro* ChIP was performed with assembled chromatin. Chromatin was incubated with or without recombinant WHIRLY1 protein expressed in *E. coli* (Miao et al., 2013) and immunoprecipitated with antibodies against WHIRLY1, H3, H3K4me3, or H3K9ac or RNAPII. The ChIP-quantitative PCR was carried out using primer containing WHIRLY1 binding sites (WRKY53II) and TATA box. The enrichment of WHIRLY1 on chromatin templates with wild-type WRKY53II and mutant TATA was obviously higher than that on chromatin templates with mutant WRKY53II (WRKY53IIm1 and WRKY53IIm2) (**Figure 4C**) (Miao et al., 2013). Interestingly, on chromatin templates with wild-type WRKY53II and mutant TATA, in the presence of WHIRLY1, H3K4me3 enrichment was inhibited while H3K9ac enrichment and RNAPII recruitment were enhanced (**Figure 4C**). Further the transcript run-on assay and quantitative RT-PCR were used to detect the transcript elongation and accumulation of *TBP* report genes. Surprisingly, the results showed that report gene *TBP* transcript elongation and accumulation were increased with the time course in the presence of WHIRLY1, while *TBP* transcription did not process when pWRKY53II were mutated or in the deficiency of WHIRLY1 (**Figures 4D,E**). Together, these results indicate that WHIRLY1 binding on WRKY53II *in vitro* represses the enrichment of H3K4me3 and enhance the enrichment of H3K9ac and RNAPII recruitment, and activate the *TBP* transcription.

H3K4me3 has been reported to involve in the preinitiation complex (PIC) assembly during transcription (Lauberth et al., 2013; Songa et al., 2014). We wondered WHIRLY1 occupancy on the *WRKY53* promoter impact H3K4me3 before or after PIC formation, therefore, 0.01% sarkosyl, which inhibits PIC assembly but does not affect elongation by pre-formed complexes (Cai and Luse, 1987; Hawley and Roeder, 1987), or B2 RNA, which binds to RNA polymerase II and inhibits transcription before PIC formation (Espinoza et al., 2004), was used to treat assembled chromatin in presence or absence of WHIRLY1 (**Figure 5A**). ChIP–qPCR results showed that neither sarkosyl nor B2 RNA affected the enrichment of WHIRLY1 at the *WRKY53* promoter. However, sarkosyl treatment decreased the enrichment of H3K4me3 at *WRKY53* promoter while B2 RNA treatment inhibited enrichment of H3K9ac and RNAPII

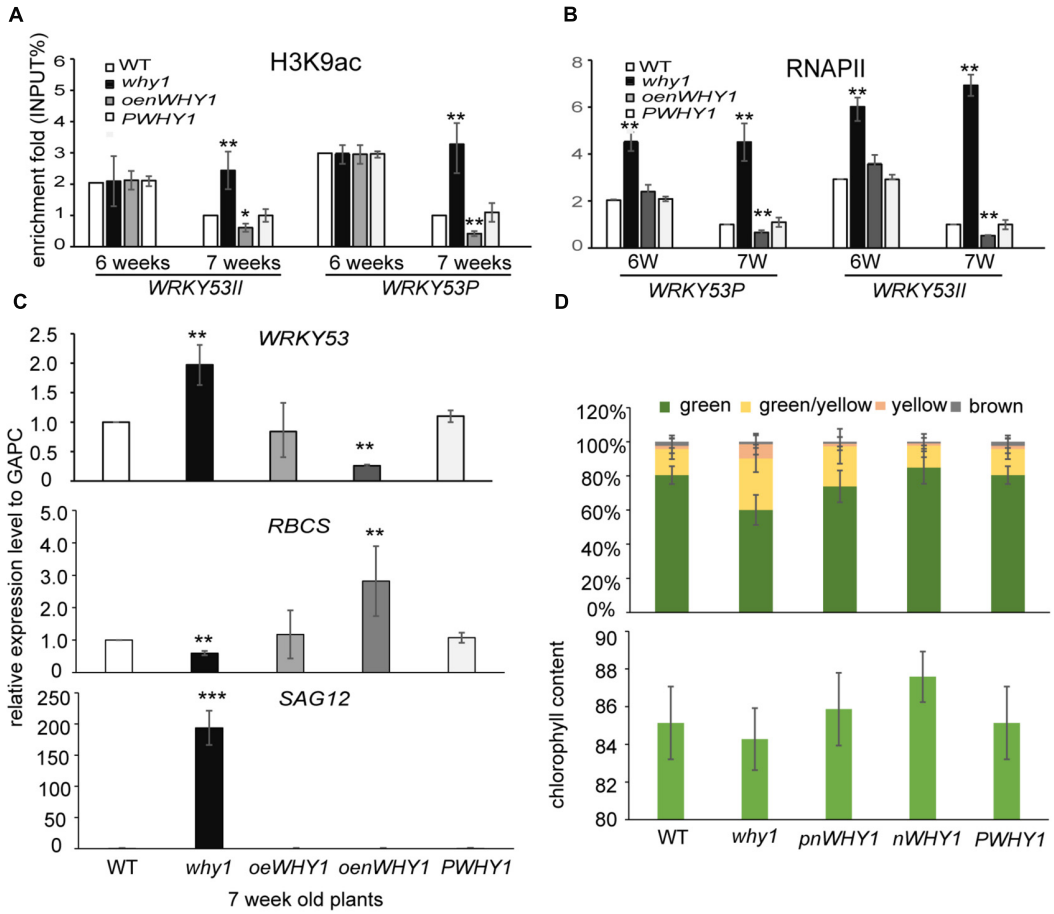

FIGURE 3 | WHIRLY1 enhances H3K9 deacetylation and represses RNAPII recruitment at *WRKY53* at leaf early senescence stage. **(A,B)** ChIP experiments were performed to assess H3K9ac **(A)** and RNAPII occupancy **(B)** at WHIRLY1 binding region (WRKY53II) and translation start region (WRKY53P) of *WRKY53* using rosette leaves of *why1*, *oenWHY1*, wild-type (WT), and *PWHY1* plants. The relative level (input %) was normalized to that of 5-week-old WT. Three biological replicates and three technique replicates were used to analyze. Error bar shows the SD ($n = 3 \times 3$). Asterisk indicate significant differences ($^*P < 0.05$ and $^{**}P < 0.01$) based on Student's *t*-test. **(C)** Expression of senescence-associated genes in 7-week-old *why1*, *oenWHY1*, WT, and *PWHY1* plants. The transcript level in each case was normalized to that of *GAPC2* as a reference gene and the expression level of WT was set as 1. Three biological replicates and three technique replicates were used to analyze. Asterisk indicate significant differences ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$) based on Student's *t*-test. **(D)** Senescent leaf fraction and chlorophyll content in 7-week-old *why1*, *oenWHY1*, WT, and *PWHY1* plants. Mean and SD of at least 12 independent measurements are shown. Error bars represent SE.

recruitment at *WRKY53* promoter (**Figure 5B**). Occupancy of WHIRLY1 on *WRKY53* promoter also inhibited the enrichment of H3K4me3 in the presence of B2 RNA and enhanced the enrichment of H3K9ac and RNAPII recruitment in the presence of sarkosyl (**Figure 5B**). The accumulation of *TBP* transcript was shown in the presence of sarkosyl (**Figure 5C**). These results suggest that WHIRLY1 binding to the *WRKY53* promoter represses the enrichment of H3K4me3 at the preinitiation stage of PIC formation, while promotes the enrichment of H3K9ac and RNAPII recruitment during the elongation by pre-formed complexes, consequently promotes the *TBP* transcription

WHIRLY1 Regulates the HDACs Expression

To investigate whether WHIRLY1 regulates H3K4me3, H3K9ac, and RNAPII recruitment at the *WRKY53* promoter in transcriptional level, differentially expression genes related to histone modifications were picked from the dataset of RNA-seq between *whirly1* and wild-type plants (Lin et al., 2018, unpublished data; **Supplementary Figure S8**). *SWI3D*, *HD2D*, *JMJ22*, *NFA2*, and *HTA4* were selected to be further analyzed in 6-week-old *why1*, *oenWHY1* mutants as well as wild-type plants by qRT-PCR. Surprisingly, our qPCR results showed the expression of *HD2D* and *JMJ22* in both *why1* mutants and *oenWHY1* mutants was lower than that of WT (**Figure 6**), which contradicts with the previous RNA-seq data. Overexpressing or knocking out *WHIRLY1* has no effect on gene expression of *SWI3D*, *NFA2*, and *HTA4*. Moreover, the expression levels of histone methyltransferase *ATX1* (*Arabidopsis Trithorax-like protein 1*), *ATX2*, *SUVH2*, and histone deacetylases *HDA15*, *HDA6*, *HDA2*, *HDA5*, and *HDA9* were also detected by qPCR. ATX1 and ATX2 play roles for trimethylating and dimethylating

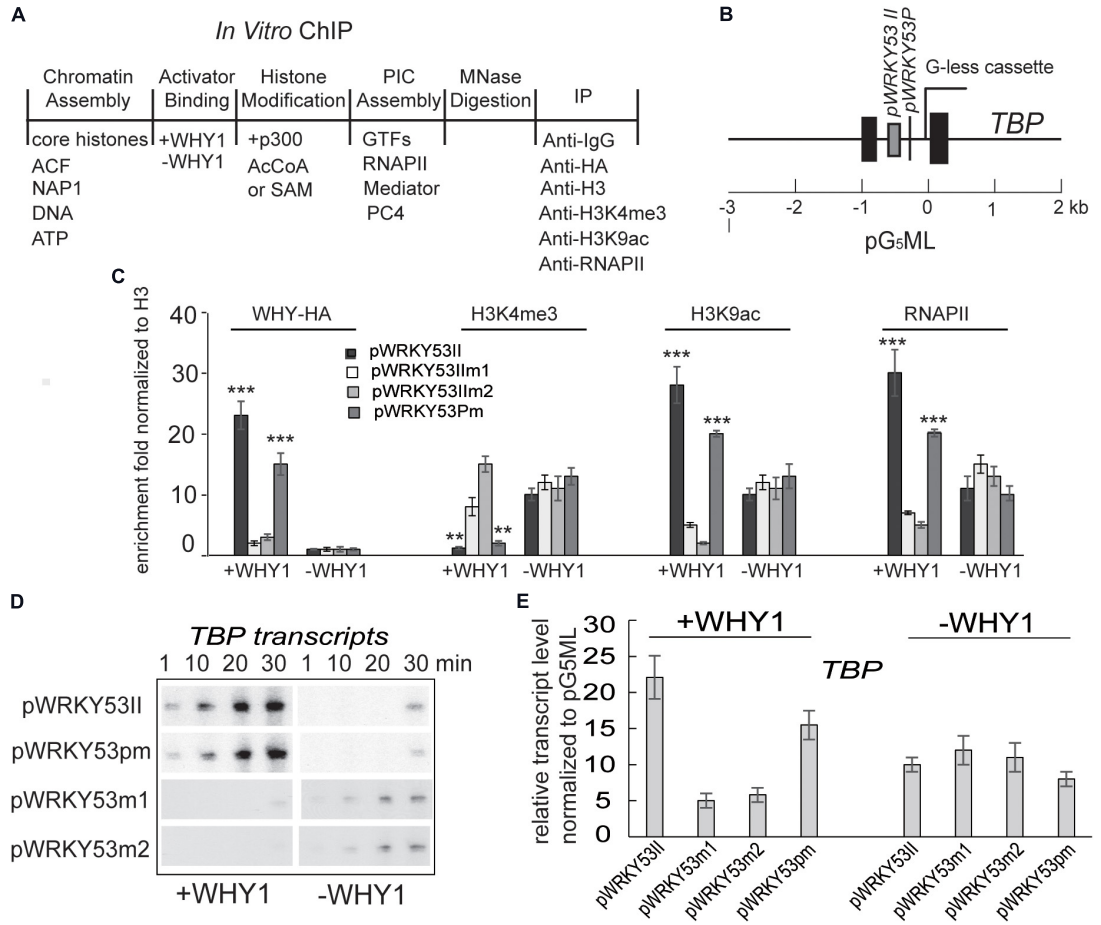

FIGURE 4 | WHIRLY1 impacts H3K4me3, H3K9ac levels on *WRKY53* promoter *in vitro*. **(A)** Schematic of the *in vitro* ChIP assays. **(B)** Schematic of the pG5ML template indicating the amplicons used for qRT-PCR. **(C)** *In vitro* ChIP was performed with assembled chromatin. Chromatin was incubated with or without recombinant WHIRLY1 protein expressed in *E. coli* (Miao et al., 2013) acetylated by p300 or methylated by *S*-adenosyl-l-methionine (SAM), digested with MNase, and immunoprecipitated with antibodies against WHIRLY1, H3, H3K4me3 or H3K9ac, or RNAPII **(A)**. The ChIP-quantitative PCR was carried out using primer containing WHIRLY1 binding sites (WRKY53II) and TATA box. H3K4me3 and H3K9ac levels were relative to H3 levels. Three biological replicates and three technique replicates were used to analyze. Asterisk indicate significant differences (**$P < 0.01$ and ***$P < 0.001$) based on Student's *t*-test. Error bars represent SE. **(D)** The report gene transcription by run-on assay. **(E)** The report gene transcription level by qRT-PCR. Three biological replicates and three technique replicates were used to analyze. Error bars represent SE.

K4 of histone H3, respectively (Saleh et al., 2008), and SUVH2 involved in regulating histone methylation marks at *WRKY53* (Ay et al., 2009). The expression of *ATX1* was slightly increased in the *why1* and decreased in the *oenWHY1* mutants, while *ATX2* and *SUVH2* showed no difference in expression between WHIRLY1 mutants and wild-type plants (**Figure 6**). Hence, WHIRLY1 is likely mediated histone methylation by regulating expression of histone methyltransferases ATX1. *HDA2*, *HDA5*, *HDA6*, *HDA9*, and *HDA15* which has been shown highly expression in leaves based on *Arabidopsis* eFP database[3] were also selected for qPCR. Interestingly, the loss of WHIRLY1 decreased the transcription level of *HDA2*, *HDA5*, *HDA6*, and *HDA9*, while overexpressing WHIRLY1 only increased *HDA6* and *HDA9* expression. These results suggest that WHIRLY1 eFP database might involve in regulating gene expression of histone deacetylases to remove the H3K9ac marks at the promoter of *WRKY53*.

DISCUSSION

WHIRLY1 was reported to be a repressor binding on the GNNNAAATT, plus an AT-rich telomeric repeat-like sequence in the *WRKY53* promoter (Miao et al., 2013). In this study, we have found that enrichment of H3K4me3 and H3K9ac at promoter region contained WHIRLY1 binding domain and TATA box and translation start region of *WRKY53*, and recruitment of RNAPII, as well as transcription of *WRKY53* are coordinated by WHRLY1 protein in a developmental manner. It demonstrates that the occupancy of WHIRLY1 represses the enrichment of H3K4me3 before senescence initiation and enhances the enrichment of H3K9ac and the recruitment of RNAPII at senescence initiation

[3]http://bbc.botany.utoronto.ca/

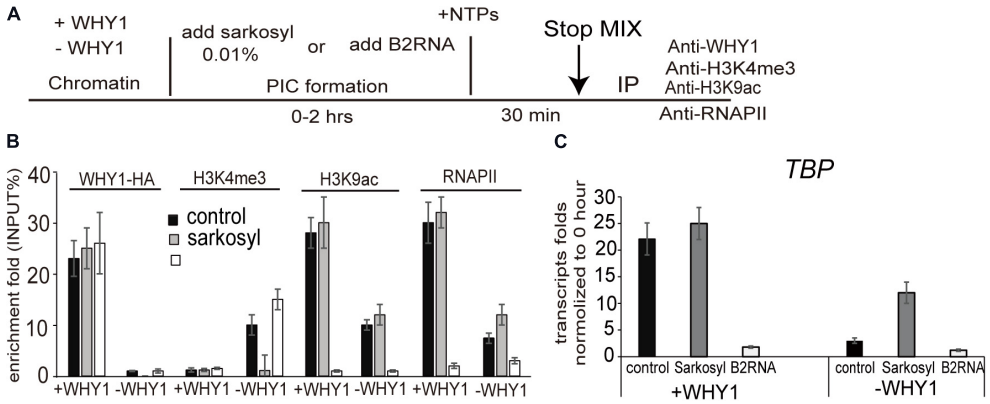

FIGURE 5 | WHIRLY1 affects H3K4me3 and H3K9ac at the preinitiation conformation stage. **(A)** Schematic of the *in vitro* transcription assay. **(B)** ChIP experiments were performed on chromatin assembled *in vitro* after transcription in presence or absence of 0.01% sarkosyl or 800 nM B2RNA with indicated antibodies. H3K4me3 and H3K9ac levels were relative to H3 levels. Three biological replicates and three technique replicates were used to analyze. Error bars represent SE. **(C)** The report gene transcription level by qRT-PCR. Three biological replicates and three technique replicates were used to analyze. Error bars represent SE.

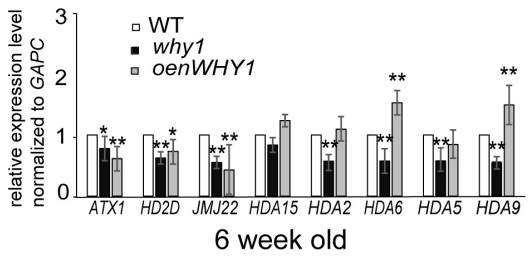

FIGURE 6 | WHIRLY1 affects the HDAs gene expression. The expression of histone modification-related genes in 6-week-old *why1*, *oenWHY1*, and wild-type (WT) plants by qRT-PCR. The transcript level in each case was normalized to that of *GAPC2* as a reference gene, and the expression level at WT was set as 1. Three biological replicates and three technique replicates were used to analyze. Error bars represent SE. Asterisk indicate significant differences (*$P < 0.05$ and **$P < 0.01$) based on Student's *t*-test.

stage, and high ratio of H3K9ac/H3K4me2-3 determines the transcription level of *WRKY53* and leaf senescence initiation. It illuminates that WHIRLY1 works as repressor of *WRKY53* transcription associated with H3K4me2-3/H3K9ac balance in the developmental manner.

WHIRLY1 Spatio-Temporally Affects H3K4 Methylation and H3K9 Acetylation at *WRKY53* During Leaf Aging

H3K4me2/3 is high at promoter or around the transcription start site (TSS) regions of active or poised genes in animals, while H3K4me2/3 is enriched at the proximal promoter and TSS site of genes in plants (Xiao et al., 2016). Both in animals and plants, H3K4me2/3 as well as H3K9ac which is also enriched at 5′-end and ATG site of genes were reported to correlate with active gene expression (Zhou et al., 2010; Songa et al., 2014). In *Arabidopsis*, H3K4me2/3 mark at 5′untranslated region and translational start region (from −155 bp to +27 bp) as well as gene body (from +367 bp to +549 bp) of *WRKY53* locus increased in senescent leaves which consisted with the expression of *WRKY53* (Ay et al., 2009). However, currently, genome-wide data of ChIP-Seq and RNA-seq showing a constant high level of H3K4me3 and H3K9ac marks in *WRKY53* gene did not directly relate to *WRKY53* expression (Brusslan et al., 2015). *WRKY53* is one example of the many genes that are marked before significant up-regulation of mRNA levels during plant aging. It was explained that this inconsistencies between H3K4me3 marks and gene expression might be down-regulated by WHIRLY1 (Miao et al., 2013; Brusslan et al., 2015), or which may explain the coincidence of low transcript levels and high levels of H3K4me3 marks, as well as the examples of posttranscriptional regulation mediated by small RNAs have been identified during leaf senescence (Kim et al., 2009; Thatcher et al., 2015; Swida-Barteczka et al., 2018). In this study, we clearly showed only some specific regions of *WRKY53* locus showed significantly change in H3K4me2-3 and H3K9ac at specific time point (**Figure 1**). The observation that the level of both H3K4me2/3 and H3K9ac at selected regions (WRKY53II and WRKY53P) of *WRKY53* from 5th to 7th week correlated with the enrichment of WHIRLY1 protein binding on *WRKY53* promoter. It indicates that WHIRLY1 spatio-temporally affect H3K4 methylation and H3K9 acetylation at *WRKY53* during leaf aging. *In vitro* assay further confirms WHIRLY1 binding on specified motifs (GNNNAAATT) of *WRKY53* promoter repressed enrichment of H3K4me3 and potentiated enrichment of H3K9ac, RNAPII recruitment, and *TBP* report gene transcription (**Figure 4**), implicating that WHIRLY1 involves in modification of local chromatin states.

WHIRLY1 Affects Coordinately the Enrichment of H3K4me3 at the PIC Formation Stage and H3K9ac at the Elongation Stage

Both H3K4me3 and H3K9ac are closely associated with active genes and play an important role in transcription. In human cells, H3K9ac potentiates the interaction of H3K4me3 and basal

TF TFIID (Vermeulen et al., 2007). There are numerous studies demonstrated a strong relationship between TF occupancy and chromatin features (Liu et al., 2016). In this study, WHIRLY1 binding on *WRKY53* promoter affected H3K4me3 and H3K9ac *in vivo* and *in vitro*. We utilized two transcription inhibitor sarkosyl and B2 RNA to address this question (Lauberth et al., 2013). Sarkosyl inhibits PIC assembly but does not affect elongation by pre-formed complexes (Cai and Luse, 1987; Hawley and Roeder, 1987), while B2 RNA binds to RNA polymerase II and inhibits transcription before PIC formation (Espinoza et al., 2004). Results of *in vitro* cell-free experiments indicated that WHIRLY1 occupied on *WRKY53* promoter inhibit enrichment of H3K4me3 at the preinitiation stage of PIC formation, in contrary, WHIRLY1 enhances the enrichment of H3K9ac and RNAPII recruitment during the elongation by pre-formed complexes (**Figure 5**).

Compass-like complex is known to facilitate PIC assembly and generate H3K4me3 (Songa et al., 2014), our results showed WHIRLY1 which did not interact with core components of compass-like complex and H3K4 methyltransferases ATX1 then affected the enrichment of H3K4me3. WHIRLY1 binding motif at *WRKY53* promoter was upstream and close to TATA box, in addition, the structure analysis of transcription initiation by RNA polymerase II showed PIC form around TATA box (Grunberg and Hahn, 2013), WHIRLY1 binding on promoter of *WRKY53* may block the site for TATA-box binding protein and repress H3K4me3 enrichment by inhibiting the PIC assembly.

A possible combinatorial interplay between posttranslational modifications on the same histone was proposed based on the patterns of H3 methylation and acetylation at promoters of specific target genes (Taverna et al., 2007). Interestingly, our study suggested a relationship between the enrichment of H3K4me3 and H3K9ac at *WRKY53* promoter region (WRKY53II). Enrichment of H3K4me3 at WRKY53II was inhibited by histone methylation inhibitor DZnep, while the enrichment of H3K9ac was improved by Dznep treatment, in turn, the enrichment of H3K9ac at WRKY53II region decreased in *oehada15* mutant plants while H3K4me3 level at WRKY53II region increased in them. Many protein complexes and epigenetic modifications in the local chromatin environment are necessary for the progress of RNAPII-mediated transcription (Lauberth et al., 2013; Stasevich et al., 2014; Gates et al., 2017). H3K4me3 and H3K9ac were reported to involve in transcription initiation and elongation (Santos-Rosa et al., 2002; Ding et al., 2012; Gates et al., 2017; Zhang et al., 2017) and the interplay between H3K4me3 and H3K9ac at gene promoter was mediated by several factors such as HATs general control nonderepressible 5 (GCN5) (Foulds et al., 2013). WHIRLY1 was involved in the transcription of *WRKY53* by interplay between H3K4me3 and H3K9ac.

WHIRLY1 Coordinates With HDACs to Modulate the Transcription of *WRKY53*

WRKY53 is a well-known transcription factor, plays remarkable role in leaf senescence and plant senescence initiation (Hinderhofer and Zentgraf, 2001; Miao et al., 2004, 2007). It regulated more than 60 senescence-related gene expressions and their related signaling networks, showing delaying senescence phenotype in the *wrky53* line, early senescence phenotype in the *oeWRKY53* lines (Miao et al., 2004, 2007). The expression of *WRKY53* is tightly controlled by multiple layers of regulation, including at the level of chromatin and transcription, as well as by ubiquitination and phosphorylation regulation. (Ay et al., 2009; Miao and Zentgraf, 2010; Zentgraf et al., 2010; Xie et al., 2014; Chen et al., 2016). In this study, WHIRLY1 had been found to bind on the promoter of *WRKY53* and regulate the enrichment of H3K4me3 and H3K9ac in a developmental manner. It may seem paradoxical that WHIRLY1 itself binding on *WRKY53* promoter enhances the enrichment of H3K9ac at leaf senescence initiation while it affects HDACs gene expression to promote the deacetylation of H3K9 at early senescence stage (**Figure 7**). Occupancy of WHIRLY1 on *WRKY53* promoter mainly occurs as *WRKY53* is being shutdown and decreases followed by aging. However, *in vitro* ChIP assay WHIRLY1 increased H3K9ac enrichment and RNAPII recruitment and upregulate downstream target gene transcription. It is a possibility that WHIRLY1 protein not only acts as a block of *WRKY53* transcription before senescence initiation, inhibits activated transcription, but also acts as an activator of other downstream genes such as *PR10a* in potato or *S40* in barley (Desveaux et al., 2000; Krupinska et al., 2014a), which might be coordinated with chromatin remodeling factor BRM or HDA6, HDA19 (Efroni et al., 2013; Chen et al., 2016). Additionally, the occupancy of WHIRLY1 may affect the accessibility of DNA by adjusting themselves to be more complicated complex under specific signals (Cappadocia et al., 2012). This may be the reason why the expression of *HD2D* and *JMJ* decreased in both *why1* mutants and *oenWHY1* mutants.

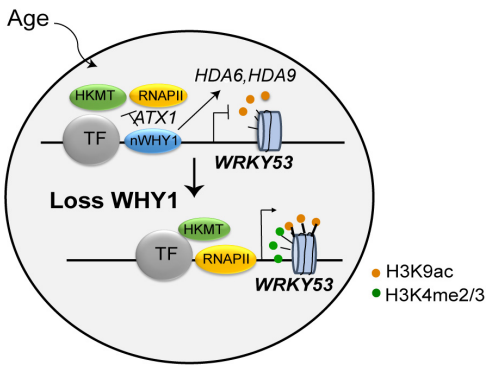

FIGURE 7 | Working model of WHIRLY1 regulates *WRKY53* transcription. WHIRLY1 binding on *WRKY53* promoter represses enrichment of H3K4 methylation by ATX1 inhibiting the PIC formation in leaf senescence initiation and modulates the enrichment of H3K9ac by association with HDACs and high-level transcription at early senescence stage. In absence of WHIRLY1, other transcription activators recruit histone lysine methyltransferase and HDACs is dissociated from *WRKY53* resulting in elevated H3K4me3, H3K9ac levels, and higher occupancy of RNAPII in *WRKY53* loci, thus increasing the transcription of *WRKY53*. TF, transcription factor; HDACs, histone deacetylases; RNAPII, RNA polymerase II; HKMT, histone lysine methyltransferase; nWHY1, nuclear WHY1 protein.

Conclusively, we propose a model that WHIRLY1 binding on *WRKY53* promoter represses enrichment of H3K4 methylation by ATX1 inhibits the PIC formation in leaf senescence initiation and modulates the enrichment of H3K9ac by association with HDAs and high-level transcription at early senescence stage (**Figure 7**). Further, what signals or factors promoted and controlled the action of WHIRLY1 on *WRKY53* promoter during leaf senescence are still speculated. The accumulation of WHIRLY1 in nucleus is mediated by CIPK14 kinase which was promoted by light conditions, sugar, cytokinin, and calcium–calmodulin signal (Gan and Amasino, 1995; Lee et al., 2005; Akaboshi et al., 2008; Qin et al., 2010; Yan et al., 2014; Ren et al., 2017). Indeed, WHIRLY1 involved in response to different light conditions (Huang D. et al., 2017; Kucharewicz et al., 2017), further revealing that their relationships might provide insights into the upstream signals of WHIRLY1 function on SSB binding on target genes.

AUTHOR CONTRIBUTIONS

YM designed the study and performed *in vitro* ChIP assay. DH and BD performed ChIP–qPCR, Western blots, and phenotyping. WL performed qRT-PCR. DL crossed double mutant and screen them, phenotyping. WfL and YR for qRT-PCR and RNA-seq data analyses. DH and YM analyzed the data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01503/full#supplementary-material

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A Novel Loop: Mutual Regulation Between Epigenetic Modification and the Circadian Clock

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In response to periodic environmental fluctuations generated by the rotation of the earth, nearly all organisms have evolved an intrinsic timekeeper, the circadian clock, which can maintain approximate 24-h rhythmic oscillations in biological processes, ultimately conferring fitness benefits. In the model plant Arabidopsis, the core mechanics of the circadian clock can be described as a complex regulatory network of three feedback loops composed of core oscillator genes. Transcriptional regulation of each oscillator gene is necessary to maintain the structure of the circadian clock. As a gene transcription regulatory mechanism, the epigenetic modification of chromatin affects the spatiotemporal expression of multiple genes. Accumulating evidence indicates that epigenetic modification is associated with circadian clock function in animals and plants. In addition, the rhythms of epigenetic modification have a significant influence on the timing of molecular processes, including gene transcription. In this review, we summarize recent progress in research on the roles of histone acetylation, methylation, and phosphorylation in the regulation of clock gene expression in Arabidopsis.

Keywords: circadian clock, epigenetic, acetylation, methylation, phosphorylation, Arabidopsis

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EPIGENETIC REGULATION OF THE CIRCADIAN CLOCK

The circadian clock is a ubiquitous molecular oscillator that provides basic timing information and regulates biochemical, physiological, and behavioral processes. In plants, the circadian clock regulatory mechanism regulates responses to the environment at the transcriptional level as well as at physiological and biochemical levels. This rhythmic oscillation of nearly 24 h decreases the unnecessary consumption of energy and organics, while increasing competitive productivity and viability.

Multiple interlocked transcriptional feedback loops formed by transcription factors are central to circadian clock function. In the model plant Arabidopsis, the circadian clock system can be described as a complex regulatory network of three loops. The core loop is composed of three important genes, namely, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), and *TIMING OF CAB EXPRESSION 1* (*TOC1*). In this loop, CCA1 and LHY inhibit the expression of *TOC1*, whereas TOC1 directly represses *CCA1* and *LHY*, thereby establishing a complete regulatory process. As a DNA-binding transcription factor, TOC1 binds directly to the promoters of *CCA1* and *LHY* to repress their expression. CCA1 and LHY, two MYB

transcription factors that are active in the morning of the subjective day, repress the expression of *PSEUDO RESPONSE REGULATOR5*, *7*, and *9* (*PRR5*, *7*, and *9*), whereas PRR5, 7, and 9 in turn suppress the expression of *CCA1* and *LHY*. The evening complex (EC) includes three other key clock components, namely, LUX ARRHYTHMO, EARLY FLOWERING3 (ELF3), and EARLY FLOWERING4 (ELF4). The EC complex can directly repress *PRR9*. In the evening loop, TOC1 suppresses the expression of *GIGANTEA* (*GI*), and GI promotes *TOC1* expression, whereas the transcription of *GI* is inhibited by CCA1 and LHY. These three negative feedback loops, together with the input–output pathway of the circadian clock, constitute a complex regulatory network that controls various physiological and crucial metabolic processes in plants (Huang et al., 2012; Nagel and Kay, 2012; Aguilar-Arnal and Sassone-Corsi, 2015; Oakenfull and Davis, 2017).

The nucleosome is a repeating unit of chromatin fiber that consists of 147 base pairs (bp) of genomic DNA wrapped around an octamer of histones. A standard octamer of histones comprises two copies of each of the four canonical histone proteins: H2A, H2B, H3, and H4. Each histone possesses a highly basic N-terminal tail, which protrudes from the surface of the histone octamer and serves as a substrate for several enzymes that lead to different post-translational modifications, including acetylation, phosphorylation, and methylation. Since histone post-translational modification constitutes an extra (*epi*) layer of gene regulation beyond that of the DNA sequence, this mechanism is termed epigenetic. Epigenetic regulation is necessary for survival and reproduction in unpredictable environments (Wang et al., 2016).

Recent studies have indicated that circadian oscillations in plants need to be monitored to facilitate the modification of oscillator regulatory mechanisms according to circumstances. Interestingly, some of the transgenerational plasticity of the plant circadian clock does not involve the alteration of clock gene DNA sequences, but instead manifests as reversible changes in the chromatin structure that determines the expression of the core oscillator genes. Chromatin reshaping depends on epigenetic factors, such as histone post-translational modifications/replacements, which create a flexible loop of gene regulation (Henriques and Mas, 2013; Baerenfaller et al., 2016; Kim et al., 2017; Hung et al., 2018; Lee and Seo, 2018; Stevenson, 2018).

Here, we provide examples of clock gene regulation mediated via epigenetic alteration, and also discuss rhythmic epigenetic changes in plants as well as the contribution of circadian clock epigenetic modification to the processes of adaptation and acclimation in plants.

EPIGENETIC MODIFICATIONS REGULATE THE CORE OSCILLATORS

In Arabidopsis, the circadian clock is composed of three interlocking transcription–translation feedback loops. The central loop, referred to as the core oscillator, was first proposed a decade ago. This loop comprises the three transcription factors *TOC1*, *CCA1*, and *LHY*. The morning-expressed CCA1 and LHY inhibit the transcription of the evening gene *TOC1*. Conversely, at dusk, TOC1 represses the transcription of *CCA1* and *LHY* (Huang et al., 2012; Oakenfull and Davis, 2017).

Previous studies have shown that histone acetylation, methylation, and phosphorylation are associated with transcriptional regulation of the core oscillator genes in the circadian clock.

EPIGENETIC MODIFICATIONS IN THE CORE LOOP

Expression of the circadian clock oscillator gene *TOC1* is modulated by dynamic changes in histone deacetylation in the *TOC1* promoter at dawn. The morning transcription factor CCA1 represses the expression of *TOC1* by binding to the *TOC1* promoter, which is accompanied by conditions favoring histone deacetylation in the *TOC1* promoter (Ni et al., 2009; Huang et al., 2012; Nagel and Kay, 2012).

Histone deacetylase (HDAC) is responsible for this histone deacetylation, which contributes to declining *TOC1* expression near dusk. In a *cca1*/*lhy* double mutant, histone H3 acetylation (H3ac) in the *TOC1* promoter was observed to be higher than that in the wild type, indicating that CCA1 has a strong inhibitory effect on *TOC1* expression and that it antagonizes H3ac to decrease the abundance of *TOC1* mRNA (Ni et al., 2009; Malapeira et al., 2012; Ng et al., 2017).

Characterization of H3ac dynamics in the *TOC1* promoter revealed an interesting regulatory mechanism. Studies examining *CCA1*-overexpressing lines indicated that a decrease in H3ac is associated with the repression of *TOC1*, whereas analysis of a *cca1*/*lhy* double mutant revealed an increase in H3ac in the *TOC1* promoter. These observations indicate that CCA1 represses *TOC1* expression by binding to the TOC1 promoter. In addition, the rhythms of histone H3 deacetylation have been found to be negatively correlated with *TOC1* transcript levels. HDACs can remove acetyl groups on lysine residues, thereby generating hypoacetylated histones, which promote chromatin fiber compaction and gene repression. In plants treated with the HDAC inhibitor trichostatin A, *TOC1* is more highly expressed after dusk (Perales and Mas, 2007; Malapeira et al., 2012), thereby indicating that the declining phase of *TOC1* is induced by HDAC activity. These results also suggest that CCA1, as a repressor of *TOC1*, might rely, at least in part, on the recruitment of HDACs to the *TOC1* promoter (Henriques and Mas, 2013; Barneche et al., 2014).

A further component contributing to chromatin modification in the *TOC1* promoter is REVEILLE 8/LHY-CCA1-LIKE 5 (RVE8/LCL5), which affects the repression of *TOC1*. Similar to *CCA1* and *LHY* transcription, *RVE8* transcription peaks in the morning. Altered expression of RVE8/LCL5 in plants modifies the circadian period. Similar to CCA1, RVE8/LCL5 regulates the expression of *TOC1* by binding to the *TOC1* promoter; however, once bound, it promotes hyperacetylation of H3 in the *TOC1* promoter and subsequently activates the expression of

this gene. In contrast, CCA1 inhibits the expression of *TOC1* by promoting histone deacetylation. Thus, although their sequences and expression peaks are similar, *RVE8/LCL5* and *CCA1* have contrasting effects on the regulation of *TOC1* transcription (Farinas and Mas, 2011; Barneche et al., 2014; Horak and Farre, 2015).

Recent studies have shown that the rhythm of histone H3K4 trimethylation (H3K4me3) is related to the oscillatory expression of the core clock genes. Analysis of the correlation of clock gene expression in seedlings treated with an H3K4me3 inhibitor has revealed that, compared with the control seedlings, the circadian rhythms of *CCA1* and *TOC1* display a longer period of expression. Therefore, it is conceivable that H3K4me3 is required to ensure correct expression peaks of the clock genes. The oscillatory waveform of H3K4me3 accumulation in core oscillator gene promoters has been shown to have a phase delay compared with that of H3ac, indicating that H3K4me3 might have a different regulatory mechanism whereby it regulates the expression of clock genes (Perales and Mas, 2007; Ni et al., 2009; Barneche et al., 2014).

The successive accumulation of H3ac (H3K56ac and H3K9ac), H3K4me3, and H3K4me2 is known to exhibit circadian rhythmicity. The inhibition of acetylation and H3K4me3 suppresses the expression of the clock genes. Blocking H3K4me3 enhances the binding activity of circadian clock inhibitors, indicating that H3K4me3 could be a marker of the transformation from activation to inhibition. Specifically, the histone methyltransferase SET DOMAIN GROUP 2/ARABIDOPSIS TRITHORAX-RELATED 3 (SDG2/ATXR3) may directly or indirectly contribute to oscillatory gene expression and H3K4me3 accumulation, and altered expression of *SDG2/ATXR3* has been observed to modify the binding activity of certain clock repressors (Malapeira et al., 2012; Henriques and Mas, 2013; Barneche et al., 2014).

LYSINE-SPECIFIC DEMETHYLASE 1-LIKE 1 (LDL1) and LDL2 interact with CCA1 and LHY to repress the expression of *TOC1*. Chromatin immunoprecipitation sequencing (ChIP-Seq) analysis has shown that many circadian genes regulated by CCA1 are targeted by LDL proteins. LDL1 and LDL2 interact with the histone deacetylase HDA6, and the LDL1-HDA6 complex binds directly to the *TOC1* promoter and represses *TOC1* expression by increasing histone deacetylation and H3K4 demethylation. These findings have contributed to elucidating a pathway through which histone modifications regulate clock genes and the inner network of core oscillator genes (Hung et al., 2018).

EPIGENETIC MODIFICATIONS OF THE OTHER LOOPS

The rhythmic expression of the core oscillator gene *TOC1* is preceded by the oscillation of H3ac accumulation in its promoter. Moreover, H3ac accumulation parallels the expression of almost all circadian clock components, including *CCA1*, *LHY*, *PRR9*, *PRR7*, *LUX*, and *TOC1*. In this regard, chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) analysis has revealed that H3K9ac, H3K14ac, and H3K56ac are involved in the transcriptional activation of clock genes (Perales and Mas, 2007; Malapeira et al., 2012).

Recent studies have also revealed that *HISTONE ACETYLTRANSFERASE OF THE TAFII250 FAMILY 2 (HAF2)* is involved in circadian clock regulation. The HAF2 protein facilitates H3ac accumulation in the *LUX* and *PRR5* promoters to activate gene expression at midday, with the expression of *HAF2* being regulated by CCA1. Future studies are expected to further elucidate the HAF2-mediated temporal coordination of late-day and evening-expressed genes (Lee and Seo, 2018).

The expression of *JUMONJI C DOMAIN-CONTAINING* 5 (*JMJD5/JMJ30*), which peaks in the evening, is regulated by the circadian rhythm. In addition, the regulation of JMJD5/JMJ30 is jointly controlled by CCA1 and LHY. LHY can suppress the expression of *JMJD5/JMJ30* by directly binding to the *JMJD5/JMJ30* promoter. Furthermore, the expression of *CCA1* and *LHY* under high-intensity red light has been found to be lower in a *jmjd5/jmj30* mutant than in the wild type, indicating that JMJD5/JMJ30, CCA1, and LHY form a negative feedback loop in response to red light (Jones et al., 2010).

Although histone H3 phosphorylation is known to play a role in the regulation of gene transcription, there have been few reports regarding the function of histone H2A phosphorylation in the promoters of circadian clock genes. Recent work has, nevertheless, demonstrated that MUT9P LIKE KINASE4 (MLK4) induces *GI* expression (Su et al., 2017). In this process, MLK4 initially interacts with CCA1 at the *GI* promoter. CCA1 in turn recruits YAF9a, resulting in accumulation of the histone variant H2A.Z and the acetylation of H4 at *GI*, thereby inducing *GI* expression (Su et al., 2017).

The monoubiquitination of histone H2B (H2Bub) is widely observed in plant clock genes, and H2Bub has been shown to have substantial effects on the oscillatory expression of circadian clock genes. The loss-of-function mutant *histone mono-ubiquitination1* (*hub1-1)* exhibits reduced H2Bub accumulation. The oscillation of *CCA1* and *ELF4* is dampened in *hub1-1*, and the LHY expression phase is also advanced. Moreover, *hub1-1* mutation may enhance the expression of *TOC1* by reducing the inhibitory activity of CCA1. H2Bub appears to act as a positive regulator of *TOC1*, *PRR7*, and *GI* expression in etiolated seedlings exposed to light. On the basis of evidence obtained to date, it appears that histone H2B may affect a large number of clock components, the mRNA abundances of which are tightly regulated by intense oscillations (Bourbousse et al., 2012; Barneche et al., 2014) (**Table 1**).

DET1 may act as a transcriptional corepressor of CCA1 and LHY to repress *TOC1* transcription. Similar to *cca1/lhy* mutants, a *det1-1* mutant was shown to exhibit a shorter period of *TOC1* oscillations. Given that DET1 interacts with H2Bub, it may repress clock genes via H2Bub (He et al., 2011; Lau et al., 2011; Kang et al., 2015). A further study has revealed that PRR5, 7, and 9 can interact with *TOPLESS/TOPLESS RELATED PROTEINS (TPL/TPR)* and *HDA6* to form a complex at the promoters of *CCA1* and *LHY*, thereby repressing the expression of these two genes (Wang et al., 2010, 2013).

TABLE 1 | Chromatin modifications and related factors associated with the expression of clock genes.

Process	Histone mark	Factor	Target gene	Reference
Acetylation	H3ac	nd	*CCA1, LHY, TOC1*	Perales and Mas, 2007
				Hemmes et al., 2012
	H3K9ac	nd	*CCA1, LHY, TOC1, PRR9, PRR7, LUX*	Malapeira et al., 2012
	H3K14ac	nd	*CCA1, LHY, TOC1*	Hemmes et al., 2012
	H3K56ac	nd	*CCA1, LHY, TOC1, PRR9, PRR7, LUX*	Malapeira et al., 2012
	H3ac	HAF2	*PRR5, LUX*	Lee and Seo, 2018
Methylation	H3K4me3	SDG2/ATXR3	*CCA1, LHY, TOC1, PRR9, PRR7, LUX*	Malapeira et al., 2012
	H3K4me2	nd	*CCA1, LHY, TOC1*	Song and Noh, 2012
	H3K36me2	JMJD5/JMJ30	nd	Jones et al., 2010
Monoubiquitination	H2Bub	HUB1	*CCA1, ELF4*	Himanen et al., 2012
Deacetylation	H3ac deacetylation	HDAC (nd)	*CCA1, LHY, TOC1, PRR9, PRR7, LUX*	Malapeira et al., 2012
	H3ac deacetylation	HDA6	*TOC1*	Hung et al., 2018
Demethylation	H3K4me2	LDL1/LDL2	*TOC1*	Hung et al., 2018
Phosphorylation	H2A Serine 95	MLK4	*GI*	Su et al., 2017

THE RHYTHMIC EXPRESSION OF HISTONE-MODIFICATION ENZYMES

Histone modifications, including acetylation–deacetylation and methylation–demethylation, play a key role in regulating the expression of clock genes, and in this regard, previous studies have indicated that the rhythmic expression of epigenetic enzymes is correlated with the daily rhythms of epigenetic modification and the expression of downstream genes (Loenen and Raleigh, 2014; Baerenfaller et al., 2016).

HISTONE DEACETYLASES

Histone acetylation/deacetylation modifications are important for gene transcription, and the oscillatory expression of clock genes is known to be associated with such modifications. Although evidence indicates that HDACs can directly regulate the expression of clock genes, the effects of individual HDAC genes have yet to be elucidated. Currently, the function of HDACs is generally studied by treatment with HDAC-specific inhibitors suberoylanilide hydroxamic acid (SAHA), trichostatin A, or butyrate or by the overexpression and repression of HDAC genes in transgenic plants. Plant HDAC family can be divided into three subfamilies, namely, the *HISTONE DEACETYLASE 1 (HDA1)*, *SIRTUIN 2 (SIR2)*, and *HISTONE DEACETYLASE 2 (HD2)* subfamilies. In Arabidopsis, members of the HDA1 subfamily include *HDA2*, *5*, *6*, *7*, *8*, *9*, *10*, *14*, *15*, *17*, *18*, and *19*, whereas the SIR subfamily includes *STR1* and *STR2*, and the *HD2* subfamily includes *HDT1*, *2*, *and 3* (Hollender and Liu, 2008; Wang et al., 2014; Bourque et al., 2016). Among the HDACs in the Diurnal database of the Mockler Laboratory[1], *HD2B*, *HDA2*, *HDA4*, *HDA5*, *HDA6*, *HDA7*, *HDA8*, *HDA9*, *HDA17*, *HDA18*, *HDA19*, *SRT1*, and *SRT2* are rhythmically expressed (**Table 2**), with the expression of *HDA4*, *5*, *6*, *7*, *9*, *10*, *17*, and *18*, and *HD2B*, *SRT1*, and *SRT2* peaking at midnight, and that of *HDA2* and *8* peaking at midday (Diurnal database).

[1]http://diurnal.mocklerlab.org/diurnal_data_finders/new

TABLE 2 | The rhythmic expression of epigenetic modification enzymes.

Name	Locus	Light condition	Phase	Correlation	Reference
HDA6	AT5G63110	LL	22	0.65049	Diurnal
HDA9	AT3G44680	LL	20	0.87604	Diurnal
HDA2	AT5G26040	LL	12	0.89002	Diurnal
HD2B	AT5G22650	LL	20	0.90944	Diurnal
HDA19	AT4G38130	LL	21	0.90467	Diurnal
HDA17	AT3G44490	LL	20	0.82185	Diurnal
SRT1	AT5G55760	LL	20	0.92113	Diurnal
SRT2	AT5G09230	LL	0	0.8815	Diurnal
HDA8	AT1G08460	LL	10	0.94022	Diurnal
SDG2	AT4G15180	LL	12	0.82954	Diurnal
SDG29/ATX5	AT5G53430	LL	5	0.81682	Diurnal
SDG23/SUVH6	AT2G22740	LL	8	0.9162	Diurnal
SDG4	AT4G30860	LL	17	0.74943	Diurnal
JMJD5	AT3G20810	LL	14	–	Jones et al., 2010

HISTONE METHYLTRANSFERASES

Methylation of lysine residues in the H3 histone tail is a key mechanism contributing to the regulation of chromatin state and gene expression, and is mediated by a family of enzymes with a SET domain. One of the main functions of these enzymes is to regulate H3K4 di- and tri-methylation, which has been discovered in the *TOC1* promoter and shown to play a role in the repression of *TOC1* by CCA1 (Sanchez et al., 2010; Malapeira et al., 2012).

The SET DOMAIN GROUP (SDG) protein family in Arabidopsis contains 49 members and can be divided into five classes based on activity and structure. The five members in class III SDG, *ATX1–5*, which are homologous to the Trithorax proteins in other eukaryotes, have been shown to participate in H3K4me. Among these proteins, ATX1 (SDG27) is important for the trimethylation of H3K4. Although *ATX2/SDG30* shows

sequence homology to ATX1, it exhibits H3K4me2 rather than H3K4me3 methylation activity, whereas ATX3/SDG14, ATX4/SDG16, and ATX5/SDG29 have been observed to affect 1000s of H3K4me2 and H3K4me3 sites across the entire Arabidopsis genome.

The SDG family of Arabidopsis also contains seven ATX-related (ATXR) proteins, among which ATXR7/SDG25 and ATXR3/SDG2 have functions similar to that of ATX. The function of ATXR3/SDG2 is comparable to that of ATX3/SDG14, ATX4/SDG16, and ATX5/SDG29, and it may regulate clock gene expression by modulating H3K4me3 in promoters. However, unlike the expression of *ATX5/SDG29*, which peaks in the morning, peak expression of *ATXR3/SDG2* occurs at midday. The Diurnal database indicates that both *ATXR3/SDG2* and *ATX5/SDG29* have rhythmic expression (**Table 2**) (Malapeira et al., 2012; Chen et al., 2017).

The other three important SDG proteins, SU(VAR)3-9 HOMOLOG 4 (SUVH4), SUVH5, and SUVH6, are histone H3 lysine 9 (H3K9) methyltransferases. SUVH4 and SUVH6 are responsible for maintaining the H3K9 methylation of inverted repeats during transcription, whereas SUVH5 is necessary for the accumulation of H3K9me2 DNA methylation. Recent studies have shown that HDA6 can interact with these three histone methyltransferases, and indicate that the C-terminal region of HDA6 is important for this interaction. In this regard, two phosphorylated serine residues, S427 and S429, have been identified in the C-terminal region of HDA6, and HDA6 phosphorylation (amino acid substituents that mimic phosphorylated proteins) has been observed to lead to increased enzyme activity. Furthermore, mutation of S427 in HDA6 to alanine was found to abolish the interactions between HDA6 and SUVH5 and SUVH6, thereby indicating that the phosphorylation of HDA6 is important for its activity and function (Yu et al., 2017). The ChIP-seq result also showed that the SUVH members displayed different DNA binding preferences, deciphering the mechanism of sequence-biased non-CG methylation in plant methylomes (Li et al., 2018).

Currently, the involvement of *SUVH4*, *5*, and *6* in the circadian clock is largely unknown; however, a previous study has shown that SUVH4, 5 and 6 affect H3K9me but not H3K4me in the *TOC1* promoter. According to the Diurnal database (**Table 2**), *SUVH4*, *5*, and *6* and *HDA6* are rhythmically expressed, and given that SUVH4, 5 and 6 interact with HDA6, it is probable that they play a role in circadian clock regulation (Cho et al., 2012).

HISTONE DEMETHYLASES

Recent studies have demonstrated that histone methylation can be inhibited by at least two different types of enzymes, LSD1 and the JMJ proteins. As discussed above, JMJD5/JMJ30 is a component of the circadian clock in Arabidopsis, and the expression of *JMJD5/JMJ30* peaks 2 h after midday (Jones et al., 2010; Hemmes et al., 2012). A further two JMJ proteins, JMJ20 and JMJ22, have also been shown to be involved in the regulation of clock genes. When the important clock input pathway gene phytochrome B (PHYB) is inactive, JMJ20 and JMJ22 are directly repressed by the zinc-finger protein SOMNUS (Lu et al., 2008; Cho et al., 2012). The Diurnal database indicates that *JMJ22* is rhythmically expressed and that its expression peaks in the evening (**Table 2**).

CONCLUSION AND PERSPECTIVES

Epigenetic regulation of the circadian clock has recently been investigated via advanced molecular biology and genetic approaches. The periodic expression of core clock genes is regulated epigenetically at the chromatin level and modification of histones primarily leads to alterations in the transcriptional activity of clock genes. Interestingly, the deacetylation of

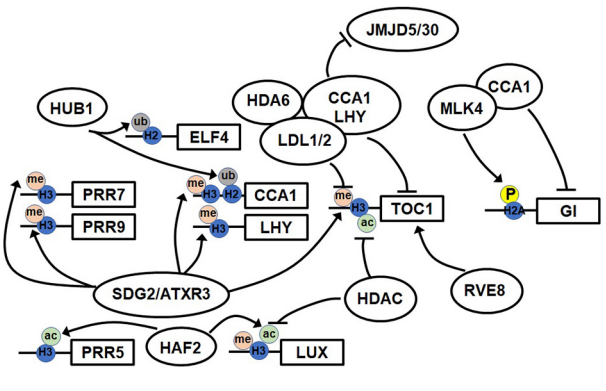

FIGURE 1 | Model of the epigenetic regulation of clock genes. The model shows known epigenetic modifications at the promoters of clock genes and factors. The accumulation of histone H3 acetylation (H3ac), H3 lysine 3 methylation (H3K4me3), and histone H2 ubiquitination (H2ub) induces the expression of clock genes. The phosphorylation of histone H2A inhibits the accumulation of histone variant H2A.Z and also the acetylation of H4.

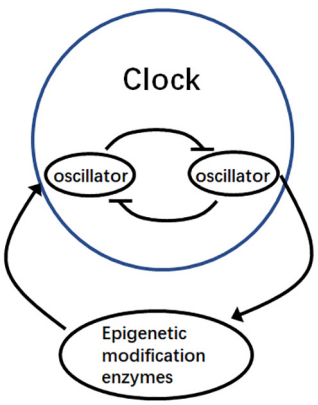

FIGURE 2 | Mutual regulation via epigenetic modification and the circadian clock. Oscillator genes regulate the expression of epigenetic modification enzymes, which in turn influence the regulation of other oscillator genes during the circadian cycle.

H3ac is related to H3K4me demethylation, which directly connects histone acetylation with methylation. In addition, the phosphorylation of H2A by MLK4 directly regulates the formation of H2A.Z and the acetylation of H4. These observations indicate that epigenetic regulation plays an important role in the regulation of the circadian clock. We also highlight that certain epigenetic modification enzymes have a rhythmic expression, suggesting that clock genes may regulate epigenetic modification enzymes (Su et al., 2017).

In addition, histone acetylation modification is a reversible dynamic process that involves both HDACs and HISTONE ACETYLTRANSFERASES (HATs). Although HAF2 is known to regulate PRR5 and LUX (Lee and Seo, 2018), the involvement of other HATs with regards to circadian rhythms remains unclear (Aquea et al., 2017). In the Diurnal database for Arabidopsis, we found that peak expression of *HISTONE ACETYLTRANSFERASE OF THE GNAT 5* (*HAG5*), *HISTONE ACETYLTRANSFERASE OF THE CBP FAMILY 12* (*HAC12*), *HAC1*, *HAC12, HAF1, HAC2*, *HAC4*, and *HAC5* occurs in the morning, whereas peak expression of *HAG2* and *HAG3* occurs in the evening, and only the expression of *HAC1* peaks near midday (Wang et al., 2014; Fina et al., 2017) (**Figure 1**).

Accumulating evidence gained from studies on the mammalian showed that the epigenetic modification is also important for the mammalian circadian clock. Similar to plants, histone acetylation and methylation also regulate the mammalian circadian clock. Consistent with the expression rhythm of clock genes, the histone acetylation H3K9ac and H3K27ac also displays circadian rhythm (Ripperger and Schibler, 2006; Feng et al., 2011; Vollmers et al., 2012). Histone methylation (H3K4me3) rhythmically oscillates at transcription start sites (TSSs) of clock genes (Le Martelot et al., 2012; Yue et al., 2017). In mammalian, the rhythmic expression of DNA methyltransferase indicated that the DNA methylation is involved in the transcription and regulation of clock genes (Benoit et al., 2013; Masri et al., 2015; Ng et al., 2017; Padmanabhan and Billaud, 2017; Kwapis et al., 2018). To date, however, no similar evidence has emerged in plants. Higher plants have three DNA methylation sites, namely, CG, CHG, and CHH (where H is A, C, or T), among which the methylation of CG and CHG sites is most important for the regulation of gene expression. DNA methyltransferases can alter the DNA methylation level of CG and CHG sites (Underwood et al., 2018), and in the Diurnal database for Arabidopsis, we found that *CHROMOMETHYLASE 3* (*CMT3*), *CMT2, DNA METHYLTRANSFE RASE 1* (*MET1*), *MET2*, *DORMANCY-ASSOCIATED PROTEIN 1* (*DRM1*), and *DRM2* have rhythmic expression patterns, with peak expression of DRM1 and DRM2 occurring near midday, that of *CMT3* and *MET1* occurring in the evening, and only that of *CMT2* occurring near midnight (Diurnal database; Xia, 2008; Wang et al., 2014).

Although the mechanisms underlying the associations between epigenetic modifications and the circadian clock have recently been a focus of research, the relationships between certain epigenetic modification enzymes and the circadian clock currently remain undetermined. Nevertheless, data obtained from ChIP-Seq analysis of the core oscillator genes in the Arabidopsis circadian clock have indicated that many epigenetic modification enzymes are rhythmically expressed. These findings provide compelling evidence that epigenetic modification enzymes are directly regulated by core oscillator genes. Thus, we hypothesize that the circadian clock can directly regulate epigenetic modification enzymes and that these enzymes in turn contribute feedback to the circadian clock, involving the mutual regulation of core oscillators (**Figure 2**). This potential output pathway might be an interesting topic of plant circadian clock study in future. The oscillator genes that directly regulate transcriptional epigenetic modification enzymes are yet to be found. The DNA methylation modification of core oscillator genes still needs to be detected. The mechanisms underlying the epigenetic modification of the core circadian clock genes remain to be further elucidated.

DATA AVAILABILITY

Publicly available datasets were analyzed in this study. This data can be found here: http://www.mocklerlab.org/supplements.

AUTHOR CONTRIBUTIONS

WH conceived the article. SD wrote the first draft. LC and LG critically revised the article. All authors were involved in the revision of the drafted manuscript and have agreed to the final content.

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Functional Coordination of the Chromatin-Remodeling Factor AtINO80 and the Histone Chaperones NRP1/2 in Inflorescence Meristem and Root Apical Meristem

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Chromatin structure requires proper modulation in face of transcriptional reprogramming in the context of organism growth and development. Chromatin-remodeling factors and histone chaperones are considered to intrinsically possess abilities to remodel chromatin structure in single or in combination. Our previous study revealed the functional synergy between the Arabidopsis chromatin-remodeling factor INOSITOL AUXOTROPHY 80 (AtINO80) and the histone chaperone NAP1-RELATED PROTEIN 1 (NRP1) and NRP2 in somatic homologous recombination, one crucial pathway involved in repairing DNA double strand breaks. Here, we report genetic interplay between *AtINO80* and *NRP1/2* in regulating inflorescence meristem (IM) and root apical meristem (RAM) activities. The triple mutant *atino80-5 m56-1* depleting of both AtINO80 (*atino80-5*) and NRP1/2 (*m56-1*) showed abnormal positioning pattern of floral primordia and enlargement of IM size. Higher mRNA levels of several genes involved in auxin pathway (e.g., *PIN1*, *FIL*) were found in the inflorescences of the triple mutant but barely in those of the single mutant *atino80-5* or the double mutant *m56-1*. In particular, the depletion of AtINO80 and NRP1/2 decreased histone H3 levels within the chromatin regions of *PIN1*, which encodes an important auxin efflux carrier. Moreover, the triple mutant displayed a severe short-root phenotype with higher sensitivity to auxin transport inhibitor NPA. Unusual high level of cell death was also found in triple mutant root tips, accompanied by double-strand break damages revealed by γ-H2A.X loci and cortex cell enlargement. Collectively, our study provides novel insight into the functional coordination of the two epigenetic factors AtINO80 and NRP1/2 in apical meristems during plant growth and development.

Keywords: *Arabidopsis thaliana*, inflorescence meristem, root apical meristem, chromatin-remodeling factor, histone chaperone

INTRODUCTION

Plant growth and development depend on a steady supply of stem cells within the meristems throughout active cell division cycles (Heidstra and Sabatini, 2014). In Arabidopsis, shoot apical meristem (SAM) can be divided into three regions: the central zone (CZ) at the apex of SAM, the peripheral zone (PZ) surrounding the CZ, and an internal ribbed meristem under the CZ. Within Arabidopsis PZ, the lateral primordia and consequent organs are generated in a Fibonacci spiral pattern, named phyllotaxis (Galvan-Ampudia et al., 2016). After transition from vegetative to reproductive growth, SAM is transformed into inflorescence meristem (IM), and then produces lateral floral primordia and organs.

The spatial distribution of the phytohormone auxin is mediated by numerous transmembrane efflux and influx carriers, and plays a crucial role in a wide variety of morphogenetic processes (Wang and Jiao, 2018). Among them, PIN-FORMED (PIN) family of auxin efflux carriers are localized in the plasma membrane on the same side of neighboring cells, and are important for the establishment and maintenance of morphogenetic auxin gradient (Adamowski and Friml, 2015). In SAM, the key PIN-family member PIN1 protein is expressed predominantly in the epidermis and provasculature (Heisler et al., 2005). The polar auxin transport mediated by PIN1 in SAM generates local auxin maxima and minima. Auxin maxima at the PZ are responsible for the specification and positioning of incipient primordia and associated lateral organs. In the mutant depleting of PIN1, the inflorescence apices are blocked in floral meristem initiation and displayed a pin-like naked stems (Reinhardt et al., 2000). Transcriptional regulation of *PIN1* has been considered to alter its protein abundance and enable regulatory cascade changes based on local auxin concentration (Reinhardt et al., 2003; Heisler et al., 2005; Habets and Offringa, 2014).

Auxin binding to the auxin receptor triggers the de-repression of downstream AUXIN-RESPONSE FACTORs (ARFs) implicated in auxin signaling (reviewed in Peer, 2013). Among them, ARF5 is a key transcription factor acting downstream of auxin perception (Reinhardt et al., 2000) and is critical for floral primordium initiation (Zhao et al., 2010). Intriguingly, *PIN1* transcription is also induced by auxin signaling through ARF5. Given the PIN1-dependent formation of auxin maxima, it may form a positive feedback that is of importance for the self-organization properties of the SAM (Wenzel et al., 2007; Krogan et al., 2016). ARF5 activates downstream genes highly expressed in organogenic regions of the reproductive shoot apex, such as *FILAMENTOUS FLOWERS* (*FIL*), and *TARGET OF MONOPTEROS 3* (*TMO3*) (Wu et al., 2015). It also represses downstream genes such as the two A-type *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes, *ARR7* and *ARR15*, which negatively regulate SAM size (Zhao et al., 2010).

In Arabidopsis primary roots, the maintenance of root apical meristem (RAM) requires two main parallel pathways. One is known as the *SHORT-ROOT* (*SHR*)/*SCARECROW* (*SCR*) pathway, two genes encoding the plant-specific GRAS family putative transcription factors (Helariutta et al., 2000; Sabatini et al., 2003). The other is the *PLETHORA1/2* (*PLT1/2*) pathway, which encode the AP2-class transcription factors (Aida et al., 2004; Blilou et al., 2005). *PLT1/2* genes are transcribed in response to auxin accumulation. Notably, members of *PIN*-family genes including *PIN1* collectively control the polar auxin distribution to determine the auxin maximum in RAM. Their combined action plays an important role in the expression pattern of *PLT* genes and further in stem cell specification.

Both chromatin-remodeling factors and histone chaperones can modulate local and global chromatin structure, playing crucial roles in DNA replication, transcription and repair (reviewed in Zhou et al., 2015; Ojolo et al., 2018). INOSITOL AUXOTROPHY 80 (INO80) is the founding member of the INO80 family chromatin-remodeling factors displaying diverse regulatory activities, such as nucleosome positioning and histone variant H2A.Z dynamics (reviewed in Gerhold and Gasser, 2014). In Arabidopsis, the *AtINO80* loss-of-function mutant *atino80-5* displays pleiotropic phenotypes including smaller organs and late flowering (Zhang et al., 2015). NAP1-RELATED PROTEIN (NRP) represents a highly conserved protein family of histone chaperones (reviewed in Zhou et al., 2015). Arabidopsis homologs *NRP1* and *NRP2* are functionally redundant, and their double mutant (*nrp1-1 nrp2-1*, abbreviated as *m56-1* in the previous study) displays short roots without any obvious phenotypes in the aerial organs (Zhu et al., 2006). Intriguingly, both *AtINO80* and *NRP1/2* are implicated in the frequency regulation of somatic homologous recombination (HR), which is an important pathway to repair DNA double-strand break (DSB), a lethal DNA damage if not repaired (Gao et al., 2012; Zhang et al., 2015). In our previous study, we generated the *atino80-5 m56-1* triple mutant, and observed a genetic epistasis of *m56-1* over *atino80-5* in the regulation of somatic HR frequency (Zhou et al., 2016). However, functional interactions between *AtINO80* and *NRP1/2* in the context of whole plant growth and development still remain largely obscure.

In this study, we report that *AtINO80* and *NRP1/2* synergistically control the proper floral primordia initiation and maintain the IM size. Transcription levels of several auxin-related genes were mis-regulated in the *atino80-5 m56-1* triple mutant. We showed the recruitment of AtINO80 and NRP1/2 as well as the decreased H3 occupancy in the chromatin regions of *PIN1*. In addition, *AtINO80* and *NRP1/2* concerted to prevent the cell death and DSB appearance in RAM and the accompanied activation of transcriptional response to DNA damage. These findings reveal their coordination in the maintenance of functional apical meristems.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wild-type (WT) and mutant lines *atino80-5* (Zhang et al., 2015) and *m56-1* (Zhu et al., 2006) are all derived from the Columbia (Col) ecotype background. The reporter lines *WOX5:GFP* (Blilou et al., 2005), *pPIN1:PIN1-GFP* (Benková et al., 2003) and *DR5rev:GFP* (Friml et al., 2003) in Col-background

have been described in previous studies. Seedlings were grown vertically on agar-solidified MS medium M0255 (Duchefa) supplemented with 0.9% sucrose at 21°C under 16 h light/8 h dark conditions. For the inhibition of polar auxin transport, *N*-naphthylphthalamic acid (NPA, 33371, Sigma-Aldrich) was added to the medium at the indicated concentrations.

Microscopy

The images of inflorescence were acquired by using a TM-3000 scanning electron microscope according to the manufacturer instructions (HITACHI). Differential interference contrast (DIC) images were taken with an Imager A2 microscope (Zeiss). For Lugol staining, roots were immersed in Lugol iodine solution containing 5% iodine for 2 min. After washing, roots were cleared with chloral hydrate solution (chloral hydrate: water: glycerol, 8:3:1, *w*/*v*/*v*). Confocal images were acquired by using a LSM710 microscope (Zeiss) with the following excitation/emission wavelengths: 561 nm/591–635 nm for Propidium Iodide (PI), 488 nm/505–530 nm for GFP. The antibody against γ-H2A.X was generated in our previous study (Zhou et al., 2016). The whole-mount root immunostaining was performed as previously described (Ma et al., 2018).

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Plant organs were dissected by using a sharp blade and quickly frozen in liquid nitrogen. We used TRIzol kit to extract RNA according to standard procedures (Invitrogen). RT was performed using Improm-II reverse transcriptase (Promega). Quantitative RT-PCR was performed in three biological replicates. *ACTIN2* (*ACT2*) was used as a reference gene to normalize the data. The gene-specific primers are listed in the **Supplementary Table 1**.

Chromatin Immuno-Precipitation (ChIP) Analysis

Chromatin immuno-precipitation was performed as described in our previous study (Zhang et al., 2015). All analysis was performed in three biological replicates. Antibodies used in this study were anti-GFP (A-11122, Invitrogen), anti-H2A.Z (Zhang et al., 2015), and anti-H3 (ab1791, Abcam). The gene-specific primers are listed in the **Supplementary Table 1**.

RESULTS

The Triple Mutant *atino80-5 m56-1* Displays a Disordered Inflorescence Phenotype

Our previous study has showed that the aerial part of *m56-1* double mutant seedling resembles that of WT, while *atino80-5 m56-1* triple mutant seedling resembles the single mutant *atino80-5* (Zhou et al., 2016). Here, we confirmed the maintenance of such epistatic effect on aerial growth throughout the whole vegetative stage. Except the decrease in leaf size observed for *atino80-5* and *atino80-5 m56-1*, no significant change of leaf phyllotaxy has been found in all the mutants (**Supplementary Figure 1**). After flowering, the WT flowers and siliques successively appeared along the branch axes in a Fibonacci spiral pattern. Such spiral pattern was not lost in *atino80-5* and *m56-1* inflorescences, albeit the spacing of *atino80-5* siliques was shortened (**Figure 1A**). Intriguingly, we found an obviously disordered positioning pattern of siliques along floral branches of the *atino80-5 m56-1* triple mutant. In many cases, several siliques appeared adjacent to each other without a spiral pattern. In addition, the development of most siliques and their fertility were greatly impaired in the triple mutant (**Figure 1A**). Notably, although the differentiation of flower organs was not generally affected in all the mutants, the organ size was reduced in *atino80-5* and more severely in *atino80-5 m56-1* (**Supplementary Figure 2**).

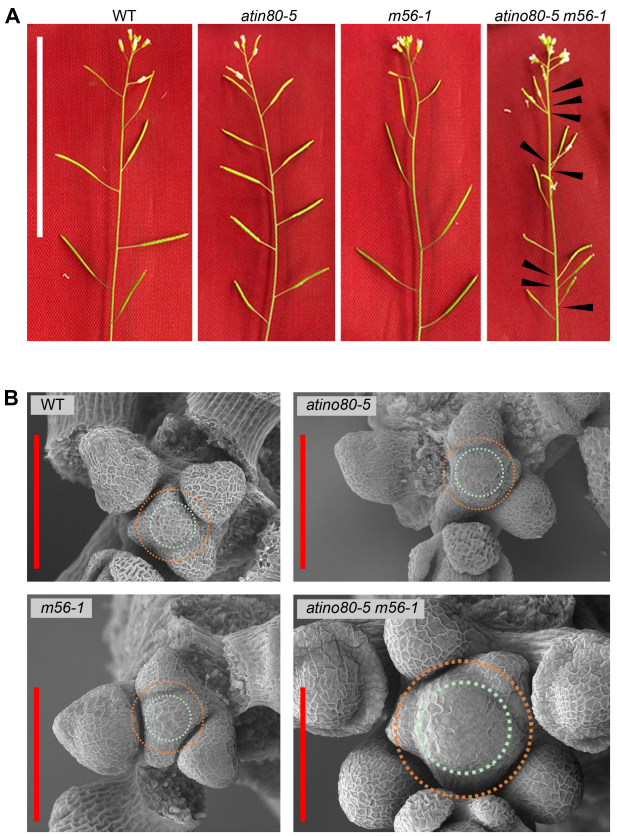

FIGURE 1 | The disordered inflorescence in *atino80-5 m56-1* triple mutant. **(A)** Comparison of floral branches in WT, *atino80-5*, *m56-1* and triple mutant. Note that the spiral positioning of siliques was disrupted in triple mutant, which are marked by black arrowheads. Bar = 50 mm. **(B)** Scanning electron microscopy of IM in WT, *atino80-5*, *m56-1* and triple mutant. For visual comparison, the CZ in each IM is outlined with bluish circle, and the PZ is outlined with orange circle. Note that the IM size is significantly enlarged in triple mutant and more floral primordia were found in the same IM. Bar = 100 μm.

We observed and compared the IM by using electron microscopy (EM) (**Figure 1B**). There was no significant difference in IMs between WT, *atino80-5* and *m56-1*, in which several floral primordia locate in a spiral pattern around the periphery zone (PZ). However, in the *atino80-5 m56-1* triple mutant IM, the CZ significantly expanded but still with isotropy, and at the same time, extraordinary number of flower primordia at various growth stages emerged concurrently around the PZ, in line with the observed disordered inflorescence phyllotaxy. Our EM observation indicated that AtINO80 and NRP1/2 play a synergistic role in the maintenance of normal IM size as well as proper pattern of lateral organ initiation in IM.

AtINO80 and NRP1/2 Modulate Chromatin Regions of *PIN1*

Both AtINO80 and NRP1/2 participate in local chromatin remodeling for transcription modulation (Zhang et al., 2015; Zhu et al., 2017). Given the vital role of auxin in determining floral primordia formation and in controlling IM size, we wonder whether auxin pathway is interrupted in the *atino80-5 m56-1* triple mutant. Therefore, we examined the transcription levels of several auxin-related genes in inflorescences. These include *PIN1*, *ARF5*, and the more downstream genes *FIL*, *TMO3*, *ARR7* and *ARR15*. Notably, the transcription levels of most examined genes are synergistically mis-regulated (fold change > 1.5) in the triple mutant (**Figure 2A**), in line with its growth abnormality in inflorescence phyllotaxy and SAM size.

In IM, PIN1 determines the polar distribution of auxin and triggers the consequent transcriptional cascade and organogenesis (Reinhardt et al., 2000). Hence, the roles of AtINO80 and NRP1/2 in *PIN1* transcriptional regulation were particularly examined in the following ChIP analysis by using inflorescences expressing EYFP-AtINO80 (Zhang et al., 2015) or EYFP-NRP1 (Zhu et al., 2017). Our ChIP results showed that EYFP-AtINO80 displayed enrichment at both 5′- and 3′-ends of the *PIN1* gene, while a single peak of EYFP-NRP1 was found after the transcription start site of *PIN1* (**Figures 2B,C**). These results indicated that *PIN1* is the target gene of chromatin-remodeling factor AtINO80 and histone chaperones NRP1/2, and at the same time suggested that the observed higher mRNA level of *PIN1* is not just the indirect result of enlarged IM size in the triple mutant inflorescence.

We also examined the occupancy of core histone H3 in WT and mutants in ChIP analysis. Relative H3 occupancy was slightly decreased in *atino80-5* and *m56-1*, but was clearly decreased (fold change > 1.5 and P-value < 0.05) in most examined regions of *PIN1* in the *atino80-5 m56-1* triple mutant when compared to WT (**Figure 2D**), which is consistent with the observed *PIN1* transcriptional change.

AtINO80 can regulate the local enrichment peak of histone variant H2A.Z within chromatin region of *FLC*, a key flowering suppressor gene (Zhang et al., 2015). Next, we analyzed the enrichment of H2A.Z relative to H3 (H2A.Z/H3) in *PIN1*. The H2A.Z/H3 peak was found near the 5′-end of *PIN1* in WT, which is largely maintained also in all the mutants (**Supplementary Figure 3**). The *atino80-5* mutant showed a reduction of H2A.Z/H3 but this reduction is compromised in *atino80-5 m56-1*, suggests that the H2A.Z dynamics is not associated with the synergistic effect of *atino80-5* and *m56-1* on the transcriptional up-regulation of *PIN1* in the *atino80-5 m56-1* triple mutant.

The Triple Mutant *atino80-5 m56-1* Exhibits Severe Root Growth Inhibition

Compared with *atino80-5* and *m56-1*, the triple mutant *atino80-5 m56-1* also displayed an additive short-root phenotype (**Figure 3A**). We measured the primary root elongation of vertically grown seedlings. The root length of *m56-1* became significantly shorter than that of WT from 8 day-after-germination (DAG), and that of *atino80-5* mutant became significantly shorter than WT from 10 DAG, which are consistent with our previous studies (Zhu et al., 2006; Zhang et al., 2015). Remarkably, as early as from 4 DAG, the triple mutant has already shown an obvious inhibition of root elongation and the synergistic effect between *atino80-5* and *m56-1* became evident along the time course of root growth analysis (**Figure 3B**).

We observed and compared the root tips through DIC microscopy (**Figure 3C**). At 6 DAG, although the root length of *atino80-5* and *m56-1* were comparable to WT, their meristem size was smaller than that of WT, and again, we observed a much smaller meristem size in the triple mutant roots. At 10 DAG, only the meristem in WT sustained the original size, whereas the corresponding size in all mutants gradually decreased when compared with their younger state. Among them, the change in the triple mutant was most severe.

Skotomorphogenesis Is Epistatic to *AtINO80* and/or *NRP1/2* Depletion

Dark treatment (skotomorphogenesis) can cause a decrease in both *PIN1* transcription level and the shoot-to-root polar auxin transport in hypocotyl, resulting in auxin depletion in the RAM as well as the consequent reduced meristem size (Sassi et al., 2012). The skotomorphogenesis-associated mechanism seems to be compatible with the observed phenotype in triple mutant, thus prompting us to examine the mutants in dark treatment.

Under dark growth conditions, the hypocotyls of all the mutants elongated as those of WT (**Supplementary Figure 4A**). Moreover, dark treatment caused similar thinner roots and much smaller RAM in all the examined roots (**Supplementary Figure 4B**). These findings indicate that skotomorphogenesis is epistatic to *AtINO80* and/or *NRP1/2* depletion. Moreover, transcriptional analysis by using RNA extracted from hypocotyls revealed that *PIN1* transcription level remained at a basal level in all the hypocotyls grown in dark. After light exposure, *PIN1* was potently transcriptionally activated in hypocotyls in the triple mutant *atino80-5 m56-1* (**Supplementary Figure 4C**), consistent with the synergistic

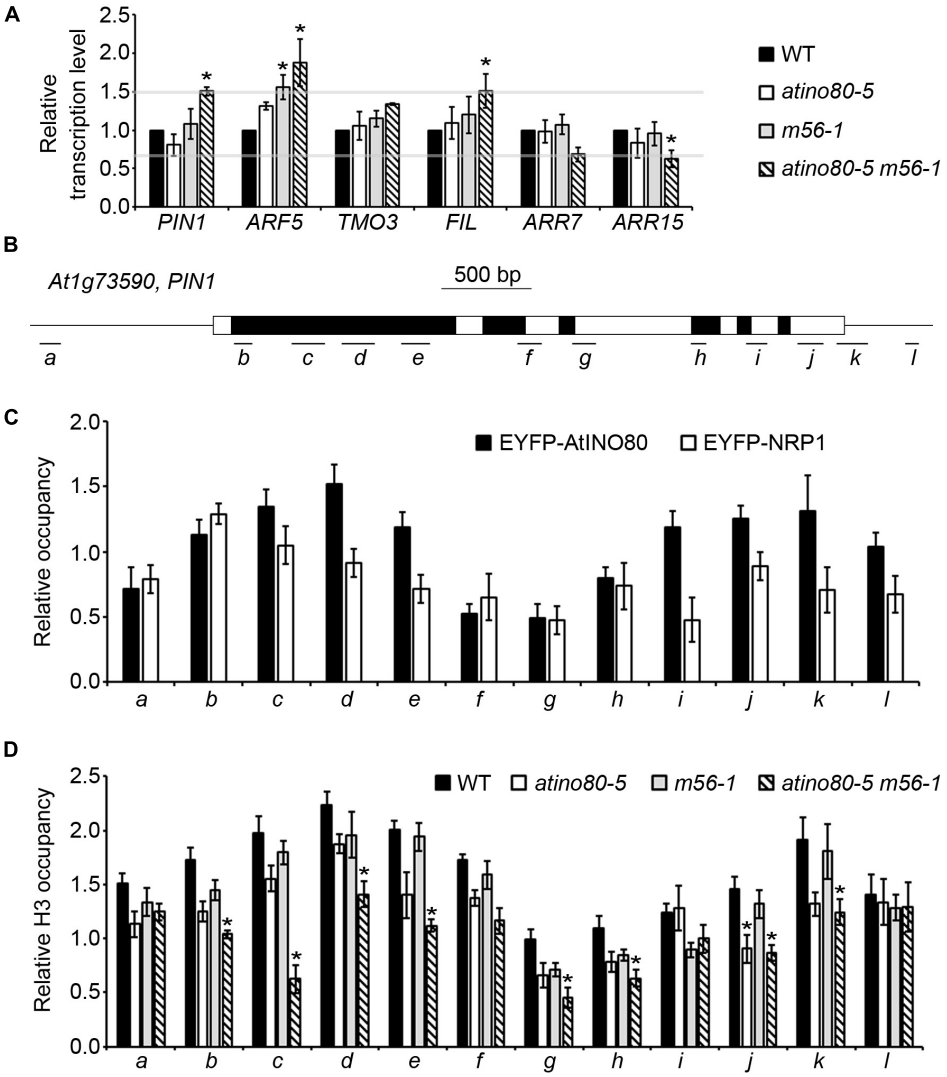

FIGURE 2 | AtINO80 and NRP1/2 synergistically regulate *PIN1* transcription levels in inflorescences. **(A)** Relative transcription level of auxin-related genes in isolated inflorescences (>10 inflorescences as one replicate). *ACT2* was used as a reference gene. Relative values were further referenced to that of WT (set as 1). Mean values are shown with error bars from three independent replicates. Asterisks indicate statistically significant differences ($P < 0.05$, *t*-test) and fold change > 1.5 in mutants when compared with WT. **(B)** Schematic representation of *PIN1* gene structure. Black boxes represent exons; white boxes represent untranslated regions and introns; lines represent the promoter and terminator; letter-labeled bars represent regions amplified by the primer pairs that correspond to the letters on the *x*-axis of the underneath graphs. **(C)** Relative occupancy of EYFP-AtINO80 and EYFP-NRP1 in *PIN1* gene regions are revealed by ChIP using GFP antibody. Inflorescences of transgenic plants were collected for the ChIP analysis. *ACT2* was used as a reference gene. Mean values from three independent experiments are shown with error bars. **(D)** Relative occupancy of H3 in *PIN1* gene regions. Inflorescences of WT, *atino80-5*, *m56-1* and triple mutant were used for the ChIP analysis. *ACT2* was used as a reference gene. Mean values from three independent experiments are shown with error bars. Asterisks indicate statistically significant differences ($P < 0.05$, *t*-test) and fold change > 1.5 in mutants when compared with WT.

role of AtINO80 and NRP1/2 in *PIN1* transcriptional repression.

Auxin Pathway Is Transcriptionally Affected in the *atino80-5 m56-1* Mutant Root Tips

We further analyzed the transcription levels of several well-studied genes involved in RAM organization. They include: *WUSCHEL-RELATED HOMEOBOX 5* (*WOX5*), *SCR*, *SHR*, *PIN1*, *PIN2*, *PLT1* and *PLT2*. *WOX5* is a homeobox gene specifically expressed in quiescent center (QC) in RAM identity (Kong et al., 2015). *PIN2* encodes another PIN-family member which plays a root-specific role of auxin transport (Luschnig et al., 1998). Notably, the transcriptional levels of *WOX5*, *PIN1* and *PLT1/2* genes were synergistically and significantly up-regulated in the *atino80-5 m56-1* triple mutant (fold change > 1.5) (**Figure 4**), suggesting that auxin pathway also undergoes a transcriptional mis-regulation in the triple mutant roots.

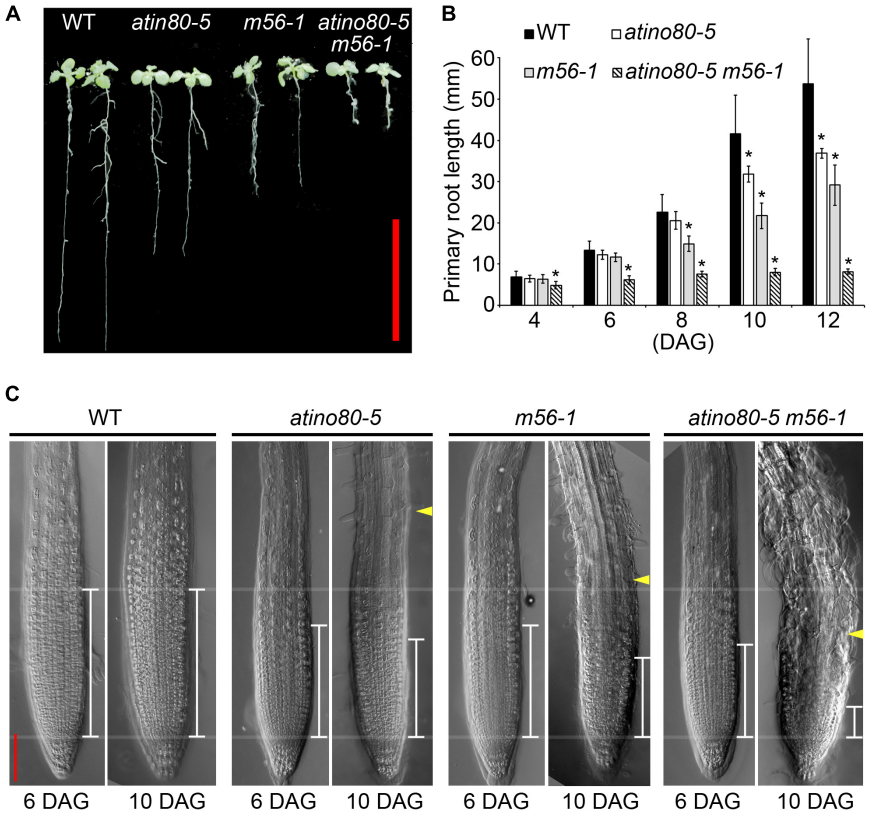

FIGURE 3 | The short-root phenotype in triple mutants. **(A)** Primary roots in WT, *atino80-5*, *m56-1* and triple mutant at 12 DAG (days after germination). Bar = 20 mm. **(B)** Comparison of the primary root elongation in WT and mutants from 4 DAG to 12 DAG. Asterisks indicate statistically significant differences between the WT and mutants ($P < 0.05$, *t*-test). **(C)** Differential interference contrast (DIC) images taken on roots at 6 DAG and 10 DAG. The white scales mark the meristems, in which cells do not enlarge as revealed by DIC. The yellow arrowheads mark the root hair protrusion. Red bar = 100 mm.

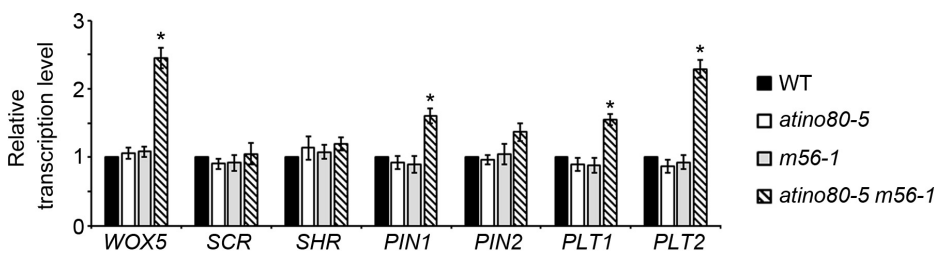

FIGURE 4 | Transcription analysis of RAM-related genes in roots. Relative transcription level of RAM-related genes using roots at 10 DAG. *ACT2* was used as a reference gene. Relative values were further referenced to that of WT (set as 1). Mean values are shown with error bars from three independent experiments. Asterisks indicate statistically significant differences ($P < 0.05$, *t*-test) and fold change > 1.5 in mutants when compared with WT.

Chromatin Immuno-Precipitation analysis by using roots as material was performed to examine whether the recruitment of AtINO80 and NRP1 in *PIN1* gene is consistent in different organs. Since transcription level of *NRP1* is lower than that of *NRP2* in Arabidopsis root (**Supplementary Figure 5**), we also introduced a transgenic plant expressing FLAG-NRP2 and included root-specific *PIN2* gene in the same ChIP analysis. The recruitments of EYFP-AtINO80 and EYFP-NRP1 in *PIN1* chromatin regions in roots was observed (**Supplementary Figures 6A,B**), with a pattern largely comparable to that previously described in inflorescences (**Figure 2C**), and the distribution pattern of FLAG-NRP2 was closely similar to that of EYFP-NRP1. In contrast, no obvious peaks of these proteins were found in *PIN2* chromatin regions (**Supplementary Figures 7A,B**). The pattern of relative H3 occupancies in *PIN1* was similar in roots with those in inflorescences (**Supplementary Figure 6C**). Meanwhile, reduction of relative H3 occupancy (fold change > 1.5 and *P*-value < 0.05) was found in some regions near the 5′-end of *PIN2* in *atino80-5 m56-1* triple mutant when compared to WT (**Supplementary Figure 7C**).

We also introgressed several fluorescent reporters including *WOX5:GFP* (Blilou et al., 2005), *pPIN1:PIN1-GFP* (Benková et al., 2003) and *DR5rev:GFP* (Friml et al., 2003) into each mutant background and observed their expression in root tips. A slightly stronger GFP signal of *WOX5:GFP* and *pPIN1:PIN1-GFP* were detected in the QC and steles in triple mutant, respectively (**Figure 5**, upper and middle panels). These findings are consistent with the above transcription analysis, and at the same time, also exclude the possibility that the severe short-root phenotype of triple mutant *atino80-5 m56-1* may be caused by the depletion of QC, which is crucial for the maintenance of stem cell niche (van den Berg et al., 1997).

In a good proportion of examined triple mutant roots (3 out of 10 samples), ectopic GFP signal of *WOX5:GFP* reporter was detected in the presumptive position of columella stem cells (**Figure 5**, upper panel). To verify the function of the columella cells, we also examined the root tips with Lugol solution and found the WT-like accumulation of starches in the columella cell layers in all the mutants (each $n > 10$) (**Supplementary Figure 8**), indicating that the differentiation of columella cells was not significantly impaired in the absence of AtINO80 or NRP1/2.

Fluorescent signal of *DR5rev:GFP* is located in QC/columella cells and enriched on the acropetal side as a polar gradient in WT. This gradient pattern was little affected in *atino80-5* and *m56-1* root tips, but was moderately interrupted in the columella cell layers in the *atino80-5 m56-1* triple mutant (**Figure 5**, lower panel), indicative of a disturbed auxin polar distribution.

Triple Mutant *atino80-5 m56-1* Is More Sensitive to NPA Treatment

To get more insight into the auxin transport in the triple mutant root, we transferred 4-day-old vertically grown seedlings to the culture medium containing different concentration of *N*-naphthylphthalamic acid (NPA), a synthetic inhibitor of auxin transport. The presence of NPA inhibited the root elongation, and this inhibitory effect is NPA-concentration dependent (**Supplementary Figure 9**). We observed the inhibitory effect at different concentrations of NPA on RAM (**Supplementary Figure 10**). Under NPA treatment at high dosage (5 μM), the WT RAM was not significantly changed even when the root length has been strongly suppressed. In comparison, the RAM structure of *atino80-5* and *m56-1* were obviously altered: the root hairs were much closer to the tips, a defect largely similar to that observed in the *atino80-5 m56-1* triple mutant under untreated conditions. Notably, although treated with a low concentration of NPA (1 μM), the triple mutant root meristem displayed already an unusual expansion, which is accompanied by a quick differentiation of epidermal cells into root hairs (**Supplementary Figure 10**). Taken together, our observations indicate that the triple mutant roots are more sensitive to exogenous NPA treatment, providing additional evidences for its defects in maintaining functional auxin distribution in RAM.

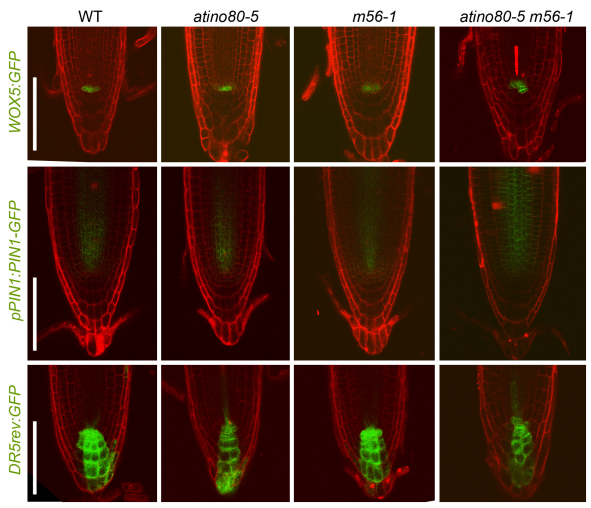

FIGURE 5 | Expression patterns of several fluorescent reporters are affected in triple mutant. Expression patterns of *WOX5:GFP* (upper panel), *pPIN1:PIN1-GFP* (middle panel) and *DR5rev:GFP* (lower panel) in WT, *atino80-5*, *m56-1* and triple mutant at 6 DAG. PI staining was simultaneously used to label cell walls in root tips. Bar = 100 μm.

AtINO80 and NRP1/2 Synergistically Prevent Programmed Cell Death and γ-H2A.X Loci Accumulation in Root Tips

It has been reported that root stem cells and their early descendants can be selectively killed by genotoxic treatment causing DSB (Fulcher and Sablowski, 2009). PI staining can enter and mark dead cells because of the interrupted membrane integrity. We noticed that the *atino80-5 m56-1* triple mutant roots have accumulated PI-marked dead cells, which were barely found in WT or the *atino80-5* and *m56-1* mutant root tips (**Figure 6A**, upper panel). This observation suggests that AtINO80 and NRP1/2 synergistically prevent programmed cell death in root tips. H2A.X phosphorylation (γ-H2A.X) at the DNA break site constitutes one of the earliest events in the DNA repair process (Friesner et al., 2005). Although our previous study showed that the whole protein extracts from the triple mutant plants grown in the normal conditions did not show an obvious γ-H2A.X accumulation in Western blot analysis (Zhou et al., 2016), our immunostaining analysis detected weak but significantly visible γ-H2A.X loci in the root tips of the *atino80-5 m56-1* triple mutant (**Figure 6A**, lower panel).

Double-strand break can also induce the early onset of endoreduplication in cortical cells, which is frequently associated with cell enlargement (Adachi et al., 2011). A plot of cortical cell area against the distance from QC revealed that the cortical cell expansion were more pronounced in the triple mutant than in *atino80-5* and *m56-1* when compared to WT (**Figure 6B**). Furthermore, we examined transcription levels of DNA damage-sensory genes *PARP1/2* and DNA repair genes *RAD51/54*. All of these tested genes were synergistically up-regulated in the triple mutant roots (**Figure 6C**). Collectively, our data indicate that *AtINO80* and *NRP1/2* coordinate to maintain chromatin stability to prevent DNA damage for genome integrity.

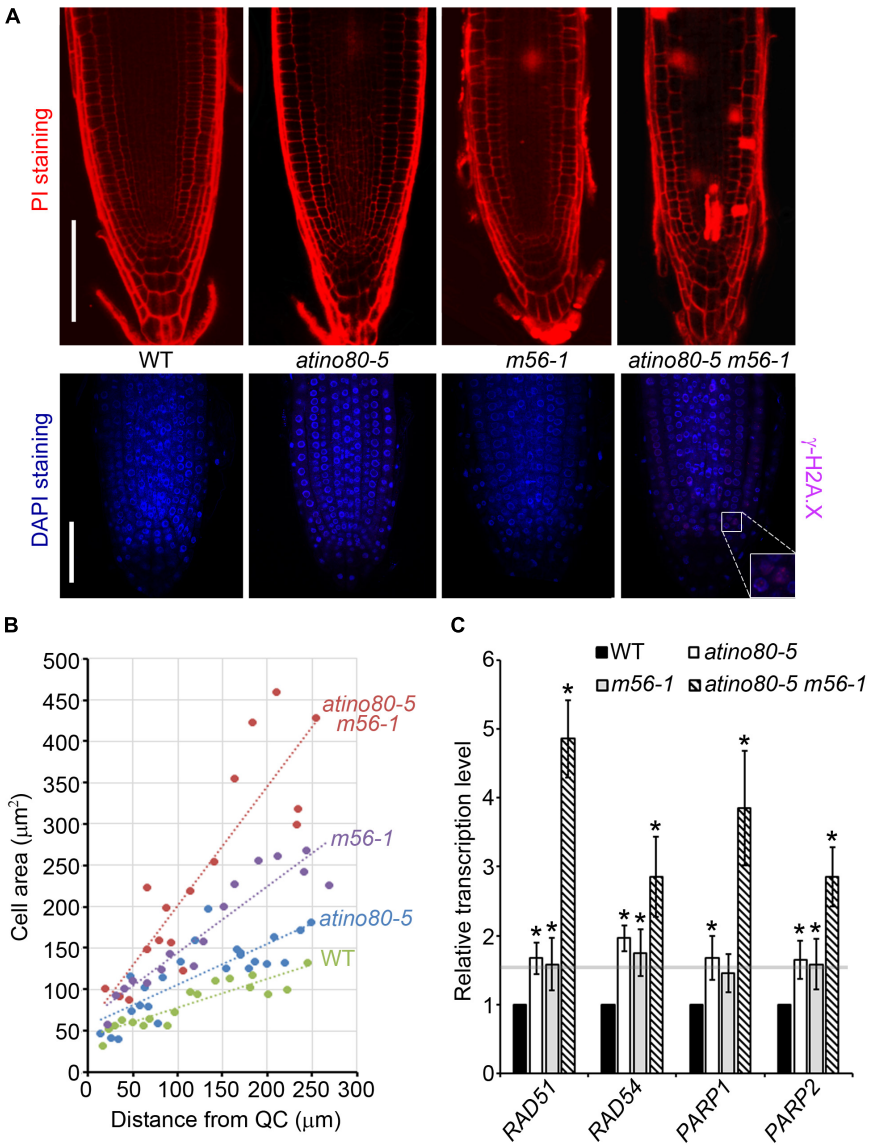

FIGURE 6 | Genome instability in triple mutant. **(A)** (Upper panel) PI-stained root tips of WT, *atino80-5*, *m56-1* and triple mutant at 6 DAG. Bar = 100 μm. (Lower panel) Whole-mount root immunofluorescence staining analysis at 6 DAG. The γ-H2A.X signal detected using specific antibody is shown in pink and DNA staining by DAPI is shown in blue. Bar = 50 μm. **(B)** The increase of the cortical cell area (mm^2) along with distance from QC cells. Regression lines are included. **(C)** Relative transcription level of DNA repair genes using roots at 6 DAG. *ACT2* was used as a reference gene. Relative values were further referenced to that of WT (set as 1). Mean values are shown with error bars from three independent experiments. Asterisks indicate statistically significant differences ($P < 0.05$, *t*-test) and fold change > 1.5 in mutants when compared with WT.

DISCUSSION

On the basis of our previous study, we expanded our genetic analysis of *AtINO80* and *NRP1/2* in plant growth and development. Here, we report on the abnormal inflorescence and severe short-root phenotypes of the triple mutant *atino80-5 m56-1*. *AtINO80* and *NRP1/2* act synergistically to maintain the proper size of IM and to control the regular positioning pattern of floral primordia. Meanwhile, both factors act together to sustain the stem cell niche as well as functional auxin distribution in RAM. In particular, the triple mutant *atino80-5 m56-1* accumulates PI-marked dead cells and shows cortical cell enlargement in root tips, which are accompanied by the transcriptional activation of key DNA damage-sensory and damage-repair genes. These findings demonstrate that *AtINO80* and *NRP1/2* exhibit complex genetic interactions in the regulation of IM and RAM functions during plant development.

Within PZ of SAM, lateral organ initiation is determined by auxin maxima. The abnormal positioning of siliques and the disordered IM observed in *atino80-5 m56-1* imply a perturbation of auxin maxima in the mutant shoot apex. In agreement with

this assumption, our RT-PCR analysis revealed an increased expression level of the auxin transporter gene *PIN1* in the *atino80-5 m56-1* triple mutant as compared to WT or to the single mutant *atino80-5* or to the double mutant *m56-1*. Although the underlying mechanism of auxin-triggered lateral organogenesis has been considered to be similar in the vegetative SAM and the reproductive IM (Wang and Jiao, 2018), the triple mutant *atino80-5 m56-1* did not show obvious lateral organ initiation defects at vegetative growth stage. One possible explanation to the absence of SAM defects but the presence of IM defects in the triple mutant is that IM may be more sensitive in auxin response than does SAM. In support of this idea, the single mutants *pin1* or *arf5* grows naked stalks without flowers but can still generate leaves (Przemeck et al., 1996). The vegetative SAM failed to form lateral leaf primordia only when *PIN1* and *ARF5* are simultaneously knocked out in the *pin1 arf5* double mutant (Schuetz et al., 2008). Alternatively, other possible explanation exists that the functional synergy of AtINO80 and NRP1/2 may be further integrated or redundant with other specific yet unknown pathways (or factors) in the organogenesis of vegetative SAM but not reproductive IM.

Both our RT-PCR and fluorescent reporter gene analyses further demonstrated up-regulation of *PIN1* and perturbed auxin maxima in the *atino80-5 m56-1* mutant roots. As the key factor in auxin transcription response, ARF5 directly interacts with the upstream regulatory region of *PIN1* and regulates the gene expression, forming an auxin gradient-trigged positive feedback in SAM self-organization (Krogan et al., 2016). Similar feedback mechanism is also used in RAM by PLT transcription factors (Blilou et al., 2005; Xu et al., 2006). In addition, more recent studies have revealed additional sequence-specific transcription factors targeting the *PIN1* gene, including MADS-domain transcription factor AGAMOUS-like 14 (AGL14) (Garay-Arroyo et al., 2013) and PIN2 PROMOTER BINDING PROTEIN 1 (PPP1), an evolutionary conserved plant-specific DNA binding protein (Benjamins et al., 2016). Currently, there is no evidence to support that AtINO80 and NRP1/2 possess any sequence-specific DNA-binding ability. Previously, NRP1 has been shown to interact with the MYB transcription factor WEREWOLF (WER) and to enrich at the WER-downstream gene *GLABRA2* (*GL2*), which encodes a homeodomain-leucine zipper transcription factor critical for root hair patterning (Zhu et al., 2017). Intriguingly, up-regulations of *ARF5* and *PLT1/2* were detected in *atino80-5 m56-1*, and enrichments of EYFP-AtINO80 and EYFP-NRP1 were observed at the *PIN1* locus. Whether AtINO80 and NRP1/2 are recruited to the *PIN1* locus through physical interaction with a specific transcription factor remains to be examined in the future.

BRAHMA (BRM), a SWI/SNF-family chromatin-remodeling factor (Clapier and Cairns, 2009), has been previously shown to play a role in Arabidopsis root development (Yang et al., 2015). Loss of function of *BRM* affected auxin distribution by reducing the transcription levels of several *PIN* genes as well as *PLT* genes. ChIP experiments showed that BRM can directly target the chromatin regions of several *PIN* genes including *PIN1* and activate their expression. BRM also antagonizes the function of Polycomb group (PcG) proteins, and down-regulates the repressive H3K27me3 chromatin mark within target genes (Yang et al., 2015). Here, our study on the synergy of AtINO80 and NRP1/2 provides evidence for the participation of chromatin-related factors other than BRM in epigenetic regulation of *PIN1*. Since the up-regulation of *PIN1* transcription in *atino80-5 m56-1* is opposite to the down-regulation of *PIN1* in the *brm* mutant, future genetic analysis will be needed to examine their functional crosstalk and epistasis, which is important for an increased comprehensive understanding of regulatory mechanisms in local transcription regulation implicated in auxin response.

Under normal growth condition, QC in the *atino80-5 m56-1* developing root tips at early stage is relatively intact, and starch normally accumulates in the columella cells. The interrupted auxin distribution could not fully explain the observed decay of RAM in the triple mutant. *AtINO80* and *NRP1/2* have been independently reported to participate in the maintenance of plant genome stability (Zhu et al., 2006; Zhang et al., 2015). Their genetic interplay has been analyzed in somatic HR and telomere length (Zhou et al., 2016). In this study, severe DNA damage was observed to accumulate in cells at the root tips of *atino80-5 m56-1*, as evidenced by the accumulation of γ-H2A.X loci and the activation of DNA damage sensory and repair genes. The PI-labeled dead cells and the accumulative cortical cell enlargement strongly point to the chromatin instability caused by *AtINO80* and *NRP1/2* depletion. It is reasonable to speculate that such chromatin instability contributes to the progressive exhaustion of normal stem cell niche and the aggravation of organ growth defects.

Our previous studies have examined the genetic interactions of *NRP1/2* with *FAS2* (Kaya et al., 2001), which encodes the second large subunit of the Arabidopsis histone chaperone Chromatin Assembly Factor-1 (CAF-1) complex (Gao et al., 2012; Ma et al., 2018). In the triple mutant *m56-1 fas2-4*, the lack of *NRP1/2* function aggravated the chromatin instability caused by the *FAS2* deletion and leads to disorganized stem cell niche, loss of stem cell identity, and constrained cell division in roots (Ma et al., 2018). We noticed some commonalities between *m56-1 fas2-4* and *atino80-5 m56-1*, such as combined gene function synergy in maintaining chromatin integrity and stability as well as growth of primary roots. Our ChIP analysis unraveled a decrease of histone H3 occupancy at *PIN1*, which is in line with the *PIN1* transcriptional activation, in the *atino80-5 m56-1* mutant. This observation may also be considered as a window reflecting defects of chromatin organization in the mutant. Previously, studies by using fluorescence *in situ* hybridization (FISH) and histone fusions with a fluorescent protein have demonstrated that histone exchange is dynamic and extensive chromatin reorganization occurs during cell differentiation in Arabidopsis roots (Costa and Shaw, 2006; Otero et al., 2016). CAF-1 plays a key function in chaperoning histone H3 during DNA replication, and consistently the *fas1* or *fas2* mutant exhibits severe defects in chromatin organization and function. In comparison, simultaneous loss of the H2A/H2B-type

histone chaperones NRP1/2 and the ATP-dependent chromatin-remodeling factor INO80 in the *atino80-5 m56-1* mutant may also impact global chromatin organization and genome function.

During last few years, techniques in Arabidopsis have been developed for isolation of nuclei tagged in specific cell types (INTACT) by affinity purification based on expression of a biotinylated nuclear envelope protein in transgenic plants (Deal and Henikoff, 2011), and for genome-wide profiling of chromatin accessibility based on DNaseI digestion (DNase-seq; Zhang et al., 2012) or Tn5 transposase cleavage (ATAC-seq; Lu et al., 2017). ATAC-seq has been successfully coupled with INTACT to establish accessible chromatin landscape in root cells expressing a tag construct driven by the constitutive CaMV 35S promoter (Tannenbaum et al., 2018). In human cells, a nicking enzyme assisted sequencing (NicE-seq) has been reported for high-resolution open chromatin profiling on both native and formaldehyde-fixed cells (Ponnaluri et al., 2017). Future exploration of these different technologies and their application to our different mutants will provide invaluable insight about mechanisms of histone chaperones and chromatin-remodeling factors in regulating chromatin organization and root cell proliferation/differentiation.

AUTHOR CONTRIBUTIONS

HK performed the laboratory work and data analysis. JM set up the genetic introgression of transgenic markers and provided technical support in microscopy analysis. DW participated in the microscopy analysis. W-HS designed the experiments and revised the manuscript. YZ designed the experiments, performed the laboratory work and data analysis, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00115/full#supplementary-material

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frontiers in Plant Science

ORIGINAL RESEARCH
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The *Arabidopsis* Sin3-HDAC Complex Facilitates Temporal Histone Deacetylation at the *CCA1* and *PRR9* Loci for Robust Circadian Oscillation

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The circadian clock synchronizes endogenous rhythmic processes with environmental cycles and maximizes plant fitness. Multiple regulatory layers shape circadian oscillation, and chromatin modification is emerging as an important scheme for precise circadian waveforms. Here, we report the role of an evolutionarily conserved Sin3-histone deacetylase complex (HDAC) in circadian oscillation in *Arabidopsis*. *SAP30 FUNCTION-RELATED 1* (*AFR1*) and *AFR2*, which are key components of Sin3-HDAC complex, are circadianly-regulated and possibly facilitate the temporal formation of the *Arabidopsis* Sin3-HDAC complex at dusk. The evening-expressed AFR proteins bind directly to the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *PSEUDO-RESPONSE REGULATOR 9* (*PRR9*) promoters and catalyze histone 3 (H3) deacetylation at the cognate regions to repress expression, allowing the declining phase of their expression at dusk. In support, the *CCA1* and *PRR9* genes were de-repressed around dusk in the *afr1-1afr2-1* double mutant. These findings indicate that periodic histone deacetylation at the morning genes by the Sin3-HDAC complex contributes to robust circadian maintenance in higher plants.

Keywords: circadian clock, chromatin modification, histone deacetylase (HDAC), Sin3 histone deacetylase and corepressor complex, CCA1, PRR9

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INTRODUCTION

The circadian clock is an internal time-keeper mechanism that ensures endogenous biological rhythms with a period of approximately 24 h, coinciding with daily environmental cycles. A large fraction of the plant transcriptome is clock-controlled, and thus the clock is globally linked to diverse signaling and metabolic pathways to ensure optimal biological functions at a specific time of day (Covington et al., 2008; Mizuno and Yamashino, 2008; Hsu and Harmer, 2012). Synchronization of the clock with the environment is closely associated with plant growth and fitness (Dodd et al., 2005; Fujiwara et al., 2008; Nusinow et al., 2011; Yoo et al., 2011; Lu et al., 2012; Nagel and Kay, 2012; Haydon et al., 2013; Zhang et al., 2013).

The circadian clock is a highly conserved system in higher eukaryotes. In *Arabidopsis*, the central oscillator is known to consist of an array of transcriptional loops. Two single-MYB

transcription factors, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), establish the central loop by repressing transcription of *TIMING OF CAB EXPRESSION 1* (*TOC1*) that in turn, represses *CCA1* and *LHY* expression (Alabadi et al., 2001; Huang et al., 2012; Pokhilko et al., 2013). The central loop is further regulated by PSEUDO-RESPONSE REGULATORs (PRR5, PRR7, and PRR9) (Nakamichi et al., 2005, 2010; Salome et al., 2010) and the evening complex (EC) consisting of EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRYTHMO/PHYTOCLOCK 1 (LUX/PCL1) (Nusinow et al., 2011; Chow et al., 2012; Herrero et al., 2012). Moreover, the TOC1 protein also plays widespread roles in transcriptionally repressing multiple core clock components, underscoring the biological importance of transcriptional regulation in circadian homeostasis (Gendron et al., 2012; Huang et al., 2012).

Accumulating evidence suggests that circadian oscillation is further shaped by additional regulatory mechanisms (Seo and Mas, 2014). In particular, chromatin modification is an important regulatory scheme underlying precise circadian waveforms (Mas, 2008; Stratmann and Mas, 2008; Kusakina and Dodd, 2012; Nagel and Kay, 2012). Transcript accumulation of core clock components correlates with rhythmic changes in accumulation of histone H3 acetylation (H3ac) in *Arabidopsis* (Hemmes et al., 2012; Malapeira et al., 2012; Song and Noh, 2012). Consistent with the fact that histone acetylation status is dynamically regulated by the antagonistic action of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Kuo and Allis, 1998; Yang and Seto, 2007), temporal association of specific sets of HATs and HDACs occurs at the loci of core clock components to shape rhythmic expression (Hemmes et al., 2012; Malapeira et al., 2012; Song and Noh, 2012). For instance, the midday-expressed HISTONE ACETYLTRANSFERASE OF THE TAFII250 FAMILY 2 (HAF2) protein catalyzes H3ac at the *PRR5* and *LUX* loci to activate expression and is responsible for the rising phase of *PRR5* and *LUX* circadian expression (Lee and Seo, 2018). In addition, the HDA6 and HDA19 proteins form protein complexes together with the TOPLESS (TPL) and PRR proteins, and repress expression of *CCA1* and *LHY* during the daytime (Wang et al., 2013). Despite the importance of diurnal histone acetylation states of core clock genes in stable circadian oscillation, the responsible epigenetic modifiers are yet to be fully characterized.

Histone deacetylase complex often form diverse types of multiprotein co-repressor complexes and play a variety of roles during plant growth and development (Buszewicz et al., 2016; Kim et al., 2016; Hung et al., 2018; Park et al., 2018; Tasset et al., 2018). One well-characterized HDAC complex in eukaryotes is the Sin3-HDAC complex (Alland et al., 2002; Kuzmichev et al., 2002; Silverstein and Ekwall, 2005; Clark et al., 2015). In *Arabidopsis*, the Sin3-HDAC complex participates in photoperiodic flowering through the periodic acetylation of the *FLOWERING LOCUS T* (*FT*) locus (Gu et al., 2013). The Sin3-HDAC complex is activated at the end of the day and is recruited to the *FT* locus by AGAMOUS LIKE 18 (AGL18) in a CONSTANS (CO)-dependent manner under long-day conditions (Gu et al., 2013). In this study, we report that the *Arabidopsis* Sin3-HDAC complex also temporally regulates *CCA1* and *PRR9* expression through catalyzing H3 deacetylation and facilitates the declining phase of their circadian expression during the evening time. These results reveal that temporal association of chromatin modifiers underlies robust rhythmic expression of clock genes and thereby stable circadian oscillation.

RESULTS

Rhythmic Expression of *AFR*s Is Shaped by CCA1

Histone deacetylase complex often form multiprotein co-repressor complexes, as exemplified by the Sin3-HDAC complex that consists of the master scaffold protein Sin3, the Reduced Potassium Dependency 3 (RPD3)-type HDAC, and Sin3-associated structural components, such as SIN3-ASSOCIATED POLYPEPTIDE 18 (SAP18) and SAP30 (Zhang et al., 1997; Laherty et al., 1998; Wu et al., 2000; Scott and Plon, 2003; Song and Galbraith, 2006). The *Arabidopsis* genome contains six Sin3 homologs, SIN3-LIKE 1-6 (SNL1-6), four RPD3 homologs (HDA19, HDA9, HDA7, and HDA6), one SAP18 homolog, and two SAP30 homologs (SAP30 FUNCTION-RELATED 1 (AFR1) and AFR2) (Wu et al., 2000; Murfett et al., 2001; Pandey et al., 2002; Gu et al., 2013).

Notably, AFR1 and AFR2 have been identified as regulators of photoperiodic flowering, which facilitate periodic histone deacetylation at the *FT* locus (Gu et al., 2013). Considering their roles in temporal histone deacetylation, we hypothesized that the *Arabidopsis* Sin3-HDAC complex may also be implicated in circadian control. To examine the possible involvement of the HDAC complex in circadian oscillation, we first checked transcript accumulation of key components of the Sin3-HDAC complex in seedlings entrained under neutral day (ND) conditions. Quantitative real-time RT-PCR (RT-qPCR) analysis revealed that only the *AFR1* and *AFR2* genes are circadianly-regulated (**Figure 1A**), while the other components are not under the control of the circadian clock (**Figure 1B**). The *AFR* genes peaked at dusk (**Figure 1A**), as reported previously (Gu et al., 2013), suggesting that clock-controlled *AFR*s presumably lead to diurnal formation of the HDAC complex.

To explore the circadian component responsible for regulation of the *AFR*s, we conducted analysis of the *cis*-elements present within the *AFR* promoters. AFRs have multiple CCA1-binding sites (CBSs, AAAATCT) and evening elements (EEs, AAATATCT) in the upstream promoters (**Figure 2A**), which are known to be bound by CCA1 and LHY (Wang et al., 1997; Harmer et al., 2000; Michael and McClung, 2003; Nagel et al., 2015). This observation raised the possibility that CCA1 may bind to the *AFR* promoters. To examine this possibility, a chromatin immunoprecipitation (ChIP) assay was performed using plants expressing epitope-tagged CCA1 under its own native promoter (*pCCA1:CCA1-HA-YFP/cca1-1*). Total protein extracts of samples collected at Zeitgeber Time 0 (ZT0) and ZT12 were immunoprecipitated with anti-HA antibody. ChIP-qPCR

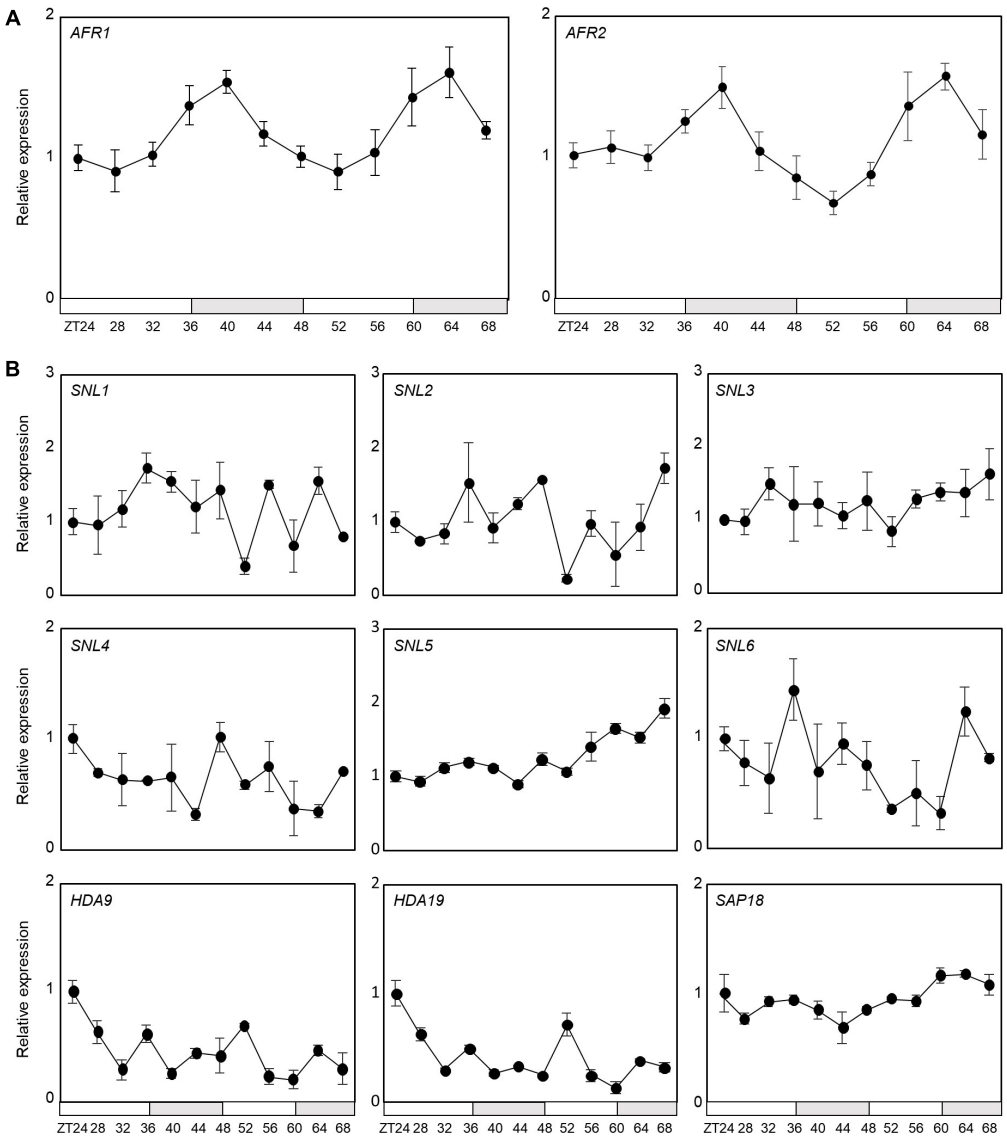

FIGURE 1 | Circadian expression of *AFR1* and *AFR2*. Seedlings grown under neutral day conditions (ND, 12 h light: 12 h dark) for 2 weeks were transferred to continuous light conditions (LL) at Zeitgeber Time 0 (ZT0). Whole seedlings were harvested from ZT24 to ZT68 to analyze transcript accumulation. Transcript levels were determined by quantitative real-time RT-PCR (RT-qPCR). Gene expression values were normalized to *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1* (*eIF4A*) expression. Three independent biological replicates were averaged. Bars represent the standard error of the mean. The white and gray boxes indicate the subjective day and night, respectively. **(A)** Expression of *AFR1* and *AFR2*. **(B)** Expression of other components of Sin3-HDAC.

analysis showed that the proximal regions of transcriptional start sites (TSSs) on the *AFR* promoters containing CBS and/or EE elements were enriched following ChIP (**Figure 2B**). Binding of CCA1 to the *AFR* promoter was specifically observed at dawn, but not at dusk (**Figure 2B**), shaping circadian expression of the *AFR*s.

To support *AFR* regulation by the transcriptional regulator CCA1, we analyzed *AFR* expression in *cca1-2* and *cca1-1lhy-21* mutant seedlings grown under ND conditions. RT-qPCR analysis showed that the peak phase of *AFR* expression was delayed in *cca1-2* and *cca1-1lhy-21*, and higher expression of *AFR*s around the end of night was observed in the *cca1-2* and *cca1-1lhy-21* mutants compared with wild-type (**Figure 3A** and **Supplementary Figure S1**). In contrast, *AFR* expression was dramatically reduced in *CCA1*-overexpressing lines (**Figure 3B**). To further support the repressive role of CCA1 in *AFR* expression, we performed transient expression assays using *Arabidopsis* mesophyll protoplasts. The GUS reporter plasmids and effector plasmids harboring 35S:*CCA1-GFP* fusion were co-transfected into protoplasts (**Supplementary Figure S2**). Co-transfection of a reporter construct with 35S:*CCA1-GFP* resulted in lower GUS activity than the control plasmid (**Supplementary Figure S2**). These results indicate that CCA1 shapes *AFR*

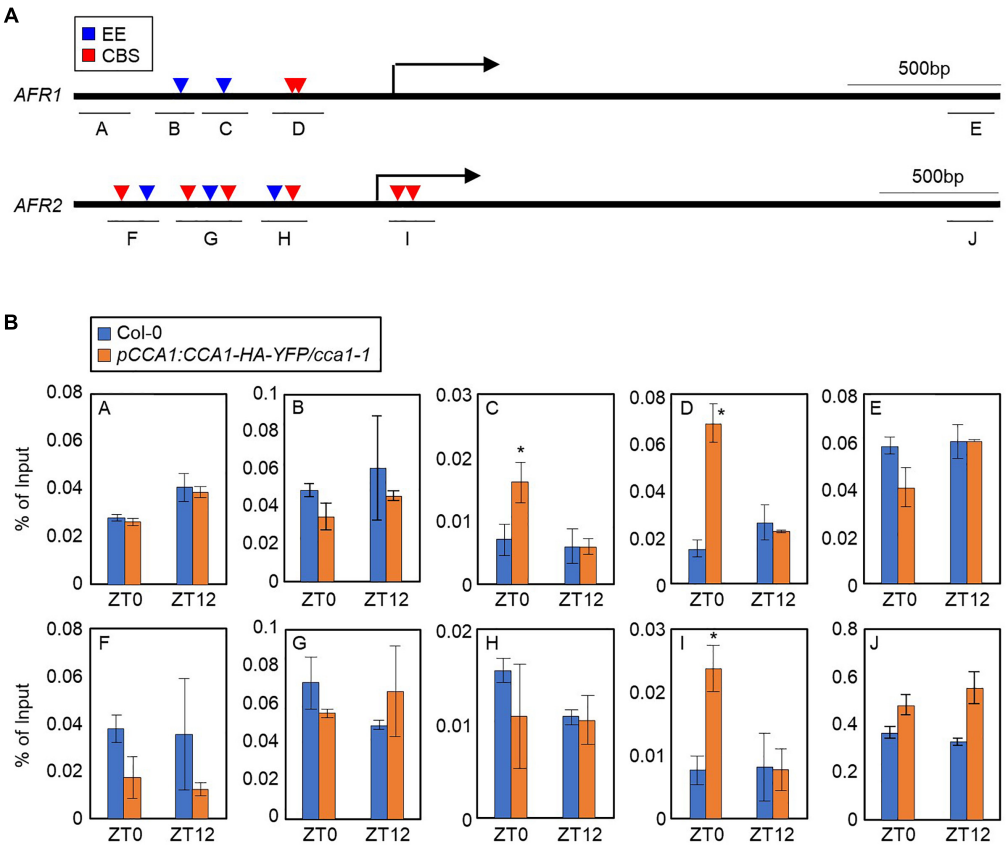

FIGURE 2 | Binding of CCA1 to *AFR* promoters. **(A)** Promoter analysis of the *AFR1* and *AFR2* genes. Underbars indicate the regions amplified by PCR after chromatin immunoprecipitation (ChIP). CBS, CCA1-binding site; EE, evening element. **(B)** Binding of CCA1 to the *AFR* loci. Two-week-old plants entrained with ND cycles were subjected to LL. Plants were harvested at ZT0 and ZT12 for ChIP analysis with anti-HA antibody. Three independent biological replicates were averaged, and statistically significant differences (Student's *t*-test, $*P < 0.05$) are indicated by asterisks. Bars indicate the standard error of the mean.

expression and enables peak expression particularly during the evening time.

AFRs Are Involved in Circadian Oscillation

Since the AFR proteins are core Sin3-HDAC components regulated by the circadian clock, we further investigated the role of AFRs in circadian oscillation. We employed the *afr1-1afr2-1* double mutant and examined endogenous circadian behavior. RT-qPCR analysis showed that circadian output genes, *COLD CIRCADIAN RHYTHM RNA BINDING 2* (*CCR2*) and *CHLOROPHYLL A/B-BINDING PROTEIN 2* (*CAB2*), were altered in *afr1-1afr2-1* mutant seedlings compared with wild-type (**Figure 4A**). We also checked several core circadian oscillator genes, including *CCA1* and *TOC1*. Again, two genes were also differentially expressed in the *afr1-1afr2-1* mutant compared with wild-type (**Figure 4B**). In particular, the morning gene expression was delayed in *afr1-1afr2-1*. The alteration patterns of the circadian genes were dissimilar in *afr1-1afr2-1* mutant. This might be due to extensive circadian feedback network that balances 24 h clock oscillation, as observed in several previous studies (Somers et al., 2004; Ding et al., 2007; Hanano et al., 2008; Li et al., 2011).

AFRs are components of the *Arabidopsis* Sin3-HDAC complex (Gu et al., 2013). To provide further support that AFR function in circadian oscillation depends on formation of the Sin3-HDAC complex, we obtained a genetic mutant of *SAP18* and analyzed circadian oscillation. Since *SAP18* is the only member of the Sin3-HDAC components that exists as a single copy in the *Arabidopsis* genome (Zhang et al., 1997; Ahringer, 2000), we suspected that the *sap18-2* mutant could be used to reflect the roles of the *Arabidopsis* Sin3-HDAC complex. Remarkably, the *sap18-2* mutant exhibited altered circadian expression of *CCA1* and *CCR2* (**Figure 4C** and **Supplementary Figure S3**), similar to *afr1-1afr2-1*, indicating that the *Arabidopsis* Sin3-HDAC complex controls circadian oscillation.

AFRs Bind to the *CCA1* and *PRR9* Loci and Catalyze H3 Deacetylation at Dusk

AFRs most likely regulate the pace of the circadian clock possibly in association with the central oscillator(s). To identify

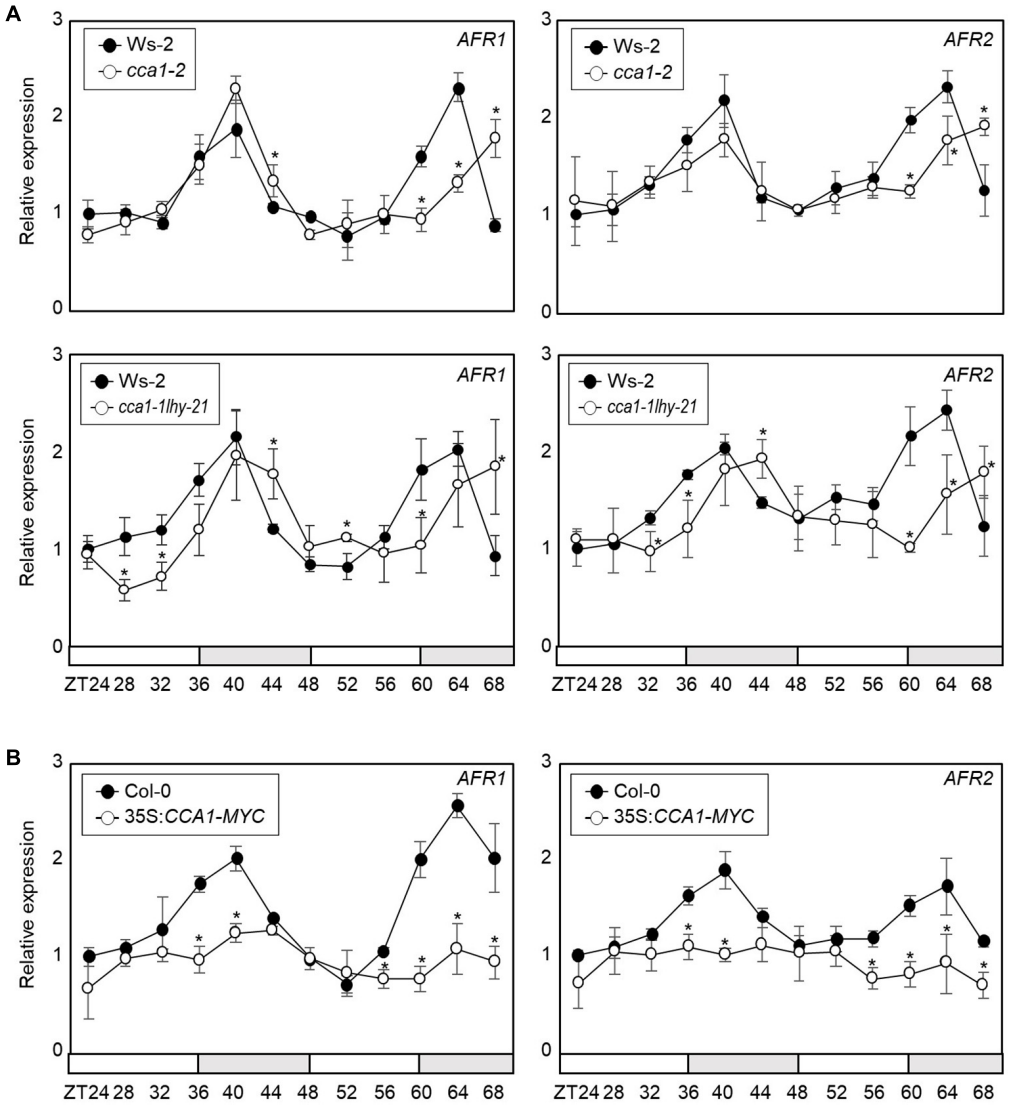

FIGURE 3 | Circadian expression of *AFR*s in *CCA1*-misexpressing plants. In **(A,B)**, seedlings grown under ND conditions for 2 weeks were transferred to LL conditions at ZT0. Whole seedlings were harvested from ZT24 to ZT68 to analyze transcript accumulation. Transcript levels were determined by RT-qPCR. Gene expression values were normalized to *eIF4A* expression. Three independent biological replicates were averaged, and statistically significant differences (Student's *t*-test, $^*P < 0.05$) are indicated by asterisks. Bars represent the standard error of the mean. The white and gray boxes indicate the subjective day and night, respectively. **(A)** Expression of *AFR*s in the *cca1-2* and *cca1-1lhy-21* mutant. **(B)** Expression of *AFR*s in 35S:*CCA1-MYC* transgenic plants.

which circadian components are regulated by the AFRs, we conducted ChIP assays using 35S:*AFR1-MYC* and 35S:*AFR2-MYC* transgenic plants. Plants were grown under ND conditions and harvested at ZT12, when AFR proteins highly accumulate (Gu et al., 2013). ChIP-qPCR analysis showed that the AFR proteins bind directly to the *CCA1* and *PRR9* loci (**Figures 5A,B**), while the other clock members examined were not targeted by the AFRs (**Supplementary Figure S4**). AFRs were primarily targeted around the TSSs of the *CCA1* and *PRR9* loci, rather than the 3′-regions of gene body (**Figure 5B**), which is consistent with previous observations that chromatin modification of core clock genes primarily occurs around TSSs (Hemmes et al., 2012; Malapeira et al., 2012). In addition, binding of AFRs to the *CCA1* and *PRR* loci was prominent at ZT12 (**Figure 5B**), when peak expression of *AFR*s was observed (**Figure 1A**).

The temporal recruitment of AFRs to the morning gene loci may cause periodic histone deacetylation. We examined H3 acetylation (H3ac) levels, which correlate to transcript accumulation of core clock genes (Hemmes et al., 2012; Malapeira et al., 2012), at the *CCA1* and *PRR9* promoters in wild-type and *afr1-1afr2-1* seedlings. ChIP with anti-H3ac antibody revealed that H3ac levels of the *CCA1* and *PRR9* genes were elevated at ZT0 but reduced at ZT12 in wild-type (**Figure 5C**), as reported previously (Hemmes et al., 2012; Malapeira et al., 2012). However, the decline of H3ac accumulation at ZT12

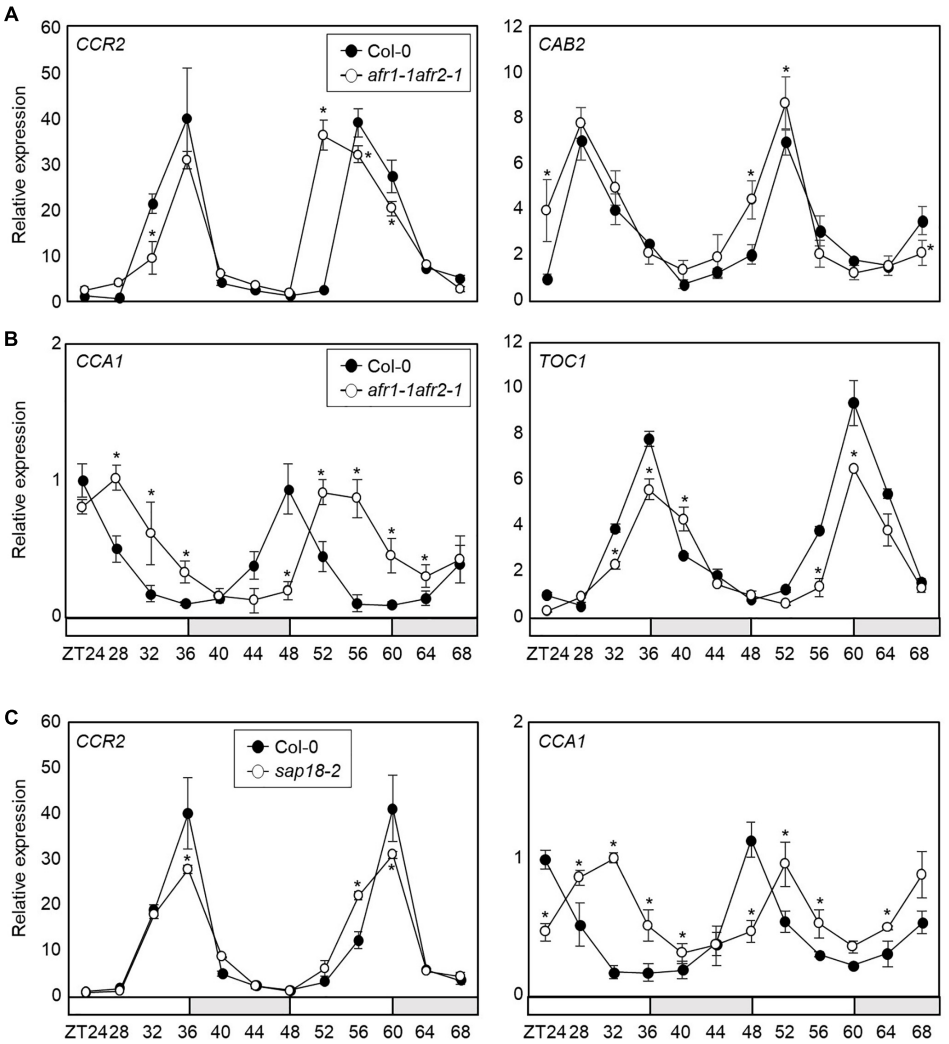

FIGURE 4 | Altered circadian rhythm in the *afr1-1afr2-1* mutant. In **(A–C)**, seedlings grown under ND were transferred to LL at ZT0. Whole seedlings were harvested from ZT24 to ZT68 to analyze transcript accumulation. Gene expression values were normalized to *eIF4A* expression and represented as *n*-fold compared to the value of the wild-type sample at ZT24. Three independent biological replicates were averaged, and statistically significant differences (Student's *t*-test, $^*P < 0.05$) are indicated by asterisks. Bars indicate the standard error of the mean. The white and pale gray boxes indicate the subjective day and night, respectively.
(A) Expression of *CCR2* and *CAB2* in *afr1-1afr2-1*. **(B)** Expression of *CCA1* and *TOC1* in *afr1-1afr2-1*. **(C)** Expression of *CCA1* and *CCR2* in *sap18-2*.

was impaired in the *afr1-1afr2-1* mutant (**Figure 5C**). Increased H3ac levels at the *CCA1* and *PRR9* loci were observed in the *afr1-1afr2-1* mutant, particularly at ZT12 (**Figure 5C**). These results indicate that AFRs mediate histone deacetylation at the morning gene loci to stably downregulate expression during evening time.

The AFR Proteins Are Responsible for the Declining Phases of *CCA1* and *PRR9*

Since the Sin3-HDAC complex catalyzes H3 deacetylation at the *CCA1* and *PRR9* loci, we speculated that circadian expression of the *CCA1* and *PRR9* genes may be shaped by diurnal H3ac accumulation. To test this possibility, we measured *CCA1* and *PRR9* expression in the *afr1-1afr2-1* mutant. In wild-type seedlings, the *CCA1* and *PRR9* genes were highly expressed in the morning, but repressed during the afternoon (**Figures 4B**, **6A**). In contrast, decrease of *CCA1* and *PRR9* expression during afternoon was compromised in the *afr1-1afr2-1* mutant (**Figures 4B**, **6A**). Circadian patterns of *CCA1* and *PRR9* expression were altered in the *afr1-1afr2-1* mutant background, and the increased expression of *CCA1* and *PRR9* was clearly observed at afternoon (**Figures 4B**, **6A**).

To further support the repressive role of AFRs in *CCA1* and *PRR9* expression, we examined the extent of AFR regulation of *CCA1* and *PRR9* transcription activity in *Arabidopsis* mesophyll protoplasts. The GUS reporter plasmids and effector plasmids harboring 35S:*AFR-MYC* fusion constructs were co-transfected into mesophyll protoplasts (**Figure 6B**). Co-transfection of a reporter construct with 35S:*AFR1-MYC*

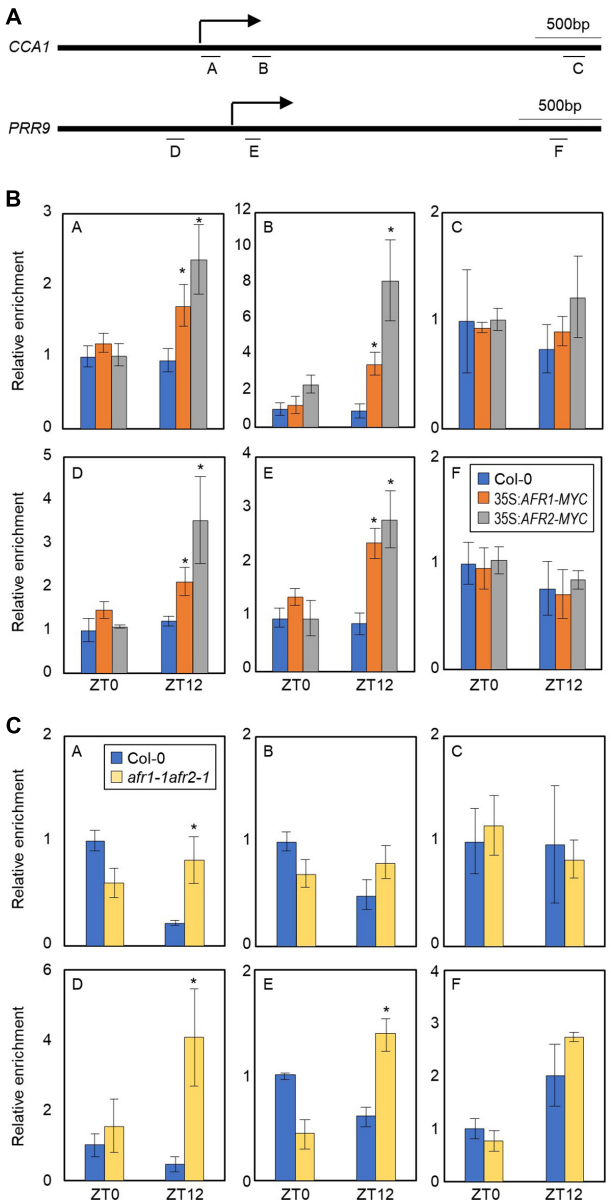

FIGURE 5 | H3 deacetylation at the *CCA1* and *PRR9* loci during evening time by AFRs. In **(B,C)**, 2-week-old seedlings grown under ND were transferred to LL and harvested at ZT0 and ZT12. Enrichment of putative binding regions of AFRs in promoters of the *CCA1* and *PRR9* genes was analyzed by ChIP-PCR. Three independent biological replicates were averaged, and statistical significance of the measurements was determined by a Student's *t*-test (*$P < 0.05$). Bars indicate the standard error of the mean. **(A)** Genomic regions for ChIP analysis. Underbars represent the amplified genomic regions. **(B)** Binding of AFRs to the *CCA1* and *PRR9* loci. **(C)** Accumulation of H3ac at the *CCA1* and *PRR9* loci in the *afr1-1afr2-1* mutant. Anti-H3ac antibody was used for ChIP to assess H3ac accumulation at the loci.

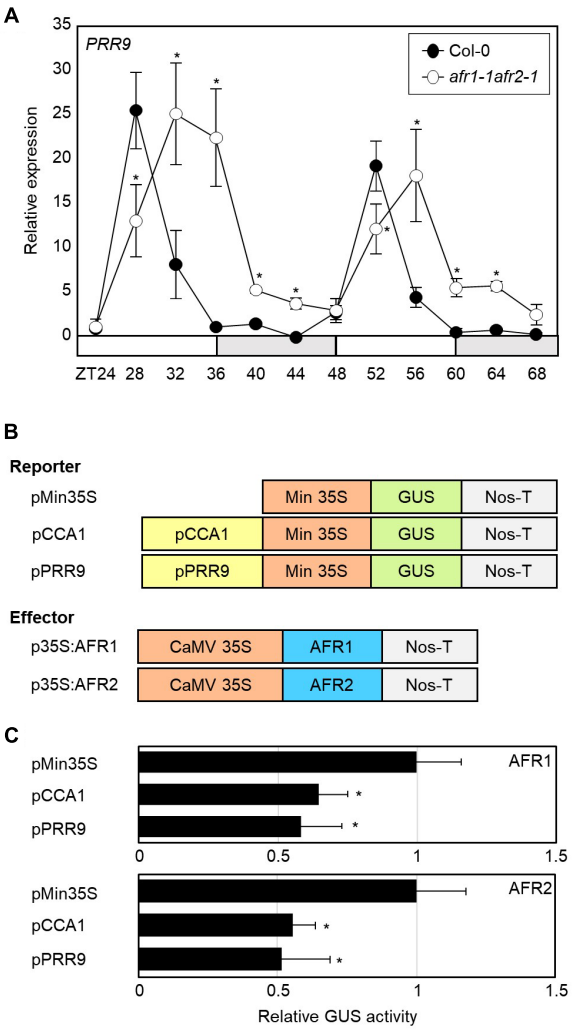

FIGURE 6 | Increased expression of *PRR9* at dusk in *afr1-1afr2-1*. **(A)** Transcript accumulation of *PRR9*. Seedlings grown under ND were transferred to LL at ZT0. Whole seedlings were harvested from ZT24 to ZT68 to analyze transcript accumulation. Gene expression values were normalized to *elF4A* expression and represented as *n*-fold compared to the value of the wild-type sample at ZT24. Three independent biological replicates were averaged, and statistical significance of the measurements was determined by a Student's *t*-test (*$P < 0.05$). Bars indicate the standard error of the mean. The white and pale gray boxes indicate the subjective day and night, respectively. **(B)** Recombinant constructs used for transient expression assays. **(C)** Transient expression analysis using *Arabidopsis* protoplasts. The core elements of *CCA1* and *PRR9* genes were inserted into the reporter plasmid. A recombinant reporter was transiently coexpressed with an effector construct containing the 35S:*AFR-MYC* construct in *Arabidopsis* protoplasts, and GUS activity was fluorimetrically determined. Luciferase gene expression was used to normalize GUS activity. Three independent measurements were averaged. Statistical significance was determined by a Student's *t*-test (*$P < 0.05$). Bars indicate the standard error of the mean.

or 35S:*AFR2-MYC* led to lower GUS activity than the control plasmid (**Figure 6C**). These results indicate that AFR activity limits expression of morning genes, *CCA1* and *PRR9*.

The AFR Proteins May Interact With LNK

In yeast, SAP30 is a key player in recruitment of the SAP30-Sin3-HDAC co-repressor complex to target loci (Ahringer, 2000). It is possible that the yeast SAP30 protein interacts extensively

with DNA-binding transcription factors. Consistently, the *Arabidopsis* AFR1 and AFR2 proteins also frequently associate with transcription factors and guide the Sin3-HDAC complex to cognate target chromatin regions (Gu et al., 2013). To identify the molecular components that recruit the Sin3-HDAC complex to the *CCA1* and *PRR9* loci, we performed yeast-two-hybrid (Y2H) assays. Clock genes were fused in-frame to the 3′-end of the activation domain (AD) of GAL4, and each construct was coexpressed in yeast cells with a recombinant plasmid containing the GAL4 DNA binding domain (BD)-AFR fusion construct. Cell growth on selective medium showed that the transcriptional corepressors NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 1 (LNK1) and LNK2 specifically bind to AFR1 and AFR2 (**Figure 7A** and **Supplementary Figure S5**). The *in vivo* interactions of LNK and AFR proteins were verified by BiFC assays. Coexpression of AFR-nYFP and LNK-cYFP constructs allowed nuclear emission of YFP fluorescence, indicating physical interactions (**Figure 7B**). Given that the LNK corepressors act along with several DNA-binding proteins such as REVEILLE 4 (RVE4) and RVE8 (Xie et al., 2014; Perez-Garcia et al., 2015), AFRs may be recruited to the *CCA1* and *PRR9* loci at least by the DNA-binding RVE-LNK complex.

Taken together, the *Arabidopsis* Sin3-HDAC complex facilitates temporal H3 deacetylation at the *CCA1* and *PRR9* loci to stably regulate circadian oscillation. The AFR proteins diurnally accumulate and possibly lead to temporal association of the Sin3-HDAC complex at evening time. The AFR proteins bind specifically to the morning gene loci and facilitate H3 deacetylation at the cognate regions at dusk. Binding of the Sin3-HDAC complex to the target promoter regions is likely specified by the RVE-LNK complex (**Figure 8**).

DISCUSSION

Chromatin Modification and the Circadian Clock

Rhythmic expression of core clock genes is intimately associated with the levels of histone modification, including H3ac and H3K4me3, at gene promoters in *Arabidopsis* (Hemmes et al., 2012; Malapeira et al., 2012). Dynamic cycles of histone modifications at the clock genes may result from transient binding of chromatin modifiers to the gene promoters. To date, several chromatin modifiers responsible for circadian control have been identified.

The SET DOMAIN GROUP 2 (SDG2)/ARABIDOPSIS TRITHORAX-RELATED 3 (ATXR3) protein is responsible for H3K4me3 deposition to activate multiple core clock genes. The H3K4me3 histone mark interferes with clock repressor binding at the core clock promoters, conferring correct timing of transcriptional repression to target clock genes (Hemmes et al., 2012; Malapeira et al., 2012). Accordingly, the *SDG2/ATXR3*-deficient mutants exhibit a global decrease in H3K4me3 levels and also a reduced amplitude of core clock gene expression (Berr et al., 2010; Malapeira et al., 2012; Yao et al., 2013; Pinon et al., 2017).

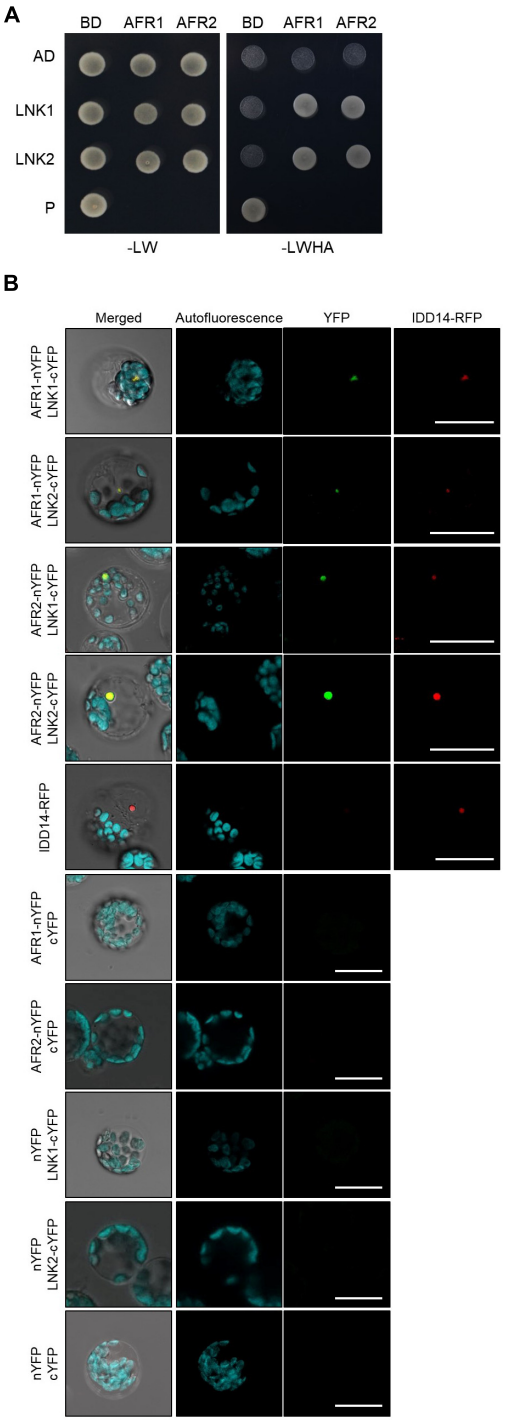

FIGURE 7 | Interactions of AFRs with LNKs. **(A)** Y2H assays. Y2H assays were performed with AFR proteins fused to the DNA-binding domain (BD) of GAL4 and LNKs fused with the transcriptional activation domain (AD) of GAL4 for analysis of interactions. Interactions were examined by cell growth on selective media. -LWHA indicates Leu, Trp, His, and Ade drop-out plates. -LW indicates Leu and Trp drop-out plates. GAL4 was used as a positive control (P). **(B)** BiFC assays. Partial fragments of YFP protein were fused with AFRs and LNKs, and co-expressed in *Arabidopsis* protoplasts. The IDD14-RFP construct was used as a nuclear marker. Reconstituted fluorescence was examined by confocal microscopy. Scale bars: 20 μm.

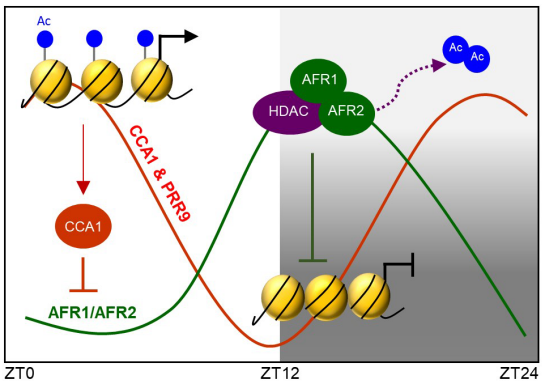

FIGURE 8 | AFRs temporally regulate *CCA1* and *PRR9* genes during evening time. *Arabidopsis* Sin3-HDAC participates in regulating rhythmic expression of the *CCA1* and *PRR9* genes. The evening-expressed AFR proteins may temporally form the Sin3-HDAC corepressor complex and bind directly to the *CCA1* and *PRR9* promoters to catalyze H3 deacetylation at the cognate regions, allowing the declining phase of *CCA1* and *PRR9* expression during evening time. Binding regions of the Sin3-HDAC complex are likely specified by LNK-associated DNA-binding factors.

Circadian expression of the *CCA1* and *LHY* genes is regulated by a couple of chromatin modifiers. The *JMJ30*/*JMJD5* gene is clock-controlled and peaks at dusk (Lu et al., 2011). This pattern of *JMJ30* expression is shaped by the central oscillators CCA1 and LHY, which directly bind to the *JMJ30* promoter (Lu et al., 2011). In turn, JMJ30 promotes expression of *CCA1* and *LHY*, presumably through its histone demethylase activity (Lu et al., 2011). In addition, HDA6 and HDA19 are also implicated in the *Arabidopsis* circadian system. The HDAC proteins form a protein complex with PRRs and TPL/TPRs (Wang et al., 2013), and repress expression of *CCA1* and *LHY* by directly binding to the *CCA1* and *LHY* promoters (Wang et al., 2013). Consistently, suppression of HDAC activity leads to circadian period lengthening and compromises the transcriptional repression activities of PRR5, PRR7, and PRR9 (Wang et al., 2013).

The *Arabidopsis* Sin3-HDAC complex is a different type of HDAC complex involved in circadian oscillation. Key members of the complex, AFR1 and AFR2, are under the control of the circadian clock and form a Sin3-HDAC complex possibly in a diurnal manner to mediate periodic histone deacetylation at the *CCA1* and *PRR9* loci. AFR-dependent H3 deacetylation at the *CCA1* and *PRR9* is relevant during the evening time and thereby dampens expression specifically at dusk. Notably, even though they share the same HDAC components, the AFR-containing Sin3-HDAC complex and HDA6/HDA19-PRR-TPL complex have different binding targets in the control of circadian oscillation. Different compositions of the protein complexes may lead to different abilities in interactive protein recognition, construction of protein interaction networks and thus target chromatin binding. For instance, the AFR proteins may specifically recruit transcriptional co-regulators, such as LNKs, and facilitate new repertoires of target gene regulation in circadian control.

A significant number of HATs and HDACs participate in circadian oscillation. Specific sets of HAT and HDAC shape circadian expression of core clock genes. For instance, HAF2 adds acetyl groups specifically to the *PRR5* and *LUX* loci to facilitate the rising phase of expression (Lee and Seo, 2018), and the Sin3-HDAC complex removes the acetyl groups at the *CCA1* and *PRR9* loci to reset the acetylation state. This is likely not an exceptional case, and many biological responses are probably diurnally shaped by means of chromatin modifications (Kouzarides, 2007; Jang et al., 2011; Seo and Mas, 2014). The opposing activities of HAT and HDAC at specific genes conceivably modulate the acetylation dynamics of target chromatin regions during a day and set gene expression at the adequate level at the right time.

Interactions of Chromatin Modifiers With DNA-Binding Transcription Factors

Histone acetyltransferases and HDACs are targeted to actively transcribed loci to control acetylation state and thereby gene expression at the genome level (Kuo and Allis, 1998; Wang et al., 2009; Peserico and Simone, 2011; Hemmes et al., 2012; Malapeira et al., 2012). However, since they have no selectivity to DNA elements, they are usually recruited to specific target loci by DNA-binding transcription factors (Todeschini et al., 2014; Bauer and Martin, 2017; Inukai et al., 2017). Interactions of chromatin modifiers with transcription factors allow elegant spatial and temporal modification of chromatin contexts (Munshi et al., 1998, 2001; Agalioti et al., 2000; Lomvardas and Thanos, 2002; Bauer and Martin, 2017).

Interactions of HDAC proteins with core clock components are crucial for refining circadian behavior in eukaryotes (Perales and Mas, 2007; Nakahata et al., 2008; Grimaldi et al., 2009). For example, in mammals, SIRT1 associates with a core transcription factor CLOCK, a positive regulator of the circadian machinery, and is recruited to the circadian gene promoters (Nakahata et al., 2008). Similarly, HDACs are associated with core clock components with DNA-binding activities in the control of circadian signaling in *Arabidopsis* (Perales and Mas, 2007). In the circadian expression of *TOC1*, the histone acetylation state seems to be regulated, at least in part, by the clock factors CCA1 and RVE8, as plants mis-expressing the MYB transcription factors exhibit an altered pattern of histone acetylation at the *TOC1* locus (Perales and Mas, 2007). CCA1 may specify repressive chromatin structures at the *TOC1* locus to regulate its expression at dawn, whereas RVE8, which has a high degree of sequence homology to CCA1, favors H3 acetylation in contrast to CCA1, most likely by antagonizing CCA1 function during the *TOC1* raising phase (Farinas and Mas, 2011). Although chromatin modifiers responsible for accumulation of H3ac at the *TOC1* locus are elusive so far, the oscillating H3ac levels are dependent on core clock transcription factors that will recruit HATs and/or HDACs to shape the waveform of *TOC1*.

AFR1 and AFR2 are recruited to the *CCA1* and *PRR9* chromatin for H3 deacetylation possibly by LNKs, although further experiments are required to prove the putative interactions. The morning-expressed LNK1 and LNK2 transcriptional coactivators lack DNA binding domains, but they interact with the *bona fide* DNA-binding proteins including CCA1, LHY, RVE4, and RVE8 to bind to core clock genes (Xie et al., 2014). Although it is unclear so far, the LNK1/2-interacting CCA1/RVEs and/or as-yet-unidentified DNA-binding proteins may transcriptionally activate *CCA1* and *PRR9* expression in the morning and also enable recruitment of the Sin3-HDAC complex to the morning gene loci to subsequently dampen expression after peak phase. The dynamic nature of histone acetylation and deacetylation depends on sophisticated interactions with transcription factors, and protein interaction networks further diversify the molecular mechanisms underlying rhythmic expression of core clock genes and thus circadian oscillation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana (Columbia-0 ecotype) was used for all experiments described, unless specified otherwise. Plants were grown under neutral day conditions (NDs; 12-h light/12-h dark cycles) with cool white fluorescent light (120 μmol photons m^{-2} s^{-1}) at 22-23°C. The *afr1-1afr2-1* mutant was previously reported (Gu et al., 2013). *sap18-2*, *cca1-1lhy-21*, and *cca1-2* mutants were obtained from *Arabidopsis* Biological Resource Center (ABRC). The lack of gene expression in mutants was verified by means of RT-PCR.

Quantitative Real-Time RT-PCR Analysis

Total RNA was extracted using the TRI reagent (TAKARA Bio, Singa, Japan) according to the manufacturer's recommendations. Reverse transcription (RT) was performed using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Dr. Protein, Seoul, South Korea) with oligo(dT18) to synthesize first-strand cDNA from 2 μg of total RNA. Total RNA samples were pretreated with an RNAse-free DNAse. cDNAs were diluted to 100 μL with TE buffer, and 1 μL of diluted cDNA was used for PCR amplification.

Quantitative RT-PCR reactions were performed in 96-well blocks using the Step-One Plus Real-Time PCR System (Applied Biosystems). The PCR primers used are listed in **Supplementary Table S1**. The values for each set of primers were normalized relative to the *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1* (*eIF4A*) gene (At3g13920). All RT-qPCR reactions were performed in three independent biological replicates using total RNA samples extracted from three independent replicate samples. The comparative $\Delta\Delta C_T$ method was employed to evaluate the relative quantities of each amplified product in the samples. The threshold cycle (C_T) was automatically determined for each reaction by the system set with default parameters. Specificity of the RT-qPCR reactions was determined by melt curve analysis of the amplified products using the standard method installed in the system.

Yeast Two-Hybrid Assays

Yeast two-hybrid (Y2H) assays were performed using the BD Matchmaker system (Clontech, Mountain View, CA, United States). The pGADT7 vector was used for GAL4-AD fusion, and the pGBKT7 vector was used for GAL4-BD fusion. The yeast strain AH109 harboring the *LacZ* and *His* reporter genes was used. PCR products were subcloned into the pGBKT7 and pGADT7 vectors. The expression constructs were cotransformed into yeast AH109 cells and transformed cells were selected by growth on SD/-Leu/-Trp medium.

Bimolecular Fluorescence Complementation (BiFC) Assays

The *LNK* genes were fused in-frame to the 5′ end of a gene sequence encoding the C-terminal half of EYFP in the pSATN-cEYFP-C1 vector (E3082). The *AFR* cDNA sequences were fused in-frame to the 5′ end of a gene sequence encoding the N-terminal half of EYFP in the pSATN-nEYFP-C1 vector (E3081). The IDD14-RFP construct was used as a nuclear marker (Seo et al., 2011). The expression constructs were cotransformed into *Arabidopsis* protoplasts. Expression of the fusion constructs was monitored by fluorescence microscopy using a Zeiss LSM510 confocal microscope (Carl Zeiss, Jena, Germany).

Chromatin Immunoprecipitation (ChIP)

pCCA1:CCA1-HA-YFP/cca1-1 and 35S:*AFR-MYC* transgenic plants were used for ChIP. Anti-MYC (06-599, Millipore), anti-HA (ab9110, Abcam), and anti-H3ac (05-724, Millipore) antibodies and salmon sperm DNA/protein A agarose beads (Millipore, Billerica, MA, United States) were used for chromatin immunoprecipitation. DNA was purified using phenol/chloroform/isoamyl alcohol and sodium acetate (pH 5.2). The level of eluted DNA fragments was quantified by quantitative real-time PCR using specific primer sets (**Supplementary Table S2**). The values were normalized to the input DNA level.

Transient Expression Assays

For transient expression assays using *Arabidopsis* protoplasts, reporter and effector plasmids were constructed. The core elements of the *CCA1* and *PRR9* promoters were inserted into the reporter plasmid, which contains a minimal 35S promoter sequence and the GUS gene. To construct the p35S:AFR effector plasmids, the *AFR1* and *AFR2* cDNAs were inserted into the effector vector containing the CaMV 35S promoter. Recombinant reporter and effector plasmids were cotransformed into *Arabidopsis* protoplasts by polyethylene glycol-mediated transformation. GUS activity was measured by a fluorometric method. A CaMV 35S promoter-luciferase construct was also cotransformed as an internal control.

The luciferase assay was performed using the Luciferase Assay System kit (Promega,[1]).

AUTHOR CONTRIBUTIONS

PJS conceived and designed the experiments. PJS wrote the manuscript with the help of HGL. HGL and CH conducted the experiments and contributed to the study design.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00171/full#supplementary-material

[1] http://www.promega.com/

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The LDL1/2-HDA6 Histone Modification Complex Interacts With TOC1 and Regulates the Core Circadian Clock Components in *Arabidopsis*

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In *Arabidopsis*, the circadian rhythm is associated with multiple important biological processes and maintained by multiple interconnected loops that generate robust rhythms. The circadian clock central loop is a negative feedback loop composed of the core circadian clock components. *TOC1* (*TIMING OF CAB EXPRESSION 1*) is highly expressed in the evening and negatively regulates the expression of *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*)/*LHY* (*LATE ELONGATED HYPOCOTYL*). CCA1/LHY also binds to the promoter of *TOC1* and represses the *TOC1* expression. Our recent research revealed that the histone modification complex comprising of LYSINE-SPECIFIC DEMETHYLASE 1 (LSD1)-LIKE 1/2 (LDL1/2) and HISTONE DEACETYLASE 6 (HDA6) can be recruited by CCA1/LHY to repress *TOC1* expression. In this study, we found that HDA6, LDL1, and LDL2 can interact with TOC1, and the LDL1/2-HDA6 complex is associate with TOC1 to repress the *CCA1/LHY* expression. Furthermore, LDL1/2-HDA6 and TOC1 co-target a subset of genes involved in the circadian rhythm. Collectively, our results indicate that the LDL1/2-HDA6 histone modification complex is important for the regulation of the core circadian clock components.

Keywords: H3K4 demethylases, HDA6, circadian clock, CCA1/LHY, TOC1, *Arabidopsis*

INTRODUCTION

The circadian rhythm is an endogenous oscillation widely observed in plants, animals, fungi, and cyanobacteria (Edgar et al., 2012). The plant circadian rhythm is highly associated with multiple important biological processes, and maintained by multiple interconnected loops that generate robust rhythms. The circadian clock central loop is a negative feedback loop composed of the core circadian clock components such as *TOC1* (*TIMING OF CAB EXPRESSION 1*) and *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*)/*LHY* (*LATE ELONGATED HYPOCOTYL*). *TOC1* is highly expressed in the evening, but low expressed at dawn (Alabadi et al., 2001). Furthermore, TOC1

was identified as a repressor of *CCA1* and *LHY* by binding to their promoters in the evening (Gendron et al., 2012; Huang et al., 2012). In contrast, *CCA1* and *LHY* are highly expressed in the morning, but low expressed at nightfall (Schaffer et al., 1998; Wang and Tobin, 1998; Alabadi et al., 2001). CCA1 and LHY bind to the evening element (EE) on the promoter of *TOC1* to inhibit its expression (Schaffer et al., 1998; Wang and Tobin, 1998; Alabadi et al., 2001; Nagel et al., 2015). CHE (CCA1 HIKING EXPEDITION) is an evening-expressed TCP-family transcription factor, which also targets the *CCA1* promoter to repress its expression. Furthermore, CCA1 and LHY were shown to repress the *CHE* expression by targeting the *CHE* promoter (Pruneda-Paz et al., 2009).

Histone modifications play important roles in the regulation of gene expression. Histone methyltransferases and demethylases determine the methylation levels, whereas histone acetylation levels are regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs or HDAs). HDACs and the H3K4 demethylase LSD1 (Lysine-Specific Demethylase 1) are the core components of the Mi2/NuRD and CoREST protein complexes in yeast and animal cells (Khochbin et al., 2001; Lee et al., 2005; Wang et al., 2009). They act co-operatively to repress gene expression in mammals (Huang et al., 2011). The interactions among the core protein components of the HDAC complexes are relatively stable and the HDAC complexes can also interact with various transcription factors under different environmental conditions (Joshi et al., 2013; Liu et al., 2014). FLD (FLOWERING LOCUS D), LDL1 (Lysine-Specific Demethylase-LIKE 1), LDL2, and LDL3 are the LSD1 homologs in *Arabidopsis* (Jiang et al., 2007). LDL1 and LDL2 act redundantly to regulate *FLC (FLOWERING LOCUS C)* by H3K4 demethylation (Jiang et al., 2007). Furthermore, *Arabidopsis* HISTONE DEACETYLASE 6 (HDA6) directly interacts with FLD to repress *FLC*, *MAF4*, and *MAF5* by reducing H3K4 methylation (H3K4me) and H3 acetylation (H3Ac) to regulate flowering time (Yu et al., 2011). In addition, HDA6 can also interact with LDL1 and LDL2 to regulate gene expression (Hung et al., 2018).

The HDAC inhibitor TSA treated plants show delayed phases and higher amplitudes of *TOC1* expression (Perales and Más, 2007). In addition, the expression of *Arabidopsis CCA1*, *LHY*, and *TOC1* is specifically associated with H3Ac and H3K4me changes (Hemmes et al., 2012; Malapeira et al., 2012), indicating that the expression of the core circadian clock components is associated with H3Ac and H3K4me level changes. Our recent study indicated that CCA1 and LHY can interact with the HDAC complex containing LDL1, LDL2, and HDA6. Furthermore, the LDL1/2-HDA6 complex can be recruit by the transcription repressors CCA1 and LHY to their target genes including *TOC1*. Since *CCA1* and *LHY* are low expressed at nightfall, the expression of *TOC1* is increased due to the release of LDL1/2-HDA6 from the *TOC1* promoter (Hung et al., 2018). In this study, we demonstrated that LDL1/2-HDA6 can also interact with TOC1 to regulate the expression of *CCA1* and *LHY*. Furthermore, LDL1/2-HDA6 and TOC1 co-target a subset of genes involved in the circadian rhythm.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* Columbia (Col-0) ecotype was used. Plants were grown at 22°C under 12/12 h light/dark conditions in growth chambers. The mutants used in this study were previously described, including *ldl1/ldl2* (Jiang et al., 2007), *hda6* (*axe1-5*) (Yu et al., 2011), *hda6/ldl1/2* (Hung et al., 2018), *toc1,* and *cca1/lhy* (Wang et al., 2011). *35Spro::LDL1:GFP*, *35Spro::GFP:HDA6*, *LDL1pro::LDL1:GFP* and *HDA6pro::HDA6:GFP* transgenic plants were previously described (Yu et al., 2011; Hung et al., 2018).

The full-length coding sequence (CDS) fragment of *TOC1* was PCR-amplified and cloned into the *pCR8/GW/TOPO* vector (Invitrogen), and then recombined into the *PK7WGF2* binary vector or *3xFLAG* Gateway vector (Invitrogen[1]). The *35S::TOC1:GFP* vector was transformed into Col-0 WT or *hda6/ldl1/2* by the floral dip method.

Bimolecular Fluorescence Complementation (BIFC) Assays

To generate the constructs for BiFC assays, the full-length coding sequence (CDS) fragment of *TOC1* was amplified by PCR and cloned into the *pCR8/GW/TOPO* vector, and then recombined into the *pEarleyGate201-YN* (Lu et al., 2010). *LDL1-YC* and *HDA6-YC* were described in the previous studies (Yu et al., 2011; Hung et al., 2018). Constructed vectors were transformed into *Arabidopsis* protoplasts or tobacco (*Nicotiana benthamiana*) leaves for transient assays. Transformed protoplasts and tobacco leaves were then examined by confocal spectral microscope imaging system (NTU-TCS SP5, Leica[2]).

Yeast Two-Hybrid (Y2H) Assays and Co-immunoprecipitation (Co-IP) Assays

Yeast two-hybrid assays were performed based on the instruction for the Matchmaker GAL4-based two-hybrid system 3 (Clontech). The *LDL1*, *LDL2*, and *TOC1* full length cDNA fragments were sub-cloned into *pGADT7* and *pGBKT7* vectors. All constructs were transformed into the yeast (*Saccharomyces cerevisiae*) strain AH109 by the lithium acetate method, and yeast cells were grown on a minimal medium/-Leu-Trp according to the manufacturer's instructions (Clontech). Transformed colonies were grown on the medium containing X-α-gal for the α-galactosidase activity assay or minimal medium/-Leu-Trp-His (3DO) with 0.25 mM 3-amino- 1,2,4-triazole (3AT).

Co-immunoprecipitation assays were performed as previously described (Yu et al., 2011). The *35S::TOC1:3xFLAG* plasmid was transformed into *Arabidopsis* protoplasts extracted from *LDL1pro::LDL1:GFP* or *35Spro::GFP* transgenic plants. Total proteins were than extracted from the transformed protoplasts. Anti-GFP (Santa Cruz Biotechnologies, catalog no. SC-9996; 1:3000 dilution) and anti-FLAG (SIGMA catalog no. M2; 1:3000

[1]https://www.psb.ugent.be/core-facilities/380-gateway-vectors

[2]https://www.leica-microsystems.com/products/confocal-microscopes/p/leica-tcs-sp5/

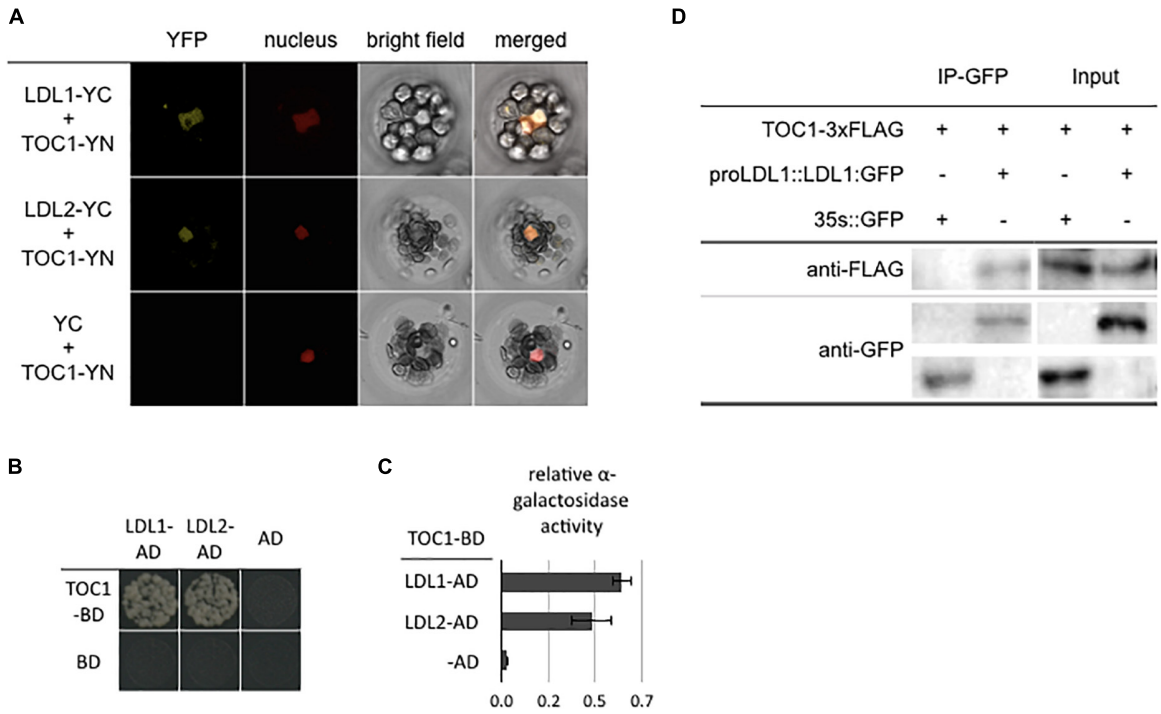

FIGURE 1 | LDL1/LDL2 interact with TOC1. **(A)** BiFC assays in *Arabidopsis* protpplasts showing interaction between LDL1/LDL2 and TOC1 in living cells. LDL1, LDL2, and TOC1 fused with the N terminus (YN) or C terminus (YC) of YFP were co-delivered into *Arabidopsis* protpplasts. The nucleus was indicated by mCherry carrying a nuclear localization signal. **(B,C)** Yeast two hybrid analysis of the interaction of LDL1/LDL2 with TOC1. LDL1-BD/LDL2-BD with CCA1-AD or LHY-AD was co-transformed into the yeast strain AH109. The transformants were plated on the SD/-Leu-Trp-His medium. **(C)** Quantitative α-galactosidase assays for protein-protein interaction in yeast. Bars indicate SD from three biological replicates. **(D)** Co-IP of the native promoter driven LDL1:GFP with TOC1 in *LDL1pro::LDL1:GFP* transformed *Arabidopsis* protoplasts. Western blot (WB) was performed with the anti-FLAG and anti-GFP antibodies.

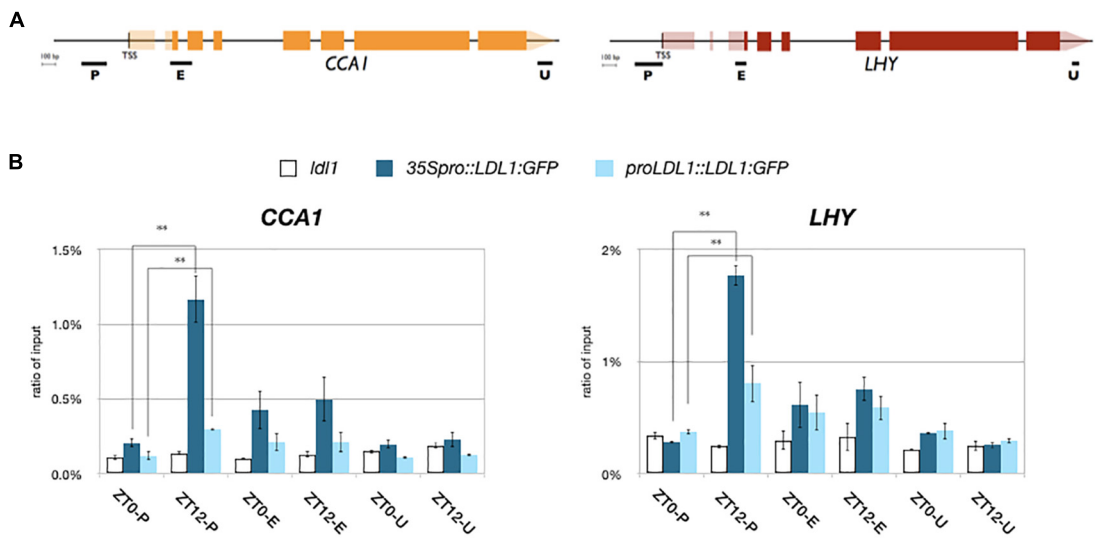

FIGURE 2 | LDL1/LDL2 target on *CCA1* and *LHY*. **(A)** Schematic diagram of *CCA1* and *LHY*. P: promoter region, E: coding region, U: 3′ UTR. **(B)** LDL1 bindis to the *CCA1* and *LHY* promoters. *35S pro::LDL1:GFP* or *LDL1pro::LDL1:GFP* was transformed into *ldl1*. 14 days-old seedlings grown under 12/12: light/dark were harvested on ZT0 or ZT12. ChIP assays were performed with the anti-GFP antibody. The amount of immunoprecipitated DNA was quantified by qRT-PCR. Values represent the average immunoprecipitation efficiencies (%) against the total input DNA. Error bars correspond to standard deviations from three biological replicates. $^{*}P < 0.05$, $^{**}P < 0.005$ (Student's *t*-test).

dilution) antibodies were used as primary antibodies for Western blot. The resulting signals were detected by using a Pierce ECL Western blotting kit (Pierce[3]).

Quantitative Real-Time PCR (qRT-PCR) Analysis

The TRIZOL reagent (Invitrogen, 15596026) was used for total RNA isolation according to the manufacturer's instructions. Total RNA treated with 2 μg of DNAse (Promega, RQ1 #M6101) were then used for cDNA synthesis (Promega, #1012891). The iQ SYBR Green Supermix solution (Bio-Rad, #170-8880) was used for real-Time quantitative PCR assays with the CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Inc.). Cycling conditions were started with 95°C/10 min, followed by 45 cycles of 95°C/15 s, 60°C/30 s, and then fluorescent detection, and melting curve detection (65–95°C, incrementing 0.5°C for 5 s, and plate reading). Each sample was normalized by calculating delta quantification cycle (Cq) to the expression of the *UBQ10* (*Ubiquitin10*) internal control and quantified at least in triplicate. The Cq and relative expression level are calculated by the Biorad CFX Manager 3.1 based on the MIQE guidelines (Bustin et al., 2009). **Supplementary Table S1** listed the gene specific primers used for qRT-PCR. Standard deviations (SD) represent at least three technical and three biological replicates. The variance in average data is represented by standard error of the mean (SEM). The SD, SEM determination and *P*-value were calculated using Student's paired *t*-test.

Protoplast Transient Assays

The *CCA1pro::LUC* plasmid construct was previously described (Wang et al., 2011). For transcriptional activity assays, the *35Spro::TOC1*, *35Spro::LDL1*, *35Spro::HDA6,* or *35Spro::GFP* effector constructs were co-transformed into protoplasts with *CCA1pro::LUC*, and the plant samples were collected at ZT0 after 12 h. The relative activities of LUC (luciferase) reporter were standardized by activities of co-expressed Renilla LUC. Experiments were repeated at least three times for each reporter-effector combination. The dual luciferase assay reagent (Promega) was used for Firefly LUC and Renilla LUC detection.

Chromatin Immunoprecipitation (ChIP) Assays and ChIP-seq Data Analyses

Chromatin immunoprecipitation assays were accomplished as previously described (Yu et al., 2011; Hung et al., 2018). Plant seedlings were treated with 1% formaldehyde for chromatin extraction. The extracted DNA was sheared to the mean length near 500 bp by sonication, proteins, and DNA fragments were then immunoprecipitated by the H3K9K14 (Millipore, catalog no. 06-599), H3K4me3 (Milipore, catalog no. 04-745), or GFP (Abcam, catalog no. ab290) antibodies. The cross-link between DNA with immunoprecipitated proteins were reversed, and then analyzed by real-time PCR using specific primers (**Supplementary Table S1**). The quantification cycle(Cq) was calculated by Biorad CFX Manager 3.1 based on the MIQE guideline (Bustin et al., 2009). Percent input was calculated as 2^[Cq(IN)-Cq(IP)]X100. Each sample was quantified at least in triplicate, and normalized by calculating delta Cq to the expression of the internal control. Standard deviations (SD) represent at least three technical and three biological replicates. The variance in average data is represented by standard error of the mean (SEM). The SD, SEM determination and *P*-value were calculated using Student's paired *t*-test.

ChIP-seq assays were performed based on previous research (Li et al., 2015, 2016; Hung et al., 2018). The LDL1 ChIP-seq data were deposited to NCBI-Gene Expression Omnibus (GEO) database (GSE118025) (Hung et al., 2018). The ChIP-Seq files from other research groups, GSE35952 (Huang et al., 2012) and (Kamioka et al., 2016), were downloaded from the NCBI-GEO database.

RESULTS

LDL1 and HDA6 Interact With TOC1 and Directly Target on *CCA1* and *LHY*

Our recent study indicated that CCA1/LHY can interact with the LDL1/2-HDA6 complex to repress *TOC1* (Hung et al., 2018). In addition, the expression of *TOC1*, *CCA1* and *LHY* is also associated with H3K4me and H3 acetylation changes (Hemmes et al., 2012; Malapeira et al., 2012). We further analyzed the functional correlation between TOC1 and the LDL1/2-HDA6 complex. TOC1 directly interacted with both LDL1 and LDL2 in BiFC assays by using *Arabidopsis* protoplasts and *Agrobacterium*-infiltrated tobacco leaves. The YFP fluorescence signal was detected in nucleus of the transformed cells (**Figure 1A** and **Supplementary Figure S1**). The interaction between LDL1, LDL2, and TOC1 was further confirmed by yeast two-hybrid assays (**Figures 1B,C**) and Co-IP assays using *Arabidopsis* protoplasts (**Figure 1D** and **Supplementary Figure S1**). Furthermore, TOC1 can also interact with HDA6 in BiFC assays (**Supplementary Figures S2A,B**). These results suggested that TOC1 may recruit the LDL1/2-HDA6 histone modification complex to its target genes such as *CCA1* and *LHY*.

We further analyzed the binding of LDL1 and HDA6 to *CCA1* and *LHY* by ChIP assays. The *LDL1:GFP* and *HDA6:GFP* transgenic plants were previously described (Yu et al., 2011; Hung et al., 2018). 14 days old plants grown under 12 h light/12 h dark condition were collected on Zeitgeber time 0 (ZT0) and ZT12. An anti-GFP antibody was used for ChIP assays, and the binding of LDL1 and HDA6 was analyzed by qPCR. We identified that both LDL1 and HDA6 can bind to the promoters of *CCA1* and *LHY*. Furthermore, the binding of LDL1 and HDA6 to the promoters of *CCA1* and *LHY* were significantly decreased on ZT0 compared to ZT12 (**Figure 2** and **Supplementary Figure S2C**). The binding of LDL1 and HDA6 to the *CCA1* and *LHY* promoters is correlated to TOC1 accumulation, since *TOC1* is highly expressed at nightfall but low expressed in the morning (Alabadi et al., 2001).

[3]https://www.lifetechnologies.com/

TOC1 and LDL1 Co-target Genes Involved in the Circadian Rhythm

Previously, we identified the global binding sites of LDL1 by ChIP-Seq assays (Hung et al., 2018). The GO-BP (Gene Ontology_Biological Process) analysis of LDL1-targeted genes revealed that LDL1 targets on a subset of circadian rhythm genes. Furthermore, LDL1 also binds to a cluster of circadian rhythm genes regulated by CCA1 (Hung et al., 2018). In this study, we further analyzed whether the LDL1 and TOC1 also co-target genes involved in the circadian rhythm.

We compared the previously published TOC1 ChIP-Seq data (Huang et al., 2012) with the LDL1 ChIP-Seq data (Hung et al., 2018). The genome browser views by Integrative Genomics Viewer (IGV) indicated that LDL1 bound to *CCA1* and *LHY*, and the binding peaks of LDL1 are highly correlated with the TOC1 binding regions on *CCA1* and *LHY* promoters (**Figure 3A**). Among 772 genes occupied by TOC1 (Huang et al., 2012), 195 of them are also co-occupied by LDL1 (P = 1.14e-16) (**Figure 3B**). Furthermore, the genomic binding regions of TOC1 are closed to the LDL1 binding regions (**Figure 3C**), indicating that TOC1 and LDL1 tend to bind to the similar genome sites. GO-BP analysis also indicated that LDL1 and TOC1 co-target on a subgroup of genes involved in circadian rhythm and response to cold (**Figure 3D**). In GO-BP analysis, the ratio of the circadian genes of LDL1/TOC1 co-targeted genes is increased when compared to the LDL1-targeted genes or the TOC1-targeted genes alone (**Supplementary Figure S3**). Interestingly, the ratio of the circadian rhythm genes is further increased in the LDL1/CCA1/TOC1 co-targeted genes (**Supplementary Figure S3**). Previous studies indicated that several *cis*-elements are enriched in the promoters of TOC1 regulated genes, including the $(AG/CT)_n$ repeat, G-box (CACGTG), Evening Element (EE)-like and TCP binding site (TBS, GGCCCA) (Gendron et al., 2012; Huang et al., 2012). Similar *cis*-elements are also enriched in the LDL1-targeted promoter regions (Hung et al., 2018).

LDL1/2-HDA6 Is Involved in the Regulation of *CCA1/LHY*

TOC1 is a repressor and targets on the promoters of *CCA1* and *LHY*. The expression of *CCA1* and *LHY* is decreased in *TOC1* over-expressing (*TOC1-OE*) plants (Gendron et al., 2012; Huang et al., 2012). Furthermore, additional *TOC1*

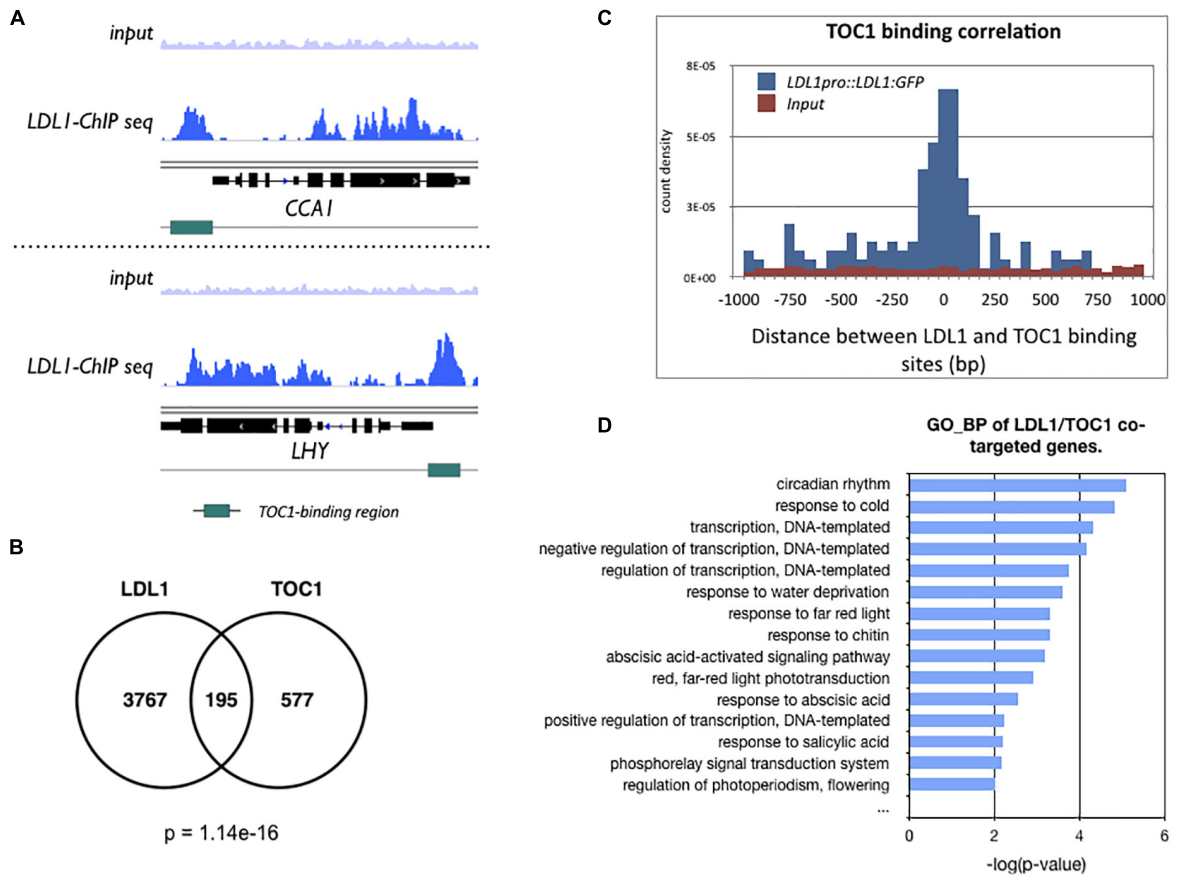

FIGURE 3 | LDL1-occupied sites in the genome identified by ChIP-seq analysis. **(A)** Integrated genome view of LDL1 binding peaks on *CCA1* and *LHY*. green BARS indicate the TOC1-binding regions form previous published data (Huang et al., 2012). **(B)** Overlap between TOC1 target genes (Huang et al., 2012) and LDL1 targeted genes (Hung et al., 2018) (hypergeometric distribution of TOC1 and LDL1 co-targeted genes: p = 1.14e–16). **(C)** Distribution of distances between the total binding sites of LDL1 and TOC1. **(D)** GO-BP annotation of LDL1/TOC1 co-occupied genes. Annotation terms with p-value < 0.01 were listed.

expression causes increased period length of *CCA1* (Mas et al., 2003a). To investigate the functional relationship between TOC1 and LDL1/2-HDA6, we generated *TOC1 over-expressing* plants in WT (*TOC1-OE*) and the *hda6/ldl1/2* background (*TOC1-OE/hda6/ldl1/2*). The binary vector containing *CaMV 35S promoter* driven *GFP:TOC1* (*35S::GFP:TOC1*) was transformed into WT or *hda6/ldl1/2*. The expression patterns of *CCA1* and *LHY* were compared by qRT-PCR in wild-type (WT), *TOC1-OE* and *ldl1/2/hda6* plants grown under 12 h light/12 h dark for 14 days. As reported previously (Gendron et al., 2012; Huang et al., 2012), the expression of *CCA1* and *LHY* was decreased in *TOC1-OE* plants. However, the expression of *CCA1* and *LHY* was not significantly decreased in *hda6/ldl1/2* compared to WT (**Figure 4A** and **Supplementary Figures S4A,B**). Furthermore, the decrease of *CCA1* and *LHY* expression was recovered when *TOC1* was over-expressed in *hda6/ldl1/2* (**Figure 4A** and **Supplementary Figures S4B,C**). We also compared the daily expression patterns of *CCA1, LHY,* and *TOC1* in *ldl1/ldl2*, *hda6*, *hda6/ldl1/2,* and WT grown under 12 h light/12 h dark conditions. The expression of *CCA1* and *LHY* was not significantly decreased or shifted in *ldl1/ldl2*, *hda6,* and *hda6/ldl1/2* compared to WT (**Supplementary Figure S4A**). The expression patterns of other TOC1 targets such as *GI*, *PRR7* and *PRR9* in *ldl1/ldl2*, *hda6,* and *hda6/ldl1/2* were analyzed in our previous study (Hung et al., 2018). *XTH27* and *AT1G10020* were previously identified to be the target genes regulated by TOC1 (Gendron et al., 2012; Huang et al., 2012), which are also targeted by LDL1 (Hung et al., 2018). The expression of *XTH27* and *AT1G10020* was increased in *ldl1/ldl2*, *hda6,* and *hda6/ldl1/2* compared to WT (**Supplementary Figure S4C**).

We further analyzed the functional correlation between LDL1, HDA6, and TOC1. *CCA1pro::CCA1:LUC* (*pCCA1:LUC*) was co-expressed with *35Spro::TOC1, 35Spro::LDL1*, *35Spro::HDA6,* or *35Spro::GFP* in *Arabidopsis* protoplasts. Although the activity of *CCA1:LUC* was only slightly reduced when co-expressed with LDL1, and activity was further decreased when TOC1 was co-expressed with LDL1 (**Figure 4B**). Similar results were also observed when TOC1 was co-expressed with HDA6 (**Figure 4C**).

We also analyzed H3K4me and H3Ac levels of *CCA1* and *LHY* in WT, *TOC1-OE* plants and *hda6/ldl1/2*. For ChIP-qPCR assays, 14-days old plants grown under 12 h light/12 h dark conditions were collected on ZT0. H3K4me and H3Ac of *CCA1* and *LHY* were decreased in *TOC1-OE* plants (**Figure 5**), indicating that TOC1 affects the levels of H3K4me and H3Ac on *CCA1* and *LHY*. We further analyzed H3Ac and H3K4me levels of *CCA1* and *LHY* in 14 days old *hda6*, *ldl1/ldl2*, *hda6/ldl1/2,* and WT on ZT0 and ZT12. The H3Ac and H3K4me levels of *CCA1* and *LHY* were not decreased in *hda6*, *ldl1/ldl2*, *hda6/ldl1/2*

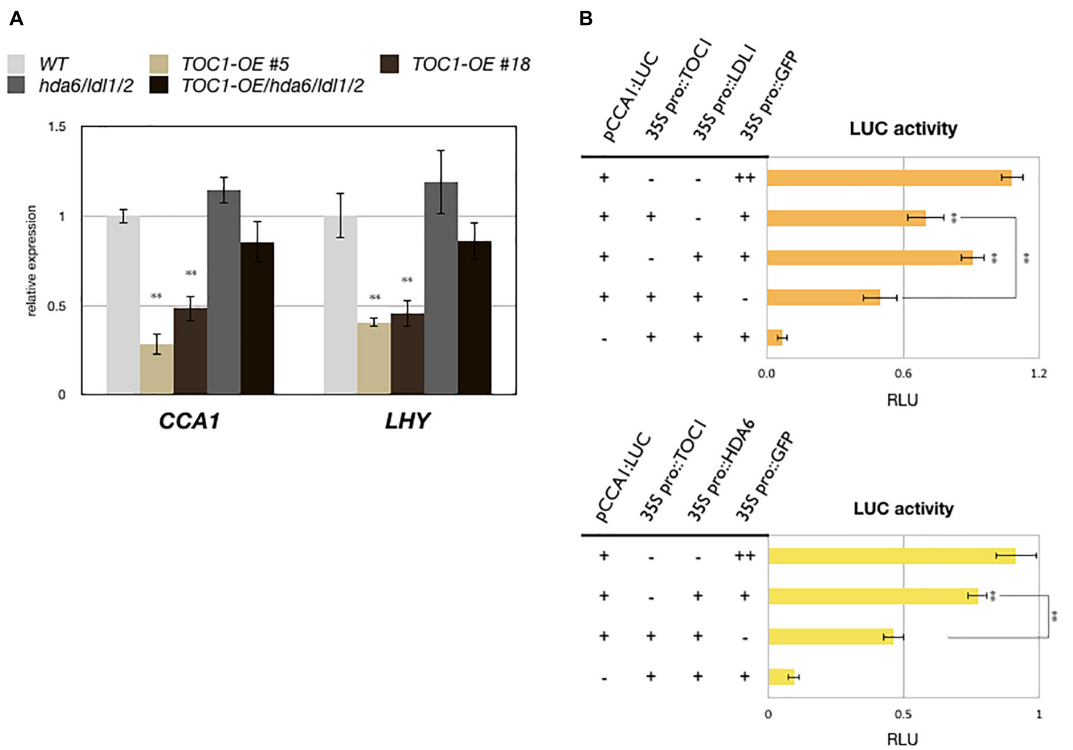

FIGURE 4 | LDL1/2-HDA6 is involved in regulation of *CCA1/LHY*. **(A)** Expression of CCA1 and LHY in *TOC1-OE* plants, *hda6/ldl1/2,* and WT. Gene expression levels were determined by qRT-PCR and normalized to *UBQ10*. Plants were grown under 12/12 light/dark for 14 days and collected on ZT0. **(B)** Transient luciferase assays in *CCA1pro::CCA1:LUC* (*pCCA1:LUC*) transformed protoplasts. CaMV 35S promoter driven *TOC1*, *HDA6,* or *LDL1* effector constructs were introduced into mesophyll protoplasts. Samples were collected on ZT0 after 12 h of transformation. Relative Light Units (RLU) represents firefly luciferase normalized by co-expressed *35S pro::Renilla luciferase*. *35Spro::GFP* transformed protoplasts were used as the negative control. Data points represent the average of three technical replicates. Error bars correspond to SD from three biological replicates. $^{*}P < 0.05$, $^{**}P < 0.005$ (Student's *t*-test).

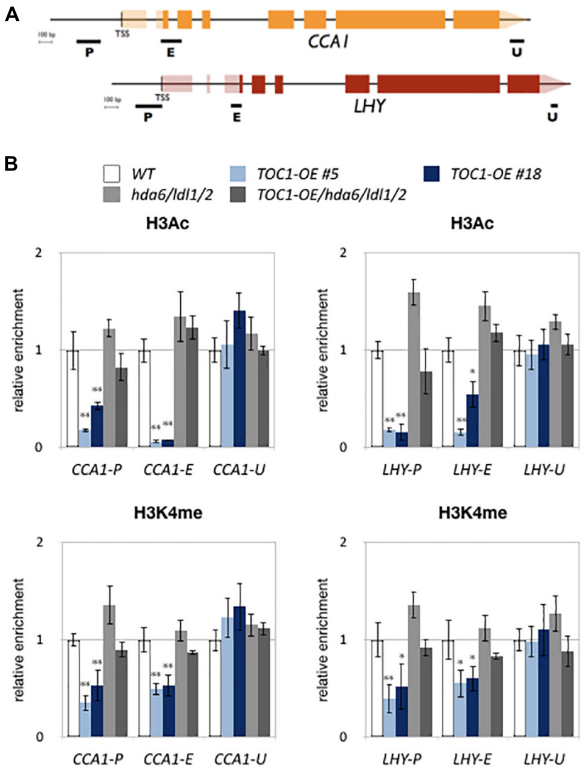

FIGURE 5 | LDL1/2-HDA6 is involved in regulation of H3Ac and H3K4me of *CCA1/LHY*. (A) Schematic diagram of CCA1 and LHY. P: promoter region, E: coding region, U: 3′ UTR. (B) ChIP analysis of H3ac and H3K4me levels of *CCA1* and *LHY* in *TOC1-OE* plants on ZT0. The amounts of DNA after ChIP were quantified by qRT-PCR and normalized to *ACT2*. Plants were grown under 12/12 : light/dark for 14 days. Data points represent the average of three technical replicates. Error bars correspond to SD from three biological replicates. $*P < 0.05$, $**P < 0.005$ (Student's *t*-test).

(**Supplementary Figure S4D**). Interestingly, decreased H3K4me and H3Ac in *TOC1-OE* were recovered in *TOC1-OE/hda6/ldl1/2,* since the H3Ac and H3K4me levels of *CCA1* and *LHY* were significant higher in *TOC1-OE/hda6/ldl1/2* compared to the *TOC1-OE* plants (**Figure 5**). These results suggested that TOC1 is involved in regulation of H3K4me and H3Ac on *CCA1* and *LHY*, and TOC1 repressed *CCA1* and *LHY* expression is dependent on the function of LDL1/2-HDA6 complex.

DISCUSSION

Arabidopsis HDA6 is a class I RPD3-like histone deacetylase associated with regulation of rRNA and transcription repression (Murfett et al., 2001; Probst et al., 2004; Earley et al., 2006; Liu et al., 2012; Yu et al., 2017). Different transcription factors can recruit HDA6 to regulate the gene expression involved in flowering, leaf development, abiotic stress response, and senescence (Wu et al., 2008; Chen et al., 2010; Yu et al., 2011; Luo et al., 2012; Liu et al., 2014). In animal and yeast cells, HDACs and LSD1 regulate gene expression cooperatively and they are both identified as the core components of Mi2/NuRD and CoREST complexes (Khochbin et al., 2001; Lee et al., 2005; Wang et al., 2009). Our recent study demonstrated that the *Arabidopsis* H3K4 demethylases LDL1 and LDL2 can interact with HDA6 to repress gene expression (Hung et al., 2018). The LDL1/2-HDA6 complex can also interact with CCA1/LHY and reduce H3Ac and H3K4me levels of the circadian core component *TOC1* (Hung et al., 2018). Furthermore, a subset of genes involved in the circadian clock are co-targeted by LDL1 and CCA1 (Hung et al., 2018).

Arabidopsis circadian clock genes are regulated by a complicate feedback regulation network forming multiple interconnected loops. The central loop is comprised of the core clock components, such as TOC1 and CCA1/LHY (Schaffer et al., 1998; Wang and Tobin, 1998; Alabadi et al., 2001; Gendron et al., 2012; Huang et al., 2012; Nagel et al., 2015). The central loop is interlocked with the evening loop and morning loop. PRR5, PRR7, PRR9, and CCA1/LHY constitute the morning loop (Nakamichi et al., 2010; Salomé et al., 2010; Pokhilko et al., 2012), whereas PRR3, GI, ZTL (ZEITLUPE), and TOC1 comprise the evening loop (Kim et al., 2003; Mas et al., 2003b; Para et al., 2007; McClung and Gutiérrez, 2010). We found that LDL1 and CCA1 co-target to a subset of circadian genes, which are repressed by CCA1 in the morning. However, LDL1 also targets to the morning expressed circadian genes, which may not be repressed by CCA1 and LHY (Nagel et al., 2015; Kamioka et al., 2016; Hung et al., 2018). Although the binding of LDL1 on the LDL1/CCA1 co-targeted genes are reduced in the *cca1/lhy* mutant, their binding is not completely abolished (Hung et al., 2018). These results suggested that in addition to CCA1 and LHY, the LDL1/2-HDA6 complex may also functionally associate with other circadian clock genes. EC (Evening Complex) is

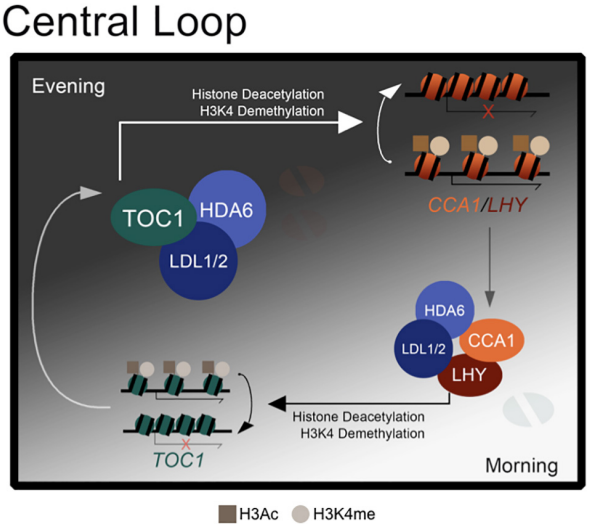

FIGURE 6 | A model for LDL1/2 and HDA6 functions in the regulation of core circadian clock components. Both morning accumulated CCA1/LHY and evening accumulated TOC1 interact with the same histone modification complex containing LDL1/2 and HDA6. CCA1/LHY act as transcription repressors and recruit the histone modification complex to their target loci such as *TOC1* in the morning. Meanwhile, TOC1 also recruits the histone modification complex to its targets such as *CCA1* and *LHY* in the evening.

also associated with regulation of the circadian genes, which is comprised of LUX (LUX ARRHYTHMO), ELF3 (EARLY FLOWERING3), and ELF4 (EARLY FLOWERING4) (Hazen et al., 2005; Nusinow et al., 2011). A previous study indicated that *Arabidopsis* HDACs are associated with PRR9 through direct interacting with TPL/TPR (TOPLESS/TOPLESS-RELATED) to regulate the expression of *CCA1* (Wang et al., 2013). Further research is required to investigate the functional correlation among LDL1/2-HDA6, PRR9, and EC.

The central loop of *Arabidopsis* circadian clock is consisted of the core clock components including CCA1, LHY, and TOC1 (Schaffer et al., 1998; Wang and Tobin, 1998; Alabadi et al., 2001; Gendron et al., 2012; Huang et al., 2012; Nagel et al., 2015). Although *CCA1* and *LHY* are low expressed at nightfall, they are highly induced at dawn (Schaffer et al., 1998; Wang and Tobin, 1998; Alabadi et al., 2001). Previously, we found that CCA1 interacts with LDL1 in the morning (Hung et al., 2018). The binding of LDL1 and HDA6 on promoter of *TOC1* is higher in the morning but decreased in the evening (Hung et al., 2018). Furthermore, *HDA6, LDL1,* and *LDL2* are constitutively expressed at different time periods. CCA1/LHY can therefore recruit the LDL1/2-HDA6 complex to suppress *TOC1* expression at dawn (Hung et al., 2018). In this study, we found that LDL1/2 and HDA6 also interact with TOC1 to regulate the expression of *CCA1* and *LHY*. In consistent with the fact that TOC1 is highly accumulated at nightfall (Alabadi et al., 2001), we found that the binding of LDL1 and HDA6 on the *CCA1* and *LHY* promoters is higher in the evening but decreased in the morning. Since TOC1 is a repressor of *CCA1* and *LHY*, the expression of *CCA1* and *LHY* is decreased with increased *TOC1* expression (Gendron et al., 2012; Huang et al., 2012). We found that histone acetylation and H3K4 methylation levels of *CCA1* and *LHY* are decreased in *TOC1-OE* plants. However, the H3Ac, H3K4me and expression levels of *CCA1* and *LHY* are significantly increased in *TOC1-OE/hda6/ldl1/2* compared to the *TOC1-OE* plants, indicating that the LDL1/2-HDA6 complex is functionally associated with the regulation of *CCA1* and *LHY* expression. Although the expression of *TOC1* is highly increased in *hda6/ldl1/2* compared to wild type, the expression of *CCA1* and *LHY* is not decreased. It is possible that in addition to LDL1/2-HDA6, other unknown proteins may also be involved in the regulation of *CCA1* and *LHY* expression.

Collectively, we propose a model to demonstrate how the core circadian clock components are regulated by H3K4 demethylation and histone deacetylation (**Figure 6**). The histone modification complex containing LDL1/2 and HDA6 can interact with both morning accumulated CCA1/LHY (Hung et al., 2018) and evening accumulated TOC1. The transcription repressors CCA1 and LHY can recruit the LDL1/2-HDA6 complex to their target loci including *TOC1* in the morning (Hung et al., 2018). Furthermore, TOC1 can also recruit the histone modification complex to its targets such as *CCA1* and *LHY* in the evening.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

F-YH, KW, and YC designed the research. F-YH, F-FC, and J-HC performed the research. F-YH, CL, CC, and KW analyzed the data. F-YH and KW wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00233/full#supplementary-material

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OPINION
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Phytohormone and Chromatin Crosstalk: The Missing Link For Developmental Plasticity?

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Plants grow continuously, forming new meristem-derived organs and tissues throughout their post-embryonic life. As sessile organisms, plants need to constantly integrate and reflect environmental fluctuations in their growth and development, which can translate into high level of developmental plasticity in response to environmental changes (Gaillochet and Lohmann, 2015). Alternatively, variable environments can select for robustness, where organisms function across a wide range of conditions with little change in phenotype. Plant growth is then governed by complex interplay of phytohormone signaling, chromatin structure remodeling and gene expression reprogramming. How these regulatory levels are interconnected remains largely enigmatic, but mechanistic evidence of crosstalk between phytohormone signaling and chromatin organization is emerging.

Here we review (1) evidences of molecular mechanisms that mediate the crosstalk between phytohormone signaling, chromatin structure and gene expression (2) how this crosstalk may link to plant developmental plasticity and robustness and finally (3) why meristems may represent central places for this crosstalk allowing plasticity and environmental memory.

CROSSTALK MECHANISMS: A CHICKEN-AND-EGG SITUATION

Phytohormone and epigenetic regulation can interact on multiple levels (**Figure 1**): (1) phytohormone signaling directly affects expression or activity of key chromatin modifiers, (2) chromatin machinery target genes of the phytohormone metabolic/signaling pathways, (3) both players interact on genes involved in developmental or stress responses.

Several examples show that components of phytohormone signaling pathways directly control the activity of key chromatin modifiers such as POLYCOMB REPRESSIVE COMPLEX (PRC) 1 and 2 with histone-methyltransferase activity playing a major role in transcriptional regulation during development (Bratzel et al., 2010; Chen et al., 2010, 2016; Ikeuchi et al., 2015; Mozgová et al., 2017). For example, the brassinosteroid (BR) signaling TFs BRASSINAZOLE-RESISTANT 1 (BZR1) recruits the H3K27me3-demethylase EARLY FLOWERING (ELF) 6 to antagonize the H3K27me3-activity of PRC2, a chromatin modifier, at the flowering repressor *FLOWERING LOCUS C* (*FLC*), preventing precocious floral transition (Yu et al., 2008; Li et al., 2018). Additionally, chromatin complexes can be post-translationally modified by components of phytohormone signaling pathways that influence their activity. For example, abscisic acid (ABA) signaling induces SnRK-mediated phosphorylation of the chromatin remodeling ATPase BRAHMA (BRM), inhibiting its repressive activity at ABA-responsive genes (Peirats-Llobet et al., 2016). These examples demonstrate that activity of chromatin modifiers can be directed to specific loci or directly modulated by phytohormone signaling cascades.

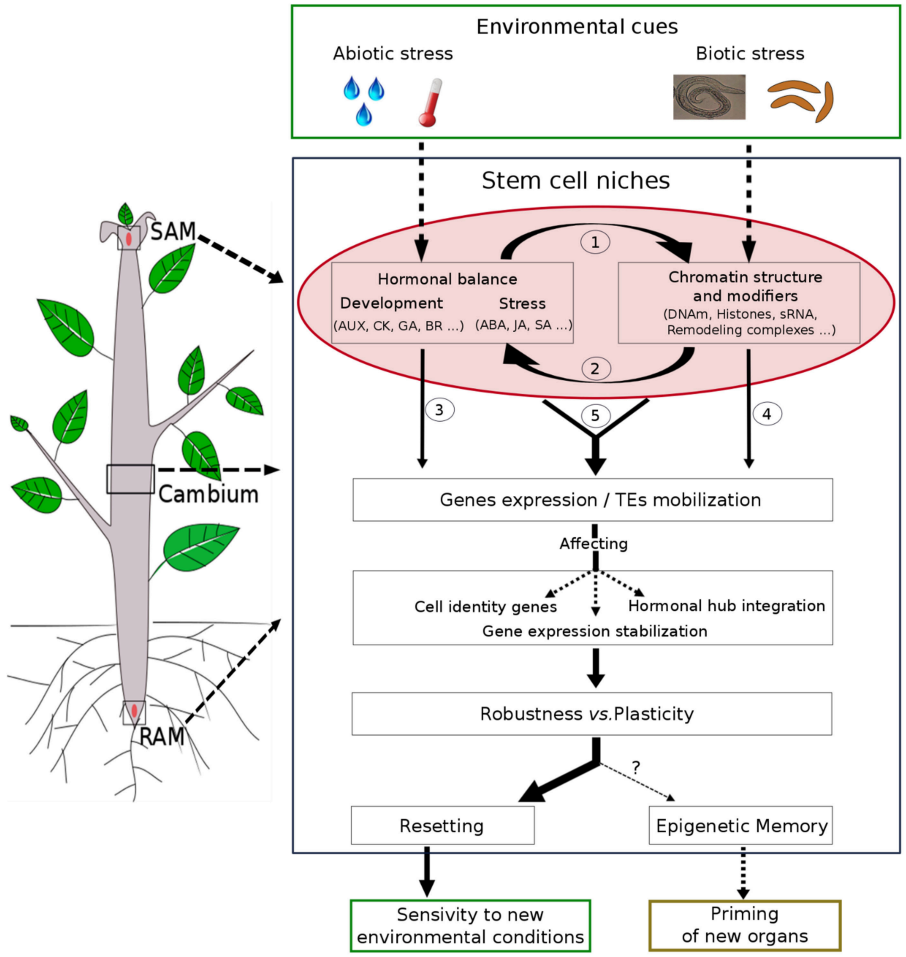

FIGURE 1 | Schematic model of phytohormones and chromatin crosstalk during plant developmental plasticity and robustness. Stem cell niches in SAM, RAM, or cambium are center of morphogenesis giving rise to the aerial and root systems or wood formation in perennials and plasticity in response to various environmental cues. Environmental signals are perceived directly or indirectly by meristems and could affect hormonal balance and/or chromatin structure in a complex crosstalk: (1) hormones can alter chromatin structure and modifiers or (2) chromatin can regulate hormones signaling/biosynthesis. These two mechanisms could then interact separately (3 and 4), jointly or successively (5) affecting genes expression and /or TEs mobilization. Thus, the hormone/chromatin crosstalk can participate in developmental choice (Robustness vs. Plasticity) by controlling cell gene identity in meristems, hormone balance integration, or chromatin stabilization of gene expression. While most of these changes are transient (resetting of hormonal and chromatin modifications) allowing the plant to be respond to new environmental conditions, chromatin states could be maintained through cell division allowing an epigenetic memory and a potential priming of new meristem-derived-organs.

Another possibility is that changes in chromatin structure control phytohormone biosynthesis, signaling and response. Variation in DNA methylation in response to water availability in poplar or among Arabidopsis epigenetic recombinant inbred lines (epiRILs) is associated with changes in jasmonic (JA), salicylic acid (SA) and ethylene responses (Latzel et al., 2012; Lafon-Placette et al., 2018). Similarly, rice plants with reduced H3K27me3 exhibit significant differences in the auxin indole-3-acetic acid (IAA), gibberellin (GA), ABA, JA, and SA content (Liu et al., 2016). Nevertheless, these effects may be pleiotropic and may reflect altered general physiological states. As more direct evidence, PRC2 activity in Arabidopsis seed coat is downregulated by fertilization-dependent auxin, and is required for repressing GA production prior to fertilization, mediating the crosstalk between two phytohormonal pathways (Figueiredo et al., 2015, 2016; Figueiredo and Köhler, 2018). PRC2 also represses auxin biosynthesis and signaling genes in the SAM and leaves of Arabidopsis (Lafos et al., 2011). Conversely, in the RAM, the expression of the auxin efflux carrier-encoding *PIN-FORMED* (*PIN*) genes is positively regulated by BRM establishing local auxin maxima and stimulating the expression of the RAM-specifying *PLETHORA* genes *PLT1* and *PLT2* (Yang et al., 2015). BRM also binds to GA-related genes to stimulate GA biosynthesis and signaling (Archacki et al., 2013).

Apart from biosynthesis and signaling, phytohormone-response genes are under direct control of chromatin modifiers. Initially described as involved in auxin homeostasis (Sorin et al., 2005), the ARGONAUTE protein AGO1, guided by small RNAs and associating with SWI/SNF complexes, was recently described to bind genes activated upon JA, auxin, and SA stimuli in

Arabidopsis (Liu C. et al., 2018). ABA-responsive genes in Arabidopsis are repressed by histone deacetylation (Perrella et al., 2013) through the action of MULTICOPY SUPRESSOR OF IRA (MSI) 1 recruiting the HISTONE DEACETYLASE (HDA)19 (Alexandre et al., 2009; Mehdi et al., 2016) and also by BRM-mediated chromatin remodeling (Han et al., 2012). Significantly, expression of 80% GA–responsive genes relies on the chromatin remodeler PICKLE (PKL) (Park et al., 2017). Consequently, plants with reduced MSI1, HDA19, or BRM levels are more sensitive to ABA, display ABA-dependent growth defects and higher tolerance to drought, and absence of PKL results in GA-reversible root swelling and embryonic lipid accumulation (Ogas et al., 1997) demonstrating the developmental importance of chromatin modifiers in phytohormone-mediated responses.

HORMONE SIGNALING AND CHROMATIN CROSSTALK CAN PARTICIPATE IN PLASTICITY AND ROBUSTNESS

Hormone signaling and chromatin crosstalk can participate in developmental paths by distinct ways: (1) control of cell identity genes in meristems, (2) chromatin-mediated stabilization of gene expression beyond the hormonal initial signal, (3) chromatin-governed integration of separate hormone signaling pathways.

Chromatin-modifying complexes target key phytohormone-regulated genes that specify meristem cell identity and whose ectopic expression can result in cell reprogramming and homeosis (Zuo et al., 2002; Galinha et al., 2007). For example, the SAM-organizing homeobox gene *WUSCHEL* (*WUS*) is regulated by cytokinin signaling, DNA methylation, H3K27me, or chromatin remodeling (Kwon, 2005; Dodsworth, 2009; Cao et al., 2015; Liu H. et al., 2018), and loss of DNA methylation in *WUS* promoter is connected to *in-vitro* shoot initiation induced by cytokinin (Li et al., 2011). Other stem cell niche-defining TFs such as *WOX4, WOX5*, *PLT1,* or *PLT2* are potential PRC2 targets (Oh et al., 2008; Lafos et al., 2011). Co-expression of these TFs can be triggered by environmental and hormone cues or ectopically induced in PRC2-depleted plants, resulting in cell reprogramming (Chanvivattana et al., 2004; Barrero et al., 2007; Ikeuchi et al., 2015; Mozgová et al., 2017). Increased or dispersed expression of cell identity-defining TFs and change and/or loss of cell identity also occurs in mutants of chromatin modifiers such as the repressive H2A-ubiquitinase complex PRC1 (Xu and Shen, 2008; Bratzel et al., 2010; Chen et al., 2010, 2016), histone deacetylases HDA6 and HAD19 (Tanaka et al., 2008; Pi et al., 2015), PKL (Ogas et al., 1999) or replication-dependent H3/H4 chaperone CHROMATIN ASSEMBLY COMPLEX (CAF)-1 (Kaya et al., 2001). Thus, chromatin structure appears to restrict expression of developmental genes to retain cell identities. Similarly, repression of ABA response by several chromatin modifiers (MSI1, HDA19, BRM) could act to prevent an ectopic stress response in favorable environmental conditions.

Chromatin structure may stabilize gene expression state beyond the duration of the environmental or phytohormone stimulus. An example is the cold-induced establishment of H3K27me3 at *FLC* during vernalization that is stable through mitosis, providing an in-cis memory system of *FLC* repression even after transfer to warmth (Berry et al., 2015; Hepworth and Dean, 2015). Persistent H3K4me2/3, H3/H4ac or local nucleosome depletion are found at genes primed for biotic or abiotic stress responses including priming by phytohormones or their analogs (Jaskiewicz et al., 2011; Lämke and Bäurle, 2017; Laura et al., 2018; Liu H. C. et al., 2018) demonstrating that also "accessible" chromatin structure contributes to mitotic memory (**Figure 1**).

Chromatin-modifying proteins may also serve as integrators defining the final outcome of interplay of various hormone signaling pathways. Phytohormone-induced change of chromatin structure may rely on multiple different chromatin modifiers as is exemplified by modulators of ABA signaling. A single chromatin modifier can also be implicated in responses to different hormones, as is exemplified by BRM (Sarnowska et al., 2016). Chromatin can thus provide a robust hub integrating different incoming cues while potentiating the persistence of the gene expression patterns through its stability during mitotic cell divisions.

MERISTEMS ARE CENTRAL PLACES FOR PHYTOHORMONE CHROMATIN CROSSTALK

The biological significance of the crosstalk in meristems is supported by (1) their central role in postembryonic morphogenesis, plasticity and memory, (2) their particularities for phytohomone signaling and chromatin remodeling, (3) first evidences reported for this crosstalk in SAM.

The meristems represent major sites of stem cell niches in plants (Scheres, 2007; Tucker and Laux, 2007; Aichinger et al., 2012). Apical meristems, together with the secondary meristem, the cambium, have the capacity to maintain and self-renew populations of undifferentiated cells, underlying continuous post-embryonic organ development modulated by environmental conditions (**Figure 1**; Gaillochet and Lohmann, 2015; Pavlovic and Radotic, 2017; Xiao et al., 2017). The SAM is also the place of epigenetic memory as reported for vernalization and some priming effects (Hepworth and Dean, 2015; Lämke and Bäurle, 2017).

Phytohormone and epigenetic pathways play overlapping/complementary roles in meristem functions and developmental plasticity or robustness, laying the basis for a biologically significant crosstalk. Importantly, meristems have been shown to be the place of epigenetic control for stem cell pluripotency, differentiation, and reprogramming (Cao et al., 2015; Gaillochet and Lohmann, 2015; Pi et al., 2015; Morao et al., 2016; Ojolo et al., 2018) whose epigenetic setup may differ from the surrounding tissues (Yadav et al., 2009; Baubec et al., 2014).

Major evidence for phytohormone-chromatin crosstalk was obtained using Arabidopsis mutants, or applying phytohormones or chemical inhibitors of chromatin modifiers in various developmental processes (Yamamuro et al., 2016; Campos-Rivero et al., 2017; Wong et al., 2017; Guo et al., 2018; Ojolo et al., 2018; Wakeel et al., 2018; Zheng et al., 2018).

Only a few reports highlight potential crosstalk directly in the meristems as exemplified by PRC2 repressing particular *PIN* genes (auxin transporters) in the SAM of Arabidopsis *clv3* mutants (Lafos et al., 2011). Recent studies in vernalized sugar beet (Hébrard et al., 2016) and in poplar under drought or cold exposure (Conde et al., 2017; Lafon-Placette et al., 2018; Le Gac et al., 2018) have recently shown that differentially expressed genes under DNA methylation control in SAM correspond to a limited developmental gene network mainly involved in growth and phytohormone pathways such as jasmonate activators and ethylene repressors. Indeed, Le Gac et al. (2018) show that hormone-related epigenome reprogramming in the SAM of poplar hybrids is stable for at least several months after the stress period in winter-dormant SAM providing evidence of an environmental epigenetic memory. Recently, this phenomenon was also described in the SAM of natural populations of black poplar under drought conditions (Sow et al., 2018a). Similarly, support for epigenetic memory of climatic conditions is found in Norway spruce trees grown from somatic embryos produced at different temperatures (Yakovlev et al., 2011, 2016). Considering the absence of post-embryonic organs, the SAM could play a major role in the transmission of the environmentally-established chromatin states during early development.

CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, phytohormone action and chromatin modifiers seem to be tightly interacting but the extent to which they act jointly or independently remains unclear (Ojolo et al., 2018). However, the multi-layered control of local chromatin structure in response to hormonal cues may provide an important hub that integrates the incoming cues, conferring developmental robustness while retaining a sufficient potential for gene transcription change, stabilization and phenotypic plasticity (Lachowiec et al., 2016).

Current knowledge leads to the opinion that this crosstalk in meristems can integrate environmental cues for developmental outcome. Erasure of this signaling may allow continuous adjustment to new environmental conditions. Its maintenance through persistent chromatin states can however stimulate mitotic memory that could prime later organ formation. How the balance between erasure and memory is achieved remains enigmatic (**Figure 1**).

While the mechanistic events could be more easily deciphered in well-established model annuals such as Arabidopsis, it is important to establish perennial models where the impact of mitotic epigenetic memory is of importance in the context of climate change. In addition to SAM and RAM, cambium, whose activity is crucial for environmentally controlled wood formation, may be an appropriate and highly relevant model (Wang et al., 2016; Oles et al., 2017; **Figure 1**). Deciphering this crosstalk in the meristems requires improving single-cell methodologies to study the dynamics of chromatin structure in response to complex phytohormone-associated environmental and developmental responses and its memory. Exploiting epigenetic variation and the potential to derive primed plants from meristem regeneration or somatic embryos (Achour et al., 2017; Gallusci et al., 2017; Springer and Schmitz, 2017; Sow et al., 2018b) seems also promising.

AUTHOR CONTRIBUTIONS

SM suggested and designed the opinion article. MDS and SM designed the **Figure 1**. IM and SM finalized and revised the article. All the authors checked and confirmed the final version of the manuscript. All the authors drafted the entire manuscript.

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REVIEW
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Chromatin Evolution-Key Innovations Underpinning Morphological Complexity

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The history of life consists of a series of major evolutionary transitions, including emergence and radiation of complex multicellular eukaryotes from unicellular ancestors. The cells of multicellular organisms, with few exceptions, contain the same genome, however, their organs are composed of a variety of cell types that differ in both structure and function. This variation is largely due to the transcriptional activity of different sets of genes in different cell types. This indicates that complex transcriptional regulation played a key role in the evolution of complexity in eukaryotes. In this review, we summarize how gene duplication and subsequent evolutionary innovations, including the structural evolution of nucleosomes and chromatin-related factors, contributed to the complexity of the transcriptional system and provided a basis for morphological diversity.

Keywords: gene duplication, evolution, chromatin, transcriptional regulation, morphological complexity, microbiota, symbiosis

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INTRODUCTION

Early organisms on Earth were microscopic, and for the first 2500 million years (Myr), living organisms rarely achieved a complexity higher than two or three cell types (Carroll, 2001). Around 500 Myr ago from the mid-Cambrian to early Ordovician, land plants that are a major focus of this review likely evolved from a lineage of unicellular eukaryotes in charophyte green algae (Stebbins and Hill, 1980; Kenrick and Crane, 1997; Harholt et al., 2016; Del-Bem, 2018; Morris et al., 2018). With the evolution of land plants, these more complex organisms colonized the Earth and transformed the biosphere providing habitable environments for terrestrial organisms by supplying sufficient oxygen and nutrients (Hori et al., 2014). Recent evolutionary analyses indicate that the cell wall, symbiotic signaling pathways, the RPB1 heptapeptide repeats, hormonal biosynthesis or signaling pathways, and desiccation and UV radiation tolerance evolved in charophyte green algae prior to land plants (Stebbins and Hill, 1980; Hajheidari et al., 2013; Hori et al., 2014; Yang and Stiller, 2014; Delaux et al., 2015; Ju et al., 2015; Harholt et al., 2016; Del-Bem, 2018). This demonstrates that charophyte green algae were preadapted to cope with harsh terrestrial environments. The greater complexity of unicellular eukaryotes and the evolution and diversification of land plants could not be possible without the existence of a high level of cellular complexity and elaborate mechanisms for gene regulation in unicellular eukaryotic ancestors (**Figure 1**).

Eukaryotes have a high degree of cellular complexity. The genomes of most eukaryotes are larger than those of prokaryotes, however, in eukaryotes, in contrast to prokaryotes, genome size does not show a good correlation with gene number (Valentine, 1978; Gregory, 2005). Furthermore, an increase in genome size or gene number is not a good criterion for developmental and morphological complexity. For example, the genome of the bryophyte *Physcomitrella patens* is

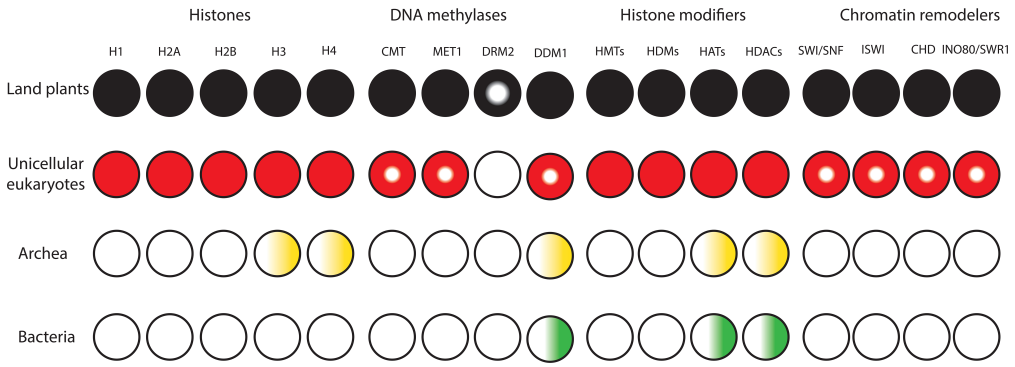

FIGURE 1 | Diversification and expansion of histones, chromatin remodelers and modifiers in the domains of life. Filled circles denote presence of orthologs in all lineages. Semi-filled circles indicate factors present in part of the lineage. Gradients indicate presence of homologs. White circles indicate the lack of homologs.

about 480 MB and possesses approximately 35,938 genes, while *Arabidopsis thaliana*, with much higher morphological complexity, has a smaller genome (∼135 MB) containing about 27,235 genes (Rensing et al., 2008). To understand the evolution and diversification of morphological complexity two questions should be addressed. First, which factors were the major genetic resources underpinning morphological complexity? And secondly, how does morphological diversity evolve? We know that all cells of a complex multicellular organism contain the same genome, however, their organs are composed of a variety of cell types that differ dramatically in both structure and function. The distinctiveness of a given cell type is determined by controlled transcriptional activity of distinct sets of genes in a cell lineage. Complexity is a term with different definitions (Carroll, 2001). However, the number of cell types is broadly considered an indicator for morphological/organismal complexity (Carroll, 2001; Levine and Tjian, 2003; Chen et al., 2014). This suggests that complex transcriptional regulation plays a key role in the evolution of complexity in eukaryotes. This is in agreement with a higher proportion of transcription factors in more complex organisms with high evolutionary distances within each kingdom (**Table 1**). Moreover, the rate of expansion of transcriptional regulators is faster than linearly for every gene added to the genome (Levine and Tjian, 2003; Charoensawan et al., 2010a; Rensing, 2014). However, in many cases evolution is not necessarily accompanied by higher morphological complexity or with an increased number of transcriptional regulators (Wolf and Koonin, 2013).

GENE DUPLICATION - A MAJOR DRIVER IN THE EVOLUTION OF MORPHOLOGICAL COMPLEXITY

Genomic studies have revealed notable increases in the number of genes, intergenic regions, intragenic regions (introns), and transposons from prokaryotes to multicellular eukaryotes. Whole-genome and small-scale duplications are known as essential sources for the evolution of functional novelty and morphological complexity (Ohno, 1970; Lynch and Conery, 2003; Gregory, 2005; Bratlie et al., 2010). Increases in organismal complexity are repeatedly coupled to short-term large-scale increases in gene number in the history of eukaryotes (Ohno, 1970; Gu et al., 2002; McLysaght et al., 2002; Maere et al., 2005; Vanneste et al., 2014). For example, eukaryotic RNA polymerases (PolI, PolII, and PolIII) evolved due to massive gene duplications during the transition from an archaeum to a fully fledged eukaryote (Koonin, 2015). Whole-genome duplications in plants normally lead to genomic instability, alteration of gene expression and cell division abnormalities (Comai, 2005). On the other hand, the genomic plasticity of polyploids is higher than diploids and this may lead to increased tolerance of polyploidy in a broader range of environmental conditions. Recent studies suggest that challenging environmental conditions may positively enhance short-term polyploid establishment and survival (for a detailed review see, Van de Peer et al., 2017). After genome duplication, duplicated genes can have different evolutionary fates. Duplicated genes predominantly become pseudogenes/silent due to non-adaptive accumulation of deleterious mutations (non-functionalization) within a few Myr (Lynch and Conery, 2003; Maere et al., 2005). In an evolutionary study in rodents, it was shown that one copy of duplicates, which is usually the novel daughter copy, experiences a fivefold higher divergence rate within 4–12 Myr after duplication. Subsequently, the divergence rate decreases and after 40.5 Myr returns to preduplication levels (Pegueroles et al., 2013). A subset of duplicates may stay active by different mechanisms. For example, an increase in the expression of duplicates can be beneficial (gene dosage) or both duplicates can be essential to keep the ancestral function (subfunctionalization). In addition, duplicates can be important to maintain the stoichiometric balance (gene balance) or to prevent interference between the products of paralogs (paralog interference). Duplication can also lead to the evolution of novel functions. Neofunctionalization arises after gene duplication resulting in one gene copy keeping the ancestral function and the second copy becoming fixed by positive selection. In addition, functional novelty can also arise due to escape from adaptive conflict (EAC). In this case, the evolution of a novel function in the ancestral copy before

TABLE 1 | Organismal/morphological complexity correlates with the proportion of transcriptional regulators within each kingdom when the evolutionary distance between organisms is high.

Kingdom	Species	No. of cell types	Genome size	No. of genes	No. of TFs	Proportion of TFs
Metazoa	*Homo sapiens*	264.5	∼ 3.3 GB	∼ 22997	∼ 1508	0.0656
	Mus musculus	130.5	∼ 2.7 GB	∼ 23873	∼ 1426	0.0597
	Tetraodon nigroviridis	119.5	∼ 390 MB	∼ 27991	∼ 1362	0.0487
	Drosophila melanogaster	59	∼ 175 MB	∼ 14141	∼ 601	0.0425
	Caenorhabditis elegans	28.5	∼ 100 MB	∼ 20140	∼ 698	0.0347
	Nematostella vectensis	22	∼ 450 MB	∼ 27273	∼ 701	0.0257
	Trichoplax adhaerens	4	∼ 50 MB	∼ 11520	∼ 233	0.0202
Viridiplantae	*Zea mays*	100	∼ 2.5 GB	∼ 45796	∼ 2689	0.0587
	Arabidopsis thaliana	27.25	∼ 135 MB	∼ 27235	∼ 1356	0.0498
	Selaginella moellendorffii	25	∼ 100 MB	∼ 22273	∼ 665	0.0299
	Physcomitrella patens	21	∼ 480 MB	∼ 35938	∼ 823	0.0229
	Marchantia Polymorpha	NA*	∼ 225 MB	∼ 32718	∼ 586	0.0179
	Klebsormidium nitens	1	∼ 117 MB	∼ 16215	∼ 273	0.0168
	Chlamydomonas reinhardtii	1	∼ 107 MB	∼ 15256	∼ 213	0.0140
	Chlorella sp. NC64A	1	∼ 46.2 MB	∼ 9791	∼ 131	0.0134

*The arrangement of organisms within each kingdom is based on organismal complexity. Proportion of transcriptional factors (TFs) represents the ratio of number of TFs to number of genes. Data were mostly obtained from transcription factor prediction database, only the longest transcript per gene was included in this study (http://www.transcriptionfactor.org) (Valentine et al., 1994; Bell and Mooers, 1997; McCarthy and Enquist, 2005; Vogel and Chothia, 2006; Charoensawan et al., 2010b; Burdo et al., 2014; Chen et al., 2014; Hori et al., 2014; Matsumoto et al., 2016; Jiao et al., 2017). *NA, not available.*

duplication has reduced the ability of the gene to carry out the original function and after duplication each copy can freely optimize the ancestral or the novel function (Ohno, 1970; Lynch, 2000; He and Zhang, 2005; Conant and Wolfe, 2008; Des Marais and Rausher, 2008). It has been shown that the decay rates of paralogs derived from small-scale duplications are considerably higher than those derived from large-scale duplications (Maere et al., 2005; Freeling, 2009). Furthermore, after whole genome duplication the retention rate of different genes is not similar. For example, genes that are involved in transcriptional regulation, signal transduction, and development have a higher retention rate than other functional categories (Blanc, 2004; Seoighe and Gehring, 2004; Maere et al., 2005). On the other hand following a large-scale duplication and emergence of polyploid organisms, most of the duplicates are deleted or non-functionalized over time and genome size reduction is accompanied by extensive genome reorganization. This process is called diploidization and leads to the conversion of polyploids to diploids over a period of several Myr and species that emerge by diploidization following polyploidization are called palaeopolyploids. All extant angiosperms are indeed palaeopolyploid (Olsen and Wendel, 2013; Dodsworth et al., 2016). It is also important to consider that whole-genome duplication in animals in general is less common than in plants (Hallinan and Lindberg, 2011; Nossa et al., 2014; Clarke et al., 2015; Schwager et al., 2017; Li et al., 2018).

ALTERATION OF GENE EXPRESSION PATTERNS AND MORPHOLOGICAL COMPLEXITY

Pioneering studies in molecular evolutionary biology revealed that there is relatively little protein divergence among mammalians such as chimps and humans, although their phenotype and behavior are very different (Britten and Davidson, 1971; Wilson et al., 1974; King and Wilson, 1975). These studies led to the proposal that the evolution of complexity occurred more by altering gene regulation than by changing protein sequences. In agreement with this proposal, later studies showed that many homologous proteins, despite long term (∼ 500 Myr) independent evolution in different lineages, are often functionally equivalent (Grens et al., 1995; Halder et al., 1995). Furthermore, vital roles are attributed to conserved protein sequences and their mutations are deleterious or lead to pleiotropic effects and are thus under purifying selection (Grens et al., 1995; Halder et al., 1995; Hoekstra and Coyne, 2007). However, alteration of their expression level or pattern is usually non-deleterious and this is mostly due to the modular nature of *cis*-regulatory elements (Hoekstra and Coyne, 2007).

The morphological complexity of multicellular organisms relies on spatio-temporal patterns of developmentally important regulatory factors (Spitz and Furlong, 2012). The precise expression patterns of master developmental regulators are mostly governed by enhancers/*cis*-regulatory modules that integrate signaling and tissue-specific inputs to specify times and locations of gene expression (Shen et al., 2012). Enhancers are short DNA sequences that contain multiple sites for sequence-specific transcription factors (Shlyueva et al., 2014). In prokaryotes, enhancer-dependent gene regulation is less common and the regulatory regions of prokaryotes and unicellular eukaryotes are usually composed of short sequences in the vicinity of the core promoter (Gralla, 1996; Wyrick and Young, 2002). However, enhancers in multicellular eukaryotes are scattered across the genome and found upstream and downstream of genes. The birth of enhancers is mediated by various mechanisms during evolution. Duplication and

rapid/subsequent diversification of enhancers is an important source for the genesis of new enhancers (Goode et al., 2011; Vlad et al., 2014). New enhancer sequences can emerge from non-regulatory sequences or older enhancer elements via random genetic drift or adaptive selection (Frankel et al., 2011; Rebeiz et al., 2011; Duque and Sinha, 2015; Villar et al., 2015). Transposable elements (TEs) are also important material for tinkering with eukaryotic transcriptional regulatory systems (Jordan et al., 2003; Cao et al., 2016). Enhancers can control genes that are located far away; therefore, one gene can be regulated by multiple distal and close enhancers with different spatiotemporal activities. Furthermore, one enhancer may regulate the activity of multiple genes. These features facilitate a vast combinatorial complexity of transcriptional regulation with a relatively limited set of genes (Long et al., 2016).

It is important to consider that alteration in heritable gene expression patterns is due either to diversification of *cis*-regulatory elements or *trans*-regulatory factors (transcription regulators and non-coding RNAs). Recent studies have quantified the relative contribution of *cis*- and *trans*-regulatory factors to the evolution of gene expression, which as shown above is a key player in the evolution of morphological complexity. These studies suggested that *trans*-regulatory factors have a higher contribution to gene expression alteration than *cis*-regulatory factors within a given species. However, as sequence divergence or evolutionary distance increase, *cis*-regulatory differences become the dominant contributor in gene expression alteration. This relative contribution of *cis*-regulatory elements and *trans*-regulatory factors in the regulation of gene expression varies amongst taxa (Metzger et al., 2017; Osada et al., 2017).

Many studies have shown evolutionary changes through diversification of regulatory elements or protein-coding sequences (Stern, 1998; Arnaud et al., 2011; Vlad et al., 2014; Kusters et al., 2015; Sicard et al., 2016; Vuolo et al., 2016; Jiang and Rausher, 2018). Reduced complexity (RCO) evolution is an interesting example in plants that shows how gene duplication and subsequent diversification in regulatory elements and coding sequences played a key role in the evolution of morphological diversity within the Brassicaceae family. Vlad et al. (2014) discovered that a tandem duplication of the *LATE MERISTEM IDENTITY 1 (LMI1)* gene has given rise to two new copies in Cardamine. One of the copies has become a pseudogene owing to accumulation of deleterious mutations, whereas another copy located immediately downstream of the *LMI1*gene locus is active. *LMI1* is expressed in the margins of leaflet, stipules, and flowers. In contrast to *LMI1*, the novel active copy *RCO* is essential for the formation of the complex leaves in *C. hirsuta*. It is expressed at the base of the leaflet and promotes leaflet formation through local growth repression. *RCO* was lost in the lineage that gave rise to *A. thaliana* leading to simplification of the leaves in this species. When the *RCO* promoter drives the expression of the *LMI1* gene at the base of leaflets, the *LMI1* gene acts similar to *RCO* and represses the growth at the flank of developing leaflets. This demonstrated that neofunctionalization has occurred due to diversification of regulatory elements. Later studies uncovered that indeed RCO enhancer evolution likely coevolved with a single amino acid change. This change led to the reduction of RCO protein stability, which is required for minimizing the pleiotropic effects of the RCO enhancer (Vuolo et al., 2016). The evolution of domesticated maize (*Zea mays* ssp. *mays*) from its wild relative teosinte (*Z. mays,* ssp. *Parviglumis*) is also an excellent example of morphological evolution through directional selection during domestication. Since the crop plant maize and teosinte are morphologically very different, taxonomists once placed them in separate genera (Doebley et al., 1997). However, later studies demonstrated that these plants are close relatives and expression alteration of a few transcription factors led to great morphological divergence and played a substantial role in the emergence of cultivated maize from teosinte. Diversification of regulatory elements of *teosinte branch1* (*tb1*) and *barren stalk1* (*ba1*), which encode bHLH transcription factors, had a great impact on positioning of the male inflorescence and conversion of lateral branches of teosinte into the maize ear (Doebley et al., 1997; Gallavotti et al., 2004; Clark et al., 2006). In teosinte, kernels are tightly sealed in a stony casing, while the kernels of crop maize are naked and could readily be consumed by animals or humans. Surprisingly, just a single amino acid change in the SBP-box transcription factor *teosinte glume architecture1* (*tga1*) was the cause of the liberation of kernels from the hardened cupulate fruitcases (Wang et al., 2005, 2015).

THE STRUCTURAL EVOLUTION OF THE NUCLEOSOME AS A PREREQUISITE STEP FOR MORPHOLOGICAL COMPLEXITY

To the best of our knowledge, all domains of life rely on DNA to store and inherit genetic information. Factors that alter the conformation of DNA to make it fit inside the cell/nucleus are present in all kingdoms of life and have the potential to influence transcription. Bacteria lack histones and contain nucleoid-associated proteins (NAPs) that are major DNA-binding factors facilitating chromosomal domain formation and organization (**Figure 1**; Luijsterburg et al., 2008). In bacterial cells, there is no inherent barrier for RNA polymerases to gain access to the DNA (Struhl, 1999; Dillon and Dorman, 2010). Archaeal cells also have circular DNA, as in bacteria. The phylum Crenarchaeota in the archaea domain generally lack histone proteins and their chromosome organization relies on Alba proteins, which are NAPs. However, the phylum Euryarchaeota in archaea mainly contain histone proteins that lack flexible tails at their N-terminus (Williams and Embley, 2014; Peeters et al., 2015). *Methanopyrus kandleri* and *Halobacterium* NRC1 in Euryarchaeota contain unusual "doublet histones" that have evolved through an end-to-end duplication of the histone fold. The ancestral gene encoding a doublet histone was split and diverged into H3 and H4 to form H3–H4 tetramers. H2A and H2B likely evolved later through a second specialization of a doublet as well (Ng et al., 2000; Malik and Henikoff, 2003).

Eukaryotic histones are derived from a common ancestor shared with Archaea. Archaeal chromatin-like structure is apparently important for DNA protection from thermal denaturation (Reeve, 2003; Sandman and Reeve, 2005). Eukaryotic cells contain very stable, compact, and at the same time very dynamic chromatin. Nucleosomes are the fundamental units of chromatin that consist of ∼147 base pairs of DNA wrapped around a core of eight histone proteins comprising two copies of histone H3, H4, H2A, and H2B. The tails of core histones protrude from the nucleosome core particle and many residues in these tails can be post-translationally modified, influencing all DNA-based processes, including transcription (Venkatesh and Workman, 2015). Chromatin also contains linker DNAs (∼10–90 bp) that connect nucleosomes and interact with histone H1 (Han and Grunstein, 1988; Szerlong and Hansen, 2011; Zhou et al., 2013). In higher eukaryotes, H1 histones have three domains, a highly conserved central globular domain, an unstructured short N-terminal domain, and a long basic C-terminal domain (Ramakrishnan et al., 1993). Linker histone-like proteins are found in eubacteria, which are likely the provenance of H1 histones (Kasinsky et al., 2001). These proteins are similar to the C-terminal domain of H1 histones in higher eukaryotes, however, they have no globular domain. Linker histones are diverse and perform various roles in processes such as chromatin organization, genome stabilization, transcriptional regulation, and embryogenesis (Hergeth and Schneider, 2015; Kotliński et al., 2016; Bayona-Feliu et al., 2017). In contrast to prokaryotes, the compact structure of chromatin in eukaryotes generated an inherent barrier for DNA-based processes. This was one of the key prerequisite steps in the evolutionary trajectory of complex multicellular organisms.

EVOLUTION OF CHROMATIN REMODELERS AND MODIFIERS

The compact structure of chromatin in eukaryotes prevents free access of transcription factors to *cis*-regulatory DNA elements. In addition to transcription factors, proteins involved in replication and repair must be able to access DNA. To tackle this barrier, it was necessary for early eukaryotes to evolve and expand classes of chromatin modifiers and remodelers to facilitate access to DNA (**Figure 1**). Due to the possession of mitochondria, Eukaryotes had more available energy to encode a higher level of proteins. This together with genome expansion likely generated evolutionary pressure for co-evolution of high density chromatin packaging and chromatin-modifying factors in early eukaryotes (Flaus et al., 2006; Lane and Martin, 2010; Garg and Martin, 2016; Koster et al., 2015; Martin and Sousa, 2016). Chromatin modifiers and remodelers further expanded and diversified in eukaryotes. This led to the establishment of distinct classes of chromatin-modifying factors with unique functional complexes that facilitate binding of transcription factors to *cis*-regulatory DNA elements in a cell-type-specific manner in higher eukaryotes (Gentry and Hennig, 2014; Sarnowska et al., 2016; Zhou et al., 2016). The major chromatin-modifying factors are DNA methyltransferases (DNMTs), histone deacetylases (HDACs), histone acetyltransferases (HATs), histone methyltransferases (HMTs), histone demethylases (HDMs), and chromatin remodelers.

DNA Methyltransferases

In prokaryotes, as a part of the restriction-modification (RM) systems DNA methylases cooperate with restriction enzymes to protect the genome against foreign DNA. Prokaryotic DNA methylases evolved from ancient RNA-modifying enzymes and are the provenance of eukaryotic DNA methylases. In eukaryotes, multiple independent duplications, losses, and divergences led to the emergence of distinct types of DNA methylases, which are involved in a range of activities, including gene and transposon silencing, imprinting, transcriptional activation, and post-transcriptional regulation (Law and Jacobsen, 2010; Blow et al., 2016; Lyko, 2018). In Arabidopsis, *de novo* cytosine methylation is catalyzed by DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) and the DNA methylation pattern is maintained by METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), as well as DRM2. Interestingly, DNA methylation could create a basis for morphological diversity by regulating DNA binding affinity of transcription factors. For example, epigenetic mutation of the *Lcyc* gene inhibits its expression and modifies the symmetry of the flowers from bilateral to radial in *Linaria vulgaris* (Cubas et al., 1999). DNA hyper-methylation in the promoter region of a SBP-box transcription factor, COLORLESS NON-RIPENING (Cnr), leads to colorless and abnormal ripening of fruits in tomato without changes in nucleotide sequence (Manning et al., 2006). DNA methylation in eukaryotes can also be guided by non-coding RNAs. Small RNA-directed DNA methylation (RdDM) pathways play a key role in maintenance of genome stability and developmental regulation (Castel and Martienssen, 2013; Matzke and Mosher, 2014). The canonical RdDM model suggests that the target loci are transcribed by Pol IV and the primary transcripts are converted to dsRNAs by RDR2. These dsRNAs are processed into mature 24nt repeat-associated siRNA (ra-siRNA) by DCL3, methylated by HEN1, and loaded into RISC-like RITS (RNA-induced transcriptional silencing) complexes containing AGO4 and Pol V, which scan the genomic DNA to drive DNA methylation at target loci carrying complementary sequences (Cao et al., 2003; Zilberman et al., 2003; Wierzbicki et al., 2008; Law and Jacobsen, 2010).

The MORC ATPase family is an evolutionary conserved protein family that is prevalent in both prokaryotes and eukaryotes (Iyer et al., 2008). However, in eukaryotes, especially in the plant kingdom it greatly expanded through gene duplication (Dong et al., 2018). Using contextual information, Iyer et al. (2008) suggested that MORC proteins may play a substantial role in the bacterial RM system. MORC proteins are required for meiotic division in animals and pathogen-associated molecular pattern (PAMP)-triggered immunity in plants (Watson et al., 1998; Kang et al., 2012; Liu et al., 2016; Dong et al., 2018). The Arabidopsis genome contains seven *MORC* genes (*AtMORC1-7*). It has been demonstrated that MORC1, MORC2, and MORC6 are involved in gene silencing and transposon suppression without changing genome-wide

DNA methylation patterns (Moissiard et al., 2012, 2014; Bordiya et al., 2016). However, MORC-mediated transcriptional silencing depends, at least in part, on the interaction with the RdDM components (Lorković et al., 2012; Brabbs et al., 2013; Liu et al., 2016).

Histone Modifiers

Post-translational modification of histones also plays a key role in the regulation of chromatin dynamics. Transcriptionally active chromatins usually contain trimethylated histone H3K4 and highly acetylated histone H3 and H4. In contrast, transcriptionally silent chromatins are enriched in the methylation of lysine 9 and/or 27 of histone H3 (Hebbes and Thorne, 1988; Jenuwein and Allis, 2001; Fischle et al., 2003). Histone methylation is catalyzed by three distinct protein families; the SET domain-containing protein family, the non-SET domain proteins Dot1/Dot1L, and the PRMT1 family. In contrast to histone acetyl/ deacetyltransferases and based on early phylogenetic analysis, it was concluded that the SET domain-containing methyltransferases evolved in the eukaryotic lineage and the bacterial SET domain was the result of horizontal gene transfer from a eukaryotic host (Stephens et al., 1998; Iyer et al., 2003). However, a recent phylogenetic study using an expanded collection of prokaryotic genomes showed that the SET domain is found in free-living bacteria as well as in pathogenic bacteria. Interestingly, these enzymes are involved in the synthesis of secondary metabolites, such as antibiotics in bacteria (Iyer et al., 2011; Alvarez-Venegas, 2014). Thus, the SET domain is also an ancient catalytic domain. The SET-domain proteins are grouped into seven families (Ng et al., 2007) and are members of different complexes with broad functions. For example, polycomb group proteins (PcG) that act as chromatin-based transcriptional repressors, generally form two multimeric complexes, the polycomb repressive complexes 1 (PRC1) and PRC2. The histone methyltransferase Enhancer of Zeste [E(z)], which is the catalytic subunit of PRC2, catalyzes the trimethylation of histone H3 lysine 27 (H3K27me3) via its SET domain (Goodrich et al., 1997; Cao et al., 2002; Czermin et al., 2002). Arabidopsis consists of three H3K27me3 HMTs, CURLY LEAF (CLF), SWINGER (SWN), and MEDEA (MEA). The loss of function mutation of *CLF* and *SWN* that act, at least in part, redundantly leads to development of embryo- or callus-like structures in Arabidopsis (Goodrich et al., 1997; Grossniklaus et al., 1998; Chanvivattana et al., 2004). The prior positioning of H3K27me3 by the PRC2 complex is normally required for the recruitment of PRC1 and subsequent monoubiquitylation of histone H2A on lysine 119 (H2AK119ub1). However, PRC2 recruitment through PRC1-dependent H2A119ub1 has also been reported (Landeira et al., 2010; Blackledge et al., 2014). In contrast to PcG, the TRithoraX Group (trxG) proteins activate transcription by catalyzing methylation of histone H3 on lysine 4 (H3K4) via their SET domain. PcG and trxG proteins are essential in establishment and maintenance of cell identity and organ development in higher eukaryotes through permanent/dynamic transcriptional regulation of developmentally important genes (Alvarez-Venegas, 2010; Schuettengruber et al., 2017). Thus, they play a substantial role in morphological complexity. Phylogenetic analysis of the SET-domain proteins suggests that four families of the SET-domain proteins were present before the divergence of plants, metazoans, and fungi and later highly expanded and diverged in each kingdom mostly due to large-scale duplication (Zhang and Ma, 2012).

Histone demethylases are classified into two distinct families, the KDM1/LSD1 and JmjC domain-containing proteins. The catalytic domain of KDM1 genes is the AOD domain. The AOD domain is found in prokaryotes suggesting that prokaryotes are the provenance of eukaryotic KMD1-type HDMs. The eubacterial *Cupin* genes are likely the ancestor of all JmjC domain-containing proteins. Whole-genome duplication was likely the major driving force for the expansion and diversification of JmjC domain-containing proteins in complex multicellular eukaryotes (Qian et al., 2015). In contrast to eubacterial proteins that contain only the JmjC domain, most of the eukaryotic proteins contain complex architectural domains (Zhou and Ma, 2008; Qian et al., 2015).

Histone acetyltransferases and deacetylases both contain ancient catalytic domains, and members of the GCN5-related N-acetyltransferase (GNAT) superfamily and the histone deacetylase superfamily are found in all kingdoms of life. However, these enzymes were greatly expanded and diversified in multicellular eukaryotes (Leipe and Landsman, 1997; Gregoretti et al., 2004; Boycheva et al., 2014; Marinov and Lynch, 2016). HATs are grouped into two classes according to their intracellular localization, i.e., into A-type and B-type. B-type HATs are localized in the cytoplasm and catalyze acetylation of free histones. However, A-type HATs are localized in the nucleus and catalyze acetylation of the nucleosome core histones. In Arabidopsis, A-type HATs are classified into four groups based on their sequence and structural similarities (Eberharter et al., 1996; Pandey et al., 2002): (1) Gcn5-related N-acetyltransferases (GNATs), (2) The MYST-related HATs, (3) cAMP-responsive element-binding protein (CBP), and (4) TATA-binding protein associated factor (TAFII250). The HDACs are also classified into four groups: (1) Reduced Potassium Dependency 3 (RDP3), (2) Histone DeAcetylase 1 (HDA1), (3) Silent Information Regulator 2 (SIR2), and (4) Histone Deacetylase 2 (HD2) (Shen et al., 2015).

Chromatin Remodelers

Transcription-relevant chromatin remodeling ATPases are classified into four distinct families (SWI/SNF, ISWI/SNF2L, CHD/Mi-2, and INO80/SWR1) that are functionally and genetically non-redundant based on their structure. The catalytic/ATPase domain of remodelers consists of two covalently linked RecA-like lobes. Chromatin remodeling complexes hydrolyze ATP and convert the chemical energy resulting from hydrolysis into mechanical motion, including sliding of the nucleosomes along the DNA, disassembling the nucleosome and exchanging histone variants (Flaus et al., 2006; Bannister and Kouzarides, 2011; Zhou et al., 2016). Phylogenetic studies have suggested that eukaryotic

chromatin remodeling ATPases have likely evolved from the ancestral Snf2-like proteins in bacteria after the innovation of chromatin-binding domains in early eukaryotes (Flaus et al., 2006; Koster et al., 2015). The Arabidopsis orthologs of yeast SWI2/SNF2 are BRM, SYD, CHR12/MINU1, and CHR23/MINU2. Structurally, BRM is the closest ortholog to yeast SWI2/SNF2. It contains a helicase/SANT-associated (HAS) domain upstream of ATPase that is a binding platform for nuclear actin-related proteins (Szerlong et al., 2008) and a C-terminal bromodomain, which is capable of binding to acetylated lysine (Dhalluin et al., 1999; Jacobson et al., 2000). In *A. thaliana*, SWI2/SNF2 proteins assemble into different large complexes and control various activities such as plant growth and development (Sarnowska et al., 2016). The ISWI complexes were initially isolated from *D. melanogaster*. In *A. thaliana*, CHROMATIN REMODELING11 (CHR11) and CHR17 are orthologs of ISWI in *D. melanogaster*. They contain an ATPase domain at their N-terminus and HAND, SANT, and SLIDE domains at their C- terminus. AtISWI proteins, which are functionally redundant, form different complexes with the AtDDT (DNA-binding homeobox and different transcription factors)-domain proteins and control multiple developmental processes (Li et al., 2014). Proteins from the CHD/Mi-2 family contain two tandemly arranged chromodomains at the N-terminus that are able to interact with methylated histones and/or DNA. The CHD/Mi-2 family evolved soon after the onset of the eukaryotic lineage and further expanded in higher eukaryotes (Hargreaves and Crabtree, 2011; Gentry and Hennig, 2014; Koster et al., 2015). *Saccharomyces cerevisiae*, *A. thaliana*, and humans consist of one, four, and nine *CHD* genes, respectively (Koster et al., 2015). CHD remodelers positively or negatively control transcription and are also involved in mRNA processing (Murawska and Brehm, 2011; Hu et al., 2014). The chromatin-remodeling complexes of the INO80 group are INO80 and SWR1 in yeast. A single *INO80* and *SWR1/PIE1* (*PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1*) are present in Arabidopsis. The INO80/SWR1 complexes similarly, to other chromatin-remodeling complexes work as transcriptional regulators. In addition, they are implicated in the DNA-repair system and are required for DNA recombination (Noh and Amasino, 2003; Fritsch et al., 2004; Gerhold and Gasser, 2014).

SYMBIOSIS AND MULTICELLULARITY

It is well documented that mitochondria and chloroplasts of eukaryotic cells, which are descended from α-proteobacteria-like and cyanobacteria-like prokaryotes, respectively, arose through endosymbiosis (Weeden, 1981; Gray et al., 1999). Thus, endosymbiosis played a crucial role in the evolution of cellular complexity. Multicellular organisms harbor a vast diversity of microbes, comprising fungi, bacteria, protists, and viruses, collectively called microbiota (Almario et al., 2017; Durán et al., 2018). Molecular clock estimates of fungal phylogeny suggest that Ascomycota, Basidiomycota, and Glomales, which are major taxonomic groups of terrestrial fungi, were present around 600 myr ago (Redecker et al., 2000) and fossilized spores and fungal hyphae that are very similar to extant arbuscular mycorrhizal fungi (AMF) with the age of 460–480 myr support molecular estimates (Selosse and Le Tacon, 1998; Redecker et al., 2000; Heckman et al., 2001). Considering that early land plants colonized poorly developed soils and did not have true roots, the establishment of AMF symbiosis supplying nutrients, water, and enhancing tolerance to biotic and abiotic stresses was a key event in the terrestrialization process (Redecker et al., 2000; Heckman et al., 2001; Rausch et al., 2001; Kenrick and Strullu-Derrien, 2014; Almario et al., 2017; Xue et al., 2018). In addition to fungi, bacterial micribiota are a substantial part of diverse assemblages of symbiotic microorganisms and are critical for plant survival (Durán et al., 2018). Surprisingly, bacterial symbiosis is required for cell division and morphogenesis in *Ulva mutabilis*, which is a green macroalgae and an important primary producer in coastal ecosystems (Wichard, 2015). Taken together, these lines of evidences suggest that symbiosis played an important role in the transition from water to land and the evolution of multicellularity. Organism-associated microbes had a great impact on phenotypic extension and host evolution. In evolutionary studies, considering the host and its associated microbiota as a biological entity, the holobiont could be key for a better understanding of the evolution of multicellular organisms (Shropshire and Bordenstein, 2016; Almario et al., 2017; Hassani et al., 2018; Haag, 2018).

CONCLUSION

In early eukaryotes, due to an increase of genome size, high density packaging of the DNA molecules into the confined space of the nucleus and simultaneous evolution of novel factors controlling the accessibility of DNA was a necessity to ensure all DNA-based processes, including transcriptional regulation. Increased genome size together with higher available energy per gene likely led to the evolution of chromatin structure and chromatin-modifying factors in early eukaryotes (Flaus et al., 2006; Lane and Martin, 2010; Koster et al., 2015; Garg and Martin, 2016; Martin and Sousa, 2016). Although, the origins of catalytic subunits of chromatin remodelers and modifiers can be traced back in prokaryotes, these catalytic subunits and their interacting partners continuously expanded and highly diversified and were finally coopted, while prokaryotes lack chromatin-remodeling and –modifying complexes. The innovation of these complexes was a key prerequisite step in the evolutionary trajectory of complex multicellular eukaryotes. Both symbiotic microbiota and epigenetics are critical for adaptation to environmental conditions, plant survival, and their evolution. However, our knowledge concerning how diversification and expansion of chromatin-related factors and recruitment of symbiotic microbiota led to the complexity of living organisms is low. In addition, the functional links between symbiotic microbiota and epigenetics is largely unknown. In future work, a combination of approaches in ecophysiology, plant-microbe

interaction, phylogenomics, molecular biology, systems biology, cell biology, and biochemistry studies on a wide range of unicellular and multicelluar organisms will shed more light on the interrelationship of chromatin-related factors and microbiota community structure and their contribution to the evolution of complex multicellular organisms and the holobiont.

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REVIEW
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Redox-Dependent Chromatin Remodeling: A New Function of Nitric Oxide as Architect of Chromatin Structure in Plants

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Nitric oxide (NO) is a key signaling molecule in all kingdoms. In plants, NO is involved in the regulation of various processes of growth and development as well as biotic and abiotic stress response. It mainly acts by modifying protein cysteine or tyrosine residues or by interacting with protein bound transition metals. Thereby, the modification of cysteine residues known as protein S-nitrosation is the predominant mechanism for transduction of NO bioactivity. Histone acetylation on N-terminal lysine residues is a very important epigenetic regulatory mechanism. The transfer of acetyl groups from acetyl-coenzyme A on histone lysine residues is catalyzed by histone acetyltransferases. This modification neutralizes the positive charge of the lysine residue and results in a loose structure of the chromatin accessible for the transcriptional machinery. Histone deacetylases, in contrast, remove the acetyl group of histone tails resulting in condensed chromatin with reduced gene expression activity. In plants, the histone acetylation level is regulated by S-nitrosation. NO inhibits HDA complexes resulting in enhanced histone acetylation and promoting a supportive chromatin state for expression of genes. Moreover, methylation of histone tails and DNA are important epigenetic modifications, too. Interestingly, methyltransferases and demethylases are described as targets for redox molecules in several biological systems suggesting that these types of chromatin modifications are also regulated by NO. In this review article, we will focus on redox-regulation of histone acetylation/methylation and DNA methylation in plants, discuss the consequences on the structural level and give an overview where NO can act to modulate chromatin structure.

Keywords: nitric oxide, redox-modification, S-nitrosation, chromatin modulation, acetylation, methylation

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SOURCES AND INTRACELLULAR LOCALIZATION OF NITRIC OXIDE

In plants, NO is formed either by reductive or oxidative pathways. In mammals, three cell-specific NO synthases (NOS) oxidize arginine to citrulline, thereby releasing NO. Although NOS-like activities have been measured in chloroplasts and peroxisomes of higher plants (He et al., 2004; Corpas and Barroso, 2018), NO synthase has only been identified in the algae (Foresi et al., 2010). Other possible substrates for oxidative NO production involve polyamines and hydroxylamine (Groß et al., 2013; Farnese et al., 2016). Reduction of nitrite

to NO constitutes the reductive route of NO production (Rockel et al., 2002; Yamamoto-Katou et al., 2006; Srivastava et al., 2009). Usually, nitrate reductase catalyzes the reduction of nitrate to nitrite. However, under low oxygen conditions, light and high nitrite levels, nitrite can be reduced to NO (Rockel et al., 2002; Planchet et al., 2005). Finally, enzyme-independent reduction of nitrite has been described in apoplast under acidic conditions (Bethke et al., 2004). Intracellular sources of NO are located in various compartments, including cytosol, peroxisomes, mitochondria, and chloroplasts (summarized in Groß et al., 2013; Farnese et al., 2016). Nuclear NO production is not described in plants. However, thiol reducing systems like thioredoxins and glutaredoxins as well as reducing molecules such as glutathione were found in the nucleus, suggesting that thiol modifications occur in this compartment (Delorme-Hinoux et al., 2016; Martins et al., 2018). Because of its lipophilic character, NO can easily cross the nuclear membrane or enter *via* nuclear pores (Toledo and Augusto, 2012; Lancaster, 2015). Moreover, NO can be transferred into the nucleus *via* S-nitrosylated proteins or S-nitrosylated low molecular weight thiols, such as S-nitrosoglutathione (GSNO) or S-nitrosocysteine. S-Nitrosylated nuclear proteins have been identified using the biotin switch technique, which labels S-nitrosylated proteins with a biotin linker allowing detection, purification, and identification of these proteins (Chaki et al., 2015). In mammals, nuclear translocation of S-nitrosylated proteins is described for gylceralaldehyd-3-phosphat-dehydrogenase and chloride intracellular channel protein CLIC4 (Hara et al., 2005; Malik et al., 2010). Nuclear localization of gylceralaldehyd-3-phosphat-dehydrogenase has been characterized in *Arabidopsis* (Holtgrefe et al., 2008; Vescovi et al., 2013; Aroca et al., 2017).

PHYSIOLOGICAL FUNCTION AND BIOCHEMISTRY OF NITRIC OXIDE

The chemical properties of nitric oxide (NO) make it highly multifunctional. Whereas some studies report toxic and harmful action of NO species, such as cell death (Pedroso et al., 2000), damage of proteins, membranes, and nucleic acids, or photosynthetic inhibition (Yamasaki, 2000), others demonstrate protective and/or signaling function of NO species. In fact, the dual function of NO is often dependent on its concentration and environment. Based on its functions, NO has been proposed as a stress-responding agent. It can counteract toxic processes induced by ROS (Beligni and Lamattina, 1998; Sun et al., 2007). It was shown that NO is involved in abiotic stress responses such as salinity, drought, UV-B radiation, temperature, and heavy metal toxicity (Mata and Lamattina, 2001; Tian et al., 2007). The role of NO in biotic stress is essential. It plays a key role in disease resistance against *Pseudomonas syringae* in *Arabidopsis* leaves, and is required for SAR induction in tobacco (Delledonne et al., 1998; Hong et al., 2008). Moreover, NO participates in plant development and physiological processes such as germination, gravitropism, root development, and flowering (Correa-Aragunde et al., 2004; He et al., 2004; Zhang et al., 2005). Although there is no doubt that NO is crucial for plant development and survival, the mechanism by which NO activates signaling function and the genes underlying this process remain to be elucidated.

NO chemical properties contribute to its role in signal transduction in a living cell (Toledo and Augusto, 2012; Lancaster, 2015). It can rapidly undergo multiple chemical reactions with enzymes, transcription factors, second messengers, or chromatin modifiers (Yu et al., 2014; Kovacs et al., 2016a). NO and its related species are able to modulate protein activities and biological function through covalent post-translational modifications (PTM) by binding to the metal centers of proteins and by affecting their cysteine and tyrosine residues. Tyrosine nitration is a post-translational modification that arises through the binding of a NO_2 into ortho carbons of aromatic ring of tyrosine residues that leads to the formation of 3-nitrotyrosine (Mata-Pérez et al., 2016; Kolbert et al., 2017). In a direct reaction termed metal nitrosylation, NO binds to transition metals, resulting in formation of metal nitrosyl complexes. In this way, activity and function of proteins can be regulated. Well studied targets for NO interaction are iron-sulfur clusters, as well as heme groups and zinc ions of proteins (Astier et al., 2010).

Examples of NO binding to iron present in heme proteins have also been observed in plants. It was suggested that two major H_2O_2-scavenging enzymes in tobacco, ascorbate peroxidase, and catalase are reversible inhibited by NO donors through the formation of an iron-nitrosyl complex (Clark et al., 2000). Plant hemoglobins were also identified as a target for NO. It was shown that *Arabidopsis* nonsymbiotic hemoglobin AHb1 binds NO and oxidizes it to nitrate, suggesting a role of hemoglobins in detoxification of NO (Perazzolli et al., 2004; Kuruthukulangarakoola et al., 2017). S-Nitrosation is the most studied redox-based post-translational modification. This modification results in the formation of S-nitrosothiols (SNO). S-Nitrosation enables a living organism to directly respond to environmental stimulus through the regulation of protein activity, protein-protein interaction, or protein localization (Hara et al., 2006; Yun et al., 2011). The release of the NO moiety from proteins and therefore the control of SNO homeostasis in a cell is maintained by two enzymes: GSNOR reductase (GSNOR), which metabolizes GSNO to a mixture of intermediates, and thioredoxins, which mediate denitrosylation (López-Sánchez et al., 2008; Cañas et al., 2012; Kneeshaw et al., 2014). Furthermore, reduced glutathione (GSH) alone is able to denitrosylate S-nitrosylated proteins. For instance, physiological levels of GSH rapidly removed the NO moiety of S-nitrosylated GAPDH resulting in the reduced and active form of GAPDH (Zaffagnini et al., 2013). For this GSH-dependent protein denitrosylation, the GSH/GSNO ratio is of relevance, but not the GSH/GSSG ratio. Although a high number of candidates for S-nitrosation were identified, only a few of them were experimentally confirmed and their functions in response to NO demonstrated (Astier et al., 2012). Most of the studies are based on biotin switch technique, where S-nitrosated cysteines are labeled with a biotinylating agent, allowing easy detection by immunoblotting using anti-biotin antibodies or purification using streptavidin matrix.

Interestingly, S-nitrosation of transcription factors can affect their function. For instance, S-nitrosation of Cys53 of the

Arabidopsis R2R3-MYB2 transcription factor inhibited its DNA-binding activity (Serpa et al., 2007). Similarly, S-nitrosation of Cys49 and Cys53 of MYB30 results are structural changes negatively affecting DNA affinity of this transcription factor (Tavares et al., 2014). In contrast, S-nitrosation of TGA1, a transcription factor involved in the activation of pathogenesis-related (PR) genes, promotes the binding to the as1-element of the PR1 promoter (Lindermayr et al., 2010). Besides the regulatory function of NO on transcription factors, NO can control gene transcription also by affecting chromatin structure and/or DNA accessibility.

REGULATION OF HISTONE ACETYLATION/METHYLATION AND DNA METHYLATION BY NITRIC OXIDE

On the genome level (nucleotide sequence), cells of multicellular organisms are identical. However, each cell differs from others through the differences in gene expression pattern that might occur in a temporally and spatially-dependent manner. Genes might be silenced/switched off and switched on again only when they are required. Such an activation/inactivation can be regulated through a direct control of regulatory elements on gene promoters. Moreover, over the last few decades, it was found that affecting the accessibility of the DNA by modification of the chromatin structure, is also a key regulator of transcription.

Genetic material of all eukaryotic organisms has to be packed into the nucleus to prevent it from becoming damaged. Since the length of eukaryotic DNA is far greater than the diameter of a nucleus, it has to be organized in a very tightly packaged structure, known as chromatin. The core subunit of chromatin is an octamer, which is composed of two copies of the histone proteins, H2A, H2B, H3, and H4, which are positively charged and enable an electrostatic interaction with negatively charged DNA. 145–147 bp of DNA are wrapped around a histone complex forming repeating nucleosomal units, which are connected with each other by short DNA fragments called "linker DNA." Linker histone H1 is located between the nucleosomes and stabilizes chromatin structure, resulting in highly condensed 30 nm fibers (Luger et al., 2012).

Chromatin structure in eukaryotic organisms is very dynamic, and can be changed during growth and development and in response to environmental stimuli. Chromatin marks are able to induce chromatin remodeling and therefore to control important molecular processes such as gene transcription, replication, repair, and recombination (Bannister and Kouzarides, 2011). DNA methylation and histone modifications are the key mediators of epigenetic modifications. DNA methylation is usually associated with long-term silencing of genes, whereas histone modifications contribute to both activation and repression of gene transcription and can be removed after several cell cycles (Jaenisch and Bird, 2003; Minard et al., 2009).

Histone modifications play an important role in the regulation of chromatin structure and in subsequent gene transcription. N-terminal histone tails, which are exposed outside the nucleosome may interact with neighboring nucleosomes and therefore manipulate the chromatin structure (Bannister and Kouzarides, 2011). Histone tails can undergo different posttranslational modifications such as acetylation, methylation, phosphorylation, and ubiquitination. These can act alone or in combination, resulting in different molecular changes that effect DNA accessibility.

Nitric Oxide Inhibits Histone Deacetylases

Histone acetylation plays a key role in regulation of gene transcription (Servet et al., 2010; Shen et al., 2015). This modification is very dynamic and is catalyzed by two families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDAs). The transfer of acetyl groups from acetyl-coenzyme A on histone lysine residues is catalyzed by histone acetyltransferases. This modification neutralizes the positive charge of the lysine residue and reduces the interaction between histones and the negatively charged DNA (Schiedel and Conway, 2018). This results in a loose chromatin structure accessible for DNA binding proteins. Histone deacetylases, in contrast, remove the acetyl group of histone tails resulting in condensed chromatin with reduced gene expression activity (Hollender and Liu, 2008; Luo et al., 2012). Therefore, histone acetylation is usually associated with gene transcription. For instance, differential acetylation at H3K9 and H3K27 and phosphorylation at H3S28 between end-of-night and end-of-day correlates with changes in diurnal transcript levels of core clock genes in *Arabidopsis* (Baerenfaller et al., 2016). In poplar, expression of carbonic anhydrase, pyruvate orthophosphate dikinase, phosphoenolpyruvate carboxykinase, and phosphoenolpyruvate carboxylase correlates with acetylation of H3K9 and H4K5 at their promoter regions (Li et al., 2017). Moreover, deacetylation of the flowering gene *AGL19* represses its transcription (Kim et al., 2013). However, there are also examples where enhanced acetylation of nearby regulatory elements and coding sequences does not generally result in higher transcription of the corresponding gene (Mengel et al., 2017). For example, comparison of ChIP-seq and transcript data of genes displaying GSNO-regulated H3K9/14 ac demonstrated that the mRNA levels of more than 60% of these genes remained unchanged (Mengel et al., 2017), concluding that histone acetylation is indeed making DNA accessible, but does not *per se* leads directly to gene transcription.

There is increasing evidence that the catalytic activity of at least some HDAs is regulated by redox modifications, which are involved in the regulation of unwinding and wrapping of chromatin. Until now, most studies of redox regulation by HDAs have been done in human and animal cells. It was reported that HDA2 in neurons gets S-nitrosated upon NO signaling triggered by brain-derived neurotrophic factor (Nott et al., 2008). S-Nitrosation of HDA2 results in chromatin acetylation and activation of gene expression that are involved in neuronal development. Notably, S-nitrosation does not affect the enzymatic activity of HDA2, but stimulates its release from chromatin. Influence of NO on HDA2 was confirmed, when redox-sensitive cysteines were mutated to alanine preventing dissociation of HDA2 from chromatin (Nott et al., 2008). S-Nitrosation of HDA2 was also demonstrated in muscles of dystrophin-deficient MDX mice (Colussi et al., 2008). The catalytic activity of this

enzyme was impaired by NO *in vivo* and *in vitro.* Additionally, protein activity in the presence of NO was also measured in purified *Escherichia coli* produced HDA1, HDA2, and HDA3. Recombinant HDA2 was highly sensitive to NO donors, and a slight reduction of protein activity was measured in HDA1 that was not caused by S-nitrosation (Colussi et al., 2008). Human HDA6 and HDA8 were also identified as potential targets for NO (Feng et al., 2001; Okuda et al., 2015). Endogenous HDA6 was identified as target for S-nitrosation using the biotin switch assay (Okuda et al., 2015). S-Nitrosation of HDA6 inhibited its catalytic activity and increased the level of acetylated alpha-tubulin suggesting that HDA6 plays a crucial regulatory function in acetylation of proteins others than histones (Okuda et al., 2015). HDA8 is S-nitrosated by GSNO *in vitro* (Feng et al., 2001). Moreover, the protein activity was significantly reduced by GSNO and another NO donor, S-nitrosocysteine, in time- and concentration-dependent manner. Interestingly, application of the NO donor sodium nitroprusside (SNP) to HDA8 had no effect on the catalytic activity of this protein, indicating that a special structural interaction is required for transferring NO (Feng et al., 2001). NO-dependent inhibition of gene expression was measured in human umbilical vein endothelial cells (Illi et al., 2008). It was demonstrated that upon NO production, protein phosphatase becomes activated and associates with a histone deacetylase complex pCamkIV/HDAs, promoting its dephosphorylation. This process leads to the shuttling of HDA4 and HDA5 (members of pCamkIV/HDAs complex) to the nucleus and deacetylation of histones. As a consequence, c-fos gene expression is inhibited. c-fos encodes for a protein with a basic leucine zipper region for dimerization and DNA-binding and a C-terminal transactivation domain (Illi et al., 2008). It is involved in important cellular events, including cell proliferation, differentiation and survival. Under non-stressed conditions, when the NO level in the cell is low, HDA4 and HDA5 remain in the cytosol, allowing hyperacetylation of chromatin (Illi et al., 2008). If similar mechanisms exist in plants as well still has to be investigated.

In *Arabidopsis* there are 18 members of HDAs, which are divided into 3 families: RPD3-like, HD-tuins, and sirtuins (Hollender and Liu, 2008; König et al., 2014; Shen et al., 2015; Bourque et al., 2016). The first family is the largest one and is composed of 12 putative members (HDA2, HDA5-10, HDA14-15, HDA17-19), which, based on their structure, can be further divided into 3 subclasses. This family of HDAs is homologous to yeast reduced potassium deficiency 3 (RPD3) proteins that are present across all eukaryotes (Hollender and Liu, 2008). All members of this family contain a specific deacetylase domain that is required for their catalytic activity. It should be highlighted that this class of HDAs is able to deacetylate more targets than just histones. Lysine acetylome profiling uncovered 91 acetylated proteins in *Arabidopsis* leaves after the treatment with deacetylase inhibitors apicidin and trichostatin A. Of these, only 14 were histone-like proteins (Hartl et al., 2017). The second family is plant-specific and contains the HD-tuins (HD2). These type of proteins was originally found in maize. The amino acid sequence of HD-tuins is related to cis-trans prolyl isomerases, which are present in other eukaryotes (Aravind and Koonin, 1998; Bourque et al., 2016). HD2s are structurally distinct from RPD3-like members, but display a sequence similarity with FK506-binding proteins. In total, four members of HD-tuins have been identified in *Arabidopsis*: HDT1 (HD2A), HDT2 (HD2B), HDT3 (HD2C), and HDT4 (HD2D). These consist of an N-terminal domain that has a conserved pentapeptide MEFWG region, which is part of a gene repression activity (Bourque et al., 2016). This region is followed by a high-charged acidic motif that is rich in glutamic and/or aspartic acid and a variable C-terminal region (Dangl et al., 2001). Moreover, HDT1 and HDT3 possess a zinc-finger motif that probably is involved in protein-protein interaction and DNA-binding (Bourque et al., 2016). The third family of plant HDAs is represented by sirtuins (SIR2-like proteins), which are homologs to yeast silent information regulator 2 (SIR2) (König et al., 2014; Bheda et al., 2016). These HDAs are unique because they require a NAD cofactor for their function and unlike RPD3 proteins, they are not inhibited by trichostatin A or sodium butyrate. Moreover, sirtuins use a wide variety of substrates beyond histones (König et al., 2014; Bheda et al., 2016).

Similar as in humans/animals redox molecules modulate histone acetylation in plants, too. Two members of the plant RPD-3 like family (HDA9 and HDA19) are sensitive to oxidation; however, the physiological function of this modification is still not understood (Liu et al., 2015). Treatment of *Arabidopsis* seedlings with the physiological NO-donor GSNO increased the abundance of several histone 3 and histone 4 acetylation marks. Presence of the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) strongly diminished the abundance of these histone mark (Mengel et al., 2017). Since, GSNO and S-nitroso-N-acetyl-DL-penicillamine (SNAP) reversibly reduced total HDA activity both *in vitro* and *in vivo*, the increased acetylation was likely caused by NO-dependent inhibition of HDA activity. Moreover, the major plant defense hormone salicylic acid, inhibited HDA activity and increased histone acetylation by inducing endogenous NO production. Additionally, genome-wide NO-dependent H3K9/14 ac profiling in *Arabidopsis* seedlings identified NO-regulated histone acetylation of genes involved in plant defense response and abiotic stress response. This includes, for example, genes encoding for TIR class nucleotide-binding site-leucine-rich repeat (TIR-NBS-LRR) class disease resistance proteins and the transcription factors WRKY27, WRKY53, TGA2 and TGA5 (Mengel et al., 2017). Plant proteins belonging to the nucleotide-binding site-leucine-rich repeat (NBS-LRR) family are used for pathogen detection. These proteins detect pathogen-associated proteins, such as the effector molecules responsible for virulence. The TIR class of plant NBS-LRR proteins contains an additional amino-terminal domain homolog to the Toll and interleukin 1 receptors (DeYoung and Innes, 2006). WRKY transcription factors are key players in modulating the transcriptome during plant defense response. WRKY27 controls the expression of genes involved in nitrogen metabolism and NO production and negatively influences symptom development of *Ralstonia solanacearum* in *Arabidopsis* (Mukhtar et al., 2008). WRKY53 acts in a transcription factor signaling network mediating

together with the EPITHIOSPECIFYING SENESCENCE REGULATOR a negative crosstalk between pathogen resistance and senescence, most likely controlled by the equilibrium between jasmonic acid and salicylic acid (Miao and Zentgraf (2007). TGA2 or TGA5 simultaneously bind to the TGACG motif of the *Pathogenesis-related1* promoter activating expression of this defense gene (Zhang et al., 2003; Hussain et al., 2018). In sum, NO regulates histone acetylation by modifying and inhibiting HDA complexes. This results in hyperacetylation of specific genes enabling their transcription. This might be an important mechanism operating in the plant stress response and facilitating expression of stress-related genes (**Figure 1**; Mengel et al., 2017).

All members of the RPD3-superfamily contain several cysteine residues and in many cases, S-nitrosation of protein cysteine residues is conserved across the kingdoms. Human HDA2 is S-nitrosated at Cys262 and Cys274, which are located close to the catalytic center (Nott et al., 2008, 2013). A comparison of the amino acid sequence of human HDA2 and HDAs from different plant species revealed that HDA6 and HDA19 are closely related to human HDA2. The HDA domains of HDA6, HDA19 and HDA2 are highly similar (ca. 65–70% identity) whereas the C-terminal parts of the sequences are divergent (**Figure 2**). The highly conserved part contains the HDA domain including six highly conserved cysteine residues (Cys112, Cys163, Cys273, Cys285, Cys296, and Cys323 of *Arabidopsis* HDA6). Additionally, Cys325 of *Arabidopsis* HDA6 is highly conserved within the plant HDa6 and HDA19. The cysteine residues, which are targeted by NO in human HDA2 (Cys262 and Cys274) are located within the region, which is conserved in plant HDA6 and HDA19 (**Figure 2**). Moreover, structural modeling of the HDA domain of *Glycine max* HDA6 and HDA19 (HDA6 68.12% sequence identity to HDA2, *Glycine max* HDA19 69.81% sequence identity to human HDA2) based on the available crystal structure of HDA2 revealed a strikingly similar 3D-fold of these proteins, where these two conserved cysteines are located close to the substrate binding site at the same positions (**Figure 3**). This makes plant HDA6 and HDA19 promising candidates for NO-affected/regulated nuclear HDA isoform(s). In sum, NO-dependent regulation of plant HDAs can be considered as a key mechanism in regulation of histone acetylation and gene expression.

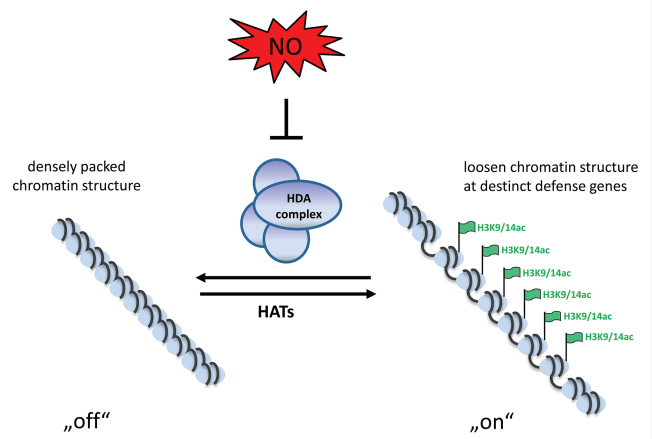

FIGURE 1 | Schematic model of NO-induced chromatin modulation. Due to the activity of HDAs the chromatin is densely packed and genes are not transcribed. Upon formation of NO, the HDA-complexes become inhibited by S-nitrosation, leading to acetylation of the chromatin. This loosen chromatin structure allows transcription in tight interplay with activating transcription factors.

Nitric Oxide Induces Expression of Demethylases and Methyltransferases and/or Affects Their Activities

Reports about the effect of NO on protein or DNA methylation in plants are rare. However, transcriptional changes in response to NO have been analyzed intensively using different techniques, for instance, cDNA-amplified fragment length polymorphism (Polverari et al., 2003), microarray (Huang et al., 2002), real-time PCR (Parani et al., 2004), and RNA-seq (Begara-Morales et al., 2014). The results of these different types of transcriptome studies demonstrated that NO is inducing a large set of genes involved in plant signal transduction, transport, defense and cell death, primary and secondary metabolism, and reactive oxygen species production and degradation. NO might regulate these genes by interacting directly with transcription factors or by regulating components of signal transduction cascades (Serpa et al., 2007; Lindermayr et al., 2010; Tavares et al., 2014; Imran et al., 2018). However, the accessibility of DNA is also an important regulatory mechanism in context of gene transcription. The accessibility of DNA can be regulated either by modification of histone tails (mainly acetylation and methylation) or by methylation of DNA. Interestingly, NO alters the expression of several methyltransferases and demethylases suggesting a regulatory role of NO in DNA and/or histone methylation (Ahlfors et al., 2009; Gibbs et al., 2014; Shi et al., 2014; Hussain et al., 2016; Kovacs et al., 2016a; Imran et al., 2018). For instance, CysNO and SNP treatment of leaves induced H3K27me3 Jumonji domain-containing histone demethylase 13 (JMJ13) expression (Ahlfors et al., 2009; Hussain et al., 2016) acting as a temperature- and photoperiod- dependent flowering repressor (Zheng et al., 2019). Enhanced endogenous levels of NO or GSNO, due to overexpression of rat neuronal NO synthase (nNOS) or knockout of GSNOR, respectively, results in downregulation of JMJ30 (Shi et al., 2014; Kovacs et al., 2016a), which demethylates H3K36me2/3, regulates period length in the circadian clock (Lu et al., 2011), and is involved in the control of flowering time (Yan et al., 2014). Transcriptomic analysis of NO-deficient *noa1-2*, *nia1nia2*, and *nia1nia2noa1-2* mutants also revealed that enzymes involved in epigenetic methylation processes are differentially expressed. For instance, chromomethylase 2 (CMT2), responsible for CHH methylation at pericentromeric heterochromatin (Stroud et al., 2013), is downregulated in each mutant (Gibbs et al., 2014). Additionally, DNA METHYLTRANSFERASE 1 (MET1) maintaining CG methylation is upregulated in *noa1-2*, but downregulated in *nia1nia2*. Further, enzymes involved in the active DNA demethylation system such as REPRESSOR of SILENCING1 (ROS1) and DEMETER-like protein 2 (DML2) (Furner and Matzke, 2011) are differently expressed in these NO-deficient mutants. Interestingly, several protein arginine methyltransferases (PRMTs) are upregulated in NO-deficient plants, for example, PRMT1a, PRMT1b, PRMT3,

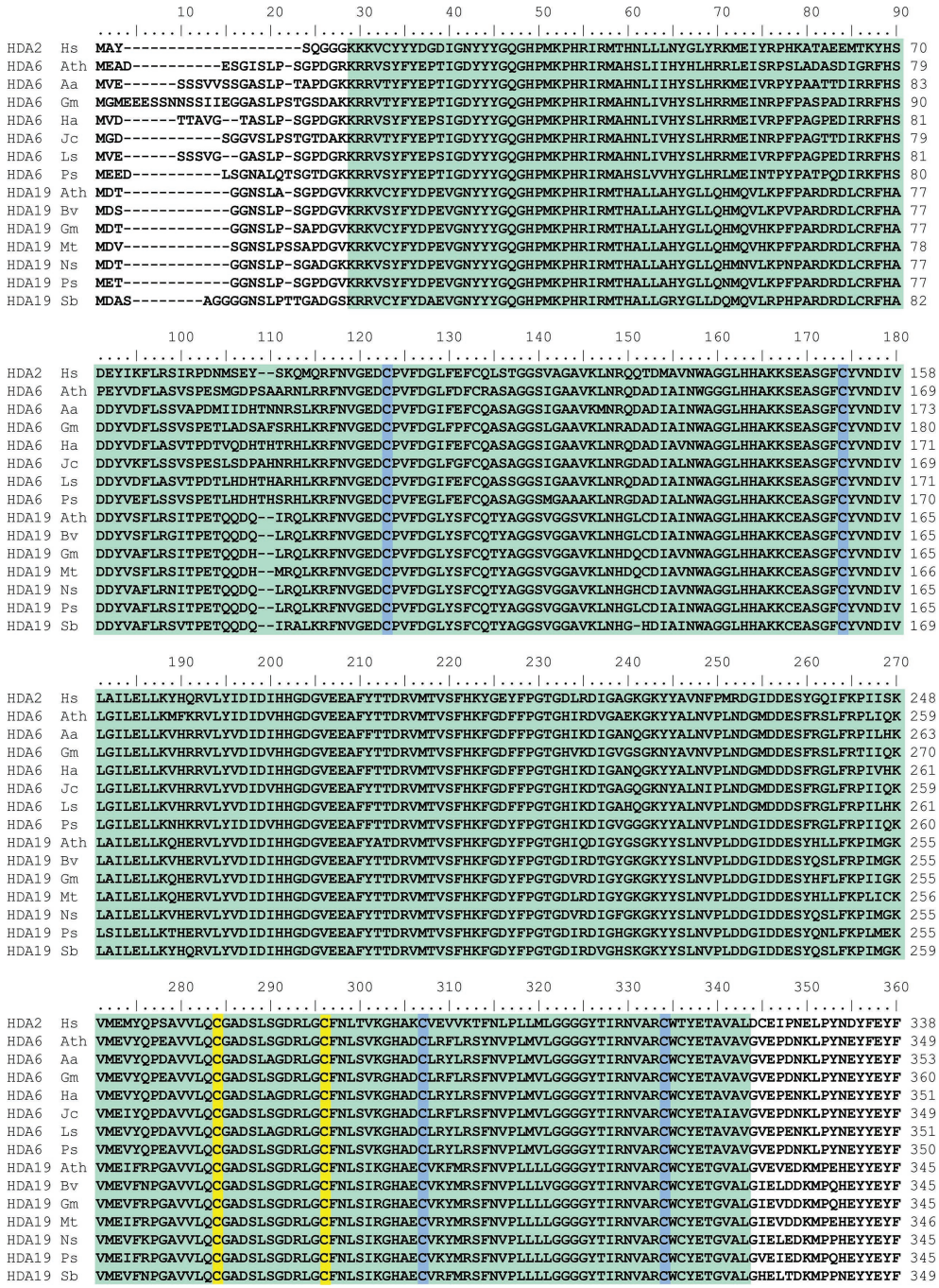

FIGURE 2 | Alignment of the amino acid sequences of the HDA domain of human HDA2 and different plant histone deacetylase 6 and 19 proteins. HDA amino acid sequences were aligned using Clustal W. The HDA domain is depicted in green. Cysteine residues of human HDA2 which are targets for S-nitrosation and the corresponding cysteine residues of plant HDAs are highlighted in yellow. Other conserved cysteine residues are marked in blue. Hs, *Homo sapiens* NP_001518.3; Ath, *Arabidopsis thaliana* AED97705.1 (HDA6) and O22446.2 (HDA19); Aa, *Artemisia annua* PWA92260.1; Gm, *Glycine max* XP_003525556.1 (HDA6) and XP_003543935.1 (HDA19); Ha, *Helianthus annuus* XP_021978414.1; Jc, *Jatropha curcas* XP_012079994.1; Ls, *Lactuca sativa* XP_023740973.1; Ps, *Papaver somniferum* XP_026387130.1 (HDA6) and XP_026455725.1 (HDA19); Bv, *Beta vulgaris* XP_010690952.1; Mt., *Medicago truncatula* XP_013462369.1; Ns, *Nicotiana sylvestris* XP_009770456.1; Sb, *Sorghum bicolor* XP_002438614.1.

PRMT10, PRMT5 in *noa1-2*; PRMT1b, PRMT3, PRMT4B in *nia1nia2*; and PRMT1b, PRMT3, PRMT4B, PRMT10, PRMT5 in *nia1nia2noa1-2* (Gibbs et al., 2014). PRMT1b, upregulated in all three NO-deficient mutants, methylates H4R3 and non-histone proteins such as the RNA methyltransferase fibrillarin 2 (Yan et al., 2007). Another example is PRMT5, which catalyze symmetric dimethylation of H4R3 *in vitro* and is essential for proper pre-mRNA splicing (Deng et al., 2010). PRMT5 is upregulated

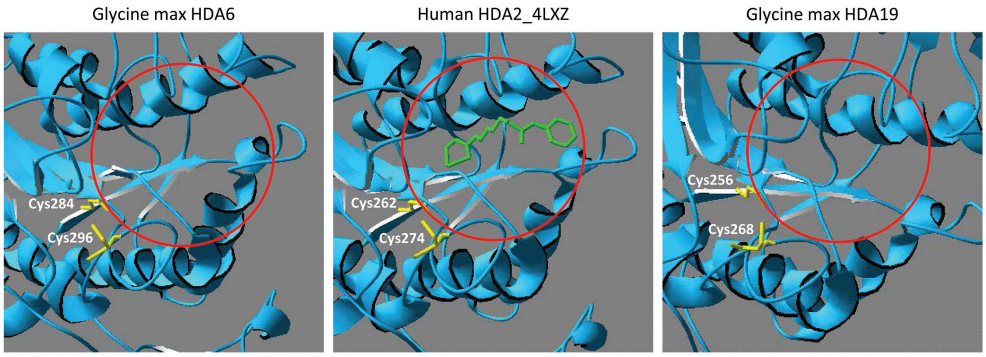

FIGURE 3 | Comparison of HDA6, HDA19 and human HDA2 substrate binding site. Structural comparison between human HDA2 and *Glycine max* HDA6 and HDA19. The HDA domains of *G. max* HDA6 (amino acids 29–397, Uniprot entry I1MTD8) and of *G. max* HDA19 (amino acids 3–371, Uniprot entry A0A0R0H2W2) were modeled using the SwissProt Modeling server with human HDA2 as template (PDB entry 4LXZ). The histone deacetylase inhibitor octanedioic acid hydroxyamide phenylamide is highlighted in green and shows the location of the active site (mark with a red circle). Cysteine residues, which are located next to the active site are marked in yellow. These cysteine residues are targets for S-nitrosation in human HDA2.

in *noa1-2* and *nia1nia2noa1-2* (Gibbs et al., 2014) and positively regulated by S-nitrosylation during stress responses (Hu et al., 2017). Regarding the late flowering phenotype of these NO-deficient mutants, it is worth mentioning that the histone demethylases JMJ18 is downregulated in each mutant. JMJ18 is a H3K4 demethylase controlling flowering time (Yang et al., 2012).

NO-dependent changes in DNA-methylation have been described in rice plants exposed to 0.5 mM NO donor sodium nitroprusside (Ou et al., 2015). The treatment resulted in stress symptoms and complete growth inhibition accompanied by hypomethylation of genomic DNA predominantly at CHG sites. As a consequence, transcription of a number of genes and transposable elements was activated and expression of several genes involved in chromatin remodeling and DNA methylation homeostasis, for example, chromomethylase 3, deficient in DNA methylation 1a and 1b, and DEMETER, was disturbed (Ou et al., 2015). In these cases, DNA methylation might be regulated *via* differential expression of DNA-methyl modifiers. However, modulation of their activity by NO-based post-translational modifications cannot be excluded.

Recently, it was shown that NO regulates protein methylation during stress responses in *Arabidopsis* plants (Hu et al., 2017). The authors demonstrated that S-nitrosation of protein arginine methyltransferase 5 (PRMT5), an enzyme catalyzing symmetric demethylation of protein arginine residues, activates its enzyme activity leading to proper splicing-specific pre-mRNA of stress-related genes (Hu et al., 2017). Although this mechanism does not evolve alteration of the chromatin structure, other studies showed that PRMT5 is a highly conserved type II protein Arg methyltransferase, which amongst others interacts with and methylates histones (Hu et al., 2017). In the *prmt5-1* mutant methylation of several proteins in the range of a molecular mass of 14 kD, including histone H4 and several core components of small nuclear ribonucleoprotein of the spliceosome, was barely detectable, while this phenotype could be rescued by a PRMT5 transgene (Hu et al., 2017). Therefore, it cannot be excluded that S-nitrosation of PRMT5 also affects chromatin structure by methylating defined histone Arg residues.

In mammals, NO exposure results in decease in global 5-methylcytosine and activation of transcriptional response. This might be linked to decreased expression of DNA methyltransferases Dnmt1 and Dnmt3a. Moreover, significant changes in the methylation level of H3K9, H3K27, H3K36, and H4K20 were observed in presence of NO. These changes are well-known as important modulators of gene transcription (Vasudevan et al., 2015) and are mainly a consequence of inhibition of KDM3A/JmjC histone demethylase activities and/or increased expression of a set of histone de-methylases (KDM1, KDM3A, KDM3B, KDM4A, KDM4B, KDM4C, KDM4D, and KDM7A). JmjC histone demethylases catalyze demethylation of mono-, di-, and trimethylated lysine residues by an oxidative, Fe(II)-dependent mechanism. NO directly inhibits the KDM3A demethylase activity by forming a nitrosyl-iron complex in the active site (Hickok et al., 2013) and exposure of mammalian cells to NO resulted in a significant increase in H3K9me2, the preferred substrate for KDM3A. Furthermore, exposure of embryonic stem cells to DETA-NO caused increased H3K4me1/2/3 and H3K9me3 methylation (Mora-Castilla et al., 2010) and concentration- and time-dependent accumulation of H3K9me2 upon DETA-NO treatment of human breast carcinoma cells was reported (Hickok et al., 2013).

Eukaryotic JmjC genes are separated in 14 subfamilies: Lysine-Specific Demethylase (KDM) 3, KDM5, JMJD6, and Putative-Lysine-Specific Demethylase (PKDM) 11, and PKDM13 subfamilies present in plants, animals, and fungi. Other subfamilies are detected only in plants and animals (PKDM12) or in animals and fungi but not in plants (KDM2 and KDM4). PKDM7-9 are plant-specific groups. The existence of Jumonji C-containing histone demethylases in plants suggested that at least some might be regulated by NO, similar the ones described in mammals.

Nitric Oxide Regulates Histone and DNA-Methylation on Metabolite Level

Methylation of histones and DNA can be regulated on two main levels–on the level of methyltransferases and de-methylases, which are catalyzing the methylation/de-methylation reactions, and on

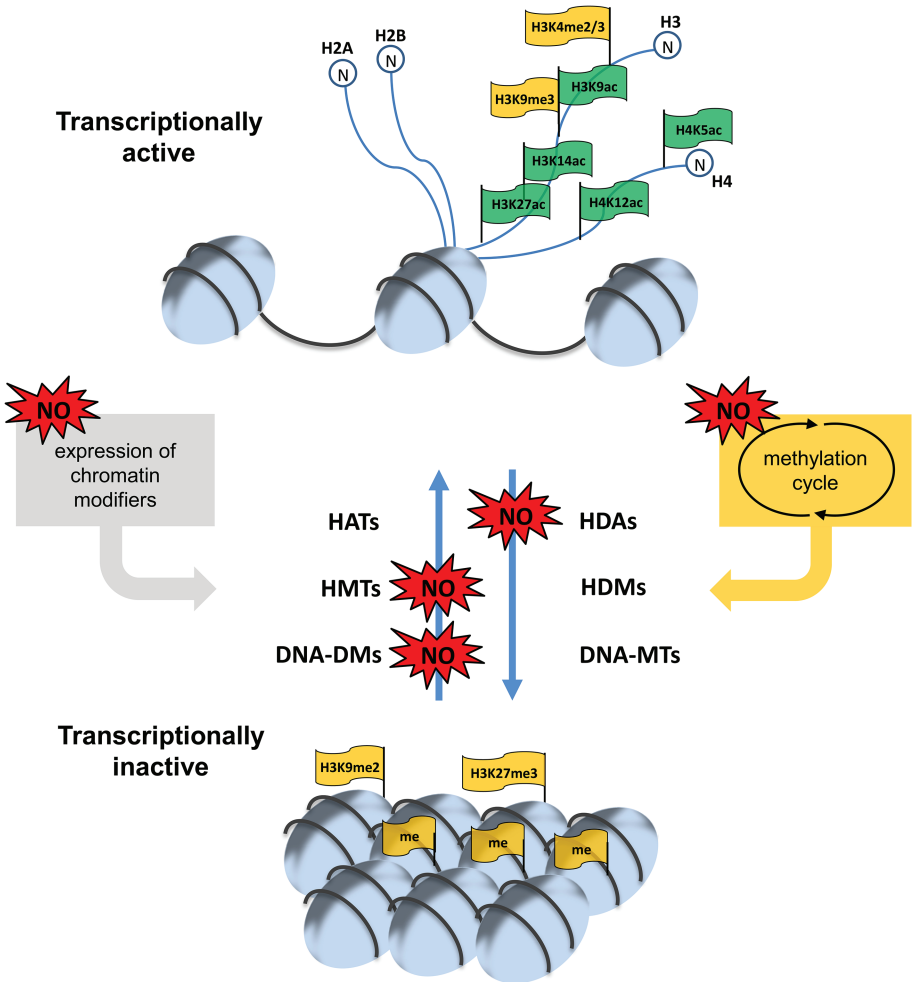

FIGURE 4 | NO-dependent regulation of chromatin modulation. Histone acetylation/methylation and DNA-methylation is controlled by different sets of acetylases/deacetylases (HATs/HDAs) and methyl transferases/demethylases (HMT/HDMs and DNA-MTs/DNA-DMs). NO can regulate the expression of some of these chromatin modifiers as well as their activity. Moreover, NO can affect the supply of the methyl group donor SAM and the level of the methyltransferase inhibitor SAH by altering the activity of enzymes of the methylation cycle and/or connected pathways. For more details see this paper.

metabolite level. S-Adenosylmethionine (SAM) is the major methyl group donor in the cell. DNA and histones are subject to methylation by specific SAM-dependent methyltransferases. Each transfer of a methyl-group generates S-adenosylhomocysteine (SAH), which is cleaved into homocysteine and adenosine (Ado) by S-adenosylhomocysteine hydrolase (SAHH) (Kovacs et al., 2016b). It is known already for a long time that the equilibrium of this reversible reaction favoring SAH synthesis (Palmer and Abeles, 1976) is driven toward hydrolysis of SAH due to removal of its products (Poulton and Butt, 1976). Methionine synthase converts homocysteine to methionine, which is in turn adenylated to SAM by SAM synthetase, whereas Ado is metabolized in the Ado salvage cycle. The ratio of SAM and SAH is considered as important regulator of cellular methylation processes.

For example, Zhou et al. (2013) reported the importance of a balanced SAM/SAH ratio for DNA and H3K9me2 methylation. The defect of folate polyglutamylation caused by mutation of folylpolyglutamate synthase affects the folate-mediated one-carbon metabolism resulting in increased SAH level, reduced SAM/SAH ratio and continuatively in a reduced DNA methylation and H3K9me2. A connection between the folate cycle, DNA methylation and redox homeostasis is also reported by Groth et al. (2016). Mutation in methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase (MTHFD1) results in loss of DNA methylation. The authors assume that reduced MTHFD1 function disturbs the cellular redox state, which might affect enzyme activities of the methylation cycle and/or methylation/demethylation reactions.

Other studies highlighted the importance of SAHH activity toward chromatin modifications (reviewed in Pikaard and Mittelsten Scheid (2014) and Vriet et al. (2015)): Mutation of the *AtSAHH1* gene resulted in reduced cytosine methylation and release of transcriptional gene silencing (Rocha et al., 2005; Mull et al., 2006; Jordan et al., 2007). Moreover, silencing of SAHH expression in tobacco plants lead to loss of DNA methylation in repetitive elements (Tanaka et al., 1997).

Furthermore, in *Arabidopsis* levels of DNA and histone methylation at endogenous repeats are reduced after treatment with the SAHH inhibitor dihydroxypropyladenine (Baubec et al., 2010).

There are several hints that the supply of SAM and the removal of SAH—the by-product inhibitor of transmethyl reactions—are at least partly regulated by NO, since the activities of key enzymes of the methylation cycle seems to be modulated by NO-dependent posttranslational modifications. In several independent proteomic studies, cobalamin-independent methionine synthase, SAMS, and SAHH were identified as targets for S-nitrosation (Lindermayr et al., 2005; Abat and Deswal, 2009; Puyaubert et al., 2014; Hu et al., 2015). In *Arabidopsis,* different SAMS isoforms are differentially inhibited by protein S-nitrosation (Lindermayr et al., 2006). While isoform SAMS1 is reversibly inhibited by GSNO, the activity of the isoforms SAMS2 or SAMS3 is not affected. Responsible for the inhibition of SAMS1 is S-nitrosation of Cys114, which is located nearby the catalytic center as part of the active site loop (Lindermayr et al., 2006). In mammals, a similar regulatory mechanism of SAMS activity is described. Here two SAMS isoforms are present. While the activity of SAMS1A is reversibly inhibited by NO, SAMS2A activity is not affected (Pérez-Mato et al., 1999).

In *Arabidopsis,* two genes encode for SAHH, but only SAHH1 seems to play a role in DNA-methylation processes (Rocha et al., 2005; Vriet et al., 2015). S-Nitrosation of *Arabidopsis* SAHH1 upon cold stress was reported, but the physiological function of S-nitrosated SAHH1 is not yet investigated (Puyaubert et al., 2014; Puyaubert and Baudouin, 2014). Beside S-nitrosation, tyrosine nitration has been observed in sunflower (*Helianthus annuus L.*) SAHH. This modification decreased the catalytic activity of SAHH (Chaki et al., 2009). The results of all these different studies suggest that NO plays an important regulatory role in allocation of the major methyl-group donor SAM and the removal of the methyltransferase inhibitor SAH, which consequently affects histone and DNA methylation.

In sum, there are different levels, where NO can affect chromatin modulation, for instance transcription and activity of chromatin modifiers or the supply of methyl group donors or methylation inhibitors (summarized in **Figure 4**). Since NO is an important signaling molecule in plant growth and development and in plant stress response all these different mechanisms discussed above allows NO to regulate physiological processes. The most important future challenges are the identification and/or verification of NO-regulated chromatin modifiers responsible for histone modifications and DNA methylation and the characterization of the mode of action of NO on these proteins. Moreover, the corresponding histone marks and the chromatin regions controlled by NO-regulated chromatin modifiers have to be identified. Such analysis would also include possible interaction between histone marks and DNA-methylation. Additionally, these NO-dependent chromatin modifications have to be analyzed in a physiological context to complement the picture of NO signaling function in plants.

Finally, the gained knowledge could be subjected to genetic/epigenetic engineering or classical breeding to improve plant traits permanently.

AUTHOR CONTRIBUTIONS

AA-K, ER and CL wrote the review article. CL coordinated the completion of the article.

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Epigenetic Clues to Better Understanding of the Asexual Embryogenesis *in planta* and *in vitro*

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HIGHLIGHTS

- One of most intriguing questions in developmental plant biology, is how the cellular totipotency is generated, and results in the asexual embryogenesis.
- Hormonal and stress signals play a key role in initiation of the embryogenic pathway by activation of cell division in somatic cells *in planta* and in *in vitro* cultured cells
- DNA hypomethylation or histone acetylation as epigenetic events activate expression of specific transcription factor, hormonal or developmental genes being responsible for totipotent stage.
- Ectopic expression of specific developmental genes can trigger somatic embryogenesis in vegetative plant organs.
- Level of DNA methylation in dedifferentiated callus tissues is lowered during embryogenesis.
- Epigenetic reprogramming is reflected by significant changes in transcript profiles during callus induction and somatic embryogenesis.

DIFFERENTIATION OF EMBRYOS OR PLANTLETS FROM VEGETATIVE ORGANS *IN PLANTA*

Formation of numerous buds and small plantlets on leaf margin of *Kalanchoe daigremontiana* is a peculiar developmental event in the plant kingdom (**Figure 1A**, Garcês et al., 2007). These organogenic or embryogenic processes start with cell divisions as responses to wounding or hormonal signals (Stage I) shown by **Figure 1B** (Guo et al., 2015; Zhu, 2017). Under formation of meristematic regions in *Kalanchoe* leaves, the chromatin status activates expression of specific key regulator and marker genes of both organogenesis (SHOOT MERISTEMLESS, STM) and embryogenesis (LEAFY COTYLEDON1, LEC1, and EMBRYONIC TEMPORAL REGULATOR, FUSCA3, Garcês et al., 2007). Suppression subtractive hybridization studies revealed that the overexpression of a large number (390) of unigenes in the asexual reproduction of *K. daigremontiana* (Zhong et al., 2013). **Figure 1B** highlights common cellular and molecular events in different stages of transition from somatic to embryogenic cell fate. In both cases (in Daucus: Grzebelus et al., 2012; Kalanchoe: Guo et al., 2015) hormonal and stress factors are involved in induction of cell division and cellular re-programming. However, the physiological machinery as well as epigenetic changes linked with these processes have been preferentially investigated in embryogenesis initiated from somatic cells. More recently, another plant species *Rhynia gwynne-vaughanii* was found to be capable for plant regeneration *in planta* (Kearney et al., 2016).

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IN VITRO RESETTING EPIGENETIC MEMORY OF EMBRYOGENIC PATHWAY WITHOUT OR WITH CALLUS INDUCTION

Thanks to the very intensive research from middle of last century, the somatic embryogenic pathway was observed in very different *in vitro* culture systems (**Figure 1A**). We see as a breakthrough in developmental biology when somatic embryo formation from root tips was observed in one of the activation tagged Arabidopsis mutants. It turned out that ectopic expression of WUSCHEL (WUS), a homeodomain protein in transgenic Arabidopsis plants caused embryo development from this vegetative organ (**Figure 1A**, Zuo et al., 2002). Similarly, overexpression of LEAFY COTYLEDON2 (LEC2) gene is sufficient to trigger the formation of somatic embryos from vegetative tissues (Stone et al., 2001). High number of somatic embryos was formed on the scutella of transgenic maize plants overexpressing transcription factors BABY BOOM (BBM) and WUSCHEL2 (WUS2) under the control of specific promoters (Lowe et al., 2018).

In contrast to the direct embryo formation from somatic tissues, frequently the callus stage is a prerequisite for cellular reprogramming that insures shutting down "old" cell fates and permitting upregulation of "new" cell fates through changing chromatin stage [see review by Fehér et al. (2003) and Fehér (2019)]. Several investigations demonstrated that the cell re-programming is accompanied by significant changes in chromatin status (DNA methylation and histone methylation/acetylation) (for review see Birnbaum and Roudier, 2017; Lee and Seo, 2018). Majority of studies was devoted for re-programming of callus cells to initiate shoot formation. Mutations in key epigenetic genes encoding for DNA METHYLTRANSFERASE (MET1), KRYPTONITE (KYP) for the histone 3 lysine 9 (H3K9) METHYLTRANSFERASE, JMJ14 for the histone 3 lysine 4 (H3K4) DEMETHYLASE, and HISTONE ACETYLTRANSFERASE (HAC1) resulted in altered WUS expression and developmental rates of regenerated shoots *in vitro* (Li et al., 2011). Clear sign for modification of the epigenetic landscape is the hypermethylation at certain genes in rice callus that was detected in CHH sequence contexts, at the promoter region of genes (**Figure 1A**, Stroud et al., 2013). Since transcriptional repression is associated with hypermethylation of DNA as a first step in developmental reprogramming, the callus stage can erase gene expression pattern by higher number of down-regulated genes (373) than the up-regulated ones (241) during callus formation from Arabidopsis root explants (Che et al., 2006). Callus formation is dependent on histone deacetylation shown by treatment of Arabidopsis leaf explants with trichostatin A (Lee et al., 2016). In addition, demethylation of H3K27me3 is critical for acquisition of callus formation from Arabidopsis leaves (He et al., 2012; Lee et al., 2018). Deposition of histone variant, H2A.Z strongly correlates with the gene activation mark H3K4me3 and genes regulated by H2A.Z may be related to environmental responses, chromatin assembly and cell cycle in callus representing undifferentiated pluripotent stem cells (Zhang et al., 2017).

However, these results cannot be directly extrapolated to reprogramming of the differentiated somatic cells to become embryogenic, there is strong experimental support for involvement of chromatin structure in this unique developmental event. Karim et al. (2018) reported that somatic embryogenesis in *Boesenbergia rotunda* (L.) was linked with relatively higher expression of *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (SERK), BBM, LEC2, and WUS genes and lower level of DNA methylation. The 5-Azacytidine (5-AzaC), an inhibitor of DNA methylation was shown to stimulate somatic embryogenesis in *Pinus pinaster, Brassica napus, Hordeum vulgare,* and *Theobroma cacao* cultures (see review by Osorio-Montalvo et al., 2018). In non-embryogenic cotton calluses, inhibition of the DNA methylation by using zebularine treatment increased the number of embryos (Li et al., 2018). Stress responsive genes as heat shock gene can be activated during embryogenic induction in cultured alfalfa callus cells (Györgyey et al., 1991).

RE-PROGRAMMING OF TERMINALLY DIFFERENTIATED CELLS-DERIVED FROM LEAF PROTOPLASTS

So far the majority of the investigations on cell re-programming was performed at level of multicellular structure. In order to avoid complexity of plant tissue in which even neighboring cells have different physiological/molecular status, homogenous population of leaf protoplast-derived cells can serve as an optimal experimental material for studies on cell re-programming. Selected Medicago genotypes offer an optimal experimental system for detailed analysis of cellular reprogramming, especially in protoplasts cultures. Comparison of embryogenic and non-embryogenic cells can provide deeper insight both at cellular and molecular levels. One experimental system was based on alfalfa (*Medicago sativa* L.) leaf protoplasts (A2 line) with unique capability to generate totipotent cells from isolated mesophyll protoplasts in culture medium with high dose ($\leq$1 mg/L) of exogenous auxin analogy, 2,4-D-dichlorophenoxy acetic acid (2,4-D) (Dudits et al., 1991; **Figure 1A**, Bögre et al., 1990). During protoplasts re-programming size of DAPI stained nuclei was significantly increased, especially on the medium with high 2,4-D concentration, that can reflect more relaxed chromatin (Pasternak et al., 2000). The embryogenic alfalfa cells could be characterized by earlier cell division, a more alkalic vacuolar pH, and non-functional chloroplasts (Pasternak et al., 2002). In parallel, in the embryogenic cells 38 up-regulated transcripts preferentially from stress responsive genes could be identified by PCR-based cDNA subtraction approach (Domoki et al., 2006). The LEC1, embryogenic gene exhibited more than seven-fold higher expression in the presence of the high 2,4-D concentration relative to cells grown in medium with low 2,4-D. This activation of LEC1 gene in embryogenic cells is linked to the reactivation of cell cycle and generation of polarity by asymmetric cell division (**Figure 1A**). These events can be clearly monitored in protoplasts cultures where auxin and oxidative stress factors can activate cyclin-dependent kinase complexes and induction of S-phase

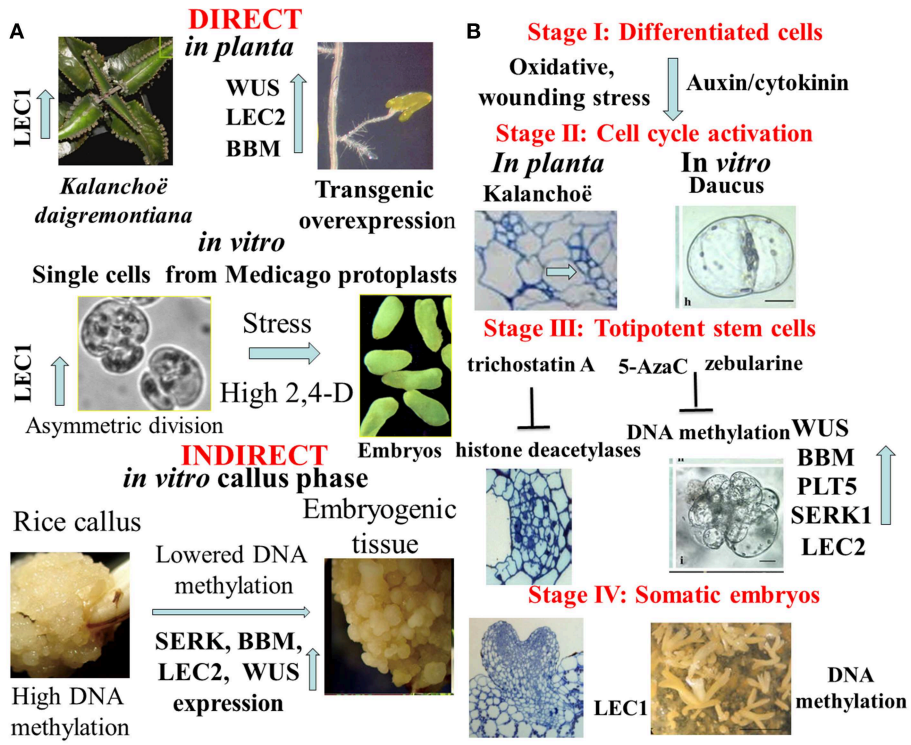

FIGURE 1 | (A) Complexity of alternative pathways in the development of somatic embryos. *In planta* direct somatic embryogenesis can result in small plantlets appearing on margin of *Kalanchoe* leaves, or in root tips overexpression of transcription factor genes as WUSCHEL (WUS); LEAFY COTYLEDON 2 (LEC2), BABY BOOM (BBM) can trigger embryo formation. *In vitro* asymmetric cell division in protoplast-derived cells exposed to high dose of synthetic auxin (2,4-D-dichlorophenoxy acetic acid) or stress signals initiates the embryogenic pathway. Frequently somatic embryogenesis occurs in callus tissues representing undifferentiated pluripotent stem cells with hypermethylation of DNA that is lowered in pro-embryogenic cells. **(B)** Representative stages of somatic embryogenesis in Kalanchoë or Daucus somatic cells in relation to hypomethylation of DNA or acetylation of histone proteins. Reactivation of cell division cycle is a prerequisite for cellular reprogramming. Trichostatin A as inhibitor of histone deacetylases or 5-azacytidine/ zebularine as inhibitors of DNA methylation can generate chromatin structure to activate expression of specific developmental genes that are involved in formation of totipotent somatic plant cells. The end products are somatic embryos to be used in micropropagation or in molecular breeding (Dudits et al., 1991; Zuo et al., 2002; Garcês et al., 2007; Wani et al., 2011; Grzebelus et al., 2012; Guo et al., 2015; Zhu, 2017).

(Ötvös et al., 2005; Pasternak et al., 2007, 2014; Fehér et al., 2008; Fehér, 2015). Non-embryogenic cell types contain big lytic vacuole (acidic one) but embryogenic cells have numerous storage protein (more alkaline). These characteristics can be seen in the de-differentiated stem cells *in planta* (Pasternak et al., 2002). The embryogenic genotype of alfalfa exhibited highly dense cytoplasm, with reduced cell expansion, and frequent asymmetric cell division (Bögre et al., 1990; Dudits et al., 1991). Dijak and Simmonds (1988) reported that in embryogenic alfalfa cells microtubule strands developed more rapidly, and microtubules were finer and more branched than in non-embrygenic protoplasts. Important signs for the embryogenic re-programming of somatic cells can be recognized during the first cell division. The gap between initiation of culture and first DNA replication events—what is much longer as normal G1 phase- and detection of increased nuclei size and stainability suggests significant role for chromatin relaxation in the process of cell cycle activation. This step is a key event in re-programming cells to reactivate division requiring auxin in the culture media (Pasternak et al., 2000, 2002).

CONCLUSIONS

Plasticity of cellular differentiation in plants is not only a very exciting biological phenomenon, but it is an important component in tissue culture-based propagation systems or in transgenic and genome editing technologies. In the present opinion paper we demonstrate that defined *in vitro* conditions with hormonal or stress effects can generate chromatin status that insures activation of specific transcription factor (WUS; LEC1, 2; BBM) genes of embryogenic program. Alternatively, transgenic overexpression of these genes can also initiate similar developmental pathway in variety of cell types. Recent publications using inhibitor of histone deacetylation or DNA methylation provide strong support for the direct involvement of chromatin status in cellular reprogramming including callus formation and asexual embryogenesis (Lee et al., 2016; Li et al., 2018; Osorio-Montalvo et al., 2018). The key role of cell division in somatic embryogenesis could be clearly shown by using of protoplast-derived homogenous cell populations for molecular and structural studies. The present analysis mainly based on some

"model" plants exhibiting somatic embryogenesis as specific trait. The present progress in discovering the underlying molecular and cellular events (see review by Fehér, 2019) is expected to extent this phenomenon to other plant species also with agronomic significance.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

CONTRIBUTION TO THE FIELD STATEMENT

Generation of totipotent stage in differentiated plant cells through molecular and cellular reprogramming attracts significant interest in the field of plant science. Plasticity of cellular differentiation/de-differentiation is an important component in the tissue culture-based propagation systems or in transgenic and genome editing technologies. Here we point out common features in plant cell re-programming from different explants with focus on the role of epigenetic mechanism and activities of developmental genes. We outline advantages of the use of protoplast-derived homogenous cell populations for the auxin/stress concentration-dependent induction of embryogenic program.

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Dynamic Changes in Genome-Wide Histone3 Lysine27 Trimethylation and Gene Expression of Soybean Roots in Response to Salt Stress

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Soybean is an important economic crop for human diet, animal feeds and biodiesel due to high protein and oil content. Its productivity is significantly hampered by salt stress, which impairs plant growth and development by affecting gene expression, in part, through epigenetic modification of chromatin status. However, little is known about epigenetic regulation of stress response in soybean roots. Here, we used RNA-seq and ChIP-seq technologies to study the dynamics of genome-wide transcription and histone methylation patterns in soybean roots under salt stress. Eight thousand seven hundred ninety eight soybean genes changed their expression under salt stress treatment. Whole-genome ChIP-seq study of an epigenetic repressive mark, histone H3 lysine 27 trimethylation (H3K27me3), revealed the changes in H3K27me3 deposition during the response to salt stress. Unexpectedly, we found that most of the inactivation of genes under salt stress is strongly correlated with the *de novo* establishment of H3K27me3 in various parts of the promoter or coding regions where there is no H3K27me3 in control plants. In addition, the soybean histone modifiers were identified which may contribute to *de novo* histone methylation and gene silencing under salt stress. Thus, dynamic chromatin regulation, switch between active and inactive modes, occur at target loci in order to respond to salt stress in soybean. Our analysis demonstrates histone methylation modifications are correlated with the activation or inactivation of salt-inducible genes in soybean roots.

Keywords: salt stress, RNA-seq, ChIP-seq, histone methylation, histone modifiers, soybean

INTRODUCTION

Environmental changes affect the organisms in a wide range of situations (Lopez-Maury et al., 2008). Among the abiotic stress factors, salt stress is a well-known factor restricting germination and growth, seriously threatens the productivity of crops. Soybean, *Glycine max*, is one of the most important crops with source of protein and oil in the human and animal diet, however its productivity is significantly affected by field condition such as soil salinity (Phang et al., 2008). In the northeast China, soybean used to be a major crop, and breeding soybean for tolerance to high sodic conditions is important in some regions of China and the world. Therefore, understanding the

molecular mechanism of the soybean tolerance to salt stress has been a major topic for crop scientists (Zhang et al., 2013).

Plants respond to abiotic stress by activation or inactivation of specific sets of genes to induce certain molecular signaling pathways which rapidly alter physiological reactions and expression initiation of responsive genes. Gene expression is directly influenced through chromatin states, which is closely associated with epigenetic regulation including histone variants, histone post-translational modifications, and DNA methylation (Schwartz et al., 2010; Henikoff and Shilatifard, 2011; Lauria and Rossi, 2011). The modifications of the histone amino-terminal tails are involved in assisting nucleosome remodeling as well as recruitment of specific transcription factors. Specific amino acids within the N-terminal regions of histones are targets for a number of covalent modifications, including methylation, phosphorylation, ubiquitination and acetylation. Some of these marks, for example, acetylation of lysine 14 of histone H3 (H3K14ac) or trimethylation of lysine 4 of Histone3 (H3K4me3), are generally associated with open, actively transcribed genomic regions, whereas others, such as H3K9me3 or H3K27me3, are indicative of a repressed chromatin state (Zhang et al., 2007; Li et al., 2008; Charron et al., 2009; Zhang et al., 2009; He et al., 2011).

The epigenetic changes including DNA methylation and/or histone modifications are associated with altered gene expression for defense responses under abiotic (e.g., salt) stress (Alexandre et al., 2009; Chinnusamy and Zhu, 2009; Ding et al., 2009; Zong et al., 2013; Chen et al., 2014). In plants, there are increasing studies of regulating gene expression by histone modification under various stresses (Chinnusamy and Zhu, 2009; Kumar and Wigge, 2010; Luo et al., 2012a; Feng et al., 2016; Deng et al., 2017). In crop breeding, it is hard to keep balance of disease resistance and yield. Recent studies showed that the rice *Pigm* locus contains a subset of genes encoding nucleotide-binding leucine-rich repeat (NLR) receptors. These receptors can lead to durable resistance to the fungus without productivity penalty through DNA methylation regulation (Deng et al., 2017). To cope with environmental stresses, plants often adopt a memory response when facing primary stress for a quicker and stronger reaction to recurring stresses. Feng et al. found that salt stress-induced proline accumulation is memorable. HY5- dependent light signaling through H3K4me3 modification on a Δ1-pyrroline-*5-carboxylate synthetase 1* (*P5CS1*) is required for such a memory response (Feng et al., 2016).

The covalent modifications were deposited or erased from target loci by the histone modifiers including histone methyltransferase (HMTs) and histone demethylases (HDMs). All the known HMTs in plants have a highly conserved domain, SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) which was also named as SDG (SET domain groups) proteins (Ng et al., 2007; Thorstensen et al., 2011). Many epigenetic modifiers' function has been well characterized. It has been reported that some modifiers have been shown to be integrated in abiotic stress signaling pathways (Schubert et al., 2006; Grini et al., 2009; Jeong et al., 2009; Guo et al., 2010; Ding et al., 2011; Lu et al., 2011; He et al., 2012; Yao et al., 2013; Gu et al., 2014; Cui et al., 2016). A plant trxG factor, *Arabidopsis* homolog of trithorax1 (ATX1) with H3K4me3 methyltransferase activity can promote transcription initiation by recruiting RNA Polymerase II (Alvarez-Venegas and Avramova, 2005; Saleh et al., 2008). ATX1 was found to be involved in drought stress signaling in both ABA dependent and ABA-independent pathways, and an *atx1* mutant was shown to be hyposensitive to drought stress (Ding et al., 2009; Ding et al., 2011). Therefore, chromatin modifications and epigenetics are directly linked to plants' responses to environmental cues.

It is important to note, however, that most of the current studies focus on epigenetic modifications at individual stress genes in plants. Second, there are more and more studies on *Arabidopsis*, rice, and maize, but limited knowledge of regulation of salt stress response through chromatin modifications in soybean plants. Moreover, there are no data on genome-wide modification patterns in regard to response to stress in soybean plants. In this study, we provide a global view of H3K27me3 patterns in chromatin isolated from soybean roots with or without salt stress treatment. Genome-wide expression patterns in control and salt stressed soybean were compared with changes in the H3K27me3 levels of nucleosomes on stress-induced differentially expressed genes. Using chromatin immunoprecipitation (ChIP) of H3K27methylation antibodies combined with genome-wide sequencing (ChIP-seq), we revealed different dynamic changes in H3K27me3 profiles taking place upon salt stress. The specific patterns of the H3K27me3 distributions including *de novo* methylation at up-regulated and down-regulated genes were identified during the stress treatment. Moreover, we provide a comprehensive overview of the histone modifiers which may work together to regulate differential H3K27me3 modification leading to activation or inactivation of gene expression during salt stress in soybean.

MATERIALS AND METHODS

Plant Materials and Growth Condition

The *Glycine* line, *Glycine max* Williams 82, was used in this study. Seeds were sterilized with 75% ethanol and then germinated in pots filled with coconut fiber. Soybean seedlings were grown in soil in an incubator with 25/20°C (light/dark) and 16/8h (light/dark) cycles until the second trifoliate leaves started expand. For the salt stress treatment, the uniformly growing plants were kept in 0, 50, 75, 100, 150, and 200 mM/L of NaCl solutions for 30 h. After the treatment, the root tissues were harvested and frozen in liquid nitrogen. As a control, the untreated seedlings (0 mM/L) were planted and harvested at the same time with the stress-treated plants. The 100 mM/L salt treated seedlings were used for RNA-seq and ChIP-seq analysis since the phenotypic differences were clear at this concentration which is also commonly used for salinity test on soybean (Belamkar et al., 2014; Zeng et al., 2019). Three replicates of the root samples both from control and 100 mM/L salt treatment were prepared for consistency of the analysis.

RNA-seq Library Construction and Analysis

Total RNA was extracted from the root of soybeans with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Library making, RNA-seq and data analysis were performed as described previously (Xu et al., 2018). PolyA$^+$ libraries were constructed using Illumina's TruSeq Stranded mRNA Library Prep Kit. The size and quality of the resulting libraries were examined using a Bioanalyzer 2100 and cDNA libraries from the RNA samples were prepared for high throughput Illumina sequencing. Paired-end

reads were generated with the Illumina HiSeq 2500 system. Three independent biological sample replicates were employed. The RNA sequencing reads were aligned to the *Glycine max* reference genome (*Glycine* max Wm82.a2.v1) using TopHat2 (Kim et al., 2013). Genes that met the criterion of a detectable expression signal in control or salt plants were further analyzed. The fold change (FC) was calculated by comparing the expression level of the salt samples to control (salt/control). Briefly, the "|Log2FC| > 1 and p-adj < 0.05" was used as the threshold to judge the significance of gene expression difference. Genes that display a greater than 2-FC in the salt-treated were designated as up- or down-regulated if the salt RNA level was higher or lower than that of control plants, respectively.

Real-Time Quantitative RT-PCR (qPCR)

RT-qPCR was performed as described previously with minor modifications (Xu et al., 2018). cDNAs were reverse transcribed with oligo (dT) from the total RNAs. RT-qPCR reaction was carried out in a QuantStudio 6 Real-Time PCR system (Applied Biosystems). At least three independent experiments employing biological replicates were performed and three technical replicates were done for each sample. Amplification of *Tubulin* (*Tub*) was used as an internal control to normalize all data. Quantification was determined by applying the $2^{-\Delta Ct}$ formula (Pu et al., 2008). All gene-specific primers are listed in **Supplementary Table 1**.

Chromatin Immunoprecipitation (Chip) Assays and ChIP-seq Analysis

ChIP assay was performed from approximately 2 g of soybean roots as previously described (Kim et al., 2012b; Xu et al., 2018). Briefly, fresh tissues of whole seedlings were infiltrated in 1% formaldehyde solution under a vacuum for 20 min to cross-link the chromatin. The reaction was stopped by adding 0.1 M glycine. Formaldehyde fixed tissues were ground in liquid nitrogen, nuclei isolated, chromatin extracted and sheared by sonication (Diagenode, Bioruptor Plus; 1 min on and 30 s off for 15 min) to generate 0.5 to 2 kb DNA fragments. The aliquot of 1–2 μl of mix DNA samples and electrophoretic was used to determine the sonication efficiency and average size of DNA fragments. A smear from 200–2,000 bp, but concentrated 500 bp was observed in the sonicated samples and for further analysis. Anti-H3K27me3 (Millipore, #07-449) antibody was used to immunoprecipitate the fragmented chromatin (IP, 200 μl of IP solution plus 1μl of antibody as 200 times dilution). Cross-linking of IP was reversed with 5 M NaCl, and DNA was precipitated with 100% EtOH. For the Input control (Input), 5M NaCl was added to 0.5% of total chromatin before immunoprecipitation to reverse the cross-linking and DNA was precipitated with 100% EtOH. The relative amount of DNA was determined using a spectrophotometer (NanoDrop, ND1000). ChIP purified DNA was amplified for 14 cycles using the Sigma Genomeplex Whole Genome Amplification (WGA2) kit following the manufacturer's protocol (Sigma-Aldrich Co, Catalog Number WGA2). More than 20 ng of IP DNA from each sample was used for library generation following the manufacturer's instructions. Three independent biological sample replicates were employed.

Library construction and deep sequencing were performed as described previously (Wang et al., 2016; Xu et al., 2018). ChIP DNA samples described above were prepared for high throughput Illumina sequencing (one hundred and fifty pair-end read sequencing). The ChIP-seq data was analyzed as described previously (Wang et al., 2016; Xu et al., 2018). The first 30 base pairs from the 5' end containing primer or adapter sequences were trimmed. The 3' end of the sequencing reads were trimmed based on base-call quality using the BWA quality trim algorithm (Li and Durbin, 2009). The sequencing reads were aligned Glycine max reference genome (Glycine max Wm82.a2.v1). Only uniquely mapped reads that mapped to one location of the genome only (as opposed to those that mapped to multiple reads) were retained for peak calling. Three biological replicates were performed for each sample. Each input was used as a control for peak calling for each sample using MACS 1.4 (Zhang et al., 2008). The statistical identification of peaks was performed for each sample using MACS with the default 10^{-5} *p*-value cutoff. The three replicates results were overlapped using BedTools (Quinlan and Hall, 2010). The resulting BED format files that contain the peak location were visualized with the Integrated Genome Viewer (Robinson et al., 2011; Thorvaldsdottir et al., 2013).

ChIP-seq results were verified by ChIP-qPCR for selected genes as previously described (Xu et al., 2018). The relative amounts of Input and IP DNA of all samples were determined using a spectrophotometer (NanoDrop, ONE C). The diluted ChIP DNA was analyzed by qPCR according to the procedure described above for RT-qPCR. Three replicates were done for each sample. Quantification was determined by applying the 2^{-Ct} formula (SuperArray ChIP-qPCR user manual; Bioscience Corporation). Average immunoprecipitates from chromatin isolated independently are expressed on graphs as percentage of corresponding input DNA, with error bars representing the standard deviations. All gene-specific primers are listed in **Supplementary Table 1**.

The *p*-value for the gene expression changes of methylated or *de novo* H3K27me3 genes in salt-treated soybean was calculated by using hypergeometric statistical test as described previously (Xu et al., 2018).

Plasmid Constructions and *Arabidopsis* Transformation

The full length coding sequence of the *Glyma.17G022500* gene was amplified, and inserted into p*CAMBIA1301*, a binary vector, under control of the 35S promoter. The resulting vector was mobilized into *Agrobacterium tumefaciens* strain GV3101. Transformation of *Arabidopsis* wild-type Columbia plants was carried out by the floral dip method as described previously (Sanchez et al., 2009). Transgenic plants were first screened on MURASHIGE and SKOOG (MS) medium supplemented with 50 mg/L hygromycin. Seeds from each T1 plant were individually collected. Selected T2 plants were propagated, and homozygous overexpression lines were confirmed by genotyping analysis. T3 progeny homozygotes were obtained for further analysis.

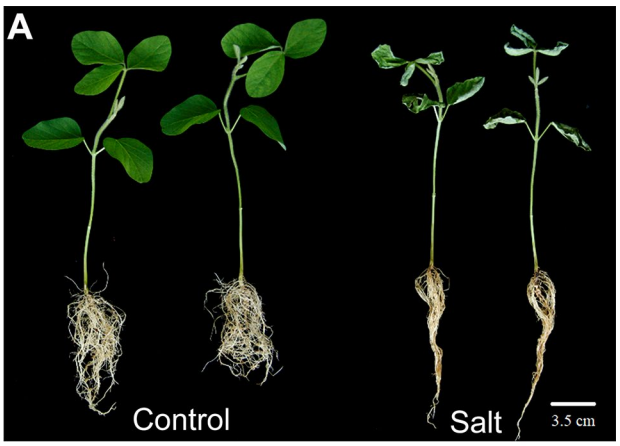
RESULTS

Gene Expression Change in Soybean in Response to Salt Treatment

Salt stress is a major abiotic stress that limits the yield of many crop species. In many plants, roots are the primary site of salinity perception. To better understand the mechanisms active in the

response of roots to salt stress, we studied salt response in soybean with different concentrations of salt treatment (see Materials and methods section). We first evaluated the salt concentration that stressed soybean growth. Three biological replicates were subjected observed the phenotypes of salt-treated plants and found as the concentration of salt stress increased, root growth was increasingly retarded. As a result, we selected to grow roots in 100 mM salt to study the impact of salt stress on gene expression in soybean (**Figure 1A**). We employed RNA-seq technology to analyze genome-wide mRNA transcript levels in soybean roots under 0 mM (control) and 100 mM of salt treatment (salt). The RNA samples from the soybean roots grown with and without salt were sequenced by the Illumina Genome Analyzer. For each sample, we obtained approximately 42–54 million reads, of which 89.15–96.65% were mapped to the soybean reference genome (**Supplementary Table 2**).

From the sequence alignment data, the expression quantification for each sample was calculated using Cufflinks (Trapnell et al., 2013). To identify the salt responsive genes, a core set of differentially expressed genes (DEGs) under salt stress in soybean were examined. We classified them as up- or down-regulated genes with statistically significant two-fold expression changes in the samples treated with 100 mM salt compared to 0 mM control plants. A total of 44,346 soybean genes with confident expression were analyzed (**Supplementary Table 3**). Out of these genes, 8,798 (19.9%) were found to be differentially expressed genes under salt treatment compared to control plants, in which 4,646 genes are up-regulated and 4,152 were down-regulated (**Figure 1B**). There are a little bit more up-regulated genes than down-regulated genes in soybean roots after salt treatment.



B

Stress	Expression change	#	%
Salt	Up	4646	10.5
	Down	4152	9.4
	Total	8798	19.9

FIGURE 1 | Soybean plants treated with salt stress and transcription profile analyzed by RNA-seq. **(A)** Salt treatment (100 mM of salt) on seedling of soybean (salt) and non-stressed control (control). **(B)** Gene expression changes in salt-treated soybean compared to control plants. Up means number and percentage (%) of genes up-regulated and Down means down-regulated relative to WT with p-value < 0.05. The total number of genes investigated is 44,346. Total indicates the total number of mis-regulated genes, i.e., total number of up- plus down-regulated genes. Bar = 2 cm.

GO Analysis of Salt Response Gene in Soybean

Gene ontology (GO) analyses showed that the DEGs under salt stresses occur in many functional groupings (**Figure 2**). The heat-map revealed different GO categories, such as transcriptional regulation, response to stress, defense response, regulation of defense response, and histone methylation represented by the up-regulated enriched genes in these categories (**Figure 2**). Compared to up-regulated genes, down-regulated genes were mainly enriched in metabolic processes. Notably, we found that except defense response, most of the GO categories of up- and down-regulated genes showed an opposite and comparable profile under salinity condition (**Figure 2**), which indicated that salt stress can cause differential and specific gene regulation in order to respond to threatening environmental factors. To explore the molecular mechanism underlying the salt response in soybean, we further analyzed mis-regulated genes whose functions are involved in salt response. Among mis-regulated genes, there were 93 genes which are closely related to salt stress response, in which 53 genes are up- and 40 are down-regulated respectively (**Supplementary Table 3**). To confirm the RNA-seq results, we examined the RNA levels of two known soybean genes, *Glyma.03G226000* and *Glyma.03G171600* (**Supplementary Figure 1**) and 11 selected salt|responsive genes, *Glyma.04G131800*, *Glyma.04G187000*, *Glyma.07G110300*, *Glyma.08G070700*, *Glyma.08G127000*, *Glyma.14G213600*, *Glyma.11G204800*, *Glyma.09G041000*, *Glyma.13G043800*, *Glyma.17g022500* and *Glyma.14G176700*, by RT-quantitative PCR (qPCR) (**Figure 3**). The expression levels determined by qPCR and those by RNA-seq analysis were highly correlated (**Figure 3**), indicating that the results obtained by the independent methods are consistent.

Transcription processes are carried out by transcription factors (TFs). To identify potential TFs involved in salt stress, we then analyzed expression patterns of genome-wide TFs. There are 3017 annotated transcription factors in soybean with expression data belonging to over 50 TF families such as homeodomain, zinc finger, WRKY, SET domain, MYB, MADS, AP2-EREBP, bHLH, NAC, bZIP and GRAS (**Table 1**) (Perez-Rodriguez et al., 2010; Jin et al., 2017). We found 513 TFs up-regulated and 491 are down-regulated under salt treatment, respectively (**Table 1**), which is consistent with the whole expression pattern of RNA-seq. Genes belonging to the bHLH, bZIP, ERF, GRAS, MYB, MYB-related, NAC, and WRKY family represent most of the differentially expressed TFs (**Table 1**). The bHLH, Ethylene Response Factor (ERF) and MYB represented the highest number of significantly expressed genes under salt treatment conditions. GO analyses showed 10 TFs investigated here

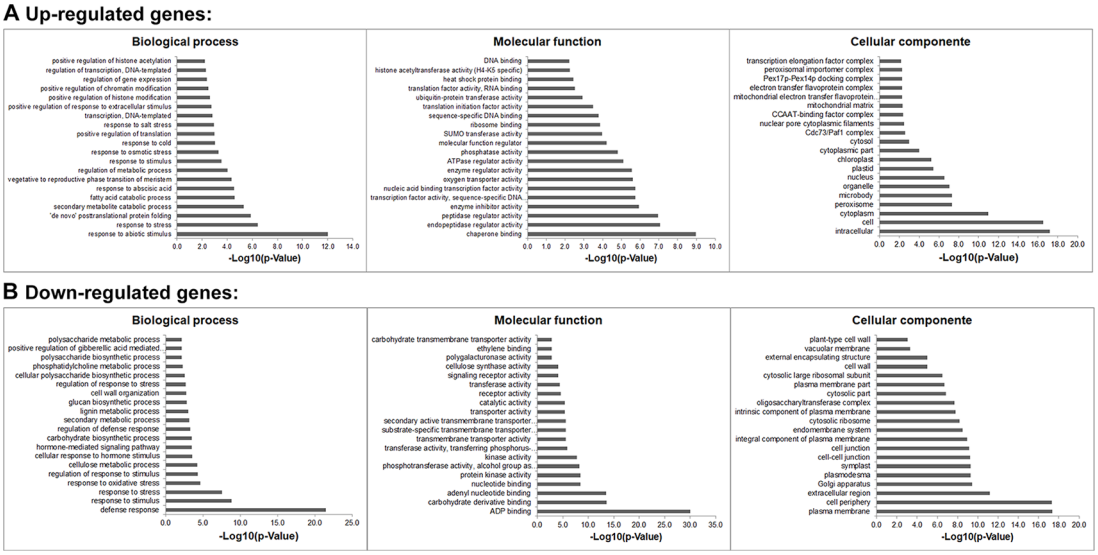


FIGURE 2 | Gene Ontology (GO) study of up- and down-regulated genes under salt stress in soybean. The agriGO program (Tian et al., 2017) was used to identify significantly enriched molecular functions, biological processes and cellular component amongst the mis-regulated (up- or down-regulated) genes (p-value < 0.01). The terms were ranked by p-value.

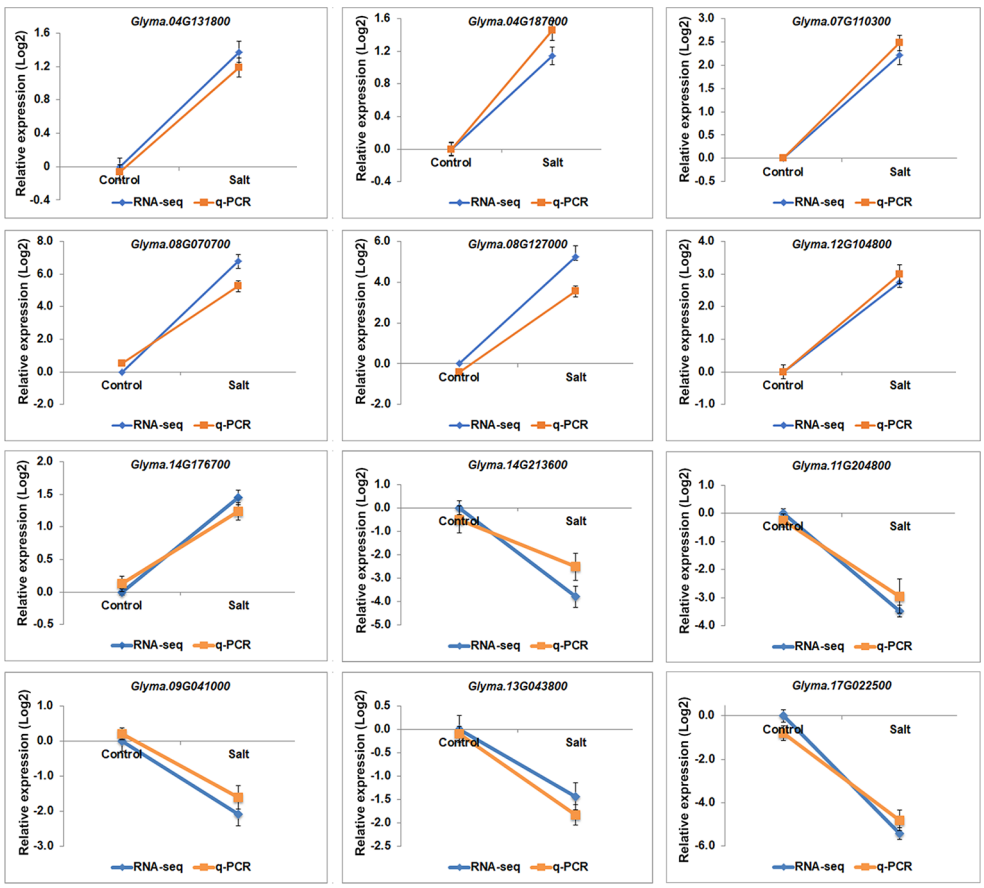


FIGURE 3 | The gene expression profile of selected salt stress genes analyzed by RNA-seq and q-PCR. mRNA expression levels of 12 selected salt stress genes with differential expression levels, *Glyma.04G131800*, *Glyma.04G187000*, *Glyma.07G110300*, *Glyma.08G070700*, *Glyma.08G127000*, *Glyma.12G104800*, *Glyma.14G213600*, *Glyma.11G204800*, *Glyma.09G041000*, *Glyma.13G043800*, *Glyma.17g022500* and *Glyma.14G176700*, in control and salt-treated soybean. Graphs show the relative expression levels analyzed by RNA-seq and by qPCR which normalized to a *Tubulin* (*Glyma.05G203800*) reference gene. Error bars represent standard deviation (SD).

TABLE 1 | Number of transcription factors under salt stress up- or down-regulated at least 2-fold in soybean.

Family	Total number	Up		Down	
		#	%	#	%
AP2	45	10	22.2	7	15.6
ARF	56	18	32.1	11	19.6
ARR-B	26	7	26.9	1	3.8
B3	42	6	14.3	4	9.5
BBR-BPC	10	2	20.0	0	0.0
BES1	15	6	40.0	1	6.7
bHLH	274	50	18.2	37	13.5
bZIP	140	20	14.3	20	14.3
C2H2	188	33	17.6	36	19.1
C3H	75	13	17.3	7	9.3
CAMTA	15	1	6.7	1	6.7
CO-like	22	3	13.6	6	27.3
CPP	12	1	8.3	1	8.3
DBB	20	6	30.0	2	10.0
Dof	71	17	23.9	7	9.9
E2F/DP	14	2	14.3	2	14.3
EIL	10	0	0.0	0	0.0
ERF	245	31	12.7	50	20.4
FAR1	49	5	10.2	1	2.0
G2-like	94	24	25.5	18	19.1
GATA	50	10	20.0	5	10.0
GeBP	8	4	50.0	1	12.5
GRAS	104	24	23.1	17	16.3
GRF	21	0	0.0	1	4.8
HB-other	18	4	22.2	2	11.1
HB-PHD	6	2	33.3	0	0.0
HD-ZIP	85	15	17.6	15	17.6
HRT-like	1	1	100.0	0	0.0
HSF	48	5	10.4	22	45.8
LBD	62	9	14.5	14	22.6
LFY	1	0	0.0	0	0.0
LSD	8	2	25.0	1	12.5
MIKC_MADS	53	11	20.8	7	13.2
M-type_MADS	14	1	7.1	2	14.3
MYB	241	32	13.3	44	18.3
MYB_related	139	19	13.7	26	18.7
NAC	167	23	13.8	33	19.8
NF-X1	5	0	0.0	0	0.0
NF-YA	21	6	28.6	5	23.8
NF-YB	29	4	13.8	5	17.2
NF-YC	17	4	23.5	1	5.9
Nin-like	21	3	14.3	7	33.3
RAV	4	3	75.0	1	25.0
S1Fa-like	4	1	25.0	0	0.0
SAP	1	0	0.0	0	0.0
SBP	38	5	13.2	4	10.5
SRS	21	7	33.3	3	14.3
STAT	1	1	100.0	0	0.0
TALE	63	12	19.0	3	4.8
TCP	44	4	9.1	9	20.5
Trihelix	67	8	11.9	10	14.9
VOZ	6	1	16.7	0	0.0
Whirly	7	1	14.3	0	0.0
WOX	18	2	11.1	0	0.0
WRKY	171	31	18.1	38	22.2
YABBY	6	0	0.0	0	0.0
ZF-HD	24	3	12.5	3	12.5
Total	3017	513	17.0	491	16.3

belong to the category of salt stress response genes, in which 8/10 of genes are MYB TFs, such as *Glyma.12G104800*, *Glyma.16G073000*, *Glyma.01G107500*, *Glyma.15G236400*, *Glyma.06G097100*. This is consistent with previous studies that MYB TFs have been known to regulate salt stress response in plants (Yang et al., 2012; Cui et al., 2013; Li et al., 2016; Wei et al., 2017). Notably, the fold changes of some TFs were significantly higher than control plants (**Supplementary Table 3**). To verify the RNA-seq results, we examined the RNA level of one MYB gene, *Glyma.12G104800*, by qPCR analysis (**Figure 3**). The expression level determined by RT-qPCR and RNA-seq were highly consistent (**Figure 3**), confirming the results of the genome-wide analysis.

Trimethylation of H3K27 Under Salt Stress in Soybean

Trimethylated histone H3 at lysine residues 27 (H3K27me3) has been detected in many organisms, including *Arabidopsis*, rice, and maize (Butenko and Ohad, 2011). It is a hallmark of gene silencing (Schubert et al., 2006; Zheng and Chen, 2011). However, whether this repressive mark is involved, and to what extent, in salt stress response in soybean is unknown. To determine the alteration of chromatin dynamics and transcriptional apparatus that respond to environmental changes, we applied ChIP-seq to monitor the changes of H3K27me3 levels at genome-wide scale under salt stress treatment in soybean (**Figure 4**). ChIP-seq was performed by using an antibody specifically recognizing H3K27me3 (Pu et al., 2013; Xu et al., 2018), and the precipitated DNAs were then sequenced. After sequencing, we obtained about 50 million of clean reads with 75–85% of the reads that could be mapped to the soybean genome (**Supplementary Table 4**). Verification of ChIP-seq results using Pearson correlation analysis showed statistically significant correlation coefficients among the biological replicates for each sample (**Supplementary Figure 2**). Genomic regions associated with H3K27me3 modification were identified by using MACS software (Zhang et al., 2008). The peak distributions of ChIP-seq are similar and average length of peaks is around 700 bp in samples of control and salt-treated plants (**Supplementary Figures 3A, B**).

The MACS peak finding program identified thousands of H3K27me3 enriched peaks in control and salt-treated samples ($p < 10^{-3}$) across the whole chromosome (**Figure 4A** and **Supplementary Figure 4**), which correspond to 1,707 and 746 annotated genes, respectively (**Supplementary Figure 3B**). As reported previously in *Arabidopsis* (Kim et al., 2012b; Xu et al., 2018), H3K27me3 peaks tend to be broad, often covering the entire transcriptional unit, hence we used a very strict statistical cutoff for peak identification. In control plants, the 1,707 genes were termed K27 genes in the next analysis vs. *de novo*_K27 genes (**Supplementary Tables 5 and 6**). We plotted the average H3K27me3 signal of the 1,707 K27 genes across the 7 kb region surrounding the transcription start site (TSS) and the transcription end site (TES) in the control soybean (**Figure 4B**). Similar to that of *Arabidopsis*, a broad H3K27me3 enrichment covers the entire transcriptional unit with the strongest signal around the TSS region, whereas the H3K27me3 signal gradually declined towards the 3' end and increased around the TES region, suggesting the conservation and divergence of epigenetic patterns across plant species (**Figure 4B**). We then checked the

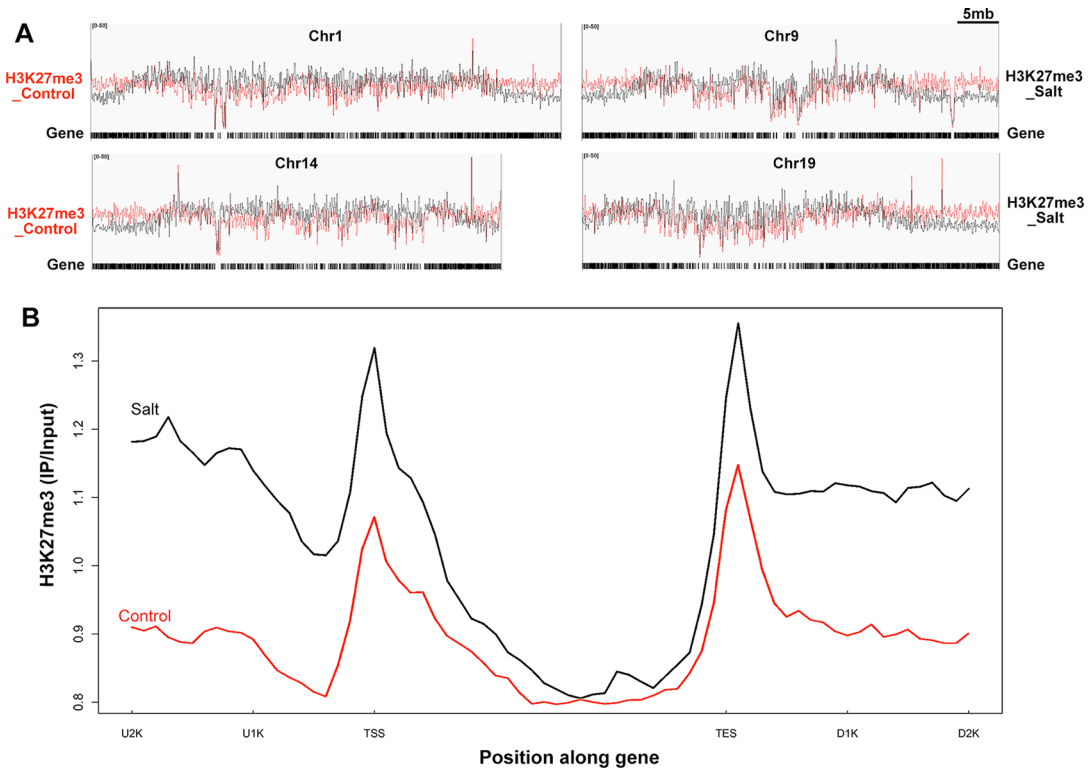


FIGURE 4 | Genome-wide H3K27me3 modification pattern in control and salt-treated soybean. **(A)** Chromosomal distribution of H3K27me3 modification sites on the randomly selected 4 soybean chromosomes. Y-axis represents the input signals for the immunoprecipitation of H3K27me3 in control on the left side (H3K27me3_Control) and salt-treated soybean on the right side (H3K27me3_Salt). The comparison of H3K27me3 marked in control (red) and salt (black) plants were shown on all chromosomes. Chr and 5mb represent chromosome and 5 megabase, respectively. Gene models shown at the bottom. **(B)**The H3K27me3 patterns of all trimethylated genes in control and salt-treated soybean. The gene sequences were aligned at the transcription start site (TSS) and average signals of the H3K27me3 enrichment 2kb upstream (U2K), 3kb gene body, and 2kb downstream of the TES (D2K) were plotted.

histone modification pattern of 746 H3K27me3 marked genes in salt-treated soybean which remained remarkably similar to that in control plants (**Figure 4B** and **Supplementary Table 6**). Compared to H3K27me3 pattern in control plants, the epigenetic marks of H3K27me3 showed a greater enrichment in salt-stressed samples (**Figure 4B**), suggests that stress caused changes in chromatin structure and histone modification which accompany changes in gene expression in response to abiotic stresses.

Relationship Between Changes in H3K27me3 and Gene Expression Under Salt Stress

H3K27me3 has been proposed to be correlated with gene silencing in many organisms (Pu and Sung, 2015). So we questioned if H3K27me3 modification is correlated with a different expression level under salt stress. We combined the specific H3K27me3 modification datasets with our DEGs to identify the relationship between H3K27me3 modification and different expression levels. We found that 829 of the 1,707 H3K27me3 specifically modified genes were not expressed in both of control and salt samples, despite some of them were not trimethylated (**Table 2**) which may be caused by our criteria used for analyzing the RNA-seq data (see Materials and methods section) as reported in our previous study (Xu et al., 2018). It is also possibly caused by the fact that not all genes expressed in soybean roots. By excluding those non-expressed genes, only 878 (51%) expressed genes were trimethylated on H3K27 in the control and salt datasets (**Table 2**). These specific H3K27me3 genes were then checked for the expression level changes in the corresponding treatment, and the numbers of up- and down-regulated genes in each of the specific H3K27me3 modification datasets were further analysed (**Table 2**). Under salt stress treatment, 170 of 336 K27 genes (50.6%) were up-regulated (**Table 2**). Statistical tests of the genome-wide relationship between reduced H3K27me3 and transcriptional deregulation (**Table 2**) in

TABLE 2 | The gene expression changes of methylated H3K27me3 genes in salt-treated soybean.

Total number of genes investigated	Number of genes with expression in RNA-seq data	Number of genes with decreased K27	Up-regulated expression		
			Number	%	*p*-value
1,707	878	336	170	50.6	1.03×10^{-15}

the salt-treated plants showed that 50.6% (p-value = 1.03x10^{-15}) of up-regulated genes also had reduced H3K27me3 levels.

We noticed that H3K27me3 is associated with expression changes of specific salt stress genes which likely contribute to response to environmental changes. Eleven genes, *Glyma.17G006800*, *Glyma.08G070700*, *Glyma.08G127000*, *Glyma.15G252200*, *Glyma.12G104800*, *Glyma.07G110300*, *Glyma.17G223600*, *Glyma.20G021200*, *Glyma.04G131800*, *Glyma.04G187000*, *Glyma.20G072600* were significantly up-regulated in salt treated samples (**Supplementary Table 3**). Six salt stress genes, *Glyma.08G070700*, *Glyma.08G127000*, *Glyma.12G104800*, *Glyma.07G110300*, *Glyma.04G131800*, *Glyma.04G187000*, showed lower H3K27me3 levels and higher mRNA expression levels after salt treatment (**Figure 3** and **Figure 5A**). To confirm the ChIP-seq results, we performed ChIP-qPCR on three selected salt response genes, *Glyma.07G110300*, *Glyma.04G131800*, *Glyma.04G187000*,

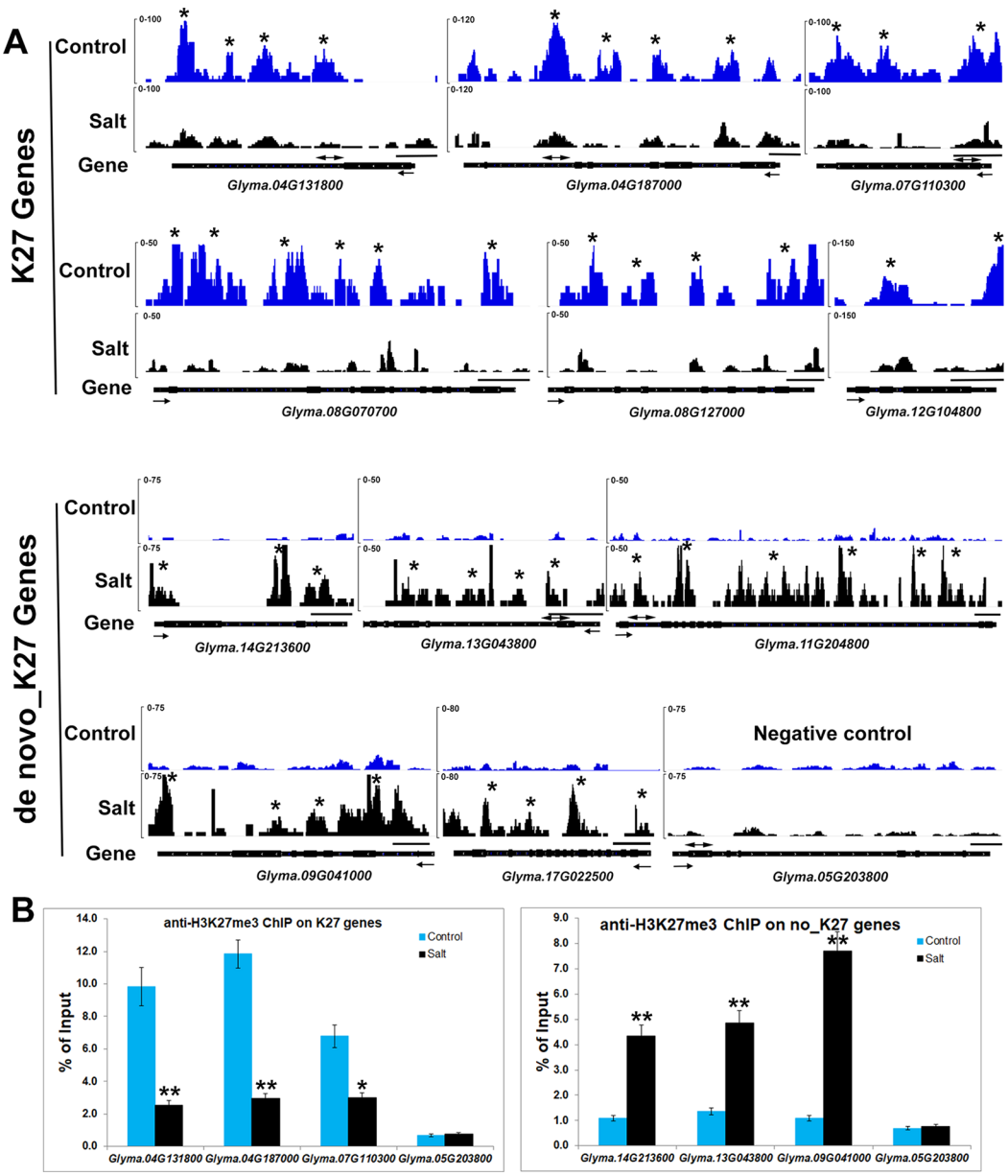


FIGURE 5 | Salt stress affects histone methylation at salt stress gene loci in soybean. **(A)** H3K27me3 patterns of K27 and *de novo*_K27 genes from ChIP-seq data in control and salt-treated soybeans. Gene models are shown at the bottom including 5' UTR (medium black line), exon (black box), intron (thin black line) and 3' UTR (medium black line). The arrow indicates transcriptional direction. The black line above gene model indicates 500bp. The "*" indicates that the MACS_peak with the statistical identification each sample using MACS with the default 10^{-5} p-value cutoff. **(B)** ChIP-qPCR analysis of H3K27me3 levels at the salt stress genes in soybean under salt stress by using *Glyma.05G203800* (*Tubulin*) gene as the negative control. ChIP-qPCR results are expressed as a percentage of input DNA, with error bars representing SD. Primers (double arrowheads) correspond to the gene regions shown in **(A)**. Significant differences from the control (Student's t test) are marked with asterisks (**P <0.01).

that showed enhanced expression levels under stress, using the un-methylated *Glyma.05G203800* (*Tubulin*) gene as the negative control (**Figure 5B**). We found that these salt response genes had much higher H3K27me3 levels in the control plants which decreased greatly under salt stress indicating that salt stress can remove the deposition of repressive chromatin marks at these loci during stress treatment (**Figure 5**). These results show that plants respond to the environmental changes through the transcriptional machinery in which transcription was turned on or shut down by changing the mode of histone modifications between activation and inactivation on all of stress response genes.

Salt Stress Causes *de novo* Histone Methylation and Gene Silencing Under Salt Stress in Soybean

Surprisingly, we found that there were only 5 of 878 K27genes (0.5%) with increased H3K27me3 marks in salt treatment. We then asked what happened to the *de novo*_K27 genes after salt stress treatment. It has been reported that *de novo* methylation can occur in a locus-specific manner during development in yeast, plant and animals (Ooi et al., 2007; Bouyer et al., 2011; Morselli et al., 2015; Stewart et al., 2015). Among 746 H3K27me3 marked genes in salt-treated soybean, we found 651 genes appeared to be marked *de novo* H3K27me3 in salt-treated plants which mainly contributed to the greater H3K27me3 pattern in salt-treated plant (**Supplementary Table 6**).

Among 651 genes, there are 294 genes with expression data in our RNA-seq analysis (**Table 3**). Statistical tests of the genome-wide relationship between increased H3K27me3 and transcriptional deregulation (**Table 3**) in the salt-treated plants showed that 33.7% (p-value = 1.9×10^{-31}) of down-regulated genes also had increased H3K27me3 levels. Some of these genes are stress-responsive genes such as *Glyma.14G213600*, *Glyma.11G204800*, *Glyma.09G041000*, *Glyma.13G043800* and *Glyma.17g022500* (**Figures 3** and **5**). Although the gain of H3K27me3 is not associated with all down-regulated genes, it contributed the greater level of H3K27me3 modification in salt stress condition compared to that in control plants.

Changes in Histone Methylation and Demethylation Contribute to Changes in H3K27me3 Modification Levels Under Salt Stress in Soybean

The H3K27 was trimethylated by histone methyl transferases (HMTs) and demethylated by HDMs (Papp and Muller, 2006; Horton et al., 2010; Pu and Sung, 2015). To explore how histone modifications were regulated when under salt stress in soybean, we used RNA-seq data to investigate the candidate causal genes of methyltransferase or demethylase for salt response. We identified 43 HMT proteins from Soybase according to the protein sequence homology with *Arabidopsis* HMTs (Grant et al., 2010). Specifically, 9 soybean genes which are homologous to *Arabidopsis* known methyltransferase genes *CURLY LEAF (CLF)*, *ATX* and *SDG*, were down-regulated, and 2 genes are up-regulated with significant p-value in salt-treated plants (**Table 4**). CLF has been well characterized in *Arabidopsis* to work specifically as H3K27 methyltransferases (Katz et al., 2004; Schatlowski et al., 2010). ATX and SDG proteins were known to methylate H3K4 and limit deposition of H3K27me3 on target loci (Ding et al., 2007; Carles and Fletcher, 2009; Grini et al., 2009; Berr et al., 2010; Guo et al., 2010; Tang et al., 2012; Yao et al., 2013). To verify the RNA-seq results, we examined the RNA levels of 4 selected methyltransferase genes, *Glyma.16G207200*, *Glyma.06G223300*, *Glyma.17G215200* and *Glyma.11G054100*, by qPCR (**Figure 6A**). The expression levels determined by qPCR and RNA-seq were highly correlated (**Figure 6A**), indicating that the results obtained by the independent methods are consistent. Therefore, these soybean proteins may function as methyltransferase to alter histone modifications of target loci for salt response.

Jumonji C (JmjC) proteins are known to demethylate all of the mono-, di and trimethylated lysines of histones (Chen et al., 2011). There are over 20 JmjC proteins have been discovered in *Arabidopsis* which are able to demethylate lysine H3K4, H3K9, H3K27, and H3K36. We checked the expression pattern of 21 JmjC proteins in salt-treated soybeans (**Table 5**). Interestingly, we found that 3 of JmjC proteins were down-regulated, and 1 was up-regulated (**Table 5** and **Figure 6B**). 2 of down-regulated genes, *Glyma.04G192000* and *Glyma.20G181000*, are homologues to *Arabidopsis Early flowering 6* (*REF6*) and *Relative of ELF6* (*ELF6*) which are known demethylases to mediate histone methylation (Yu et al., 2008; Lu et al., 2011). These results indicated the involvement of histone modifiers in the changing H3K27me3, subsequently transcript levels during salt stress.

Overexpression of One Soybean Gene Enhances the Salt Tolerance in Transgenic *Arabidopsis*

The differentially expressed genes identified through RNA-seq were considered to be preferentially genes involved in abiotic stress responses, suggesting their stress regulation in the soybean plants. To investigate whether these genes would affect the stress response, we selected one of mis-regulated genes, *Glyma.17G022500* and studied its effect on salt tolerance or sensitivity in *Arabidopsis*. The transgenic *Arabidopsis* plants containing *Glyma.17G022500* under the control of the *CaMV35S* promoter in the *pCAMBIA1301* vector were generated. Independent transgenic lines were obtained by Hygromycin-resistance selection and confirmed by genotyping PCR. The homozygous T3 generation of three independent overexpression lines, namely OE-1, OE-2, OE-3, and the control line (WT) were used for further analysis (**Supplementary Figure 5**). To avoid adverse effects of salt treatment on germination, we transferred 5 days' seedlings of WT, OE-1, OE-2, and OE-3, from MS plates to MS medium containing 150 mM of salt and grew them for an additional 5 days under SD conditions. After 5 days salt stress, the transgenic *Glyma.17G022500* lines displayed a higher salt tolerance than the WT plants (**Figure 7**). As shown in

TABLE 3 | The gene expression changes of *de novo* methylated H3K27me3 genes in salt-treated soybean.

Total number of genes investigated	Number of genes with expression in RNA-seq data	Down-regulated expression		
		Number	%	p-value
651	294	99	33.7	1.9×10^{-31}

TABLE 4 | Expression profile of histone methyltransferases in soybean.

Gene	*Arabidopsis* homologues and Annotation		Log_2 FC	*p-value*
Glyma.17G215200	*AT1G05830*	ATX2|trithorax-like protein 2	–5.58	1.89E-05
Glyma.16G100200	*AT4G13460*	SDG22, SUVH9, SET22|SU(VAR)3-9 homolog 9	–3.83	0.068396
Glyma.11G054100	*AT2G23380*	CLF, ICU1, SDG1, SET1|SET domain-containing protein	–3.38	2.52E-06
Glyma.13G186800	*AT5G04940*	SUVH1|SU(VAR)3-9 homolog 1	–2.60	0.001074
Glyma.07G056000	*AT3G61740*	SDG14, ATX3|SET domain protein 14	–2.28	0.003279
Glyma.06G151500	*AT5G09790*	ATXR5, SDG15|ARABIDOPSIS TRITHORAX-RELATED PROTEIN 5	–2.14	0.032018
Glyma.01G188000	*AT2G23380*	CLF, ICU1, SDG1, SET1|SET domain-containing protein	–1.84	0.012066
Glyma.15G224400	*AT1G73100*	SUVH3, SDG19|SU(VAR)3-9 homolog 3	–1.81	0.020458
Glyma.20G005400	*AT4G13460*	SDG22, SUVH9, SET22|SU(VAR)3-9 homolog 9	–1.71	0.028072
Glyma.12G196800	*AT4G15180*	SDG2, ATXR3|SET domain protein 2	–1.67	0.007215
Glyma.13G306800	*AT3G21820*	ATXR2, SDG36|histone-lysine N-methyltransferase ATXR2	–1.41	0.643551
Glyma.02G095600	*AT5G24330*	ATXR6, SDG34|ARABIDOPSIS TRITHORAX-RELATED PROTEIN 6	–1.27	0.79442
Glyma.04G236500	*AT5G53430*	SDG29, SET29, ATX5|SET domain group 29	–1.21	0.127662
Glyma.11G038000	*AT2G22740*	SUVH6, SDG23|SU(VAR)3-9 homolog 6	–1.20	0.147649
Glyma.19G124100	*AT1G73100*	SUVH3, SDG19|SU(VAR)3-9 homolog 3	–1.11	0.179543
Glyma.07G157400	*AT4G13460*	SDG22, SUVH9, SET22|SU(VAR)3-9 homolog 9	–0.96	0.173577
Glyma.15G158500	*AT5G42400*	ATXR7, SDG25|SET domain protein 25	–0.78	0.263644
Glyma.04G125500	*AT5G04940*	SUVH1|SU(VAR)3-9 homolog 1	–0.66	0.353851
Glyma.16G024900	*AT3G61740*	SDG14, ATX3|SET domain protein 14	–0.55	0.580097
Glyma.03G119900	*AT1G73100*	SUVH3, SDG19|SU(VAR)3-9 homolog 3	–0.46	0.436713
Glyma.13G305000	*AT4G15180*	SDG2, ATXR3|SET domain protein 2	–0.46	0.467736
Glyma.09G156500	*AT2G44150*	ASHH3, SDG7|histone-lysine N-methyltransferase ASHH3	–0.28	0.645242
Glyma.02G012100	*AT4G02020*	EZA1, SWN, SDG10|SET domain-containing protein	–0.23	0.840641
Glyma.10G222800	*AT1G76710*	ASHH1|SET domain group 26	–0.18	0.806477
Glyma.19G066800	*AT4G27910*	ATX4, SDG16|SET domain protein 16	–0.11	0.871815
Glyma.10G012600	*AT4G02020*	EZA1, SWN, SDG10|SET domain-containing protein	–0.04	0.953137
Glyma.03G215600	*AT3G61740*	SDG14, ATX3|SET domain protein 14	0.06	0.943206
Glyma.06G301900	*AT4G15180*	SDG2, ATXR3|SET domain protein 2	0.18	0.765767
Glyma.04G245400	*AT1G77300*	EFS, SDG8, CCR1, ASHH2, LAZ2|histone methyltransferases(H3-K4 specific);histone methyltransferases(H3-K36 specific)	0.26	0.651076
Glyma.04G214600	*AT5G09790*	ATXR5, SDG15|ARABIDOPSIS TRITHORAX-RELATED PROTEIN 5	0.29	0.887053
Glyma.11G040100	*AT2G22740*	SUVH6, SDG23|SU(VAR)3-9 homolog 6	0.37	0.869301
Glyma.12G195700	*AT3G21820*	ATXR2, SDG36|histone-lysine N-methyltransferase ATXR2	0.38	0.54887
Glyma.01G204900	*AT2G22740*	SUVH6, SDG23|SU(VAR)3-9 homolog 6	0.40	0.514298
Glyma.18G282700	*AT1G05830*	ATX2|trithorax-like protein 2	0.40	0.524246
Glyma.12G102400	*AT4G15180*	SDG2, ATXR3|SET domain protein 2	0.45	0.446104
Glyma.09G052200	*AT5G42400*	ATXR7, SDG25|SET domain protein 25	0.49	0.445646
Glyma.20G168900	*AT1G76710*	ASHH1|SET domain group 26	0.55	0.376492
Glyma.06G117700	*AT1G77300*	EFS, SDG8, CCR1, ASHH2, LAZ2|histone methyltransferases(H3-K4 specific);histone methyltransferases(H3-K36 specific)	0.60	0.345329
Glyma.08G258500	*AT1G05830*	ATX2|trithorax-like protein 2	0.76	0.545905
Glyma.18G285900	*AT5G24330*	ATXR6, SDG34|ARABIDOPSIS TRITHORAX-RELATED PROTEIN 6	1.08	0.247913
Glyma.16G207200	*AT2G44150*	ASHH3, SDG7|histone-lysine N-methyltransferase ASHH3	1.65	0.024473
Glyma.01G202700	*AT2G22740*	SUVH6, SDG23|SU(VAR)3-9 homolog 6	2.64	0.077437
Glyma.06G223300	*AT4G30860*	ASHR3, SDG4|SET domain group 4	2.79	0.003304

Figure 7A, the WT plants became severely wilted and impaired with white cotyledons and leaves after salt stress. However, the transgenic *Glyma.17G022500* lines showed more open, green leaves in all three independent lines (**Figure 7A**). The *Glyma.17G022500* transgenic lines displayed significantly higher survival rate than the WT plants after salt treatment (**Figure 7B**). Furthermore, we found that the roots in the transgenic lines grew longer than that in the WT plants on salt plates (**Figure 7C**). These results indicate that overexpression of *Glyma.17G022500* enhances *Arabidopsis* salt stress tolerance which confirmed our RNA-seq results.

DISCUSSION

The soybean gene methylation pattern is characteristic of plant methylation pattern. Here, we investigated the modification profiles of H3K27me3 after salt stress treatment in soybean. H3K27me3 was detected mainly in TSS and TES regions and 1,707 annotated genes were identified with H3K27me3 marks (**Figure 4**), which displayed the conservation and divergence of epigenetic patterns to previous studies in *Arabidopsis* (Zhang et al., 2007; Kim et al., 2012b). We further analyzed K27, *de novo*_K27 genes as well as DEGs and revealed different dynamic changes in H3K27me3 profiles taking place upon salt stress. The specific H3K27me3 patterns including *de novo* methylation at up-regulated and down-regulated genes were identified during the stress treatment. In addition, a comprehensive overview of the histone modifiers were identified which may regulate differential H3K27me3 modification leading to activation or inactivation of gene expression during salt stress in soybean.The certain proportion of H3K27me3-modified genes without expression support also implies that the H3K27me3

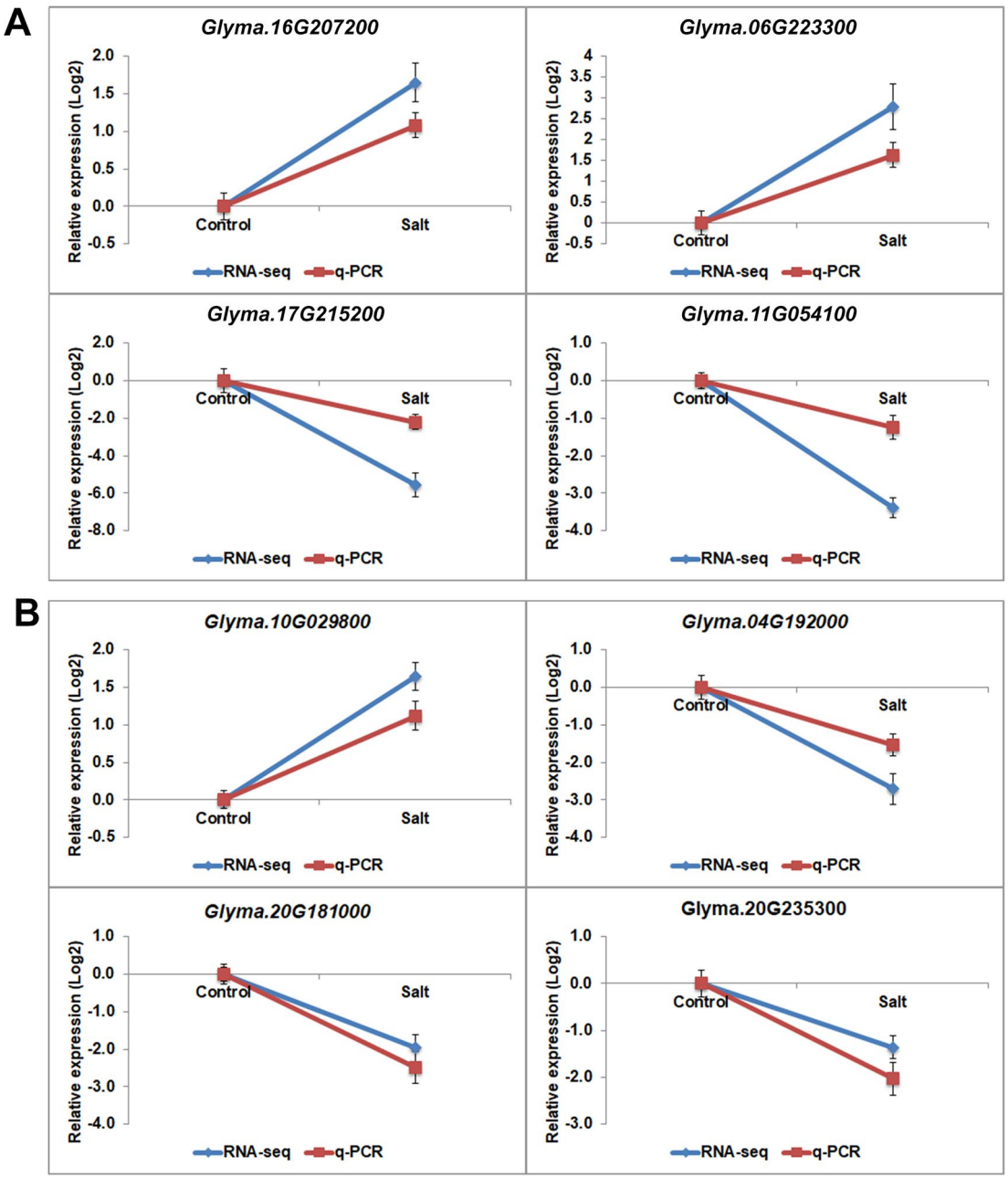


FIGURE 6 | The gene expression pattern of selected histone modifier genes analyzed by RNA-seq and q-PCR. mRNA expression levels of 4 histone methyltransferases **(A)** and histone demethylasesgenes **(B)** with differential expression levels in salt-treated soybean compared to control plants. Graphs show the relative expression levels analyzed by RNA-seq and by qPCR which normalized to a *Tubulin* (*Glyma.05G203800*) reference gene. Error bars represent standard deviation (SD).

level may be associated with expression levels of a subset of genes in soybean genome by working together with other factors, such as the HMT, SDG proteins which bind the H3K27me3 site (Papp and Muller, 2006; Schuettengruber et al., 2011; Thorstensen et al., 2011).

The differentially expressed genes identified in this study were considered to be the key genes involved in the stress response mechanism in the plants. Some of them have been shown to be related to salt response in soybean. For example, *GmSALT3/GmCHX1* (*Glyma.03G171600*) which is a gene associated with salt tolerance with great potential for soybean improvement showed down-regulated expression pattern after salt treatment (Guan et al., 2014; Qi et al., 2014; Liu et al., 2016). The Na +/H+ antiporter gene *GmNHX1* (*Glyma.20G229900*) which can enhance salt tolerance of soybean roots (Li et al., 2006; Sun et al., 2006; Yang et al., 2017). A soybean glycogen synthase kinase 3 gene which can enhance tolerance to salt was up-regulated in salt

TABLE 5 | Expression profile of the histone demethylases in soybean.

Gene	*Arabidopsis* homologues and Annotation		Log_2 FC	*p*-value
Glyma.04G192000	*AT3G48430*	REF6|relative of early flowering	–2.71	0.0017
Glyma.20G181000	*AT5G04240*	ELF6|Zinc finger (C2H2 type) family protein/transcription factor jumonji (jmj) family prote	–1.97	0.0032
Glyma.04G185900	*AT5G63080*	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	–1.87	0.1304
Glyma.06G174000	*AT3G48430*	REF6|relative of early flowering 6	–1.48	0.1951
Glyma.19G064000	*AT1G62310*	Transcription factor jumonji (jmjC) domain-containing protein	–1.43	0.0825
Glyma.20G235300	*AT1G09060*	Zinc finger, RING-type;Transcription factor jumonji/aspartyl beta-hydroxylas	–1.36	0.0271
Glyma.14G159400	*AT1G11950*	Transcription factor jumonji (jmjC) domain-containing protein	–0.99	0.5115
Glyma.12G055000	*AT3G20810*	JMJD5|2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protei	–0.25	0.6778
Glyma.11G130600	*AT3G20810*	JMJD5|2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	–0.03	0.9667
Glyma.10G209600	*AT5G04240*	ELF6|Zinc finger (C2H2 type) family protein/transcription factor jumonji (jmj) family protein	0.13	0.8604
Glyma.19G068800	*AT4G00990*	Transcription factor jumonji (jmjC) domain-containing protein	0.16	0.7779
Glyma.02G144300	*AT5G19840*	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.16	0.8740
Glyma.09G207400	*AT5G46910*	Transcription factor jumonji (jmj) family protein/zinc finger (C5HC2 type) family protein	0.26	0.6988
Glyma.11G023700	*AT1G63490*	transcription factor jumonji (jmjC) domain-containing protein	0.36	0.5608
Glyma.20G104900	*AT4G00990*	Transcription factor jumonji (jmjC) domain-containing protein	0.48	0.6567
Glyma.07G263200	*AT1G11950*	Transcription factor jumonji (jmjC) domain-containing protein	0.60	0.4016
Glyma.10G284500	*AT4G00990*	Transcription factor jumonji (jmjC) domain-containing protein	0.74	0.4306
Glyma.10G153000	*AT1G09060*	Zinc finger, RING-type;Transcription factor jumonji	0.88	0.1398
Glyma.01G219800	*AT1G63490*	transcription factor jumonji (jmjC) domain-containing protein	0.92	0.1816
Glyma.01G014700	*AT5G46910*	Transcription factor jumonji (jmj) family protein/zinc finger (C5HC2 type) family protei	1.36	0.0890
Glyma.10G029800	*AT5G19840*	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	1.65	0.0037

treated soybean in this study (Wang et al., 2018). Other known salt responsive genes identified through RNA-seq analysis (Zeng et al., 2019) such as *Glyma.02G228100*, *Glyma.04G180400*, *Glyma.03G226000*, *Glyma.08G189600*, *Glyma.02G228100* et al., were also identified in this study. We also identified new candidate genes for salt response in soybean. For example, the gene on Chr. 7, *Glyma.07G110300*, which was up-regulated in the salt-treated plants (**Figure 3**) was annotated as "UDP-glucosyltransferase superfamily protein" in this study, which was in agreement with earlier observations that the glucosyltransferase modulates abiotic stress tolerance in *Arabidopsis* (Tognetti et al., 2010; Liu et al., 2015). The gene *Glyma.04G131800*, which was annotated as "prohibitin-3, mitochondrial", was also up-regulated in the salt-treated soybeans (**Figure 3**). The members of prohibitin family acted in stress response (Wang et al., 2010; Seguel et al., 2018). The gene *Glyma.04G187000* encodes a histone deacetylase which is a histone modifier with direct function in regulation of stress response in plants (Chen and Wu, 2010; Chen et al., 2010; Luo et al., 2012b; Zheng et al., 2016). Overexpression of one mis-regulated gene, *Glyma.17G022500*, in *Arabidopsis* resulted in higher survival rates than those in WT lines under salt stress (**Figure 7B**), and the resistance to salt was significantly different (**Figure 7**). Therefore, we conclude that *Glyma.17G022500* has an important effect on resistance to salt stress. This analysis of gene expression patterns between control and salt plants provides a number of candidate genes which might be directly or indirectly involved in the stress response trait. The further genetic analysis and transformation experiments could be used to confirm their roles in salt stress response in the soybean genotypes.

The repression of genes in development mediated by H3K27me3 modification is a highly conserved mechanism in both plants and animals. There are several thousand genes, ~ 20% of all transcribed genes, are marked by such modifications in *Arabidopsis* (Zhang et al., 2007; Hennig and Derkacheva, 2009; Lu et al., 2011; Kim et al., 2012b; Xu et al., 2018). Here, there are only ~2,000 (5%) genes identified as H3K27me3 marked genes in soybean which is lower than the average percentage in *Arabidopsis*. Our results showed that H3K27me3 was correlated with only small parts of genome-wide transcript changes of mis-regulated genes during salt stress response in soybean (**Tables 2** and **3**). This may be due to H3K27me3 not being the only repressive histone modification marks for gene silencing in soybean since other repressive or active histone modification marks may play a vital role in regulating gene expression in response to stress (Chinnusamy and Zhu, 2009; Kim et al., 2012a; Liew et al., 2013; Zong et al., 2013; Yang et al., 2014; Liu et al., 2018). It has been reported that many histone modification marks such as active marks: H3K27ac, H3K4me3, H3K36me3, H3K9ac, and repressive marks: H3K9me3, H2K119ub (Bratzel et al., 2010; Gu et al., 2013; Yang et al., 2014; Xu et al., 2016), are known to be positively or negatively correlated with active or silencing transcription in plants. Indeed, here we found the gene of *Glyma.04G187000* which encodes a histone deacetylase was up-regulated in soybean under salt stress (**Figure 3**), suggesting that it may regulate gene expression through histone acetylation.

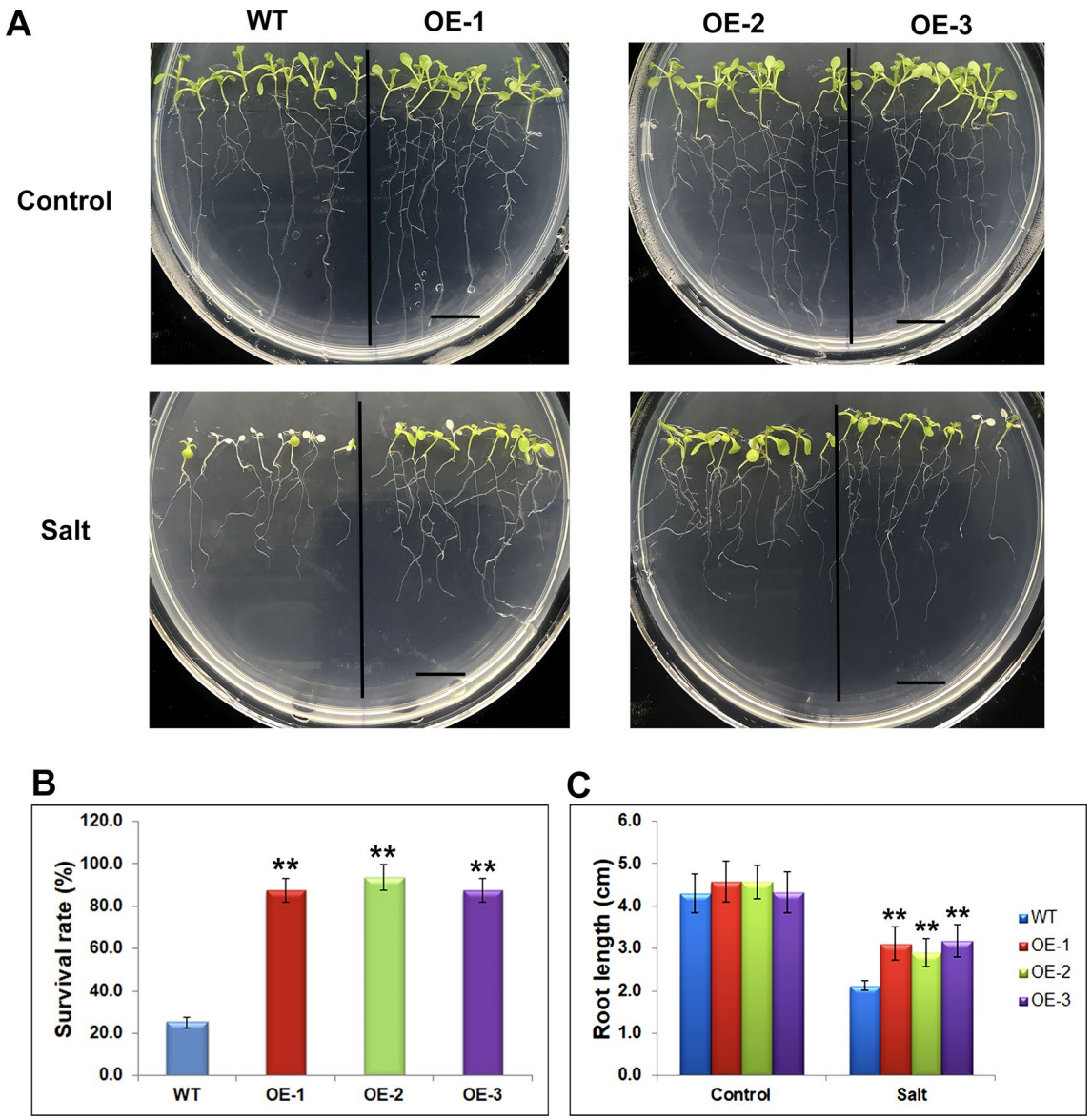


FIGURE 7 | Phenotypes of *Glyma.17g022500* transgenic plants under salt stress. **(A)** Salt tolerance assay of the *Glyma.17g022500* overexpression (OE) lines, OE-1, OE-2, OE-3 plants. WT, OE-1, OE-2 and OE-3 seedlings at 5 DAG were transferred from MS medium to MS medium containing 150 mM salt and grown for an additional 5 days. Bar = 1 cm. **(B)** Survival rate of the plants in **(A)** under salt stress. The data presented are the mean ± SD (n = 50). **(C)** Root length of seedlings grown on medium with and without salt. Root length was measured after 5 days of growth on MS or MS with salt (n = 50). Significant differences from the WT (Student's t test) are marked with asterisks (**P <0.01).

Chromatin accessibility is defined as the availability of DNA sequences for molecular interactions, typically mediated through by DNA binding factors and nucleosomes that are the major factors of chromatin accessibility (van Steensel, 2011). Nucleosome-free regions have been observed in many organisms and are associated with transcriptionally active genes (Henikoff and Shilatifard, 2011). How do these H3K27me3 marks induce silencing of genes expression? The H3K27me3 marks are mainly mediated by Polycomb group (PcG) proteins which cause gene expression by histone modification and nucleosome condensation. Recent studies reported that PcG-mediated H3K27me3 can spread on chromosome and lead to chromatin compaction (Xu et al., 2018). In the absence of PcG genes, the maintenance of chromatin integrity with gene repression by directly associating with target gene loci became lesser extent. The chromatin cannot be a tightly folded structure with lower levels of H3K27me3 modifications (Becker and Workman, 2013; Kingston and Tamkun, 2014). Therefore, more repressive histone marks on a given gene lead to lower transcript levels, whereas less marks cause higher expression levels, which is consistent with our results in this study. Despite K27 or *de novo*_K27 genes, the decreasing in H3K27me3 levels accompanies the de-regulation of gene expression in salt stress treatment (**Figures 3** and **5**). However, *de novo* enrichment of H3K27me3 on target genes leads to repression of expression

(**Figures 3** and **5**). These results indicated that H3K27me3 play vital roles in maintaining the appropriate chromatin conformation and integrity, thereby avoiding uncontrolled transcriptional activity when response to abiotic stresses (**Figure 8**).

Indeed, H3K27me3 was negatively correlated with transcript levels in all organisms. A high level of histone H3K27 methylation results in low transcript levels and low H3K27me3 modification levels, lead to actively transcribed genes (Pu and Sung, 2015). In our study, we noticed that most H3K27me3 marked genes were identified in control plants with the basal expression levels (**Supplementary Table 3**). The decrease in repressive H3K27me3 marks of most H3K27me3 genes identified in control plants with up-regulated expression in salt stress is consistent with the notion that the absence of repressive chromatin marks could result in the activation of transcript (**Table 2**). However, the fact that the whole H3K27me3 pattern in salt stress plants was greater than that in control plants was unexpected, although consistent with the differential gene expression pattern (**Figure 4**). In other words, most K27me3 marked genes were mainly those with low expression levels, under stressed conditions. In contrast, genome-wide H3K27me3 pattern in salt treated plants did not show such a trend, indicating that new or *de novo* H3K27me3 marks occurred after salt treatment which may underlie the association of salt-responsive patterns, down-regulation, with differential expression levels. Indeed, we found 650 H3K27me3 marked genes which were not trimethylated in control plants gained H3K27me3 marks after salt stress treatment. The *de novo* methylation has been reported that the new modification can occur in a locus-specific manner during development in yeast, plant and animals (Ooi et al., 2007; Bouyer et al., 2011; Morselli et al., 2015; Stewart et al., 2015). This *de novo* methylation event observed in our study largely shapes the methylation pattern of K27 genes after salt treatment, with additional changes occurring in gene expression required for stress response.

In plants and animals, the histone modification of H3K27me3 maintains the developmentally regulated genes in silenced chromatin status. The removal and establishment of H3K27me3 marks at specific target genes is a dynamic and reversible process and is therefore critically important for normal development. These modifications are carried out by the histone modifiers — histone methyltransferases and histone demethylases. To address how methylation and demethylation were well established in salt stress, we identified some modifiers which may cause changes of H3K27me3 pattern and gene expression observed in our study (**Tables 4** and **5**). In plants, all the HMTs have a well-known conserved SET domain and also named as SET domain groups (SDG) proteins (Thorstensen et al., 2011). In *Arabidopsis*, the methyltransferases trimethylate H3K27, including CLF, MEDEA (MEA) and SWINGER (SWN) (Hennig and Derkacheva, 2009; Liu et al., 2010; Butenko and Ohad, 2011; Zheng and Chen, 2011). Here, we found 43 potential HMT genes with expression in control plants which correspond with different *Arabidopsis* HMTs, such as *CLF*, *ATX*, *ATXR*, *SDG* and *SUVH* (**Table 4**). There are 11 genes which showed different expression pattern in salt stress (**Table 4** and **Figure 6A**). Histone methylation was reversible through the JmjC Jumonji C domain containing proteins and the lysine-specific demethylase (LSD1). Interestingly, we found 21 JmjC genes were expressed in control plants and 4 of them showed differential expression levels (**Table 5** and **Figure 6B**). Among them, *Glyma.04G192000* and *Glyma.20G181000* are homologues of *Arabidopsis REF6* and *ELF6* respectively, which are well characterized demethylases. It has been reported that the REF6 protein, also known as JMJ12, can specifically demethylate H3K27me3 at its target gene loci to active gene expression (Lu et al., 2011). The *REF6* mutants cause the ectopic accumulation of H3K27me3 at hundreds of genes and a number of defective developmental phenotypes (Yu et al., 2008; Cui et al., 2016; Li et al., 2016). ELF6 identified

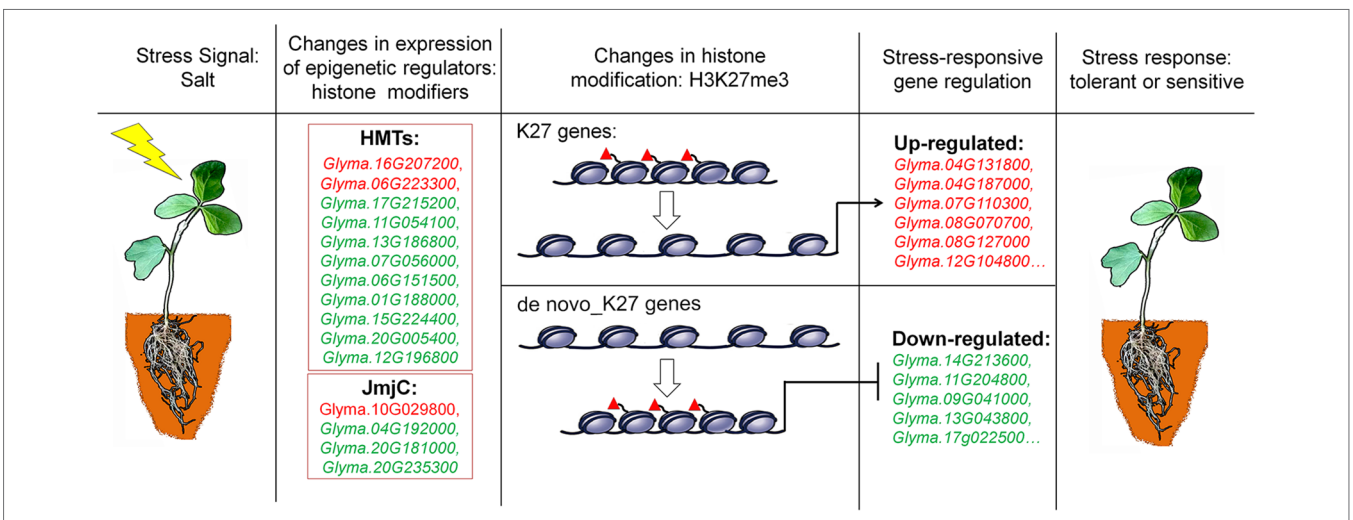


FIGURE 8 | A proposed model for epigenetic regulation of stress response in soybean. Salt stress signals induce changes in the expression of epigenetic regulators of histone modifiers such as histone methyltransferases (HMTs) and histone demethylases (JmjC). These epigenetic regulators specifically modify histone modifications of H3K27me3 on K27 or *de novo*_k27 genes which lead to the expression changes of salt-responsive genes. The different behavior of methylation marks during the response process illustrates that they have distinct roles in the transcriptional response of implicated genes. Genes up-regulated in salt-treated soybean are marked red, and down-regulated are green respectively. Red triangles represent H3K27me3 marks.

as an H3K27me2/3-specific demethylase closely related to *REF6* was rquired for removal of H3K27me3 from the *Flowering Locus C* (*FLC*) locus in developing embryos in vernalized plants (Crevillen et al., 2014). These results suggested that these potential HMT or JmjC protein may function as similar roles to response for establishment or removal of H3K27me3 with conserved mechanisms of the dynamic regulation of H3K27me3 between *Arabidopsis* and soybean.

Based on our results and previous studies, we proposed a hypothesis to illustrate epigenetic regulation of salt stress response in soybean (**Figure 8**). The DNA sequence of genes with low expression levels may be tightly wrapped around the nucleosome and blocked from transcript activation by an unknown mechanism. When plants are subjected to salt stress, for K27 marked genes, decreased levels of H3K27me3 mediated by JmjC proteins release the DNA sequence from the nucleosome for the induced transcription process. DNA sequences of genes with high expression levels are often maintained with a low density of nucleosomes and low levels of inactive histone modification. In contrast, for *de novo*_K27 genes under stress conditions, increased modification levels of inactive marks mediated by HMT proteins on target genes can cause chromatin compaction, thus reducing the gene expression level. However, many details, such as how these JmjC and HMT genes find the proper context and being recruited to establish repressive modification in this hypothesis, need to be clarified by further experiments.

Taken together, our findings described here support a model in which H3K27me3 was closely associated to salt responsive genes under stress conditions in soybean. H3K27me3 modification levels were negatively correlated with the expression level changes of a portion of the salt-responsive genes in soybean. The salt stress can cause *de novo* methylation events in gene regulation for stress response. We identified histone methyltransferases and JmjC domain-containing demethylases in soybean, providing an overview of H3K27me3-mediated salt responsive network. These results suggest that histone modifications may play important but largely unknown roles in the stress responses. It will be of interest to determine and explore how these proteins play roles at specific target genes to mediate local histone methylation enrichment when responding to abiotic stress. The information gathered here will be of particular interest for future studies on the evolution of epigenetic-mediated stress mechanism and the divergence of functionality in crop plants. In addition to the potential roles of histone modifications in influencing stress response, the combination of technical innovation such as synthetic biology and genome editing, will allow greater control of conferring stress tolerance for crop improvement in future agriculture.

DATA AVAILABILITY

The RNA-seq and histone modification ChIP-seq data sets from this article have been deposited in Gene Expression Omnibus (GEO) with accession number GSE133575.

AUTHOR CONTRIBUTIONS

LP and YD conceived and designed the experiments. SL, SG, WW, HZ, TG, QL, XY and FX performed the experiments. LP, LS, GS and WG analyzed data. LP, LS and GS wrote the paper. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01031/full#supplementary-material

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