

EPIGENETIC MODIFICATIONS ASSOCIATED WITH ABIOTIC AND BIOTIC STRESSES IN PLANTS: AN IMPLICATION FOR UNDERSTANDING PLANT EVOLUTION

EDITED BY : Mahmoud W. Yaish, Heribert Hirt and Barbara Hohn
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EPIGENETIC MODIFICATIONS ASSOCIATED WITH ABIOTIC AND BIOTIC STRESSES IN PLANTS: AN IMPLICATION FOR UNDERSTANDING PLANT EVOLUTION

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A plant defends itself, using epigenetic weapons, against the rough environment. Image designed by Thomas and Barbara Hohn.

Cover image: Piyawat Hirunwattanasuk/Shutterstock.com.

Alterations in gene expression are essential during growth and development phases and when plants are exposed to environmental challenges. Stress conditions induce gene expression modifications, which are associated with changes in the biochemical and physiological processes that help plants to avoid or reduce potential damage resulting from these stresses.

After exposure to stress, surviving plants tend to flower earlier than normal and therefore transfer the accumulated epigenetic information to their progenies, given that seeds, where this information is stored, are formed at a later stage of plant development.

DNA methylation is correlated with expression repression. Likewise, miRNA produced in the cell can reduce the transcript abundance or even prevent translation of mRNA. However, histone modulation, such as histone acetylation, methylation, and ubiquitination, can show distinct effects on gene expression. These alterations can be inherited, especially if the plants are consistently exposed to a particular environmental stress. Retrotransposons and retroviruses are foreign movable DNA elements that play an important role in plant evolution. Recent studies have shown that epigenetic alterations control the movement and the expression of genes harbored within these elements. These epigenetic modifications have an impact on the morphology, and biotic and abiotic tolerance in the subsequent generations because they can be inherited through the transgenerational memory in plants. Therefore, epigenetic modifications, including DNA methylation, histone modifications, and small RNA interference, serve not only to alter gene expression but also may enhance the evolutionary process in eukaryotes.

In this E-book, original research and review articles that cover issues related to the role of DNA methylation, histone modifications, and small RNA in plant transgenerational epigenetic memory were published.

The knowledge published on this topic may add new insight on the involvement of epigenetic factors in natural selection and environmental adaptation. This information may also help to generate a modeling system to study the epigenetic role in evolution.

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Editorial: Epigenetic Modifications Associated with Abiotic and Biotic Stresses in Plants: An Implication for Understanding Plant Evolution

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Keywords: stress, epigenetics, evolution, molecular, plants, DNA methylation

Editorial on the Research Topic

Epigenetic Modifications Associated with Abiotic and Biotic Stresses in Plants: An Implication for Understanding Plant Evolution

Epigenetics in modern definition refers to the heritable alterations in gene expression which may lead to a variation in the phenotype without a change in the DNA sequence (Morris, 2001). Molecular events in epigenetics can take place naturally in cells but can also be modulated by environmental stressors. A combination of epigenetics and environmental stress dynamics lead scientists to investigate the impact of epigenetics on evolution in plants. The aim of the current research topic was to explore and update our understanding on epigenetic mechanism which may drive evolution in plants. The present edited volume includes original research and review articles describing epigenetic changes and their impact on the evolutionary adaptation mechanisms.

The adjustment of gene expression is a key mechanism used by plants during growth and developmental processes. Because plants are immobile organisms, the control of gene expression becomes more essential when plants are subjected to inescapable environmental stressors (Yaish et al., 2017). The expression level of a particular gene is manipulated by a series of coordinated epigenetic events on the nucleosomes, which involve DNA methylation, histone post-translational modifications and small RNA interference (Baulcombe and Dean, 2014).

When exposed to stresses, plants tend to flower earlier than normal in order to skip the unfavorable conditions and produce seeds as soon as possible in order to conserve the species (Yaish et al., 2011). The resultant seeds may transfer the accumulated epigenetic information of the stressed plants to their progenies. Thus, this process may eventually lead to adaptive evolution. The transfer of epigenetic information over generations is called epigenetic transgenerational memory (Molinier et al., 2006). Such transgenerational epigenetic changes may be the result of heritable epialleles (Johannes et al., 2009), as well as through nucleosome recycling during cell division (Alabert et al., 2015). These findings are corroborated by the conclusions of the contribution by Iglesias and Cerdán that emphasized the essential role of nucleosome assembly on DNA replication processes and the stable epigenetic inheritance in plants. However, Iwasaki and Tricker, found in two other separate reviews published in this topic that the changes in chromatin can also be reverted to the default standard in order to reduce potentially negative impacts on the respective phenotype. On the other hand, Latzel et al. showed that epigenetic memory could be an essential motor which empowers environmental adaptation or intelligent behavior of clonal plants.

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The relationship between DNA methylation and gene expression is not direct, and not yet completely understood (Law and Jacobsen, 2010), partially due to the involvement of other epigenetic factors in controlling gene expression. Small non-coding RNA sequences (sRNA), generated by the epigenetic machinery of cells, can alter gene expression or even block the translation of the targeted mRNA in the ribosomes (Carrington and Ambros, 2003). Bioinformatic analysis work carried out by Yakovlev and Fossdal, have confirmed that sRNA can play an important role in the establishment of epigenetic memory in Norway spruce. Another study published in this topic has shown that there is a crosstalk mechanism between DNA methylation and sRNA expression in *Populus simonii* plants when exposed to temperature stress; thus, DNA methylation influences miRNA expression and, in turn, may regulate the expression of their natural mRNA targets (Ci et al.). Additionally, Matsunaga et al. showed that sRNA has the ability to regulate the expression of the Ty1/copia retrotransposon, ONSEN, when Arabidopsis is grown under heat stress. It is worth saying here that different epigenetic players can be involved in regulating a tolerance mechanism in plants. For example, Liu et al. highlighted in their review the critical regulatory role of different epigenetic players including DNA methylation, histone modifications and sRNA in modulating the expression of thermotolerant genes, a complicated mechanism, which requires further investigation. Interestingly, the contribution by Espinas et al. revealed that transgenerational transfer of pathogen defense signaling and priming is also controlled by epigenetic mechanisms similar to those found in plants, due to abiotic stresses. In addition, Han and Luan illustrated in their review that sRNA species can be horizontally transferred from plants to mammals and from microbes to plants, and these cross-kingdoms sRNA molecules are important in host gene silencing mechanisms. Horizontal movement of sRNA may represent an additional mechanism of plant evolution through epigenetics.

Environmental stresses on some plant species lead to DNA methylation/demethylation (Yaish, 2013; Al-Lawati et al., 2016). These changes can be maintained and transmitted to the offspring, even in the subsequent absence of the original stress however, the DNA methylation alteration is not constant among the different stress episodes and plant species. For example, Eichten and Springer have proven that DNA methylation patterns in corn are lacking consistency when the plants are subjected to cold, heat and ultraviolet irradiation. Therefore, the inheritance of changes in DNA methylation in corn, possibly related to changes in phenotypes, is probably not robust. However, in other experiments, DNA methylation has shown some consistency in terms of heritability. For example, methyl-sensitive amplified fragment polymorphism analysis has revealed that DNA methylation, when induced by laser irradiation, is heritable and has induced the expression of miniature inverted-repeat transposable elements (MITEs) in rice (Li et al.).

The Arabidopsis histone H3K4 methyltransferase PRDM9 was shown to exert a clear effect on the distribution of meiotic recombination hotspots (Choi et al., 2013). DNA methylation patterns may also affect chromosome recombination events during meiosis in plants. Recent studies have shown that

DNA hypomethylation in Arabidopsis, due to the loss of function in DNA methyl transferase (*MET1*), leads to alterations in the distribution of the crossover events throughout the chromosomes during mitotic cell division (Mirouze et al., 2012; Yelina et al., 2012). Given this notion, DNA methylation will theoretically affect the production of new alleles and eventually the appearance of new phenotypes and species, a process that could promote evolution in plants. However, recent work on genomes of different plant species revealed that the evolutionary factors that control changes in DNA methylation significantly vary across plant species, genes and methylated sequence contexts (Takuno et al., 2016).

The issues being raised here concern how far we are from decoding the evolutionary mechanism based on epigenetics and whether stress facilitates or accelerates the appearance of new species. Since the days of Lamarck (1744–1829 AD), who introduced the theory of the heritability of acquired characters, scientists tried unsuccessfully to prove that epigenetic inheritance is the reason behind the appearance of new phenotypes under stress conditions (Pigliucci and Finkelman, 2014). The situation has not changed much, even after recent discoveries in molecular epigenetics, which indeed reveal some evidence on the fluctuating relationship between environmental stresses and the appearance of novel phenotypes.

The use of artificially induced methylation changes led to the establishment of stable epialleles measurable as epiQTLs (Cortijo et al., 2014), and epigenetic recombinant inbred lines (epiRILs) (Johannes et al., 2009). These set-ups permitted to test the appearance, amongst these libraries, of phenotypic variants, thereby establishing correlations between phenotype and epigenotype. However, this example in Arabidopsis cannot be generalized to all plant species growing under natural conditions. The difficulties in considering epimutations in adaptive evolution processes stem from the fact that deciphering the effects of epigenetic alterations on gene expression and DNA recombination frequency is complex and even elusive.

Epigenetic variations are basically controlled by DNA-coded chromatin modifier proteins. This point was emphasized when Ding and Mou studied the ELONGATOR, multiprotein complex involved in epigenetic regulation in animals and plants, and found that this protein plays a role in histone acetylation and DNA methylation in plants. Consequently, mutations within the corresponding genes led to abnormal growth and development, and deviant susceptibility to various stressors in *Arabidopsis thaliana*. Similarly, in another review article, Moraga and Aquea have concluded that members of the Spt-Ada-Gcn5 ACETYLTRANSFERASE (SAGA) protein family complexes may regulate gene expression under various stress conditions in plants through the posttranslational modification of some histones. Therefore, epigenetic changes are not independent agents of adaptation and evolution; rather, they can mediate the mechanism that controls this process (Kronholm, 2017).

In conclusion, research that seeks to understand epigenetic mechanisms and their relation to stress adaptation may help, to a certain degree, to appreciate the evolution scenarios of some species under specific conditions; however, the information

yielded by different branches of epigenetic research is unable to provide epimutations with a clear role in evolutionary adaptation mechanisms. This fact suggests the presence of other unknown epigenetic players, which control this process and, thus, clearly warrant more in-depth investigations.

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The author confirms being the sole contributor of this work and approved it for publication.

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Epigenetic Control of Defense Signaling and Priming in Plants

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Immune recognition of pathogen-associated molecular patterns or effectors leads to defense activation at the pathogen challenged sites. This is followed by systemic defense activation at distant non-challenged sites, termed systemic acquired resistance (SAR). These inducible defenses are accompanied by extensive transcriptional reprogramming of defense-related genes. SAR is associated with priming, in which a subset of these genes is kept at a poised state to facilitate subsequent transcriptional regulation. Transgenerational inheritance of defense-related priming in plants indicates the stability of such primed states. Recent studies have revealed the importance and dynamic engagement of epigenetic mechanisms, such as DNA methylation and histone modifications that are closely linked to chromatin reconfiguration, in plant adaptation to different biotic stresses. Herein we review current knowledge regarding the biological significance and underlying mechanisms of epigenetic control for immune responses in plants. We also argue for the importance of host transposable elements as critical regulators of interactions in the evolutionary “arms race” between plants and pathogens.

Keywords: epigenetic control, plant immunity, defense priming, DNA methylation, histone modification, transposable elements, plant-microbe interactions

INTRODUCTION

Plants have evolved sophisticated mechanisms to adapt to fluctuating environments, including immune systems for dealing with diverse infectious microbes that threaten plant growth and survival. In response, plant pathogens have evolved a substantial degree of phenotypic plasticity to avoid and/or suppress recognition by the host (Gomez-Diaz et al., 2012). Such dynamic interactions compel the evolution of plant mechanisms that link pathogen sensing to rapid and effective defense activation to minimize fitness costs.

COMPONENTS OF THE PLANT INNATE IMMUNE SYSTEM

Plants have evolved innate immune systems that recognize and respond to pathogens. These systems consist of two tiers of inducible resistance mechanisms, namely pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Chisholm et al., 2006; Jones and Dangl, 2006; Dodds and Rathjen, 2010). PTI represents the first tier of plant immunity and is conferred by pattern recognition receptors (PRRs) that recognize PAMPs or endogenous elicitors, termed damage-associated molecular patterns (DAMPs),

generated by pathogen assaults (Boller and Felix, 2009; Macho and Zipfel, 2014; Zipfel, 2014). On the other hand, ETI is typically mediated by nucleotide binding (NB)-leucine rich repeat (LRR) receptors (NLRs) (Takken and Govers, 2012; Wu et al., 2014; Cui et al., 2015). Plant immunity is characterized by such multilayered structures, which likely enable fine-tuning of defense responses. Fine control of receptor-mediated pathogen recognition and defense signaling downstream of the receptor are fundamental to avoid precocious activation of immune responses that negatively influence plant growth. How do plants mount effective immune response at a minimal fitness cost?

DNA METHYLATION: A DYNAMIC REGULATOR OF DEFENSE GENES

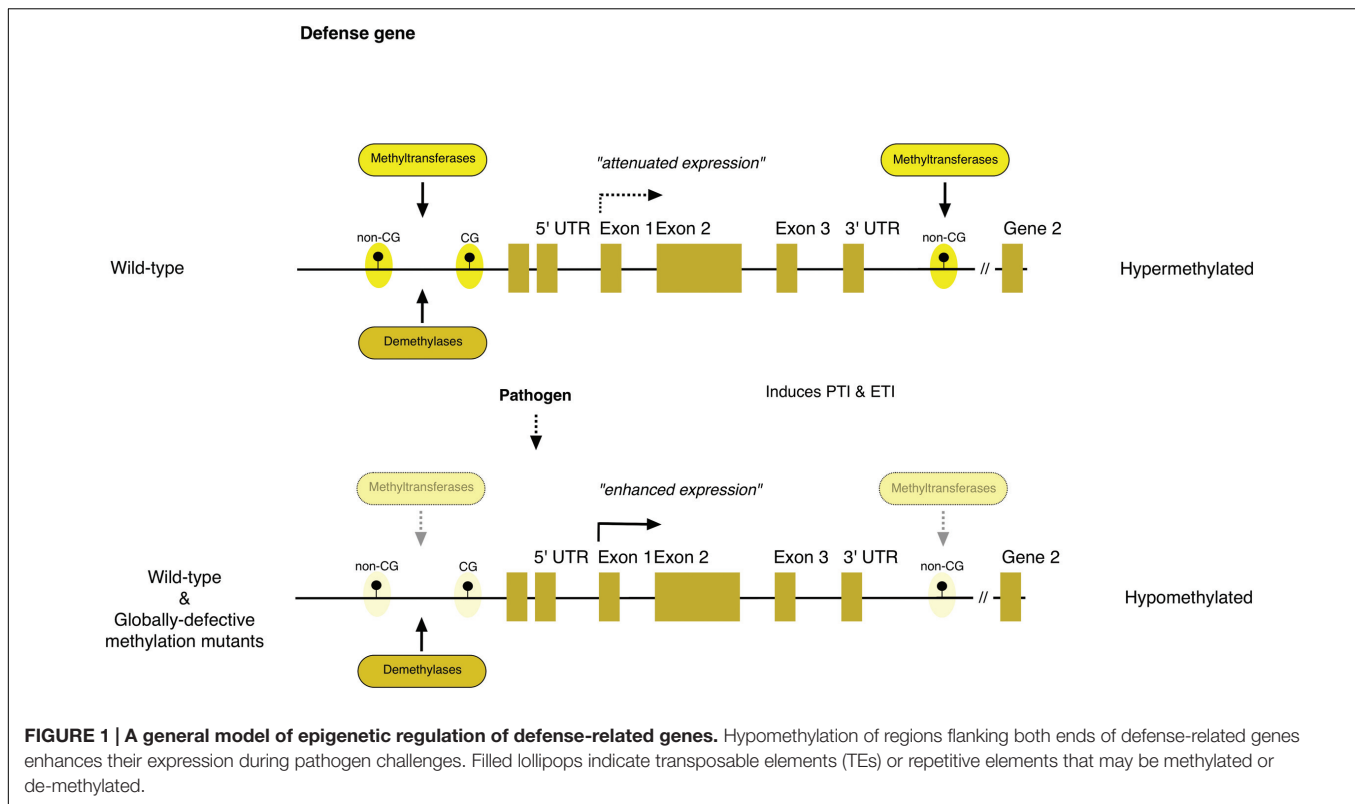
Cytosine methylation of the DNA bases in all sequence contexts, CG and non-CG (CHG and CHH, where H is non-G), is triggered by small interfering RNAs (siRNAs) via a *de novo* methylation pathway termed RNA-directed DNA methylation (RdDM). Canonical RdDM begins by production of RNAs by Polymerase (Pol) IV via NUCLEAR RNA POLYMERASE D (NRPD) subunits, and after several processing steps, the processed RNAs are loaded into ARGONAUTE 4 (AGO4) and base-paired with an RNA scaffold produced by Pol V. Recruitment of AGO4 involves its interaction with NUCLEAR RNA POLYMERASE E1 (NRPE1) of Pol V. Subsequent interaction with DOMAINS REARRANGED METHYLTRANSFERASE (DRM) leads to methylation of DNA target sequences. On the other hand, in the non-canonical Pol II-RDR6-dependent RdDM pathway, Pol II-transcribed single-stranded RNA (ssRNA) is converted into double-stranded RNA (dsRNA) by RNA-DEPENDENT RNA POLYMERASE 6 (RDR6), and then processed into 21–22nt siRNA. The siRNA is loaded into AGO6 that can be directed to the scaffold RNA transcribed by Pol V, which establishes DNA methylation. These methylation marks are maintained through mitosis and meiosis via a pathway catalyzed by METHYLTRANSFERASE1 (MET1) and CHROMOMETHYLASE3 (CMT3) methyltransferases, while REPRESSOR OF SILENCING1 (ROS1), DEMETER-LIKE2 (DML2), and DML3 are DNA glycosylases that dynamically erase DNA methylation via a base excision repair process (details of the RdDM pathway are referred to Law and Jacobsen, 2010; Matzke and Mosher, 2014; Du et al., 2015; Matzke et al., 2015). DNA methylation is a vital process that is also linked to other epigenetic pathways, such as histone methylation and acetylation (Eden et al., 1998; Qian et al., 2012; Du et al., 2015).

Recent studies have extended our understanding of epigenetic control of plant immunity (Alvarez et al., 2010; Sahu et al., 2013; Saijo and Reimer-Michalski, 2013; Ding and Wang, 2015). High-resolution DNA methylation profiling by Downen et al. (2012) provides the first genome-wide insight into biotic stress-responsive genes in *Arabidopsis*, expression of which is modulated by DNA methylation and demethylation. *met1-3* and *ddc* (*drm1-2 drm2-2 cmt3-11*) plants that are globally defective in maintaining CG and non-CG methylation, respectively, show enhanced defense responses when exposed

to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). The same results were obtained in mutants partially defective in CG and non-CG methylation. Moreover, in rice, application of 5-azadeoxycytidine, a DNA demethylating agent, enhances bacterial resistance to *Xanthomonas* (Akimoto et al., 2007). These results are consistent with findings that enhanced RdDM in *ros1-4* plants leads to lowered resistance to *Pst* DC3000 (Yu et al., 2013). In addition, flg22 treatment results in inhibition of transcriptional gene silencing (TGS) as it de-represses RdDM targets. Yu et al. (2013) also confirmed increased bacterial resistance in *ddc* and *met1 nrpd2* plants. *met1 nrpd2* plants also exhibit hypersensitivity response (HR)-like cell death and high *PR1* expression, pointing to de-repression of ETI-like defenses. Furthermore, *ros1 dml2 dml3* (*rdd*) plants, simultaneously disrupted for the three DNA demethylases, show lowered fungal resistance (Le et al., 2014).

Pol V, but not Pol IV, has been implicated in plant immunity (Lopez et al., 2011; Matzke and Mosher, 2014). However, Le et al. (2014) showed an overlap of down-regulated genes between *rdd* and the RdDM mutants, *nrpe1* and *nrpd1*, suggesting that Pol V and Pol IV both regulate defense responsive genes. In addition, fungal infection is enhanced in *nrpe1* and *ago4* plants, while it is slightly reduced in *nrpd1* plants. These results clearly suggest that genome-wide disruption of DNA methylation leads to defense activation, in a way reminiscent of ETI, and that DNA methylation down-regulates immune responses. However, this is not the case for all defense-related genes, as evident in the blast resistance gene, *Pib*, in rice (Li et al., 2011) and in the genome-wide methylation analysis of tobacco plants infected with *Tobacco mosaic virus* (TMV) (Kathiria et al., 2010). Future investigation will be required to determine whether RdDM pathways play a distinctive role in different plant species, between different target genes, against different pathogens or combinations thereof. It is of particular importance to elucidate the regulatory components, the mode of control, and specific target sites in the genome for canonical and non-canonical RdDM pathways in plant immunity, not only in *Arabidopsis* but also in other plant models.

These *Arabidopsis* studies also offer insight into methylation states in plant genomes and how changes influence immune responses. In response to *Pst* challenge or flg22 application, DNA methylation levels are globally reduced in all sequence contexts, while the decrease following SA application is restricted to CG and CHG contexts (Figure 1). Intergenic transposable elements (TEs) seem to be among the main targets for both canonical and non-canonical RdDM pathways during pathogen challenge. Stress-associated differential methylation in the CG context occurs predominantly ~1 kb upstream of transcriptional start sites (TSS) for protein-coding genes, whereas such methylation in the CHH context occurs high in intergenic regions. Differential methylation in both contexts is over-represented at both ends of protein coding genes. *At3g50480*, a locus encoding a homolog of RPW8 disease-resistance (*R*) protein, undergoes differential methylation changes during pathogen infection. Another *R* gene, *RMG1* (*At4g11170*), is highly induced in response to flg22 and in *met1 nrpd2* plants, while it is compromised in *ros1* plants in which TSS-flanking regions are highly methylated. In *rdd* plants, TEs inserted adjacent to or within 200 bp of promoters



and gene bodies, represent major targets of methylation. It is important to note that not only TEs, but also sequences surrounding them are methylated. This is particularly true for those inserted in promoter regions, as shown for *CC-NBS-LRR* (*At1g58602*) and *jacalin lectin* (*At5g38550*). Work on cytosine DNA methylation (mC) in rice and *Arabidopsis* also indicates that proximal regions of TEs, when they are within or in proximity to stress-inducible genes, play a critical role in responsiveness to environmental stress cues (Secco et al., 2015). These findings suggest that regulatory processes modulating methylation at or near gene boundaries, particularly in *R* gene loci, help to fine-tune defense responses, at least in these plant models. Future studies will be required to determine the precise function of these DNA sequences and the molecular mechanisms underlying their recognition and modification.

TRANSPOSABLE ELEMENTS (TEs) IN PLANT IMMUNITY

A major class of *R* proteins are the NLR immune receptors that mediate ETI to various pathogens. *NLR* genes often form gene clusters in the genome that contain repetitive sequences and TEs (Meyers et al., 2003). The repetitive nature of *NLR*-gene clusters is thought to facilitate rapid expansion and sequence diversification of these genes, possibly by promoting unequal recombination (Friedman and Baker, 2007). It is well documented that TEs inserted in the promoter region often regulate neighboring genes in both animals and plants

by changing their epigenetic states (Slotkin and Martienssen, 2007). A recent report shows that TEs in intronic regions can regulate *NLR* expression in *Arabidopsis* (Tsuchiya and Eulgem, 2013). *Arabidopsis RPP7* encodes a CC-NBS-LRR class of NLR that confers resistance to downy mildew, *Hyaloperonospora arabidopsidis* (*Hpa*) (Eulgem et al., 2007). Proper transcription and splicing of *RPP7* requires a protein named ENHANCED DOWNY MILDEW2 (*EDM2*), which encompasses PHD domains that recognize H3K9 methylation and a putative RNA methyltransferase domain at the C-terminus (Lei et al., 2014; Tsuchiya and Eulgem, 2014). In the *edm2* mutant, transcription of *RPP7* is attenuated due to premature termination of the transcripts at the TE within the 1st intron, termed *ECL* (exon 1-containing LTR-terminated transcript). Interestingly, intronic TEs, including COPIA-type retrotransposon in the 1st intron, are targeted by repressive epigenetic marks, such as DNA methylation and H3K9 methylation, as are their intergenic copies, even though they are embedded within the actively transcribed gene unit (Saze et al., 2013; Tsuchiya and Eulgem, 2013). Maintenance of repressive epigenetic marks in intronic TEs seems to be important for proper expression of *RPP7*, since *RPP7*-mediated ETI to *Hpa* is impaired in plants deficient for H3K9 methylation, recapitulating the immunocompromised phenotype of *edm2* plants. Similarly, reduced DNA methylation in *DECREASE IN DNA METHYLATION1* (*DDM1*) mutants or *CMT3* results in a transcription defect of *RPP7* (Le et al., 2015). Interestingly, even though *RPP7* shows sequence polymorphism among different *Arabidopsis* accessions due to TE insertions within intronic regions (Tsuchiya

and Eulgem, 2013), most of these natural accessions harbor the COPIA element in the 1st exon. This implies that TE insertion has selective advantages, possibly by providing a fine-tuning mechanism for *RPP7* expression (McDowell and Meyers, 2013). As reported, epigenetic states of TEs are dynamically altered in response to biotic stress (Downen et al., 2012). Epigenetic control of intragenic TEs may thus act as a regulatory mechanism for *NLR* gene expression in plant-pathogen interactions.

HISTONE MODIFICATION AND ITS ROLE IN SYSTEMIC ACQUIRED RESISTANCE

Defense activation at recognition sites for PAMPs or effectors generates and delivers systemic signals throughout the plant, which result in enhanced immunity to a broad spectrum of pathogens, called systemic acquired resistance (SAR) (Conrath, 2011; Fu and Dong, 2013; Kachroo and Robin, 2013; Conrath et al., 2015). During and after SAR, defense-related genes become sensitized to subsequent pathogen attack at distal, non-challenged sites, known as defense priming. Defense-primed plants are enabled to mount a swift defense response, which involves “kick starting” of up- and down-regulation for priming target genes.

Among potential mechanisms underlying defense priming, histone modifications are of particular interest since they affect the landscape of transcription of defense-related genes through evolutionarily highly conserved functions (Waterborg, 2011). Recent studies in plants have implicated H3K4me3, H3K4me2, H3K9ac, H4K5ac, H4K8ac, and H4K12ac in defense priming. In particular, H3K4me3 is considered a primary chromatin marker of stress memory (Conrath et al., 2015). Recent studies on heat stress acclimation in *Arabidopsis* present a model in which transient binding of the heat-inducible transcription factor HsfA2 leads to sustained H3K4 methylation and thus the maintenance of heat stress memory, i.e., acquired thermotolerance (Lamke et al., 2016). Notably, HsfA2 function is dispensable for the acquisition of thermotolerance *per se*, but indispensable for its maintenance (Charnig et al., 2007). On the other hand, Mozgová et al. (2015) have shown that the histone chaperone, CAF-1, mediates a repressive chromatin state of defense genes, by retaining nucleosome occupancy and suppressing H3K4me3 marking. However, loss of CAF-1 alone is insufficient to activate SA-related defense genes. These findings suggest that CAF-1-conditioned chromatin modification prevents inappropriate defense activation. Further investigation will be required into the mechanisms by which defense signaling triggered upon pathogen recognition overcomes this barrier and leads to a priming state, partly through increasing H3K4me3 deposition, at both challenged and non-challenged sites.

Histone acetyltransferases (HATs) and deacetylases (HDACs) also participate in control of defense priming. *hac1-1* (histone acetyltransferase 1) plants are compromised in bacterial resistance and defense priming following PTI (Singh et al., 2014). This is the first evidence that an HAC1-dependent pathway

is responsible for defense priming after exposure to recurring abiotic stress cues. HAC1 does not seem to direct resistance to *Pst per se*, suggesting that HAC1 links recurring stress response activation to defense priming. It remains to be shown how HAC1 establishes the epigenetically primed states at open chromatin target sites. Consistent with a positive role for histone acetylation in defense activation, loss of HDA19 results in de-repression of SA-based defenses (Choi et al., 2012) and depletion of the HDAC HDT701 enhances H4 acetylation and resistance to both fungal and bacterial infection (Ding et al., 2012).

It has been reported that defense priming and these histone marks are transgenerationally inherited (Heard and Martienssen, 2014; Iwasaki and Paszkowski, 2014; Kinoshita and Seki, 2014; Crisp et al., 2016). A recent study in yeast has proven for the first time that H3K9 methylation is heritable over several generations (Audergon et al., 2015). Given the evolutionary conservation for functions of these histone marks, it is conceivable that histone modifications provide a basis for heritable immune response memory.

A subset of, if not all, defense genes activated in SAR, seems to be primed as a consequence of interplay between different histone modifications, via mechanisms that are still poorly understood (Conrath, 2011; Gutzat and Mittelsten Scheid, 2012; Spoel and Dong, 2012; Saijo and Reimer-Michalski, 2013; Conrath et al., 2015; Ding and Wang, 2015). Priming of defense-related genes has a fitness advantage compared to their substantial activation (van Hulst et al., 2006). It is tempting to speculate that this has contributed to the evolution of genomic regions that undergo histone modifications to establish such a priming state at target genes, which enables effective transcriptional reprogramming toward enhanced resistance in response to second challenge. In animals, enhancer and promoter sites are often marked with H3K4me1/H3K27ac and H3K4me3/H3K27me3, respectively (Azuara et al., 2006; Bernstein et al., 2006; Zhou et al., 2011; Calo and Wysocka, 2013; Voigt et al., 2013) (**Figure 2**). These combinatorial histone marks can occur in a gene-autonomous manner, and seem to exert complex regulatory effects, as is the case of H3K4me3/H3K27me3 in the promoter region (called a bivalent promoter) (Bernstein et al., 2006). It should be noted, however, that bivalency is not restricted to narrow genomic regions, as enhancers can influence target genes as much as a million bases distant (Pennacchio et al., 2013). Thus, cautions need to be taken when considering bivalency, which can occur at the same nucleosome unit harboring two antagonizing marks in different histone molecules or in one histone molecule (e.g., H3K4me3/H3K27me3 in promoters; **Figure 2A**), or in separate nucleosome units (e.g., H3K27me3/H3K27ac in promoters and enhancers, respectively; **Figure 2B**). In acclimation to abiotic stress, an increase of transcription-permissive H3K4me3 occurs when plants are exposed to recurring stress cues without removing transcription-repressive H3K27me3 (Saleh et al., 2007; Avramova, 2015). Given that not only pathogen recognition, but also adverse abiotic conditions can induce defense priming in plants (Singh et al., 2014; Vivancos et al., 2015), it is of high interest to test whether bivalent histone modification also plays a role in defense priming. Future

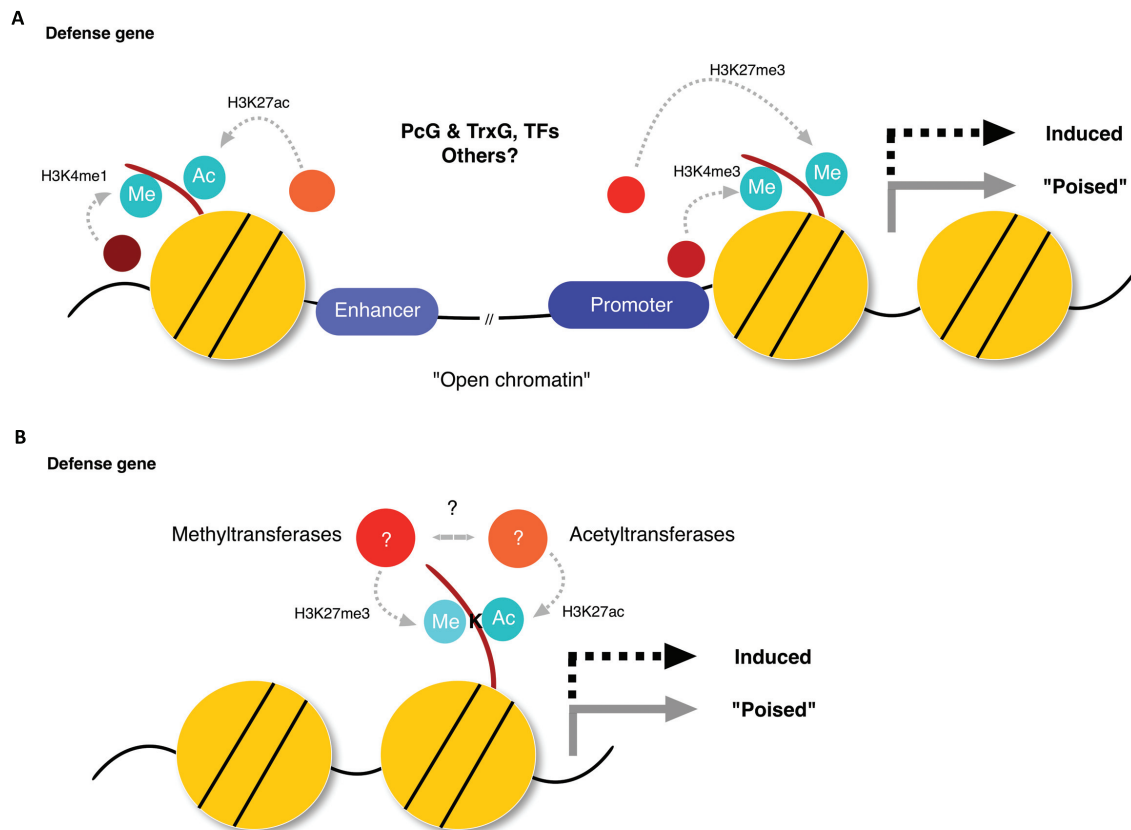


FIGURE 2 | Bivalent phenomena hypothesis in plant defense priming. (A) An interplay of opposing histone modification marks in enhancer and promoter regions modulate the expression status of defense-related genes. Polycomb-group (PcG) and Trithorax (TrxG) proteins may assemble with interacting proteins, such as transcription factors (TFs). **(B)** Opposing histone marks on the same lysine-site (K-site) act as a switch to modulate the expression status of defense-related genes.

studies will be required to clarify the functional significance of bivalent modification, which may be distinct from that of either transcription-permissive or -repressive modification alone.

SUMMARY AND FUTURE PERSPECTIVES

In this review, we have integrated recent advances in epigenetic control of defense-related transcriptional reprogramming and priming.

Earlier findings about the role of DNA methylation in modulating defense responses (Pavet et al., 2006; Luna et al., 2012) and recent epigenome analysis (Dowen et al., 2012; Yu et al., 2013; Le et al., 2014) have revealed the importance of fine control of DNA methylation near the boundaries of defense-related genes, repetitive sequences, and TEs during immune responses in plants. It is still unclear whether methylation patterns established in the host genome reflect specific infection strategies and/or infection states of pathogens. If this is the case, however, it predicts that pathogen-induced changes in DNA

methylation status, possibly at specific genomic sites, can be sensed by a surveillance system in the host. In this context, RdDM components may act as part of such immune sensory systems, much as alterations in RdDM efficiency following pathogen challenge lead to hypomethylation of defense-related genes and to immune activation.

Histone acetylation and methylation have emerged as critical regulators of defense priming. These modifications occur at specific histone residues in concert with or as a consequence of transcriptional reprogramming in response to pathogen challenge or environmental cues, which result in sustainable reconfiguration of the nucleosome. It is also interesting to see whether and how histone acetylation and methylation are established and coordinated with each other during defense activation and priming. At present, we know little about histone modifications of target regions in the genome, the dynamic changes they induce, and histone-modifying enzymes involved in plant immunity. Findings for HAC1-mediated priming, for instance, provide a good start toward a deeper understanding of the significance and modes of actions of these histone modifications in plant-pathogen interactions. Moreover, these efforts need to be integrated with elucidation of the signals that

link pathogen recognition to epigenetic modifiers in both cell-autonomous and non-cell autonomous contexts (as reviewed in Spoel and Dong, 2012; Conrath et al., 2015). Emerging data suggest common adaptive strategies in plant acclimation to different biotic/abiotic stressors, which seem to involve cooption of evolutionarily conserved epigenetic regulation in a manner unique to plants.

Last but not least, it should be noted that our review particularly focuses on DNA methylation, TE control and histone modification in the contexts of defense-related gene expression, NLR receptor expression and SAR/priming, respectively, do not indicate restriction of these epigenetic controls to the corresponding aspects of plant immunity. These and other epigenetic mechanisms may play a role in fine control of different steps in plant immunity, and thereby contribute to its multilayered structure.

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AUTHOR CONTRIBUTIONS

NE conceptualized the contents of the manuscript, wrote, and edited the manuscript. HS wrote the manuscript. YS wrote, edited the manuscript, and directed the contents of the manuscript.

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Epigenetic Memory as a Basis for Intelligent Behavior in Clonal Plants

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Environmentally induced epigenetic change enables plants to remember past environmental interactions. If this memory capability is exploited to prepare plants for future challenges, it can provide a basis for highly sophisticated behavior, considered intelligent by some. Against the backdrop of an overview of plant intelligence, we hypothesize: (1) that the capability of plants to engage in such intelligent behavior increases with the additional level of complexity afforded by clonality, and; (2) that more faithful inheritance of epigenetic information in clonal plants, in conjunction with information exchange and coordination between connected ramets, is likely to enable especially advanced intelligent behavior in this group. We therefore further hypothesize that this behavior provides ecological and evolutionary advantages to clonal plants, possibly explaining, at least in part, their widespread success. Finally, we suggest avenues of inquiry to enable assessing intelligent behavior and the role of epigenetic memory in clonal species.

Keywords: asexual reproduction, DNA methylation, plant memory, between ramets communication, 5-azacytidine, zebularine

INTRODUCTION

Although it has historical antecedents in remarks by Darwin (1880), the more recent and not-universally held characterization of plants as intelligent organisms is largely attributable to a series of publications by Trewavas (2003, 2004, 2005, 2014). Indeed, Trewavas's arguments for plant intelligence, coupled with counterarguments (Firn, 2004) to his viewpoint, capture the overall discussion of this subject quite well. In the present paper, we propose investigation of a system that is likely to especially strongly display those behaviors that Trewavas – and we – would characterize as intelligent; moreover, this system is particularly well-suited to address the arguments put forth to dispute plant intelligence. Finally, we hypothesize that to the extent that intelligence is manifested in the behavior of the focal organisms – clonal plants – it may at least in part explain the ecological and evolutionary success of their life strategy.

First, a brief overview of Trewavas's arguments and the proffered rebuttals is in order. As he noted (Trewavas, 2003), the traditionally prevailing presumption that plants lack intelligence is likely largely due to methodological difficulties and biases in applying to plants concepts originally developed in the context of animals. In particular, because the traditional, zoocentric measure of behavior has been in terms of movement, plants' sessile lifestyle and different reaction time-scale has probably helped create the widespread view of plants as passive and therefore incapable of behaving, let alone exhibiting intelligence. Similarly, Trewavas (2003) noted that because many plant responses to environmental conditions are manifested in terms of growth, or through such cellular-level processes as changes in turgor pressure, it is tempting to dismiss these responses as

developmental or physiological processes, rather than behavior. However, given their sessile nature, it is through such directed growth that plants can explore and preferentially exploit favorable environments, akin to the movement shown by animals.

Therefore, Trewavas (2003) employed Stenhouse's (1974) objective definition of intelligence as "adaptively variable behavior within the lifetime of the individual", because it is not *a priori* inapplicable to plants; however, he did so with the understanding that "behavior" be limited to responses on the level of the whole plant (i.e., excluding direct responses of a single organ or tissue to the environment with which it is interacting). Along with many examples of intelligence so defined, he described (Trewavas, 2003) various plant capabilities that make such intelligence possible, including plant learning abilities (e.g., Shen-Miller, 1973; Ishikawa et al., 1991) and communication and decision-making capabilities (Addicott, 1982; Griffiths and McIntyre, 1993; Takayama and Sakagami, 2002).

Trewavas's characterization of plants as intelligent was disputed (Firn, 2004) on multiple bases, the most salient of which can be distilled down to the contentions that the concept of the individual is largely inapplicable to plants (as they are not only modular, but organs can exist on their own), that in any case they do not respond as individuals (with responses made instead at the organ level and without coordination), that memory such as it exists in plants is similarly localized (i.e., there is no repository of plant-wide memories analogous to a brain) and simply consists of linear sequences of developmental experiences, and that, unsurprisingly, given these limitations, plants do not make meaningful choices. In turn, Trewavas (2004) rebutted these assertions by, among other things, citing evidence that generally plant parts cannot survive on their own; that responses are coordinated between plant parts (e.g., roots controlling shoot growth or vice versa), relying upon communication between them; that memory – albeit not residing in a central repository – operates at a plant-wide level; and that plant decision-making goes well beyond merely achieving homeostasis or making simple binary choices. Moreover, much more evidence of the complexity and coordination of plant behavior has been adduced over the years since Trewavas's initial publications.

Of special relevance to the study system that we discuss, Trewavas (2003), rather than seeing modularity as an obstacle to intelligent behavior, suggested that "[a] consequence of a repetitive modular structure is that the individual ramets might be regarded as being like parallel processors contributing different experiences resulting from different ages to present day decisions." Following up on this idea, we hypothesize that the additional level of modularity displayed by clonal plants that occur as networks of connected individuals could confer a greater capacity for intelligent behavior in comparison to otherwise similar plants that occur as fully separate individuals (with the relevant comparisons being between plants of the same growth form and similar size, such that, e.g., clonal herb individuals can be compared to those of non-clonal herbs, clonal shrub individuals to non-clonal shrub individuals and clonal tree individuals to non-clonal trees). The plants in such a network would benefit not only from the varied

experiences of the constituent individuals but also from modes of communication and coordination that rely upon physical connections (and which would complement those modes that are also available to fully separate individuals). Additionally, we hypothesize that epigenetic modification, which has already been suggested as a plant memory mechanism (Gagliano et al., 2014; Trewavas, 2014) may be especially important in clonal plants, potentially contributing further to heightened capacity for intelligent behavior. Here we present these hypotheses against an overview of recent advances elucidating plant learning and communication capabilities, and then propose particular research approaches to ascertain whether epigenetic modification does in fact contribute to adaptive behavior in clonal plants. Finally, we briefly explore the possible role of the potential, associated fitness advantage of heightened intelligence in the proliferation of clonality in plants.

REMEMBERING AND LEARNING FROM ENVIRONMENTAL INTERACTIONS

There have been ample demonstrations of plant abilities to not only store information about their environmental interactions, even for several generations, but to also properly evaluate that information in the context of ambient conditions (Galloway and Etterson, 2007; Sultan et al., 2009; Whittle et al., 2009; Latzel et al., 2010, 2014). Moreover, plants have even been shown to predict future conditions based upon past experiences. Thus, plants can perform quantum computing in chloroplasts to anticipate future light quality changes based on former light quality, enabling them to adjust photosynthetic apparatus accordingly (Karpinski and Szechynska-Hebda, 2010; Szechynska-Hebda et al., 2010). Similarly, plants can also modify their growth in accordance with expected future resource competition based on previous experience (Shemesh et al., 2010; Novoplansky, 2016). Finally, memories of previous environmental stress can also help plants better overcome future stress (Ding et al., 2012).

Learning from past experiences requires memory. Although some memory mechanisms such as accumulation or stable modification of key regulatory proteins cannot be separated from developmental processes, most plant memories are likely enabled by molecular mechanisms, of which epigenetic modification is probably the most prominent (Bruce et al., 2007; Ginsburg and Jablonka, 2009; Ding et al., 2012; Thellier and Lüttge, 2013; Müller-Xing et al., 2014). Epigenetic mechanisms such as histone modifications and reversible methylation of cytosine alter gene expression and thus also phenotypes (Liu and Wendel, 2003; Bird, 2007). Because epigenetic variation is environmentally inducible and mediates phenotypic plasticity (Jablonka and Lamb, 1995; Alvarez et al., 2010), it is very likely that many of the environmental interactions of an individual are reflected in epigenetic change.

Environmentally induced epigenetic change can become fixed for a period (Chinnusamy and Zhu, 2009) and eventually may become heritable, thus altering plant-environment interactions in subsequent generations (Molinier et al., 2006; Boyko et al., 2010; Verhoeven et al., 2010). Epigenetic variation can thus

enable plants to remember their former interactions with the environment, learn from them, and apply the acquired knowledge to improve their performance in the future (e.g., Boyko et al., 2010; Kou et al., 2011; Bilichak et al., 2012; Ding et al., 2012; Müller-Xing et al., 2014). Moreover, such behavior, because it can be performed at any stage of plant development, is clearly independent of developmental progression, and thus overcomes the objection that supposed behavior is actually just development and therefore not a manifestation of intelligence.

THE POTENTIAL FOR CLONALITY AND EPIGENETICS TO COMBINE IN FACILITATING PLANT INTELLIGENCE

Clonal reproduction can yield genets comprising up to several hundred connected ramets (Kemperman and Barnes, 1976). Each ramet essentially constitutes an individual plant, generally capable of persisting independently if it becomes disconnected from the rest of the genet spontaneously or by injury. However, the interconnected genet functions in some ways as a single entity, with stressed ramets able to avert death by drawing on the support of stronger ramets (Fischer and Stöcklin, 1997), labour division among ramets (Alpert and Stuefer, 1997), and also with the overall growth of a genet (Stuefer and Hutchings, 1994) increased by its capability to share resources among ramets.

Genets also have the ability to adjust to resource heterogeneity by placing ramets in favorable patches (Bell, 1984; Waters and Watson, 2015), showing that they take account of spatial environmental variation, which is especially likely to be encountered as they spread. Additionally, because a genet typically produces new, connected ramets over time, such that there is coexistence of ramets of different ages (as mentioned by Trewavas, 2003), the ramets within a genet are likely to have been produced not only over spatially varying environmental conditions, but also over temporally varying conditions. If information on such variation can be stored, shared, and translated into appropriate responses, then this can form the basis for particularly intelligent behavior on the part of clonal plants.

Clonal plants do appear to have greater ability than non-clonal plants to remember past environmental interactions (Jablonka and Lamb, 2005; Latzel and Klimešová, 2010; Verhoeven and Preite, 2014). This may be due at least in part because of their more effective transfer of epigenetic information among clonal generations (Jablonka and Lamb, 2005; Latzel and Klimešová, 2010; Verhoeven and Preite, 2014), in contrast to the resetting of most environmentally induced epigenetic information during meiosis during sexual reproduction (reviewed by Paszkowski and Grossniklaus, 2011). In fact, the more faithful intergenerational transmission of epigenetic information would provide an advantage to ramets (relative to sexually produced individuals) even if they become detached from their parent clone and are living independently.

Nevertheless, it is within connected genets that epigenetic memory is likely to have its greatest effect in terms of facilitating

intelligent behavior of clonal plants. This is because although even populations of non-clonal plants can communicate via root exudates or volatiles (e.g., Falik et al., 2011), the physical connections maintained within such genets allow communication by additional means. Communication within interconnected ramets has been demonstrated (Stuefer et al., 2004), particularly with regard to early warning systems in which information of an herbivore attack can be spread throughout the clone, enabling those ramets not yet attacked to produce defenses (Gómez et al., 2007). Hormones such as jasmonic and salicylic acids are thought to carry such signals within a clonal network. Although they can provide information advantage for clonal plants, these messengers would carry information only about the situation currently or very recently encountered by the genet, and would not convey information about events or conditions from the more distant past.

Based on studies by Raj et al. (2011) and Richards et al. (2012) showing that clonal, genetically homogeneous populations of *Populus* and *Fallopia*, respectively, remember former environments via epigenetic variation, we propose that environmentally induced epigenetic change might be the mechanism that can carry precise information about environmental interactions from the more distant past. We suggest that given the absence of meiotic resetting of epigenetic modification in clonal reproduction, newly emerging offspring ramets can inherit epigenetic information of previous environmental interactions from the maternal ramet. Moreover, because an offspring ramet can encounter different environments than those experienced by the maternal ramet, the epigenetic information that will ultimately be conveyed by the offspring ramet to the generation subsequent to it (i.e., third generation) can represent a combination of maternal (first generation) and offspring (second generation) environmental interactions.

Indeed, from studies on *Arabidopsis thaliana*, we know that a single genotype can have more than 250 distinct stable epigenetic states (Johannes et al., 2009; Cortijo et al., 2014). Thus, in a genet consisting of tens or hundreds of interconnected ramets, each ramet is likely to have a different epigenetic state depending not only upon the environmental conditions experienced by itself and its progenitors but also upon the interactions between these conditions and the phenotypes in each of these generations. This variation among connected individuals, each of which consists of a whole, potentially independent plant, in combination with the enhanced capability for communication among them, would, we suggest, provide the basis for “swarm intelligence”, an emergent property of cooperating groups that enables them to solve problems that are beyond the abilities of a single member (Bonabeau et al., 1999). Indeed, in seeking to explain the decentralized intelligence exhibited by individual plants, Trewavas (2005) suggested looking at “other decentralized intelligence systems such as those found in social insects.” This analogy is even more appropriate when considering the potential swarm intelligence of ramets within a connected genet.

Although we are primarily concerned with the relative capacity for intelligent behavior of a connected genet vs an array of non-clonal, yet comparable individuals, the ability for

constituent ramets to process information and act somewhat autonomously does raise the question (with possible evolutionary implications) of whether a genet would have an intelligence advantage over a single plant of similar size to the entire genet. In more fully considering this possibility, which is beyond the scope of this paper, an important issue would be whether the possible communications advantages enabled by greater physical integration within a single, large individual would offset the benefit of having a “swarm” of largely autonomous ramets.

One possible product of epigenetically mediated intelligence in clonal plants is pre-programming of offspring ramet phenotypes for particular types of growth based on the previous interactions of the genet with the environment. This can significantly increase overall success of the genet, particularly in cases when environmental conditions are predictable. For instance, adaptive responses to drought may encompass increased investment in roots to better tap ground water and/or reduced leaf growth to reduce water loss (Kozłowski and Pallardy, 2002; Chaves et al., 2003). In the case of simple phenotypic plasticity, the newly emerging offspring ramet would respond to the ambient water level and adjust its growth accordingly. Thus, in the scenario in which rainfall temporarily moistens soil of an otherwise dry environment, a newly emerging ramet would adjust its phenotype to wet conditions and grow shallow roots and large leaves, which could easily become inefficient or even costly for the genet when this moisture disappears. However, knowledge previously acquired by the genet could theoretically modify the plastic response of such a new ramet to better take account of long-term environmental conditions. Such intergenerational phenotypic adjustments are well-documented for sexual generations (Galloway and Etterson, 2007; Sultan et al., 2009; Latzel et al., 2010, Latzel et al., 2014), and we propose that similar effects are likely to occur in clonal plants. Of course, it is possible that constraints imposed on plastic responses to contemporaneous conditions could be detrimental if they reflect conditions that are no longer at all relevant. Nevertheless, we suggest that, the ongoing communication among ramets from differing periods may allow, via swarm intelligence, the genet to evaluate the suitability of differing responses and then, translate this, through signaling and resource allocation, into decisions to favorable outcomes for constituent ramets. Evolutionarily, such intelligent behavior may actually enable maintenance of high phenotypic plasticity of clonal species because educated control of offspring ramet phenotypes by reducing risks associated with high phenotypic plasticity, as described above.

Indirect support for this idea may have been provided by Louapre et al. (2012), who in an experiment on *Potentilla* and *P. anserina* found that as a stolon lengthened, the decision about where to place successive rooting ramets depended not only on resource richness experienced by the newest ramet but also by those preceding it. Additionally, the placement of new *P. reptans* ramets was influenced by the variability in richness among the rooting sites of previous ramets. Thus, for this species, their model system demonstrated that not only the average amount of resources obtained by older ramets, but also the variability of

resources among ramets can alter the growing strategy of a whole genet (Louapre et al., 2012). Although they considered only plant hormones or resource molecules as potential messengers in their system, epigenetic variation could also underlie the behavior of the clones in their study.

Variation among epigenetic memories of interconnected ramets can also modify the information values of signals that are sent within the genet. Because epigenetic variation alters plant responses to hormones and other messengers (Latzel et al., 2012), potentially each ramet in a genet could respond differently to signals received. It is also plausible that epigenetically distinct ramets can differently modulate the intensity of signals that they are sending. Thus, the interaction of interconnected ramets can form the basis for a unique system in which the information spread is evaluated and modulated on the basis of epigenetic memory.

For all of the abovementioned reasons, we suggest that epigenetic memory within connected genets can enable information storage and evaluation such that it can provide the basis for variability in decision-making that would overcome objections (e.g., Firn, 2004) that plant decision-making is trivial, thus not manifesting intelligence. Moreover, such variability could enable genetically and even developmentally identical genets to react differently to environmental stimuli such as attack by insect or microbial infection.

ASSESSING INTELLIGENT BEHAVIOR OF CLONAL PLANTS

Although we do not propose a test to quantify the overall intelligence of clonal plants, i.e., something akin to an IQ test, we believe that the extent to which information is stored epigenetically, transmitted across space and time within connected genets, and ultimately incorporated into the decision making of constituent ramets should all be able to be assessed. Thus, conceptually, to test whether epigenetically conveyed information from the past is useful to the plants in making better decisions (i.e., that will yield greater fitness or a proxy thereof), we can compare two genetically identical and developmentally equivalent genets, one naïve, i.e., without such information and one possessing such information about past environmental interactions. If the plants' behavior were guided only by presently sensed information, without input from acquired memory, then both genets should react similarly to present environmental stimuli. However, if the plants have the ability to evaluate new, ambient signals against a backdrop of stored environmental information, shared through clonal network connections, the genet with relevant experience should behave differently, giving it a considerable advantage over the naïve one.

To specifically examine the role of epigenetic modification in furnishing such memory for decision-making by clonal plants, perhaps the most straightforward way to make this comparison would be to use a demethylating agent that would disrupt already-acquired epigenetic modification. Using this approach, equivalent genets would be exposed to the same sequence of temporally varied environmental conditions, but for one set,

their epigenetic memory would be disrupted continuously via DNA demethylation using a demethylating agent such as 5-azacytidine or zebularine. Demethylation has been successfully applied in studies testing the role of epigenetic variation in phenotypic plasticity or adaptation to changed environments (e.g., Tatra et al., 2000; Boyko et al., 2010). These studies germinated seeds in a 5-azacytidine solution, an approach inapplicable to mature clonal plants. However, we have developed a new approach in which mature clonal plants are sprayed repeatedly with an aqueous solution containing 5-azacytidine and surfactant for months. Azacytidine is absorbed by leaves similarly to nutrients from a foliar fertilizer. Because of the constant exposure of growing plants to the demethylating agent, the newly created offspring ramets have modified methylation patterns. Our first results show that the method can lead to about 4–8% reduction in global methylation level of DNA in *Trifolium repens* (Gonzalez et al., unpublished) and *Taraxacum brevicorniculatum* (Dvořáková et al., unpublished) without significant effects on plant phenotypes. Although this method may have side effects that can create unknown artifacts, such an approach can serve at least as a proof-of-principle, and if the side effects are negligible or can be accounted for, it can yield useful observations.

A more sophisticated (and complicated) approach would be to grow genetically identical, developmentally equivalent genets, and subject them to innocuous variation in environmental conditions that could serve as a signal for subsequent, more consequential environmental changes (i.e., equivalent to such signals used for conditioning in behavioral experiments on animals). By providing such genets with various environmental signals and consequences (e.g., varying herbivory intensity), and then seeing whether past experience favorably alters responses to present conditions, we can assess whether genets are learning from experience and applying this knowledge. The latter approach can be combined with experimental demethylation to determine the mechanistic role of epigenetic modification.

Additionally, to examine the extent to which information is shared and has impacts across a genet, individual ramets can be treated differently (e.g., be subjected to different levels of herbivory) and an assessment can be made of whether and how much their neighbors show responses (over a longer term than would be accounted for by hormones). Similarly, although ramets of different developmental stages may respond differently to particular environmental conditions, effects on neighbors of those at sensitive stages can be assessed, to determine whether a ramet can learn from a connected ramet's experience. Finally, the extent to which epigenetic variation within a genet contributes to variation among ramets in responses to an environmental stimulus can be assessed by demethylation, which by eliminating some of the epigenetic variation could convert a pattern of highly individualistic responses to one of more uniform responses.

Importantly, all the suggested approaches should try to compare behavior of genets consisting of different number of ramets to test the prediction that intelligent behavior increases with increasing complexity of clonal plants. Additionally, because we propose that intelligence in clonal plants would arise from multiple factors including intergenerational transmission of

epigenetic modification and ongoing, enhanced communication through physical connections among ramets with experiences from different times and places, we suggest that research also explicitly examine the role of such communication. Thus, perhaps, comparison can be made between intact clonal networks and networks with connections severed (after the network has already been subjected to varying environments). Or, less intrusively, similar comparisons could be done between networks and equivalent numbers of single ramets of the same species. Additionally, to directly address the question of clonal vs. non-clonal intelligence, the ability of (connected) genets to respond to environmental variation could be compared with that of arrays of similar (and phylogenetically close) non-clonal individuals. Although all of these comparisons would have to overcome challenges, we believe that it is worthwhile to at least suggest such possible avenues of future research.

CONCLUSION

Trewavas (2014) suggested that intelligence must be understood in terms of the interaction of the individual with the environment, with the environment posing the problems that intelligence is needed to solve. As examples from the literature show, plants are able to draw lessons from their interactions with the environment and can respond in ways that cannot be characterized as simple physiological or developmental consequences. Moreover, we suggest that clonal networks, especially in conjunction with epigenetic modification, can facilitate intelligent behavior, with epigenetics enabling long-term information storage in response to the environment and the latter permitting not only unaltered transmission of this information to subsequent generations, but also integration of information across multiple units experiencing different conditions.

A longstanding question in plant ecology has been why the clonal lifestyle, despite some likely disadvantages in terms of reduced population genetic variation, is so widespread, and is indeed predominant in some habitats (Klimeš et al., 1997). The posited facilitation of intelligent behavior, if actually present, might at least in part explain the success of clonal species, by enabling them to have more flexible, accurate and efficient responses to varied environments than do non-clonal species.

Not only might clonal plants gain an advantage over non-clonal plants, but it is likely that there are differences among clonal species in their abilities to accumulate and/or employ stored information – i.e., their intelligence. In particular, their intelligence could depend on life-history characteristics such as clonal growth type (e.g., phalanx vs. guerrilla strategy), integration level of ramets, level of information exchange among ramets, and persistence of clonal connections.

Results from our first (as yet unpublished) studies on this subject have shown that memories of former environments can persist across several cohorts of ramets and can be erased by demethylation (Rendina González et al., in press). Because the research that our group can conduct is finite and the questions, especially regarding potential advantages that

enhanced intelligence could provide to clonal vs. non-clonal plants, would require major undertakings to definitively answer, we hope that this essay will stimulate discussion about our ideas and possibly inspire other researchers to help determine their overall validity.

AUTHOR CONTRIBUTIONS

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

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Genetic and epigenetic control of plant heat responses

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Plants have evolved sophisticated genetic and epigenetic regulatory systems to respond quickly to unfavorable environmental conditions such as heat, cold, drought, and pathogen infections. In particular, heat greatly affects plant growth and development, immunity and circadian rhythm, and poses a serious threat to the global food supply. According to temperatures exposing, heat can be usually classified as warm ambient temperature (about 22–27°C), high temperature (27–30°C) and extremely high temperature (37–42°C, also known as heat stress) for the model plant *Arabidopsis thaliana*. The genetic mechanisms of plant responses to heat have been well studied, mainly focusing on elevated ambient temperature-mediated morphological acclimation and acceleration of flowering, modulation of circadian clock and plant immunity by high temperatures, and thermotolerance to heat stress. Recently, great progress has been achieved on epigenetic regulation of heat responses, including DNA methylation, histone modifications, histone variants, ATP-dependent chromatin remodeling, histone chaperones, small RNAs, long non-coding RNAs and other undefined epigenetic mechanisms. These epigenetic modifications regulate the expression of heat-responsive genes and function to prevent heat-related damages. This review focuses on recent progresses regarding the genetic and epigenetic control of heat responses in plants, and pays more attention to the role of the major epigenetic mechanisms in plant heat responses. Further research perspectives are also discussed.

Keywords: heat, genetic mechanism, epigenetic regulation, small RNAs, transgenerational memory

Introduction

Owing to the global warming, the annual mean maximum and minimum temperatures have been reported to increase by 0.35 and 1.13°C, respectively, for the period 1979–2003 (Peng et al., 2004). It is probable that the growing season temperatures in the tropics and subtropics by the end of the 21st century will exceed the most extreme seasonal temperatures recorded from 1900 to 2006 (Battisti and Naylor, 2009). Global warming has profound and diverse effects on plants. Warmer temperature has advanced the average first flowering date of 385 British plant species by 4.5 days (Fitter and Fitter, 2002). Besides, recent climate warming (2001–2008) has shifted vascular plant species' ranges to higher altitudes in European mountainous regions (Pauli et al., 2012). Furthermore, it is noteworthy that climate warming poses a serious threat to the global crop yields. Over the past three decades (1980–2008), heat has caused a decrease of 3.8 and 5.5% in the global maize and wheat production (Lobell et al., 2011). It is estimated that global

yields of the six most widely grown crops (wheat, rice, maize, soybeans, barley, and sorghum) drop by 0.6 ~ 8.9% for every 1°C the temperature increases (Lobell and Field, 2007). In the dry season, global warming has been estimated to cause a ~10% reduction in rice yield for every 1°C increase in growing-season minimum temperature (Peng et al., 2004). Thus, it is critical to dissect the heat sensing and signal transduction pathways in plants.

Genetic knowledge of plant responses to heat stress has been accumulating, including several putative heat sensors, HSFs and HSPs (heat shock factors and proteins) response pathways, and the network of phytohormones, chaperones, and secondary metabolites (Bokszczanin and Fragkostefanakis, 2013; Qu et al., 2013). However, our understanding of plant responses to warm temperature is limited, despite recent discoveries implicating the central role of the basic helix-loop-helix (bHLH) transcription factor *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*) in warmth-mediated morphological acclimation and acceleration of flowering (Proveniers and van Zanten, 2013). The modulation of circadian clock and immunity by high temperature also remains largely unknown. Recently, epigenetic regulations of heat responses have attracted increasing interests. The epigenetic mechanisms in response to heat include covalent modifications of DNA and histones, histone variants, ATP-dependent chromatin remodeling, histone chaperones, small RNAs, long non-coding RNAs (lncRNAs), and other undefined mechanisms (Table 1). This review briefly introduces the genetic mechanisms of plant responses to heat and highlights recent progresses regarding the underlying epigenetic regulations mainly in the *Arabidopsis* model, with aspects of some important physiological processes.

Genetic Mechanisms of Plant Responses to Heat

Warm Temperature-Mediated Morphological Acclimation and Acceleration of Flowering

The responses of *Arabidopsis* plants to warm temperature include hypocotyl and petiole elongation, leaf hyponasty, and early flowering (Gray et al., 1998; Balasubramanian et al., 2006; Koini et al., 2009). Warm temperature promotes auxin accumulation and activate the gibberellin (GA) and brassinosteroids (BRs) pathway resulting in hypocotyl elongation (Gray et al., 1998; Stavang et al., 2009). *PIF4* plays a central positive role in the acclimation to increased ambient temperature (Figure 1A; Proveniers and van Zanten, 2013). Warm temperature induces transient expression of *PIF4* (Koini et al., 2009; Kumar et al., 2012). *PIF4* has been demonstrated to control morphological acclimation to warm temperature via auxin. *PIF4* binds to the promoters of the key auxin biosynthesis genes in a temperature-dependent manner (Franklin et al., 2011; Sun et al., 2012). *PIF4* may also target the auxin-responsive gene *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*) at warm temperature (Koini et al., 2009). Moreover, *PIF4* directly or indirectly stimulates the expression of auxin target genes *SMALL AUXIN UP RNA* (*SAUR*) 19–24, which drive warmth-induced hypocotyl elongation and probably petiole elongation and leaf hyponasty (Franklin et al., 2011). In addition

to morphological acclimation, *PIF4* controls warm temperature-mediated floral induction through direct activation of the floral pathway integrator gene *FLOWERING LOCUS T* (*FT*) by binding its promoter (Kumar et al., 2012). A receptor-like kinase *SCRAMBLED/STRUBBELIG* (*SCM/SUB*) also plays a role in coordinating cell proliferation and differentiation during leaf development under increased ambient temperature (Lin et al., 2012).

Besides *PIF4*, MADS-box genes *SHORT VEGETATIVE PHASE* (*SVP*) and *FLOWERING LOCUS M* (*FLM*)/*MADS AFFECTING FLOWERING* (*MAF*) 1–5 modulate flowering time in response to ambient temperature changes (Balasubramanian et al., 2006; Lee et al., 2007; Gu et al., 2013). These genes act as flowering repressors and loss of their function leads to accelerated flowering independent of the photoperiod pathway. Interestingly, the RNA processing-related gene products are enriched upon thermal induction, suggesting that temperature might affect RNA processing in *Arabidopsis*. For instance, *FLM* is subject to temperature-dependent alternative splicing (Balasubramanian et al., 2006). The *SVP-FLM-β* complex is predominately formed at 17°C and prevents precocious flowering. By contrast, the competing *SVP-FLM-δ* complex is impaired in DNA binding and acts as a dominant-negative activator of flowering at 27°C (Figure 1A; Lee et al., 2013; Pose et al., 2013). Therefore, *PIF4* is an activator of *FT* while the MADS-box genes are repressors. However, how the two antagonistic pathways are integrated to modulate flowering time at warm conditions still needs to be genetically dissected.

The Effect of High Temperature on Circadian Clock

As a cellular time-keeper mechanism, the circadian clock allows plants to coordinate environmental time cues, such as photocycles (light/dark) and thermocycles (warm/cold), with endogenous biological rhythms with a period of ~24 h. Recent studies have suggested that plant circadian clock consists of three interlocked transcriptional feedback loops, i.e., a core oscillator loop, a morning loop, and an evening loop (Hsu and Harmer, 2014). Key players in this interconnected network are two MYB transcription factors *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), and *TIMING OF CAB EXPRESSION1/PSEUDO-RESPONSE REGULATOR1* (*TOC1/PRR1*). These three components repress the activity of each other and direct temporal regulation of most other clock components (Hsu and Harmer, 2014).

The two key responses of circadian clock to high temperatures are temperature entrainment and temperature compensation (Figure 1B). Thermocycles are able to entrain the clock in constant light with shorter periods than photocycles in *Arabidopsis* (Boikoglou et al., 2011). The genes governing temperature entrainment remain largely unknown, except for the evening loop component *EARLY FLOWERING 3* (*ELF3*), and the morning loop components *PRR7* and *PRR9*. The etiolated *elf3-1* seedlings are unable to exhibit classic indicators of entrainment by temperature cycles in darkness (Thines and Harmon, 2010). The *prp7-3 prp9-1* double mutants fail to entrain to thermocycles of 22/12°C, but can entrain to 28/22°C thermocycles without a robust oscillation (Salome and McClung,

TABLE 1 | Different epigenetic regulations involved in different heat responses.

Plants	Heat treatment	Major effects	Major epigenetic regulations	Reference
<i>Saccharina japonica</i>	20°C for 3 h	Regulation of tolerance to heat stress	miRNAs	Liu et al. (2014)
<i>Arabidopsis thaliana</i>	Grown at 26°C	Elevated survival of <i>Turnip Crinkle Virus</i> -infected Plants	siRNAs	Zhang et al. (2012)
<i>A. thaliana</i>	Grown at 27°C	Early flowering, hypocotyl and petiole elongation	H2A.Z	Kumar and Wigge (2010), Kumar et al. (2012)
<i>Nicotiana benthamiana</i>	Grown at 27°C	Enhanced antiviral defense	siRNAs	Qu et al. (2005)
<i>N. benthamiana</i> <i>Manihot esculenta</i>	Grown at 25–30°C	Enhanced antiviral defense	siRNAs	Chellappan et al. (2005), Andika et al. (2013)
<i>A. thaliana</i>	Grown at 30°C	Warmth-induced PTGS release with transgenerational memory	miRNAs and siRNAs	Zhong et al. (2013)
<i>Oryza sativa</i>	34°C for 48 h	Smaller seed size	DNA methylation, H3K9me2	Folsom et al. (2014)
<i>Hordeum vulgare</i>	35.5°C for 24 h	Regulation of tolerance to heat stress	miRNAs	Kruszka et al. (2014)
<i>Gossypium hirsutum</i>	35–39°C /29–31°C day/night for 7 days	Regulation of anther development	DNA methylation, histone modifications	Min et al. (2014)
<i>A. thaliana</i>	37°C for 3 h/day during the day for 1 week	Increase in homologous recombination frequency with transgenerational memory	DNA methylation, small RNAs	Boyko et al. (2010)
<i>A. thaliana</i>	37°C for 1–4 h	Regulation of tolerance to heat stress	Ta-siRNAs and miRNAs	Guan et al. (2013), Li et al. (2014b)
<i>Populus tomentosa</i>	37°C for 8 h	Regulation of tolerance to heat stress	miRNAs	Chen et al. (2012)
<i>A. thaliana</i>	37°C for 12 h	Regulation of tolerance to heat stress	long non-coding RNAs	(Di et al., 2014)
<i>A. thaliana</i>	37°C for 12 h	Heat stress-induced alternative splicing of <i>miR400</i>	<i>MIR400</i>	Yan et al. (2012)
<i>A. thaliana</i>	37°C for 16 h	Mediating the temporary growth arrest	ATP-dependent chromatin remodeling	Mlynarova et al. (2007)
<i>A. thaliana</i>	37°C for 24 h	Transgenerational retrotransposition of ONSEN	siRNAs	Ito et al. (2011)
<i>Helianthus annuus</i>	37°C for 24 h	Regulation of tolerance to heat stress	<i>miRNA396</i>	Giacomelli et al. (2012)
<i>P. trichocarpa</i>	37°C for 24 h	Regulation of tolerance to heat stress	miRNAs	Lu et al. (2008)
<i>M. esculenta</i>	37°C for 24 h	Regulation of tolerance to heat stress	miRNAs	Ballen-Taborda et al. (2013)
<i>A. thaliana</i>	37°C for 30 h	Release of TGS with reduced nucleosome occupancy and loss of chromocenter organization	CAF-1-dependent chromatin assembly complex	Pecinka et al. (2010)
<i>A. thaliana</i>	4°C for 1 week and then 37°C for 15 h	Release of TGS	Unorthodox and potentially new mechanisms	Tittel-Elmer et al. (2010)
<i>A. thaliana</i>	37°C for 4 days or 44°C for 30 min	Reorganization of chromatin and release of transcriptional gene silencing,	HIT4-dependent TGS regulation pathway	Wang et al. (2014)
<i>A. thaliana</i>	38°C for 1 h	Down regulation of <i>HSFB2a</i> involved in gametophyte development	Long non-coding antisense RNA <i>asHSFB2a</i>	Wunderlich et al. (2014)
<i>Apium graveolens</i>	38°C for 1 h	Regulation of tolerance to heat stress	miRNAs	Li et al. (2014a)
<i>A. thaliana</i>	38°C for 1.5 h per day in the dark and then returned to normal growth conditions; for 7 consecutive days	Modulation of pattern-triggered immunity	Histone modifications	Singh et al. (2014)
<i>A. thaliana</i>	Several heat cycles (37°C for 12 h in the light and 21°C for 12 h in the dark)	Activation of the imprinted gene <i>SDC</i>	An undefined epigenetic mechanism	Sanchez and Paszkowski (2014)

(Continued)

TABLE 1 | Continued

Plants	Heat treatment	Major effects	Major epigenetic regulations	Reference
<i>Triticum aestivum</i>	40°C for 1 h	Regulation of tolerance to heat stress	miRNAs and long non-coding RNAs	Xin et al. (2010), Xin et al. (2011)
<i>Chlamydomonas reinhardtii</i>	40°C for 1 h, for three times	Regulation of tolerance to heat stress	Histone modifications and chromatin remodeling	Strenkert et al. (2011)
<i>O. sativa</i>	42°C day/36 °C night for 24 h	Regulation of tolerance to heat stress	miRNAs	Sailaja et al. (2014)
<i>T. aestivum</i>	42°C for 2 h	Regulation of tolerance to heat stress	miRNAs	Kumar et al. (2014)
<i>Brassica rapa</i>	42°C for 3 h per day for 7 days	Stress-induced transgenerational inheritance	<i>miR168</i> and <i>braAGO1</i>	Bilichak et al. (2015)
<i>A. thaliana</i>	42°C for 16 h	Transcriptional reprogramming	DNA methylation, histone acetylation	Popova et al. (2013)
<i>A. thaliana</i>	42°C for 48 h	Stress-induced release of GUS silencing	H3K9ac1 and H3K9/14ac2	Lang-Mladek et al. (2010)
<i>A. thaliana</i>	BT and AT ^a	Gene transcription activation	Histone chaperone ASF1	Weng et al. (2014)
<i>A. thaliana</i>	BT and AT ^a	Regulation of tolerance to recurring environmental stress	The <i>miR156-SPL</i> module	Stief et al. (2014)
<i>Brassica rapa</i>	46°C for 1 h	Regulation of tolerance to heat stress	miRNAs, nat-siRNAs, chloroplast small RNAs	Wang et al. (2011), Yu et al. (2012b, 2013)
<i>A. thaliana</i>	50°C for 3 h/day for 5 day	Transgenerational phenotypic and epigenetic changes	H3K9 methylation and DNA methylation	Migicovsky et al. (2014)
<i>Quercus suber</i>	Temperature increases by 10°C every 3 days from 25–55°C	Acclimation to high temperature	DNA methylation, histone acetylation	Correia et al. (2013)

^aBT, basal thermotolerance, 45°C for 2 h; AT, acquired thermotolerance, pretreated at 37°C for 1–1.5 h and returned to 22°C for several hours or days for recovery, treated at 45°C for 2 h.

2005; Salome et al., 2010). Temperature compensation refers to the ability of maintaining a relatively constant period over a range of environmental temperatures. As temperature increases from 12 to 27°C, the periodicity has no significant changes in *Arabidopsis* (Gould et al., 2006). High temperature enhances the CCA1 binding affinity to the promoters of the oscillator genes, which is precisely antagonized by protein kinase CASEIN KINASE2 (CK2; Portoles and Mas, 2010) and transcription factor FLOWERING BASIC HELIX-LOOP-HELIX 1 (FBH1; Nagel et al., 2014) to maintain the circadian period. At high temperatures, the activities of CCA1 and LHY are counterbalanced by the temperature-dependent regulation of TOC1 and GI (Gould et al., 2006) as well as PRR7 and PRR9 (Salome et al., 2010). *REVEILLE8* (*RVE8*), a homolog of *CCA1* and *LHY*, is also required for temperature compensation, as *rve8* mutants have long-period and its overexpression lines have short-period phenotypes under high temperature (Rawat et al., 2011). Moreover, the activity of PRR7 is regulated by HEAT SHOCK FACTOR B2b (HsfB2b; Kolmos et al., 2014) and the evening complex night-time repressor consisting of ELF3, ELF4, and LUX ARRHYTHMO (LUX; Mizuno et al., 2014). The night-time repressor also mediate temperature responses of the clock transcriptional circuitry by regulating other targets *GI*, *LUX* and *PIF4/5*. This activity of night-time repressor is antagonized by warm temperature, suggesting that the PIF4-mediated morphological acclimation may be regulated by the night-time repressor under warm temperature (Mizuno et al., 2014). It is noteworthy that heat-induced alternative splicing of clock components such as *CCA1*, *PRR7*, *TOC1*, and *ELF3* may be an important

mechanism in temperature compensation (Kwon et al., 2014; Filichkin et al., 2015). Overall, our knowledge on the response of circadian clock to high temperature is rather limited. How plants integrate circadian clock with immunity under high temperature remains elusive.

Modulation of Plant Immunity by High Temperature

The effect of high temperatures on plant immunity has been well summarized recently (Hua, 2013). Two major influences of high temperature on plant immunity are that high temperature often inhibits the effector triggered immunity (ETI) and enhances RNA-silencing mediated resistance (Figure 1C). In ETI, pathogen effectors are recognized by the host proteins encoded by resistance (*R*) genes, of which most are nucleotide binding-leucine rich repeat (NB-LRR) class of proteins (Martin et al., 2003). *SUPPRESSOR OF npr1-1*, *CONSTITUTIVE 1* (*SNC1*) is the first identified *R* gene mediating high temperature inhibition of resistance (Yang and Hua, 2004), which is negatively regulated by *BONZAI1* (*BON1*; Zhu et al., 2010). At 22°C, the *bon1-1* loss-of-function mutation activates *SNC1*, which induces constitutive salicylic acid (SA)-mediated defense responses and inhibits plant growth. While at 28°C, the nuclear accumulation of *SNC1* protein is reduced by high temperature, which may inhibit the activity of *SNC1* protein and suppress the defense responses (Zhu et al., 2010). Besides *BON1*, other negative regulators of *SNC1* have been identified, such as *BON1-ASSOCIATED PROTEIN 1* (*BAP1*), *BAK1-INTERACTING RECEPTOR-LIKE KINASE 1* (*BIR1*), *SUPPRESSOR OF rps4-RD*

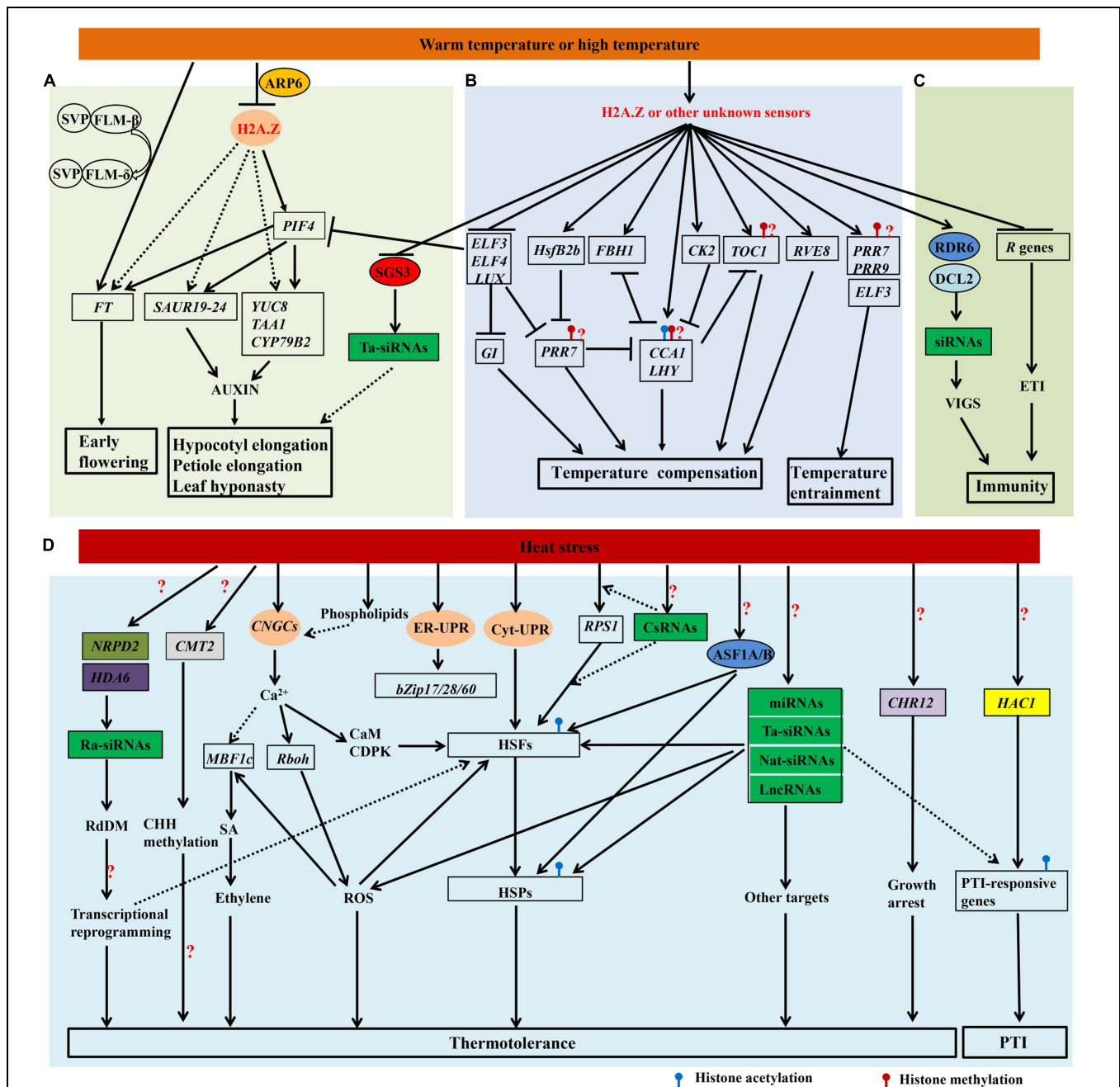


FIGURE 1 | Proposed model integrating genetic and epigenetic controls of heat responses. Genes and proteins are represented in boxes and circles, respectively. The genes and proteins in color are involved in the epigenetic regulation of heat responses. The four putative heat sensors, H2A.Z, the calcium channel in the plasma membrane (CNGCs), two unfolded protein sensors in ER (ER-UPR) and the cytosol (Cyt-UPR), are indicated. The speculative regulatory paths are indicated with broken arrows. **(A)** Warm temperature mediates the morphological acclimation and acceleration of flowering. Under warm temperatures, the expression of *PIF4* could be induced by the eviction of H2A.Z at its promoter. *PIF4* binds to the promoters of target genes and plays a central role in the morphological acclimation and acceleration of flowering. Warm temperature also induces the transition from SVP-FLM-β to the competing SVP-FLM-δ complex, the latter is then released from the promoter of *FT*. The inhibition of ta-siRNAs (green box) through the down-regulation of SGS3 protein (red circle) by warm temperature may be also

involved in the morphological acclimation. **(B)** The genetic mechanisms of temperature entrainment and temperature compensation are proposed. *ELF3*, *PRR7* and *PRR9* are involved in temperature entrainment, while *CCA1*, *LHY*, *PRR7*, *PRR9*, *GI*, *CK2*, *RVE8*, *FBH1* and *HsfB2b* are proved to play roles in temperature compensation. Note that histone modifications of *LHY*, *CCA1*, *TOC1*, *PRR7* and *PRR9*, such as H3K56ac, H3K9/14ac, H3K4me3 and H3K4me2, may (question mark) be regulated by high temperatures. **(C)** High temperature inhibits *R* genes-mediated ETI and enhances RNA-silencing mediated resistance. Reduced H2A.Z-containing nucleosome occupancy or other unknown mechanism are likely involved in the modulation of clock (B) and immunity (C). **(D)** Heat sensors and main signal transduction pathways in heat stress responses (HSR) are shown. Heat stress activates CNGCs, ER-UPR, and Cyt-UPR, and triggers signaling through multiple kinases as well as transcriptional regulators of the HSR, such as HSFs, MBF1c, and Rboh. RPS1 (Continued)

FIGURE 1 | Continued

in the chloroplast also responds to heat stress, and generates a retrograde signal to activate *HsfA2*-dependent heat-responsive genes in the nucleus. Some csRNAs are highly sensitive to heat stress and may regulate *RPS1*-mediated heat stress responses. Heat stress also affects the production of some ra-siRNAs, miRNAs, ta-siRNAs, nat-siRNAs, and lncRNAs. These non-coding RNAs may regulate *HSFs*, *HSPs*, and other target genes that function in heat acclimation. The *NRPD2* (olive box) and *HDA6* (purple box)-dependent RdDM pathway and the *CMT2* (gray box)-dependent CHH methylation may be required for thermotolerance. AtASF1A/B proteins (blue circle) are recruited onto chromatin and facilitate H3K56ac, which promotes the activation of some *HSFs* and *HSPs*. The chromatin-remodeling gene *CHR12* (light purple box) plays a vital role in mediating the temporary growth arrest of *Arabidopsis* under heat stress. Repetitive heat stress has also been reported to modulate PTI in a *HAC1* (yellow box)-dependent manner. Many unknown steps (?) remain to be recognized in this model.

(*SRFR1*), *CONSTITUTIVE EXPRESSOR OF PR GENES 1* (*CPRI*) and *MAP KINASE PHOSPHATASE 1* (*MKP1*; Gou and Hua, 2012). These genes tightly control *SNC1* activities at both the transcriptional and posttranscriptional levels. Besides, abscisic acid (ABA) plays a positive role in the high temperature-mediated inhibition of disease resistance, as ABA deficiency promotes nuclear accumulation of *SNC1* and potentiates defense responses at 28°C (Mang et al., 2012; Zhu et al., 2012b). In contrast, nitric oxide (NO) may act as a negative regulator in the high temperature-mediated inhibition of disease resistance (Wang et al., 2012a). It will be interesting to further dissect the interplay between ABA, NO, and SA-mediated defense responses under high temperature. Another major effect of high temperature in plant immunity is the enhancement of RNA-silencing mediated resistance. The underlying mechanisms will be discussed later in this review (see Small Interfering RNAs).

Thermotolerance in Plants

The mechanisms of thermotolerance to heat stress in plants have been elaborated, including the *HSFs* and *HSPs*, ROS, phospholipids and calcium signaling pathways, and the network of hormones (Figure 1D; Qu et al., 2013). The thermotolerance in *Arabidopsis* consists of basal and acquired thermotolerance. The basal thermotolerance is an inherent ability for plants to survive in exposure to temperatures above the optimal for growth, while acquired thermotolerance refers to the ability to cope with lethal high temperatures after acclimatization to mild high temperatures (Clarke et al., 2004). It is reported that SA, jasmonic acid (JA) and ethylene signaling pathways and ROS scavenging are required for basal thermotolerance (Miller et al., 2008; Clarke et al., 2009). The transcriptional co-activator *MULTIPROTEIN BRIDGING FACTOR 1C* (*MBF1c*) is required for basal thermotolerance and functions upstream of SA, trehalose and ethylene signaling pathways during heat stress (Suzuki et al., 2008). Moreover, NADPH oxidases *RESPIRATORY BURST OXIDASE HOMOLOGUE* (*Rboh*) enhances the production and maintenance of ROS, which is important for basal thermotolerance (Miller et al., 2008). *HSFs* and *HSPs* play central roles in the acquired thermotolerance in plants. *HSFs* are the central regulators responsible for the expression of *HSP* genes. The *Arabidopsis* genome contains 21 *Hsf* members that can be sorted into classes A, B, and C (Baniwal et al., 2004). *HsfA1a* is a master regulator for acquired thermotolerance that triggers the heat stress response through the induction of *HsfA1b* and *HsfA2* expression, while *HsfA2* is a major heat stress factor and induces the expression of *HSPs* under

heat stress. *HsfB1* acts as a co-regulator enhancing the activity of *HsfA1a* and *HsfA2* (Baniwal et al., 2004). *HSPs* are categorized into five classes based on their approximate molecular masses: *Hsp100*, *Hsp90*, *Hsp70*, *Hsp60*, and small *Hsps* (*sHsps*). These *HSPs* function as molecular chaperones and play complementary and sometimes overlapping roles in stabilizing proteins and membranes and assisting in protein refolding under heat stress (Wang et al., 2004). A variety of signaling molecules, such as ABA, H_2O_2 , ethylene, SA, calcium, and phospholipids, are also involved in acquired thermotolerance as well. These signaling molecules regulate the expression of *HSFs* and *HSPs* and protect cells against heat stress-induced oxidative damage (Song et al., 2012).

Although great progress has been achieved in the elucidation of molecular mechanisms of thermotolerance, how plants sense and transduce the signal of heat stress is still an important topic to be addressed. It is hard to define the primary heat sensor(s) as heat stress simultaneously poses a threat to almost all macromolecules and all organelles in the cells. Several putative heat sensors have been proposed, including a plasma membrane cyclic nucleotide gated calcium channel (CNGC) and two unfolded protein sensors in the endoplasmic reticulum (ER) and the cytosol (Figure 1D). The *CNGC2* gene in *Arabidopsis* and its ortholog *CNGCb* from *Physcomitrella patens* act as the primary heat sensors of land plant cells (Saidi et al., 2009; Finka et al., 2012). Heat shock impairs the protein stability and activates the unfolded protein response (UPR) in the ER and the cytosol. The cytosolic UPR is triggered by unfolded proteins in the cytosol and is notably regulated by *HsfA2* (Sugio et al., 2009). Heat promotes the translocation of two basic leucine-zipper domain-containing transcription factors *bZIP17* and *bZIP28* to the nucleus. The nuclear-localized *bZIPs* not only activate ER chaperone genes and induce the ER-UPR, but also activate BR signaling, which is required for heat stress acclimation and growth (Che et al., 2010). Heat-induced cleavage of *bZIP60* by the RNA splicing factor *IRE1b* also triggers the ER-UPR (Deng et al., 2011). Taken together, these results suggest that the primary heat sensor may lie in the plasma membrane, ER or cytosol. However, a heat-responsive retrograde pathway in chloroplast has recently been reported (Yu et al., 2012a). The photosynthetic apparatus in the chloroplast are the primary susceptible targets of heat stress. Through proteomic screening, the chloroplast ribosomal protein *S1* (*RPS1*) is also identified as a heat-responsive protein. Under heat stress, *RPS1* plays a critical role in modulating the translational efficiency of thylakoid proteins to maintain the stability and integrity of thylakoid membranes. The capacity of protein translation in chloroplasts generates the retrograde signals to activate

HsfA2-dependent heat-responsive genes in the nucleus (Yu et al., 2012a). Thus, the chloroplasts are proposed as heat sensors as well.

Epigenetic Regulation of Heat Responses in Plants

DNA Methylation

DNA methylation is a biological process by which a methyl group is added to the cytosine bases of DNA to form 5-methylcytosine. In plants, DNA methylation occurs frequently in all three sequence contexts: the symmetric CG and CHG contexts (where H = A, T or C) and the asymmetric CHH context. In *Arabidopsis*, overall genome-wide levels of 24% CG, 6.7% CHG and 1.7% CHH methylation are observed (Cokus et al., 2008). DNA methylation in plants predominantly occurs on transposons and other repetitive DNA elements (Zhang et al., 2006). Different proteins are involved in the establishment, maintenance and removal of DNA methylation. *De novo* methylation in all sequence contexts is catalyzed by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), and DNA methylation is maintained by three different pathways: CG methylation is maintained by METHYLTRANSFERASE 1 (MET1) and DECREASE IN DNA METHYLATION 1 (DDM1); CHG methylation is maintained by CHROMOMETHYLASE 3 (CMT3), a plant-specific DNA methyltransferase; and asymmetric CHH methylation is maintained by DRM2 (Chan et al., 2005). The DRM2 activity is regulated by the RNA-directed DNA methylation (RdDM) pathway (Matzke and Mosher, 2014). The plant-specific RNA polymerase IV (Pol IV) transcribes heterochromatic regions to generate single-stranded RNA (ssRNAs). RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) then synthesizes double-stranded RNA intermediates (dsRNAs) as precursors for RNase III-class DICER-LIKE 3 (DCL3) to process into 24-nt small interfering RNAs (siRNAs). Following incorporation into ARGONAUTE 4 (AGO4), the 24-nt siRNAs base-pair with Pol V scaffold transcripts, which results in DRM2 recruitment and DNA methylation at the source loci (Matzke and Mosher, 2014). In plants, four bifunctional 5-methylcytosine glycosylases, REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DME-like 2 (DML2) and DML3, have been implicated in the active removal of 5-methylcytosine from DNA through the base excision repair pathway (Zhang and Zhu, 2012). DNA methylation has two main roles in plants: defending the genome against selfish DNA elements and regulating gene expression. DNA methylation induces the transcriptional gene silencing (TGS) of transgene as well as endogenous transposons and retrotransposons to maintain genome stability (Chan et al., 2005). DNA methylation of promoter regions usually inhibits transcription initiation, while methylation within the gene body quantitatively impedes transcript elongation in *Arabidopsis* (Zilberman et al., 2007).

The global methylation can be differently affected by heat in different species. Exposure of *Arabidopsis* plants to heat stress results in an increased global methylation and a higher homologous recombination frequency (HRF; Boyko et al., 2010).

The up-regulation of *DRM2*, *NUCLEAR RNA POLYMERASE D 1* (*NRPD1*) and *NRPE1* in response to heat stress may contribute to the increased genome methylation in *Arabidopsis* (Naydenov et al., 2015). An increase in global methylation is also observed in Cork oak (*Quercus suber* L.) grown at 55°C (Correia et al., 2013). In *Brassica napus*, the DNA methylation levels increase more in the heat-sensitive genotype than in the heat-tolerant genotype under heat treatment (Gao et al., 2014). However, in cotton (*Gossypium hirsutum*) anthers, high temperature significantly decreases the expression of *S-ADENOSYL-L-HOMOCYSTEINE HYDROLASE1* (*SAHH1*) and DNA methyltransferases (*DRM1* and *DRM3*), resulting in the genome-wide hypomethylation at the tetrad stage and the tapetal degradation stage (Min et al., 2014). It appears that there is no consistent trend in the changes of DNA methylation under heat in different species. The methylation status of certain loci may be affected by heat stress. In developing rice seeds, the DNA methylation level of *Fertilization-Independent Endosperm1* (*OsFIE1*), a member of Polycomb Repressive Complex 2 (*PRC2*), is reduced and the transcript abundance of *OsCMT3* is repressed by a moderate heat stress (34°C) at 48 h after fertilization, which may lead to the misregulation of *OsFIE1* (Folsom et al., 2014).

Heat stress induces transcriptional activation of various transgenes that are previously silenced via TGS, such as the 35S promoter of *Cauliflower Mosaic Virus* and β -glucuronidase (*GUS*), which occurs without detectable changes in the levels of DNA methylation (Lang-Mladek et al., 2010; Pecinka et al., 2010; Tittel-Elmer et al., 2010). Warm temperature slightly increases the methylation level in some regions of *GUS* but decreases it in other regions (Zhong et al., 2013). Thus, it seems that DNA methylation is not involved in the regulation of heat responses. However, an analysis of the heat tolerance of mutants defective in DNA methylation reveals that the RdDM pathway is required for basal thermotolerance (Popova et al., 2013). Plants deficient in *NRPD2*, the second-largest subunit of Pol IV and V, are hypersensitive to heat stress, while *rdr2*, *dcl3* and *ago4* mutants are less sensitive. In *nrpd2* mutants recovering from heat, the misexpression of some protein-coding genes is associated with the epigenetic regulation of adjacent transposon remnants (transposons and retrotransposons; Popova et al., 2013). For example, the expression of the *COPIA*-like transposon *At1g29475* is induced by heat but not decreases during recovery in *nrpd2* plants, which may repress the adjacent six highly homologous auxin-responsive genes during recovery (Popova et al., 2013). Another study has also reported that the expression of *Calmodulin-like 41* (*CML41*) gene is up-regulated by high temperature with reduced DNA methylation level in the TE insertion very closely to the transcriptional start site (Naydenov et al., 2015). However, the activation of *ONSEN*, an LTR-copia type retrotransposon, could not be explained by the reduction of DNA methylation at the promoter upon heat stress (Cavrak et al., 2014). Whether the changes in DNA methylation of TEs play a causal role in the heat-induced activation of nearby genes needs to be explored.

Interestingly, in a genome-wide association analysis to detect loci with plastic response to climate, *CMT2* has been found to

be associated with temperature seasonality in *Arabidopsis* (Shen et al., 2014). The accessions with *CMT2_{STOP}* allele, which contains a premature stop codon, have broader geographic distribution than accessions with the wild-type allele. The *CMT2_{STOP}* allele can alter the genome-wide CHH-methylation pattern and *cmt2* mutant plants have an improved heat-stress tolerance, suggesting that *CMT2*-dependent CHH methylation may act as an important alleviator of heat stress responses. Moreover, the *CMT2_{STOP}* allele is associated with increased leaf serration and higher disease presence after bacterial inoculation (Shen et al., 2014). In summary, the above studies have not elaborated on the role of DNA methylation in heat responses. *cmt2* mutant plants have an improved heat-stress tolerance while *nrpd2* mutant plants are hypersensitive to heat stress, suggesting the different roles of *CMT2*-dependent CHH methylation and the RdDM pathway in response to heat (Figure 1D). Whether DNA methylation regulate plant immunity and circadian clock under heat needs to be investigated.

Histone Covalent Modification

In eukaryotic cells, genomic DNA is packaged into chromatin. The fundamental unit of chromatin is the nucleosome composed of ~147 bp- DNA wrapped around a histone octamer consisting of two copies of H2A, H2B, H3, and H4. Histone tails can be covalently modified at various amino acids and via different types, such as acetylation, mono/di/trimethylation, phosphorylation, ubiquitination, glycosylation, ADP ribosylation, carbonylation, sumoylation, and biotinylation. These modifications can activate or repress transcription by generating either 'open' or 'closed' chromatin configurations, respectively, thereby regulating the accessibility of chromatin to transcriptional regulators (Li et al., 2007). In plants, histone methylation and acetylation have been well characterized. As one of the most complex modifications, histone methylation not only occurs at distinct sites of lysine and arginine residues but also differs in the number of methyl groups added. In *Arabidopsis*, histone methylation mainly occurs at Lys4 (K4), Lys9 (K9), Lys27 (K27), Lys36 (K36), and Arg17 (R17) of histone H3, and Arg3 (R3) of histone H4 (Liu et al., 2010). These methylation types have different roles. H3K4me and H3K36me mainly generate 'open' chromatin configurations and activate transcription, whereas H3K9me and H3K27me create a "closed" chromatin and transcriptional repression. H3K9me2 functions as a silencing mark linked to DNA methylation, while H3K27me3 represses the expression of many genes targeted by PRC2. The effects of histone methylation on genome management, transcriptional regulation, and development in plants have been well reviewed (Liu et al., 2010; He et al., 2011). Histone acetylation and deacetylation are catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Histone acetylation are directly connected with transcriptional activation and affect a variety of biological processes in plant growth and development as well as biotic and abiotic stress responses (Chen and Tian, 2007).

Similar to DNA methylation, the histone modifications can be differently affected by heat in different species. In the unicellular green alga *Chlamydomonas reinhardtii*, there are higher levels of histone H3/4 acetylation and lower levels of H3K4me1

at promoter regions of active genes compared with inactive promoters and transcribed and intergenic regions after heat stress (Strenkert et al., 2011). The transcription factor *HSF1* may mediate the acetylation of histones H3/4, remodeling of the H3K4 methylation, and transcription initiation/elongation upon heat stress (Strenkert et al., 2011). However, temperature shift from 25 to 45°C decreases the acetylated histone H3 levels in the forest tree Cork oak (Correia et al., 2013). The deacetylated H3 may be responsible for repressive chromatin in gene promoters and repression of gene transcription. Histone modifications are also involved in rice seed and cotton anther development at high temperature. The H3K9me2 level of *OsFIE1* is sensitive to moderate heat stress and may be an important component involved in regulating *OsFIE1* when developing rice seeds are exposed to a moderate heat stress (Folsom et al., 2014). In cotton anthers, one histone methyltransferase, one histone monoubiquitination gene and two jumonji C (jmc) domain-containing genes are down-regulated upon high temperature (Min et al., 2014). The roles of the differently regulated histone modifications in heat responses remain unknown.

After heat stress, the levels of H3K9me2, H3K27me1 and H3K4me3 at a transcriptionally silenced GUS transgene (TS-GUS) and a non-LTR retrotransposon LINE039 showed only minor changes or remain unchanged (Lang-Mladek et al., 2010). But the amounts of H3K9ac1 and H3K9/14ac2 significantly increased in response to heat. The histone deacetylase HDA6 may be involved in this process as the TS-GUS activity showed a pronounced increase in *hda6* mutants (Lang-Mladek et al., 2010). Similarly, another study also demonstrates that levels of H3K4me3, H3K9me2, H3K27me2, and H3K27me3 were unaffected by temperature shift from 4 to 37°C for 15 h while a modest enrichment in H3K9ac-K14ac was detected at 5S rDNA, 106B long terminal-like dispersed repeats and a *Mutator*-like transposable element related locus MULE-F19G14 (Tittel-Elmer et al., 2010). Thus, the heat-induced release of silencing seems to be associated with histone acetylation but not histone methylation. However, similar to the wild-type control, the transcripts from these three targets over-accumulated in *hda6* mutants exposed to temperature shift, but reverted to the initial level after 2 days of recovery. These results exclude the possibility that HDA6 activity is required for the release of gene silencing (Tittel-Elmer et al., 2010). Interestingly, both the levels of repressive H3K9me2 and active H3K4me3 significantly reduced directly after long heat stress (37°C for 30 h) and returned to the initial level after 7 days of recovery (Pecinka et al., 2010). After long heat stress, nucleosomes and all the histone modifications on them were partially removed through unknown mechanisms, and then reloaded to the chromatin upon returned to ambient temperatures, while the levels of histone modifications on the remaining histones remained relatively unchanged (Pecinka et al., 2010). Thus, histone modifications may not play an important role in the heat-induced release of silencing.

Recently, environmental history of repetitive heat stress has been reported to modulate *Arabidopsis* pattern-triggered immunity (PTI) in a HISTONE ACETYLTRANSFERASE1 (HAC1)-dependent manner (Figure 1D; Singh et al., 2014). *Arabidopsis*

plants exposed to repetitive heat stress were more resistant to virulent bacteria than plants grown in a more stable environment. The enhanced resistance in repetitively stress-challenged plants occurred with priming of PTI-responsive genes and the potentiation of PTI-mediated callose deposition. The transcriptional activation of PTI-responsive genes was associated with enrichment of H3K9/14ac, H3K4me2 and H3K4me3, indicating a positive relationship between the bacterial resistance and histone modifications after heat stress. In *hac1-1* mutants, repetitively heat stress failed to induce enhanced resistance to bacteria, priming of PTI, and enrichment of H3K9/14ac, H3K4me2 and H3K4me3. These findings reveal that HAC1 is a necessary component for bacterial resistance, priming of PTI, and open chromatin configurations mediated by repetitive heat stress exposure. Whether H3K4 methylation have a similar role needs to be further analyzed (Singh et al., 2014). Multiple histone modifications, such as H3K56ac, H3K9/14ac, H3K4me3, and H3K4me2, have been found to closely correlate with the rhythmic expression of *LHY*, *CCA1*, *TOC1*, *PRR7*, and *PRR9* in *Arabidopsis* (Seo and Mas, 2014). Histone acetylation may contribute to the circadian peak of expression by influencing transcription factor accessibility under different temperature conditions, while H3K4me3 may antagonize clock repressor binding, ensuring a proper timing and duration of gene activation (Figure 1B). Overall, the roles of histone modifications in response to heat stress are largely obscure and need to be further recognized.

Histone Chaperones

Histone chaperones are a group of proteins that bind histones and prevent non-productive aggregation between highly positive charged histones and highly negative charged DNA without using the energy of ATP (Zhu et al., 2012a). They play a crucial role in nucleosome assembly during different processes such as DNA replication, repair, and transcription. In general, histone chaperones can be classified as either H3–H4 or H2A–H2B chaperones on the basis of their preferential histone binding. In plants, the well-studied chaperones include H3–H4 chaperones ANTI-SILENCING FUNCTION 1 (ASF1), CHROMATIN ASSEMBLY FACTOR-1 (CAF-1) and HISTONE REGULATORY HOMOLOG A (HIRA), and the H2A–H2B chaperones NUCLEOSOME ASSEMBLY PROTEIN1 (NAP1), NAP1-RELATED PROTEIN (NRP) and FACILITATES CHROMATIN TRANSCRIPTION (FACT; Zhu et al., 2012a). Only a few studies have reported the role of histone chaperones in heat responses. The reload of nucleosome, whose occupancy is reduced by long heat stress, requires the CAF-1-dependent chromatin assembly complex (Pecinka et al., 2010). Wild-type plants lost nucleosomes immediately after heat stress and restored the original level during recovery. By contrast, the *fasciata1* (*fas1*) and *fas2* mutants that lack different subunits of CAF-1, had the already reduced nucleosome occupancy before heat treatment. The nucleosome occupancy was further reduced by long heat stress in the mutants, and there was no restoration even after 7 days of recovery. The heat stress-induced loss of nucleosomes and heterochromatin decondensation led to the

activation of transcriptionally silenced repetitive elements. The CAF-1-dependent chromatin assembly complex may provide a safeguarding mechanism to minimize the heat-induced epigenetic damage in the germ line (Pecinka et al., 2010). *AtASF1A* and *AtASF1B* have also been reported to participate in basal and acquired thermotolerance (Figure 1D; Weng et al., 2014). Upon heat stress, *AtASF1A/B* proteins were recruited onto chromatin, and their enrichment was correlated with nucleosome removal and RNA polymerase II accumulation at the promoter and coding regions of some *HSF* and *HSP* genes. Moreover, *AtASF1A/B* facilitated H3K56ac, which also promotes the activation of some *HSFs* and *HSPs* (Weng et al., 2014).

Histone Variants

In addition to the conventional histones, which are deposited mostly during the S phase of the cell cycle, all eukaryotes have non-allelic histone variants that can be incorporated into nucleosomes in a DNA replication-independent manner during the entire cell cycle. Histone variants can alter the properties of the nucleosomes they occupy and play important roles in maintenance of genome stability, transcriptional activation and repression (Kamakaka and Biggins, 2005). There are 15 histone H3 genes in the *Arabidopsis* genome, including six canonical H3.1 or H3.1-like genes, eight H3.3 or H3.3-like genes and one centromeric histone H3 gene (Okada et al., 2005). The roles of H3 variants in heat responses have not been reported hitherto. Thirteen H2A-encoding genes have been identified in *Arabidopsis*, including four canonical H2A genes, two H2A.X genes, three H2A.Z genes and other four less categorized genes (March-Diaz and Reyes, 2009). Recently, an important study revealed that H2A.Z-containing nucleosomes mediate the thermosensory response in *Arabidopsis* (Kumar and Wigge, 2010). In a genetic screen of mutants defective in heat sensing, the *ARP6* gene was identified to mediate the response to increased temperature. The *ARP6* protein is an essential component of the SWR1 complex required for H2A.Z incorporation into chromatin (March-Diaz and Reyes, 2009). When grown at 22°C, the *arp6* mutants display phenotypes similar to wild-type plants grown at 27°C, such as hypocotyl and petiole elongation, leaf hyponasty, and early flowering. It is proposed that H2A.Z occupancy represses gene expression by creating a physical block to transcription or by preventing the binding of transcription activators at cooler temperatures, and eviction of H2A.Z at higher temperatures would thereby facilitate transcription of target genes. This temperature-induced H2A.Z nucleosome dynamics has been proved to regulate the binding of PIF4 to the FT promoter, thereby controlling the thermosensory activation of flowering. Based on these results, H2A.Z-containing nucleosomes are recognized as temperature sensors in the nucleus (Figure 1A; Kumar et al., 2012). However, it is unclear whether this mechanism is also responsible for the regulation of other heat-induced genes, such as auxin biosynthesis genes required for warm temperature-mediated morphological acclimation, *HSF* and *HSP* genes in acquired thermotolerance, and genes involved in the modulation of plant immunity and circadian clock by high temperature.

ATP-Dependent Chromatin Remodeling

ATP-dependent chromatin remodeling complexes use the energy of ATP hydrolysis to alter the structure of chromatin by destabilizing histone–DNA interactions, moving histone octamers or catalyzing the incorporation of histone variants. According to ATPases used, the complexes can be grouped into four main classes: the SWItch/Sucrose Non-Fermentable (SWI/SNF) class, the imitation switch (ISWI) class, the inositol requiring 80 (INO80) class, and the chromodomain and helicase-like domain (CHD) class (Clapier and Cairns, 2009). The SWI/SNF complex is the first ATP-dependent chromatin remodeling complex identified, and 41 SNF2 proteins in *Arabidopsis* have yet been identified. Functional analysis indicates that many of these proteins play important roles in plant development and stress response. Moreover, some of these proteins are involved in epigenetic regulation, such as TGS (dependent or independent of DNA methylation), H2A.Z deposition and histone modifications (Knizewski et al., 2008).

The Swi2/Snf2-related (SWR1) complex regulates transcription by replacing the H2A–H2B histone dimers in nucleosome with dimers containing the H2A.Z variant. As mentioned above, the ARP6 protein, which is an essential component of the SWR1 complex, plays an important role in temperature sensing (Figure 1A; Kumar and Wigge, 2010). The SNF2/Brahma-type chromatin-remodeling gene *CHROMATIN REMODELING* (*CHR12*) also plays a vital role in mediating the temporary growth arrest of *Arabidopsis* under heat, drought and salinity stresses (Figure 1D; Mlynarova et al., 2007). When exposed to stress conditions, a gain-of-function mutant overexpressing *AtCHR12* showed growth arrest of normally active primary buds and reduced growth of the primary stem. In contrast, the loss-of-function mutant showed less growth arrest than the wild-type when exposed to moderate stress (Mlynarova et al., 2007). In *Chlamydomonas reinhardtii*, heat stress induces low nucleosome occupancy at promoter regions of active genes, which is mediated by HSF1 and other unknown chromatin remodeling complexes (Strenkert et al., 2011). However, the heat stress-mediated release of TGS is at least partly independent of the activity of MORPHEUS' MOLECULE 1 (*MOM1*)/*CHR15*, a well-known DNA methylation-independent transcriptional silencer, and DECREASED DNA METHYLATION 1 (*DDM1*)/*CHR1*, a component required for DNA methylation and H3K9me2 (Tittel-Elmer et al., 2010). Whether other ATP-dependent chromatin remodeling complexes play roles in heat responses remains elusive.

Small RNAs

Small RNAs are 18–30 nt non-protein-coding RNAs, which have emerged as key guide molecules in the control of gene expression. Two major types of small RNAs in plants, microRNAs (miRNAs) and siRNAs, are distinguished by the different proteins involved in their biogenesis and the modes of regulation (Ghildiyal and Zamore, 2009).

microRNAs

Plant miRNAs are a class of 20–24 nt endogenous small RNAs that derive from the miRNA genes (*MIR*; Rogers and Chen,

2013). *MIR* genes are transcribed by Pol II to generate primary miRNA transcripts called pri-miRNAs. The pri-miRNAs are processed into stem-loop precursor pre-miRNA and further excised as miRNA/miRNA* duplex by the endonuclease activity of the DCL1 protein complex in the nucleus. The mature miRNAs are exported to the cytoplasm and incorporated into AGO proteins, mediating posttranscriptional gene silencing (PTGS) through slicing or translational inhibition, or TGS by targeting chromatin for cytosine methylation (Rogers and Chen, 2013).

A diversity of conserved and non-conserved heat-responsive miRNAs have been identified by small RNA deep-sequencing in different species, but few of them have been validated by either northern blots or real time PCR. As listed in Table 2, most of the conserved heat-responsive miRNAs are differently regulated in various species, except for miR159, 166 and 472 families. *miR159* has been found to be down-regulated by heat in *Arabidopsis* (Zhong et al., 2013), wheat (*Triticum aestivum*; Wang et al., 2012b; Kumar et al., 2014) and cassava (*Manihot esculenta*; Ballen-Taborda et al., 2013). The main targets of *miR159* are MYB transcription factors. *Tae-miR159* has been demonstrated to direct the cleavage of *TaGAMYB1* and *TaGAMYB2* (Wang et al., 2012b). The *tae-miR159* overexpression rice lines and *Arabidopsis myb33myb65* double mutants are more sensitive to heat stress relative to the wild-types, indicating that the down-regulation of *miR159* and up-regulation of its targets after heat stress might participate in a heat stress-related signaling pathway and contribute to heat stress tolerance (Wang et al., 2012b). *miR166*, which targets HD-Zip transcription factors, is up-regulated by heat in *Arabidopsis* (Zhong et al., 2013), wheat (Xin et al., 2010), and barley (*Hordeum vulgare*; Kruszká et al., 2014). The heat-induced up-regulation of *hvu-miR166a* and down-regulation of its targets, *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*) and a homeobox-leucine zipper protein *HOX9*-like gene, might influence the leaf morphology (Kruszká et al., 2014). *miR472* may be down-regulated by heat in *Arabidopsis* (Zhong et al., 2013) and Chinese white poplar (*Populus tomentosa*; Chen et al., 2012), but need to be further validated.

Several miRNA families seem to be responsive to heat in at least four species, including miR156, 160, 167, 168, 169, 171, 395, 398, 408, and 827 families (Table 2). Some members of the miR156 family are induced by heat in *Arabidopsis* (Zhong et al., 2013; Stief et al., 2014), *Brassica rapa* (Yu et al., 2012b), and wheat (Xin et al., 2010; Kumar et al., 2014), but are repressed by heat in rice (Sailaja et al., 2014) and cassava (Ballen-Taborda et al., 2013). In *Arabidopsis*, *miR156* isoforms are highly induced after heat stress and target *SQUAMOSA-PROMOTER BINDING-LIKE* (*SPL*) transcription factor genes (especially *SPL2* and *SPL11*) that are master regulators of developmental transitions (Stief et al., 2014). AGO1 acting through *miR156* and its target *SPLs* appears to mediate the adaptation to recurring heat stress (HS memory) by inducing the expression of HS memory-related genes (Stief et al., 2014). *Bra-miR156h* and *bra-miR156g* were also heat-induced and their putative target *BracSPL2* was down-regulated (Yu et al., 2012b). The up-regulation of *tae-miR156* and down-regulation of its putative target *SPL* genes *Ta3711* and *Ta7012* were also validated in wheat (Xin et al., 2010). However, the roles

TABLE 2 | The conserved heat-responsive miRNAs in different plant species.

Family	miRNA ^a	Heat treatment	Regulation	Validation ^c	Target proteins
156	<i>ath-miR156h</i>	AT ^b	Up-regulation	Yes	SPL transcription factor
	<i>ath-miR156g,h</i>	Grown at 30°C	Up-regulation	No	
	<i>bra-miR156g,h</i>	46°C for 1 h	Up-regulation	Yes	BracSPL2
	<i>osa-miR156a,g,h</i>	42°C day/36°C night for 24 h	Down-regulation in roots and shoots	Yes	SPL transcription factor
	<i>tae-miR156a-g</i>	40°C for 2 h	Up-regulation	Yes	SPL transcription factor
	<i>tae-miR156</i>	42°C for 2 h	Up-regulation	Yes	Heat shock protein 90
	<i>mes-miR156a</i>	37°C for 24 h	Down-regulation	Yes	SPL transcription factor
159	<i>ath-miR159a,b</i>	Grown at 30°C	Down-regulation	No	MYB and TCP transcription factors
	<i>tae-miR159</i>	40°C for 2 h	Down-regulation	Yes	GAMYB1 and GAMYB2
	<i>tae-miR159a,b</i>	42°C for 2 h	Down-regulation	Yes	WRKY transcription factor; MYB3; alkaline phosphatase family protein, cytochrome P450, cobalamine adenosyl transferase, Mob1-like protein and TLD family protein
160	<i>mes-miR159a</i>	37°C for 24 h	Down-regulation	Yes	myb-like HTH transcriptional regulators
	<i>ath-miR160a-c</i>	Grown at 30°C	Up-regulation	No	Auxin response factors
	<i>osa-miR160a</i>	42°C day/36°C night for 24 h	Down-regulation in roots, up-regulation in shoots	Yes	
	<i>pto-miR160a-d</i>	37°C for 8 h	Down-regulation	No	
	<i>tae-miR160</i>	40°C for 2 h	Up-regulation	No	Heat shock protein 70; ARF; tetrapeptide repeat (TPR)
	<i>tae-miR160</i>	42°C for 2 h	Down-regulation	Yes	
	<i>hvu-miR160a</i>	35.5°C for 24 h	Up-regulation	Yes	ARF17 and ARF13
	<i>mes-miR160a</i>	37°C for 24 h	Down-regulation	Yes	Auxin response factor
	<i>celery-miR160</i>	38°C for 1 h	Up-regulation	Yes	Auxin response factor
162	<i>osa-miR162a</i>	42°C day/36°C night for 24 h	Down-regulation in both roots and shoots	Yes	Endoribonuclease DCL1
164	<i>ath-miR164a-c</i>	Grown at 30°C	Up-regulation	No	NAC domain containing transcription factors
	<i>tae-miR164</i>	42°C for 2 h	Down-regulation	Yes	Small heat shock proteins 17; NAC transcription factor; target genes involved in mitogen-activated protein kinase (MAPK) signaling pathways
166	<i>celery-miR164</i>	38°C for 1 h	Up-regulation	Yes	NAC domain containing transcription factors
	<i>ath-miR166a</i>	Grown at 30°C	Up-regulation	No	HD-Zip transcription factors including PHV and REVOLUTA
	<i>tae-miR166a-d</i>	40°C for 2 h	Up-regulation	Yes	Unknown
	<i>hvu-miR166a</i>	35.5°C for 24 h	Up-regulation	Yes	HD-Zip transcription factors including PHV and REVOLUTA; a homeoboxleucine zipper protein HOX9-like gene
167	<i>ath-miR167c,d</i>	Grown at 30°C	Up-regulation	No	HD-Zip transcription factors including PHV and PHB
	<i>ath-miR167d</i>	AT ^b	Down-regulation	No	
	<i>bra-miR167</i>	42°C for 3 h per day for 7 days	Up-regulation	No	TOM1-like protein 2; Tudor domain-containing protein 3
	<i>bra-miR167*</i>	42°C for 3 h per day for 7 days	Up-regulation	No	GDSL esterase/lipase; Ribulose biphosphate carboxylase/oxygenase activase
	<i>bra-miR167</i>	46°C for 1 h	Up-regulation	No	BracARF6
	<i>osa-miR167a,c,d</i>	42°C day/36°C night for 24 h	Down-regulation in both roots and shoots	Yes	Class III HD-Zip protein 4; heat repeat family protein
	<i>sja-miR167a</i>	20°C for 3h	Down-regulation	No	Unknown
	<i>pto-miR167c,d,f,g</i>	37°C for 8 h	Up-regulation	Yes	Unknown
	<i>tae-miR167</i>	42°C for 2 h	Up-regulation	Yes	Dnaj heat shock n-terminal domain-containing protein
	<i>hvu-miR167h</i>	35.5°C for 24 h	Up-regulation	Yes	ARF8 and a serine/threonine-protein kinase Nek5-like gene

(Continued)

TABLE 2 | Continued

Family	miRNA ^a	Heat treatment	Regulation	Validation ^c	Target proteins
168	<i>bra-miR168</i>	42°C for 3h per day for 7 days	Up-regulation	Yes	BraAGO1
	<i>osa-miR168a</i>	42°C day /36°C night for 24 h	Down-regulation in shoots, no expression in roots	Yes	AGO1
	<i>sja-miR168a</i>	20°C for 3 h	Down-regulation	No	Unknown
	<i>pto-miR168a,b</i>	37°C for 8 h	Down-regulation	Yes	Unknown
	<i>tae-miR168</i>	40°C for 2 h	Up-regulation	No	Unknown
	<i>celery-miR168</i>	38°C for 1 h	Up-regulation	Yes	Unknown
169	<i>ath-miR169a,d-n</i>	Grown at 30°C	Down-regulation	No	CCAAT Binding Factor (CBF) and HAP2-like transcription factors
	<i>ath-miR169d,e,k,i,m</i>	AT ^b	Down-regulation	No	
	<i>ath-miR169b,c</i>	Grown at 30°C	Up-regulation	No	
	<i>osa-miR169a,b,g</i>	42°C day/36°C night for 24 h	Down-regulation in roots, up-regulation in shoots	Yes	Nuclear transcription factor Y subunit
	<i>pto-miR169j-m</i>	37°C for 8 h	Up-regulation	No	Unknown
	<i>pto-miR169n-t</i>	37°C for 8 h	Down-regulation	No	Unknown
	<i>tae-miR169a-d</i>	40°C for 2 h	Up-regulation	No	Unknown
	<i>ath-miR171a-c</i>	Grown at 30°C	Up-regulation	No	Scarecrow-like transcription factors
	<i>ath-miR171b,c</i>	AT ^b	Up-regulation	No	
	<i>bra-miR171a-1</i>	42°C for 3 h per day for 7 days	Up-regulation	No	26S protease regulatory subunit 6A homolog
171	<i>pto-miR171a-i</i>	37°C for 8 h	Down-regulation	No	Unknown
	<i>tae-miR171a</i>	42°C for 2 h	Down-regulation	Yes	Scarecrow-like protein
	<i>ptc-miR171l-n</i>	37°C for 24 h	Down-regulation	Yes	SCL, clathrin assembly protein
	<i>ath-miR172b*</i>	Grown at 30°C	Down-regulation	No	Eukaryotic translation initiation factor 5, putative; calcium-transporting ATPase
	<i>ath-miR172c,d,e</i>	Grown at 30°C	Up-regulation	No	AP2 transcription factors
	<i>ath-miR172a,e</i>	AT ^b	Up-regulation	No	
	<i>tae-miR172a,b</i>	40°C for 2 h	Down-regulation	Yes	AP2 transcription factors; floral homeotic protein
	<i>ath-miR319a,b</i>	Grown at 30°C	Up-regulation	No	MYB and TCP transcription factors
	<i>ath-miR319a,c</i>	AT ^b	Up-regulation	No	
	<i>ath-miR319c</i>	Grown at 30°C	Down-regulation	No	
319	<i>tae-miR319</i>	42°C for 2 h	Down-regulation	Yes	MYB3; histone protein-associated genes
	<i>ath-miR393a,b</i>	Grown at 30°C	Down-regulation	No	F-box proteins and bHLH transcription factors
	<i>tae-miR393</i>	40°C for 2 h	Up-regulation	Yes	Genes involved in auxin signaling pathway and basal defense
393	<i>ath-miR394a,b</i>	Grown at 30°C	Down-regulation	No	F-box proteins
	<i>celery-miR394</i>	38°C for 1 h	Up-regulation	Yes	Unknown
	<i>pto-miR394a,b</i>	37°C for 8 h	Down-regulation	No	Unknown
394	<i>ath-miR395a-f</i>	Grown at 30°C	Up-regulation	No	ATP sulphurylases; leucine-rich repeat family protein
	<i>ath-miR395d</i>	AT ^b	Up-regulation	No	
	<i>pto-miR395a-j</i>	37°C for 8 h	Down-regulation	Yes	Unknown
	<i>sja-miR395x</i>	20°C for 3 h	Up-regulation	No	Unknown
	<i>tae-miR395b</i>	42°C for 2 h	Up-regulation	No	sulfur transporters and ATP sulphurylases
	<i>celery-miR395</i>	38°C for 1 h	Up-regulation	Yes	Unknown
	<i>ath-miR396a</i>	Grown at 30°C	Up-regulation	No	Growth-regulating factor (GRF) transcription factors; rhodenase-like proteins; kinesin-like protein B
396	<i>han-miR396</i>	37°C for 24 h	Down-regulation	Yes	HaWRKY6
	<i>ath-miR397a,b</i>	Grown at 30°C	Up-regulation	No	Laccases and beta-6 tubulin
397	<i>osa-miR397b.2</i>	42°C for 8 h	Up-regulation	Yes	L-ascorbate oxidase

(Continued)

TABLE 2 | Continued

Family	miRNA ^a	Heat treatment	Regulation	Validation ^c	Target proteins
398	<i>osa-miR397</i>	42°C day/36°C night for 24 h	Down-regulation in shoots, no expression in roots	Yes	L-ascorbate oxidase precursor; F-box domain containing protein
	<i>mes-miR397a</i>	37°C for 24 h	Down-regulation	Yes	Laccase/Diphenol oxidase family protein
	<i>ath-miR398b</i>	Grown at 30°C	Up-regulation	No	CSD1, CSD2 and CCS
	<i>ath-miR398</i>	37°C for 4 h	Up-regulation	Yes	
	<i>bra-miR398a,b</i>	46°C for 1 h	Down-regulation	Yes	BracCSD1
399	<i>osa-miR398</i>	42°C day/36°C night for 24 h	Down-regulation in both roots and shoots	Yes	Superoxide dismutase (SOD) gene family
	<i>sja-miR398a-5p</i>	20°C for 3 h	Up-regulation	No	Unknown
	<i>tae-miR398</i>	42°C for 2 h	Up-regulation	Yes	Superoxide dismutase (SOD) gene family
	<i>ath-miR399b-d, f</i>	Grown at 30°C	Up-regulation	No	Phosphatase transporter
	<i>ath-miR399c,d</i>	AT ^b	Down-regulation	No	
400	<i>bra-miR399b</i>	46°C for 1 h	Down-regulation	No	BracPHO2
	<i>ath-miR400</i>	37°C for 12 h	Down-regulation	Yes	Pentatricopeptide (PPR) repeat-containing protein
408	<i>ath-miR408</i>	Grown at 30°C	Up-regulation	No	Peptide chain release factor; plantacyanin
	<i>sja-miR408b-5p</i>	20°C for 3 h	Down-regulation	Yes	An unknown conserved protein
	<i>pto-miR408</i>	37°C for 8 h	Down-regulation	Yes	Unknown
	<i>mes-miR408</i>	37°C for 24 h	Down-regulation	Yes	Plantacyanin
	<i>celery-miR408</i>	38°C for 1 h	Up-regulation	Yes	Unknown
472	<i>ath-miR472</i>	Grown at 30°C	Down-regulation	No	RFL1 (RPS5-LIKE 1)
	<i>pto-miR472a,b</i>	37°C for 8 h	Down-regulation	No	Disease resistance protein; F-box protein
482	<i>pto-miR482</i>	37°C for 8 h	Down-regulation	Yes	Unknown
827	<i>ath-miR827</i>	Grown at 30°C	Down-regulation	No	SPX (SYG1/Pho81/XPR1) domain-containing protein; DNA-binding storekeeper protein-related
1117	<i>ath-miR827</i>	AT ^b	Down-regulation	No	
	<i>bra-miR827</i>	46°C for 1 h	Down-regulation	Yes	Unknown
	<i>tae-miR827</i>	40°C for 2 h	Up-regulation	No	Unknown
	<i>ptc-miR827</i>	37°C for 24 h	Down-regulation	Yes	Sec14 cytosolic factor family protein
	<i>tae-miR1117</i>	42°C for 2 h	Down-regulation	Yes	Calcium dependent protein kinase 1
1450	<i>pto-miR1450</i>	37°C for 8 h	Up-regulation	No	Unknown
	<i>ptc-miR1450</i>	37°C for 24 h	Down-regulation	Yes	Leucine-rich repeat transmembrane protein kinase

The miRNA family, kinds of heat treatment, up or down-regulation, validated by either northern blot or realtime PCR, and their target proteins are presented. ^a*ath*: *Arabidopsis thaliana* (Yan et al., 2012; Guan et al., 2013; Zhong et al., 2013; Stief et al., 2014); *bra*: *Brassica rapa* (Yu et al., 2012b; Bilichak et al., 2015); *celery*: *Apium graveolens* (Li et al., 2014a); *han*: *Helianthus annuus* (Giacomelli et al., 2012); *hvu*: *Hordeum vulgare* (Kruszka et al., 2014); *mes*: *Manihot esculenta* (Ballen-Taborda et al., 2013); *osa*: *Oryza sativa* (Jeong et al., 2011; Sailaja et al., 2014); *ptc*: *Populus trichocarpa* (Lu et al., 2008); *pto*: *P. tomentosa* (Chen et al., 2012); *sja*: *Saccharina japonica* (Liu et al., 2014); *tae*: *Triticum aestivum* (Xin et al., 2010; Wang et al., 2012b; Kumar et al., 2014). ^bAT: acquired thermotolerance, pretreated at 37°C for 1 h and returned to 22°C for 1.5 h for recovery, treated at 44°C for 45 min. ^cvalidated by either northern blot or realtime PCR.

of the down-regulated *miR156* in rice (Sailaja et al., 2014) and cassava (Ballen-Taborda et al., 2013) remain unknown. The regulation of *miR160* family by heat is quite different in various species, although they all target auxin response factors (ARFs). After heat stress, *miR160* was up-regulated in *Arabidopsis* (Zhong et al., 2013), barley (Kruszka et al., 2014) and celery (*Apium graveolens*; Li et al., 2014a), but down-regulated in cassava (Ballen-Taborda et al., 2013) and Chinese white poplar (Chen et al., 2012). An increase in barley *miR160a* during heat stress down-regulated the expression level of *ARF17* and *ARF13*, which might affect shoot morphology and root growth (Kruszka et al., 2014). *miR160* as well as *miR169* in rice showed differential expression in roots and shoots under heat stress, suggesting the different regulation of the target genes by heat in this two different tissues (Sailaja

et al., 2014). It is also amazing that *miR160* in wheat was up-regulated by 40°C for 2 h in the heat tolerant genotype TAM107 (Xin et al., 2010), but down-regulated with the up-regulation of its putative target *HSP70* by 42°C for 2 h in another heat tolerant cultivar HD2985 (Kumar et al., 2014). *miR167* was proved to be up-regulated in Chinese white poplar (Chen et al., 2012), wheat (Kumar et al., 2014) and barley (Kruszka et al., 2014) but down-regulated in rice (Sailaja et al., 2014). Heat stress enhanced the *miR167h*-guided cleavage of the *ARF8* and *NEK5* transcript in barley (Kruszka et al., 2014). *miR168* has also been shown to be up-regulated by heat in *Brassica rapa* (Bilichak et al., 2015) and celery (Li et al., 2014a), but down-regulated in Chinese white poplar (Chen et al., 2012) and rice (Sailaja et al., 2014). A differential expression of *bra-miR168* following heat shock in the parental

tissues was observed to be negatively correlated with transcript levels of its putative target *braAGO1* in the corresponding tissues, suggesting the important role of *bra-miR168* in heat responses (Bilichak et al., 2015).

The miR398 family have been validated to be up-regulated in *Arabidopsis* (Guan et al., 2013) and wheat (Kumar et al., 2014), but down-regulated in rice (Sailaja et al., 2014) and *Brassica rapa* (Yu et al., 2012b). Heat stress rapidly induced *ath-miR398* and reduced transcripts of its target genes *COPPER/ZINC SUPEROXIDE DISMUTASE 1* (*CSD1*), *CSD2* and *COPPER CHAPERONE FOR SOD 1* (*CCS*) that control ROS accumulation (Guan et al., 2013). The altered redox status contributed to the consequent accumulation of HSFs and HSPs that are critical for thermotolerance. Transgenic plants overexpressing miR398-resistant versions of *CSD1*, *CSD2*, or *CCS* under the control of their native promoters were hypersensitive to heat stress, and the expression of many HSF and HSP genes under heat stress was reduced in these plants. In contrast, *csd1*, *csd2*, and *ccs* plants were more tolerant to heat stress than wild-type plants with the increased expression levels of HSF and HSP genes. Moreover, HSF1b and HSF1b were found to be responsible for heat induction of miR398. Thus, HSFs, miR398 and its target genes *CSD1*, *CSD2*, and *CCS* form an essential regulatory loop for thermotolerance in *Arabidopsis* (Guan et al., 2013). However, in *Brassica rapa*, heat stress reduced the expression of the conserved miRNAs *bra-miR398a* and *bra-miR398b*, which guides heat response of their target gene *BracCSD1* (Yu et al., 2012b). The expression of most members in miR169, 171, 395, and 827 families have not been experimentally validated, and their targets remain largely unknown (Table 2). In addition to the above miRNA families, a lot of conserved miRNA families response to heat only in 1–3 species (Table 2). Among them, *han-miR396* in sunflower (*Helianthus annuus*) was found to be repressed by high temperature, which results in the up-regulation of the putative target *HaWRKY6* (Giacomelli et al., 2012). But plants overexpressing miR396-resistant versions of *HaWRKY6* were hypersensitive to heat shock, indicating that *HaWRKY6* is involved in a fine modulation in response to heat (Giacomelli et al., 2012).

In addition to the conserved miRNAs, many non-conserved and novel heat-responsive miRNAs have been validated. For instance, *ptc-miR1445*, *1446a-e* and *1447* were down-regulated by heat in *P. trichocarpa* (Lu et al., 2008); *pto-smR7*, 8, and 9 were down-regulated by heat in *P. tomentosa* (Chen et al., 2012); *osa-miR1884* was down-regulated in roots but up-regulated in shoots by heat (Sailaja et al., 2014); *tae-candidate_3466* and *5064* were up-regulated in wheat by heat (Kumar et al., 2014). Interestingly, the splicing of introns hosting *miR160a* and *miR5175a* in barley was heat induced, but the roles of these spliced isoforms in response to heat stress are unclear (Kruszka et al., 2014). Such heat stress-induced alternative splicing also regulates the *miR400* expression in *Arabidopsis* (Yan et al., 2012). The intronic *MIR400* is co-transcribed with its host gene *At1g32583*. Upon heat stress, a specific alternative splicing occurred at the first intron of *At1g32583* containing the *miR400* hairpin, which led to a decrease of mature *miR400*, but did not affect the host gene expression. This alternative splicing event may be favorable for

thermotolerance, as overexpression of *MIR400* made the plants more sensitive to heat stress (Yan et al., 2012). These findings extend our view about the regulatory mechanism linking miRNAs and heat stress.

It is worth noting that some heat-responsive miRNAs also function in other biotic and abiotic stresses. The *miR156-SPL* pathway in rice also functions in other stresses such as cold, salt and drought stress, suggesting a vital role of *miR156* in modulating plant development and responses to abiotic stress (Cui et al., 2014). *tae-miR827* and *2005* were up-regulated in wheat by both powdery mildew infection and heat stress (Xin et al., 2010). *ptc-miR 171l-n*, *530a*, *1445*, *1446a-e*, and *1447* were down-regulated in response to heat as well as cold, salt and dehydration in *P. trichocarpa* (Lu et al., 2008). *mes-miR156a*, *159a*, *160a*, *397a*, and *408* were down-regulated by heat and drought stresses in cassava (Ballen-Taborda et al., 2013). Thus, miRNAs may integrate the regulatory networks of heat stress with that of other biotic and abiotic stresses.

Small Interfering RNAs

Plant siRNAs are processed by DCL2-4 from long dsRNAs, which are generated directly from virus replication and inverted repeats (IRs), or converted from ssRNAs by RDRs, or by annealing of two complementary and separately transcribed RNA strands (Bologna and Voinnet, 2014). siRNAs guide a silencing effector complex to homologous DNA loci to trigger TGS or target mRNAs for transcript cleavage. Several exogenously triggered PTGS pathways resulting in transcript cleavage have been reported. These PTGS pathways can be induced by sense transgenes (S-PTGS), antisense transgenes (A-PTGS), inverted-repeat transgenes (IR-PTGS) and virus replication (VIGS). The diverse PTGS pathways play important roles in plant immunity and silencing of transgenes (Brodersen and Voinnet, 2006). In *Arabidopsis*, several distinct classes of endogenous siRNAs have also been uncovered, including repeat-associated siRNAs (ra-siRNAs), trans-acting siRNAs (ta-siRNAs), natural antisense transcript-derived siRNAs (nat-siRNAs), endogenous IR-derived siRNAs, and double-strand-break-induced RNAs (diRNAs; Bologna and Voinnet, 2014). Ra-siRNAs are typically 24-nt small RNAs that are derived from genomic repetitive sequences, which usually direct DNA methylation through the RdDM pathway (Matzke and Mosher, 2014). The ta-siRNAs arise from eight recognized *Arabidopsis* TAS loci (*TAS1a-c*, *TAS2*, *TAS3a-c*, and *TAS4*) through a miRNA-dependent biogenesis pathway (Fei et al., 2013). Ta-siRNAs are distinguished for the ability to function in *trans* to suppress the expression of target genes, such as disease resistance genes and transcription factors. Nat-siRNAs, which originate from the overlapping region of a pair of natural antisense transcripts (NAT), have been found a role in stress responses. In the case of two published nat-siRNAs, one transcript of the NAT pair is constitutively expressed and the other is induced by salt or bacterial pathogen, which induce the production of nat-siRNAs (Borsani et al., 2005; Katiyar-Agarwal et al., 2006). Nat-siRNAs target the constitutive expressed transcript for cleavage, which confers tolerance to the inductive

stress. Endogenous IR-derived siRNAs are processed by DCLs from genomic loci rearranged to form extended IRs that produce perfect or near-perfect dsRNA molecules (Dunoyer et al., 2010). IR-derived siRNAs can drive non-cell-autonomous silencing at both transcriptional and posttranscriptional levels and may have adaptive value by integrating temporally and/or spatially restricted stresses or environmental signals at the whole-plant level and perhaps in progenies (Dunoyer et al., 2010). DiRNAs derive from both sense and antisense strands around double-strand-break sites and may function as guide molecules directing chromatin modifications or the recruitment of protein complexes to source sites to facilitate repair (Wei et al., 2012).

In addition to miRNAs, the diverse exogenous and endogenous siRNAs are affected by heat. An increase in growth temperature from 22 to 30°C effectively inhibited S-PTGS and A-PTGS but not IR-PTGS in *Arabidopsis* (Zhong et al., 2013). The warmth-induced PTGS release most likely occurred during a critical step that leads to the formation of stable dsRNAs involving RDR6 and SUPPRESSOR OF GENE SILENCING 3 (SGS3). The abundance of many endogenous tasiRNAs was also significantly reduced by the 30°C growth, consistent with increased transcript levels of *TAS* and tasiRNA-target genes, which may affect the morphological acclimation (Figure 1A). The temperature increase reduced the protein abundance of SGS3, as a consequence, attenuating the formation of stable dsRNAs. Overexpression of SGS3 could release such warmth-triggered inhibition of siRNA biogenesis (Zhong et al., 2013). Heat shock (37°C for 1 h) also decreased the accumulation of *TAS1*-derived siRNAs, whereas their target genes *HEAT-INDUCED TAS1 TARGET1* (*HTT1*) and *HTT2* were highly up-regulated by heat shock (Li et al., 2014b). Meanwhile, *HTT1* and *HTT2* were directly activated by HsfA1a through binding to their promoters. *HTT1* mediated thermotolerance by acting as a cofactor of Hsp70-14 complexes (Li et al., 2014b; Figure 1D). Some nat-siRNAs are responsive to heat stress in *Brassica rapa* and *Arabidopsis*. For example, nat-siRNAs derived from Bra018216/Bra018217 and its homologous NAT pair AT3G46230/AT3G46220 were induced by heat, leading to the induction of the former gene (*Bra018216* and *AT3G46230*) and the repression of the latter gene (*Bra018217* and *AT3G46220*; Yu et al., 2013; Figure 1D). A novel class of heat-responsive small RNAs derived from the chloroplast genome of *Brassica rapa* has been reported (Wang et al., 2011). Many members of chloroplast small RNAs (csRNAs) families are highly sensitive to heat stress, and some csRNAs respond to heat stress by silencing target genes (Wang et al., 2011). It will be interesting to investigate the role of these csRNAs in RPS1-mediated heat-responsive retrograde pathway (Figure 1D). Although ra-siRNAs-mediated RdDM pathway is required for basal thermotolerance (Popova et al., 2013), the underlying mechanism is still not clear (Figure 1D). The study of *ONSEN* reveals a novel regulation mechanism via siRNAs in heat responses (Ito et al., 2011). In *Arabidopsis* seedlings, *ONSEN* is transiently activated by heat stress and re-silenced during the recovery period. A surprisingly high frequency of retrotransposition, which produces new *ONSEN* insertions, is observed in the progeny of stressed

nripd1 plants but not of the wild-type plants, suggesting a crucial role of the ra-siRNA pathway in restricting transgenerational retrotransposition triggered by heat stress. Moreover, natural and experimentally induced variants in endogenous loci harboring new *ONSEN* insertions confer heat responsiveness to nearby genes. Therefore, heat-induced mobility bursts of *ONSEN* may generate novel, stress-responsive regulatory gene networks (Ito et al., 2011). A recent study in maize has also demonstrated that allelic variation for insertions of the TEs associated with heat stress-responsive expression can contribute to variation in the regulation of nearby genes, probably by providing binding sites for transcription factors or influencing chromatin (Makarevitch et al., 2015). The roles of endogenous IR-derived siRNAs and diRNAs in heat responses remain to be investigated.

High temperatures often enhance the VIGS-mediated disease resistance (Figure 1C). The temperature shift from 25 to 30°C induced the accumulation of siRNAs and increases the cassava geminivirus-induced RNA silencing in plants (Chellappan et al., 2005). Temperature-dependent survival of *Turnip crinkle virus*-infected *Arabidopsis* plants relies on an RNA silencing-based defense that requires DCL2, AGO2, and HEN1 (Zhang et al., 2012). The activity of DCL2 was up-regulated by high temperature, suggesting that DCL2 protein may be a temperature-sensitive component responsible for modulation of RNA silencing pathway (Zhang et al., 2012). In addition, RDR6 may be closely related to the temperature sensitivity of the silencing pathway in *Nicotiana benthamiana* (Qu et al., 2005). Plants with reduced expression of NbRDR6 were more susceptible to various viruses and this effect was more pronounced at higher growth temperatures (Qu et al., 2005). Moreover, NbRDR6 plays a root-specific role in the inhibition of *Chinese wheat mosaic virus* (CWMV) accumulation and biogenesis of CWMV siRNAs at higher temperatures (Andika et al., 2013). It will be interesting to investigate whether other components of RNA silencing also affect virus resistance under high temperatures.

Other Epigenetic Regulation of Heat Responses

In recent years, new epigenetic mechanisms have been revealed to regulate the heat responses, including lncRNAs, HEAT INTOLERANT 4 (HIT4)-mediated non-canonical TGS regulation and other two unorthodox pathways. LncRNAs, with size larger than 200 nt, are precursors for siRNA biogenesis and act as scaffolds for the establishment of DNA methylation and histone modifications (Wierzbicki, 2012). In *Arabidopsis*, the expression of *HSFB2a* was counteracted by a natural and heat-inducible long non-coding antisense RNA, *asHSFB2a* (Wunderlich et al., 2014). In leaves, the antisense RNA gene was only expressed after heat stress and dependent on the activity of HSF1a/HSF1b. *HSFB2a* and *asHSFB2a* RNAs were also present in the absence of heat stress in the female gametophyte. *HSFB2a* activity temporarily repressed vegetative growth during development and after heat stress, the antisense regulation by *asHSFB2a* counteracted this effect to restore growth and further development (Figure 1D; Wunderlich et al., 2014). Other 15 heat-responsive lncRNAs have been found in *Arabidopsis*, but their functions are still unknown

(Di et al., 2014). Seventy-seven putative heat-responsive lncRNAs, which are not conserved among plant species, have been identified in wheat (Xin et al., 2011). Among them, TahlnRNA27 and TahnRNA5 are the precursors of *tae-miR2010* and *tae-miR2004*, respectively. These two lncRNAs and miRNAs were up-regulated after heat stress in heat sensitive genotype Chinese Spring (CS) and heat tolerant genotype TAM107. Nine heat-responsive lncRNAs such as TahnRNA21 may be precursors of siRNAs. TahnRNA9 and TahnRNA12 are identified as signal recognition particle (SRP) 7S RNA variants and can be regulated by siRNAs. Besides, three lncRNAs (TahlnRNA12, TahlnRNA23, and TahlnRNA29) are characterized as U3 snoRNAs (Xin et al., 2011). Interestingly, lnc-508 was down-regulated by heat and cold, while lnc-168 was down-regulated by heat and salt in *Arabidopsis* (Di et al., 2014). Twenty-three lncRNAs respond to both powdery mildew infection and heat stress in wheat (Xin et al., 2011). These results suggest that like miRNAs, lncRNAs may also integrate the regulatory networks of heat stress with that of other biotic and abiotic stresses.

HIT4 has been reported to mediate heat-induced decondensation of chromocenters and release from TGS with no change in the level of DNA methylation (Wang et al., 2013, 2014). HIT4 acts independent of MOM1 at the level of heterochromatin organization and this activity is essential for basal thermotolerance in plants. Thus, HIT4 delineates a novel TGS regulation pathway, involving a currently unidentified component that links HIT4 relocation and the large-scale reorganization of chromatin (Wang et al., 2013, 2014). A special inductive temperature shift released the heterochromatin-associated silencing in *Arabidopsis* plants in a genome-wide manner (Tittel-Elmer et al., 2010). This occurred without alteration of repressive epigenetic modifications and did not involve common epigenetic mechanisms. Such induced release of silencing was rapidly restored, without the involvement of factors known to be required for silencing initiation. Therefore, stress-induced destabilization of heterochromatic TGS and its re-establishment may involve novel mechanisms that repress transcription (Tittel-Elmer et al., 2010). In a recent study, long-term heat stress activated the *Arabidopsis* imprinted gene *SUPPRESSOR OF DRM1 DRM2 CMT3 (SDC)*, which encodes a putative F-Box protein and is silent during vegetative growth due to DNA methylation (Sanchez and Paszkowski, 2014). The heat-mediated transcriptional induction of *SDC* occurred only above a particular window of absolute temperature and was proportional to the level of stress. After heat stress, *SDC* was slowly re-silenced, allowing a temporal extension of *SDC* activity to contribute to the recovery of plant biomass. The *SDC* activation seems to occur independently and in parallel to canonical heat-shock perception and signaling, but rely on a yet undefined epigenetic mechanism (Sanchez and Paszkowski, 2014).

Transgenerational Memory and Evolutionary Adaptation

Transgenerational memory, also known as epigenetic inheritance, refers to the transmittance of epigenetic states and

certain environmental responses from one generation to the next. These transgenerational effects may offer the offspring an adaptive advantage or genomic flexibility for better fitness. Recent evidence suggests that some abiotic and biotic stress responses are transgenerational in plants. For example, exposure of *Arabidopsis* plants to UV-C and flagellin can induce transgenerational increases in HRF (Molinier et al., 2006). Heat responses also exhibit transgenerational epigenetic inheritance (Boyko et al., 2010; Lang-Mladek et al., 2010; Ito et al., 2011; Zhong et al., 2013; Iwasaki and Paszkowski, 2014; Migicovsky et al., 2014). The immediate progeny of heat-stressed *Arabidopsis* plants have fewer, but larger leaves, and tend to bolt earlier (Migicovsky et al., 2014). These plants have increased expression of *HSA2*, but reduced expression of *ROS1* and several *Su(var)3-9* homologs (*SUVH*) genes involved in H3K9 methylation and DNA methylation. These phenotypic and epigenetic changes are partially deficient in the offspring of heat-stressed *dcl2* and *dcl3* mutants (Migicovsky et al., 2014). It is also reported that transgenerational adaptation of *Arabidopsis* to stress requires DNA methylation and the function of Dicer-like proteins (Boyko et al., 2010). However, the transgenerational retrotransposition of *ONSEN* is prevented by the siRNAs pathway (Matsunaga et al., 2012), while *SGS3* overexpression could decrease the warmth-induced transgenerational memory (Zhong et al., 2013). Thus, the role of siRNAs pathway in transgenerational memory of heat responses remains controversial. The involvement of AGO1 and the *miR156-SPL* pathway has been demonstrated to maintain the short memory of acquired thermotolerance in the adaptation to recurring heat stress at the physiological and molecular level in *Arabidopsis* (Stief et al., 2014). *Bra-miR168* and its target *braAGO1* are also suggested to be putative messengers that mediate meiotic epigenetic inheritance in *Brassica rapa* (Bilichak et al., 2015). Further experiments on transgenerational heat stress in the hypomorphic *ago1* mutants may shade a new light on the contribution of AGO1 and the miRNA pathway to epigenetic inheritance in plants.

The role of DNA methylation in transgenerational memory is also obscure. It is reported that there seems no consistent correlation between DNA methylation changes of transgene and the warmth-induced transgenerational release of PTGS (Zhong et al., 2013). Similarly, DNA methylation may be also not involved in the release of transgene TGS by heat stress (Lang-Mladek et al., 2010; Pecinka et al., 2010; Tittel-Elmer et al., 2010). However, it remains a possibility that changes in DNA methylation at certain sites of a silenced target gene or at certain loci of the genome are responsible for the transgenerational memory, as CG methylation plays a central role in transgenerational stability of the *Arabidopsis* epigenome (Mathieu et al., 2007). Loss of CG methylation triggers genome-wide aberrant *de novo* non-CG methylation by interfering with the RdDM process and expression of DNA demethylases, as well as progressive H3K9 remethylation of heterochromatin. It is proposed that immediate, non-heritable stress responses may be associated with alteration of non-CG methylation patterns mediated by siRNAs/RdDM and ROS1/DME, while long-term, heritable adaptation to a changing environment would require modulation of CG patterns (Mathieu et al., 2007). Recently, a forward

genetic screen revealed that DDM1 and MOM1 act redundantly in preventing the transmission of stress-induced transcriptional changes to progeny of the stressed plants (Iwasaki and Paszkowski, 2014). Such DDM1- and MOM1-mediated or other mechanisms of chromatin resetting could prevent the transgenerational transmission of environmentally induced epigenetic traits. The roles of other epigenetic mechanisms in transgenerational memory, such as H2A.Z and H3K27me3, are worth further investigation.

Although the mechanisms remain to be elucidated, the transgenerational memory of heat responses may contribute to evolutionary adaptation. The warmth-induced epigenetic memory was maintained for at least three generations with gradually declining (Zhong et al., 2013). Heat stress also induced transgenerational phenotypic changes over three generations (Suter and Widmer, 2013a,b). Ancestral exposure to elevated temperatures over P and F1 generations resulted in increased fitness in the F3 heat-treated *Arabidopsis* plants (Whittle et al., 2009). Thus, the transgenerational memory of heat responses may allow potentially long-term adaptation and rapid evolution, as chromatin modifications can be mitotically or meiotically heritable. Stress-induced epigenetic changes may lead to the formation of heritable epialleles and transcriptional activation of TEs (Mirouze and Paszkowski, 2011). The epialleles and transposon-driven variation in gene expression may contribute to the phenotypic diversity of different individuals in a population or a species that can be subject to natural selection (Mirouze and Paszkowski, 2011). The transgenerational retrotransposition of *ONSEN* may reshape gene regulatory networks and potentially create novel traits for adaptation to heat stress, as genes in the vicinity of *ONSEN* neo-insertions acquired heat-responsiveness (Ito et al., 2011). Given that the activity of transposable element is important for adaptive plant evolution (Lisch, 2013), and *ONSEN* is evolutionarily conserved and transcriptionally activated by environmental heat stress in some Brassicaceae species (Ito et al., 2013), it will be interesting to explore the possible role of *ONSEN* and other TEs in plant evolution. Whether heat mediates the formation of heritable epialleles still needs to be investigated. Random RdDM-mediated epiallele formation is suggested to play a greater role in evolution than genetic variation (Matzke and Mosher, 2014). Epigenetic variation in DNA methylation among epigenetic recombinant inbred lines (epiRILs) that are nearly isogenic but highly variable at the level of DNA methylation, can cause substantial heritable variation of drought tolerance and nutrient plasticity (Zhang et al., 2013). It will be worth investigating whether heat-induced changes of global methylation creates potential for the evolution of phenotypic plasticity.

Concluding Remarks

Heat greatly affects the growth, development, and productivity of plants. Several heat sensors have been reported, including the calcium channel in the plasma membrane, H2A.Z-containing nucleosomes in the nucleus, and two unfolded protein sensors in

ER and the cytosol. Importantly, different epigenetic regulations may also be involved in the responses to different heat treatments (Table 1). The epigenetic regulation of warm and high temperatures mainly involves warmth-induced PTGS release, enhanced VIGS-mediated resistance, and H2A.Z-mediated morphological acclimation and acceleration of flowering. Various epigenetic mechanisms (known or unknown) are involved in response to heat stress. It is notable that different lengths (from 1 h to 4 days) of heat treatment at 37°C have diverse effects on the epigenome, suggesting the complexity in the epigenetic regulation of heat stress.

Despite recent advances in our understanding of the genetic and epigenetic mechanisms involved in heat stress sensing in plants, many questions remain to be answered by future research (Box 1). Perhaps the most important questions in the genetic mechanisms of heat responses are: what are the primary heat sensors? In addition to the four heat sensors mentioned above, a list of other components like mRNAs, miRNAs and hormonal import and antiport channels may also be plausible thermometers based on physical capacities (McClung and Davis, 2010). Systematic analysis of the changes in the genome, transcriptome, microme, and proteome by omic approaches may help to identify novel transcriptional, translational, and posttranslational regulation components and underlying mechanisms in plant heat responses (Bokszczanin and Fragkostefanakis, 2013; Hasanuzzaman et al., 2013). Perhaps plants sense heat through different organelles in different phases of the response, and then these signaling pathways are integrated and work synergistically or differentially to defend plants from heat-induced deleterious effects.

As listed in Box 1, a set of questions concerning the epigenetic regulations of heat responses are proposed. One of the major challenge ahead may be to discover the mechanisms of transgenerational memory heat responses. Systemic screening for factors regulating transgenerational memory of heat may address the long-term controversial issue. Besides, the roles of

BOX 1 | Proposals of future researches.

- What are the primary heat sensors? Are the CNGCs, H2A.Z or the unfolded protein sensors the true heat sensor?
- How are the different heat-sensing pathways integrated in plant cells?
- What are the roles of the RdDM pathway in response to heat in crop plants?
- How do the different histone modifications regulate the heat responses in different plants?
- Do histone modifications regulate the response of circadian clock to high temperature and heat stress?
- By which precise mechanism is the H2A.Z occupancy regulated by high temperature? Is H2A.Z occupancy regulated by heat stress? Is H2A.Z occupancy involved in the modulation of plant immunity and circadian clock by high temperature?
- How are the heat-responsive miRNAs, siRNAs and lncRNAs regulated by heat? What are the regulatory networks of their targets in plants?
- How is the epigenetic regulation of heat responses integrated with the epigenetic regulation of other biotic and abiotic responses?
- How is the transgenerational memory of heat responses controlled?
- How to improve the thermotolerance of crops without sacrificing growth?

DNA methylation and histone modifications in response to heat need to be defined. miRNAs, lncRNAs and the chromatin-remodeling gene *AtCHR12* have been suggested to integrate the epigenetic regulation of heat stress with the regulation of other biotic and abiotic stresses. Other epigenetic regulations may also have similar functions. Further investigations should be focused on the epigenetic regulatory networks between heat stress and other biotic and abiotic stresses. We should note that most experiments on the role of genetic and epigenetic regulation in heat responses are limited to the model *Arabidopsis* plants in laboratory conditions with short-term heat treatment. As temperatures

in the field change seasonally and fluctuate daily, further studies should also be centered on the genetic and epigenetic regulations of heat responses in crop plants in the field, which may produce practical approaches to develop crop plants with improved thermotolerance.

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Maintaining Epigenetic Inheritance During DNA Replication in Plants

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Biotic and abiotic stresses alter the pattern of gene expression in plants. Depending on the frequency and duration of stress events, the effects on the transcriptional state of genes are “remembered” temporally or transmitted to daughter cells and, in some instances, even to offspring (transgenerational epigenetic inheritance). This “memory” effect, which can be found even in the absence of the original stress, has an epigenetic basis, through molecular mechanisms that take place at the chromatin and DNA level but do not imply changes in the DNA sequence. Many epigenetic mechanisms have been described and involve covalent modifications on the DNA and histones, such as DNA methylation, histone acetylation and methylation, and RNAi dependent silencing mechanisms. Some of these chromatin modifications need to be stable through cell division in order to be truly epigenetic. During DNA replication, histones are recycled during the formation of the new nucleosomes and this process is tightly regulated. Perturbations to the DNA replication process and/or the recycling of histones lead to epigenetic changes. In this mini-review, we discuss recent evidence aimed at linking DNA replication process to epigenetic inheritance in plants.

Keywords: epigenetic inheritance, DNA replication, nucleosome assembly, DNA polymerases, plant development, *Arabidopsis thaliana*

INTRODUCTION

A myriad of studies have shown that plants are exposed to stress episodes, such as cold, heat and drought, that lead to transcriptional reprogramming of gene expression and phenotypic changes. This reprogramming can be “memorized” for short as well as for long periods of time, enabling plants to aid responses when these events recur. The former depends on the half-life of stress-induced proteins, RNAs and metabolites, whereas the latter depends on epigenetic processes. Epigenetic inheritance of changes in gene function means that they: (i) are mitotically and/or meiotically heritable; (ii) cannot be explained by changes in the DNA sequence; and (iii) are maintained without the influence of the initial *stimulus*. Memory occurs by multiple mechanisms, but requires chromatin changes, such as DNA methylation, histone modifications and non-coding RNAs. However, whether these chromatin modifications are the cause of heritable transcriptional changes remains controversial. In this mini-review, we discuss recent evidence relating the coupling of epigenetic inheritance with DNA replication in plants. On the other hand, how the replication of DNA is influenced by the chromatin landscape has been recently reviewed (Sequeira-Mendes and Gutierrez, 2015).

NUCLEOSOME ASSEMBLY AT THE REPLICATION FORK LINKS DNA REPLICATION WITH EPIGENETIC INHERITANCE

The nucleosome is the basic chromatin unit, containing about 147 bp of DNA wrapped around an octameric protein core composed of two subunits of each histone: H2A, H2B, H3 and H4 (Luger et al., 1997). This octameric structure is disassembled in front of the advancing replication fork as two H2A–H2B dimers and one (H3–H4)₂ tetramer and reassembly occurs behind the replication fork on both leading and lagging strands with both parental and newly synthesized histones (Gruss et al., 1993). The replication machinery has been reviewed elsewhere (Sequeira-Mendes and Gutierrez, 2015). The newly synthesized H3–H4 dimers are transported into the nucleus by the ANTI-SILENCING FUNCTION1 (ASF1) chaperone and then transferred to the CAF-1 chaperone. CAF-1 is bound to PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) at the replication fork, where the formation of the novo (H3–H4)₂ tetramers takes place after successive transfer of H3–H4 dimers (**Figure 1**) (Wittmeyer and Formosa, 1997; Tyler et al., 1999; Wittmeyer et al., 1999; Moyer et al., 2006; Groth et al., 2007; Abe et al., 2011; Huang et al., 2013b; MacAlpine and Almouzni, 2013). The newly synthesized H4 is acetylated at K5 and K12 positions, which is a highly conserved histone modification, whereas the residues of H3 acetylation differ among species (Annunziato, 2013). Recent evidence sheds light on the segregation of parental nucleosomes at the replication fork. In yeast, the FACILITATES CHROMATIN TRANSCRIPTION complex (FACT) and the MINICHROMOSOME MAINTENANCE COMPLEX COMPONENT 2 (Mcm2) act together during their association with the replisome to bind and manage the parental histone complexes released from chromatin (Foltman et al., 2013). In this way, deposition is closely coupled to the replication machinery, as nucleosomes re-appear about 400 bp behind the replication fork on both leading and lagging strands (Radman-Livaja et al., 2011; Smith and Whitehouse, 2012). Hence, pre-existing parental nucleosomes contribute to approximately half of the histones on nascent DNA, suggesting an important contribution to the epigenome of daughter cells (Radman-Livaja et al., 2011; Alabert and Groth, 2012; Sarkies and Sale, 2012; Whitehouse and Smith, 2013; Campos et al., 2014). Chromatin modifications decorating the nucleosome core are thus believed to serve as carriers of epigenetic information.

Far from being complete, the understanding of this process in plants has been possible through diverse genetic screens in *Arabidopsis* (Liu and Gong, 2011). CAF-1 is a highly conserved complex in eukaryotes. It is recruited by PCNA to the replication fork and is involved in supplying *de novo* synthesized H3–H4 dimers to the DNA replication machinery (**Figure 1**). *Arabidopsis* CAF-1 is composed of three subunits encoded by *FASCIATA1* (*FAS1*), *FASCIATA2* (*FAS2*) and *MULTICOPY SUPPRESSOR OF IRA1* (*MSI1*) (Kaya et al., 2001; Kirik et al., 2006; Ono et al.,

2006; Ramirez-Parra and Gutierrez, 2007). Mutants in *FAS1*, *FAS2* and *MSI1* show severe developmental defects, such as smaller leaves, fasciated stems, abnormal phyllotaxis, reduced fertility, more open euchromatin and reduced heterochromatin content. The *Arabidopsis* *ASF1* homologues *AtASF1A* and *AtASF1B* have an important role during development. In contrast to single mutants, the double mutant *asf1a asf1b* shows severe defects in growth and vegetative and reproductive development, together with constitutive activation of DNA replication stress genes (Zhu et al., 2011). In addition, they have a role in the heat shock response, by participating in the activation of heat stress genes, supposedly through nucleosome removal and H3K56ac accumulation (Weng et al., 2014). The role of the primary chaperone of H2A–H2B dimers, NUCLEOSOME ASSEMBLY PROTEIN1 (NAP1), is conserved in Eukaryotes (Burgess and Zhang, 2013). In *Arabidopsis*, NAP1-RELATED PROTEIN 1 and 2 (NRP1 and NPR2) chaperone *de novo* synthesized H2A and H2B to be assembled into the nucleosomes during replication of DNA and they are also required for the formation of heterochromatin (Zhu et al., 2006).

Topoisomerases break one or both DNA strands to relax DNA conformation, and mutant analysis suggested they have a role in the maintenance of chromatin structure. Loss of function mutants of topoisomerase MGOON1 (TOP1-β) displayed a stochastic occurrence of ectopic *AGAMOUS* (*AG*):*GUS* expression, suggesting that maintenance of chromatin marks is unstable in the absence of topoisomerase function, allowing random switches between on/off states. Once established, such expression states appear to be copied to daughter cells during cell divisions, but occasionally switch back to a repressed state as evidenced by revertant *AG:GUS* sectors (Graf et al., 2010). Dimethylation of Lysine 9 of H3 (H3K9me2) and DNA methylation are reduced in plants treated with camptothecin, an inhibitor of the DNA topoisomerase 1α (TOP1α), and in *top1a* loss of function mutants, leading to the de-repression of Pol V target loci (Dinh et al., 2014). In agreement to that, in studies performed with the *top1a-2* allele, it was demonstrated that TOP1α is required for the deposition of H3 trimethylated on K27 (H3K27me3) at PcG target genes (Liu et al., 2014). However, a simple relationship between TOP1α and gene repression was not found since another subgroup of PcG genes was downregulated in *top1a-2* mutants. It was also shown that TOP1α is important to decrease nucleosome density and hence, TOP1α could act as a promoter of gene expression in a subset of genes (Liu et al., 2014).

DNA REPLICATION DYNAMICS AND EPIGENETICS

In *Arabidopsis*, as in other eukaryotes, three replicative DNA polymerases duplicate DNA: Polα (with primase activity), Pole (leading strand extension) and Polδ (lagging strand extension). Their catalytic subunits are encoded by *POLA1* (also known as *ICU2*), *POLE1* and *POLD1* and their

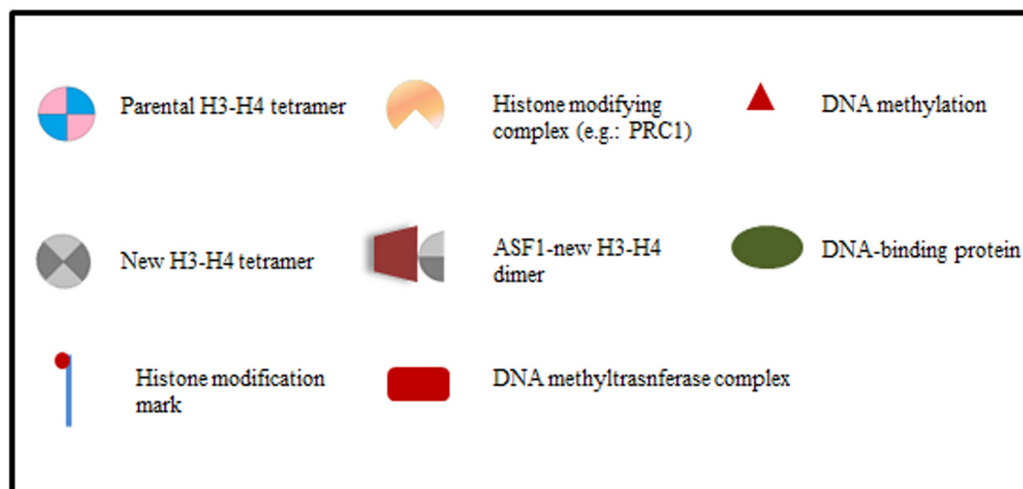
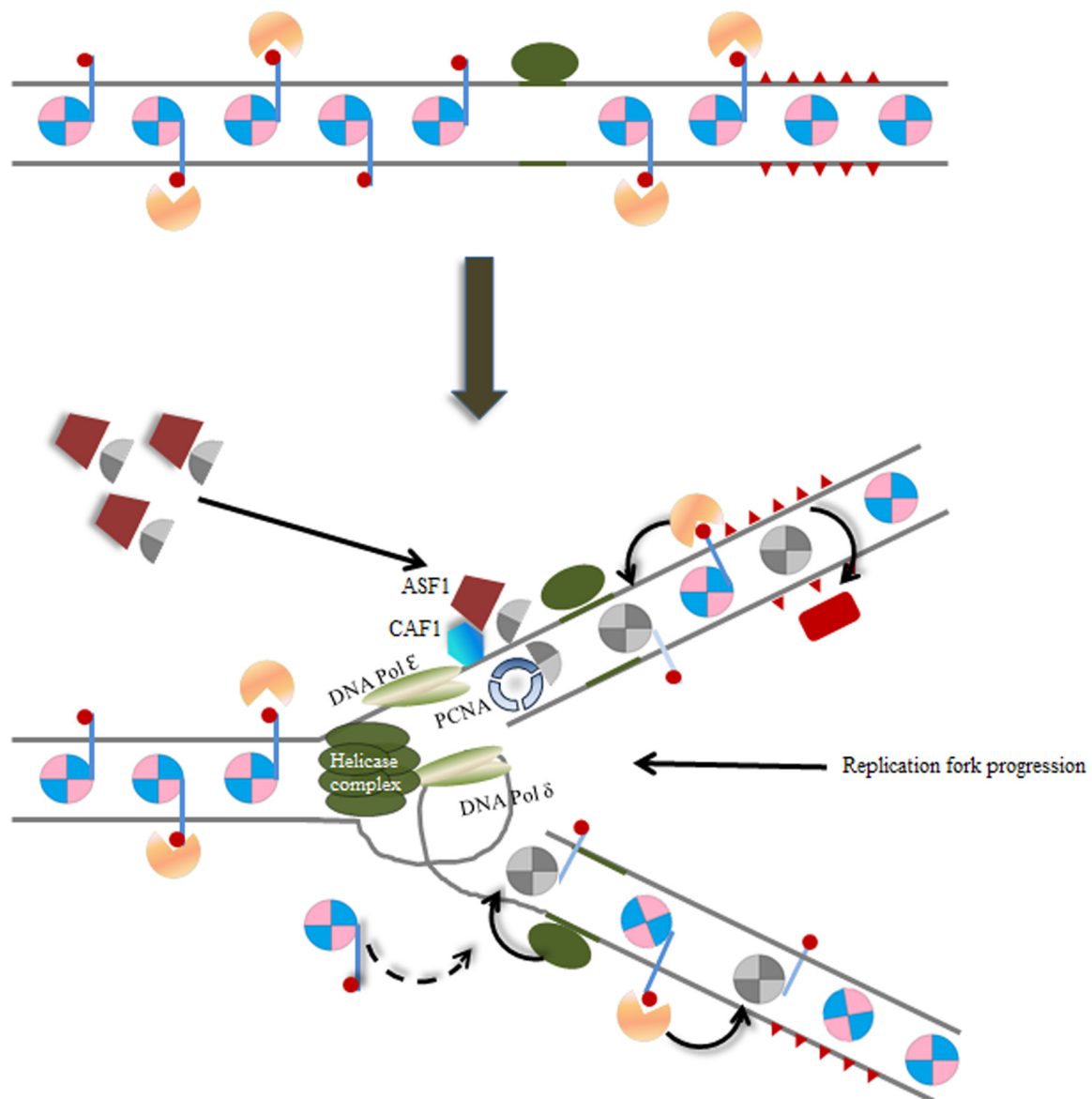


FIGURE 1 | Continued

FIGURE 1 | Continued

Schematic depiction of mechanisms by which epigenetic inheritance may be maintained during DNA replication. As replication fork proceeds, (i) newly deposited histones are modified by protein complexes (e.g.: PRC2) targeted by inherited modifications on parental histones; (ii) DNA binding proteins recruit/repel modification protein complexes; (iii) cross-talk between the two complementary mechanisms is also possible. For simplicity, H3–H4 nucleosomes are depicted.

roles in epigenetic inheritance were evidenced in different genetic screenings. The *polα/icu2-1* missense mutation leads to reactivation of a silenced *35S-NPTII* transgene and repetitive elements due to decreased H3K9me2, but not DNA methylation (Liu et al., 2010). *icu2-1* interacts genetically with *CURLY LEAF (CLF)*, which encodes a SET-domain component of Polycomb Repressive Complex 2 (PRC2) which methylates H3K27, and also interacts with *FAS1* and with *LIKE-HETEROCHROMATIN PROTEIN 1 (LHP1)*, which encodes a protein with PRC1 activity in *Arabidopsis* (Barrero et al., 2007). POLA/ICU2 is required for the stable inheritance of vernalization memory through mitoses. The flowering repressor *FLOWERING LOCUS C (FLC)* is stably repressed after vernalization. However, the *icu2-1* allele induces a mosaic de-repression of an *FLC:GUS* marker after vernalization (Hyun et al., 2013). A similar mosaic de-repression was also shown for an *AG:GUS* marker (Barrero et al., 2007). The variability in the degree of derepression was explained by the role of POLA/ICU2 in the mitotic inheritance of the H3K27me3 marks and further, the *icu2-1* allele led to reduced binding of LHP1 at target loci (Hyun et al., 2013). The similarities among mutants affected in chromatin assembly factors and topoisomerases with mutants in POLA/ICU2 underscores its role in the process of chromatin assembly and the maintenance of repressive histone marks through mitosis.

Mutants for the POLE1 and POLD1 catalytic subunits have also been described. *early in short days7-1 (esd7-1)* (del Olmo et al., 2010) and *ABA overly sensitive 4-1 (abo4-1)* (Yin et al., 2009) are hypomorphic alleles of *POLE*. *gigantea suppressor5 (gis5)* (Iglesias et al., 2015) and the *polδ1* (Schuermann et al., 2009) are hypomorphic or RNA interference alleles of *POLD*. They share similar developmental abnormalities, display early flowering and leaf incurvature. *POLE/ESD7* interacts genetically with *PRC2* and *PRC1* genes, shows more severe phenotypes when combined with *clf* or *tfl2* mutants (del Olmo et al., 2010). In addition, *POLE/ESD7* interacts with *TFL2* *in vitro*. These findings agree with the fact that discrete regions at the *FLOWERING LOCUS T (FT)* chromatin are enriched in H3K4me3 and H3 acetylation (H3Ac) in *esd7* and *abo4-1* mutants, and the *FLC* first intron is enriched in H3K27me3 in *abo4-1* mutants, correlating with higher *FT* and lower *FLC* expression levels (del Olmo et al., 2010).

In *gis5*, early flowering and curly leaves correlate with higher levels of H3K4me3 and H3Ac, and higher expression of *SEP3* in phloem cells. Furthermore, as the *gis5* allele is thermosensitive, the strong increase in *SEP3* expression and H3K4me3 at the *SEP3* locus were both temperature-dependent and correlated well with the DNA replication stress responses and the increase in homologous recombination (HR). Taking

into account that the *gis5* mutation affected an Ala residue poorly accessible to the solvent and close to the active site, the changes in *SEP3* epigenetic marks and expression could be produced by the changes in the dynamics of DNA replication, a hypothesis that needs further testing (Iglesias et al., 2015).

EPIGENETIC INHERITANCE RELIES ON HISTONE AND NON-HISTONE PROTEINS

The evidence supporting the link between changes in chromatin marks and epigenetic inheritance is mainly based on correlations between changes in gene expression patterns and changes in the abundance of chromatin modifications. Nevertheless, correlation does not mean cause and therefore, the changes in chromatin states may reflect the transcriptional level rather than be the cause of it (Henikoff and Shilatifard, 2011). If chromatin marks are truly epigenetic, looking at parental histones distribution during the replication of DNA should give an insight into the mechanisms of epigenetic inheritance (Figure 1).

As H3–H4 tetramers are twofold diluted and are inherited randomly at each daughter strand, chromatin marks on this nucleosomes are considered to contribute to the inheritance of chromatin states and somehow inherited during DNA replication. This contribution may be related to: (i) the extension of the domain (number of nucleosomes enriched with the chromatin marks), (ii) the turnover of histones modifications during the cell cycle, (iii) the recognition and binding of chromatin marks on the parental (H3–H4)₂ tetramers by the protein complexes involved in their own deposition, (iv) physical interaction with the DNA replication machinery, and (v) the kinetics for the reestablishment of chromatin marks on newly deposited histones (Sarkies and Sale, 2012). In this regard, H3K27me3, H3K9me3 and DNA methylation have been proposed to contribute themselves to the maintenance of epigenetic inheritance (Huang et al., 2013a).

Interactions between the DNA replication machinery and protein complexes involved in the deposition of chromatin modifications are necessary for the maintenance of the chromatin states (Margueron and Reinberg, 2010; Alabert and Groth, 2012). During DNA replication, nascent DNA is hemimethylated. In animals, parental DNA methylation is reestablished at CG motifs in daughter strands by recruiting the maintenance DNA-methylase DNMT1 to the replication fork by PCNA and UHRF1 (Bostick et al., 2007; Woo et al., 2008). Also, CAF1 and UHRF1 recruit H3K9 methyltransferase at the PCNA to monomethylate H3K9 at heterochromatin domains (Esteve et al., 2006; Scharf et al., 2009; Xu et al., 2012).

In plants, VIM proteins (the orthologs of mammalian UHRF proteins) recruit MET1, the ortholog of mammalian DNMT1, suggesting that plants share a similar mechanism with mammals for CG reestablishment (Woo et al., 2008). In *Arabidopsis*, the CHROMOMETHYLASE 3 (CMT3) is involved in the maintenance of CHG methylation and is recruited by H3K9me2 (Bartee et al., 2001; Lindroth et al., 2004). On the other hand, the histone methyltransferase KYP/SUVH4 binds, and is recruited onto, methylated cytosines (Johnson et al., 2007; Du et al., 2012; Johnson et al., 2014), showing the existence of a positive feedback loop between DNA methylation and histone marks in plants (Figure 1).

Recently, a direct physical and functional link between DNA replication, small RNA generation and H3K9 dimethylation was shown to occur in yeast. The Cdc20 subunit of Pole is conserved among eukaryotes. Cdc20 interacts directly with the silencing factor Dos2 forming a complex with Mms19, which is a transcriptional activator that promotes heterochromatin transcription by Pol II, a necessary step to establish heterochromatin silencing by H3K9 methylation during DNA replication (Li et al., 2011). If a similar process exists in plants is currently unknown, but a Pole mediated mechanism of recruiting silencing factors is possibly present in *Arabidopsis*. It has been found that LHP1 interacts with ICU2/POLA and ESD7/POLE subunits (Barrero et al., 2007; Yin et al., 2009; Hyun et al., 2013), as discussed above. Contrary to other systems, plants possess two other RNA Polymerases, RNA PolIV and RNA PolV, which are involved in DNA silencing. Recently, a link between HISTONE DEACETYLASE6 (HDA6) and MET1 has been established and helps to describe a truly epigenetic process (Blevins et al., 2014). In *hda6* mutants a subset of loci are derepressed at similar levels than in *polIV* single and *hda6 polIV* double mutants suggesting that both are in the same pathway for the silencing of these groups of genes (Blevins et al., 2014). However, *POLIV* transgenes complement the *polIV* mutants whereas *HDA6* transgenes do not complement *hda6* mutants only for this specific subset of genes, suggesting the loss of epigenetic information at these loci, which cannot be restored after complementation with *HDA6*. This epigenetic information is maintained by MET1 and *HDA6* by conferring a silent status to these loci which depend on certain level of GC methylation. Further downstream, the silent status is somehow recognized to ensure Pol IV recruitment and RNA dependent DNA methylation to further silencing (Blevins et al., 2014). It is speculated that *HDA6* is necessary for erasing acetyl groups on newly deposited histones, before recruiting Pol IV and MET1 to methylate DNA.

These examples show that a close interrelationship between chromatin modifications, chromatin modifiers and the DNA replication machinery is required for proper perpetuation of the chromatin states. Moreover, this association is dynamic. Recent comprehensive studies from mammals and *Drosophila* systems, using nascent chromatin capture (NCC) to profile chromatin proteome dynamics from newly synthesized DNA, show association dynamics for about 4,000 proteins when comparing nascent chromatin with mature post-replicative

chromatin (Alabert et al., 2014). The authors suggested that histone modifications, such as H3K9me3 and H3K27me3 serve as truly epigenetic marks, given their levels are relatively stable from duplicating to maturing chromatin. On the other hand, it has been shown that there is no evidence for these histone modifications to be considered themselves truly epigenetic marks given their low levels in the nascent chromatin (about 400 bp from the replication fork) (Petruck et al., 2013). These results agree with previous reports in *Drosophila* showing that H3K27me3 levels are reestablished at G1/M phase (Lanzuolo et al., 2011). Nevertheless, there is consensus from both studies to consider trithorax (TrxG) and PcG complexes as truly epigenetic carriers, as they are in close proximity to the replication fork (Follmer et al., 2012; Fonseca et al., 2012; Lo et al., 2012; Petruck et al., 2012). Similar approaches are not currently available for plant systems.

The inheritance of the vernalized state is a model of epigenetic memory in plants. In plants exposed to long periods of cold, *FLC* chromatin is enriched in H3K27me3 and PRC1 complex, which stably silence the *FLC* chromatin (Xiao and Wagner, 2015). This process requires the PRC2 complex to spread H3K27me3 across the whole *FLC* locus. This takes place when H3K27me3 is recognized by PRC2. During DNA replication, parental nucleosomes with this histone modification recruit PRC2 proteins which catalyze the trimethylation on neighboring, H3K27me3 depleted nucleosomes (Hansen et al., 2008; Margueron et al., 2009). Similar spreading from the initial nucleation site to adjacent sequences has been observed for reporter systems in plants (see above). Thus, at both *AG:GUS* and *FLC:GUS* transgenic lines strong enrichment of H3K27me3 was found not only at both *AG* (and *FLC*) but also at *GUS* sequences (Hyun et al., 2013). It was proposed that a buffer system helps to overcome the fluctuations in the levels of epigenetic marks on target loci, especially when these marks are diluted during DNA replication, and then restored during the maturation of chromatin (Huang et al., 2013a). On the other hand, it is unlikely that every single histone modification will be self-copied. There is evidence that supports the existence of primary histone modifications that influence the inheritance of “secondary” histone modifications, as suggested for monomethylated forms of H3K4, H3K9 and H3K27 with respect to the trimethylated forms (Sarkies and Sale, 2012).

PRC2 and TRXG protein complexes bind DNA sequences known as Polycomb (PRE) and Trithorax (TRE) response elements, respectively (Margueron and Reinberg, 2010). By binding DNA sequence targets they contribute to inhibit (PRC2) or activate (TRXG) gene transcription (Steffen and Ringrose, 2014). Recent mechanistic evidence in *Arabidopsis* suggests that cell division provides a window of opportunity to change fate. The flower development protein *AG* induces *KNUCKLES* (*KNU*) expression by displacing PcG proteins from PRE sequences on *KNU* locus, a process that takes about 2 days to be completed, leading to a failure in the maintenance of repressive histone modifications (Sun et al., 2014). It has been recently demonstrated in yeast that strongly bound transcription factors that remain associated to DNA cognate sites during DNA replication can influence nucleosome organization

during the maturation of Okazaki fragments by Pold δ (Smith and Whitehouse, 2012). Interestingly, the VERNALIZATION1 transcription factor, which is involved in epigenetic regulation of *FLC*, remains associated with euchromatin during mitosis (King et al., 2013).

In summary, plants are an interesting model system to study the impact that DNA replication dynamics has on the maintenance of epigenetic inheritance due to the high conservation of the DNA replication machinery among eukaryotes and the high tolerance of plants to missense mutations on DNA replication components. Additional studies tending to investigate the functional and biochemical relationship between DNA polymerases, DNA replication factors and protein complexes that modify chromatin will provide further insights into DNA replication-coupled epigenetic inheritance.

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Transgenerational inheritance or resetting of stress-induced epigenetic modifications: two sides of the same coin

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The transgenerational inheritance of stress-induced epigenetic modifications is still controversial. Despite several examples of defense “priming” and induced genetic rearrangements, the involvement and persistence of transgenerational epigenetic modifications is not known to be general. Here I argue that non-transmission of epigenetic marks through meiosis may be regarded as an epigenetic modification in itself, and that we should understand the implications for plant evolution in the context of both selection for and selection against transgenerational epigenetic memory. Recent data suggest that both epigenetic inheritance and resetting are mechanistically directed and targeted. Stress-induced epigenetic modifications may buffer against DNA sequence-based evolution to maintain plasticity, or may form part of plasticity’s adaptive potential. To date we have tended to concentrate on the question of whether and for how long epigenetic memory persists. I argue that we should now re-direct our question to investigate the differences between where it persists and where it does not, to understand the higher order evolutionary methods in play and their contribution.

Keywords: transgenerational, epigenetic, stress, re-setting, evolution, methylation, transposable elements

Introduction

Molinier et al. (2006) demonstrated that stress-induced epigenetic modification could be inherited through several generations in plants, causing considerable excitement. It had long been recognized that such capacity could allow for epigenetic priming of the progeny with important implications for improving crop plants (Mirouze and Paszkowski, 2011; Rodríguez López and Wilkinson, 2015), releasing cryptic variation (Grant-Downton and Dickinson, 2006), for population level adaptation (Richards, 2008) and adaptive evolution (Jablonka and Raz, 2009). Further examples of transgenerational epigenetic effects have been discovered including phenotypic inheritance (Verhoeven and Van Gurp, 2012) and the inheritance of gene expression (Scoville et al., 2011). Although we now have greater mechanistic understanding of transgenerational epigenetic inheritance (e.g., Crevillén et al., 2014; Kuhlmann et al., 2014) there are still few, multi-generational population or species-level studies (Richards, 2006; Bossdorf et al., 2008; Johannes et al., 2008). These few, however, have allowed us to begin to understand the evolutionary importance of stress-induced epigenetic modifications (Rapp and Wendel, 2005; Bräutigam et al., 2013; Kooke et al., 2015).

DNA cytosine methylation is an important epigenetic modification and is demonstrably heritable through mitosis. *Arabidopsis* epigenetic recombinant inbred line (epiRIL) populations that have no

DNA sequence variation but epigenetic variation in DNA methylation were created by crossing wild-type “Columbia” with mutants deficient in DNA methylation. The patterns created by recombination in these *Arabidopsis* epiRILs led to a range of stress-tolerance and phenotypes similar to the natural range in accessions within six to nine generations (Roux et al., 2011). Highly heritable epigenetic quantitative trait loci (epiQTL) for flowering time and primary root length were found, associated with the loss of DNA methylation at differentially methylated regions in the founder line (Cortijo et al., 2014) and amenable for artificial selection. These epiRILs also displayed increased plasticity in response to drought, nutrient and salt stresses (Zhang et al., 2013; Kooke et al., 2015) and associated epiQTL were highly heritable, illustrating the stability of epigenetic modification.

Stress-Induced Priming

Biotic and abiotic stresses trigger epigenetic modifications in the genome. In particular these modifications regulate the “open-ness” of chromatin to suppress or allow gene transcription, transposition of transposable elements (TEs), nucleosome occupancy and recombination. The effect is an altered epigenome that regulates stress response. In some cases the signature of the stress experience remains in the epigenome after relief from the stress, providing a “memory.” If this memory conditions the response to stress during subsequent development, the organism is said to be epigenetically primed. If the memory of the stress experienced by a parent conditions the response of its progeny, this epigenetic priming may be transgenerational. Following Molinier et al.’s (2006) demonstration of heritable epigenetic response to both biotic and abiotic stresses, transgenerational epigenetic priming of plants has been reported in response to pest and pathogen attacks (Luna et al., 2012; Rasmann et al., 2012; Slaughter et al., 2012) and to abiotic stresses including growth in high salt, UVC, heavy metal contamination, increased evaporative demand, heat and oxidative stresses (Boyko et al., 2010; Rahavi et al., 2011; Tricker et al., 2013; Matsunaga et al., 2015). Experimenters typically repeat experiments with clean seed stocks in controlled conditions and yet reproduce the same epigenetic responses, for example the priming of antibacterial defense (Dowen et al., 2012; Yu et al., 2013). These results suggest that epigenetic priming is targeted.

In *Arabidopsis*, priming for antibacterial defense involves active demethylation of TEs that leads to transcriptional activation of defense regulators via hormonal signals (Yu et al., 2013). DNA hyper- and hypo-methylation are observed epigenetic changes in stress response and priming (Boyko et al., 2010; Verhoeven et al., 2010; Tricker et al., 2012), and the recruitment of stress-induced methylation is regulated by small, non-coding RNAs (short-interfering RNAs/siRNAs; Boyko et al., 2010). This RNA-directed DNA methylation (RdDM) may provide DNA sequence specificity to epigenetic modifications via sequence-complementarity of the siRNAs that recruit DNA methylation. However, DNA methylation is reversible and demethylation is also an important response and priming strategy (Yu et al., 2013; Iwasaki and Paszkowski, 2014). Molecular mechanisms that prevent the transgenerational memory of stress have been

discovered (Iwasaki and Paszkowski, 2014) and these can be entrained by repeated cycles of stress (Sanchez and Paszkowski, 2014).

The challenge is to understand the dynamics of epigenetic modifications in response to stress and how these interplay with intra- or inter-generational memory to target priming.

Epigenetic Inheritance of Memory

The regulation of response to plant growth environment is clearly heritable when heritable is defined as passed from the parent to the progeny. One of the best-known examples is the regulation of vernalization requirement in winter annual *Arabidopsis thaliana*. The requirement for vernalizing temperatures to induce flowering is determined in the pathway involving the flowering repressor FLOWERING LOCUS C (FLC), its silencing and the epigenetic maintenance of silencing during warmer temperatures (reviewed in Baulcombe and Dean, 2014). The epigenetic regulation of vernalization in *Arabidopsis* is passed from parent to progeny, i.e., it is an inherited pathway. However, the accumulated epigenetic modifications themselves are reset in each sexual generation, and it is this re-setting that determines the vernalization requirement anew.

The heritable memory of epigenomic regulation is open to selection. In breeding for the epigenetic component of energy efficiency and stress tolerance in *Brassica rapa* (Hauben et al., 2009), the efficiency advantage of the original population and its epigenome component (phenotype, methylome, transcriptome, histone modification) was highly heritable in successive generations undergoing recurrent selection. High and low efficiency selections had distinct profiles of DNA methylation, histone methylation and acetylation different from the parent and from each other. Epigenomic profiles changed during development in opposite directions but were heritable in a cross. This did not indicate that the epigenomic profile had reached reproductive cells because it could not be fixed in the first rounds of selection. The influence of fluctuations in the environment was not explicitly investigated during this experiment, but lines bred for the epigenetic component of energy efficiency were also more drought tolerant.

Re-Setting the Epigenome

Transcriptional gene silencing is maintained by DNA methylation and histone modifications. These epigenetic modifiers are regulated during gametogenesis and are correlated with the dynamics of chromatin condensation that produce permissive and repressive states of transcriptional activation. At imprinted genes that display parent-of-origin, allele-specific expression, regulation by cytosine methylation and a Polycomb-Repressive Complex determines differential expression through cell divisions with time (reviewed in García-Aguilar and Gillmor, 2015). Methylation is re-programmed in the different nuclei during gametogenesis (Calarco et al., 2012; Jullien et al., 2012). It has been suggested that this re-programming allows the generation of mobile siRNA signals in companion cells that reinforce silencing of TEs in the embryo. In sperm and

male germline microspore cells, asymmetric (CHH sequence) methylation is reduced and 24 nucleotide siRNA from imprinted, maternally expressed genes accumulate in sperm cells. CHH methylation is restored after fertilization, during embryogenesis, and the pattern of DNA methylation and silencing is restored at many TEs and epialleles. However, this inheritance of silencing is progressive and incomplete in the male germline cells before fertilization (Calarco et al., 2012). Likewise, the transitions from spore mother cell to megaspore and gametophyte in the female reproductive lineage are also marked by different repressive and permissive histone composition and by chromatin remodeling, suggesting a pre-meiotic epigenetic influence on post-meiotic development (reviewed in Baroux and Autran, 2015). This re-setting during gametogenesis might allow for the removal of epigenetic modifications accumulated in response to stress or growth conditions during development of the parent. Additionally, it provides a window of opportunity to relax epigenetic suppression of transcription and transposition.

The Genomic Basis of Transgenerational Epigenetic Response to Stress

Natural variation in DNA methylation has been assayed genome-wide in *Arabidopsis* accessions, maize and soybean inbred lines (Vaughn et al., 2007; Eichten et al., 2013; Schmitz et al., 2013a). Along with other repressive chromatin states, DNA methylation is often associated with transposon-rich centromeric regions of the genomes, recently inserted TEs or duplicated regions, and often accompanied by high concentrations of siRNAs that generate RdDM at retrotransposons (Lister et al., 2008). It has been proposed that these epigenetic mechanisms exist primarily as defenses against potentially harmful genomic elements such as TEs (reviewed in Johnson, 2007).

A number of stresses can mobilize TEs (Grandbastien, 1998) and suppression of heat stress-induced retrotransposition of the *ONSEN* element requires the siRNA biogenesis pathway (Ito et al., 2011). Transposons may cycle between active and silenced states and the invasion of a new TE and eventual silencing can establish epiallelism at proximal genes (Marí-Ordóñez et al., 2013). Variation from new insertions may also create new, regulatory inserts responsive to the inducing stress (Ito et al., 2011) or even new, environmentally-responsive genes (reviewed in Oliver et al., 2013). Federoff (2012) has argued eloquently that, in contrast to the view that epigenetic mechanisms exist to suppress TEs, they have evolved and been preserved precisely to allow expansion, duplication and complexity derived from transposition within genomes, whilst repressing illegitimate recombination. Such a scenario requires that the suppression of TE activation by epigenetic means is relaxed or fluctuating. Marí-Ordóñez et al. (2013) found that the epigenetic suppression of the newly invasive retrotransposon *Evadé* (*EVD*) was sequential so that initial, incomplete post-transcriptional silencing shifted to transcriptional silencing over generations once a copy number of 40 was reached. *EVD* bore the seeds of its own destruction; its molecular suppression of post-transcriptional silencing generated RdDM that led to its transcriptional silencing. These findings (and

others reviewed in Ito and Kakutani, 2014) are consistent with the idea that TE-activation and epigenetic suppressors act in concert to allow fluctuation and complexity. It can be proposed that the re-setting of epigenetic states at gametogenesis exists to allow this relaxation.

The Adaptive Potential of Transgenerational Epigenetic Responses

Although the majority of stress-induced chromatin modifications do not persist past gametogenesis (reviewed in Pecinka and Mittelsten Scheid, 2012), others are faithfully re-acquired, albeit limited to one or a few progeny generations not exposed to the same stress (Boyko et al., 2010; Lang-Mladek et al., 2010). Pecinka and Mittelsten Scheid (2012) argued cogently that there is no conclusive evidence yet for the transgenerational epigenetic inheritance of stress-induced memory in plants, and that such evidence would need to document long-lasting changes of more than two generations that significantly influenced the plant's stress-responsiveness or adaptation. I argue that there is evidence for long-lasting epigenetically-induced change in stress-responsiveness encoded in the genome, but that it is hard to spot.

If the re-setting of stress-induced epigenetic modification at gametogenesis exists to allow encoding of transgenerational memory at new, responsive elements how might we see its signature in the genome? In some known cases, the epigenetic regulation of stress response is fixed in the genome at TE-derived sequences and heritable: Examples include the siRNA-based silencing of the *UBP1b* gene in *Arabidopsis*, the *AltSB* aluminum tolerance locus of sorghum, and the regulation of desiccation tolerance via inducible siRNAs at the *CDT-1* element of *Craterostigma plantagineum* (Magalhaes et al., 2007; Hilbricht et al., 2008; McCue et al., 2012). In addition, the feedback system that generated the *Mu* killer locus in maize may be highly prevalent. Via siRNAs, *Mu* killer heritably silences the *MuDR* transposon (Slotkin et al., 2003). *Mu* killer derives from an inverted duplication of a partially deleted *MuDR* element (Slotkin et al., 2005) and this derivation of the means of epigenetic silencing from the target is common in many genomes (reviewed in Lisch, 2013) and is subject to purifying selection, at least in rice (Hanada et al., 2009). Coupled with evidence that TE insertions increase the number of stress-responsive genes (Naito et al., 2009) these reports suggest that the relaxation of epigenetic suppression of TEs forms part of an evolvable genomic memory, but that this is largely invisible over evolutionary timescales (Lisch, 2013).

Reversible epigenetic regulation may have advantages in fluctuating environments. Burggren (2015) suggested that the immediate “sunsetting” of a stress-induced epigenetic modification once the stress was removed, could allow for bet-hedging against the possible return of the stress. The progeny of one generation exposed to a stress would maintain the epigenetic capacity to respond but not the stressed phenotype. Being poised for fluctuation—that is having an extra layer of regulation ready for release—would benefit survival if the stress was encountered again. Alternatively, epigenetic phenotypic modifications might “wash-out” over generations so that the phenotypic effects would

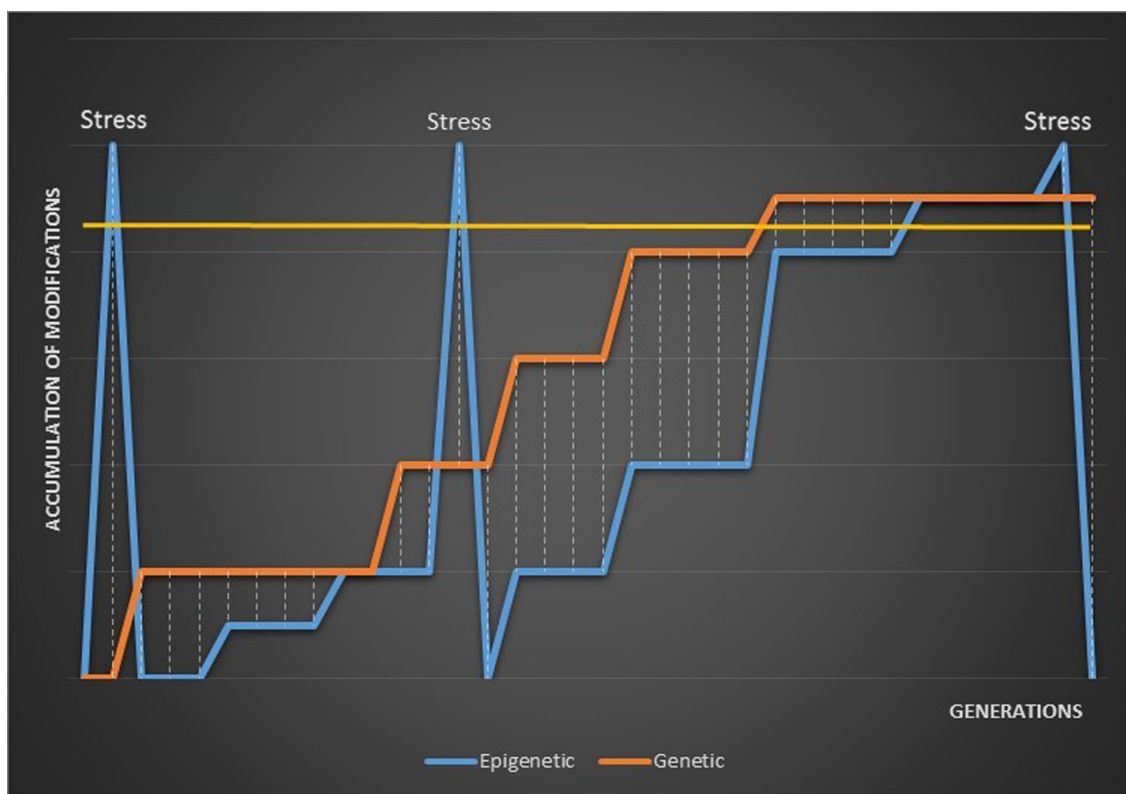


FIGURE 1 | A model of the distance between epigenetic and genetic variation when epigenetic modifications accumulate following stress and are re-set between generations. Phenotypic variation is only visible above the gold, horizontal line. The distance between epigenetic modifications and accumulating genetic mutations is illustrated by the dashed lines.

become undetectable or disappear at population scale (Burggren, 2015).

Epigenetic and genetic variation co-evolve (Schmitz et al., 2013b). This needs to happen so that epigenetic plasticity does not completely buffer evolvability and reduce the correlation between fitness and genotype, slowing selection. Klironomos et al. (2013) modeled the effect of selection on epigenetic as well as genetic variation. They showed that early selection of epigenetic variation could allow for the build-up of neutral genetic variation and faster adaptation in comparison with selection via genetic variation alone. This would allow for population survival by epigenetic adaptation following stress, and act as a stepping-stone to increased genetic fitness. Once genetic fitness had increased and been fixed, epigenetic variations would accumulate neutrally.

These models might account for the seeming variability in observations of epigenetic phenotypic plasticity and transgenerational epigenetic responses to stress. A rapid accumulation of epigenetic variation in response to stress would be visible in the phenotype of the first one or several generations if washed-out or rapidly sunset. Alternatively, a stress-induced burst of epigenetic modification might be visible in the first generation but largely disappear after re-setting with only a proportion fixed following the re-set generation. A rapid re-setting of stress-induced epigenetic variation followed by neutral accumulation to a new fitness maximum would only become

visible once epigenetic variation exceeded genetic variation, or following another stress-induced burst. This model is illustrated in **Figure 1**.

Theoretically a brief, transgenerational epigenetic memory ensures plasticity, but the dual inheritance of genetic and epigenetic variation ensures adaptation (Pal, 1998). If re-setting allows a window of opportunity for increased epigenetic variation in response to stress, then it could form a vital part of a species' evolvability. Importantly, mechanistic investigation of re-setting over only a few generations should elucidate the contribution of re-setting to adaptation where, over longer timescales, the evidence of new stress-responsiveness from the activation or exapction of TEs will be invisible. Likewise the recent discovery of molecular mechanisms that restrict transgenerational epigenetic inheritance (Iwasaki and Paszkowski, 2014) will contribute to our understanding of the targeting of epigenetic re-setting or inheritance.

Conclusion

When we consider the evidence for epigenetic transgenerational inheritance in response to stress we should consider whether it will be visible in an individual epigenome and whether the ebb and flow of visible epigenetic modifications limits or contributes to plasticity. The framework for population and species-level studies

of genetics can now be applied in epigenetics to inform our understanding (Richards, 2006; Johnson and Tricker, 2010). It is to be hoped that the combination of mechanistic and theoretical understanding advancing side by side, and the recognition that the persistence or reversibility of transgenerational epigenetic modifications are really two sides of the same coin, will allow us to exploit the undoubted potential of epigenetic regulation of plant stress response for the future.

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Laser Irradiation-Induced DNA Methylation Changes Are Heritable and Accompanied with Transpositional Activation of *mPing* in Rice

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DNA methylation is an integral component of the epigenetic code in most higher eukaryotes. Exploring the extent to which DNA methylation can be altered under a specific condition and its heritability is important for elucidating the biological functions of this epigenetic modification. Here, we conducted MSAP analysis of rice plants with altered phenotypes subsequent to a low-dose Nd³⁺YAG laser irradiation. We found that all four methylation patterns at the 5'-CCGG sites that are analyzable by MSAP showed substantial changes in the immediately treated M0 plants. Interestingly, the frequencies of hypo- and hypermethylation were of similar extents, which largely offset each other and render the total methylation levels unchanged. Further analysis revealed that the altered methylation patterns were meiotically heritable to at least the M2 generation but accompanied with further changes in each generation. The methylation changes and their heritability of the metastable epigenetic state were verified by bisulfite sequencing of portion of the retrotransposon, *Tos17*, an established locus for assessing DNA methylation liability in rice. Real-time PCR assay indicated that the expression of various methylation-related chromatin genes was perturbed, and a Pearson correlation analysis showed that many of these genes, especially two AGOs (*AGO4-1* and *AGO4-2*), were significantly correlated with the methylation pattern alterations. In addition, excisions of a MITE transposon, *mPing*, occurred rampantly in the laser irradiated plants and their progenies. Together, our results indicate that heritable DNA methylation changes can be readily induced by low-dose laser irradiation, and which can be accompanied by transpositional activation of transposable elements.

Keywords: Nd³⁺YAG laser irradiation, DNA methylation, transposable elements, epigenetic inheritance, rice

INTRODUCTION

DNA methylation is an important epigenetic marker that occurs frequently at cytosine bases, and plays important roles in orchestrating gene expression and maintaining genome stability. In plants, cytosine methylation occurs in three different sequence contexts: CG, CHG, and CHH (H is any nucleotide except G), but CG methylation stands as the most predominant pattern (Lister et al., 2008). A large body of studies have indicated that although DNA cytosine methylation was relatively stable and transgenerationally inheritable, it can be perturbed to change under certain conditions (Kou et al., 2011; Ou et al., 2012).

DNA methylation level and pattern for a given organism are the results of dynamic interplay between methylation and demethylation. An established function of cytosine methylation at promoter regions is to repress gene expression transcriptionally, and methylation within the gene body is involved in alternative splicing (Wang et al., 2016). In plants, *de novo* methylation is controlled by an RNA-dependent methylation (RdDM) pathway (Matzke and Mosher, 2014). Once established, DNA methylation is maintained through various mechanisms depending on sequence context. DNA methyltransferase 1 (MET1, homolog of animal Dnmt1) and chromomethylases (CMT3, a plant-specific DNA methyltransferase) target and maintain CG and CHG methylations, respectively (Law et al., 2010). Null mutation of the MET1-coding gene cause genome-wide loss of ^mCGs and pleiotropic developmental defects both in *Arabidopsis* and rice (Stroud et al., 2013; Hu et al., 2014). RdDM mainly targets euchromatic regions and is excluded from pericentrometric heterochromatin regions surrounding centromeres (Zemach et al., 2013). DNA methylation in heterochromatic sequences preferentially require a plant-specific SWI2/SNF2-like chromatin-remodeling protein called DDM1 (Decrease in DNA Methylation 1). The mutation of DDM1 leads to strong transcriptional activation of transposable elements (TEs) (Lippman et al., 2004).

Active DNA demethylation plays important roles in the gene regulation, and the 5-methylcytosine (5-mC) can be demethylated passively during cell replication, or actively due to action of bi-functional DNA glycosylases that not only recognize and remove 5-mC from dsDNA, but also show lyase activity (Law et al., 2010). In rice, active DNA demethylation is initiated by the REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DEM), and DEMETER-LIKE (DML) family (Ooi and Bestor, 2008).

In plants, the total 5-^mC contents vary extensively among species, ranging from 6% in *Arabidopsis* to 30% in tobacco, which is probably related to differences in genome size (C-value) and abundance of TEs and other types of repetitive sequences (Alonso et al., 2015). TEs account for 35% of the rice genome, and many of which are retrotransposons, including roughly 14% LTR and 1% non-LTR retrotransposons, respectively. Some TEs in the rice genome, including both DNA transposons and retrotransposons, are still capable of reactivation at the transcriptional or transpositional levels. TE activation at both levels may have phenotypic consequences by

influencing expression of their neighbor genes or insertional mutagenesis (Lisch, 2013). Ample evidence indicates that DNA methylation is a major mechanism repressing TE activity under normal conditions, while loss of methylation due to genetic or environmental perturbations may lead to their de-repression (Wessler, 1996; Kashkush and Khasdan, 2007; Lisch, 2013).

The miniature inverted-repeat transposable elements (MITEs) are a kind of TEs, which predominate numerically in the rice genome (Feschotte et al., 2002; Casacuberta and Santiago, 2003; Jiang et al., 2004). The miniature *Ping* (*mPing*) (Feschotte et al., 2002; Kikuchi et al., 2003; Nakazaki et al., 2003; Jiang et al., 2004) is a quiescent MITE transposon in most rice cultivars under natural growing conditions, but was found to be activated by strong perturbing conditions like tissue culture and hydrostatic pressurization (Lin et al., 2006; Ngezahayo et al., 2009). Being devoid of the transposase-encoding sequences, the mobility of *mPing* is commonly dependent on other autonomous transposons, such as *Ping* and *Pong*, to provide the transposase required for its transposition (Feschotte et al., 2002; Kikuchi et al., 2003; Nakazaki et al., 2003; Jiang et al., 2004).

Tos17, a Ty1-Copia class I autonomous LTR retrotransposon, is one of the few active retrotransposons identified in the rice genome (Hirochika et al., 1996). *Tos17* has two copies in the rice reference genome (cv. Nipponbare) (Sabot, 2014), and both copies are silenced under normal conditions. However, the *Tos17* copy located on chromosome 7 can be activated to transpose by tissue culture and other conditions (Ding et al., 2007; Ou et al., 2015). Rampant mobilization of *Tos17* was detected in rice lines derived from introgressive hybridization with *Zizania latifolia* (Wang H. Y. et al., 2010). The body region of *Tos17* is generally accepted as an established locus in the rice genome for assaying cytosine methylation liability (La et al., 2011).

Heavy-ion beam irradiation and Ethyl methanesulfonate (EMS) induced mutagenesis were frequently used for breeding proposes and molecular biology (Serrat et al., 2014; Hirano et al., 2015). Low-dose laser irradiation has been successfully used in boosting crop performance, including promoting germination, growth and yield, positively influence physiological and biochemical parameters, and improving the disease resistance (Xu et al., 2008; Liu et al., 2016). Being an unusual condition, we suspect that low-dose laser irradiation might also influence epigenetic stability of the treated plants. Indeed, Wang H. et al. (2010) found that laser irradiation induced cytosine methylation changes in sorghum, and the alteration frequency in the inter-line F1 hybrids was higher than that of their pure-line parents, suggesting an interaction of hybridity and laser irradiation. Nevertheless, no information is yet available regarding if cytosine DNA methylation patterns and transposon activity could be affected subsequent to laser irradiation, and for this purpose, rice is the testing plant of choice. The present study was designed to address this issue.

Here, we report that low-dose laser irradiation induced extensive changes in DNA methylation patterns in rice plants that showed phenotypic alterations. The methylation changes correlate with altered expression of some of the chromatin-related genes according correlation analysis. Importantly,

mPing was mobilized in the methylation-altered plants and their progenies. Transgenerational analysis indicated that the altered methylation patterns could be inherited to organismal progenies. Together, our results indicate that low-dose laser irradiation could readily induce heritable epigenetic changes and transpositional activity of TEs in plants.

MATERIALS AND METHODS

Plant Materials and Laser Irradiation

A pure-line rice cultivar, Jinongda18 (Ma et al., 2003) was used in this study. Uniform germinating seeds were pretreated with 355, 532, and 1,064 nm Nd³⁺ YAG pulsed laser for 6, 10, 15, 20 times, respectively (Table 1). Eleven treatment groups with different cumulative doses of laser irradiation were set, and the untreated wild-type (WT) plants were used as the mock control. More than 1000 plants were used for the mock and 11 stress treatments, and the treated seeds were allowed to continue germinating in petri-dishes for 5d in a growth cabinet (30°C during the day and 25°C during the night, 16/8 h photoperiod at 50 $\mu\text{molm}^{-2}\text{s}^{-1}$). Seedlings were then transferred to a homogeneous experimental paddy-field plot at Jilin Agricultural University, Changchun, China, in accord with season. Plant showing prominent phenotypic changes especially in plant height and heading date were tagged. The heading date was recorded as the first plant showing panicle emergence. Five plants were randomly chosen to measure their height. A single treated M0 individual with the shortest height and the latest heading date, designated as M0#11, was selected to generate the M1 progeny by self-pollination, then, a single M1 individual, designated as M1#1, was chosen to produce the M2 generation by self-pollination.

TABLE 1 | Phenotypes for plant height and heading date of WT (untreated) and various groups of laser-treated rice.

Groups	Device	Power (kv)	Wavelength (nm)	Exposure frequency (min)	Plant height (cm)	Heading date (days)
WT [#]	—	—	—	—	101 \pm 1.58	84
1	Nd ³⁺ YAG pulsed laser	4	355	10	102 \pm 2.12	83
2				15	100 \pm 3.08	84
3				20	98 \pm 3.39	84
4				6	101 \pm 2.45	83
5			532	10	102 \pm 2.00	84
6				15	99 \pm 2.24	87
7			1,064	20	95 \pm 2.12**	88
8				6	100 \pm 2.24	87
9				10	99 \pm 2.24	87
10				15	95 \pm 1.58**	88
11 ^{##}				20	93 \pm 3.00**	89

Data were presented as *t* mean \pm standard deviation, **P* < 0.05, ***P* < 0.01.

[#] WT of rice cultivar Jinongda18.

^{##} M0-11 was chosen for transgenerational study.

Methylation-Sensitive Amplified Polymorphism (MSAP) Analysis

The flag-leaves of rice plants in WT group and the 11 low-dose laser-treated groups were sampled as pools, while the M0#11, M1, and M2 progeny were sampled as individuals for DNA isolation at the grain filling stage. DNA was isolated using the modified CTAB method and purified by phenol extractions. MSAP is a modified version of the amplified fragment length polymorphism (AFLP) to detect the stability and alteration in cytosine DNA methylation at the 5'-CCGG sites (Yaish et al., 2014). Genomic DNA was digested with *EcoRI* combined with *HpaII* or *MspI* (New England Biolabs, Beverly, Massachusetts), ligated with *EcoRI* and H/M adapters, and then amplified with one pair of pre-selective and 20 pairs of selective primers (Supplementary Table 1). The amplification products of MSAP were resolved by 5% denaturing polyacrylamide gel electrophoresis and visualized by silver staining. Only clear and completely reproducible bands in two independent experiments were scored.

Rational for MSAP Data Tabulation

MSAP is performed using *HpaII* and *MspI*, a pair of isoschizomers that recognize the same restriction site (5'-CCGG) but have different sensitivities to methylation of the cytosines. *HpaII* will not cut if either of the cytosines is fully (double-strand) methylated, whereas *MspI* will not cut if the external cytosine is fully- or hemi- (single-strand) methylated (McClelland et al., 1994). Thus, for a given DNA sample, H0M1 indicates CG methylation; H1M0 indicates CHG methylation; H1M1 indicates no methylation; and H0M0 indicates CG/CHG methylation. Based on the principles, the changes of MSAP patterns were divided into four categories: (a) CG hyper: H1M1 to H0M1, H1M0 to H0M0; (b) CHG hyper: H1M1 to H1M0, H0M1 to H0M0; (c) CG hypo: H0M1 to H1M1, H0M0 to H1M0; and (d) CHG hypo: H1M0 to H1M1, H0M0 to H0M1.

Isolation and Sequencing of Variant MSAP Bands

Bands showing alteration in methylation patterns in the MSAP profiles in the laser-irradiated M0 plants and/or their progenies relative to WT were isolated, boiled with ddH₂O, and amplified with the pre-selective primers used in the original MASP analysis. The PCR products were purified with Wizard PCR Preps Purification System (Promega). Then the purified PCR products were ligated into pM18-T vector (Promega), and then sequenced. Homology analysis was performed by BlastX at the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Sequence alignment was done by the CLUSTALW program using the Genedoc software.

Real-Time Reverse Transcriptase (RT)-PCR Analysis

Total RNA was isolated from the flag-leaves at the same developmental stage as that used for DNA methylation analysis by the Trizol Reagent (Invitrogen). Then RNA was treated with DNaseI (Invitrogen), reverse-transcribed by the SuperScript TMRNase H-Reverse Transcriptase

(Invitrogen), and subjected to qRT-PCR analysis using gene-specific primers (Roche LightCycler 480). Genes encoding DNA methyltransferase1 or *MET1* (LOC_Os07g08500, DMT707), *CMT3* (LOC_Os05g13790, DMT703), *DRM2-1* (LOC_Os03g02010, DMT706), and *DRM2-2* (LOC_Os05g04330, DMT710), *DME1* (LOC_Os01g011900, DNG702), *DME2* (LOC_Os05g37350, DNG701), *DDM1* (LOC_Os03g51230, CHR741), Argonaute *AGO1-1* (LOC_Os02g45070, AG0711), *AGO1-2* (LOC_Os04g47870, AG0708), *AGO4-1* (LOC_Os04g06770, AG0705), *AGO4-2* (LOC_Os01g16870, AG0703) were analyzed in WT, M0, M1, and M2 progeny to interrogate the impacts of laser irradiation on their steady-state transcript abundance. These gene-specific primers were designed by the Primer Premier 5 software (Supplementary Table 2). A β -actin gene of rice (LOC_Os05g0438800) was used as an internal control. DNA contamination was tested by inclusion of RNAs without RT. Three batches of independently isolated RNAs were used as biological replications. The relative amounts of the gene transcripts were determined using the Ct (threshold cycle) method and the fold-change data were analyzed by the $2^{-\Delta\Delta C_t}$ method.

Locus-Specific PCR Assay on *mPing* Excision

Based on the principle that presence vs. absence of *mPing* produces 433 bp difference in length in given locus, excision of *mPing* from a known locus can be easily detected by locus-specific PCR amplification. Thus, a set of 53 pairs of locus-specific primers was designed by the Primer Premier 5 software (Supplementary Table 3), and each pair of primers bracketed an intact *mPing* based on the rice reference genome sequence of the standard laboratory genotype Nipponbare (<http://rgp.dna.affrc.go.jp>). The amplicons were visualized by ethidium bromide staining after electrophoresis through 2% agarose gels. A set of empty donor sites for *mPing* excision were identified, isolated and sequenced, together with their corresponding *mPing*-containing loci.

Transposon Display

Transposon display (TD) was performed by combining the *MseI*-adaptor-specific primers either with a *mPing* subterminal-specific primer or with a set of inter-simple sequence repeat (ISSR) exactly as previously reported (Jiang et al., 2003). Major procedures, i.e., digestion, ligation, pre-selective amplification, selective amplification were the same as for MSAP. And the restriction enzyme *MseI* (New England Biolabs) was used, the pre-selective primers were *MseI* +0 (5'-GATGAGTCCTGAGTA A) and *mPing* internal amplification primer (5'-GCTGACGAG TTTCACCAGGATG), the selective primers were 8 *MseI* +2 (Supplementary Table 4) and *mPing* proximal ends amplification primers (5'-TGTGCATGACACACCAGTG). The novel bands in TD gels of laser irradiated plants (M0, M1, and M2) relative to WT were considered as putative *mPing de novo* insertions.

Southern Blot Analysis

Genomic DNA (3 μ g per lane) of the various plants was digested by *HindIII* or *XbaI* (New England Biolabs). Digested DNA was

run through 1% agarose gel and transferred onto Hybond-N⁺ nylon membranes (Amersham Pharmacia Biotech, Piscataway, New Jersey) by the alkaline transfer recommended by the supplier. And the *mPing* (positions: 6–430), *Pong* (BK000586.1), and *Ping* (AB087616.1) probes were prepared as previously described (Lin et al., 2006). The probe-fragments were gel-purified and labeled with fluorescein-11-dUTP by the Gene Images random prime-labeling module (Amersham Pharmacia Biotech). Hybridization signal was detected by the Gene Images CDP-Star detection module (Amersham Pharmacia Biotech). The filters were exposed to X-ray films.

Bisulfite Sequencing of a Mark Locus

Genomic DNA was modified using an EZ DNA Methylation-Gold kit (Zymo Research) according to the manufacturer's recommendations. Modified DNA was purified using a Zymo-Spin IC column (Zymo Research). The primers of retrotransposon *Tos17*, an established locus in the rice genome for assessing DNA methylation liability, for bisulfite sequencing were designed using the MethPrimer program (<http://www.urogene.org/methprimer/>) (Supplementary Table 5). For each PCR, 1.0–3.0 μ l of bisulfite-treated DNA was used, and the PCR products were cloned into the pMD18-T vector and sequenced. More than 20 clones were sequenced for each sample. Analyses of the bisulfate sequencing results were conducted at the Kismeth website (<http://katahdin.mssm.edu/kismeth>). The methylation levels per site (CG, CHG, and asymmetric CHH) were calculated by dividing the number of non-converted (methylated) cytosines by the total number of cytosines within the assay (Ngezhahayo et al., 2009).

Statistics

Statistical significance was determined using SPSS 11.5 for Windows (<http://www.spss.com/statistics/>). Continuous variables e.g., plants height, fold-change data in qRT-PCR were presented as the mean \pm standard deviation and statistically tested by an unpaired, two-tailed *t*-test. A value of $P < 0.05$ was considered significant and $P < 0.01$ was very significant. The Pearson correlation analysis between the DNA methylation pattern variations (detected by MASP) and the expression levels of methylation-related genes (detected by qRT-PCR) was calculated by the range method using SPSS. $-1 \leq$ Pearson's $r \leq 1$. Pearson's $r > 0$ indicates the two variables have a positive correlation, Pearson's $r < 0$ indicates the two variables have a negative correlation, Pearson's $r = 0$ indicates the two variables have no correlation. The color depth symbolizes the values of Pearson's r , and the red and blue blocks denote positive and negative correlations, respectively.

RESULTS

Plant Growth Inhibition by the Laser Irradiation Treatments

Phenotyping was conducted at the reproduction stage after the laser treatment in rice cultivar Jinongda 18. Phenotypic variations especially plant height and heading dates were observed in all the 11 laser treatment groups with variable extents (Table 1).

Of 1,000 laser irradiated germinating seeds, *ca.* 990 plants grew to maturity in the experimental paddy-field. The heading date was recorded as the first plant showing panicle emergence in one group, and it was 84 days in WT, and which was delayed in most laser-treated groups. For example, heading date was 88 days in groups 7 and 10, and 89 days in group 11 (**Figure 1**). These observations indicated that long-wave laser treatment was more effective than short-wave laser, and the treatment frequency was a key factor which influences the degree of phenotypic variations in the same wavelength.

Five plants were randomly chosen to quantitatively measure the plant height, and the mean plant height was 101 ± 1.58 cm in WT (**Table 1**), and the common feature was dwarfing in all the 11 laser-treated groups. For example, it was 95 ± 2.12 cm in group 7 ($P < 0.01$), 95 ± 1.58 cm in group 10 ($P < 0.01$), the shortest height was 93 ± 3.00 cm in group 11 ($P < 0.01$). It indicated that laser irradiation negatively influenced plant growth and development, and the degrees of impact were largely dose-dependent.

Alteration of Cytosine Methylation Patterns in the Laser-Treated Rice Plants and Its Transgenerational Inheritance

A total of 1,140 clear and reproducible bands (between two technical replicates, starting from the first step, i.e., DNA isolation) were scored using 20 pairs of selective primers in the MSAP analysis (Supplementary Table 1) for the 11 selected M0 individuals that showed clear phenotypic variations, along with WT (**Figure 2A**). The tabulated methylation level was 21.2% ($^m\text{CG} + ^m\text{CHG}$) in WT, and all but one (M0-1) of the 11 M0 plants showed similar levels of methylation to that of WT, which collectively ranged from 19.4 to 21.1% (**Figure 2B**). This suggests that the laser irradiation treatments did not cause large changes in total DNA methylation level at the 5'-CCGG sites. Notably, however, for the single M0 plant (M0-1) that did show clear loss of methylation, it mainly occurred at the CG sites (**Figure 2B**). In contrast to the general stability in methylation level, we found that all four methylation patterns (CG hyper, CG hypo, CHG hyper, and CHG hypo) that are discernible based on the MSAP data (Materials and methods) showed significant changes in all 11 laser irradiated M0 plants relative to WT. The frequencies of these methylation pattern alterations ranged from 1 to > 4% (**Figure 2C**). The similar frequencies of hyper vs. hypo methylation changes at both CG and CHG sites clearly have

offset each other and render the total methylation levels broadly constant (**Figure 2B**). Notably, in all the MSAP profiles we did not find parallel band changes between the two isoschizomers, *HpaII* and *MspI*, indicating nucleotide sequence changes at the 5'-CCGG sites should be rare if occurred at all in these low-dose laser treated rice plants (**Figure 2A**).

Next, we asked whether the methylation changes were inheritable to the next organismal generation. For this purpose, we chose plant M0-11 which showed the most obvious phenotypic changes (**Table 1**) to produce the M1 generation via self-pollination. A total of 543 reproducible bands (between two technical replicates) were scored using 10 informative selective MSAP primers in 18 randomly chosen M1 plants (**Figure 3A**). Results showed that the total methylation level of M0#11 was 20.3%, and which ranged from 19.7 to 22.8% in the M1 plants (**Figure 3B**), suggesting a moderate fluctuation in methylation level occurred in the M1 plants, which contrasted with the broad methylation level stability among the M0 plants (**Figure 2B**). Analysis of the four methylation patterns revealed the important observation that a great majority of the altered methylation patterns in the M0 plant (M0-11) was inherited to all 18 plants of the M1 generation (**Figure 3A**). Nevertheless, it is important to note that heritability of the alterations was clearly much less than 100% and variable across the 18 M1 individuals (**Figure 3C**). Moreover, gaining of additional methylation pattern alterations, especially CG hypo and both CG hyper and CHG hyper patterns, were apparent in most of the M1 plants (**Figure 3C**). The less than 100% heritability, together with unbalanced gain vs. loss of additional alteration, is consistent with the discernible fluctuations in total methylation level in the M1 plants (**Figure 3B**).

The still dynamic nature of methylation patterns in the M1 plants promoted us to address the issue further. We thus chose a single M1 plant (M1-1) to produce the M2-generation plants by self-pollination. A total of 543 reproducible bands (between two technical replicates) were scored by using seven pairs of selective MSAP primers across 16 randomly selected M2 individuals (**Figure 4A**). Results indicated that the methylation levels of all 16 M2 plants remained similar to that of the M1 plant (**Figure 4B**). Importantly, except for one M2 plant (M2-2), the two hyper-methylation patterns (CG hyper and CHG hyper) were uniform and the same as those of M0-11 and M1-1 (**Figure 4C**), pointing to their stabilization. By contrast, the two

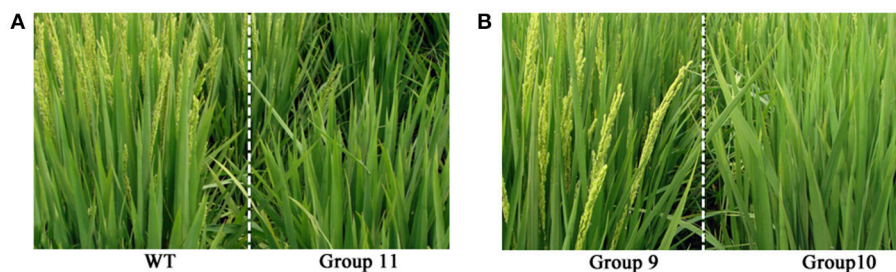


FIGURE 1 | Phenotypic changes in the M0 generation of Jinong 18 after low-dose laser-irradiation. (A) WT vs. Group 11; (B) Group 9 vs. Group 10.

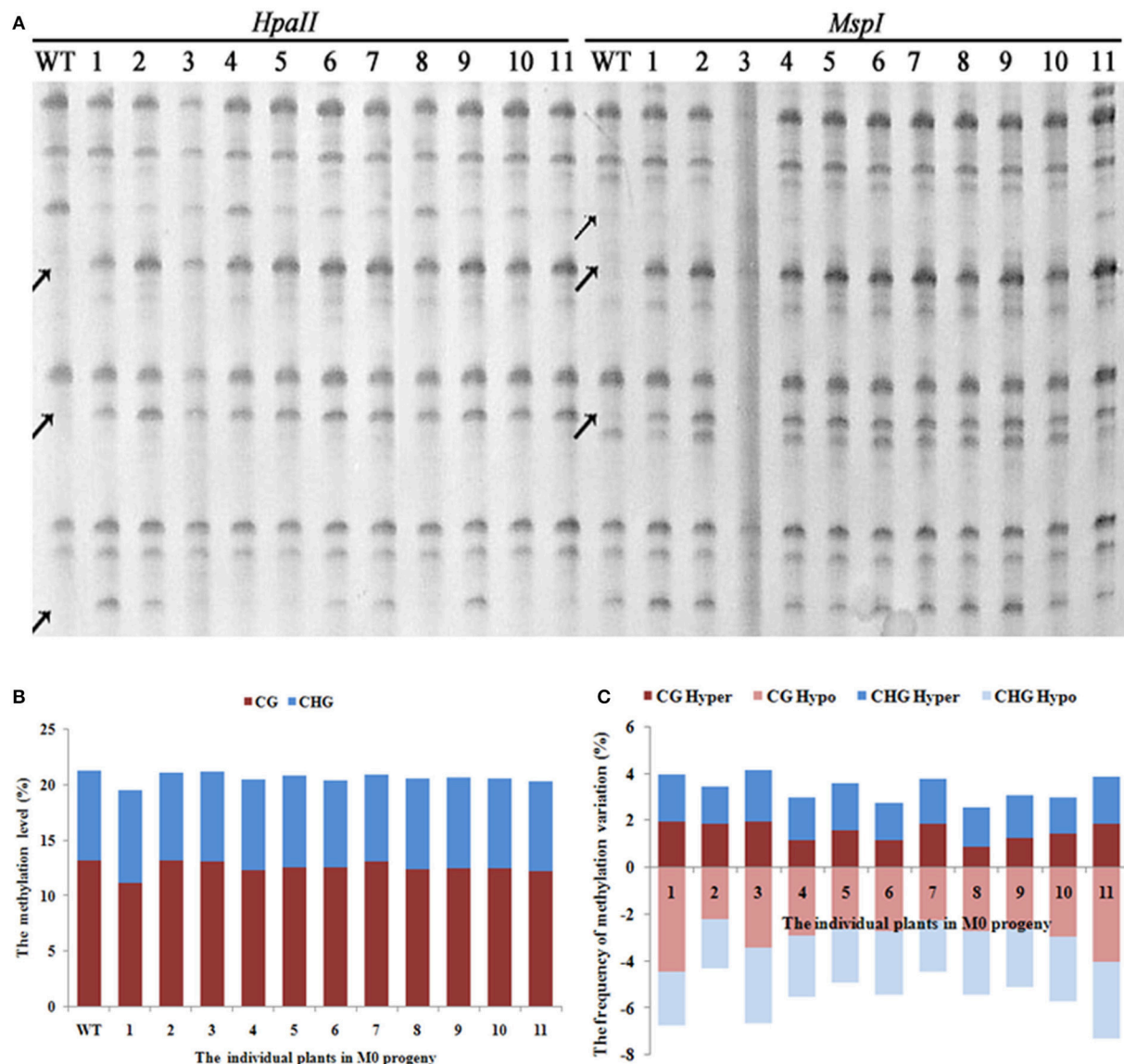


FIGURE 2 | (A) Examples of MSAP gels and **(B)** the total methylation levels **(C)**, variations occurred in CG and CHG sites in M0 generation after laser-irradiation. The arrows shows methylation variations.

hypomethylation patterns (CG hypo and CHG hypo) were still as fluctuating among the M2 plants (**Figure 4C**) as they were among the M1 plants (**Figure 3C**). Together, our results suggest that although some of the newly acquired methylation pattern alterations due to the treatment were inherited and stabilized by the M2 generation, DNA methylation patterns in general are still dynamic in the M2 generation.

Validation of DNA Methylation Alteration and Its Transgenerational Dynamics by Locus-Specific Bisulfite Sequencing

Tos17 is an endogenous long terminal repeat (LTR) retrotransposon in the rice genome, and which is usually heavily

methylated over its entire length under normal conditions. The methylation state of *Tos17* however is highly labile and loss of methylated cytosines (^mCs) may occur under various conditions, which may (but not necessarily) lead to its transpositional activation (Liu et al., 2004). Thus, *Tos17* has been widely accepted as a sensitive mark to assess methylation liability in rice (Liu et al., 2004; Ding et al., 2007). To verify that laser irradiation has caused changes in DNA methylation patterns and their transgenerational heritability by an independent approach, we analyzed methylation state of the 5' portion of *Tos17* together with its immediate flank (**Table 2**) by locus-specific bisulfite sequencing in several M1 and M2 individuals that showed clear methylation changes in the MSAP profiles along with their WT. First, for the entire sequenced segment, there are

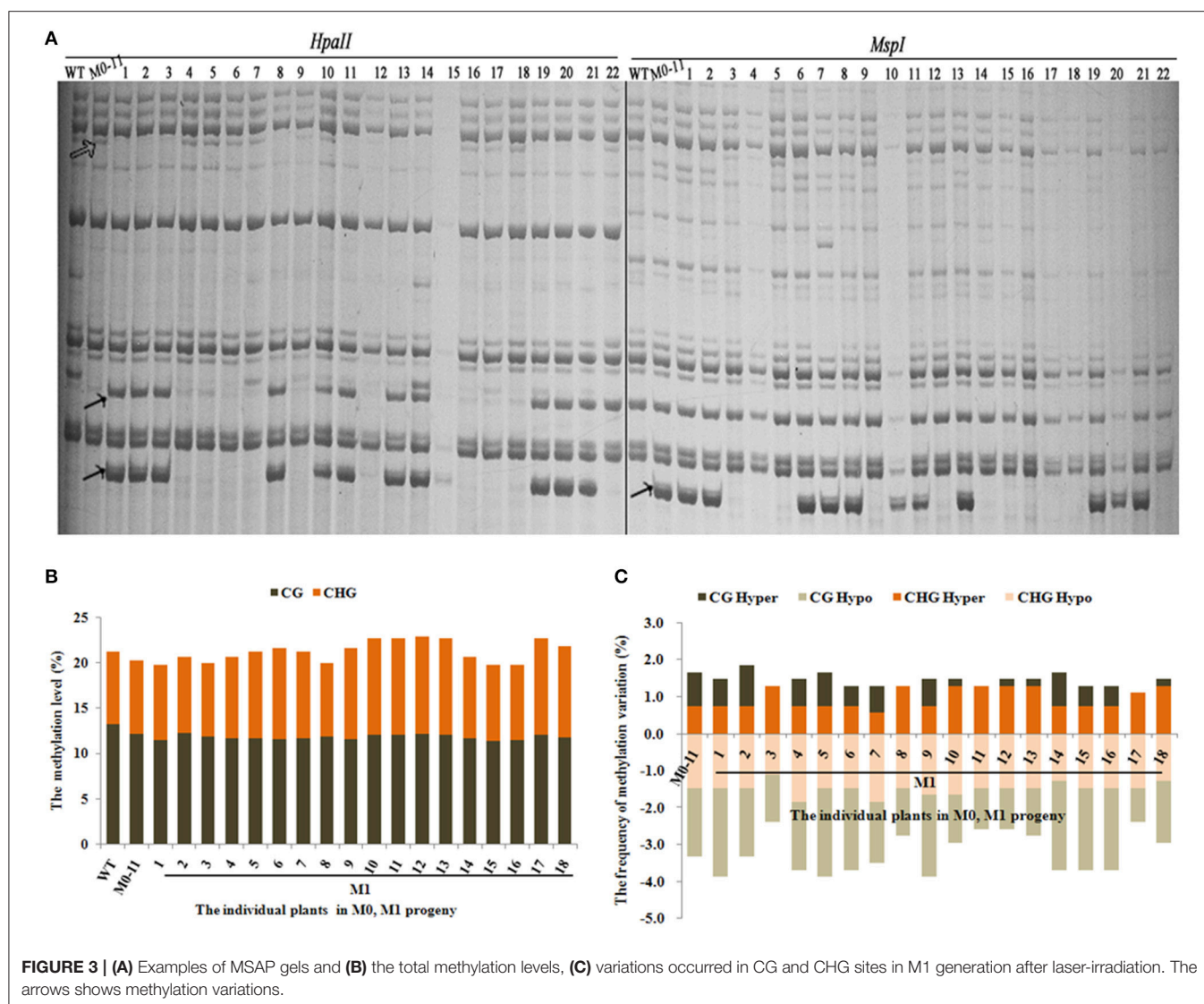


FIGURE 3 | (A) Examples of MSAP gels and **(B)** the total methylation levels, **(C)** variations occurred in CG and CHG sites in M1 generation after laser-irradiation. The arrows shows methylation variations.

two CG sites located in the flank region (Table 2), which are slightly methylated (*ca.* 10%) in WT, but the methylation was completely lost in all analyzed progeny individuals derived from the low-dose laser-treated mother plant (Figure 5A). There are nine CHG sites in the sequenced segment, four in the flank and five in the 5' portion of *Tos17* (Table 2). In addition, there are 50 CHH sites in the sequenced segment, 27 in the flank and 23 in the body region of *Tos17* (Table 2). Analysis of these sequences found that (i) in WT, CG, CHG, and CHH sites were low-methylated, while only a small fraction of CHG sites had a high frequency of methylation. (ii) In the M1 plant, CG sites were slightly or moderately methylated compared with WT, most CHG sites were maintained the methylation patterns of M0, while CHH sites showed inheritance of M0 or reversal of the methylation patterns to those of WT. (iii) In the M2 plant, nearly all three kinds of methylation at CG, CHG and CHH were identical with those of M1 (Figure 5), denoting largely stable epigenetic inheritance thereafter.

Transgenerational Changes in Expression of Chromatin Genes Encoding Enzymes Directly Related to DNA Methylation

Establishment, erasure and maintenance of DNA methylation patterns are accomplished by the interlaced action of multiple enzymes encoded by a large set of genes collected termed chromatin genes (McCue et al., 2015; Rigal et al., 2016; Tan et al., 2016). To test whether the laser irradiation-induced heritable changes of DNA methylation patterns might be related to altered expression of the chromatin genes, especially those directly related to DNA methylation, we analyzed expression levels of a set of genes encoding DNA methyltransferases (*MET1-1*, *CMT3-1*, *DRM2-1*, and *DRM2-2*), 5-methylcytosine DNA glycosylases (*DME1*, *DME2*), the *SWI/SNF* chromatin remodeler (*DDM1*), and four siRNA pathway-related AGO proteins (*AGO1-1*, *AGO1-2*, *AGO4-1*, *AGO4-2*) in all the 11 M0 plants (Table 1). Real-time PCR results indicated that all the analyzed genes showed significantly perturbed expression in these M0 plants

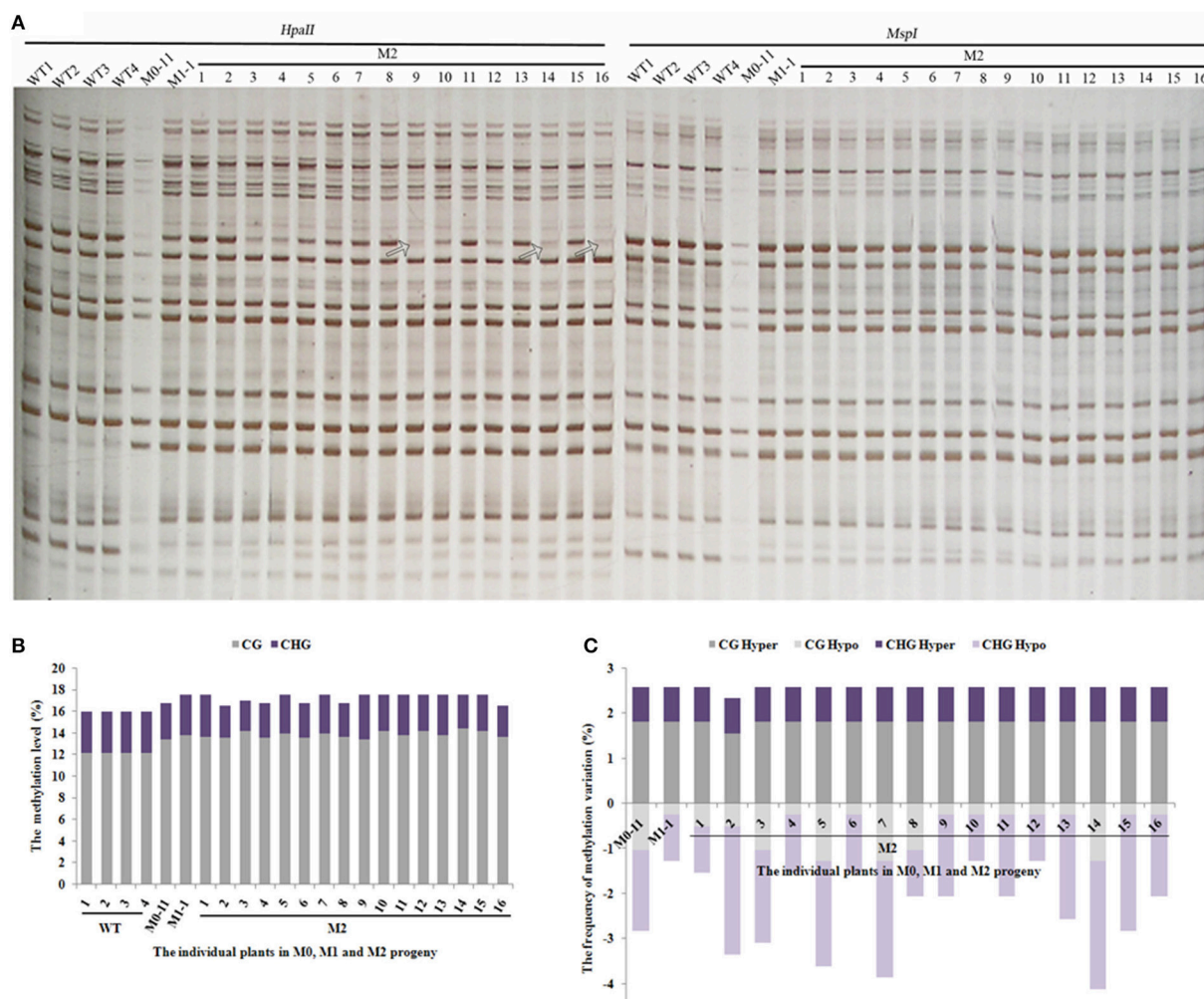


FIGURE 4 | (A) Examples of MSAP gels and (B) the total methylation levels, (C) variations occurred in CG and CHG sites in M2 generation after laser-irradiation. The arrows shows methylation variations.

(Supplementary Table 6). Specifically, genes coding for DNA methyltransferases *CMT3-1*, *DRM2-1*, and *DRM2-2* were all significantly down-regulated in the M0 plants. Similarly, genes coding for 5-methylcytosine DNA glycosylases *DME1* and *DME2* were also down-regulated in the M0 plants, and so were genes coding for the siRNA pathway-related AGO proteins *AGO1-1*, *AGO1-2*, *AGO4-1*, and *AGO4-2* (Supplementary Table 6). In contrast, the gene coding for *SWI/SNF* chromatin remodeler *DDM1* was significantly up-regulated in all the M0 plants (Supplementary Table 6).

To test whether the significantly perturbed expression states of these chromatin genes in the M0 plants were also transgenerationally persistent, all the 18 M1 plants and 16 M2 plants were subjected to the same qRT-PCR analysis (Supplementary Table 7). Results indicated that *DDM1*, which was significantly up-regulated in M0, displayed a further up-regulation in virtually all the M1 plants, whereas *MET1-1*, *CMT3-1*, *DRM2-1*, *DME1*, *DME2*, *AGO1-1*, *AGO1-2*, *AGO4-1*,

TABLE 2 | The nucleotide sequence of the bisulfite sequenced region of *Tos17*.

Sequence name	Sequence
<i>Tos17</i>	(431)GACCATCTACATCGTTGCTGGCTTGCTGCACAGGTCGAG GCCCTGTTCCTCCCATTTGCTGCTGCGGTGCTGCCGTTCTGC TTTCTGCTTCCAGAAAATCAGAGGAGGAGGCTCTCTGCTATCAG ATAAAAGATCAGACTCAAGTAGCTGAGTTGCTAGTGATCTTTGG (601)TTTATACAATTGTTTTGATATATCATGGTGTGAGATGGAATT CTTGGTCCCTATCCATGTGCTGGTATTGCCCTCATGTTTCATCTATA TCAACTGTTACCCCAATTGCCTCCCAACCATGGATTTTGGACT CAGGAGCCTCCTTTTCATATGTGATTTGATGAT(762)

The analyzed region for *Tos17* is 332 bp in length, with the first 170 bp being a portion of the 5'-LTR whereas the rest 162 bp being a portion of body-region (underlined).

AGO4-2, which were down-regulated in M0 (Supplementary Table 6), showed up-regulated expression in nearly all the M1 plants (Supplementary Table 7). *DRM2-2*, which was also

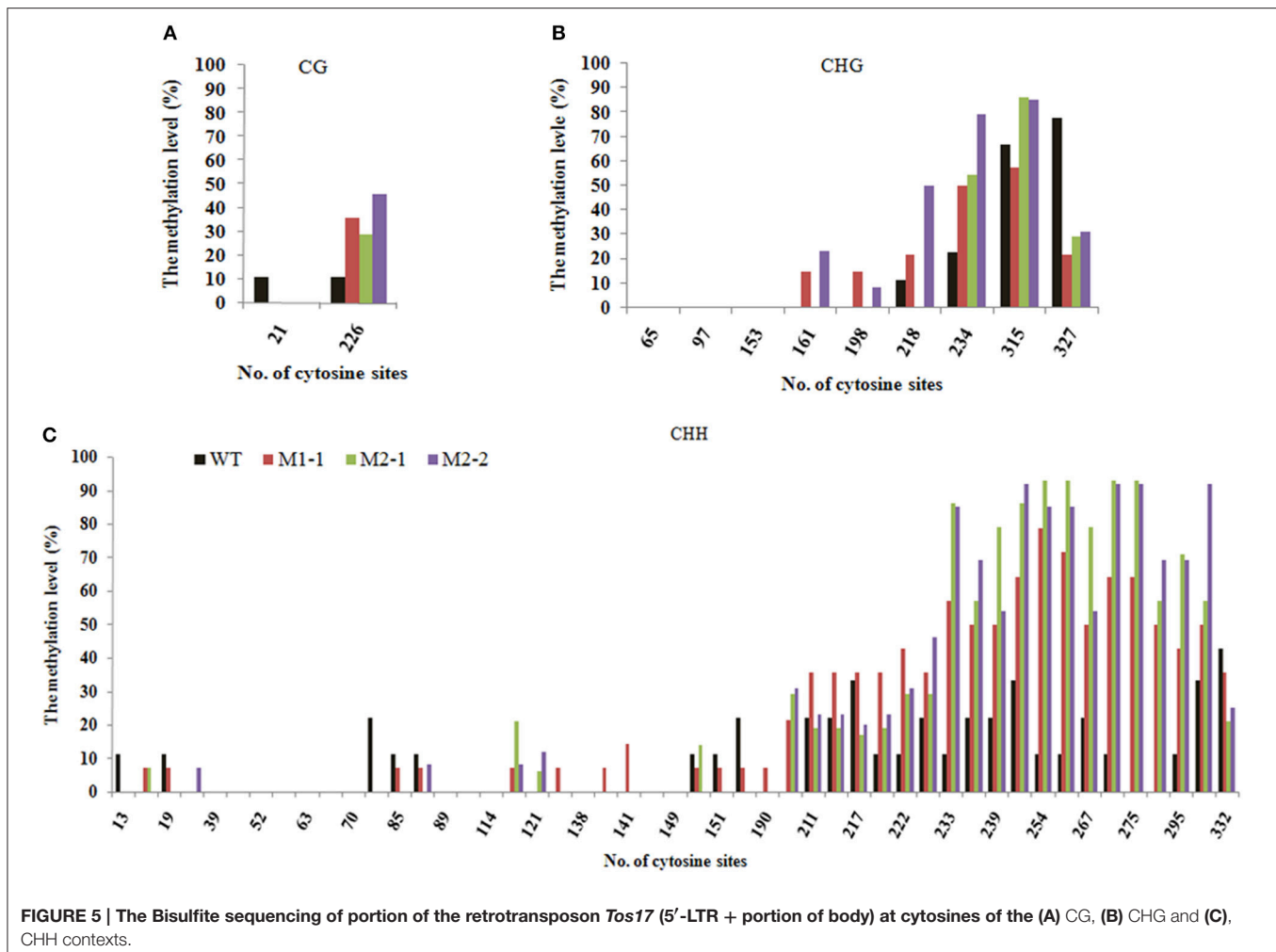


FIGURE 5 | The Bisulfite sequencing of portion of the retrotransposon *Tos17* (5'-LTR + portion of body) at cytosines of the (A) CG, (B) CHG and (C), CHH contexts.

down-regulated in M0 (Supplementary Table 6), showed largely reversion to the expression state of WT (Supplementary Table 7). Notably, *DRM2-1* and *DRM2-2* showed a dramatic upregulation in two (M1-11 and M1-12) of the M1 plants (Supplementary Table 7). Further analysis of the M2 generation plants indicated that *CMT3*, *DRM2-1*, *DME1*, and *AGO1-1* showed stable inheritance of the expression states of their M1 mother plant (Figure 6A), while *MET1*, *DRM2-2*, *DME2*, *AGO4-1*, and *AGO4-2* showed largely reversion of expression states to those of the M0 plant, which nevertheless were still significantly different from those of WT (Figure 6B). *DDM1* which was significantly up-regulated in the M0 and M1 plants, showed a tendency of down-regulation in all but two (M2-5 and M2-6) of the studied M2 plants, and in these two plants it showed a highly outlier upregulation (Supplementary Table 8).

In addition, A Pearson correlation analysis was used to analyze the possible correlations between the DNA methylation patterns (detected by MSAP) and the perturbed expression of chromatin genes (detected by qRT-PCR). The value of Pearson's r symbolizes the correlation tendency between the two variables. We found that, in M0 plants, CG hypermethylation was positively

correlated with expression *AGO4-2* ($r = 0.71$, $P < 0.05$) (Figure 7A). In M1 plants, CG hypomethylation was positively correlated with expression of *AGO4-1* ($r = 0.60$, $P < 0.01$), *AGO4-2* ($r = 0.69$, $P < 0.01$) and *MET1-1* ($r = 0.51$, $P < 0.05$), while CHG hypermethylation was negatively correlated with expression *AGO4-1* ($r = -0.52$, $P < 0.05$) and *AGO4-2* ($r = -0.55$, $P < 0.01$) (Figure 7B). However, no correlation was detected in M2 plants, consistent with the MSAP profiles showing that the DNA methylation patterns were stabilized by large in the M2 generation (Figure 4C). Together, the above data of transgenerational expression dynamics suggested that the immediate changes, inheritance, additional changes, reversion to parental state as well as stabilization of the altered CG and CHG methylation patterns in the laser irradiated plants and their offspring are closely linked to the transgenerationally perturbed expression states of at least some of the chromatin genes analyzed.

It should be cautioned, however, that the expression dynamics of these chromatin-related genes both among the laser-irradiated individuals and between generations clearly did not follow a persistent trend. Therefore, we cannot rule out the possibility that

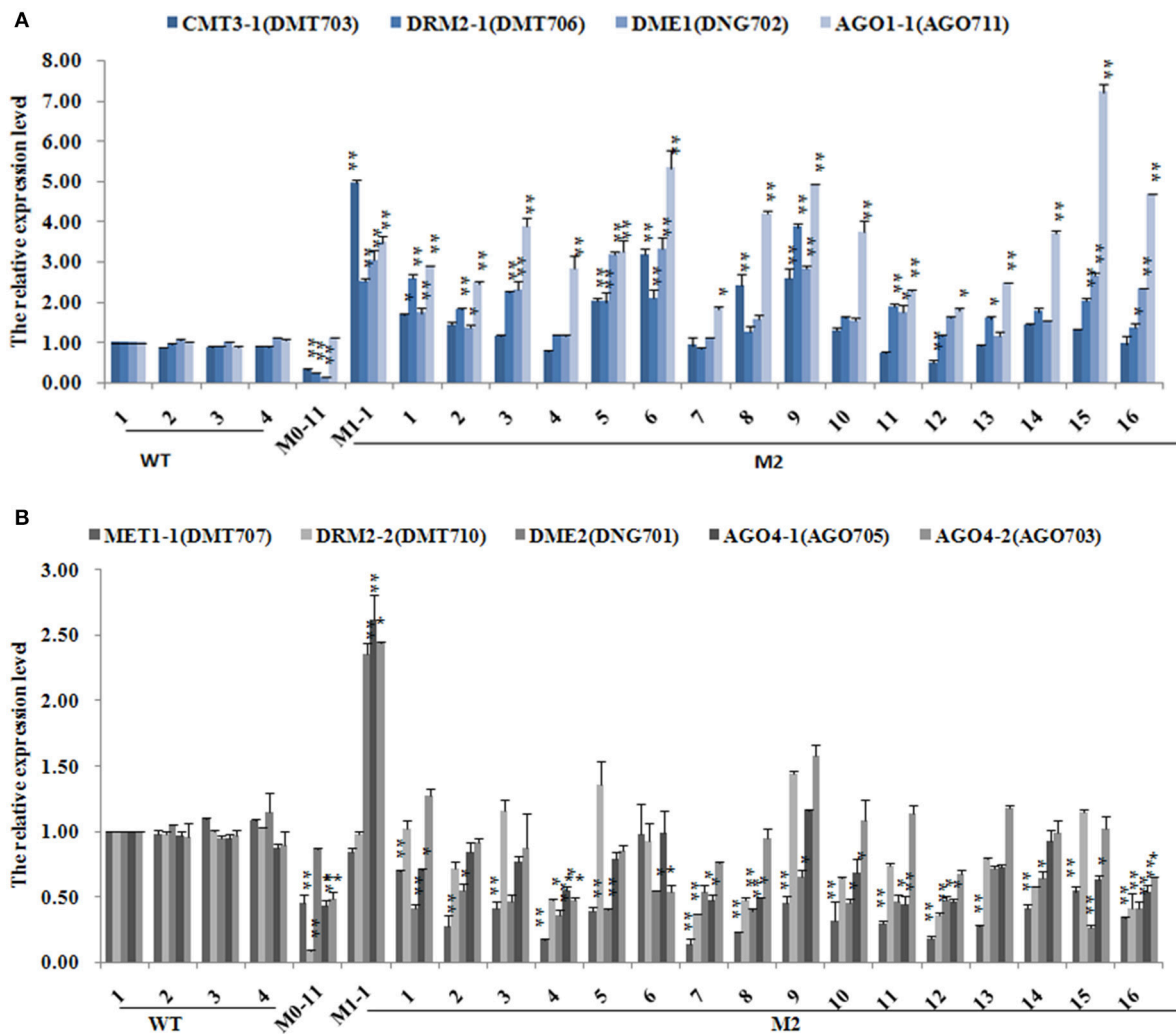


FIGURE 6 | The expression levels of different DNA methylation-related genes subsequent to the low-dose laser irradiation in M0, M1, M2 generation of Jinong 18. (A) CMT3-1, DRM2-1, DME1, AGO1-1; (B) MET1-1, DRM2-2, DME2, AGO4-1, AGO4-2.

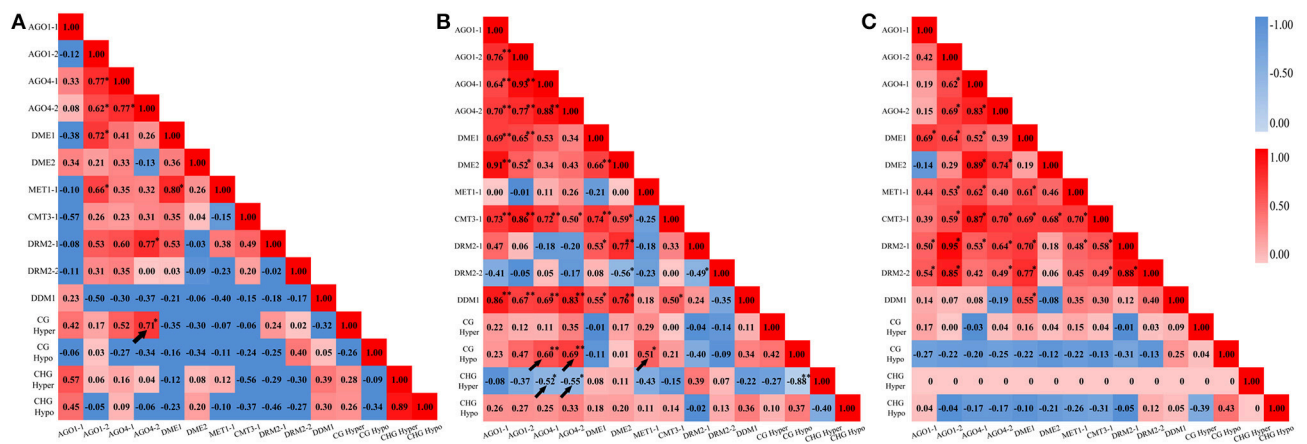


FIGURE 7 | Correlation analysis between cytosine methylation variations and the dynamic expression levels of the various genes related to DNA methylation. (A) M0 generation; (B) M1 generation; (C) M2 generation.

stochastic factors like expression noise may have also contributed to the expression dynamics.

Characterization of Variant MASP Loci in the Laser-Irradiated Rice Plants and Progenies

To get some insights into the functional relevance of the genomic loci underwent methylation pattern changes as a result of laser-irradiation, 36 reproducible variant bands (shared among at least three M0 individuals) were eluted from the MSAP gel profile, cloned and sequenced. A BlastX query indicated that 16 loci were homologous to known or predicted protein-coding genes, three loci were TEs, and the other 15 loci showed no similarity with known sequences in the database (Supplementary Table 9), suggesting that at least certain variant methylation loci are likely functionally consequential.

Transpositional Activation of *mPing* in the Laser-Irradiated Rice and Its Selfed Progenies

mPing (430 bp in length) is the most active MITE-type TE in the rice genome, and can be induced to transpose under various stress conditions, and its mobility can be associated with DNA methylation of itself and/or its flanks (Ngezhahayo et al., 2009). Given the extensive DNA methylation pattern changes induced by the laser irradiation, we sought to test the possibility that *mPing* had been mobilized by the treatment. For this purpose, 53 *mPing*-containing loci identified in the WT were analyzed by locus-specific PCR, and the results showed that *mPing* excision occurred in 21 (ca. 40%) loci in one or more of the 11 selected laser-irradiated M0 plants (Table 3), and *mPing* mobility was most active in M0#11 (Figure 8A). It has been documented previously that excision of *mPing* is not necessarily accompanied by reinsertions of the mobilized copies (Shan et al., 2005). To test if *mPing* reinsertions occurred in these plants, transposon-display (TD) was performed. Although exhaustive TD was conducted, only six reinsertion events in the M0#11 plant or some of its 19 M1 progenies (bands appeared *de novo* relative to WT), and all six loci were isolated and confirmed by newly designed PCR primers (e.g., Figure 8B). This suggests that although excisions of original *mPing* copies occurred abundantly (ca. 40%, 21 out of 53 loci), most mobilized copies failed to reinsert back to the rice genome. To confirm this possibility further, Southern blotting was conducted using the full-length *mPing* as a probe. Indeed, only loss of bands were detected in M0#11 and its progenies at the resolution of Southern blotting (Figure 8C), confirming the possibility, which is consistent with a previous study (Shan et al., 2005).

DISCUSSION

Both in the evolutionary time-scale and during ontogenic lifecycle, plants encounter various internal and external stresses that may have negative impacts on their growth, development, reproduction and evolutionary success. These adverse conditions include recurring situations like abiotic and biotic stresses, as

TABLE 3 | The statistical of dap sites of *mPing* in the M0 generation of Jinong18.

Primers	WT	1	2	3	4	5	6	7	8	9	10	11
mPL2	+	–	–	–		–	–	+	–	–	+	–
mPL5	+	–	–	–		–	–	–	–	–	–	–
mPL7	+	+	+	+		+	+		+	+		+
mPL9	+	+	+	–		+	+	–	–	–	–	±
mPL10	+	+	+			+	+	+		+	+	+
mPL11	+	+	–	+	+	+	+	–	–		+	–
mPL12	+	–	–	–		–	–	–	–	–		–
mPL13	+	+	+	–		+	+	–	–	–	–	±
mPL16	+	–	–	–	–	–	–	–	–	–	–	–
mPL20	+	+	+	–	–	+	+	–	–	–	–	+
mPL21	+	–	–	–	–	–	–	–	–	–	–	–
mPL22	+	–	–	–	–	–	–	–	–	–	–	–
mPL23	+	–	–	–		+	+	+	+	+	–	–
mPL30	+	+	+	+		+	+		+	–	–	+
mPL31	+	+	+	–		+	+	–	–	–	–	±
mPL33	+	+	+	–		+	+					+
mPL37	+	–	–	–		–	–		–	–	–	–
mPL39	+	–	+	+		–	+		+	+		+
mPL42	+	–	–	–		–	+	–	–	–	–	–
mPL 45	+	–	–	+		–	–	+	+	+	+	–
mPL 48	+	–	–	–		–	–		–	–	–	–

“+”represents existence of *mPing* at the locus (~700 bp), “–”represents absence of *mPing* at the locus and hence a lower band (~230 bp), space represents no bands amplified, “±” represents a locus with existence of *mPing* in a heterozygous state.

well as less frequent insults like interspecific hybridization and geological disasters. Conceivably, the strategies evolved in plants to cope with the conditions are diverse. Cong and Whitelaw have suggested that epigenetic modification is an efficiently evolved system that may enable rapid tuning by plants to enable adaptation to new environments (Youngson and Whitelaw, 2008). Previous researches showed that pathogen infection, nutrition or water deficiency, environmental pollution and tissue culture all induced DNA cytosine methylation variations (Kou et al., 2011; Ou et al., 2012; Gao et al., 2013; Wang et al., 2013; Zhang et al., 2014).

Low-dose laser irradiation has long been considered as a non-mutagenic treatment and widely used in agriculture to promote seed germination. Paradoxically, new crop cultivars were often selected from the treatments, which would entail the occurrence of heritable variations (Xu et al., 2008; Liu et al., 2016). Here we show that low-dose laser-irradiation could generate heritable phenotypic variants such as dwarfing and delayed development in rice in a dose-dependent manner. Apparently, either genetic mutation or heritable epigenetic changes or both must have occurred to these plants. Although we not rule out the possibility that the variant phenotypes were due to genetic mutations, given the well established notion, discussed above, that epigenetic mechanisms are readily responsive to all kinds of stressful conditions, and that the low-dose irradiation is certainly an unfamiliar condition that is not normally experienced by the rice plants, we suspect that

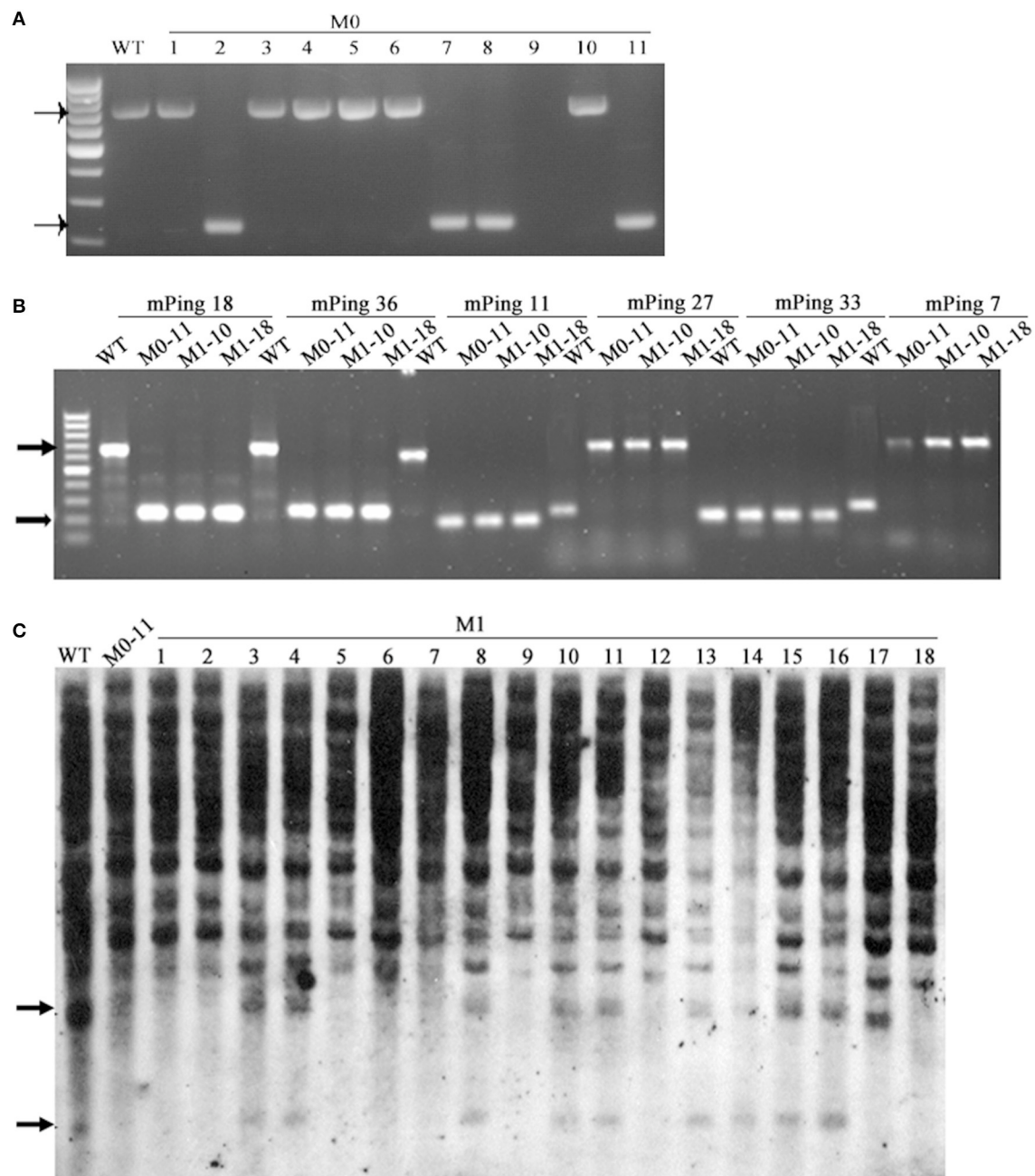


FIGURE 8 | The mobility of *mPing* induced by laser irradiation in Jinong 18. (A) The *mPing* excisions detected in M0. **(B)** The *mPing* insertions and excisions detected in M1. **(C)** Southern blot analysis of the *mPing* mobility using separate probes.

the treatment that may have more likely instigated epigenetic instabilities. Indeed, we found extensive changes of DNA methylation patterns occurred in these phenotypically altered plants based on the MSAP analysis which in principle randomly samples at 5'CCGG sites across the genome. Importantly, we document that a significant proportion of the altered methylation patterns is inherited to organismal progenies of the laser-irradiated plants, and forming new epigenetic alleles. This kind

of epigenetic variation and inheritance of unusual parental experience is known as evolutionarily consequential (Jablonka and Lamb, 2015). By contrast, in the MSAP profiles we did not observe identical variant bands that appeared in digests by the two isoschizomers (*HpaII* and *MspI*), suggesting that if nucleotide sequence mutations were generated by the low-dose laser treatments, they were insignificant compared with changes in DNA methylation.

We show that chromatin-related genes encoding DNA methyl-transferase CMT3, the *de novo* methylase DRM2-1, DNA glycosylase DME1 and the siRNA-related proteins AGO1-1 were down-regulated immediately after the laser-irradiation in the M0 plants, but they were up-regulated in the M1 plants, and which was then followed by relatively stable inheritance of the elevated expression state in the M2 plants (**Figure 6A**). Similarly, genes encoding DNA methyl-transferase MET1, the *de novo* methylase DRM2-2 and the siRNA-related proteins AGO4-1 and AGO4-2 were down-regulated after laser-irradiation in M0, and up-regulated in M1, but then largely reversed to the expression levels of M0 in M2 (**Figure 6B**). These observations suggest that the heritable changes of DNA methylation patterns is likely due to transgenerationally perturbed expression of these chromatin-regulation genes by the low-dose laser irradiation. Conceivably, with time, the expression of these genes will be reversed to the original default states (i.e., in the WT), but the newly acquired methylation patterns will be perpetuated, and thus different from that of the WT. Indeed, a correlation analysis indicated that at least some of the methylation pattern changes are significantly correlated with expression dynamics of some of the chromatin genes. If the heritable changes of DNA methylation bear functional impacts, then some of their readout phenotypes, if adaptive, can be fixed under natural settings or selected artificially for breeding purposes. The fact that at least some of the variant MSAP loci are associated with known or predicted protein-coding genes lent support to this possibility.

TEs account for 35% of the rice genome. Although the great majority of TEs have become defective due to sequence changes (e.g., truncation or nested insertion by other TEs), a small fraction of TEs, being primarily repressed by epigenetic mechanisms, are largely intact at the sequence level and potentially active. Indeed, the majority of DNA methylation was found to map to TEs in the rice genome (Law et al., 2010). In this respect, we note that the bisulfite results mainly showing hypermethylation at cytosines of all sequence contexts (CG, CHG, and CHH) in virtually all the analyzed *Tos17* region in progenies of the laser-treated plant, which is consistent with the consistently elevated transcript abundance of DDM1 that is known as required to establish and perpetuate cytosine methylation at TEs of all sequence contexts (Saze and Kakutani, 2007; Ito et al., 2015). Therefore, its significant and persistent upregulation may have contributed to the increased methylation of TEs like *Tos17*. Notably, under various internal and external stress conditions, the normally dormant TEs can be re-weakened and become mobilized (McClintock, 1984), and which often is accompanied by disruption of repressive epigenetic marks, e.g., in the case of *mPing* (Ngezhahayo et al., 2009). Specifically, it

was documented that the transpositional activity of *mPing* was closely correlated altered methylation states both of itself and its upstream flanks under tissue culture conditions (Ngezhahayo et al., 2009). We show here that *mPing* is also mobilized in the laser-irradiated rice plants which showed alterations in DNA methylation and phenotypic variations. It is therefore likely that mobilization of *mPing* by the treatment is also correlated with DNA methylation changes. An interesting observation is that great majority of the excised *mPing* copies were not re-integrated into the rice genome, consistent with an earlier report in other situations (Shan et al., 2009). Still the excisions alone may have phenotypic consequences because *mPing* contains regulatory sequences which impact regulation of genes residing at its flanks under various stress conditions (Naito et al., 2009; Ito et al., 2016). It will be interesting to test if the progeny plants derived from the laser-irradiated mother plants, which have lost many original copies of *mPing*, will respond differently to stress conditions.

AUTHOR CONTRIBUTIONS

SL, QX, and XY performed most of the experiments. JM and HK contributed to seedling planting, sample preparation and phenotyping, XL contributed to data collection. XG and BL coordinated the project, and conceived the study. FW, XG, and BL analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Minimal evidence for consistent changes in maize DNA methylation patterns following environmental stress

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DNA methylation is a chromatin modification that is sometimes associated with epigenetic regulation of gene expression. As DNA methylation can be reversible at some loci, it is possible that methylation patterns may change within an organism that is subjected to environmental stress. In order to assess the effects of abiotic stress on DNA methylation patterns in maize (*Zea mays*), seeding plants were subjected to heat, cold, and UV stress treatments. Tissue was later collected from individual adult plants that had been subjected to stress or control treatments and used to perform DNA methylation profiling to determine whether there were consistent changes in DNA methylation triggered by specific stress treatments. DNA methylation profiling was performed by immunoprecipitation of methylated DNA followed by microarray hybridization to allow for quantitative estimates of DNA methylation abundance throughout the low-copy portion of the maize genome. By comparing the DNA methylation profiles of each individual plant to the average of the control plants it was possible to identify regions of the genome with variable DNA methylation. However, we did not find evidence of consistent DNA methylation changes resulting from the stress treatments used in this study. Instead, the data suggest that there is a low-rate of stochastic variation that is present in both control and stressed plants.

Keywords: DNA methylation, maize, abiotic stress, epigenetics, tissue culture

Introduction

Plants, like all organisms, must respond to environmental stresses imposed throughout their life. These responses occur at all levels from fine-scale gene expression changes to large morphological changes allowing the plant to cope with environmental pressures. There is substantial interest in the potential role that epigenetics may play in plant responses to stress (reviewed in Finnegan, 2002; Boyko and Kovalchuk, 2011; Mirouze and Paszkowski, 2011; Richards, 2011). While there is evidence for a role of chromatin and small RNAs in responses to abiotic stress (Chinnusamy and Zhu, 2009; Ruiz-Ferrer and Voinnet, 2009; Gutzat and Mittelsten Scheid, 2012; Bond and Baulcombe, 2014), there is less evidence that heritable changes in DNA methylation—mitotic or meiotic—are associated with stress responses (reviewed in Gutzat and Mittelsten Scheid, 2012; Pecinka and Mittelsten Scheid, 2012; Eichten et al., 2014). DNA methylation is a highly heritable chromatin modification that can influence gene expression and transposon activity (Chan et al., 2005; Law and Jacobsen, 2010; Matzke and Mosher, 2014). There is a complex set of

cellular machinery that is involved in *de novo* and maintenance DNA methylation activities (Law and Jacobsen, 2010; Matzke and Mosher, 2014) as well as a set of enzymes that can perform demethylation (Zhang and Zhu, 2012). It is possible that DNA methylation could be altered due to abiotic stress, leading to novel regulation of genes (Lukens and Zhan, 2007; Chinnusamy and Zhu, 2009). As DNA methylation is often mitotically heritable (Law and Jacobsen, 2010), it is possible that stress-induced DNA methylation changes may be maintained through mitotic cellular division allowing a continued regulation of genomic features into later stages of life.

There are a number of studies that have investigated DNA methylation following biotic or abiotic stress. These studies vary substantially in terms of the treatments that have been used, the methods for assessing DNA methylation, and the interpretation of the findings. A number of studies (Lira-Medeiros et al., 2010; Tan, 2010; Verhoeven et al., 2010; Wang et al., 2011; Bilichak et al., 2012; Karan et al., 2012; Colaneri and Jones, 2013; Zheng et al., 2013) have used methyl-sensitive AFLP (MSAP) approaches to identify changes in DNA methylation in plants growing in different environments. This approach can have some limitations in quantifying DNA methylation changes (Pecinka and Mittelsten Scheid, 2012) and in many studies it was not clear whether observed changes are reproducible in multiple individuals. Genome-wide profiles of DNA methylation have provided evidence for consistent changes in DNA methylation in response to biotic stress (Downen et al., 2012; Le et al., 2014) or tissue culture (Stroud et al., 2013; Stelpflug et al., 2014). A recent high-resolution study of both DNA sequence and DNA methylation changes in response to abiotic stress (Jiang et al., 2014) revealed elevated rates of both mutation and epimutation in response to salt stress in *Arabidopsis*.

Our primary goal was to search for perturbations to the maize methylome following exposure to abiotic stress. In particular we sought to test two related hypotheses. The first hypothesis suggests that there would be targeted, specific alterations of the methylome that would be reproducible in multiple individuals subjected to the same stress. The second hypothesis is that stress may destabilize the methylome and result in more variation relative to controls. In order to test these hypotheses we profiled DNA methylation in individual plants and control siblings subjected to several different stress conditions including heat, cold, and UV. While some changes in DNA methylation are observed it is not apparent that these are the result of either consistent responses to a stress or even increased instability of DNA methylation in stressed plants.

Methods

Plant Growth Conditions

Seeds from a self-fertilized B73 inbred line were planted in individual 8" pots and grown in a growth chamber (16 h light, 26°C). After 14 days, plants were randomly assigned to experimental conditions (12 plants per condition) consisting of controls grown in the greenhouse (16 h light, 26°C temp), growth chamber (16 h light, 26°C), heated growth chamber (50°C), cold room (4°C), or a growth chamber with supplemented UV light

(60–64 $\mu\text{moles m}^{-2} \text{s}^{-1}$) for 4 h at a time. Stress plants were maintained in growth chambers (16 h light, 26°C) in between stress treatments. Stress was repeated every other day for a total of four treatments. After the stress regimen, plants were moved to a greenhouse to grow to maturity (16 h light, 26°C). All plants that successfully grew to maturity and formed reproductive organs (tassel and ear formation) were used for analysis.

Tissue Harvest and meDIP Profiling

Final (flag) leaf tissue was harvested and DNA was isolated using cetyltrimethylammonium bromide method (CTAB; Doyle and Doyle, 1987). DNA was prepared for meDIP profiling as described (Eichten et al., 2011). Labeled DNA was hybridized to a NimbleGen 3 × 1.4M long oligonucleotide array consisting of 1.4 million single copy probes based on the B73 reference genome sequence (RefGenV2; GEO platform GPL15621). The microarray contains ~1.4 million probes and includes a probe every ~200 bp for the low-copy portion of the maize genome (Eichten et al., 2011, 2013). Slides were hybridized for 16–20 h at 42°C per manufacturer's guidelines. Slides were washed and scanned on the Nimblegen MS200 array scanner, aligned, and quantified using NimbleScan software (Roche Nimblegen, Madison WI) per manufacturer's instructions. This resulted in raw data reports for each of the ~1.4M probes found on the array. Tissue culture samples were developed as described (Stelpflug et al., 2014).

Data Normalization and Discovery of Differentially Methylated Regions

Data was processed as described in Eichten et al. (2011). Briefly, raw data (pair) files were exported from NimbleScan into the Bioconductor statistical environment in R (Gentleman et al., 2004). Array data was normalized using variance stabilization normalization (vsd; Huber et al., 2002). For all samples methylated DNA immunoprecipitation (meDIP) enrichments were estimated for each probe in a linear model accounting for array, dye, and sample effects using the limma package (Smyth, 2004). Statistical contrasts were then fit between IP samples and genomic DNA control samples (input) for each sample. Moderated *t*-statistics and the log-odds score for differential meDIP enrichment was computed by empirical Bayes shrinkage of the standard errors with the false discovery rate controlled to 0.05. Resulting scaled and normalized methylation levels (meDIP enrichment) for each probe for each sample were used for subsequent analyses.

Two different approaches were implemented to discover differentially methylated regions (DMRs). The first approach compared the average DNA methylation level for all samples from a treatment to the average of all control samples for each probe and then utilized DNA copy (Venkatraman and Olshen, 2007) to search for segments (regions including at least three adjacent probes) with consistent differences in DNA methylation. The second approach calculated the differences in DNA methylation level for each sample relative to the average DNA methylation level for the six control plants. DNACopy was then run to identify DMRs present in a single sample relative to the control. For both approaches, DMRs were required to exhibit at least 2-fold change in DNA methylation relative to the control

average and include at least three adjacent probes. A list of non-redundant DMRs was determined by combining the coordinates for the DMRs discovered in each of the samples into a single list.

Data Access

Control, hot, cold, and UV stress microarray raw (pair) files were deposited with the National Center for Biotechnology Information GEO under accessions (GSE65266). Tissue culture data files are deposited under accession (GSE56479).

Results

The primary set of biological materials utilized for this study was obtained from inbred B73 sibling plants (**Figure 1**). The inbred B73 was selected due to the availability of a reference genome sequence (Schnable et al., 2009). Sibling B73 seedlings were grown and subjected to control conditions or distinct environmental stresses 14 days after planting. The treatments included 4-h exposure to heat (50°C), cold (4°C) or elevated UV-A/B (60–64 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and were repeated every other day for a total of four treatments. The temperatures were chosen to result in severe, near-lethal, stress to maximize the potential for a response. After the final seedling stress treatment, the plants were grown to maturity using standard greenhouse conditions and DNA was extracted from the last adult leaf of the plant to use for DNA methylation profiling. In B73 the transition from vegetative to floral meristem does not occur until ~28 days after planting (Thompson et al., 2014) and therefore our profiling of DNA isolated from the last adult leaf of the plant is expected to represent the descendants of cells that had been in the meristem at the time of the stress providing the ability to search for mitotically heritable changes in DNA methylation. DNA methylation profiles were obtained for six individuals grown in control conditions, five individuals subjected to cold stress, four individuals subjected to UV stress and three heat-stressed individuals.

The DNA methylation profiles were obtained by performing meDIP followed by hybridization to an oligonucleotide microarray. This method compares the signal for meDIP samples (enriched for methylated DNA) to input DNA to gage the relative level of DNA methylation for any region of interest. This method is quite useful for detecting large differences in DNA methylation for a particular region but is limited in its ability to detect minor differences in DNA methylation (Eichten et al., 2013). In particular, this approach often finds differences in CG or CHG methylation in which one “allele” is highly methylated in these contexts and the other “allele” has low or no methylation in any context.

The DNA methylation profiles obtained for each sample were normalized and compared to each other. In general, the DNA methylation profiles obtained from these samples are quite similar to each other. The majority of the genome exhibits very similar levels of DNA methylation in all control and stress samples. Several approaches were utilized to search for DMRs. The first approach compared all samples from each treatment with all control samples to search for regions that exhibited consistent changes in methylation from a particular treatment.

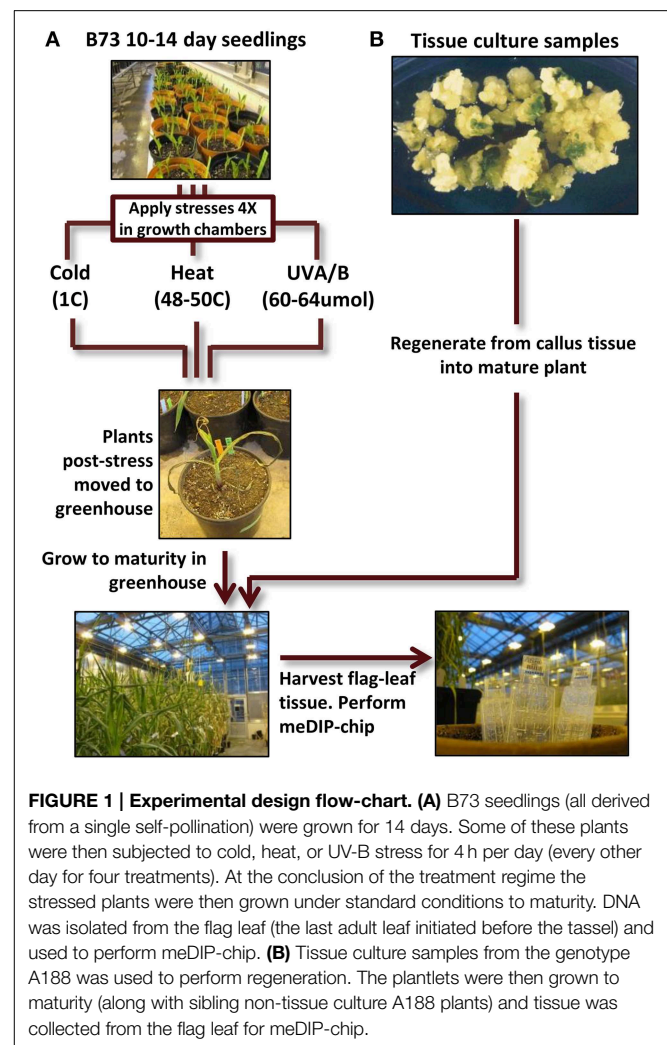


FIGURE 1 | Experimental design flow-chart. (A) B73 seedlings (all derived from a single self-pollination) were grown for 14 days. Some of these plants were then subjected to cold, heat, or UV-B stress for 4 h per day (every other day for four treatments). At the conclusion of the treatment regime the stressed plants were then grown under standard conditions to maturity. DNA was isolated from the flag leaf (the last adult leaf initiated before the tassel) and used to perform meDIP-chip. **(B)** Tissue culture samples from the genotype A188 was used to perform regeneration. The plantlets were then grown to maturity (along with sibling non-tissue culture A188 plants) and tissue was collected from the flag leaf for meDIP-chip.

TABLE 1 | Treatment-specific DMR identification.

Treatment	DMRs	#Hypo-methylated	#Hyper-methylated
Cold	0	0	0
Heat	4	3	1
UV	1	0	1

Very few DMRs were found when comparing all samples from a treatment to the control plants (**Table 1**). The number of DMRs identified using this approach is quite small with none for cold, four for heat and one for UV. This suggests that there are very few, if any, genomic regions that exhibit consistent changes in DNA methylation in response to the stresses used in this experiment.

Although we did not observe evidence for consistent changes in DNA methylation in response to these abiotic stress treatments it is possible that the stress treatments would result in greater rates of DMRs in individual plants. In order to assess whether the stress treatments resulted in a higher “epimutation” rate we attempted to identify DMRs that were present in individual

TABLE 2 | Individual-specific DMR identification.

Sample	DMRs	#Hypo-methylated	#Hyper-methylated
Cold_1	70	10	60
Cold_2	104	66	38
Cold_3	607	3	604
Cold_4	84	83	1
Cold_5	66	1	65
Hot_1	38	32	6
Hot_2	122	69	53
Hot_3	58	5	53
UV_1	33	3	30
UV_2	800	270	530
UV_3	9	0	9
UV_4	199	90	109
Control_1	3	3	0
Control_2	412	295	117
Control_3	284	236	48
Control_4	282	19	263
Control_5	253	2	251
Control_6	58	1	57
Non-redundant	2589	944	1935

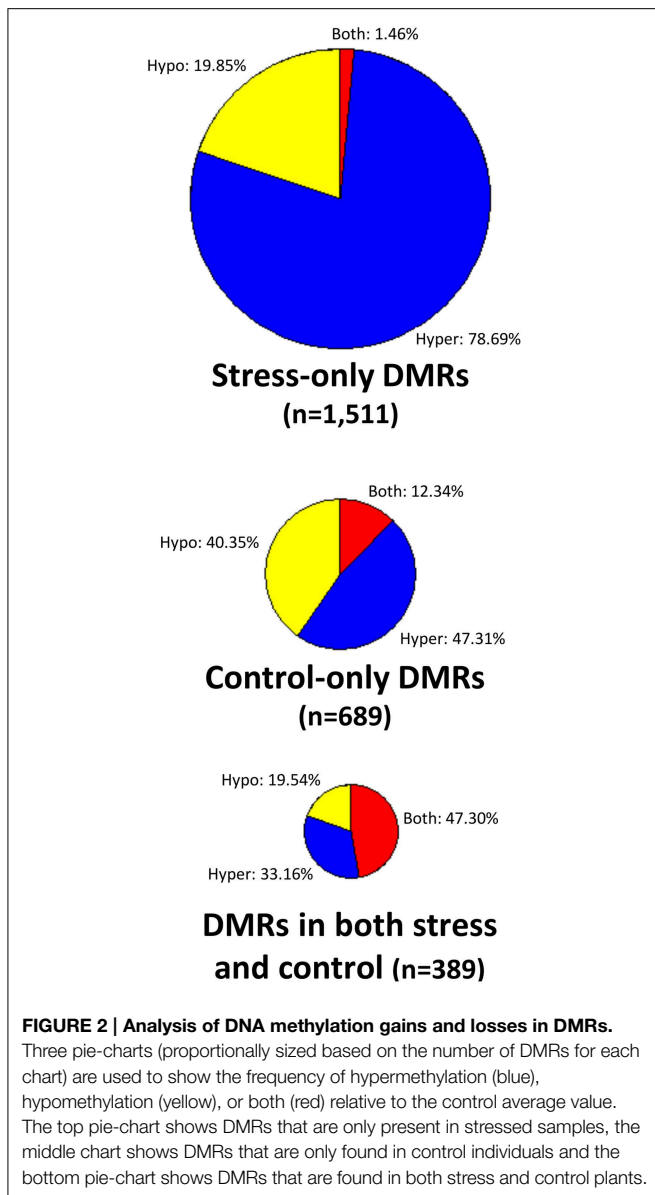
plants. There is an inherent difficulty in identifying plant specific DMRs as we do not have biological replication since each plant it being compared as a single individual. However, our search for DMRs required that multiple probes from the same genomic region exhibit consistent changes in DNA methylation. The individual DNA methylation profiles for each plant were contrasted with the average methylation profile of the six control plants and segments (regions including at least three adjacent probes) that exhibit differences in DNA methylation were identified using DNAcopy (Venkatraman and Olshen, 2007). This allowed us to identify DMRs in a single control plant relative to the average of all controls (stochastic variation) as well as DMRs in individual stressed plants relative to the control average (stress-induced variation). A non-redundant list of DMRs that were identified in at least one individual was generated and further filtered to require a minimum of 2-fold change (Supplemental Table 1). A wide range of significant DMRs for individual plants was observed (Table 2). While there may be some biological variation in the number of DMRs there are also technical aspects that can influence the discovery of DMRs. Hybridization quality can affect signal strength and signal:noise ratios resulting in substantial differences in the numbers of DMRs that are detected.

There are no clear trends in the number of DMRs or the proportion of methylation gains (hypermethylation) and losses (hypomethylation) across samples (Table 2). Overall, hypermethylation (gains compared to control average) was more prominent than hypomethylation in the full set of non-redundant DMRs. However, individual plants sometimes varied in terms of the relative number of hyper- and hypo-methylation events (Table 2). Many of the DMRs were only identified in a single individual (80% of hypomethylated DMRs and 84%

of hypermethylated DMRs). However, some of the DMRs are identified in 2–5 plants (Supplemental Table 1). The DMRs were classified based on whether they exhibit a change only in the stressed plants, the control plants, or whether they change in both stressed and control individuals (Figure 2). If we limit our analysis to DMRs that are found in more than one individual plant ($n = 618$) we find that 63% are found in both control and stress individuals while 23% are observed only in stressed plants and 14% are only observed in control plants. The fact that many of the DMRs that are observed in multiple individuals are observed in both stress and control plants suggests that many of these likely reflect stochastic variation. While there are slightly more examples of DMRs in plants subjected to stress than in control plants this could be an artifact of the experimental design. This study includes 12 stressed individuals and 6 control plants so there is a greater chance for discovery in the stress group than in the control group. Given the sampling of twice as many stress plants, these numbers suggest similar frequencies of rare methylation changes in stressed and un-stressed plants. The analysis of the frequencies of DNA methylation loss and gain in these DMRs suggests some differences between the DMRs found in stress and control plants (Figure 2). The majority of the DMRs only observed in the stressed plants are hypermethylation events. DMRs that exhibit both gains and losses are quite rare in the stressed plants. In the control plants, hypomethylation events are most common.

The location of the DMRs relative to genes and transposable elements (TEs) was assessed (Figure 3). The DMRs were first classified based on the directionality of the methylation change (hypomethylated, hypermethylated, or both relative to the control average) and then based on whether they exhibited changes in stressed plants, control plants or both. In each of these nine groups we calculated the proportion of the DMRs located near (within 500 bp) of genes, TEs, both genes and TEs, or neither (Figure 3). The comparison of the DMRs only observed in stress plants that show either hypermethylation or hypomethylation reveals interesting differences. The hypermethylation DMRs are much more likely to occur near genes and are depleted near TEs. This may suggest differences in DNA methylation gain and loss for TEs relative to genes in stressed samples. Similar trends are observed if this analysis is restricted only to the DMRs that are observed in more than one plant.

Although the DMRs tended to only be observed in a small number of plants it is possible that many of these DMRs show smaller changes in DNA methylation that did not reach the cut-offs used for our DMR calling. In order to assess whether there are quantitative trends in DNA methylation at these regions that are consistent in multiple plants from the same treatment we performed three different hierarchical clustering analyses using the stress-only DMRs, the control-only DMRs and the DMRs found in both stress and control individuals (Figure 4). In each case the relationships between samples are not heavily influenced by the treatments. This suggests that the small number of regions that do show changes in DNA methylation among plants do not show consistent behavior in multiple plants subjected to the same treatment.



A separate clustering was performed that included DNA methylation profiles from individual plants subjected to tissue culture and their appropriate controls (**Figure 5**). In previous work (Stelpflug et al., 2014) tissue culture was performed using the A188 inbred resulting in the generation of multiple R_0 plants. The same tissue (last adult leaf) was harvested from 10 plants subjected to tissue culture and three control sibling plants that were not subjected to tissue culture (**Figure 1**). DNA methylation profiling and DMR calling was performed using the same methods used for the abiotic stress analysis. The A188 genotype was used for these experiments rather than B73 due to the fact that A188 plants are largely successful when processed through tissue-culture whereas B73 performs poorly. In the analysis of these samples we found evidence for consistent alterations of DNA methylation in tissue-cultured individuals relative to controls (Stelpflug et al., 2014). The DMRs from both

studies were combined to generate a non-redundant list of DMRs from tissue culture or abiotic stress. We further filtered this list to remove all regions that exhibit >0.25 difference between the B73 control average and the A188 average control to remove regions that might have genetic variation or genotype-specific differences in DNA methylation. The combined clustering of the DMRs from both abiotic stress and tissue-culture provide evidence for consistent changes within the tissue-culture samples. However, the A188 controls and B73 controls are interspersed with the individuals subjected to heat, cold or UV stress (**Figure 5**). This analysis suggests that we can detect treatment specific effects (tissue-culture) but that the abiotic stresses used in this study did not have substantial treatment effects.

Discussion

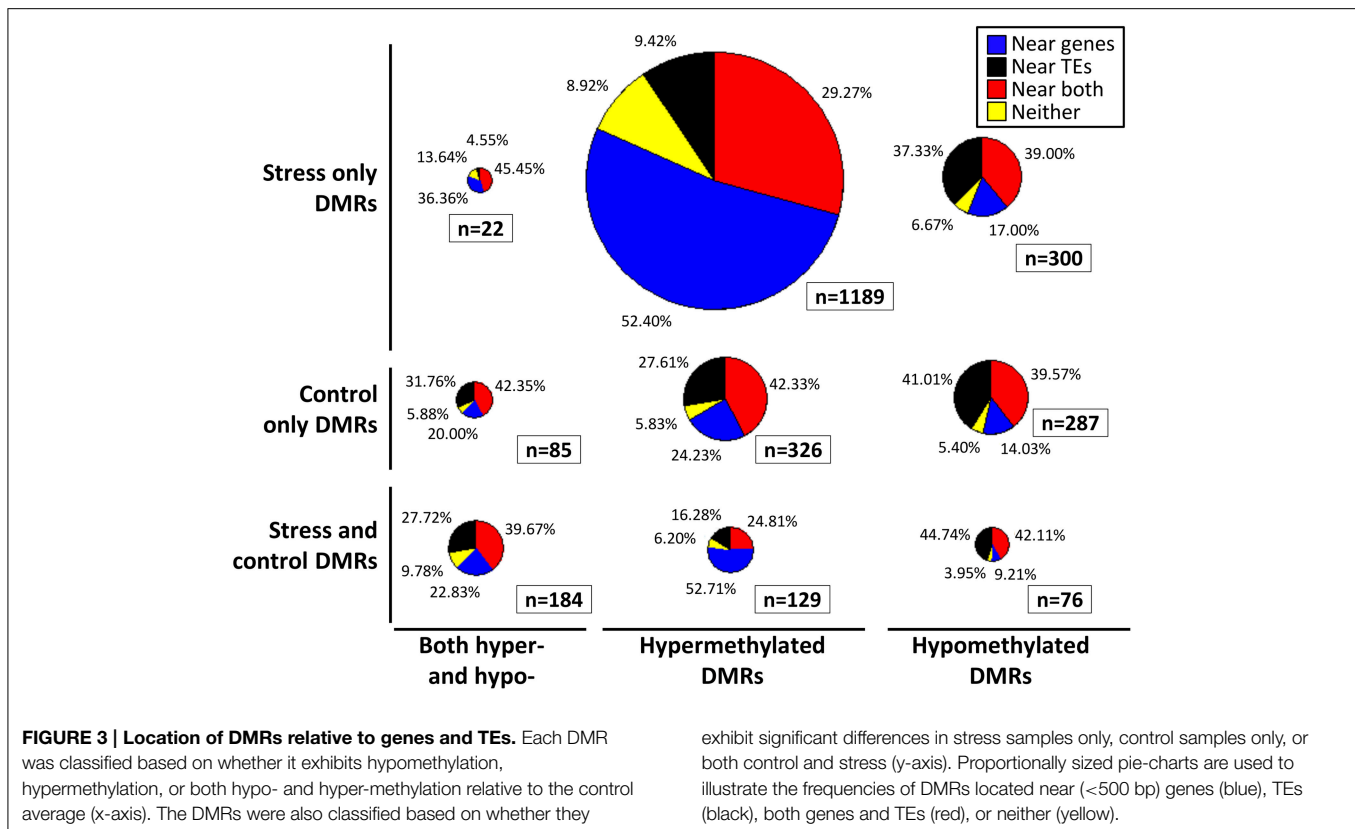
Our experiment was designed to address two potential hypotheses about DNA methylation changes in response to abiotic stress. One hypothesis is that a particular stress will result in consistent DNA methylation alterations. The second hypothesis is that abiotic stress will result in increased variation in DNA methylation. In both cases the expectation is that these changes in DNA methylation will be mitotically heritable and maintained through the life of the plant. Prior to discussing our findings relative to these specific hypotheses it is worthwhile to consider the limitations of our study.

Limitations to Study Design and Interpretation

One limitation is that we are only studying mitotically heritable changes. The tissue that was used (last adult leaf) should be composed of mitotically derived descendants of cells that were still located within the meristem during the stress treatments. It is possible that treatments could result in DMRs that are only transiently formed or only “remembered” in meristematic cells and therefore would not be present in the tissue we sampled. This would reduce our power to detect methylation changes, as they would be present only in some of the cells.

A second limitation arises from the methodology used to profile DNA methylation. Compared to whole-genome bisulfite sequencing (WGBS) the meDIP-array profiling is relatively low-cost and still produces genome-wide scans. The lower cost enabled us to profile a greater number of individuals in this experiment. However, this method also has reduced resolution to detect DMRs, especially if DNA methylation changes are context-specific. There are many possible ways that DNA methylation could change in response to a treatment and we will consider our power to detect these changes in homozygous or heterozygous conditions.

A genomic region that begins as completely unmethylated and then gains DNA methylation in response to abiotic stress (or stochastic events) may be relatively easier to detect, especially in the heterozygous condition as there would be a strong gain of meDIP signal. Initially, these events are likely to be *de novo* DNA methylation events involving small RNA targeting of a DRM methyltransferase (Law and Jacobsen, 2010; Matzke and Mosher, 2014). This will result in moderate levels of DNA methylation in all sequence contexts. If there is substantial gain



in DNA methylation then these regions might be detected as they would be effectively immunoprecipitated. However, if the *de novo* methylation level is low then these regions may not be strongly enriched following meDIP and may not provide enough signal for detection. *De novo* methylation of a region is often followed by stable maintenance of DNA methylation only in CG and CHG contexts catalyzed by MET1 and CMT enzymes, respectively (Law and Jacobsen, 2010). This maintenance DNA methylation often results in quite high levels of CG and CHG methylation and if some plants have triggered this pathway while others have not then this difference would likely be detectable in the experimental procedures we have used.

Losses of DNA methylation might be more difficult to detect, especially in the heterozygous condition. Genomic regions that begin with high levels of DNA methylation on both alleles could lose DNA methylation in one or both alleles. These losses could occur passively due to failure of maintenance DNA methylation activities or could occur actively through action of demethylase enzymes such as ROS1 (Zhang and Zhu, 2012). For both passive and active losses of DNA methylation it is quite possible for events to occur on one allele and not the other. This would result in heterozygosity for DNA methylation levels and these are difficult to detect using meDIP-array analysis. Since both fully methylated and heterozygote individuals contain methylated DNA at the locus you will recover meDIP signal in both and the quantitative differences in signal may not meet our criteria.

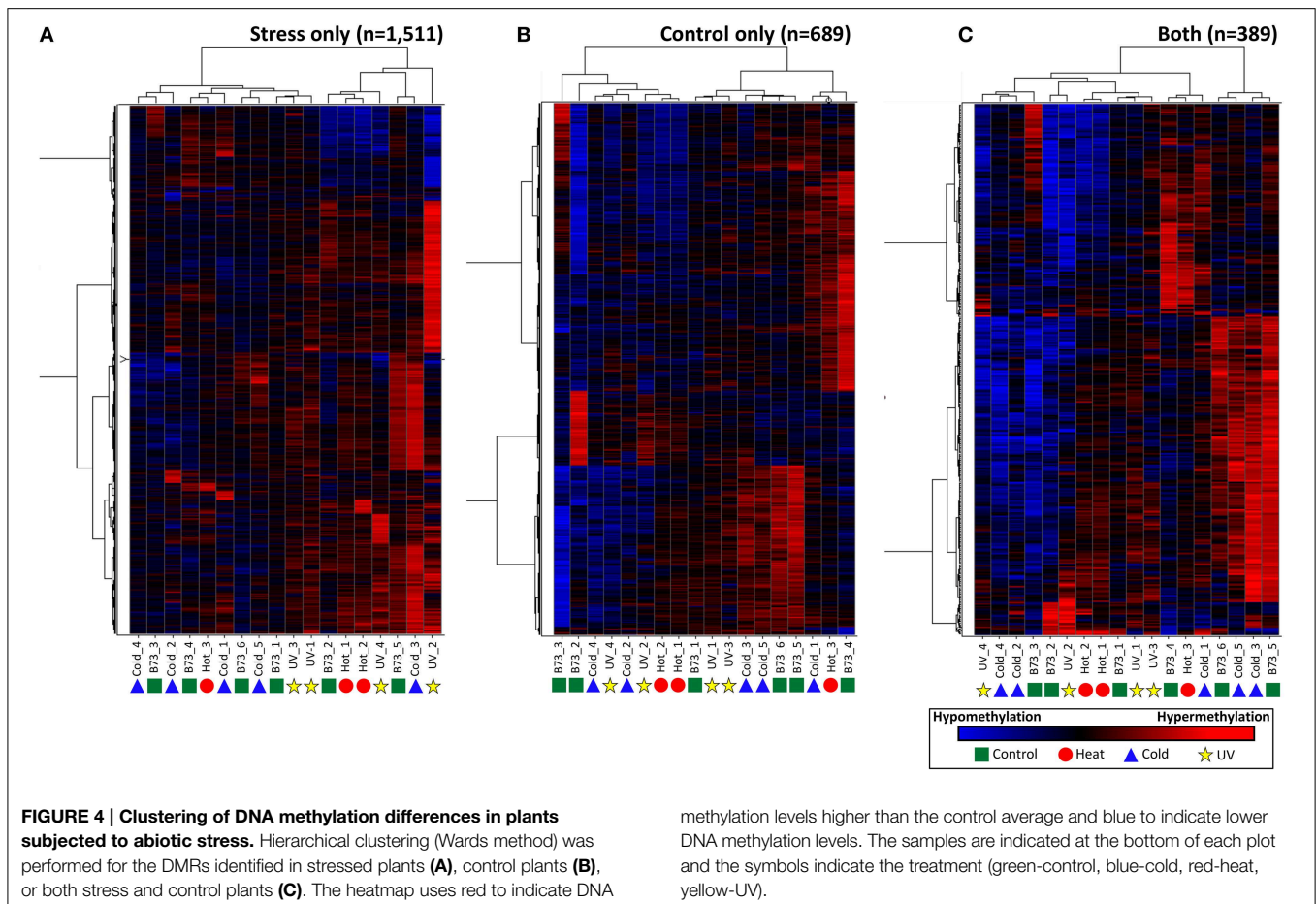
The gain, or loss, of CHH methylation may be difficult to detect using the meDIP approach. If two individuals both contain

highly methylated DNA in the CG and CHG context at a particular locus and only differ in one having CHH methylation there are not likely to be differences in meDIP signal. The DNA from both individuals will be effectively immunoprecipitated and the extra CHH in one individual likely won't result in significantly more immunoprecipitation or signal. This means that our approach will struggle to identify most examples of changes in targeting of *de novo* methylation. Only in cases that go from completely unmethylated DNA to partially methylated CHH (as well as CG and CHG) will we potentially detect the DMR.

Despite the limitations described above, it is worth noting that our approach certainly would be able to detect major changes in DNA methylation patterns. If there was substantial perturbation of genomic DNA methylation this experiment would be able to detect those changes. In addition, if specific genomic regions went from being completely unmethylated to having moderate levels of DNA methylation this would have been readily detected. Similarly, if genomic regions had gone from moderate levels of DNA methylation to being unmethylated at both alleles this also would have been readily detected.

Evaluating Evidence for Consistent Stress-Induced Methylome Perturbations

One hypothesis is that a particular stress, such as heat, would consistently result in specific changes to the methylome. There is evidence that this occurs in tissue-culture in both rice (Stroud et al., 2013) and maize (Stelpflug et al., 2014). However, in



our analysis of cold, heat and UV-stressed maize seedlings we did not find evidence for consistent methylome perturbations. There are very few, if any, DMRs that are consistently observed in multiple individuals subjected to the same stress. Clustering of overall DNA methylation profiles do not find evidence for relationships of different individuals subjected to the same stress with the exception of plants subjected to tissue culture (Figure 5). This may not be that surprising. In order for consistent changes to occur in multiple individuals it would require that DNA methylation, or demethylation, machinery be targeted to specific genomic locations in response to particular environmental cues. Consistent changes to specific loci would also be expected to be homozygous as the methylation machinery would likely be targeted to both alleles. These types of changes should be easier to detect both due to the consistent presence in multiple individuals (providing greater statistical power) and due to the homozygous change (providing greater detection power). However, we did not observe clear evidence for consistent DNA methylation changes following the environmental conditions used in this study.

Evaluating Evidence for Increase Rate of Changes in Methylome following Stress

A second hypothesis is that environmental stresses may result in increased stochastic variation for DNA methylation patterns.

Stress would destabilize the methylation, or demethylation, machinery and this would essentially result in higher “epimutation” rates in stressed plants. This is a more difficult hypothesis to evaluate within the limitations of the current study. Stochastic epimutations are likely to be heterozygous and we would have limited power to detect these changes, especially for DNA methylation losses on only one allele. In addition, due to variance in the signal:noise ratio for the experimental data for different individuals the simple count of the number of DMRs per individual can be difficult to interpret. We did find more DMRs in the stressed plants than in the control plants. However, there are more stressed plants that were evaluated and the control plants were compared to the control average (which they contribute to). One interesting observation is that the relative frequency of hypermethylation events and hypomethylation events differs in the stress and control plants. The stressed plants have an excess of hypermethylated events. These events should be easier to detect in our experimental design and may indicate increased rates of DNA methylation gain in stress plants. Overall, our experiments suggest that there is not a major change in the rate of stochastic methylation changes but do suggest that there might be some differences. The recent study by Jiang et al. (2014) similarly found slight changes in both epimutation and mutation rate in Arabidopsis plants subjected to abiotic stress.

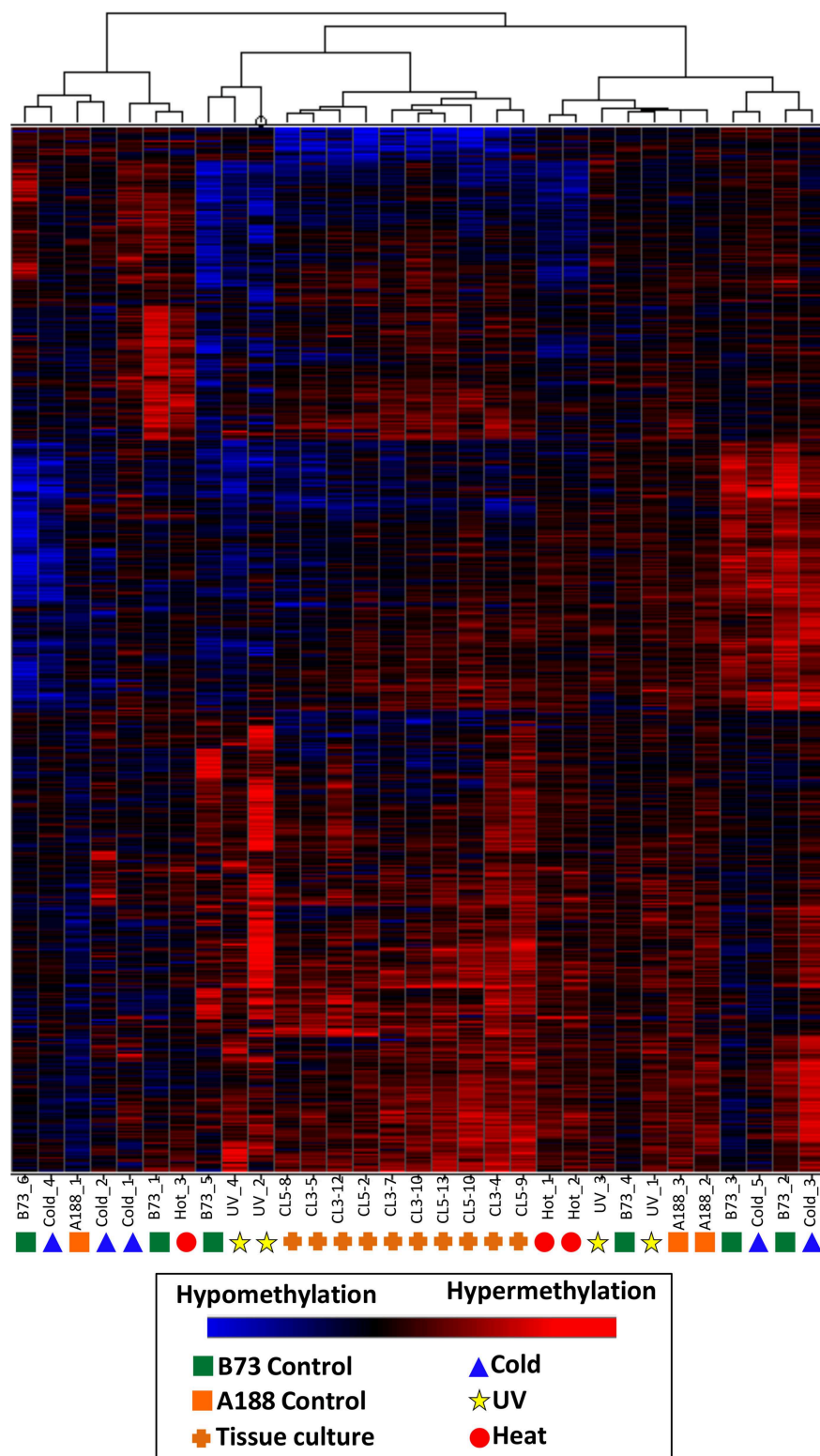


FIGURE 5 | Clustering of DNA methylation differences in plants subjected to abiotic stress or tissue culture. Hierarchical clustering (Wards method) was performed for the DMRs identified in the analysis of individual plants subjected to abiotic stress or tissue culture. These DMRs are the subset that do not exhibit differences in B73 (used for abiotic stresses) relative to A188 (used for tissue culture). The heatmap uses red

to indicate DNA methylation levels higher than the control average and blue to indicate lower DNA methylation levels. The samples are indicated at the bottom of each plot and the symbols indicate the treatment (green squares, B73 control; orange squares, A188 control; blue triangles, cold; red circles, heat; yellow stars, UV; orange pluses, tissue culture regenerants).

Experiments that sample larger numbers of both control and stressed plants will be important in determining the exact rate for increased stochastic methylation changes.

Acknowledgments

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00308/abstract>

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A small RNA mediated regulation of a stress-activated retrotransposon and the tissue specific transposition during the reproductive period in *Arabidopsis*

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Transposable elements (TEs) are key elements that facilitate genome evolution of the host organism. A number of studies have assessed the functions of TEs, which change gene expression in the host genome. Activation of TEs is controlled by epigenetic modifications such as DNA methylation and histone modifications. Several recent studies have reported that TEs can also be activated by biotic or abiotic stress in some plants. We focused on a Ty1/copia retrotransposon, *ONSEN*, that is activated by heat stress (HS) in *Arabidopsis*. We found that transcriptional activation of *ONSEN* was regulated by a small interfering RNA (siRNA)-related pathway, and the activation could also be induced by oxidative stress. Mutants deficient in siRNA biogenesis that were exposed to HS at the initial stages of vegetative growth showed transgenerational transposition. The transposition was also detected in the progeny, which originated from tissue that had differentiated after exposure to the HS. The results indicated that in some undifferentiated cells, transpositional activity could be maintained quite long after exposure to the HS.

Keywords: transposon, small RNA, environmental stress, *Arabidopsis thaliana*, *ONSEN*

INTRODUCTION

Plant genomes contain a large number of transposable elements (TEs) (Feschotte et al., 2002). In particular, retrotransposons that use RNA-mediated amplification constitute a large part of the plant genome (Kumar and Bennetzen, 1999). Retrotransposons are classified into two major subclasses on the basis of their sequence and structural similarity. The first class is long terminal repeat (LTR) retrotransposons, which contain LTRs on both sides. The second class is non-LTR retrotransposons, which include long interspersed nuclear elements and short interspersed nuclear elements (Schmidt, 1999). LTR retrotransposons are further divided into two families according to the order of the coding genes. Ty3/gypsy retrotransposons are enriched in intergenic regions that include centromeric heterochromatin in some plants (Jiang et al., 2003). The second family, Ty1/copia retrotransposons, are conserved in the euchromatic regions of some plant species (Kumar, 1996).

Although all plants contain various TEs, most of these are transcriptionally silenced through epigenetic regulation (Lippman et al., 2003). DNA methylation is one of the major regulators of transposon expression. TEs are activated in DNA hypomethylation mutants (Miura et al., 2001; Kato et al., 2003; Mirouze et al., 2009; Tsukahara et al., 2009), which represents a defense mechanism against ectopic transposon activation. One of the best-studied mechanisms for the regulation of TEs is RNA-directed DNA methylation (RdDM), which regulates TEs through small

interfering RNA (siRNA)-mediated DNA methylation (Wierzbicki et al., 2008; Gao et al., 2010). Higher plants possess specific DNA-dependent RNA polymerases known as RNA polymerase IV (*PolIV*) and RNA polymerase V (*PolV*; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005). *PolIV* produces initial RNA transcripts for RNA silencing and *PolV* transcribes a messenger RNA for siRNA-induced DNA methylation on the target site.

In *Arabidopsis*, an ectopic transcript produced from TEs is transcribed to double-stranded RNA by RNA-dependent RNA polymerase 2 (*RDR2*) and subsequently processed in 24–26 nt siRNAs by *DICER-LIKE 3* (*DCL3*; Zhang et al., 2007b; Mosher et al., 2008). The siRNAs bind to an RNA-induced silencing complex containing *ARGONAUTE 4* (*AGO4*) that interacts with *PolV* to recruit the DNA methyltransferase *DOMAINS REARRANGED METHYLTRANSFERASE 2* (*DRM2*), leading to *de novo* DNA methylation of the target TEs (Cao and Jacobsen, 2002; Matzke and Birchler, 2005).

It remains unknown when the TEs are activated or what triggers TE activation in nature. The answer may lie in accumulated evidence that TEs are activated by environmental stress. Some TEs were activated by abiotic stress in combination with DNA demethylation. For example, in corn, cold stress induced hypomethylation and activated the *ZmM1* element that was similar at the LTR region of the putative retroelement (Steward et al., 2002).

In other cases, the activation of LTR retrotransposons is independent of DNA methylation and is under the control of *cis*-regulatory sequences in the LTRs. The best-characterized examples are the *Tnt1* and *Tto1* retrotransposons in tobacco. *Tnt1* and *Tto1* are activated by biotic stresses, such as tissue culturing, wounding, and pathogen infections, and by abiotic stresses, including salicylic acid and jasmonate (Peschke et al., 1987; Hirochika, 1993; Pouteau et al., 1994; Takeda et al., 1998). The stress-induced activation is controlled by *cis*-acting motifs in the U3 region of the LTR sequence that are similar to those of plant defense genes (Grandbastien et al., 1997; Mhiri et al., 1997; Vernhettes et al., 1997; Takeda et al., 1999).

Recently, a Ty1/copia retrotransposon named *ONSEN* was found to be activated by heat stress (HS) in *Arabidopsis* (Ito et al., 2011). The LTR of *ONSEN* contains a sequence that is recognized by the plant's heat response transcription factor, *HsfA2* (Cavrak et al., 2014). *HsfA2* has a conserved N-terminal DNA-binding domain that binds a heat response element (HRE; Schramm et al., 2006). An electrophoretic mobility shift assay demonstrated that *HsfA2* bound to an HRE in the *ONSEN* LTR (Cavrak et al., 2014), indicating that *HsfA2* was directly involved in transcriptional activation. *ONSEN* is more strongly activated in a mutant that was deficient in a pathway for siRNA biogenesis than in the wild-type plant (WT), indicating that an siRNA-related pathway modulates transcriptional activation of *ONSEN* (Ito et al., 2011). Although heat-induced expression was enhanced by a mutant of DNA methyltransferase (Cavrak et al., 2014), *ONSEN* was not expressed in a hypomethylation mutant or a mutant lacking RdDM component without HS (Ito et al., 2011). This finding indicates that the initiation of the transcriptional activation is independent of DNA methylation.

In addition to transcriptional regulation, TEs may be controlled by transpositional processes in the host plant. A high frequency of new *ONSEN* insertions was observed in the progeny of stressed plants that were deficient in siRNA biogenesis (Ito et al., 2011). *ONSEN* was also expressed in the WT and in other epigenetic mutants subjected to HS; however, transgenerational transposition was not observed in the progeny (Ito et al., 2011). The results suggested that siRNA-mediated pathways regulated transgenerational transposition. Although the mechanism of *ONSEN* activation has been studied at the transcriptional level (Ito et al., 2011), the precise mechanism of transpositional regulation remains unknown. Here, we provide insight into siRNA-mediated regulation and the initiation of transgenerational transposition of *ONSEN*.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWING CONDITIONS

The *Arabidopsis thaliana* plants used in the experiments included WT plants, *npr1* mutants (Herr et al., 2005), and transgenic plants that possessed a full-length LTR (genome position; 4212570–4213146) of *ONSEN* (*AT5G13205*) fused with a GFP gene. The plants were grown on Murashige and Skoog (MS) plates under continuous light at 21°C. For the analysis of high light and oxidative stress, WT, *npr1*, and *hsfA2* T-DNA insertion mutant (Nishizawa et al., 2006) plants were grown on MS at 25°C under continuous light (irradiance 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). In order to

analyze their reactions to short days and long days, plants were grown on MS in pots for 7 days with continuous light at 21°C and moved to a long-day chamber (16 h light and 8 h dark) or a short-day chamber (8 h light and 16 h dark). All WT and mutant plants were *A. thaliana* ecotype *Columbia*.

STRESS TREATMENTS

For HS treatment, 7-day-old seedlings were subjected to a temperature shift from 21°C to 37°C for 24 h except some experiments. After undergoing heat treatment, plants were transplanted to soil and allowed to grow at 21°C. For the high light and oxidative stress treatments, 7-day-old WT, *npr1*, and *hsfA2* plants were exposed to high light at 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 25°C or sprayed with 50 μM methylviologen.

SOUTHERN BLOT ANALYSIS

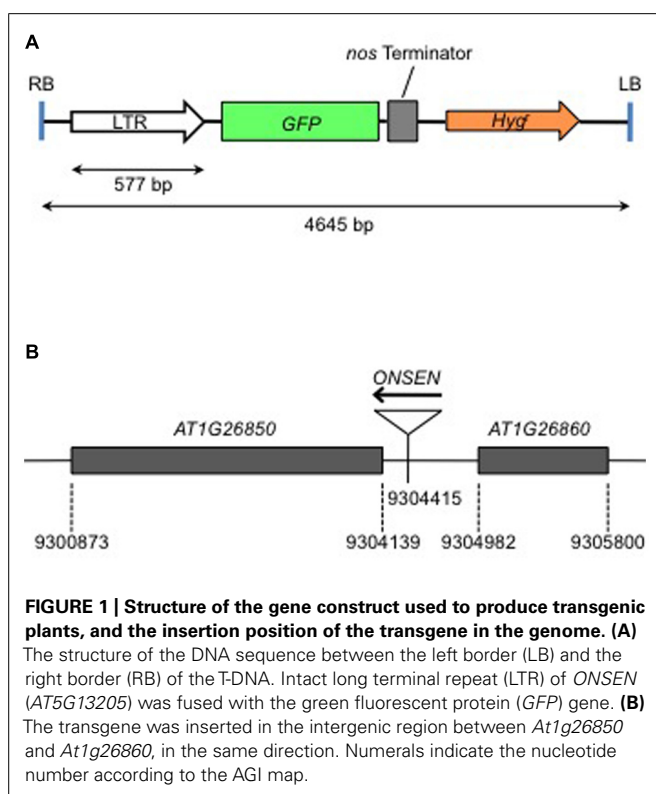
Arabidopsis genomic DNA was isolated using a Nucleon PhytoPure DNA extraction kit (GE Healthcare Life Science). Blotting of genomic DNA was performed as described (Miura et al., 2004). Hybridization signals were detected using a radio-labeled *ONSEN*-specific probe (Table 1) that was generated with the Megaprime DNA Labeling System (GE Healthcare Life Science) in a high-SDS hybridization buffer (Church and Gilbert, 1984).

REAL-TIME PCR

In order to analyze the TE expression resulting from heat treatment, total RNA was extracted from seedlings or leaves using TRI Reagent (Sigma T9424), according to the supplier's recommendations. The five individual plants were pooled prior to RNA extraction. Around 3 to 5 μg of total RNA was treated with RQ1 RNase-free DNase (Promega) and reverse-transcribed using the ReverTraAce qPCR RT Kit (TOYOBO FSQ-101) with an oligo dT primer. In order to quantify the amount of *ONSEN* DNA, genomic DNA was extracted from seedlings or leaves using the DNeasy Plant Mini Kit (QIAGEN 69104), according to the supplier's recommendations. Real-time PCR was performed using the Applied Biosystems 7300 Real Time PCR System with the THUNDERBIRD SYBR qPCR Mix (TOYOBO QPS-201). Three biological repetitions were performed and SD was determined (Figure 2). Similarly, three technical repetitions were performed

Table 1 | Primer sequences.

Experiment	Primer	Sequence (5'-3')
ONSEN probe	ONSEN-F	TAATGTTCCCTTCCAAGTCCC
	ONSEN-R	GCTTGAATGACCCAAGAAGT
ONSEN RT-PCR	COPIA78-4129F_RT	CCACAAGAGGAACCAACGAA
	COPIA78-4300R_RT	TTCGATCATGGAAGACCGG
18S RT-PCR	18Sf-FW	CGTCCCTGCCCTTTGTACAC
	18Sf-RV	CGAACACTTCACCGGATCATT
GFP RT-PCR	GFP_qPCR_F	TGGCTTTGATGCCGTTCTTTTG
	GFP_qPCR_R	CGATTTCAAGGAGGACGGAACAT
Cloning	AT5G13205-GFP-F1	CACCTGAGAAGCAGCAGAAACCAA
	AT5G13205-GFP-R1	AGGGAACATTGTTACTCGCCA



and SD was determined (Figures 4 and 10). Following the high light and oxidative stress experiments, RNA was extracted using the Plant RNA Reagent (Life Technologies). A volume containing 2 μ g of total RNA was subjected to RNase-free DNaseI (Thermo Scientific) and reverse-transcribed using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa 6210A). Quantitative real-time PCR was performed with a LightCycler 96 System (Roche), using the FastStart Universal SYBR Green Master (ROX; Roche). Quantities of DNA were determined from a standard curve and were normalized to the amount of 18s rDNA. At least three biological repetitions were performed and SE were determined.

LASER CAPTURE MICRODISSECTION AND RNA EXTRACTION

Arabidopsis seedlings were fixed in Farmer's fixative (3:1 ethanol:acetic acid) overnight at 4°C. Subsequently, fixation, dehydration, and paraffin infiltration were performed using a microwave processor (H2850 EBS). Paraffin-embedded sections were cut to a thickness of 12 μ m and mounted on a PEN membrane glass slide (Applied Biosystems LCM0522). To remove the paraffin, slides were immersed in Histo-Clear II (National Diagnostics HS-202; 2 \times 5 min) and then air-dried at room temperature. Laser capture microdissection was performed using the ArcturusXT LCM system (Applied Biosystems). Selected areas were captured by an infrared laser onto Arcturus CapSure Macro LCM Cap (LCM0211 Applied Biosystems) and subsequently cut with a UV laser. Total RNA was extracted using the PicoPure RNA Isolation Kit (Applied Biosystems KIT0204) and quantified using the Agilent RNA 6000 Pico Kit (Agilent 5067-1513).

RESULTS

HEAT ACTIVATION OF TRANSGENE

The heat-activation of *ONSEN* is controlled by the promoter in the LTRs that involves a *cis*-regulatory sequence for HREs (Cavrak et al., 2014). HREs are bound by the heat shock transcription factor (HSF) that controls heat-induced genes (Nover et al., 2001). We produced a transgenic *Arabidopsis* that possessed an intact LTR of *ONSEN* (*AT5G13205*) fused with a gene for green fluorescent protein (*GFP*) (Figure 1A). A transgenic plant with a single-copy insert was analyzed for expression of the reporter gene. The insertion was mapped on an intergenic region between *AT1G26850* and *AT1G26860* on chromosome 1 (Figure 1B). The *GFP* signal was analyzed immediately after a group of 10 1-week-old seedlings was subjected to heat treatment and again 3 days after the treatment (Figure 2A). The *GFP* signals were detected throughout the plants that were subjected to the heat treatment, but gradually decayed and were below the detection limit at 3 days after heat treatment (Figure 2B).

To understand the role of siRNA for transcriptional silencing of the transgene, the transgenic plant was crossed with *nrpd1*, a mutant that was deficient in siRNA biogenesis. The resulting transgenic line involved an LTR fused with *GFP* in a *nrpd1* background. The intensity of the *GFP* signal was stronger in the *nrpd1* transgenic line than in the WT line; however, the *GFP* signal fell below the detection level by 3 days after the heat treatment (Figure 2B). The intensity of the *GFP* signals was consistent with the transcription level of *GFP* (Figure 2C) and the endogenous *ONSEN* (Figure 2D). These results indicated that the transcriptional regulation of the transgene was controlled by an siRNA-related pathway that controls endogenous *ONSEN* copies.

ONSEN ACTIVATION BY STRESS

The LTR promoter of *ONSEN* contains a *cis*-regulatory sequence for HREs that binds to a heat-induced transcriptional factor, HsfA2 (Cavrak et al., 2014). HsfA2 was reported to play an important role in the response to environmental stresses, including high light stress (Nishizawa et al., 2006). To analyze the effect of high light stress on *ONSEN*, young seedlings were exposed to high light (800 μ mol photons $m^{-2}s^{-1}$) for 6 h. The *ONSEN* mRNA level started to increase at 1 h and continued to gradually increase to the 6-h point, indicating that *ONSEN* was activated by high light stress (Figure 3A). The expression level of *ONSEN* was considerably higher in the *nrpd1* mutant than in the WT (Figure 3A), suggesting that the transcriptional activation was controlled by siRNA-mediated mechanisms.

To analyze the response of high light stress on *ONSEN* more precisely, we applied N,N'-dimethyl-4,4'-bipyridinium dichloride (Paraquat) to young seedlings. Paraquat produces hydrogen peroxide, which causes oxidative stress to the plant, similar to that caused by high light stress. Approximately 50 7-day-old seedlings were exposed to 50 μ M Paraquat for 6 h. *ONSEN* was activated and at 6 h after the treatment its mRNA levels were increased (Figure 3B). In the *nrpd1* mutant, *ONSEN* was highly activated in response to oxidative stress (Figure 3B). Transcriptional activation was not detected in the *hsfa2* mutant that was subjected to neither high light stress nor to oxidative stress (Figures 3A,B). The results

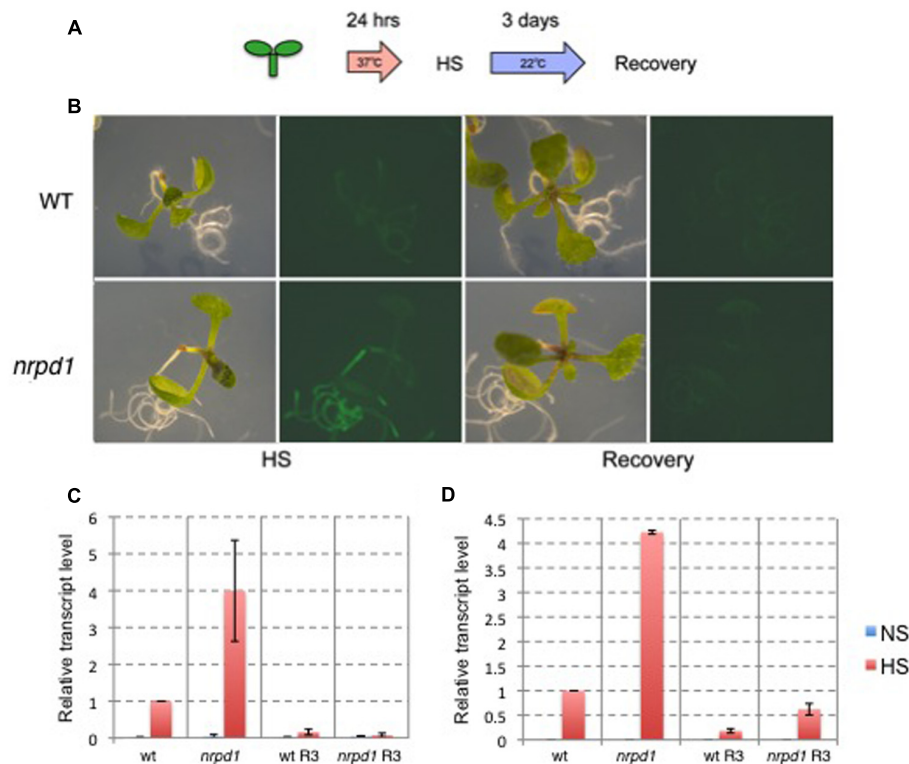


FIGURE 2 | Expression analysis of GFP driven by the *ONSEN* promoter.

(A) Scheme of the heat stress (HS) experiment. **(B)** Photographs of bright field and GFP fluorescence images. Wild-type plants (WT) and *nprp1* plants (*nprp1*) were observed immediately after HS and after the recovery phase (Recovery). **(C)** Relative transcription level of GFP. WT and *nprp1* plants

(*nprp1*) were analyzed immediately after HS and after the recovery phase

(R3). **(D)** Relative transcription level of endogenous *ONSEN*. The same samples as those used in c were used. NS, non-stress control samples. Error bars represent the mean \pm SEM, $n = 3$; values are relative to heat-stressed wild-type (WT HS).

indicated that *ONSEN* was activated by oxidative stress via the HsfA2 transcriptional factor and that the activation was regulated by an siRNA-mediated pathway.

THE QUANTITY OF ACTIVE *ONSEN* AFFECTED TRANSGENERATIONAL TRANSDUCTION

To verify the effect of transcriptional activity and transposition frequency in *nprp1*, 1-week-old seedlings were exposed to 37°C

for 6 h and 24 h, respectively. Quantitative analysis showed that after the 24-h HS, the transcription level had increased by approximately six times in both WT plants and *nprp1* (Figure 4A). In addition, the number of synthesized extrachromosomal DNA copies was six times as high in the WT and 14 times as high in *nprp1* plants (Figure 4B) as in these same seedling groups exposed to the 6-h treatment. New insertions of *ONSEN* were detected in the progeny of *nprp1* that were subjected to HS for 24 h; however,

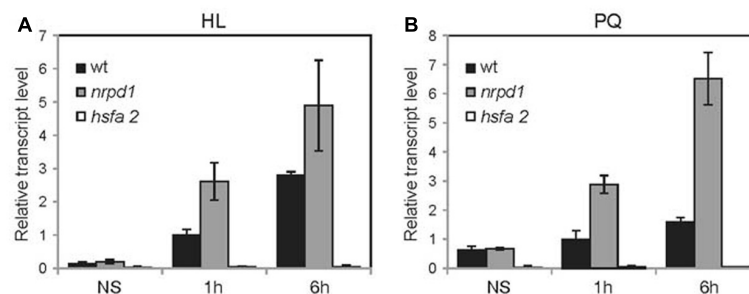
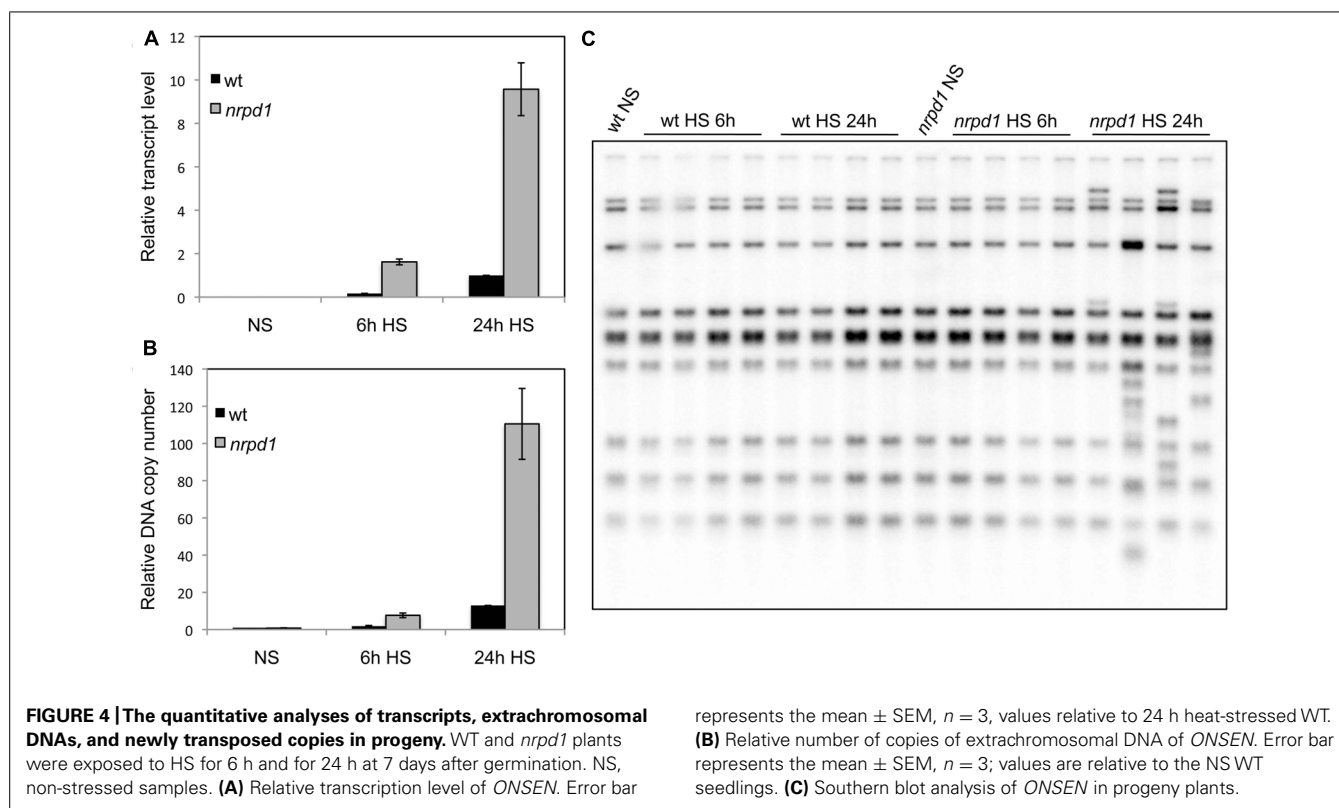


FIGURE 3 | Activation of *ONSEN* by high light stress and oxidative stress. Plants before stress treatment, after continuous stress treatment for 1 h and after continuous stress treatment for 6 h were analyzed. **(A)** Relative

transcription level of *ONSEN* immediately after high light stress (HL).

(B) Relative transcription level of *ONSEN* immediately after Paraquat treatment (PQ). Error bars represent SDs ($n = 3$).



they were not detected in the progeny of *nrpd1* that were subjected to HS for 6 h. A transposition was not detected in the progeny of WT plants subjected to HS (Figure 4C). These results suggested that a high amount of active *ONSEN* was important for transgenerational transposition to occur in the mutant that was impaired in the biogenesis of siRNAs.

HEAT STRESS-INDUCED TRANSGENERATIONAL TRANSPOSITION IN SEEDLINGS

To investigate how the timing of the HS response in young seedlings affected transgenerational transposition, seedlings of the *nrpd1* and WT plants, at ages ranging from 1 to 6 days after germination, were exposed to 37°C for 24 h (Figure 5A). Transgenerational transposition was analyzed in the progenies of heat-stressed plants. The new copies were not detected in the progeny of WT plants (Figure 5B), but they were detected in the progeny of stressed *nrpd1* plants (Figure 5C). Interestingly, transgenerational transposition was observed in seedlings whose parents were stressed at a very early developmental stage, as young as 1 day old (Figure 5C).

TRANSGENERATIONAL TRANSPOSITION WAS INDEPENDENT OF FLOWERING TIME

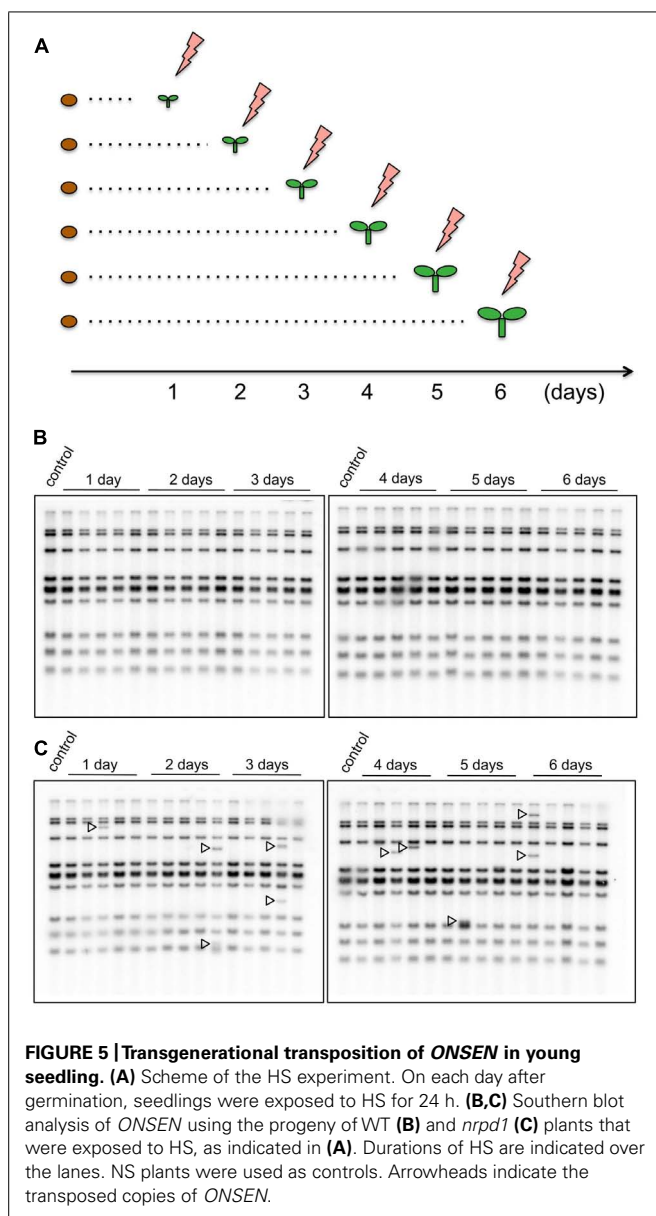
Next, we analyzed whether transposition frequency was affected by flowering time. In *Arabidopsis*, flowering is controlled by photoperiod and is promoted by long day lengths. Under long-day conditions, bolting was observed 30 days after germination in both WT and *nrpd1* mutant plants. Under short-day conditions, bolting

started approximately 50 days and 40 days after germination in WT and *nrpd1* plant, respectively (Figure 6).

Seedlings that were growing under either long-day or short-day conditions were exposed to heat treatment 7, 10, 14, and 21 days after germination. The flowering time of the stressed *nrpd1* was slightly affected by the treatment (Figure 6). No new copy of *ONSEN* was detected in the WT progeny, which were growing under conditions that were neither long day nor short day (Figure 7A). Transgenerational transposition was observed in the progeny of *nrpd1* plants that were grown under either long-day or short-day conditions (Figure 7B). These results suggested that transposition frequency is not affected by flowering time and that the new insertions were transmitted into the reproductive tissue even when 3-week-old *nrpd1* plants were exposed to HS.

STRESS MEMORY COULD BE REGULATED IN UNDIFFERENTIATED TISSUES DURING DEVELOPMENT

To better understand the regulation of *ONSEN*, we observed new insertions of *ONSEN* in the siblings of a single flower on a single branch of a heat-stressed *nrpd1* plant. The pattern of new insertions was similar among the progeny within a single flower, although it differed among flowers on the same branch (Figures 8A,B). To understand the maintenance of heat activation of *ONSEN* during branch development, we analyzed transgenerational transposition in the secondarily produced branches. Surprisingly, new insertions were detected in an *nrpd1* progeny originating from the side shoots that was produced after cutting the initial shoot that was subjected to HS (Figure 9). This result



indicated that the transposition activity could be maintained for a long period of time after HS.

REGULATION OF *ONSEN* BY AN siRNA-RELATED MECHANISM IN UNDIFFERENTIATED TISSUES

To understand the mechanism of the transgenerational transposition of *ONSEN*, we analyzed the transcriptional activity of *ONSEN* in the shoot apex. The shoot apex, including the apical meristem, was fixed in paraffin (**Figure 10A**) and isolated by laser capture microdissection (**Figure 10B**). In *nrpd1* plants, *ONSEN* was highly activated in the shoot apex after heat treatment; however, the activity level returned to the baseline value 5 days after HS. In the WT plant, *ONSEN* was expressed at relatively low levels even after heat treatment (**Figure 10C**). These results indicated that an siRNA-related pathway regulated transcriptional activation of *ONSEN* in the shoot apex.

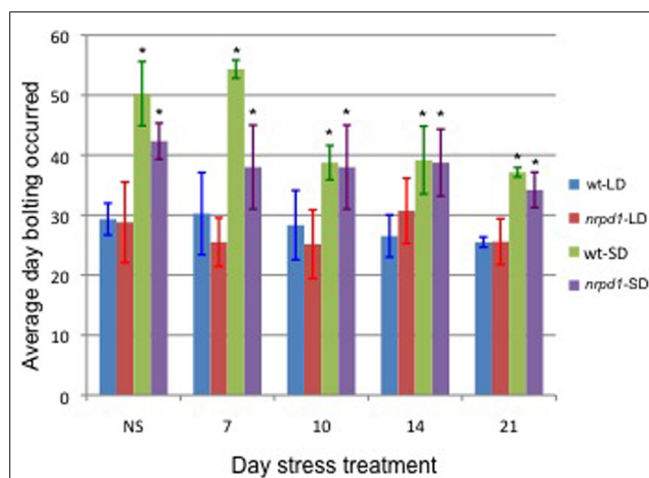


FIGURE 6 | The effect of daylight conditions on flowering time. The average of the day on which bolting occurred was measured in 3 to 8 plants that grew under long-day (LD) or short-day (SD) conditions. Plants were exposed to HS for 24 h at 7, 10, 14, and 21 days after germination. The WT and *nrpd1* plants grew under LD and SD were compared, respectively, using a *t*-test. An asterisk indicates significantly different, $p < 0.05$. NS control plants.

DISCUSSION

Transposons are abundant in plant genomes and show great diversity among species. Large numbers of transposon copies may occur in a host genome as a result of activation of TEs by an external stimulus or environmental stress (Wessler, 1996; Grandbastien, 1998; Capy et al., 2000). We analyzed a Ty1/copia retrotransposon, *ONSEN*, as a model for stress-activated transposon. *ONSEN* is a relatively young element with conserved LTR sequences and functional coding genes in *Arabidopsis* (Ito et al., 2011). The LTR sequence contains conserved HREs that become functional stress-responding promoters of *ONSEN* (Cavrak et al., 2014). It is not known how *ONSEN* acquired the stress-responding promoter in the LTR; however, after a TE has gained a functional promoter that responds to environmental stress, the number of copies of activated TE may be amplified in the host genome when environmental changes occur in nature.

We analyzed the heat-induced promoter of *ONSEN* using a reporter gene assay. Gene expression was enhanced in a mutant that was deficient in siRNA biogenesis. Reduction of DNA methylation in the LTR has been shown to favor the HS response (Cavrak et al., 2014). However, we showed that the activity level of the transgene gradually declined and was no longer detected after 3 days, indicating that the activation of *ONSEN* was initially regulated by transcriptional factors and that the resilencing of *ONSEN* was controlled independently of RdDM. In *Arabidopsis*, heat-activation of several repetitive elements can occur without epigenetic changes such as DNA methylation and histone modifications but is accompanied by heterochromatin decondensation (Pecinka et al., 2010). The transcriptional silencing of the activated elements delayed in mutants of CAF-1 which loads nucleosomes onto replicated DNA suggested that CAF-1

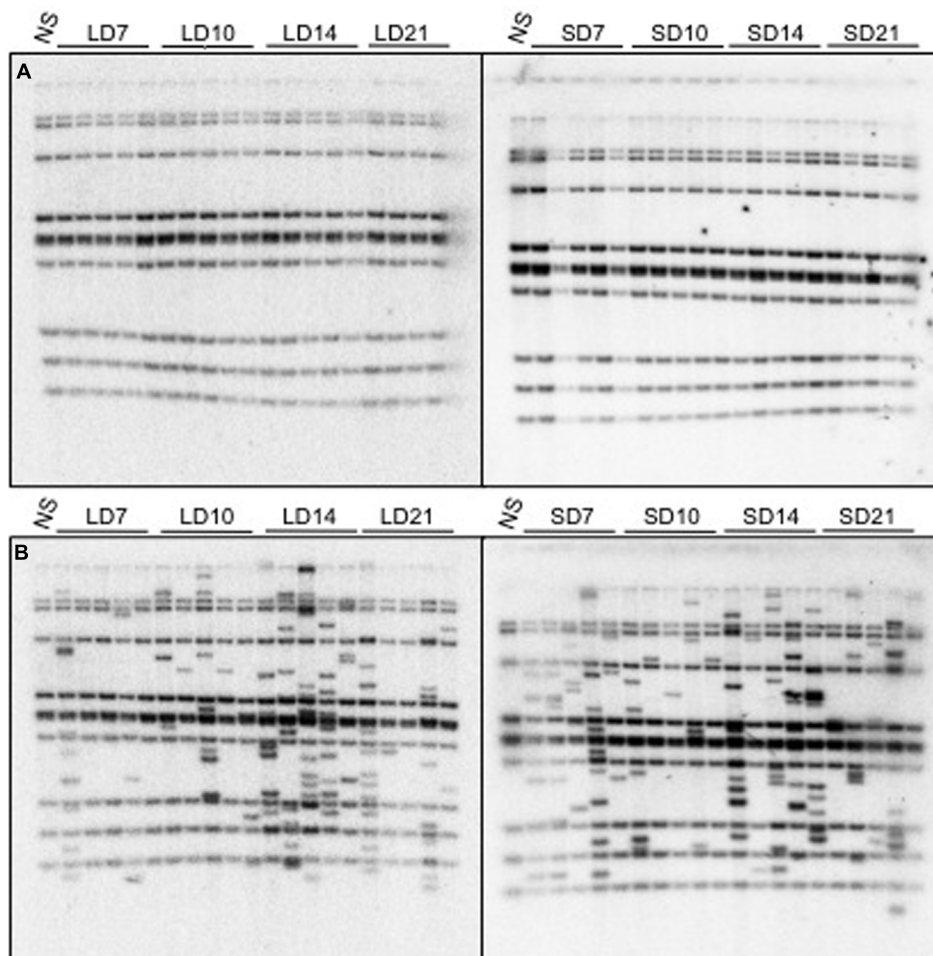


FIGURE 7 | Transgenerational transpositions in the WT (A) and *nrpd1* (B) plants. Data for plants grown under long-day (LD) and short-day (SD) conditions are shown in the left panels and right panels, respectively. 7, 10, 14, and 21 denote plants that were exposed to HS for 24 h at 7, 10, 14, and 21 days after germination, respectively. NS, non-stressed plants.

was important for efficient restoration of silencing after HS (Pecinka et al., 2010). Recently, it is reported that a chromatin-remodeling factor, Decrease in DNA Methylation 1 (DDM1) and Morpheus Molecule 1 (MOM1) act redundantly to restore silencing after HS in *Arabidopsis* (Iwasaki and Paszkowski, 2014). The heat-induced transcriptional activation of some genes in *ddm1mom1* double mutants persisted longer than in WT and was transgenerationally inherited. These findings indicated that heat-induced chromatin changes might play a role for the rapid resetting of *ONSEN* expression in *nrpd1* with impaired siRNA repression.

Transposable elements could be activated in a harsh environment and could affect the host genome. For example, in *Arabidopsis*, activated *Athila* retrotransposons produce a small RNA that regulates a stress-response gene, *OLIGOURIDYLATE BINDING PROTEIN 1* in trans (McCue et al., 2012). We determined that *ONSEN* was activated not only by HS but also by oxidative stress. Activity levels were enhanced in the mutant that was deficient in siRNA biogenesis, indicating that the

activation was controlled by RdDM. The activation of *ONSEN* was not detected in the mutant lacking heat-inducible transcription factor, suggesting that activation of *ONSEN* induced by oxidative stress is regulated by the same pathway as heat-induced activation.

The mobility of *ONSEN* was regulated tightly by the siRNA-mediated pathway. In a mutant of siRNA biogenesis, a transgenerational transposition was observed, indicating that *ONSEN* transposition was transmitted to a reproductive cell. In this report, we analyzed whether transcriptional activation timing was an important factor for transposition frequency during plant development. Transposition occurred when the plant was heat-stressed during a period of the developmental phase. The same transposition pattern on the progeny within a single flower indicated that some transposition occurred prior to flower differentiation. Further, the same transposition pattern among the progenies from different branches indicated that some transposition occurred before branch development. Conversely, the different transposition pattern in

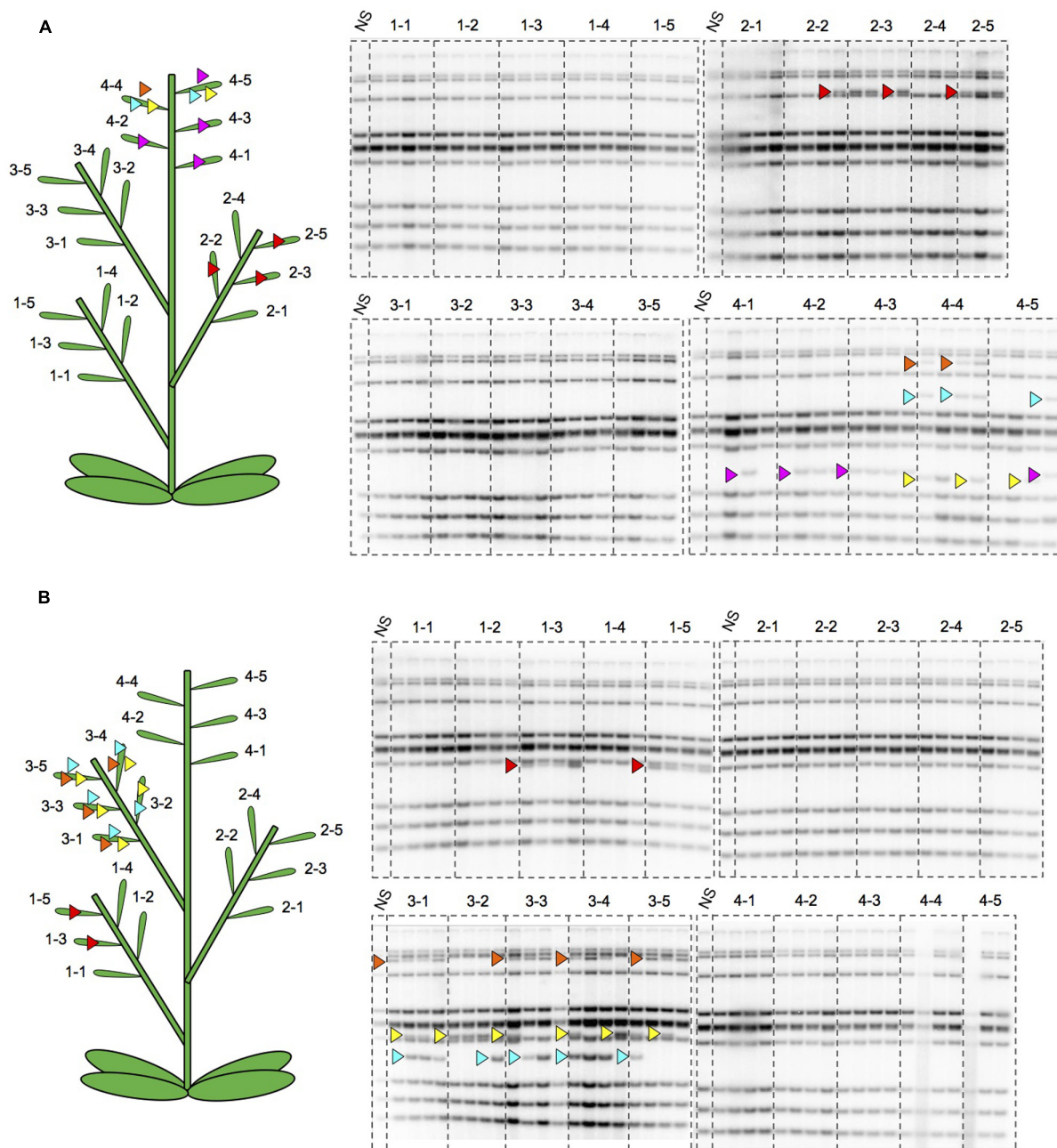


FIGURE 8 | Southern hybridization of *ONSEN* using the progeny of heat-stressed plants. (A) DNAs were extracted from the progeny of one *nrpd1* plant. Numbers over lanes indicate the seed pods having seeds to grow progeny. Four progeny from the same seed pod were analyzed. The location of each seed pod on the parent plant is

shown on the illustrated plant. Arrowheads indicate the new copies of *ONSEN* and the same color of arrowhead denotes that they transposed to the same loci. **(B)** DNAs were extracted from the progeny of another *nrpd1* plant. Symbols are the same as those used in **(A)**.

the progeny of adjacent flowers indicated that transposition occurred just before flower bud initiation. The observation that transposition occurred in the progenies produced from flowers that were differentiated after HS indicated that transposition activation might be maintained over a long period of time, although the transcriptional activation was transient.

One question that remains to be resolved concerns the regulatory mechanism of the transposition that occurred during the period of flower differentiation. One possibility is that transposition requires HS during the period of inflorescence meristem formation. We found that transposition occurred when HS was applied to 1 day-old seedling in which the inflorescence meristem has not been formed, suggesting that the presence of inflorescence

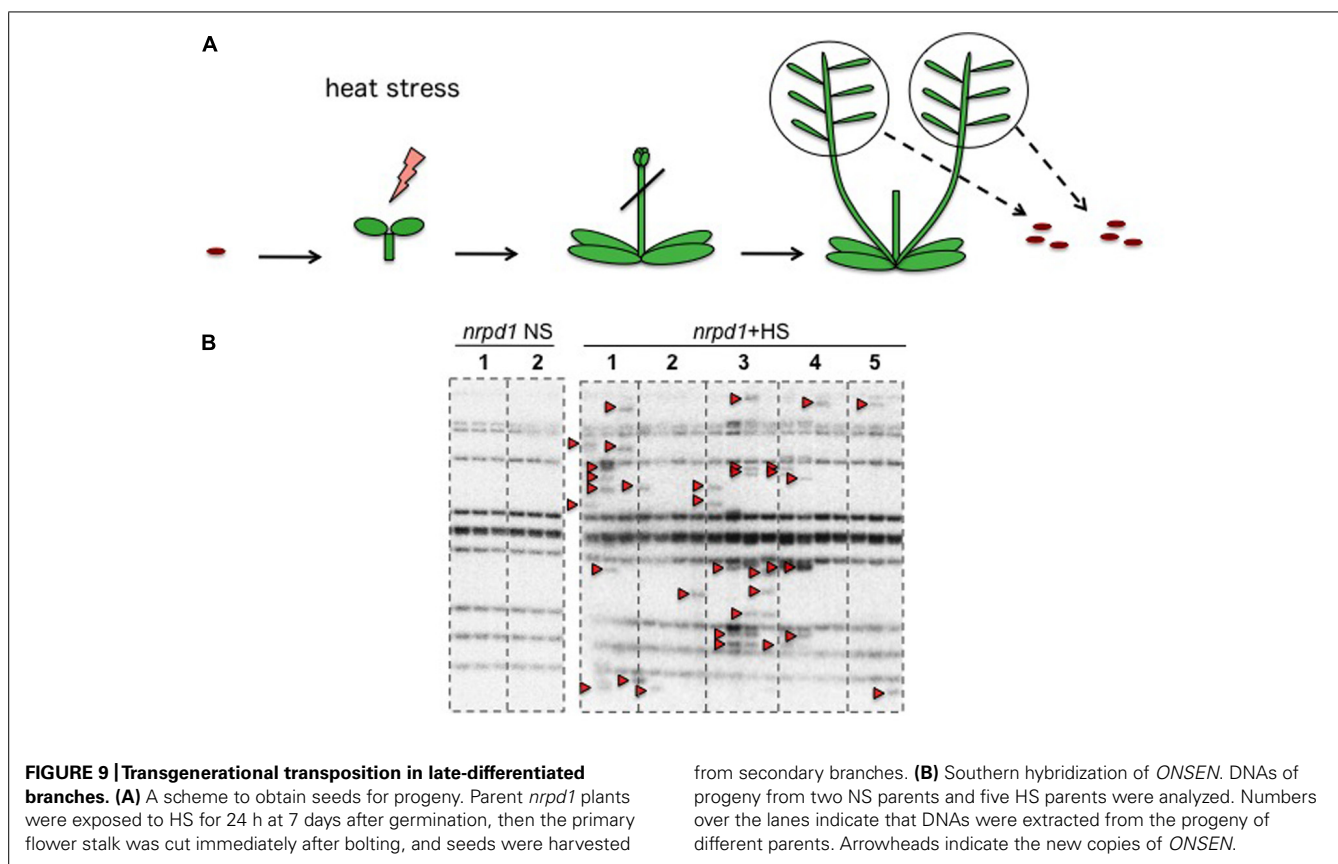


FIGURE 9 | Transgenerational transposition in late-differentiated branches. (A) A scheme to obtain seeds for progeny. Parent *nrpd1* plants were exposed to HS for 24 h at 7 days after germination, then the primary flower stalk was cut immediately after bolting, and seeds were harvested

from secondary branches. **(B)** Southern hybridization of *ONSEN*. DNAs of progeny from two NS parents and five HS parents were analyzed. Numbers over the lanes indicate that DNAs were extracted from the progeny of different parents. Arrowheads indicate the new copies of *ONSEN*.

meristem cells is not necessary for HS-induced transposition. Another possibility is that the activity levels of *ONSEN* may be maintained in the shoot apical meristem and transposition may have occurred at the point when the cell's fate changes during the reproductive growth of a stressed mutant. A further possibility is that *ONSEN* RNA was maintained in the shoot apical meristem during plant development. We found that the transcriptional activity levels of *ONSEN* returned to baseline levels within 5 days in the shoot apex of both WT and *nrpd1* mutant plants. This result indicated that neither the activity level of *ONSEN* nor *ONSEN* RNA was maintained in the shoot apex during plant development. We could not exclude the possibility that the transcriptional activity of *ONSEN* was sustainable only in specific tissues, such as the shoot apical meristem of *nrpd1* plants.

The transposition of *ONSEN* during flower differentiation might require an active mark in addition to the transient activation by HS. The transposition of *ONSEN* was independent of DNA methylation and *ONSEN* was not transposed in *ddm1* hypomethylation mutation subjected to HS (Ito et al., 2011). The HS might induce active chromatin in *ONSEN*; however, subsequently, siRNA could induce *ONSEN* as a silenced chromatin. A transgenerational transposition of heat-activated *ONSEN* was not observed in a mutant deficient in histone H3K9 methyltransferases (*SUVH4/KRYPTONITE*) (Ito et al., 2011), indicating that the regulation of transposition was independent of histone H3K9 dimethylation. Histone H3K27 trimethylation

(H3K27me3) is associated with gene repression and the target genes are tissue-specifically activated during differentiation processes or induced by abiotic or biotic stresses in *Arabidopsis* (Zhang et al., 2007a). H3K27me3 dynamically regulated the target gene expression during plant differentiation and the targets were enriched in TEs in meristem indicating that stem cells must be protected from TE activation to form germline (Lafos et al., 2011). *ONSEN* was a target of H3K27me3, however, the methylation level was not significantly different in meristem and leaves in WT (Lafos et al., 2011). It is interesting to know whether HS in *nrpd1* mutant could affect the modification of H3K27me3 on *ONSEN* in a tissue-specific manner.

In *nrpd1* mutant plants, HS could change chromatin structures, thereby releasing *ONSEN* transposition, and the active chromatin state could be maintained until the period of flower differentiation. A large-scale reorganization of chromatin was observed during floral transition in *Arabidopsis* (Tessadori et al., 2007). The pericentric heterochromatin reduced prior to bolting and recovered after elongation of the floral stem. Also decondensation of chromatin in gene-rich regions coincided with the floral transition. We still do not know the meristem-specific regulation of transposition during floral transition; however, transposon silencing in plant germ cells has been reported in *Arabidopsis* (Slotkin et al., 2009; Olmedo-Monfil et al., 2010; Ibarra et al., 2012). It is worth noting that chromatin decondensation was not observed in nuclei from meristematic tissue after HS (Pecinka

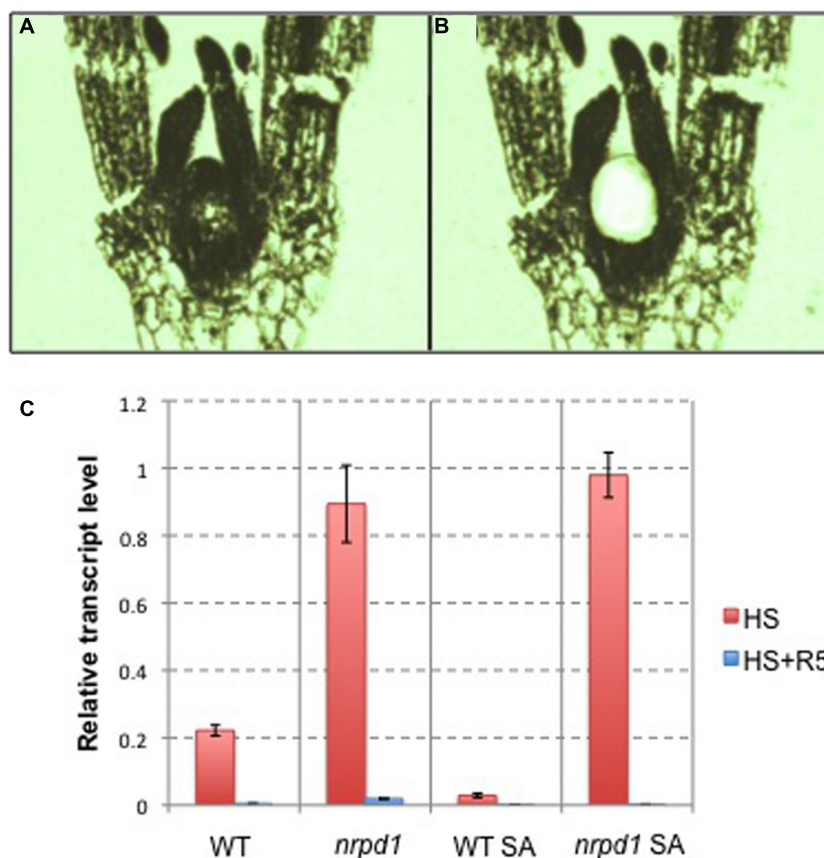


FIGURE 10 | Tissue-specific expression of *ONSEN*. (A) A paraffin section of a young seedling. (B) A paraffin section of a young seedling after the shoot apex was isolated by laser capture microdissection. (C) Relative transcription level of *ONSEN* in young leaf tissue and the shoot apex immediately after being subjected to

HS and 5 days after the heat-stressed (HS + R5). WT: hypocotyl tissue in WT plants. *nprp1*: hypocotyl tissue in *nprp1* mutant plants. WT SA: the shoot apex in WT plants. *nprp1* SA: the shoot apex in *nprp1* mutant plants. Error bars represent the mean \pm SEM, $n = 3$; values are relative to heat-stressed WT.

et al., 2010). Recently, it has been reported that RdDM is function specific in the shoot apical meristem and reinforces the silencing of TEs during early vegetative growth (Baubec et al., 2014). These findings demonstrated the importance of TE regulation during vegetative growth prior to the formation of the next generation.

It is also worth noting that the progeny of heat-stressed *Arabidopsis* had fewer but large leaves and tended to bolt earlier to increased plant biomass (Migicovsky et al., 2014). In the stressed progeny, the expression of *HsfA2* and *ONSEN* was elevated with decreasing of global DNA methylation. The transgenerational phenotypic and epigenetic changes were partially deficient in the Dicer-like mutant, however, *ONSEN* expression increased in the progeny of heat-stressed plants regardless of mutant type (Migicovsky et al., 2014). It will be worthwhile to investigate the mechanism by which the stress memory is maintained during plant development.

In this study, siRNA-mediated regulation functioned to control transposons that were ectopically activated by environmental stress. Plant has evolved the regulation mechanisms that were

independent of suppression by constitutive heterochromatin. The regulation was important during the process of plant development and may have a function in undifferentiated cells.

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Horizontal Transfer of Small RNAs to and from Plants

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Genetic information is traditionally thought to be transferred from parents to offspring. However, there is evidence indicating that gene transfer can also occur from microbes to higher species, such as plants, invertebrates, and vertebrates. This horizontal transfer can be carried out by small RNAs (sRNAs). sRNAs have been recently reported to move across kingdoms as mobile signals, spreading silencing information toward targeted genes. sRNAs, especially microRNAs (miRNAs) and small interfering RNAs (siRNAs), are non-coding molecules that control gene expression at the transcriptional or post-transcriptional level. Some sRNAs act in a cross-kingdom manner between animals and their parasites, but little is known about such sRNAs associated with plants. In this report, we provide a brief introduction to miRNAs that are transferred from plants to mammals/viruses and siRNAs that are transferred from microbes to plants. Both miRNAs and siRNAs can exert corresponding functions in the target organisms. Additionally, we provide information concerning a host-induced gene silencing system as a potential application that utilizes the transgenic trafficking of RNA molecules to silence the genes of interacting organisms. Moreover, we lay out the controversial views regarding cross-kingdom miRNAs and call for better methodology and experimental design to confirm this unique function of miRNAs.

Keywords: miRNA, siRNA, horizontal gene transfer, plant-microbe interactions, controversy of cross-kingdom miRNA

INTRODUCTION

The fundamental concept of gene transfer is that it occurs from parents to offspring. In addition to this vertical transfer, horizontal gene transfer has also been shown to exist in bacteria and simple eukaryotes (Gogarten et al., 2002; Anderson, 2009). Recently, studies have indicated that genes from viruses, prokaryotes and fungi can be transferred to higher species, a phenomenon that has received a great attention (Yue et al., 2012; Crisp et al., 2015). As the products of gene transcription, small RNAs (sRNAs) have been reported to move horizontally between different species. sRNAs of approximately 19–25 nucleotides in length belong mainly to two classes: microRNAs (miRNAs) and siRNAs. Acting as regulatory molecules, sRNAs are involved in a wide range of biological processes that are essential for organ morphogenesis, genome modification, and adaptive responses to biotic and abiotic stresses (Reinhart et al., 2002; Carrington and Ambros, 2003; Lai, 2003; Bartel, 2004). In plants and animals, sRNAs direct the cleavage of endogenous mRNAs or repress their translation (Hamilton et al., 2002; Llave et al., 2002). In addition, sRNAs protect plants and animals from viral infections through the RNA interference (RNAi) system (Wang et al., 2004). It is believed that RNAi also functions in communication among different kingdoms. Recently, both animals and plants have been reported to exchange sRNAs with parasites, pathogens, or symbiotic organisms.

Many studies have reported the introduction of cross-kingdom sRNAs between animals and parasites. For example, miRNAs traffic from human sickle cells to malarial parasites (LaMonte et al., 2012) and from helminth nematodes to mouse cells (Buck et al., 2014). However, compared with animals, trafficking of sRNAs has not been widely observed between plants and other organisms. Additionally, plant sRNAs are mobilized through the phloem and are carried to distinct target cells, where the sRNAs induce a reduction of gene expression (Molnar et al., 2010). Therefore, it is of great value to investigate the mobility of sRNAs between different species.

According to computational analysis and experimental validation, certain types of plant-associated sRNAs have been shown to cross kingdoms and play a role in improving immunity and the defense against viruses or the aggravation of viral symptoms. In this report, we describe the processes and effects of plant-derived miRNAs that move to animals/viruses and microbe-derived siRNAs that move to plants, including host-induced gene silencing (HIGS) system that utilize sRNAs to silence parasite genes in plants. Moreover, we discuss the competing view of cross-kingdom miRNAs and introduce the main techniques employed to measure exogenous miRNAs, and call for better methodology and experimental design to confirm this unique function of miRNAs.

ROLES OF HORIZONTALLY TRANSFERRED miRNAs

miRNAs Transferred from Plants to Animals

Dietetically absorbed plant miRNAs have been confirmed to exist stably in human plasma (Liang et al., 2014a). Thus, it is an intriguing question whether these evolutionarily conserved plant miRNAs can enter into mammalian cells and exert physiological functions. Indeed, it has been demonstrated by high-throughput sequencing that certain miRNAs from plants such as *Zea mays* (*Z. mays*), *Arabidopsis thaliana* (*A. thaliana*), *Oryza sativa* (*O. sativa*), and *Citrus trifoliata* (*C. trifoliata*) can exist stably in human plasma and breast milk exosomes, including 35 miRNAs from 25 MIR families (Lukasik and Zielenkiewicz, 2014). Targets of the aforementioned miRNAs included the mRNAs of proteins associated with transcription factors (e.g., low-density lipoprotein receptor, LDLR), immune system functions (e.g., zinc finger e-box-binding homeobox 1, ZEB1), saccharometabolism (e.g., glycogen debranching enzyme, GDE) and hormone responses (e.g., melanocortin receptor 4, MC4R; **Table 1, Figure 1**). Due to the vital role of breast milk in infant growth and nutrition, it is of great value to explore the effects of foreign miRNAs on infants. It has been proven that plant miRNAs are consistently present in the umbilical cord blood and amniotic fluid of humans (Li et al., 2015). This suggests that those plant miRNAs may transfer through the placenta to the fetus. Moreover, a greater number of immune-related miRNAs have been detected in the colostrum than in mature milk (Gu et al., 2012). Therefore,

certain immune-related exogenous miRNAs that may exist in the colostrum are expected to influence an infant's immune system.

In another study, Zhang et al. (2012a) demonstrated that single-stranded mature miRNAs present in rice can exist stably in the sera and tissues of various animals and humans. One of these miRNAs, osa-miR168a, has been shown to be selectively packed into microvesicles (MVs; Liang et al., 2010) that were shed from intestinal epithelial cells and then released into the circulatory system. These MVs efficiently delivered the miRNAs into recipient cells, in which osa-miR168a suppressed the expression of a target gene, low-density lipoprotein receptor adapter protein 1 (LDLRAP1), in the livers of humans and mice, thereby decreasing the removal of low-density lipoprotein from the plasma (**Table 1, Figure 1**). In addition to miR168a, another type of plant miRNA, miR172, has been observed in the stomach, intestine, serum, and feces of mice after being fed total RNA extracted from *Brassica oleracea* (Liang et al., 2014b), which suggests that plant miRNAs can survive in the circulatory system and gastrointestinal (GI) tract in mice. Interestingly, synthetic tumor suppressor miRNAs that mimic plant miRNAs can be absorbed by GI tract and functions in reducing tumor burden of mice (Mlotshwa et al., 2015). This method could be utilized to produce edible plants that contain therapeutic tumor miRNAs, which may be applied as clinical small molecules for patient treatment.

miRNAs Transferred from Plants to Viruses

In addition to plant miRNAs that can transfer into the bodies of humans and mice, there are other types of miRNAs that have been speculated to move in a trans-kingdom manner from plants to viruses. Feng and Chen (2013) predicted a total of 38 and 37 tomato miRNA/miRNA* sequences that mostly shared high complementarity with the open reading frames (ORFs) of the genomic RNAs of CMV-Fny (severe subgroup 1A strain) and CMV-Q (mild CMV subgroup strain), respectively, which may result in repression of translation or the cleavage of target genes. Importantly, some of these genes act in CMV replication (e.g., CMV protein 2a) and movement (e.g., CMV protein 3a; **Table 1, Figure 1**). It has been assumed that plants utilize the mechanism of post-transcriptional gene silencing (PTGS) to prevent the replication and spread of CMV virions. Similar results have been obtained in *Tomato leaf curl New Delhi virus* (ToLCNDV), in which six encoded ORFs of DNA-A and DNA-B were shown to be targeted by eight miRNA/miRNA* sequences (Naqvi et al., 2010; **Table 1**).

Computational prediction alone cannot demonstrate that certain plant miRNAs crossover to viruses. Plant miR2911, the only miRNA that exists stably in honeysuckle decoction (HS decoction) due to its special high G-C content, has been shown to target the genes of *Influenza A viruses* (IAVs) with the help of MVs in humans and mice (Zhou et al., 2014). Drinking HS decoction results in a significant increase in miR2911 levels in the plasma and lungs of mice. Plant miR2911 can directly bind to the target genes *PB2* and *NSI*, which are essential for influenza replication, thereby inhibiting their amplification (**Table 1, Figure 1**). Notably, the transport of miR2911 has been

TABLE 1 | Trans-kingdom small RNAs.

Number/Name of sRNAs	Derivation	Target species	Target genes/ORFs	Reference
35 miRNAs (e.g., zma-miR156a, ath-miR319b, osa-miR444.2, ctr-miR167)	<i>Z. mays</i> , <i>A. thaliana</i> , <i>O. sativa</i> , <i>C. trifoliata</i> , etc.	<i>H. sapiens</i>	LDLR, ZEB1, GDE, MC4R, etc.	Lukasik and Zielenkiewicz, 2014
38 miRNA/miRNA* (e.g., miR171b*, miR156)	<i>S. lycopersicum</i>	CMV-Fny	2a, 3a ORFs	Feng and Chen, 2013
37 miRNA/miRNA* (e.g., miR171b*, miR395)	<i>S. lycopersicum</i>	CMV-Q	2a, 3a ORFs	Feng and Chen, 2013
8 miRNA/miRNA* (e.g., miR1918, <u>miR156b*</u> , miR164a, miR172b*, miR166a*)	<i>S. lycopersicum</i>	ToLCNDV	AC1, <u>AC2</u> , <u>AC3</u> , AV1, AV2, BV1 ORFs	Naqvi et al., 2010
miR168a	<i>O. sativa</i>	<i>H. sapiens</i> / <i>M. musculus</i>	LDLRAP1	Zhang et al., 2012a
miR2911	<i>L. ponica</i>	IAVs	PB2 and NS1	Zhou et al., 2014
vsr1378	GFKV	<i>V. vinifera</i>	S2P metalloprotease	Miozzi et al., 2013
vsr6978	GRSPaV	<i>V. vinifera</i>	VPS55	Miozzi et al., 2013
siR221	TMV-Cg	<i>A. thaliana</i>	CPSE30	Qi et al., 2009
siR118	TMV-Cg	<i>A. thaliana</i>	TRAP α	Qi et al., 2009
vsRNA (termed as sRCC1)	CaMV	<i>A. thaliana</i>	<i>At1g76950</i> (awaits functional characterization)	Moissiard and Voinnet, 2006
Bc-siR3.2	<i>B. cinerea</i>	<i>A. thaliana</i>	MPK2 and MPK1	Weiberg et al., 2013
Bc-siR3.1	<i>B. cinerea</i>	<i>A. thaliana</i>	PRXIF	Weiberg et al., 2013
Bc-siR5	<i>B. cinerea</i>	<i>A. thaliana</i>	WAK	Weiberg et al., 2013
Bc-siR3.2	<i>B. cinerea</i>	<i>S. lycopersicum</i> .	MAPKKK4	Weiberg et al., 2013
Y-sat derived siRNA	CMV	<i>N. tabacum</i>	<i>Chll</i>	Shimura et al., 2011; Smith et al., 2011

Predicted sRNAs of shadow area and validated sRNAs are listed in the first column. The direction of trans-kingdom sRNAs above is from 'Derivation' to 'Target Species.' Some predicted sRNAs are of large quantities and we do not spread them in the table, just give the number and representative ones, each corresponding to their target genes/ORFs, except of the underlined *miR156b** targeting two ORFs, *AC2*, and *AC3*.

shown to be similar to that of miR168a, as both are packed into MVs and go through the GI tract. Afterwards, these miRNAs are transported to target cells through the circulatory system.

The Probable Process of the Horizontal Transfer of miRNAs from Plants to Mammals

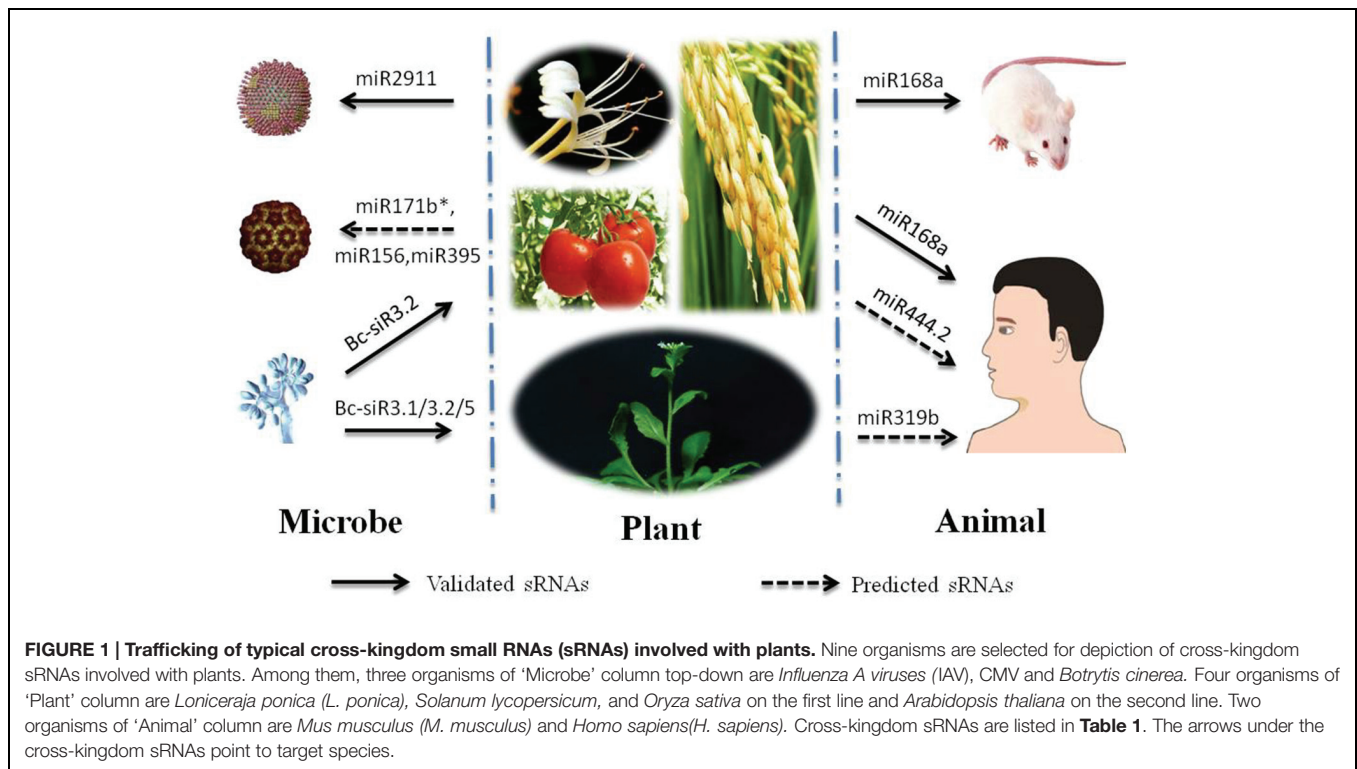
When we ingest plant materials, they are preliminarily crushed into debris by the mechanical action of the oral cavity and stomach and simultaneously catabolized into glucose by various digestive enzymes. It has been assumed that, in this process, mature miRNAs are released from the destroyed plant cells and transferred to the small intestine in the gut (Zhang et al., 2012a). Along with AGO2, these miRNAs have been observed to selectively pack into shedding vesicles or exosomes (both called MVs) and are secreted to the outer space by epithelial cells (Cocucci et al., 2009; Elhassan et al., 2012; Zhang et al., 2012a; Zhou et al., 2014). It is worth noting that not all of the miRNA-AGO2 complexes are packed into MVs when entering into the intestinal epithelial cells, and only a minority of the complexes in the MVs can gain access to the recipient cells (Collino et al., 2010). Indeed, exosomal miRNAs are derived from a particular subset of genes (Valadi et al., 2007). In some

cases, the packaging of miRNAs into MVs is driven by particular substances, such as an antigen (Mittelbrunn et al., 2011). Via endocytosis and exocytosis, MVs translocate through the vascular wall and are transported to the target cells via the circulatory system, with the ensuing step of releasing the miRNA-AGO2 complexes. Nevertheless, MVs do not interact with all types of cells and interact only with those cells that the MVs specifically recognize. Because foreign miRNAs have high G-C content and specific 2'-O-methylated 3' ends (Yu et al., 2005), and their transportation is with the help of binding to AGO2 and shielding in MVs (Mitchell et al., 2008; Arroyo et al., 2011; Zhang et al., 2012a; Zhou et al., 2014), they can exist stably under different temperatures, acidification and RNase activity during the process of digestion and transportation.

ROLES OF HORIZONTALLY TRANSFERRED siRNAs

siRNAs Transferred from Viruses/Fungi to Plants

Virus-derived siRNAs bind to plant transcripts. vsRNAs from *Grapevine fleck virus* (GFKV) and *Grapevine rupestris stem*



pitting-associated virus (GRSPaV) have been predicted to target plant transcripts according to genome-wide identification (Miozzi et al., 2013). It has been reported that 24/26 different grapevine transcripts could be targeted by 27/30 vsRNAs from the GfKv genome and GRSPaV genome, respectively. To test whether the decrease in grapevine transcripts described above was related to the infection of viruses, Miozzi et al. (2013) carried out quantitative real-time polymerase chain reaction (qRT-PCR) and 5'-RACE analyses. The results supported the idea that a lower accumulation of transcripts, such as S2P metalloprotease and vacuolar protein-sorting 55 (VPS55), was associated with the cleavage of vsRNA from GfKv (vsR1378) and GRSPaV (vsR6978) (**Table 1**). The former transcript is implicated in the control of regulated intramembrane proteolysis, and the latter is involved in the regulation of the endosomal trafficking of proteins. In the same manner, *Tobacco Mosaic Virus* (TMV-Cg) siR221 and siR118 target cleavage and polyadenylation specificity factor (CPSE30) and translocon-associated protein alpha (TRAP α) in *A. thaliana*, respectively (Qi et al., 2009) (**Table 1**). Two targets of the siRNAs were validated through modified RNA ligase-mediated 5'-RACE experiments. In addition to the RNA viruses described above, sRNAs from the DNA virus *Cauliflower Mosaic Virus* (CaMV) can also silence plant transcripts (Moissiard and Voinnet, 2006). The CaMV-derived sRNAs mostly come from the polycistronic 35S RNA sequence, which exhibits an extensive secondary structure known as a translational leader. By employing the entire leader sequence in a BLAST search against cDNAs and ESTs from *Arabidopsis*, three transcripts (*At4g05190*, *At4g17710*, and *At4g76950*) were retrieved. One of these transcripts, *At4g76950*,

was confirmed to bind the corresponding vsRNA (termed as sRCC1; **Table 1**).

Fungus-derived siRNAs bind to plant transcripts as well. *Botrytis cinerea* (*B. cinerea*) sRNAs (Bc-sRNAs) that silence plant genes involved in immunity are an example. Normally, pathogens deliver protein effectors into plant cells to suppress plant immunity. However, sRNAs derived from *B. cinerea* may also act as effectors (Weiberg et al., 2013). In *A. thaliana*, three Bc-sRNAs (Bc-siR3.1, Bc-siR3.2, and Bc-siR5) that structurally mimic plant sRNAs can be loaded into the plant AGO1 protein, after which the Bc-sRNAs target genes with complementary sequences, such as *mitogen-activated protein kinase 2* (MPK2) and MPK1 (by Bc-siR3.2); an oxidative stress-related gene, *peroxiredoxin* (PRXIIIF; by Bc-siR3.1); and *cell wall-associated kinase* (WAK; by Bc-siR5), which are involved in the plant's immunity against *B. cinerea*. Similar results have been obtained in *Solanum lycopersicum*, where MAPKKK4 was targeted by Bc-siR3.2 (**Table 1**, **Figure 1**).

siRNAs from CMV Satellite RNAs Transferred to Plants

Satellite RNAs (satRNAs), a type of subviral RNA, are encapsulated by their helper viruses, such as CMV. SatRNAs are dispensable for the replication of the genome/subgenome of viruses but have the ability to aggravate or attenuate disease symptoms (Simon et al., 2004). CMV exhibits a tripartite genome whose components are termed RNA1, RNA2, and RNA3, which are mainly required for its replication, virulence, and movement (Kouakou et al., 2013). CMV also contains subgenomic RNAs (RNA4 and RNA4A), which are each responsible for the

translation of the coat protein and protein 2b. In addition to genomic and subgenomic RNAs, CMV strains encompass satRNAs, and one type of satRNA, Y satRNAs (Y-sat), can produce siRNAs that may be associated with yellowing symptoms caused by an RNAi mechanism in *Nicotiana tabacum* (*N. tabacum*). Because subviral RNA has no ability to encode proteins, it is reasonable to postulate that RNA silencing mediates satellite pathogenicity (Wang et al., 2004). Shimura et al. (2011) and Smith et al. (2011) demonstrated that CMV Y-sat-derived siRNAs could interfere with the mRNA of the host magnesium protoporphyrin chelatase subunit I (*ChlI*) gene, leading to inhibition of chlorophyll biosynthesis, thus causing the yellow phenotype (Table 1).

HOST-INDUCED GENE SILENCING (HIGS) ACTS AS A TOOL IN THE DEFENSE AGAINST BIOTIC STRESS

It has been noted that plant inverted-repeat transgenic constructs, usually with a sense-intron-antisense palindromic structure, can be employed to produce dsRNAs and siRNAs and further silence the transcripts of parasitic organisms. Hence, such RNAi constructs could be designed to test whether HIGS can affect the interaction between plants and parasitic organisms. Based on experimental data, dsRNAs and siRNAs derived from transgenic constructs in host cells would be transferred to fungi/nematodes to achieve silencing of their genes (Nowara et al., 2010; Ibrahim et al., 2011; Yin et al., 2011; Koch et al., 2013; Ghag et al., 2014; Vega-Arreguin et al., 2014). HIGS, which is as an effective transgenic tool, has been used extensively to protect plants from infection by parasitic organisms (Nowara et al., 2010; Nunes and Dean, 2012; Ghag et al., 2014). It is still unknown how these RNA molecules are transferred to the interacting organisms. Nowara et al. (2010) considered it likely that these molecules may travel to the fungi via an exosomal pathway. On the one hand, exosomes are accumulating at plant-fungus contact sites and vesicle fusion/budding have been observed at the haustoria complex, which is responsible for the transfer of nutrients to the fungi. On the other hand, plant multivesicular bodies have been shown to contain small RNAs as well as components of the silencing machinery (Valadi et al., 2007). However, how RNA molecules gain access to the bodies of nematodes and target specific genes remains unknown and requires further elucidation.

CONTROVERSIES RELATED TO HORIZONTALLY TRANSFERRED miRNAs THAT REMAIN TO BE VERIFIED

The crossover of miRNAs is not universal between plants and animals. For example, insignificant plant miRNA levels have been detected in the plasma of healthy athletes and mice fed with fruits/vegetables (Snow et al., 2013). In addition, some plant miR168 family members were nearly undetectable when organisms were fed monocot plants in which miR168 levels were relatively higher (Zhang et al., 2012b). Besides, conflicting studies

identified low levels of the aforementioned osa-miR168a that did not result in an RNAi-mediated decrease of LDLRAP1 in mouse livers (Dickinson et al., 2013; Witwer et al., 2013). Due to the controversies described above, we mainly focus on the research of Zhang et al. (2012a), who support the cross-kingdom function of miRNAs, and the research of Dickinson et al. (2013), who dispute this function, to examine the causes of the controversy. The experimental methods both show that deep sequencing and qRT-PCR are the main tools for measuring exogenous miRNAs. However, Zhang's group conducted a more detailed experiment. The sequencing results obtained by Dickson may present a bias in plants, as only a thousand reads per million raw reads of rice sRNAs were detected in rice-containing chow, which is inconsistent with previous high-throughput sequencing studies showing that the miRNA reads obtained from rice generally represent 10% of total reads (Zhu et al., 2008; Jeong et al., 2011; Yi et al., 2013). Thus, it is not surprising that plant miRNAs cannot be detected in mouse sera and livers. As plant miRNAs bear 2'-O-methylated 3' ends, which can result in a decreased ligation efficiency (Munafo and Robb, 2010), the deep sequencing results reported by Dickson need to be further verified, such as oxidized deep sequencing used by Zhang's group to test genuine plant miRNAs, with the exception of those based on the qRT-PCR technique (Chen et al., 2013; Dickinson et al., 2013).

Apart from the two major controversial studies, many other studies involving cross-kingdom miRNAs have been conducted using similar methods. Some authors have been unable to detect or have only detected very small amounts of plant-derived miRNAs in mammals (Snow et al., 2013; Witwer et al., 2013), while others, who hold different views, claim that plant miRNAs exist in silkworms but do not play roles in physiological progress (Ling et al., 2015). It is worth noting that new techniques, known as next generation sequencing (NGS) and digital droplet PCR (dPCR), has been applied to test exogenous miRNAs successfully (Wang et al., 2012; Ling et al., 2015). Thus, the existing techniques and experimental design need to be modified for searching more exogenous miRNAs. Regarding the functions of exogenous miRNAs, studies have shown that the concentration of miRNAs affects their ability to target corresponding genes (Mullokandov et al., 2012). Therefore, the concentration of miRNAs in the species of origin has been a major limitation in the detection of miRNAs in other kingdoms thus far. Moreover, Dietary MicroRNA Database (DMD) presents for researchers the types and functions of dietary derived miRNAs, which will be a great tool to explore more of dietary miRNAs in the future (Chiang et al., 2015).

CONCLUSION

The mobility of sRNA molecules is the key to understanding how sRNA molecules function in regulatory roles between one kingdom and another. In this process, the transportation of sRNAs through the MV pathway is shared both from plants to mammals or from plants to fungi. Trafficking sRNAs derive from various species, including plants, viruses, and

fungi. In most cases, crossover by these RNAs occurs in host-parasites interactions. Unlike miRNAs transferred from plants to mammals, these interactions are not one-sided, but bidirectional. It should be noted that the movement of RNA molecules has been used as a transgenic tool to control plant disease, such as through HIGS. Therefore, the elucidation of cross-kingdom sRNA mechanisms between two interacting organisms is of great interest. The horizontal transfer of sRNAs extends our understanding of sRNAs. In humans, this unique feature may be utilized to control viruses, such as *Influenza A* viruses, and improve infant immunity when consuming colostrum. For plants, the horizontal transfer of sRNAs can be used to control diseases caused by

parasitic organisms. Although the number of plant-associated trafficking sRNAs is fewer compared with those of animals, and controversial views associated with dietary-derived miRNAs await validation, we still remain confident that an increasing number of foreign sRNAs will be studied and utilized in the future.

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In Silico Analysis of Small RNAs Suggest Roles for Novel and Conserved miRNAs in the Formation of Epigenetic Memory in Somatic Embryos of Norway Spruce

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Epigenetic memory in Norway spruce affects the timing of bud burst and bud set, vitally important adaptive traits for this long-lived forest species. Epigenetic memory is established in response to the temperature conditions during embryogenesis. Somatic embryogenesis at different epitype inducing (Epl) temperatures closely mimics the natural processes of epigenetic memory formation in seeds, giving rise to epigenetically different clonal plants in a reproducible and predictable manner, with respect to altered bud phenology. MicroRNAs (miRNAs) and other small non-coding RNAs (sRNAs) play an essential role in the regulation of plant gene expression and may affect this epigenetic mechanism. We used NGS sequencing and computational *in silico* methods to identify and profile conserved and novel miRNAs among small RNAs in embryogenic tissues of Norway spruce at three Epl temperatures (18, 23 and 28°C). We detected three predominant classes of sRNAs related to a length of 24 nt, followed by a 21–22 nt class and a third 31 nt class of sRNAs. More than 2100 different miRNAs within the prevailing length 21–22 nt were identified. Profiling these putative miRNAs allowed identification of 1053 highly expressed miRNAs, including 523 conserved and 530 novels. 654 of these miRNAs were found to be differentially expressed (DEM) depending on Epl temperature. For most DEMs, we defined their putative mRNA targets. The targets represented mostly by transcripts of multiple-repeats proteins, like TIR, NBS-LRR, PPR and TPR repeat, Clathrin/VPS proteins, Myb-like, AP2, etc. Notably, 124 DE miRNAs targeted 203 differentially expressed epigenetic regulators. Developing Norway spruce embryos possess a more complex sRNA structure than that reported for somatic tissues. A variety of the predicted miRNAs showed distinct Epl temperature dependent expression patterns. These putative Epl miRNAs target spruce genes with a wide range of functions, including genes known to be involved in epigenetic regulation, which in turn could provide a feedback process leading to the formation of epigenetic marks. We suggest that TIR, NBS and LRR domain containing proteins could fulfill more general functions for signal transduction from external environmental stimuli and conversion them into molecular response. Fine-tuning of the miRNA production likely participates in both developmental regulation and epigenetic memory formation in Norway spruce.

Keywords: conifers, *Picea*, epigenetic memory, epigenetic regulators; miRNAs, somatic embryos

INTRODUCTION

Adaptation to the changing environments is vitally important for long-lived plant species like forest trees. Epigenetic modifications and specifically epigenetic memory could be important mechanisms for diversifying environmental responses and widening the total plasticity of populations. The epigenetic memory of a plant is defined by the reproducible set of modifications of DNA and chromatin (without alteration of the DNA sequence) induced by external stimuli, which alter gene expression and therefore the properties and behavior of the plant. Memorizing of specific responses, laid down by epigenetic mechanisms, could provide significant strategic benefits to those plants, since the most successful response could be tuned or reenacted in response to a modified environmental condition and this would be retained in future cell lineages, and potentially inherited and altered by selection in future generations (Bräutigam et al., 2013; Baulcombe and Dean, 2014; Iwasaki and Paszkowski, 2014; Kinoshita and Seki, 2014). Much remains to be known about the enigmatic repertoire of epigenetic mechanisms that operate in forest trees but earlier studies firmly confirmed the presence of epitype inducing (Epi) temperature-dependent plant phenotypes (Yakovlev et al., 2012; Liu et al., 2015) and significant transcriptomic changes in such epitypes (Yakovlev et al., 2016).

Both long non-coding RNAs (lncRNAs) and small RNAs (sRNAs) such as short non-coding RNAs are known to be core components of signaling networks involved in epigenetic modification, transcription regulation and participate in transgenerational epigenetic inheritance in plants and animals (Hauser et al., 2011; Heard and Martienssen, 2014). Epigenetic regulation can be mediated through a dynamic interplay between sRNAs, DNA methylation, histone modifications, histone variants, and chromatin architecture, which together modulate transcriptional silencing, activation and the accessibility of DNA in variety of ways (Heo and Sung, 2011; Simon and Meyers, 2011; Lee, 2012; Mirouze, 2012; Bond and Baulcombe, 2014).

MicroRNAs (miRNAs) are a specific class of sRNA regulators, with having roles in phenotypic plasticity, plant development and as well as metabolism, all of which act through posttranscriptional regulation of gene expression. miRNAs are widely recognized as having a 20–24 nt length, and characteristically originate from a hairpin-folded single-stranded RNA precursor structure (Meyers et al., 2008). MicroRNA precursors are transcribed from specific miRNA genes (MIR), and are processed predominantly by a multi-functional DICER-LIKE1 (DCL1). The miRNAs in turn negatively regulate gene expression by forming miRNA-inducing silencing complex (miRISC) in association with the ARGONAUTE (AGO) proteins (Bartel, 2004). miRNAs have potential to regulate virtually all cellular mechanisms (Sun, 2012; Wu, 2013), and they do so by restricting translation or cleaving multiple target transcripts. In some instances, they have already been identified as key players

in producing rapid adaptation to changing environmental conditions (Sunkar et al., 2012; Ferdous et al., 2015; Nguyen et al., 2015). As miRNAs target transcripts through the imperfect match of sequence composition between miRNA and target, the consequence of gene regulation by miRNAs is that a few miRNAs can specifically change the expression pattern, or fine tune, many specific genes simultaneously. The consequence of gene regulation by miRNAs is similar to that by transcription factors (TFs) (Morris and Mattick, 2014). Moreover, a regulatory cascade essential for appropriate execution of several biological events is triggered through the combinatorial network action of both miRNAs and TFs (Sunkar, 2010; Arora et al., 2013). Besides TFs being among miRNA targets there are known epigenetic regulators (Pikaard and Mittelsten Scheid, 2014), and these in turn, regulate the expression of the individual miRNAs (Gruber and Zavolan, 2013; Song et al., 2015). More specifically, miRNAs are shown to be directly involved in epigenetic regulation and memorizing the responses to different types of stress both in plants and animals (Khraiwesh et al., 2012; Osella et al., 2014; Stief et al., 2014; Hilker et al., 2016). Thus miRNAs have the ability to regulate many target genes, initiate transcriptional and silencing cascades, provide feedback loops, and split transcriptional regulation off into a separate dedicated parallel tracks including those already known to be in the epigenetic regulatory pathway itself.

Conserved and novel miRNAs were identified in angiosperm and gymnosperm species (Montes et al., 2014), including multiple conifers—pines (Lu et al., 2007; Oh et al., 2008; Wan et al., 2012b; Quinn et al., 2014), spruces (Yakovlev et al., 2010; Nystedt et al., 2013), and larches (Zhang et al., 2012, 2013), but their diversity, abundance and regulatory functions are still largely to be studied. Norway spruce is a suitable *Pinaceae* member to conduct experimental studies for epigenetic modification in gymnosperms since a variety of resources exist, including the possibilities for *in vitro* propagation of plant material (Kvaalen and Johnsen, 2008) and draft genome (Nystedt et al., 2013). Furthermore, epigenetic memory in Norway spruce affects vitally important adaptive traits such as the timing of bud burst and bud set, adaptive traits crucial for this species environmental success. Epigenetic alteration of these traits are presumed to be established or modified in response to the Epi temperature conditions prevailing during early seed formation, based phenotypic and molecular responses (Kvaalen and Johnsen, 2008; Johnsen et al., 2009). Moreover, *Picea abies* somatic embryogenesis (SE) is an ideal experimental system for studying this phenomenon since these responses are recapitulated through *in vitro* SE. SE at different temperatures closely mimic the processes of epigenetic memory formation, naturally occurring in zygotic seeds, and give rise to epigenetically different plants (epitypes), which have a clonal character, with a reproducible and predictable temperature-dependent altered bud phenology.

Some progress has already been achieved toward understanding molecular mechanisms underlying the epigenetic memory formation. A significant number of epigenetic regulators, including sRNA biogenesis pathways, are differentially expressed at different Epi conditions, supporting

Abbreviations: DEG, differentially expressed gene; DEM, differentially expressed defined miRNA; SE, somatic embryogenesis; sRNA, small non-coding RNA; miRNA, microRNA; isomiRs, isoform microRNAs; Epi, epitype inducing.

that methylation of DNA, histones modifications and sRNAs are pivotal for the establishment of the epigenetic memory (Yakovlev et al., 2014, 2016). We found several miRNAs differentially expressed in buds of different epitypes and suggesting their involvement in the epigenetic memory (Yakovlev et al., 2010), and this stimulated the need for a much deeper study of the various miRNA during SE in spruce, the life stage at which the epigenetic memory is laid down. The main goal of the current study was to further characterize and identify the extensive miRNA fraction in the small non-coding part of Norway spruce transcriptome. We aimed to identify the key miRNAs regulating differentially expressed genes (DEGs) and those especially related to epigenetic regulators that could potentially establish the epigenetic memory process during embryogenesis, by examining their expression profiles during SE at contrasting Epl temperature conditions. To our knowledge, this is also the first genome-wide *in silico* characterization of miRNAs and study of their transcript profiles during SE in spruce. We show an extensive number of miRNAs that can target epigenetic regulators including those modifying DNA and histone methylation, and sRNA pathways genes, supporting the notion that these predicted miRNAs and their target genes could be among central players in epigenetic memory formation.

METHODS

Plant Material and RNA Extraction

Somatic embryos, and their induction and growth, used in this analysis are already previously described (Kvaalen and Johnsen, 2008; Yakovlev et al., 2014, 2016). Embryogenic samples were those obtained from two seeds (genotypes) originating from a controlled cross of a defined female (♀#2650) and male (♂#2707) of Norway spruce parents, with those crosses being performed either in outdoor conditions; a cold originated genotype, denoted as A2C, or in greenhouse conditions as a warm originated genotype, denoted as B10W. With the current analysis, nine samples were collected from each of the two different genotypes, representing three maturation stages and three different temperatures in which the epitypes form, providing 18 samples in total. Twenty to Thirty milligram of embryo containing callus or 2–5 embryos were collected per sample, combined and immediately snap-frozen and stored in liquid nitrogen until RNA extraction. Embryo tissues were ground in 1.5 ml Eppendorf tubes by pestle and the total RNA extracted using an Epicentre MasterPure™ Plant RNA Purification Kit (Epicentre, Madison, WI, USA, #MCR85102) according to the manufacture's instruction. Total RNA preparations were stored at -80°C and the integrity and quantity of total RNA was assessed by Agilent 2100 Bioanalyzer with RNA 6000 Nano Kit and also the Small RNA kit (Agilent, Santa Clara, CA, USA #5067-1511 and #5067-1548 respectively).

Small RNA Library Construction, Sequencing and Bioinformatics

The 18 small RNA libraries were each constructed from 1 μg of total RNA, using the Ion Total RNA-Seq Kit v2 for Small RNA Libraries (#4476289), with the enrichment steps

as outlined in the Ion RNA-Seq Library Preparation guide (#4476286 revision E). Quality and quantity of amplified libraries were analyzed with the Agilent Technologies 2100 Bioanalyzer with Agilent High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA, #5067-4626). Template-positive Ion Sphere™ Particles (ISPs) containing clonally amplified DNA were prepared with the Ion OneTouch™ 2 Instrument using the Ion PGM Template OT2 200 Kit (#4480974) according manufacturer instructions. Sequencing was performed using Ion Personal Genome Machine® (PGM™) Sequencer (Thermo Fisher Scientific Inc.) and each library was sequenced individually on 316v2 chips using the Ion PGM™ sequencing 200 Kit v2 (#4482006). Data was initially processed on Ion Torrent Server using Torrent Suite software (v.4.2) and fastq formatted files were analyzed using CLC Genomics Workbench software (V 8.+) (QIAGEN, Aarhus A/S, Denmark).

In Silico Identification of Conserved and Novel Mirnas and miRNA Genes in Spruce

All the single-read and sRNA sequences beyond 19–29 bp from the 18 libraries were filtered out to remove rare and too short or too long reads. To search for conserved miRNAs, the filtered sRNA sequences were compared to known miRNAs in the miRBase v21.0 (Kozomara and Griffiths-Jones, 2011) restricted to all tree species in addition to miRNAs from the genomes of *Arabidopsis*, *Vitis*, and *Physcomitrella* allowing up to 2 nucleotides mismatch using the sRNA module of CLC genomics workbench software (v.8). To identify miRNA gene loci and novel miRNAs, we mapped all the filtered sRNA reads to the gene models encompassing high-, medium- and low-confidence as defined in the *Picea abies* genome v1 (<http://congenie.org/>) (Nystedt et al., 2013). A gene model was considered a putative miRNA gene loci when at least 100 reads of distinct sRNA tags mapped to the loci or gene model with a minimum of 0–2 mismatches. Gene sequences having 80–300 nt flanking the candidate miRNA sequence were manually scrutinized based on the criteria for miRNA definition described by Meyers et al. (2008). In addition, secondary structures of putative miRNA genes were predicted using different folding algorithms by the CLC genomics workbench software. When the stem-loop hairpin for the putative MIR was confirmed, then the existence of putative novel miRNAs was estimated. sRNA(s) with higher frequency was/were considered as guide miRNAs, sRNA(s) on the opposite strand of the loop was/were considered as star-miRNAs (*miRNA). We allowed shifting of star sequences relative to miRNA guide sequences for 1–6 bp.

During analysis, we established that some miRNA candidates (guide and star sequences) were determined to have considerably long hairpin structures, so we extended our search for gene models within the fragments for up to 1 kb, using the same procedure for miRNA detection as those used in the shorter fragments.

Expression Analysis of Predicted MiRNAs and *In Silico* Identification of their Targets

Expression analysis was performed using RNA-Seq tool of the CLC Genomic Workbench v8 with defined lists of miRNAs for annotation of the sRNA reads. Prediction of miRNA targets was

carried out by searching for complementary regions between the identified miRNAs in this study and by using all the *Picea abies* gene models v1 as the transcript sequence input using online web server psRobot—Plant Small RNA Analysis Toolbox (Wu et al., 2012), and the psRNATarget—Plant Small RNA Target Analysis Server (Dai and Zhao, 2011).

To substantiate putative targets and to refine potential miRNA-mRNA target pairs, we additionally analyzed correlations between transcript amounts of miRNA and their defined targets at three different EpI temperatures. mRNA transcript amounts were taken based on our previous study (Yakovlev et al., 2016).

qRT-PCR for miRNAs

To validate sequencing data we quantified transcript levels for 10 selected conserved and novel miRNAs with quantitative real-time RT-PCR. For analysis we used the same small RNA extracts which were used for sequencing. cDNAs were synthesized from 600 ng of the small RNA extracts with the Mir-XTM miRNA First-Strand Synthesis kit (Clontech, #638315) following manufacturer recommendations. Real-time RT-PCR amplification was performed using Mir-XTM miRNA qRT-PCR SYBR[®] Kit (Clontech, #638314) in a 25 µl reaction volume, using 2 µl of a diluted cDNA solution described above as template and 200 nM of each primer. qPCRs were performed on a ViiATM 7 Real-Time PCR System (Applied Biosystems, USA) following the manufacturer's instructions. After PCR, dissociation curves were carried out to verify the specificity of the amplification. There were three biological replicates for each sample. All expression levels were normalized to geometric mean of three selected ribosomal and transfer RNA genes (Pa4.5S, Pa5S and PatRNA-R), showing most stable expression profiles as describe previously (Yakovlev et al., 2010). Forward primers were designed based on mature miRNA sequence. If T_m of mature miRNA was less than 60°C, it had been adjusted by adding G's to the 5' end of the miRNA sequence. The list of studied miRNAs and their primer sequences are shown in Table S10. The 3' primer for qPCR was the mRQ 3' Primer supplied with the kit.

Data acquisition and analysis were done using ViiATM 7-system SDS software for absolute quantification and MS Excel software.

Data Submission

Unique transcripts from 18 libraries sequenced using Ion Torrent PGMTM Sequencer were deposited to the SRA (Short Read Archive, NCBI) and got the following accession: submission ID SUB1781210; BioProject ID PRJNA339513 and accession IDs: SAMN05592191–SAMN05592208.

RESULTS

Small RNA Library Sequencing

In total, we sequenced 18 small RNA (sRNA) libraries, representing three stages of *in vitro* spruce embryo development and three different EpI temperature treatments. This produced nearly 50 million reads in the length range from 7 to 50 nt (Table S1). Three clear read length peaks were found in the embryonic

sRNA pool after the trimming—and these corresponded with the lengths of 24–23, 21–20, and 31–32 nt (**Figure 1A**). The 31–32 nt group of sRNAs consist of ~14,5 thousands sRNAs among more than 3 million reads. To reduce complexity and focus attention on the canonical miRNA population, we filtered all reads to 19–27 nt and removed all single reads to avoid sequencing and stochastic errors. In total, over 13 million reads were retained for further analysis with two clear peak classes—prevailing with length of 24–23 nt and then of 21–20 nt, in both genotypes A2K and B10W (**Figure 1B**).

In Silico Identification of Conserved and Novel miRNAs in Spruce

Using CLC Genomics Workbench a sRNA analysis was conducted. Search and annotation of conserved miRNAs was based on the miRBase v21 database using a criteria allowing up to two mismatches. A total of 636 conserved miRNAs were defined and these belonged to 51 miRNA families. These could originate from 99 defined precursors. Not all precursors for the conserved miRNA members were found, but at least one precursor was identified for the majority of miRNA families and we considered that sufficiently supported the internal origin of the defined class of conserved miRNAs (Table S2).

Additionally we defined 1316 novel miRNAs that had no homology to miRBase v21 annotations. They could belong to 630 families and could originate from 740 predicted precursors (Table S3).

The majority of identified miRNAs in spruce embryos were 21 nt (41%) and 22 nt (34%) in length, all other length classes count less than 10% (**Figure 2**) (Figure S1). More often the miRNAs at the initial positions contain uridine (U–37%) and adenine (A–25%) and less C and G (16 and 22% correspondingly).

For nearly all conserved miRNA families we found large numbers of miRNA members (isomiRs). In average, there were 15 isomiRs per family, but varied from 1 to 102. The largest quantity of isomiRs was found for the highly conserved family miRNA166, with 102 miRNAs. More than 40 miRNAs were found across the identified miRNA families—of miR156, miR159, miR946, miR950, miR951, miR1311 and miR3701. Eleven families had more than 10 but less than 40 member miRNAs and these included miR319, miR390, miR396, miR397, miR482, miR947, miR1312, miR1316, miR3705, miR3710, and miR3712. The remaining 39 families had less than 10 isomiRs (**Table 1**).

Quantification of Transcripts and Identification of Differentially Expressed miRNA Profiles

For expression analysis, we used all miRNAs with average read counts greater or equal to 10 in at least one of the sequenced libraries. From the 2267 miRNAs we defined in the transcriptome of Norway spruce embryos, 1115 miRNAs were further used for differential expression analysis.

Differentially expressed miRNAs (DEMs) were identified through pair-wise comparison of libraries by setting the threshold $|\log_2 \text{RPKM ratio}|$ to more than 1 and p -value <

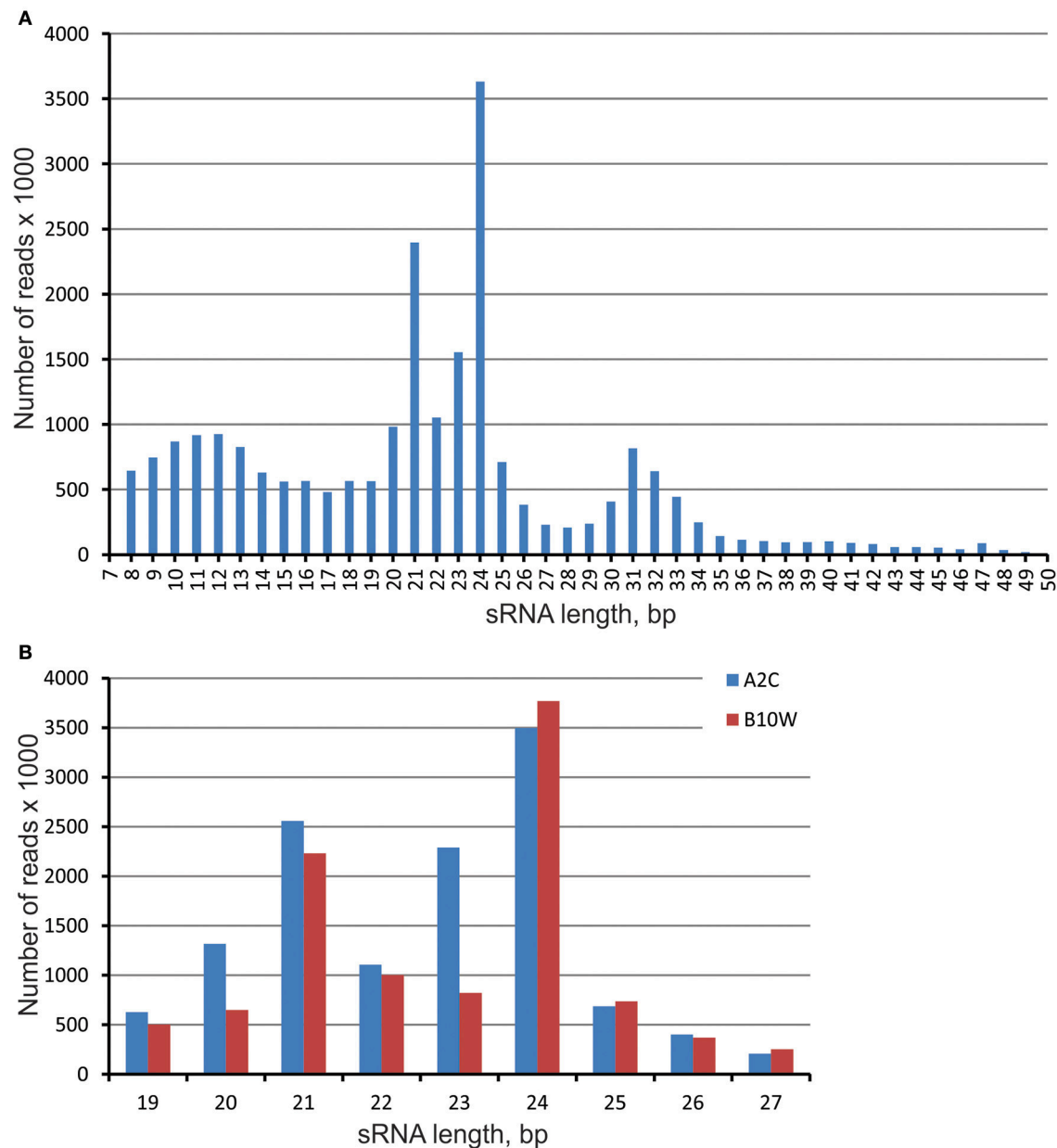


FIGURE 1 | Length distribution of sRNA in libraries prepared from the embryos of two genotypes of the full-sib family of Norway spruce from cross ♀#2650 × ♂#2707 produced in outdoor conditions—A2C and produced in greenhouse conditions—B10W. **(A)** averaged sRNA length distribution in all libraries; **(B)** sRNA length distribution in miRNA length range—19–27 bp in two genotypes.

0.05. Temperature responsive miRNAs displaying more than two fold difference between EpI temperatures were considered as differentially expressed and these were further examined. In total, we detected 676 DEMs while the remaining 439 miRNAs, did not show any differences in transcript numbers at different EpI temperatures. Most of the defined miRNAs were present in all treatments. Among the DEMs, only one miRNA—Pab-miRn931 was expressed solely at 28°C and 15 miRNAs were expressed at two temperatures and were absent at third particular temperature.

Based on their transcript profiles at the three different EpI temperatures, 654 DEMs could be grouped into 12 clusters. Main transcription profiles shown on **Figure 1** and detailed description of clusters presented on Table S4. The first two clusters included 159 miRNAs significantly upregulated at 28°C and decreasing in abundance with decreasing the temperature. The most abundant here were conserved miRNAs from miR156, miR159, miR166, miR167, miR396, miR1311, miR3701, and miR951, as well as 26 novel miRNAs. Two other clusters (5–6) contained 50 miRNAs significantly upregulated at 18°C and decreasing in abundance

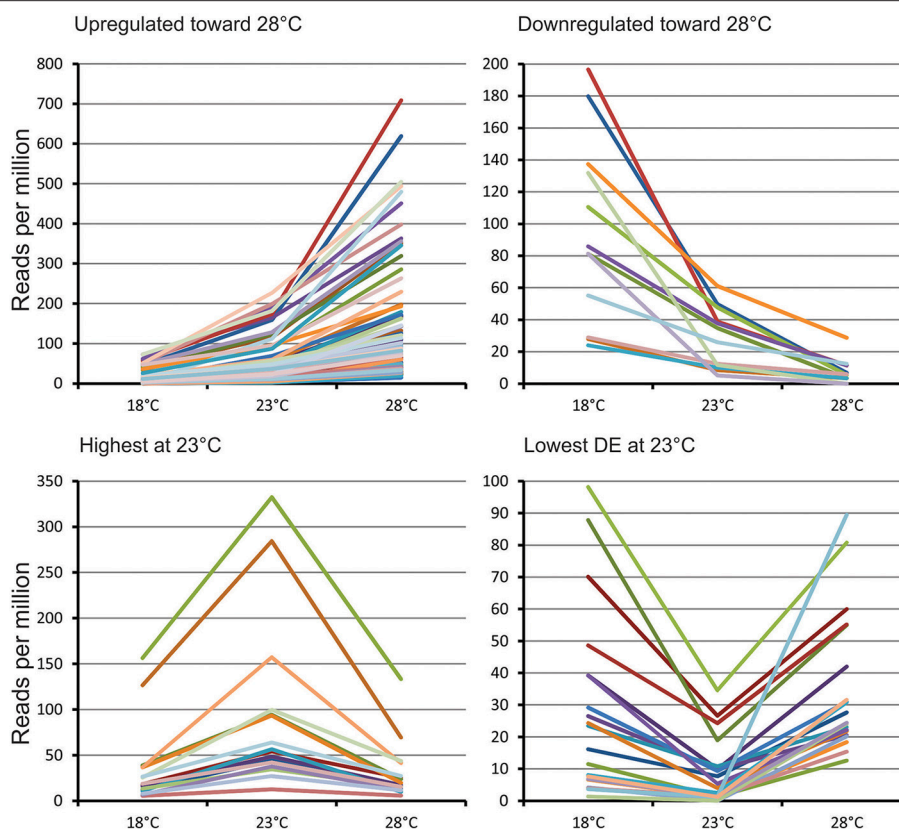


FIGURE 2 | Main expression profiles of predicted differentially expressed miRNAs in Norway spruce embryos cultivating at three different Epl temperatures.

with increasing temperature. Most of miRNAs in this cluster were novel, and only conserved miRNAs from miR950 and miR482 families were identified based on sequence similarities. Two clusters (10–11) encompass 22 miRNAs found most abundant at 23°C (**Figure 1**). Two conserved miRNAs from miR319 and miR3701 families were found here, yet all the other discovered miRNAs were found to be novel. Other clusters consisted of various other miRNAs having similar transcript profiles, the largest of which were clusters 3 and 4. Cluster 3 encompassed 101 miRNAs that were highly expressed at 28°C, yet were of equally low expression at 18° and 23°C, while cluster 4 had an opposing profile, and this encompassed 151 miRNAs showing equal high expression at 28° and 23°C, while showing low expression at 18°C. Both clusters include different miRNAs from the families of miR156, miR159, miR166, miR167, miR396, miR946, miR1311, miR1312, miR3701 miR951 as well as many novel miRNAs (Table S4).

Additionally, we specifically analyzed the changes in sequence and abundance of DEMs from conserved families at different Epl temperatures. Within each family, we found wide range of modifications, including nucleotide substitutions, 5' and 3' uridylation and adenylation, trimming and tailing. In addition, we found quite variable transcription patterns for different family members, sometimes opposite. However, we did not find any Epl temperature specific isomiRs presented only at one specific

culturing temperature and did not find any clear influence of Epl temperature on modification type. Some examples of miRNA diversity within families, their expression patterns and their corresponding stem-loop RNA secondary structure of hairpin-forming precursors presented at Figure S2.

In Silico Prediction of Targets of Conserved and Novel Norway Spruce miRNAs

For the assignment of functional roles to the whole set of defined miRNAs, the target gene transcripts were predicted by the online web server psRobot—Plant Small RNA Analysis Toolbox (Wu et al., 2012) and the psRNATarget—Plant Small RNA Target Analysis Server (Dai and Zhao, 2011). In the first instance, we searched for the respective target genes for all miRNAs as defined in Norway spruce v1 coding sequences (Nystedt et al., 2013) irrespective of their transcript profiles. This resulted in 2050 miRNAs being identified as the cognate miRNAs to 6058 annotated gene models from around 1414 gene families with diverse biological functions and 4701 gene models without matches to the database. The largest number of gene models, which could be regulated by miRNAs, were in gene families containing following Pfam domains: Leucine Rich Repeat (LRR) protein genes, protein kinase domain, pentatricopeptide (PPR) repeat, NB-ARC (nucleotide-binding adaptor R-gene shared) domain, ATPase family associated with

TABLE 1 | Family description, targets and putative functions of predicted conserved miRNAs families defined in within the sRNAs of Norway spruce embryos in comparison with previously published data.

Conserved miRNA family	Number of miRNAs (isoforms)	Number of highly expressed miRNAs*	Number of putative target gene models	Targets of miRNAs defined in our study	Described and putative targets of miRNAs	Putative functions	References
1 Pab-miR1311	41	29	6	DnaJ domain proteins, NBS-LRRs	unknown	Environmental signal transduction and response mechanisms; gymnosperm-specific	Wan et al., 2012b
2 Pab-miR1312	19	7	12	TIR domain; NBS-LRRs; AAA proteins	Argonaute/ Zwiille-like family; Flagellin-sensing 2-like family	Involved in miRNA biogenesis pathway regulation, mediates the innate response to bacterial pathogens	Wan et al., 2012b
3 Pab-miR1313	5	2	9	Protein tyrosine kinase with LRR domains	unknown	Cellular signaling pathways, gymnosperm-specific	Wan et al., 2012b
4 Pab-miR1314	9	4	11	Unknown; RNA polymerase; transcription factor ICE1-like	Brassinosteroid Insensitive 1-LRR receptor Serine/Threonine kinase	extracellular stress signal transduction, mediate the response to brassinosteroid (BR) hormones	Wan et al., 2012b
5 Pab-miR1315	4	4	39	DRF autoregulatory domain; LRR domains	unknown	Part of signaling mechanisms, upstream GTPase signals to regulate cellular processes - cytokinesis, cell polarity, and organelle motility; gymnosperm-specific	Wan et al., 2012b
6 Pab-miR1316	10	6	26	Ribosomal protein S7p/S5e; Region in Clathrin and VPS; Lipase (class 3)	unknown	vesicular sorting and trafficking pathways and essential for body plan development, defense and response to the environment; gymnosperm-specific	Wan et al., 2012b
7 Pab-miR156	62	44	56	SBP domain; Glycosyl hydrolases family 16	Squamosa promoter-binding protein (SBP)-like family	Tissues development and maturation	Huang et al., 2013
8 Pab-miR159	52	23	9	MYB family; Plant transcription factor NOZZLE; bZIP transcription factor	MYB family	Floral initiation and anther development; seed germination, GA and ABA signaling pathways	Reyes and Chua, 2007; Zhang et al., 2010
9 Pab-miR160	5	3	No DEGs	-	Auxin response factors - ARF	Modulates expression of auxin-response genes during tissues development; connected with miR167	Liu et al., 2007
10 Pab-miR162	7	5	No DEGs	-	DCL1-like	DCL1 miRNA biogenesis transcriptional feedback loop	Zhang et al., 2012
11 Pab-miR164	1	-	No DEGs	-	NAC domain containing family	polar auxin transport (PAT) and transduces auxin signals to promote root development	Lu et al., 2015
12 Pab-miR165	2	1	21	Homeobox; bZIP transcription factor; Exostosin; Protein kinase	Class III HD-ZIP TFs	polar auxin transport and maintenance of shoot apical meristem and vascular patterning	Huang et al., 2013; Barik et al., 2014

(Continued)

TABLE 1 | Continued

	Conserved miRNA family	Number of miRNAs (isoforms)	Number of highly expressed miRNAs*	Number of putative target gene models	Targets of miRNAs defined in our study	Described and putative targets of miRNAs	Putative functions	References
13	Pab-miR166	102	74					
14	Pab-miR167	9	6	2	Auxin response factors - ARF	Auxin response factors - ARF	control of stress/ temperature and auxin responsive signaling during development, phenotypic plasticity, somatic embryogenesis	Wu et al., 2006; Burkew et al., 2014
15	Pab-miR168	3	2	No DEGs	–	Argonaute family (AGO1 YA) family	AGO1-mediated post-transcriptional silencing, virus resistance	Jagtap and Shivaprasad, 2014
16	Pab-miR169	9	3	1	CCAAT-binding transcription factor	CCAAT-binding transcription factor (CBF-B/NF-YA) family	Abiotic stress response, ABA signaling pathways	Zhang et al., 2010
17	Pab-miR171	8	4	1	GRAS family transcription factor	SU(VAR)3-9 homolog; scarecrow-like (SCL) and GRAS transcription factors	Response to exogenous auxin and very early cellular differentiation and development	Zhang et al., 2010; Wan et al., 2012b
18	Pab-miR172	3	3	4	AP2 domain; unknown	apetala2 - AP2	Seed development and maturation, response to cold	Zhang et al., 2010
19	Pab-miR218	1	–	No DEGs	–	Anion exchanger family - AE1	Disease resistance	Wan et al., 2012b
20	Pab-miR2916	6	2	No DEGs	–	unknown	–	Huang et al., 2013
21	Pab-miR319	25	15	No DEGs	–	Myb and TCP family transcription factors	versatile functions in multiple aspects of plant growth and development and abiotic stress response	Reichel and Millar, 2015
22	Pab-miR3630	2	–	No DEGs	–	LRR receptor-like family kinase; auxin-responsive gh3 family	multiple functions in plant growth and development	Pantaleo et al., 2010; Boke et al., 2015
23	Pab-miR3693	3	1	No DEGs	–	NBS-LRR family	Extracellular signal transduction, disease resistance	Yakovlev et al., 2010
24	Pab-miR3694	4	2	No DEGs	–	Unknown	–	Yakovlev et al., 2010
25	Pab-miR3695	4	1	No DEGs	–	Unknown	–	Yakovlev et al., 2010
26	Pab-miR3696	5	4	No DEGs	–	Unknown	–	Yakovlev et al., 2010
27	Pab-miR3697	6	5	19	TIR domain; NBS-LRR proteins; BRCA2 repeat	NBS-LRR family	Extracellular signal transduction, disease resistance	Fossdal et al., 2012
28	Pab-miR3699	4	1	1	Unknown proteins	Unknown	–	Yakovlev et al., 2010
29	Pab-miR3700	5	4	1	Unknown proteins	Unknown	–	Yakovlev et al., 2010
30	Pab-miR3701	50	26	27	NBS-LRR proteins; Cellulose synthase	Unknown	–	Yakovlev et al., 2010

(Continued)

TABLE 1 | Continued

	Conserved miRNA family	Number of miRNAs (isoforms)	Number of highly expressed miRNAs*	Number of putative target gene models	Targets of miRNAs defined in our study	Described and putative targets of miRNAs	Putative functions	References
31	Pab-miR3702	4	3	No DEGs	–	SPT4	involved in RNA polymerase V-mediated transcriptional gene silencing	Yakovlev et al., 2010; Köllen et al., 2015
32	Pab-miR3703	8	7	2	MutS domain V mismatch repair	Unknown	gymnosperm-specific	Yakovlev et al., 2010; Köllen et al., 2015
33	Pab-miR3705	10	5	1	Pyridine nucleotide-disulphide oxidoreductase	NBS-LRR family	Extracellular signal transduction, disease resistance	Fossdal et al., 2012
34	Pab-miR3706	4	3	3	Protein tyrosine kinase; Leucine Rich Repeat; ABC1 family	Unknown	Stress signaling; gymnosperm-specific	Yakovlev et al., 2010
35	Pab-miR3707	6	6	14	TIR domain; NBS-LRR proteins	Unknown	Stress signaling; gymnosperm-specific	Yakovlev et al., 2010
36	Pab-miR3708	5	5	No DEGs	–	Unknown	–	Yakovlev et al., 2010
37	Pab-miR3709	9	4	5	NBS-LRR proteins	Unknown	Stress signaling; gymnosperm-specific	Yakovlev et al., 2010
38	Pab-miR3710	21	17	256	NBS-LRR proteins; DRF autoregulatory domain; IPP transferase; Rdx family	Unknown	Stress signaling; gymnosperm-specific	Yakovlev et al., 2010
39	Pab-miR3711	3	2	–	–	Unknown	–	Yakovlev et al., 2010
40	Pab-miR3712	13	8	9	TIR domain; NBS-LRR family, AAA domain	Unknown	–	Yakovlev et al., 2010
41	Pab-miR390	10	5	No DEGs	–	TAS3 transcripts	Ta-siRNA biogenesis, developmental timing and patterning	Yoshikawa, 2013
42	Pab-miR394	4	3	4	hAT family dimerisation domain; LRR domains	LCR (Leaf Curling Responsive-ness) F-box family	leaf morphology, regulation of the cell cycle, response to abiotic stresses	Knauer et al., 2013
43	Pab-miR396	35	14	12	F-box family; UDP-glucosyl transferases;	growth-regulating factors (GRFs)	cell proliferation	Rodriguez et al., 2010; Knauer et al., 2013
44	Pab-miR397	11	2	21	Multicopper oxidases	ATP sulfurylase (ATPS)	involved in oxidative stress response, photosynthesis, and cellular respiration, mediating copper homeostasis	Burkew et al., 2014
45	Pab-miR398	1	1	No DEGs	–	copper/zinc superoxide dismutase (SOD)	–	–
46	Pab-miR399	1	–	No DEGs	–	phosphate/E2 ubiquitin-conjugating family (PE2U)	maintaining phosphate homeostasis, temperature dependent regulation of flowering	Kim et al., 2011; Burkew et al., 2014

(Continued)

TABLE 1 | Continued

	Conserved miRNA family	Number of miRNAs (isoforms)	Number of highly expressed miRNAs*	Number of putative target gene models	Targets of miRNAs defined in our study	Described and putative targets of miRNAs	Putative functions	References
47	Pab-miR408	3	2	1	unknown	Laccase 3; thiamin pyrophosphokinase 1	mediating copper homeostasis, abiotic stress responses, alkaloids biosynthesis	Boke et al., 2015; Ma et al., 2015
48	Pab-miR4414	1	1	No DEGs	–	Tetratricopeptide repeat (TPR)-like superfamily	Protein interactions during transcription and protein transportation	Wang et al., 2011; Liu et al., 2014
49	Pab-miR482	39	26	54	NBS-LRR family; Myb-like DNA-binding domain	NBS-LRR disease resistance family; Histone deacetylase	Signal transduction, resistance to disease or other stresses	Shivaprasad et al., 2012
50	Pab-miR535	7	5	4	ACT domain with acetolactate synthase	Squamosa promoter-binding family-like – SPL2; DNA/RNA helicase family	low temperature-responsive miRNA, regulation of timing of transition from vegetative to reproductive phase	An et al., 2011
51	Pab-miR6478	4	2	No DEGs	–	Protein of unknown function (DUF3537)	Unknown	Liu et al., 2014
52	Pab-miR828	2	–	No DEGs	–	TAS4 transcript; MYB-like and WER TFs	Ta-siRNA biogenesis, regulation of transcription, stress response	Guan et al., 2014
53	Pab-miR946	46	31	27	NBS-LRR family; RING-type zinc-finger; HEAT repeats	Unknown	involved in extracellular signal transduction, protein bindings and intracellular transport	Wan et al., 2012b
54	Pab-miR947	10	5	3	Unknown	Unknown	–	Wan et al., 2012b
55	Pab-miR948	3	3	4	Protein tyrosine kinase	Unknown	modulates enzymatic activity and creates binding sites for the recruitment of downstream signaling proteins	Wan et al., 2012b
56	Pab-miR949	7	5	6	Unknown	Unknown	–	Wan et al., 2012b
57	Pab-miR950	77	51	196	TIR domain; NBS-LRR family; AP2 domain; 50S ribosome-binding GTPase	Cytoplasmic ribosomal family S13-like; NBS-LRR family	Extracellular signal transduction, disease resistance	Wan et al., 2012b
58	Pab-miR951	42	25	34	TIR domain; NBS-LRR family; Cytochrome P450 family	NBS-LRR family	Extracellular signal transduction, disease resistance	Fossdal et al., 2012
		851	522	901				

*With more than 10 reads.

various cellular activities (AAA), Toll-Interleukin receptor (TIR) domain, Clathrin heavy chains/VPS (vacuolar protein sorting-associated), tetratricopeptide (TPR), Myb-like DNA-binding domain, mTERF (Mitochondrial transcription termination factor), Multicopper oxidase, AP2 domain, Cytochrome P450, F-box domain and many others (Table S5). TIR and NBS-LRR comprise one of the largest groups of genes in spruce. We found more than 1900 gene models containing different LRR domains and more than 740 gene models containing NB-ARC domain and close to 370 models containing TIR domain. In total, 2594 for the TIR or NB-ARC LRR gene models were found.

Differential Expression of miRNAs and their Predicted Targets

Afterwards we selected putative targets among DEGs for the 522 conserved and 593 novel DEMs under the inductive conditions required of epitype differentiation. We additionally analyzed correlations between miRNA and their target transcription patterns at different EpI temperatures to refine potential cognate miRNA-mRNA target pairs. All target pairs with correlation below -0.6 were considered as prospective miRNA regulated gene models, especially as correlations between target transcript and miRNAs at all temperatures could help build a robust definition functional pairs, providing further insight into temperature-dependent processes leading to the formation of epigenetic memory in developing embryos (Tables S6–S8).

In total, we defined 1921 miRNA—mRNAs (DEM—DEG) pairs, consist of 470 miRNAs and 1139 target genes, incl. 930 annotated gene models from around 212 gene families with diverse biological functions and 209 gene models without match at the NCBI databases. Similar to the whole set of miRNAs, the largest number of DEM/DEG pairs were found in gene families coding for tandem repeat domain (TRD) containing proteins. Among the gene families are the 166 LRR gene models, which could be targeted by 278 miRNAs; 90 NB-ARC—by 169 miRNAs, and 52 TIRs—by 138 miRNAs. TIR, NBS-LRR proteins could be targeted by both conserved (miR482, miR946, miR950, miR1311-1316, miR3710, etc.), and novel miRNAs, like Pab-miRnY45_str, Pab-miRn00543, Pab-miRn00468, Pab-miRn00930, Pab-miRn00202_3p, Pab-miRn00386, Pab-miRn00492, Pab-miRn00930, Pab-miRn01804_5p, Pab-miRnB5, etc. Clathrin and vacuolar protein sorting (VPS) domain proteins often contain PPR and TPR repeat domains, and these could be the targets for regulation by miRNAs based on duplex sequence similarity. 30 Clathrin/VPS and 128 PPR/TPR gene models could be targeted by 202 miRNAs, including Pab-miRn00676, which could regulate the translation of 43 genes (Table 2; Table S6).

DEMs were also showed to target several transcription factors such as Squamosa promoter-binding (SPB) protein (target of miR156), plant transcription factor NOZZLE-like (miR159), Myb-like (miR159, miR482), Homeobox domain bZIP transcription factor (miR166), HD-ZIP protein (miR165/miR166), CCAAT-binding transcription factor (miR169) and AP2-like transcription factors (miR172, miR950), involved in developmental timing and transition from juvenility.

TABLE 2 | Enrichment of Pfam domains based on the preliminary functional characterization of most abundant differentially expressed gene families, which could be regulated by highly differentially expressed miRNAs.

Pfam ID	Pfam Description	Number of targeted genes models	Number of miRNAs
PF00560	Leucine Rich Repeat (LRR)	163	274
PF00069	Protein kinase domain	97	139
PF00931	NB-ARC (nucleotide-binding adaptor R-gene shared) domain	90	169
PF01535	Pentatricopeptide (PPR) repeat	84	105
PF01582	Toll-Interleukin receptor (TIR) domain	52	138
PF00004	ATPase family associated with various cellular activities (AAA)	52	85
PF00515	Tetratricopeptide (TPR) repeat	44	57
PF00637	Clathrin heavy chain/VPS (vacuolar protein sorting-associated)	30	40
PF00394	Multicopper oxidase	15	44
PF02536	mTERF (Mitochondrial transcription termination factor)	11	15
PF00646	F-box domain	11	11
PF00249	Myb-like DNA-binding domain	10	12
PF00847	AP2 domain	8	8
PF01397	Terpene synthase, N-terminal domain	8	8
PF00418	Microtubule-associated protein (MAP) Tau, tubulin-binding repeat	5	7
PF06345	DRF (Diaphanous-related formins) autoregulatory domain	4	11
PF00566	Rab-GTPase-TBC domain	4	5
PF01715	tRNA Delta(2)-isopentenylpyrophosphate transferase (IPP transferase)	3	15
PF00046	Homeobox domain	3	11
PF00201	UDP-glucuronosyl and UDP-glucosyl transferase	3	5
PF08263	Leucine rich repeat N-terminal domain	3	4
PF11721	Di-glucose binding within endoplasmic reticulum	3	4
PF04937	Protein of unknown function (DUF 659)	3	3
PF08744	Plant transcription factor NOZZLE	3	3
PF03110	SBP (SQUAMOSA promoter binding protein-like) domain	2	40
PF00106	short chain dehydrogenase	2	7
PF00514	Armadillo/beta-catenin-like repeat	2	5
PF00227	Proteasome subunit	2	4
PF01764	Lipase (class 3)	2	4
-	Pfam domains families with less than 3 genes, total	211	
-	Not annotated gene models	209	
Total number of target gene models		1,139	

Besides transcription factors, other targets included F-box protein (miR396), laccase (miR397), plant U-box (PUB) proteins (miR946).

We found that 317 DEGs could be regulated by several miRNAs and 290 DEMs could regulate more than one gene (Figures S3.1–20). For example, six DEMs—Pab-miR950.59, Pab-miR950.67, Pab-miR950.68, Pab-miR950.69, Pab-miR950.70 and Pab-miR950.72 from the miR950 family could target the same gene model MA_10433003g0010, coding for multi-domain TIR-NBS P-loop containing Nucleoside Triphosphate Hydrolase domain and involved in signal transduction and response to different external stimuli (Figure S3.1). Pab-miRn0282.1, Pab-miRn0054.4_5p, Pab-miR950.59, and Pab-miR950.72 could target the same gene model MA_55143g0010 at four different target sites (Figure S3.2). MA_55143g0010 is coding for multi-domain TIR-NBS-LRR protein, containing Helix-hairpin-helix motif and probably in addition to signal sensing, monitoring and transduction functions could directly bind to DNA and could fulfill “reader” functions.

Similarly, the novel Pab-miRn0539_5p was found to potentially regulate 12 different gene transcripts of NBS-LRR and ATPases; while conserved Pab-miR1315.1 could regulate 9 genes, mostly LRRs and cytoskeleton remodeling proteins participating in regulation of cellular processes such as cytokinesis, cell polarity, and organelle motility. In most cases, such redundant targeting occurs in gene families of repeat proteins, such as multiple multi-domain TIR-NBS-LRR proteins, proteins kinases, HEAT-repeat proteins and tetra- (TPR) or pentatricopeptide (PPR) repeat proteins (Figures S3.4,5).

In Silico Prediction of Differentially Expressed miRNAs Targeting Epigenetic Regulators

To evaluate miRNAs regulating epigenetic regulators and the potential for feedback loops within the sRNA biogenesis pathways we focused on the putative miRNA targets encoded within genes of the known epigenetic pathways described earlier (Yakovlev et al., 2016). All miRNAs and their predicted targets were analyzed, irrespective of their expression levels and distributing the target gene models by the type of epigenetic modification, they could be related to (Table 3, Table S9).

In total, we found 84 conserved miRNAs from 32 families and 280 novel miRNAs that could target and post-transcriptionally regulate 683 gene models spanning all pathways of epigenetic regulation. Among them, 22 conserved miRNAs from 12 families and 98 novel miRNAs, with 197 of predicted targets, showed opposite transcription patterns and considered as involved into post-transcriptional silencing of epigenetic regulators during epitype formation in Norway spruce embryos (Table 3). As the miRNAs targets, we found several gene families, coding for genes involved in epigenetic regulation, like WD domain, G-beta repeat; SNF2 family N-terminal domain; DEAD/DEAH box helicase; BRCA1 C Terminus (BRCT) domain; KH (K Homology) RNA-binding domain; PHD-finger; Core histone H2A/H2B/H3/H4; SET domain; BTB/POZ (BR-C, ttk and bab/Pox virus and Zinc finger) domain; E1-E2 ATPase; ThiF family of Ubiquitin-activating enzyme (E1 enzyme) and some others.

TABLE 3 | Predicted miRNAs targeting epigenetic regulator with the distribution of the target gene models by the type of epigenetic modification.

	Number of miRNAs	Number of target gene models	Number of DEMs	Number of target DEGs
DNA methylation	4	5	3	3
Histone methylation	108	119	34	37
Histone acetylation	25	26	7	7
Histone (protein) phosphorylation	210	476	69	133
Histone ubiquitination (sumoylation)	24	29	9	9
Chromatin remodeling	17	17	7	7
sRNA pathways	21	16	4	4
Thermosensing	9	6	1	1
Total	364	683	120	197

We found only 4 miRNAs targeting the genes involved in DNA methylation, including methylation marks setting, erasing or reading (Table S9-1) and 3 miRNA-target pairs showed opposite transcription profiles, considering repression of target transcripts by high levels of miRNA transcripts and *vice versa*. 108 miRNAs were found to target 119 epigenetic regulators related to histone methylation (Table S9-2). Among them, 34 miRNA targeting 37 gene models, showed opposing transcriptional profiles. At least three SET-domain and one polycomb-like protein genes look to be regulated by conserved Pab-miR156.61 and three novel miRs—Pab-miRn0254.1_5p, Pab-miRn0305.2_3p, and Pab-miRn0252.1_3p correspondingly. In addition, large number of histone methylation readers could be regulated by miRNAs.

Phosphorylation was also an over-represented processes which appears to be highly regulated by miRNAs. Protein kinases (PK) were abundantly found among the gene families and had the highest number of total miRNA targets. We found 210 miRNAs, which could target 476 PK gene models, from which 69 miRNAs had opposite transcription profiles with 133 targets. Here we note, the conserved miRNAs from families’ miR162, miR390, miR948, miR1313 and miR 3706 and abundant number of novel miRNAs, including Pab-miRn0117.1_3p; Pab-miRn0117.3_3p; Pab-miRn0165_3p; Pab-miRn0286.2_5p; Pab-miRn0301; Pab-miRn0367.1; Pab-miRn0441; Pab-miRn0592; Pab-miR1313.2 and others. All of them could target multiple gene models (Table S9-4).

Gene models related to histone acetylation were also revealed to be miRNA targets, but only 25 miRNAs were predicted to target such genes, and seven of these miRNA-target pairs had negatively correlated transcription profiles, indication miRNA regulatory effect (Table S9-3). Similarly, genes involved in ubiquitination/de-ubiquitination were infrequent amongst the miRNA targets. In this case, we found 24 miRNA targeting 29 gene models and 9 miRNA-target pairs with opposing transcriptional profiles (Table S9-5). Only 17 miRNAs were found to target chromatin remodelers, and these occurred mainly from

the SNF2 family and in one case from a SWI gene family. In the latter case, 7 miRNA-target pairs had opposing transcription profiles, characteristic of a miRNA-target pair, and in each case these miRNAs were all of novel sequence composition (Table S9-6).

Conserved miRNAs were found to target genes participating in miRNA and other sRNAs biogenesis pathways. Several conserved miRNAs from miR156, miR162 and miR482 could target *DCL1-like* gene transcripts but transcription patterns did not confirm any regulatory effect. While, novels Pab-miRn miRn0030.3 shown to regulate *ARGONAUTE7 (AGO7)*, Pab-miRn0009.3_3p—3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMG1*) and Pab-miRn0305.2_3p; Pab-miR482.29 shown to regulate different *SUPPRESSOR OF GENE SILENCING 3 (SGS3)* genes (Table S9-7).

Another important and noteworthy observation was that a few genes were related to those already identified to be involved in thermosensing. Six genes were found to be potentially regulated by 9 miRNAs. We found just one pair Pab-miRn0407—Calcium-activated BK potassium channel (MA_10433576g0020) with opposite transcript profiles. Most of other genes belong to the Ankyrin repeat family and Myb-like DNA-binding domain, which could be targeted by numerous miRNAs, so it is difficult to establish clear transcription patterns using whole embryo samples (Table S9-8).

Verification of miRNA-Seq Expressions by qRT-PCR

To validate the miRNA-Seq expression data, a subset of 10 specific DEMs (Table S1) was selected for verification by qRT-PCR. The selected miRNAs (5 conserved and 5 novel) showed a distinct differential profiles during embryo development at different epitype-inducing temperatures. For all of the 10 studied miRNAs the qRT-PCR outcomes closely correlated with the transcript abundance estimated by miRNA-Seq (Figure S4).

DISCUSSION

Different EpI temperatures of SE result in epigenetically different plants (epitypes) with altered bud phenology observed phenotypically later in the sporophytes annual growth cycle, in a predictable and reproducible manner (Kvaalen and Johnsen, 2008). During early development, complex gene expression, together with epigenetic changes, control and determine the embryonic cell fates (de Vega-Bartol et al., 2013; De-la-Peña et al., 2015). Some of these processes are determined by epigenetic modifications and networks of gene expression are directed and mediated by non-coding RNAs (Simon and Meyers, 2011; Lee, 2012; Mirouze, 2012). To focus on the genetic pathways initiating and establishing the epigenetic memory response, we obtained sRNAs using deep miRNA sequencing on embryos from contrasting EpI temperatures. Our sRNA analysis revealed over 3000 miRNA candidates from somatic embryonic tissues, including those of the 24-nt miRNA class. This result reconfirms their earlier found presence (Nystedt et al., 2013), in other gymnosperm species (Wan et al., 2012a,b; Wang et al., 2015),

and firmly reject the earlier hypothesis that the 24 nt miRNA and siRNA class are missing and the notion that the DCL4-mediated miRNA biogenesis is absent within gymnosperm plants. We found a more complex structure of sRNA pool in embryonic tissues, compared to developed plant tissues, with the presence of additional longer fraction of sRNAs of 31–32 nt length. These sRNAs were not reported earlier in any plant species, but a same size class were described in animals germ line cells as PIWI-related sRNA (Le Thomas et al., 2014). Appearance of such longer fraction was very recently described during callus formation in tobacco plants Lunerova, (personal communication). Therefore, we could speculate that appearance of longer sRNA fraction could be related to pluripotent state of cells, presented in callus or developing SE. This longer sRNA fraction was not the topic of our present study and should be pursued further in a separate study. Aside from this, our *in silico analysis* uncovers numerous predicted novel miRNA families and identifies miRNAs with potential involvement in epigenetic regulation and thermosensing based on anti-correlated expression patterns of miRNA—target pairs.

Whilst this sRNA sequencing effort and *in silico analysis* is not an exhaustive search or analysis of the complex spruce sRNA population as we were limited only to one tissue type, it is the most exhaustive examination of 24–23 nt and 21–20 nt small RNAs in spruce to date. We limited our miRNA candidates' pool to predicted novel and conserved miRNAs with more than 10 reads in any libraries. In total, we identified 1115 miRNAs; nearly half of which (593) were putatively novel miRNAs and 522 conserved miRNAs. This conserved pool included 21 spruce specific miRNAs defined in previous studies (Yakovlev et al., 2010).

Analysis of the conserved miRNAs pool revealed a high number of miRNAs isoforms (isomiRs) nearly for all of the 58 conserved miRNA families. Various mechanisms are associated with the diversification of miRNA sequences, including imprecise DCL processing or post-transcriptional modifications, like trimming and tailing (Li et al., 2014). As we did not find specific genomic fragments with precursors for the most of isomiRs, most probably, they were originate from the common precursors and modified post-transcriptionally. Existence of isomiRs could be also explained by the possibility of miRNAs to originate from several MIRs. In this case, any SNP changes in miRNA genes would cause the creation of specific isomiRs. Generation of isomiRs from the same miRNA locus may extend its functional influence. As miRNA isoforms vary in size and sequence from the canonical miRNA, alternative targets may be regulated and differential AGO loading could arise, resulting in diverse regulatory outcomes (Nielsen et al., 2012; Ameres and Zamore, 2013; Li et al., 2014). Large amount of isoforms for conserved miRNAs are present in non-model species (e.g., Mica et al., 2009; Lin and Lai, 2013; Liu et al., 2014), but these isomiRs are not well characterized and, in most cases, their origins and functions if any remains unknown (Nielsen et al., 2012). It has previously been demonstrated that temperature treatments altered the expression of a specific subset of mature miRNAs and displayed differential expression of numbers of miRNA isoforms (Baev et al., 2014).

This is the first report of *miRNAs in gymnosperms. Even if our predictions detect the presence of both guide and *miRNA, we have not equivalently identified specific*miRNAs since such identification should be supported with more exact experimental knowledge of each miRNAs origin precursor. For most putative miRNA we found several potential precursors and we cannot at present define which exact precursor of origin or which arm of these precursors is the guiding (functional) mature miRNA and which is the passenger (non-functional) ones. We have defined miRNAs closer to 5' end as -5p and putative *miRNA on the opposite strand of the predicted precursor (closer to 3' end) as -3p. In many cases both predicted miRNAs (guide and *miRNA) from both strands were expressed and occasionally in opposite manner. Our sRNAs originate from whole embryos containing various tissues and cell types, and since different tissues and cell types may preferentially express either the -5p or the -3p variant (or vice versa), it is impossible in the present material to define with sufficient certainty which variant is main miRNA and which is star. Future cell type specific studies should be performed to clear up this matter.

Our target prediction revealed a large range of gene families with diverse biological functions. Usually, miRNAs regulate posttranslational repression of mRNAs via two different mechanisms: the miRNAs induce mRNA translational repression and the miRNAs induce mRNA cleavage under the help of ARGONAUTE protein (Bartel, 2009). Due to lack of necessity of full complementarity between miRNA and its target, it is difficult to establish clear relations between miRNA transcript profiles and their putative targets and transcript profiles as in many cases same gene transcript could be regulated by several miRNAs and, opposite, same miRNA could target several gene transcripts. High redundancy of miRNA—mRNA interplay and the multiplicity of miRNA genes and miRNA binding sites in the UTR of target genes may play a synergistic or additive function in the regulation of such targets (Bartel, 2009). Hence, the role of miRNA in gene expression would most likely be that of a fine-tuning process rather than an ON/OFF switch. One gene may be targeted by up and downregulated miRNA at the same time in order to attain the optimum concentration required for a specific function (Herranz and Cohen, 2010). This is the case for the mediation of cell fate decisions, where miRNAs act in synergy with other transcription regulators to establish gene regulatory networks (Herranz and Cohen, 2010).

We want to highlight the predictive nature of our *in silico* predictions using psRobot and psRNATarget analysis server and that future experimental validation studies are needed to confirm or refute our predictions. Notwithstanding, in most cases when we found inverse relations between miRNAs transcript levels and transcript levels of their predicted targets we presently considered them as a likely functional miRNA target pairs and have predicted their putative functional importance.

The highest number of predicted target gene models, which could be regulated by miRNAs, were found among multiple repeats containing proteins gene families, like: Leucine Rich Repeat (LRR) protein genes, protein kinase domain, NB-ARC (nucleotide-binding adaptor R-gene shared) domain, ATPase family associated with various cellular activities

(AAA), Toll-Interleukin receptor (TIR) domain, Clathrin heavy chains/VPS (vacuolar protein sorting-associated), tetra- (TPR) and pentatricopeptide (PPR) repeat protein genes and some others. TIR-, NBS-LRR genes are considered as one of the first lines of defense against pathogen infection (Dangl and Jones, 2001; Meyers et al., 2005). However, our *in vitro* culture was axenic, free from any pathogens, and should not initiate any defense-related responses. Even so, we could see a large amount of differentially expressed LRR-containing genes showing clear dependence on epitype inducing temperature, and this imply their involvement in processes far removed from pathogen-mediated interactions. We consider that TIR, NBS, and LRR domain containing proteins may fulfill more general role in signals transduction from external environment (both biotic and abiotic) and conversion into molecular responses of diverse nature. TPR proteins can promote the formation of highly specific multiprotein complexes and can support the binding of different ligands (Zeytuni and Zariwach, 2012). A typical PPR protein could binds one or several organellar transcripts, and influences their expression by altering RNA sequence, turnover, processing, or translation (Barkan and Small, 2014). Clathrin and vacuolar protein sorting (VPS) domain proteins are another large group of protein involved in the vesicular sorting and trafficking pathways and essential for body plan development, defense and response to the environment (Chen et al., 2011). They often contain penta- and tetratricopeptide repeat domains, which could be the targets for regulation by miRNAs. In response to temperature differences, these may help fine tune intracellular traffic or the delivery of signaling molecules, but it is hard to envisage otherwise how they may directly contribute to temperature-dependent formation of epigenetic memory in the spruce embryos.

More specifically, we were looking for the genes involved in epigenetic regulation. It is shown, that a significant part of sRNA can serve as a pointer and participate in chromatin modification of promoters or DNA methylation, preventing, or activating the transcription of the individual is often remotely located sRNA coding genes or clusters (Mirouze, 2012). It is remarkably, that from the more than 700 gene models of putative epigenetic regulators described in developing embryos (Yakovlev et al., 2016), more than half are predicted targets by miRNAs. Moreover, we found that in EpI temperature dependent manner 197 DEGs of epigenetic regulators could be post-transcriptionally regulated by 120 miRNAs, including 22 conserved miRNAs from 12 families. miRNAs were mostly involved in regulation of genes related to methylation modifications, both in DNA and histones. In addition, several miRNAs were shown to target sRNA biogenesis pathway's gene models, confirming the existence of tight regulatory feedback loops within the miRNA and siRNA pathways in both gymnosperms and angiosperms (Henderson and Jacobsen, 2007; Niu et al., 2015). The opposite may occur, that miRNAs expression could be regulated by specific genes in response to changes in an extracellular microenvironment and considered as one of the major mechanisms for epigenetic modifications of the cell. It was shown that ion channels/transporters could transduce extracellular signals into miRNA transcript level changes,

which, in turn, regulating target genes, and proposed potential link between cells and their microenvironment through ion channels/transporters (Jiang et al., 2012).

Most of miRNAs targeting epigenetic regulators predicted here were novel, so their possible existence also in other plant species should be pursued to verify their general importance. However, the fact that we find miRNA directly targeting all types of epigenetic modifiers indicated that miRNAs are central players involved in formation of epigenetic memory or at least in regulating the expression of the epigenetic machinery. In light of their important functions in the epigenetic memory formation, future validation work on these miRNAs and their targets is required.

CONCLUSION

In this *in silico* analysis, we defined a predicted repertoire of conserved and novel miRNAs that could play crucial roles in regulating embryo development and epigenetic memory formation in Norway spruce. We showed that developing Norway spruce embryos possess a more complex sRNA structure than reported for somatic tissues. A variety of the predicted miRNAs showed distinct EpI temperature dependent expression patterns. These putative EpI miRNAs target spruce genes with a wide range of functions, including genes known to be involved in epigenetic regulation, which in turn could provide a feedback process leading to the formation of epigenetic marks. We suggest that TIR, NBS, and LRR domain containing proteins could fulfill more general functions for signal transduction from external environmental stimuli and conversion them into molecular response. Fine-tuning of the miRNA production likely participates in both developmental regulation and epigenetic memory formation in Norway spruce. This study also provides

important information for comparative studies of miRNAs with other plant species and their predicted involvement in epigenetic regulation.

AUTHOR CONTRIBUTIONS

IY and CF conceived and designed research. IY conducted experiments. IY analyzed data and wrote the manuscript together with CF. Both authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fphys.2017.00674/full#supplementary-material>

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Methylation of miRNA genes in the response to temperature stress in *Populus simonii*

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DNA methylation and miRNAs provide crucial regulation of the transcriptional and post-transcriptional responses to abiotic stress. In this study, we used methylation-sensitive amplification polymorphisms to identify 1066 sites that were differentially methylated in response to temperature stress in *Populus simonii*. Among these loci, BLAST searches of miRBase identified seven miRNA genes. Expression analysis by quantitative real-time PCR suggested that the methylation pattern of these miRNA genes probably influences their expression. Annotation of these miRNA genes in the sequenced genome of *Populus trichocarpa* found three target genes (*Potri.007G090400*, *Potri.014G042200*, and *Potri.010G176000*) for the miRNAs produced from five genes (*Ptc-MIR396e* and *g*, *Ptc-MIR156i* and *j*, and *Ptc-MIR390c*) respectively. The products of these target genes function in lipid metabolism to deplete lipid peroxide. We also constructed a network based on the interactions between DNA methylation and miRNAs, miRNAs and target genes, and the products of target genes and the metabolic factors that they affect, including H₂O₂, malondialdehyde, catalase (CAT), and superoxide dismutase. Our results suggested that DNA methylation probably regulates the expression of miRNA genes, thus affecting expression of their target genes, likely through the gene-silencing function of miRNAs, to maintain cell survival under abiotic stress conditions.

Keywords: DNA methylation, lipid metabolism, miRNA, *Populus simonii*, temperature stress

INTRODUCTION

Temperature has major effects on plant growth and development in the field, where temperatures can change frequently, potentially causing stress on the plant. Heat or cold stress can negatively affect many physiological processes; thus plants have evolved complex signaling pathways that perceive and transduce signals in response to particular stresses (You et al., 2014). These signals act through H₂O₂, malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD), which affect the degree of response to abiotic stress (Nie et al., 2015; Zhang et al., 2015). Some of these signals require transcription and are broadly regulated by a variety of factors, including cytosine methylation, covalent modification of DNA by 5-methylcytosines. For example, alterations in methylation might be critical to energy metabolism in the Antarctic polychaete *Spiophanes tcherniai* (Marsh and Pasqualone, 2014). Rakei et al. (2015) identified specific DNA sequences that play an important role in cold tolerance with possible responsive components correlated with cold stress in plants, suggesting that DNA methylation regulates cold stress signals. In addition, the altered methylation state of *CycD3-1* and *Nt-EXPA5* shifted their expression during heat stress in tobacco (Centomani et al., 2015).

MicroRNAs (miRNAs) also regulate expression of genes directly and indirectly related to the response to temperature stress. MiRNAs are small (21–24 nucleotides), non-coding, single-stranded RNAs derived predominantly from intergenic regions, and function as key regulators of gene expression (Sunkar and Zhu, 2004). For instance, work in *Arabidopsis thaliana* identified 29 miRNAs that regulate gene expression in response to drought stress (Hajdarpašić and Ruggenthaler, 2012). Also, in *Larix leptolepis*, four miRNA families (miR159, miRNA169, miRNA171, and miRNA172) are all induced by abiotic stress and their targets regulate genes crucial to cell development, including MYB transcription factors (miR159), an NF-YA transcription factor (miR169), a scarecrow-like transcription factor (miR171) and *apetala2* (miR172; Zhang et al., 2010). In addition, Ishikawa et al. (2014) found that a genotoxic stress-responsive miRNA, miR574-3p, delays cell growth by suppressing the enhancer of a rudimentary homolog gene *in vitro*.

Thus, miRNAs and DNA methylation have crucial functions in regulating gene expression in response to abiotic stress in plants. However, so far, few studies have examined possible mutual adjustments between DNA methylation and miRNAs. Rykov et al. (2013) found that the methylation of *MIR125b-1* and *MIR137* genes was correlated with non-small-cell lung cancer progression. Also, repression of miRNAs was correlated to hypermethylation of their promoters in human cancer cells (Li et al., 2011). Additional research has begun to examine the potential interaction between DNA methylation and miRNAs in response to abiotic stress in plants, but so far this interaction remains unclear.

In comparison to annual plants, perennial plants undergo more temperature changes over their longer lives. Here, we chose *Populus simonii* as an experimental system to examine the possible interaction between cytosine methylation and miRNAs. The advantages of using *Populus* species as genomic models for tree molecular biology have been extensively reported. Among *Populus* species, *P. simonii* shows broad geographic distribution and a strong ability to survive, even in extreme temperatures (−41 to +43°C) and under other abiotic stresses. It is one of the most important native tree species in northern China, for its commercial and ecological value (Wang et al., 2012).

Considering global climate change and frequent extreme weather, including very low and high temperatures, studying the plant response to temperature stress may provide important information for future agricultural and ecological studies. Given the increasing evidence for miRNAs and DNA methylation as important regulators of gene expression in response to abiotic stresses, we hypothesized that the potential interaction of miRNAs and DNA methylation plays a critical role in stress-responsive gene expression. Here, we used *P. simonii*, a resistant and adaptable species, for methylation-sensitive amplification polymorphism (MSAP) and transcript level analysis through quantitative real-time PCR (qRT-PCR) to uncover changes of methylation and expression of miRNA genes in response to heat and cold stress. This study provides new insights on different DNA methylation-mediated regulatory

mechanisms in the response to temperature stresses in plants.

MATERIALS AND METHODS

Plant Materials and Treatments

Populus simonii 'QL9' was planted in pots under natural light conditions (1,250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation), $25 \pm 1^\circ\text{C}$, $50\% \pm 1$ relative humidity and 12 h day/night in an air-conditioned greenhouse. In this study, thirty annual clones of the same size (50 cm in height) were divided into three groups; one group was chosen to act as the control group and other two groups were treated by heat or cold stress, respectively. These treatment groups were exposed to 42 and 4°C for 3, 6, 12, and 24 h for heat, and cold stress treatments, respectively. The 3- and 6-h time points were chosen to capture early responsive genes, and the 24-h time point for late responsive genes (Lee et al., 2005). Clones not exposed to abiotic stress were used as the control group. Three biological replicates were used for each treatment time point, including the control group. For physiological and gene expression analysis, fresh leaves were collected from the five groups, then immediately frozen in liquid nitrogen and stored at -80°C until analyzed.

Measurement of Physiological and Biochemical Characteristics

Endogenous H_2O_2 levels were detected by measuring luminol-dependent chemiluminescence according to the method described by Dat et al. (1998) and the H_2O_2 -specific fluorescent probe H2DCF-DA (green; Molecular Probes, Eugene, OR, USA, prepared in a MES-KCl buffer, pH 5.7). The amount of malondialdehyde (MDA), and the activities of SOD and catalase (CAT) were measured by absorption photometry using a spectrophotometer. The details were as described by Giannopolitis and Ries (1977), Carrillo et al. (1992), and Song et al. (2013), respectively.

DNA and RNA Extraction

Plant materials were stored in liquid nitrogen and total genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen China, Shanghai), according to the manufacturer's protocol. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, China, Shanghai) following the manufacturer's protocol. Genomic DNA and RNA were measured with a Nano Vue UV/visible spectrophotometer (GE Healthcare Company) and stored at -80°C .

Methylation-sensitive Amplification Polymorphism (MSAP) Analysis

Methylation-sensitive amplification polymorphism analysis was carried out based on an established protocol (Sha et al., 2005; Peredo et al., 2006), and the isoschizomers *Hpa* II and *Msp* I were employed as frequent-cutter enzymes. During the selective PCR step, *EcoR* I and *Hpa* II/*Msp*

I primers adding three additional selective nucleotides were used. The selective PCR products were resolved by electrophoresis on 6% sequencing gels and detected with silver staining (Tixier et al., 1997). The differentially amplified fragments represent stress-responsive differentially methylated regions.

The isolation of polymorphic bands was performed as described previously (Sha et al., 2005). Briefly, polymorphic fragments were excised from the gels, hydrated in 50 μ l of water, and incubated at 42°C for 30 min. The eluted DNA was amplified with the same primer pairs and under the same conditions used for selective amplification. Sequence information was obtained by cloning the fragments into vector pMD18-T (Takara Bio, Inc., Tokyo, Japan), and three positive clones for each individual were selected for sequencing.

The methylation patterns of specific cytosine loci (5'-CCGG-3') obtained by MSAP have four types: unknown, unmethylated, methylated at CG (m CG) and hemi-methylated at CNG (m CNG; Supplementary Figure S1). The relative methylation levels were calculated using the following equations: relative methylated level ($\frac{^m\text{CG}}{\text{unknown}+^m\text{CG}+^m\text{CNG}+\text{unmethylated}}$), relative hemi-methylated level ($\frac{^m\text{CNG}}{\text{unknown}+^m\text{CG}+^m\text{CNG}+\text{unmethylated}}$), and relative total methylation level ($\frac{^m\text{CG}+^m\text{CNG}}{\text{unknown}+^m\text{CG}+^m\text{CNG}+\text{unmethylated}}$). In these formulas, m CG represents the number of MSAP markers with the m CG methylation pattern. Similarly, m CNG, unknown, and unmethylated represent the number of MSAP markers with m CNG, unknown, and unmethylated methylation patterns, respectively.

Bisulfite Sequencing of Candidate Differentially Methylated Sequences

For analysis of the candidate differentially methylated sequences (DMSs), genomic DNA was treated with bisulfite and used as template for amplification, which was carried out for 35 cycles. The assay primers span the region that contains the 5'-CCGG-3' sites and are listed in Supplementary Table S3. PCR products were then purified using a Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the vector pMD18-T (Takara Bio, Inc., Tokyo, Japan). 20 positive clones for each individual were selected for sequencing through an ABI sequencer (PRISM BigDye Terminator, ABI, Sunnyvale, CA, USA).

Annotation of miRNA and Target Genes

The DMSs were analyzed using miRBase¹ to find the mapped miRNA genes and psRNATarget tools² to map the target genes in the sequenced reference genome of *P. trichocarpa*. The annotation information for target genes was obtained from PopGenIE³ and pathway analysis of biological process from KEGG⁴.

¹<http://www.mirbase.org/search.shtml>

²<http://plantgrn.noble.org/psRNATarget>

³<http://popgenie.org>

⁴<http://www.kegg.jp/kegg/tool/mappathway2.html>

Real-time Quantitative PCR of Mature miRNAs

Quantitative PCR analysis of miRNA was carried out following a high-stringency protocol where a poly A tail was added by using poly A polymerase. The Power SYBR Green PCR Master Mix (ABI) and the StepOne+ Real-Time PCR System (ABI) were used to perform quantitative PCR according to the standard protocol. The forward primers were designed based on miRNA sequences in miRBase 21.0⁵. The reverse primer was designed based on the poly(T) adapter, which was always the same (5'-GTCGTATTAATTCTGTGCTCGC-3'). The internal reference gene was a 5.8S rRNA (forward primers: 5'-GTCTGCCTGGGTGTCACGCAA-3'; Supplementary Table S2).

Gene Expression Analysis by qRT-PCR

For quantitative PCR analysis, the TaKaRa ExTaq R PCR Kit, SYBR green dye (TaKaRa, Dalian, China) and a DNA Engine Opticon 2 machine (MJ Research) were used. Gene-specific primers were designed to target the 3' UTR of each gene (Supplementary Table S1). A melting curve was used to check the specificity of each amplified fragment. For all reactions, triplicate technical and biological repetitions of each individual were performed; the PCR was performed according to Song et al. (2014). After amplification, the PCR products were sequenced to check the specificity of the primer sets. Relative expression levels of candidate genes were standardized to the transcript levels for *PsiACTIN*, which shows stable expression under abiotic stress calculated by the $2^{-\Delta\Delta C_t}$ method.

Validation by Degradome Sequencing

We used the fastx toolkit to exclude low-quality reads from the degradome sequencing and to remove adapter sequences. The Cleaveland 2.0 software was used to further analyze the reads. Briefly, the *P. trichocarpa* transcripts database of JGI Phytozome V 7.0 was mapped with the reads. Next, the true miRNA cleavage site was distinguished from background noise with a target plot. We used the default parameters and performed 100 randomized sequence shuffles with Cleaveland 2.0. The cleaved targets were categorized into five categories using the following criteria: (1) the read abundance of the cleavage site had the maximum value on the transcript; (2) the read abundance of the cleavage site had the maximum value on the transcript but was not unique; (3) the read abundance of the cleavage site did not have the maximum value but was higher than the median on the transcript; (4) the read abundance of the cleavage site was equal to or less than the median on the transcript; and (5) only one raw read existed at the cleavage site. Only the targets that were verified by degradome sequencing were recorded in this study.

Statistical Analysis

Significance of differences in enzyme activities were determined with the Least Significant Difference test using SPSS 20 (Copyright IBM Corporation 1989). Differences were considered

⁵<http://www.mirbase.org/>

statistically significant when $P < 0.05$. Asterisks “*” represent $P < 0.05$, “**” represent $P < 0.01$ compared with control group.

RESULTS

Changes of Physiological and Biological Parameters in Response to Temperature Stress

To evaluate the dynamic biological and physiological reactions that occur during temperature treatment, we treated 30-cm cuttings from 1-year-old branches at 42 or 4°C for 0, 3, 6, 12, and 24 h and measured four parameters of the cuttings: H_2O_2 contents, and activities of catalase (CAT), SOD, and malondialdehyde (MDA; **Figure 1**). In both heat and cold stress treatments, the activity of CAT and SOD reached a peak at 6 h; also, CAT and SOD activities were significantly higher under heat stress than cold at 6 h. In addition, the amounts of MDA and H_2O_2 significantly increased from 12 to 24 h in cold- and heat-treated plants ($P < 0.01$; **Figure 1**).

Relative Levels and Patterns of Cytosine Methylation under Temperature Stress

We used samples at 6 h of treatment to examine DNA methylation changes and gene expression responses underlying these treatments. MSAP analysis, which can reveal different

methylation sites in the genome, was used to examine the genome-wide patterns of DNA methylation in response to temperature stress treatments. We used 240 primer combinations with 15 *Hpa* II/*Msp* I and 20 *Eco*R I primers (Excel S1) to detect the sites of DNA methylation at the 5'-CCGG-3' sequence from *P. simonii*. In total, MSAP produced 4199 methylation bands, including 1066 polymorphic loci (25.39%; **Figure 2A**). Among these, 70.73 and 46.90% polymorphic loci were from cold stress and heat stress, respectively.

To estimate the relative methylation levels under different abiotic stresses, we next examined the methylation state (unknown, unmethylated, methylated at CG, or hemi-methylated at CNG) of the methylation bands (Supplementary Figure S1). Relative total methylation levels ranged from 39.59% (heat) to 64.54% (cold) and the relative total methylation level of the control group was 50.84%. The relative methylation level (m CG) was 10.98% in heat-treated plants, 14.82% in cold-treated plants, and 11.91% in control plants. In addition, the relative hemi-methylation level of *P. simonii* in different treatments was 28.61% (heat), 49.72% (cold), and 38.93% (control). These results showed that plants showed the highest relative methylation in all methylation patterns under cold stress treatment, and the lowest level under heat stress treatment (**Figures 2B–D**). Comparison of methylation band types under heat and cold stress treatments also detected differences in the cytosine methylation patterns under different abiotic stresses. This identified 175 heat-specific bands (94 m CG and 81 m CNG), 211 cold-specific methylated bands (89

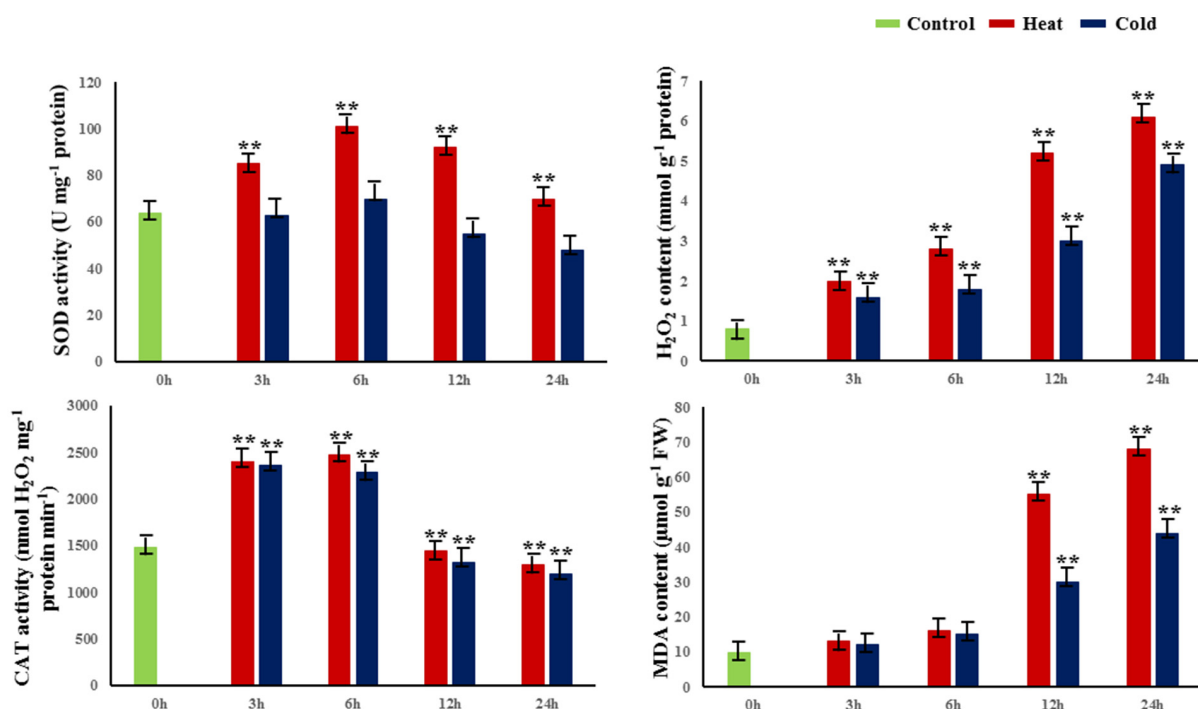
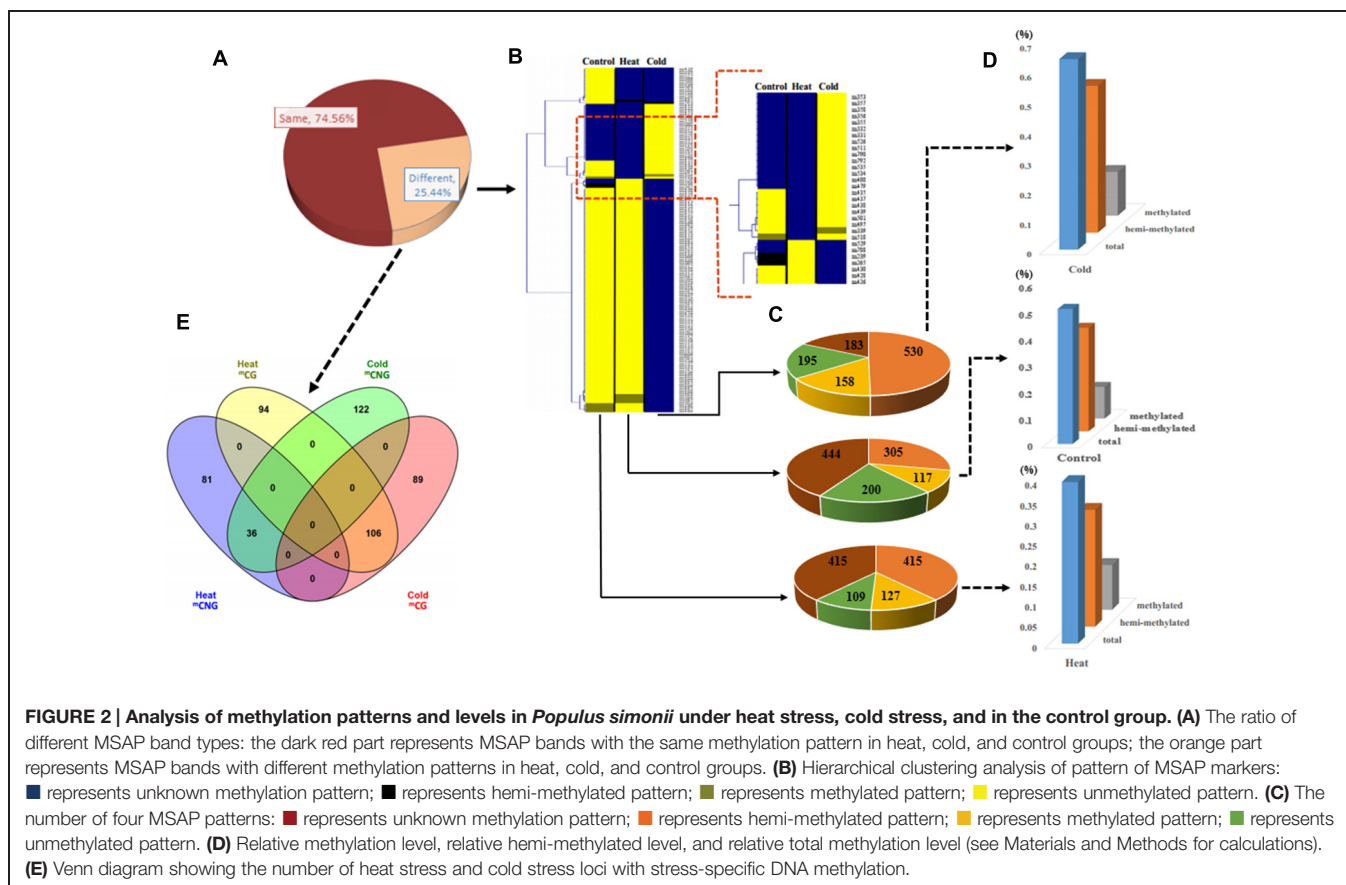


FIGURE 1 | Changes in physiological and biochemical properties in response to abiotic stress. SOD superoxide dismutase, H_2O_2 hydrogen peroxide, MDA malondialdehyde, CAT catalase. 0 h indicates the control group without stress treatment. 3–24 h indicate different times of exposure to heat and cold stress. Error bars represent standard error. Activities are presented as mean \pm SE, and $n = 3$. An asterisk indicates differences at $*P < 0.05$ compared with the control group, and two asterisks indicates significant differences at $**P < 0.01$ compared with the control group.



^mCG and 122 ^mCNG), and 142 bands present in both heat and cold (106 ^mCG and 36 ^mCNG; **Figure 2E** and Excel S1).

Identification of miRNA Genes Methylated in Response to Temperature Stress

Based on the polymorphisms observed in MSAP bands under different temperature stresses, we isolated and sequenced a subset of the stress-specific DMSs. We focused on two classes: Class I includes DMSs present in both heat and cold stresses, but not in the control group; Class II includes DMSs specific to heat or cold stress. From the 1066 differentially methylated bands, we isolated and sequenced 400 bands, including 150 stress-specific MSAP bands for each stress and 100 bands common to both stresses. After removing low-quality sequences and redundant fragments, we finally obtained 259 DMSs.

To identify miRNA genes potentially affected by methylation in response to temperature stress, the DMSs were annotated by BLAST searches against miRBase. This identified seven DMSs that mapped to conserved miRNA genes in the sequenced *P. trichocarpa* genome: *Ptc-MIR156i*, *Ptc-MIR156j*, *Ptc-MIR167h*, *Ptc-MIR390c*, *Ptc-MIR393a*, *Ptc-MIR396e*, and *Ptc-MIR396g* (Excel S2); these are homologs of the *P. simonii* miRNA genes. The psRNATarget algorithm predicted 111 targets of the miRNAs produced by these methylated miRNA genes (Excel S3) and gene ontology (GO) classification of these miRNA

targets showed enrichment of hydrolase activity, acting on acid anhydrides, ribonucleotide binding, purine nucleotide binding, DNA binding, and purine nucleoside binding in molecular function GO terms (Supplementary Figure S3A). In biological process, regulation of cellular biosynthetic process, RNA metabolic process, regulations of macromolecule biosynthetic process, and regulation of gene expression were enriched (Supplementary Figure S3B).

Methylation of miRNA Genes in Response to Temperature Stress

Annotation analysis of all target genes based on their homologs in the sequenced reference genome of *P. trichocarpa* showed that of the three miRNA genes are located in genic regions. Examination of the local methylation patterns of these miRNA genes revealed that *Ptc-MIR393a* shows different cytosine methylation patterns among control group (^mCNG), cold (^mCG), and heat stress (^mCNG) and is located in the 5' untranslated regions (UTR) and first exon of *Potri.008G062800*, which has two exons and one intron. Also, *Ptc-MIR396e* is located in the 5' UTR and first exon of *Potri.018G127000*, which has two exons and one intron and was unmethylated in the control group, ^mCG in cold-treated plants, and ^mCNG in heat-treated plants. Moreover, *Ptc-MIR396g* is located in the promoter of *Potri.003G160700*, which contains four exons and three introns, and was unmethylated in the control group, ^mCG

To confirm the status of the methylation of DMSs, we performed bisulfite sequencing for each of the temperature stresses (treated for 6 h) and the control group. The methylation level of CHG on candidate DMSs ranged from 34.2 to 100.0% in the control, CHH ranged from 48.6 to 69.5%, and CG ranged from 51.3 to 90.6%. The methylation levels in the temperature treated samples are listed in **Table 2**, and the sequences contained less CG and CHG methylation than CHH methylation. In treated individuals, *de novo* methylation and demethylation occurred simultaneously (Excel S4 and Supplementary Figure S5), but methylation of these candidate sequences showed a decreasing trend, with the methylation under heat treatment declining more than under cold treatment, and the methylation level in ^mCNG context being lower than in ^mCG and unmethylated.

Expression of miRNA Genes in Response to Temperature Stress

To verify the possible relationship between DNA methylation pattern and expression of candidate miRNA genes, we used quantitative real-time PCR (qRT-PCR) to detect the expression levels of these miRNA genes in control and treated samples (Supplementary Table S2). Five miRNA genes (*Ptc-MIR156i*, *Ptc-MIR167h*, *Ptc-MIR393a*, *Ptc-MIR396e*, and *Ptc-MIR396g*) showed ^mCG modification under cold stress and ^mCNG modification under heat stress but were unmethylated in the control group (**Table 1**). The qRT-PCR analysis showed that the

expression of miRNA genes with ^mCNG was significantly higher than the miRNA genes with ^mCG and also higher than the non-methylated genes under temperature stress. *Ptc-MIR390c* showed ^mCG under heat stress and was unmethylated in cold-treated and control groups and also showed lower expression when modified with ^mCG, compared with the unmethylated control (**Table 1**; **Figures 3B,C**). Thus, generally, under temperature stress, the candidate methylated miRNA genes that had ^mCNG showed higher expression than those that had ^mCG. However, miRNA genes with the same methylation pattern under heat and cold stresses showed different expression levels under these two conditions. For instance, *MIR156j* had the same methylation pattern (^mCG) in both heat and cold stresses, but it showed higher expression under heat stress than under cold stress (**Table 1**).

Expression of Target Genes of miRNAs

After we measured the expression of the miRNA genes (Supplementary Table S1), we next measured the expression of their target genes to examine the regulatory relationship between the miRNAs and their targets. In total, we found 111 target genes, including 38 targets of *Ptc-miR156i/j*, 30 of *Ptc-miR167h*, 11 of *Ptc-miR393a*, and 32 of *Ptc-miR396e/g*. To detect whether the changes in miRNA gene expression affected target gene expression, we used qRT-PCR to measure the mRNA levels of the targets of these miRNA genes (**Figure 3E** and Excel S3). The results showed that the targets' expression

TABLE 2 | Methylation level in different sequence contexts.

Sequence	Sample	Methylation pattern ^b	^m CG/total CG	Number of CG	^m CHG/total CHG	Number of CHG	^m CHH/total CHH	Number of CHH
1 ^a	Control	Unmethylated	105/140 (75.0) ^c	7	123/140 (87.9)	7	623/900 (69.2)	45
	Heat-treated	^m CNG	99/140 (70.7)	7	81/140 (57.9)	7	400/900 (44.4)	45
	Cold-treated	^m CG	63/140 (45.0)	7	120/140 (85.7)	7	601/900 (66.8)	45
2 ^a	Control	Unmethylated	145/160 (90.6)	8	64/120 (53.3)	6	308/560 (55.0)	28
	Heat-treated	^m CNG	101/160 (63.1)	8	66/120 (55.0)	6	199/560 (35.5)	28
	Cold-treated	^m CNG	116/160 (72.5)	8	78/120 (65.0)	6	411/560 (73.4)	28
3 ^a	Control	Unmethylated	144/200 (72.0)	10	57/80 (71.3)	4	261/420 (62.1)	21
	Heat-treated	^m CNG	145/200 (72.5)	10	18/80 (22.5)	4	162/420 (38.6)	21
	Cold-treated	^m CG	116/200 (58.0)	10	55/80 (68.8)	4	288/420 (68.6)	21
4 ^a	Control	Unmethylated	226/260 (86.9)	13	100/100 (100.0)	5	482/760 (63.4)	38
	Heat-treated	^m CG	167/260 (64.2)	13	38/100 (38.0)	5	383/760 (50.4)	38
	Cold-treated	Unmethylated	199/260 (76.5)	13	84/100 (84.0)	5	517/760 (68.0)	38
5 ^a	Control	^m CNG	82/160 (51.3)	8	123/140 (87.9)	7	343/580 (59.1)	29
	Heat-treated	^m CNG	80/160 (50.0)	8	120/140 (85.7)	7	144/580 (24.8)	29
	Cold-treated	^m CG	118/160 (73.8)	8	99/140 (70.7)	7	247/580 (42.6)	29
6 ^a	Control	Unmethylated	222/260 (85.4)	13	41/120 (34.2)	6	243/500 (48.6)	25
	Heat-treated	^m CNG	149/260 (57.3)	13	35/120 (29.2)	6	138/500 (27.6)	25
	Cold-treated	^m CG	156/260 (60.0)	13	15/120 (12.5)	6	269/500 (53.8)	25
7 ^a	Control	Unmethylated	120/220 (54.5)	11	62/120 (51.7)	6	445/640 (69.5)	32
	Heat-treated	^m CNG	63/220 (28.6)	11	41/120 (34.2)	6	280/640 (43.8)	32
	Cold-treated	^m CG	105/220 (47.7)	11	63/120 (52.5)	6	361/640 (56.4)	32

^aThe number represents candidate differentially methylated sequences (DMSs), seen in Excel S4. ^bThe methylation pattern refers to the pattern derived from DMSs cut by *EcoR* I and *Hpa* II/*Msp* I. ^cThe sum of ^m5C in the total methylatable Cs in the indicated sequences in a total clones examined are presented as numbers observed and as percentages (%) in parentheses. For example, the 140 CG sites were calculated by multiplying 20 clones by seven sites derived from candidate DMS 1 overlapping with *Ptc-MIR156i* (Excel S2).

varied with the expression changes in the corresponding miRNA genes in response to heat and cold stresses. For example, *Potri.001G294400* encoding a homolog of *Arabidopsis* VACUOLAR SORTING RECEPTOR 5 (VSR5), a target of Ptc-miR156i and j with ^mCG methylation modification, was downregulated in response to cold stress, when *Ptc-MIR156i* and *j* were upregulated. *Potri.001G323100* encodes a homolog of *Arabidopsis* AUXIN SIGNALING F-BOX 2 (AFB2), is a target of Ptc-miR393a, showed ^mCNG, and was repressed under heat stress, in contrast to *Ptc-MIR393a*. In short, expression of the target genes decreased as the expression of the corresponding miRNA increased, consistent with the gene-silencing function of miRNAs (Figure 3D).

Regulatory Network of DNA Methylation in the Response to Temperature Stress

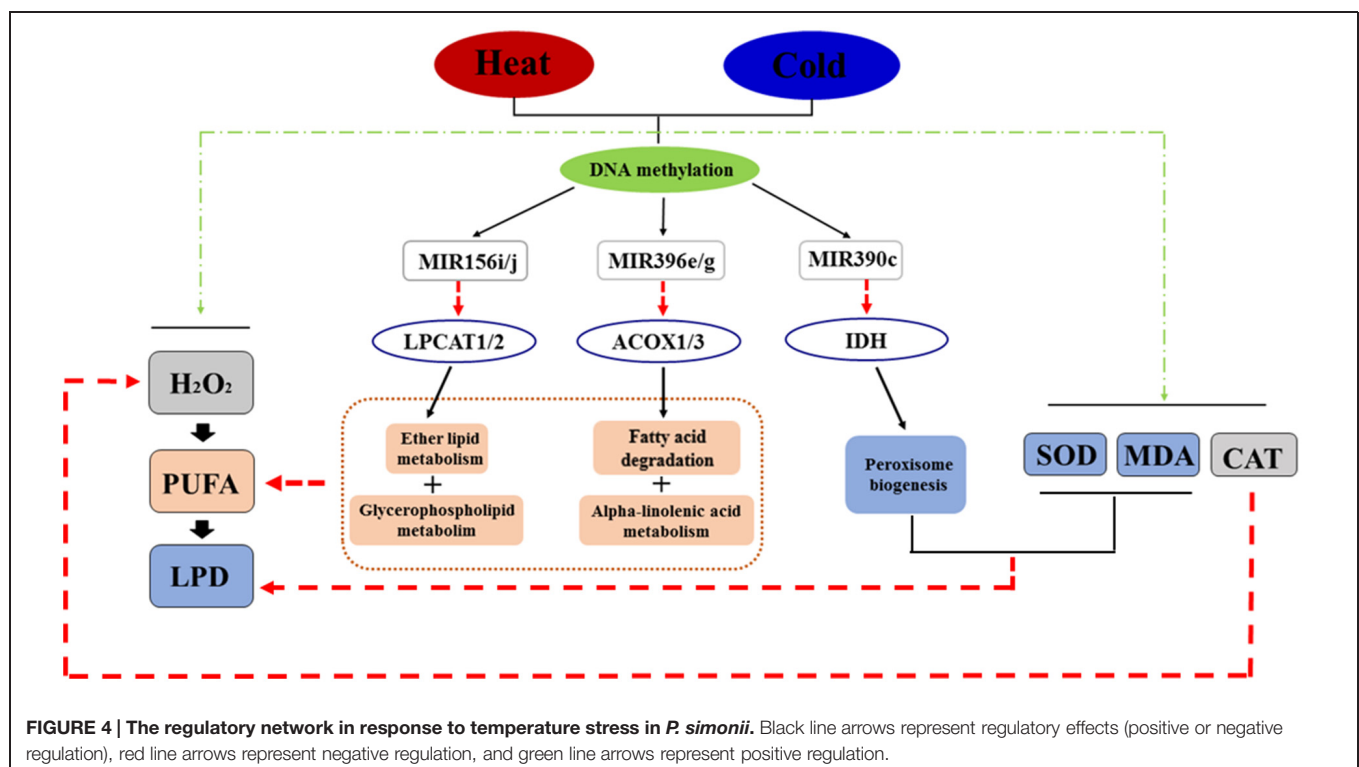
KEGG pathway analysis was used to characterize the functions of the target genes and indicated that target genes generally participate in lipid metabolism, including fatty acid degradation. For example, *Potri.007G090400*, a target of Ptc-miR396e and g, encodes a protein that functions in glycerophospholipid metabolism. *Potri.014G042200*, a target gene of Ptc-miR156i and j, encodes a protein involved in ether lipid metabolism. Other predicted products function in alpha-linolenic acid metabolism [ACYL-COA OXIDASE (ACOX1 and ACOX3)] and biosynthesis of unsaturated fatty acids [ACYL-COA OXIDASE (ACOX1 and ACOX3)], which all deplete lipid peroxide. Also, ISOCITRATE DEHYDROGENASE (IDH) participates in peroxisome biogenesis (a target gene of Ptc-miR390c; Biswas and Mano, 2015; Figure 4 and Excel S3). To validate the predicted

target genes of miRNAs, degradome sequencing was performed, which confirmed *LPCAT1/2*, *ACOX1/3*, and *IDH* as targets of *MIR156i/j*, *MIR396e/g*, and *MIR390c*, respectively, in lipid metabolism or peroxidation (Supplementary Figure S4).

Based on these pathways and the interactions between DNA methylation, miRNAs, and target genes, we constructed a regulatory network showing the changes of DNA methylation in response to temperature stress (Figure 4). In this network, temperature stress (heat and cold) induced production of H₂O₂, which produced more oxyradicals attacking polyunsaturated fatty acids, which induced lipid peroxidation to destroy cells. Moreover, heat and cold stresses induced SOD, MDA, and CAT activities to decrease superoxide, lipid peroxide, and H₂O₂ levels, respectively. Temperature stress also changed the methylation patterns of specific loci associated with miRNA genes responding to stress treatments, including *Ptc-MIR156i* and *j*, *Ptc-MIR390c*, and *Ptc-MIR396e* and *g*, which decrease the products of ACYL-COA OXIDASE (ACOX1 and ACOX3), PHOSPHOLIPID/GLYCEROL ACYLTRANSFERASE FAMILY PROTEIN (LPCAT1 and LPCAT2), and ISOCITRATE DEHYDROGENASE (IDH) from *Potri.007G090400*, *Potri.014G042200*, and *Potri.010G176000*, respectively (Excel S3). These products participate in lipid metabolism and peroxisome biogenesis; these reversible pathways can consume polyunsaturated fatty acids and lipid peroxides to prevent cell death (Figure 4).

Verification of MSAP Data

To make sure the MSAP data are reliable, we used methylation-sensitive PCR (MS-PCR) to confirm the data produced by



MSAP. For this purpose, we design MS-PCR primers for 45 MSAP sequences for 15 stress-specific MSAP bands and 15 common MSAP bands in both heat and cold stress treatments (Supplementary Figure S2). The MS-PCR results indicate that MSAP is a stable, effective, and reproducible technology for detecting methylated sites that change in response to temperature stress in the genome of *P. simonii*.

DISCUSSION

Catalase, SOD, and MDA are three important factors participating in general physiological and biochemical processes in plants. H_2O_2 produces oxyradicals that attack polyunsaturated fatty acids, thus inducing lipid peroxidation to destroy cells (Roach et al., 2015). CAT metabolizes H_2O_2 to prevent or reduce these harmful effects (Nie et al., 2015; Zhang et al., 2015), and provides a parameter allowing evaluation of the degree of physiological and biochemical effects of stress. The decomposition products of lipid peroxide induce cell damage and can be measured by the amount of MDA, which reflects the extent of lipid peroxidation and thus the extent of cell damage. Measurements of MDA and SOD complement each other, as the SOD activity indirectly reflects the oxygen free radical-scavenging ability of the cell, and the level of MDA indirectly reflects the severity of the effects of free radicals on the cell. In this study, we detected changes in CAT activity, SOD activity, MDA contents, and H_2O_2 contents in response to temperature stress. These results showed that the physiological reaction of poplar is the strongest after 6-h temperature stress treatment. So, we chose *P. simonii* after 6-h stress treatment as the experimental material for analysis of DNA methylation.

The Variation of DNA Methylation in Response to Heat and Cold Stress

In this study, we found that 25.38% of methylation sites changed in response to abiotic stress. This was consistent with the observations of Alonso et al. (2015), who examined 49 studies on different abiotic stresses in 18 species; in 78% of studies, the results agreed with the hypothesis that stress elicited changes in global DNA methylation. This suggests that DNA methylation might function in genomic regulation in response to abiotic stress. In addition, Tang et al. (2014) found that cytosine methylation at various loci decreased 10.28% due to drought exposure in *Lolium perenne*. Our results showed that 70.73 and 46.90% of differentially methylated loci responded to cold or heat stress, respectively, suggesting that methylated loci might respond differently to different abiotic stresses. The $mCHG$ and $mCHH$ modifications also showed site-specific methylation between male and female flowers, suggesting that different DNA methylation patterns might have different influences on flower development (Song et al., 2014). By contrast, in this study, we obtained 175 methylated bands (94 mCG and 81 $mCNG$) that were specific to heat treatment, and 211 methylated bands (89 mCG and 122 $mCNG$) that were specific to cold stress (Figure 2E and Excel S1), suggesting that methylation patterns might differ in the responses to heat and cold stress.

The Effect of Methylated miRNA Genes on the Expression of Target Genes

Different DNA methylation patterns have different effects on gene expression (Sunkar and Zhu, 2004). *MIR164a* associated with flower development showed significantly lower methylation levels in female flowers than in male flowers and induced expression in male flowers; by contrast, *MIR164a* was only methylated in male flowers and its CHH methylation level was higher than its CG and CHG levels (Song et al., 2014). These results indicated that the expression of miRNA genes might be regulated by their methylation level or pattern. Here, five candidate miRNA genes (*MIR156i*, *MIR167h*, *MIR393a*, *MIR396e*, and *MIR396g*) related to the response to temperature stress showed mCG modification and repressed expression under cold stress; these loci also showed $mCNG$ modification and induced expression under heat stress, compared with the expression of miRNA genes that were unmethylated under control conditions (Table 1). This furthermore indicated that the different cytosine methylation patterns of MSAP markers probably associate with different expression levels of miRNA genes.

Moreover, the methylation levels under heat treatment declined more than under cold treatment, which might explain the observation that miRNA expression increased most in heat-treated samples. The CNG methylation was generally lower than the other methylation patterns (mCG and unmethylated), which indicated that the results of methylation sequencing support the results of the enzyme digestion. These findings suggest that in poplar, methylation regulates miRNA gene expression in the response to abiotic stress.

In addition, to detect the influence of these miRNA genes on their target genes, we used qRT-PCR to survey the transcript levels of target genes. The results showed that *ACYL-COA OXIDASE 1 (ACOX1)* and *ACYL-COA OXIDASE 3 (ACOX3)*, targets of Ptc-miR396e and g, were induced in cold stress. The *IDH* gene, a target of Ptc-miR390c, was highly expressed in cold stress. Two targets of Ptc-miR156i and j, *PHOSPHOLIPID/GLYCEROL ACYLTRANSFERASE FAMILY PROTEIN (LPCAT1 and LPCAT2)*, were repressed in heat stress (Excel S3). Degradome sequencing confirmed that these targets produce cleavage products consistent with regulation by the corresponding miRNAs. These variations of expression were consistent with the gene-silencing function of miRNAs, where miRNA levels negatively correlate with the transcript levels of their target genes. Our results suggested that the function of DNA methylation in response to temperature stress might be implemented by affecting expression of miRNAs and their targets.

The Regulatory Network of *P. simonii* in the Response to Abiotic Stress

Recent work showed that lipid peroxide-derived toxic carbonyl compounds mediate environment stress-induced damage of plants (Biswas and Mano, 2015), suggesting that lipid peroxide might also negatively affect plants in response to abiotic stress. Lipid peroxide derives from lipid peroxidation due to

oxyradicals that attack polyunsaturated fatty acids when plants suffer temperature stress that induces oxidative stress. Abiotic stress signals result directly or indirectly from gene expression regulated by many factors, including DNA methylation (Rakei et al., 2015).

Here, we focused on the effect of DNA methylation on regulation of miRNAs as critical to changes in target gene expression to adapt to abiotic stress. Numerous genes related to oxidative stress play crucial roles in maintaining reactive oxygen species homeostasis and levels in organisms. In this study, we found *Ptc-MIR156i* and *j*, and *Ptc-MIR396e* and *g* affected genes related to lipid metabolism and depletion of polyunsaturated fatty acids preventing the production of lipid peroxide. However, miRNAs with different methylation patterns were differentially expressed under different stress treatments, which led to higher expression of *ACOX1* and 3, and *LPCAT1* and 2 under cold stress with ¹³C pattern than under heat stress with ¹³CNG pattern. This pattern was reversed for *IDH*, which functions in peroxisome biogenesis directly reducing lipid peroxides. The *ACOX* gene encodes a peroxisomal acyl-CoA oxidase that is thought to catalyze the first reaction during biosynthesis of the fatty acid component of daumones (Joo et al., 2010). The transcript levels of *ACOX* did not show significant changes following oxidative stress induced by perfluorododecanoic acid (Liu et al., 2008). *IDH* is resistant to denaturation, which reduces its catalytic efficiency in high temperature regimes (Bergmann and Gregorius, 1993). Rzezniczak and Merritt (2012) found that *IDH* seem to be induced by oxidative stress in the enzyme network responsible for the reduction of nicotinamide adenine dinucleotide phosphate and further mentioned that biological network interactions were strongly influenced by environmental conditions. In our study, transcript levels of *ACOX* decreased 2.4-fold and *IDH* decreased over 5-fold in response to heat treatment (Excel S3); these changes of expression might relate to the variation in DNA methylation. Plant *LPCAT* enzymes are crucial in regulating the acyl-CoA composition of cells by transferring polyunsaturated and hydroxy fatty acids produced on phosphatidylcholine straight to the acyl-CoA pool for further

metabolism or catabolism (Lager et al., 2013). *Arabidopsis* *LPCATs* were measured in the reverse reaction from which phosphatidylcholine was transferred to acyl-CoA to a similar extent. Here, *LPCAT1/2* decreased 2.66-fold under heat treatment (Excel S3), while the methylation level of candidate sequences overlapping *Ptc-MIR156i* and *Ptc-MIR156j* declined (Table 2), suggesting that the variation of DNA methylation might suppress *LPCAT* correlated with reduced PC for fatty acid desaturation to protect cells from temperature stress. These results indicated that methylated miRNAs might play a key role in *P. simonii* under temperature stress. Our data also provide a series of candidate miRNA genes for research into epigenetic regulation of abiotic stress responses.

AUTHOR CONTRIBUTIONS

DZ conceived and designed the experiment. DC performed the DNA and RNA extractions and performed MSAP analyses and drafted the manuscript. YS carried out the gene expression analysis. MT participated in the statistical analyses. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00921>

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Chromatin resetting mechanisms preventing transgenerational inheritance of epigenetic states

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Epigenetic regulation can be altered by environmental cues including abiotic and biotic stresses. In most cases, environmentally-induced epigenetic changes are transient, but in some cases they are maintained for extensive periods of time and may even be transmitted to the next generation. However, the underlying mechanisms of transgenerational transmission of environmentally-induced epigenetic states remain largely unknown. Such traits can be adaptive, but also can have negative consequences if the parentally inherited epigenetic memory interferes with canonical environmental responses of the progeny. This review highlights recent insights into the mechanisms preventing transgenerational transmission of environmentally-induced epigenetic states in plants, which resemble those of germline reprogramming in mammals.

Keywords: transgenerational epigenetic memory, chromatin regulation, vernalization, FLC, ELF6, DDM1, MOM1

Introduction

Epigenetic marks such as DNA methylation, histone modifications, or histone variants influence the chromatin structure and transcriptional states. These epigenetic marks can be stably maintained, but also can be dynamically altered during development or in response to environmental stimuli.

In most cases, environmentally-induced epigenetic changes are transient. However, in some cases they are stably maintained through mitotic cell divisions and therefore can be regarded as a type of long-term cellular memory. The best understood example of such epigenetic memory in plants is that of vernalization, which involves the epigenetic silencing of *FLOWERING LOCUS C (FLC)* gene by prolonged cold (Kim and Sung, 2012; Song et al., 2012). During embryogenesis, the *FLC* epigenetic state is reset thus allowing the next generation to respond to vernalization signals (further described below).

Epigenetic changes can be meiotically inherited (i.e., transgenerationally transmitted). The inheritance of epigenetic changes is especially well documented in plants where DNA methylation is stably maintained mitotically and meiotically (Law and Jacobsen, 2010). In *Arabidopsis*, DNA METHYLTRANSFERASE1 (*MET1*) and chromatin remodeling factor DECREASE IN DNA METHYLATION 1 (*DDM1*) are essential for global maintenance of DNA methylation as shown by the whole genome hypomethylation occurring in *met1* and *ddm1* mutants (Finnegan and Dennis, 1993; Vongs et al., 1993; Jeddeloh et al., 1998; Saze et al., 2003). Many of these hypomethylated loci are stably inherited for many generations even after reintroduction of a functional *MET1* or *DDM1* allele (Johannes et al., 2009; Reinders et al., 2009). Furthermore, transgenes, viral infection, or specific plant tissue culture conditions can also alter DNA methylation patterns in some genes, thus inducing so-called “epialleles” (Vaucheret et al., 1998; Baulcombe, 1999; Vaucheret and Fagard, 2001; Krizova et al., 2009; Rhee et al., 2010).

progeny of non-vernalized plants in the mutants, suggesting that removal of H3K27me3 by ELF6 is required for restoring the pre-vernalization chromatin state associated with *FLC* (Figure 1B). Intriguingly, different *ELF6* alleles are associated with distinct phenotypic responses. Loss-of-function *elf6* alleles are early flowering irrespective of the occurrence of vernalization due to the increased expression of the flowering regulator gene *FT* (Noh et al., 2004). Thus, it was suggested that ELF6 has a broader function, and that the particular hypomorphic mutation reveals a specific aspect of ELF6's activity to restore the pre-vernalization chromatin state of *FLC* during embryogenesis.

In mammals, reprogramming of epigenetic marks, including H3K27me3, occurs in germ cells and early embryo (Cantone and Fisher, 2013). The reprogramming mediated by ELF6 would be a relevant mechanism conserved in evolution. In *Arabidopsis* genome, H3K27me3 is found in 15% of all genes (Zhang et al., 2007). It would be interesting to address whether other genes are reprogrammed by ELF6 or related proteins.

Resetting Chromatin Changes Induced by Heat Stress

In 2010, three independent research groups reported the influence of environmental stresses on epigenetically silenced loci in *Arabidopsis* (Lang-Mladek et al., 2010; Pecinka et al., 2010; Tittel-Elmer et al., 2010). They exposed plants to various stress conditions such as temperature shift, drought, elevated salinity, or UV radiation, and examined activities of transcriptionally silenced reporter genes. It was found that heat stress (37 or 42°C) or UV-B radiation releases silencing and activates reporter genes. The release of transcriptional silencing induced by stress occurs at various endogenous loci (Tittel-Elmer et al., 2010). However, this activation is transient since the loci are re-silenced within a few days after stress (Lang-Mladek et al., 2010; Pecinka et al., 2010; Tittel-Elmer et al., 2010). The rapid re-silencing appears to involve nucleosome loading since it is delayed in mutants with impaired chromatin assembly (Pecinka et al., 2010). These results suggest that chromatin non-permissive to transcription displays plasticity in response to stress, but also that there is a robust buffering system that resets chromatin changes to the initial ground state. Interestingly, stress-induced transcriptional activation occurs in differentiated tissues but not in meristematic tissues, suggesting the existence of a mechanism protecting germline cells from epigenetic damage (Pecinka et al., 2010).

Interestingly, the LTR-type retroelement *ONSEN* was found to behave rather exceptionally in response to heat stress. Unlike other heterochromatic loci destabilized by heat, the transcriptional activation persists for longer periods of time (Pecinka et al., 2010; Tittel-Elmer et al., 2010). The heat stress-induced transcriptional activation is enhanced in siRNA defective mutants, however, eventually *ONSEN* transcripts gradually decay as the plant pursues its growth, and no transpositions can be detected in vegetative tissues. Surprisingly, high frequency of transposition is observed in the progeny of siRNA defective mutants subjected to heat stress, suggesting that the siRNA pathway prevents transgenerational transposition of *ONSEN* (Ito et al., 2011).

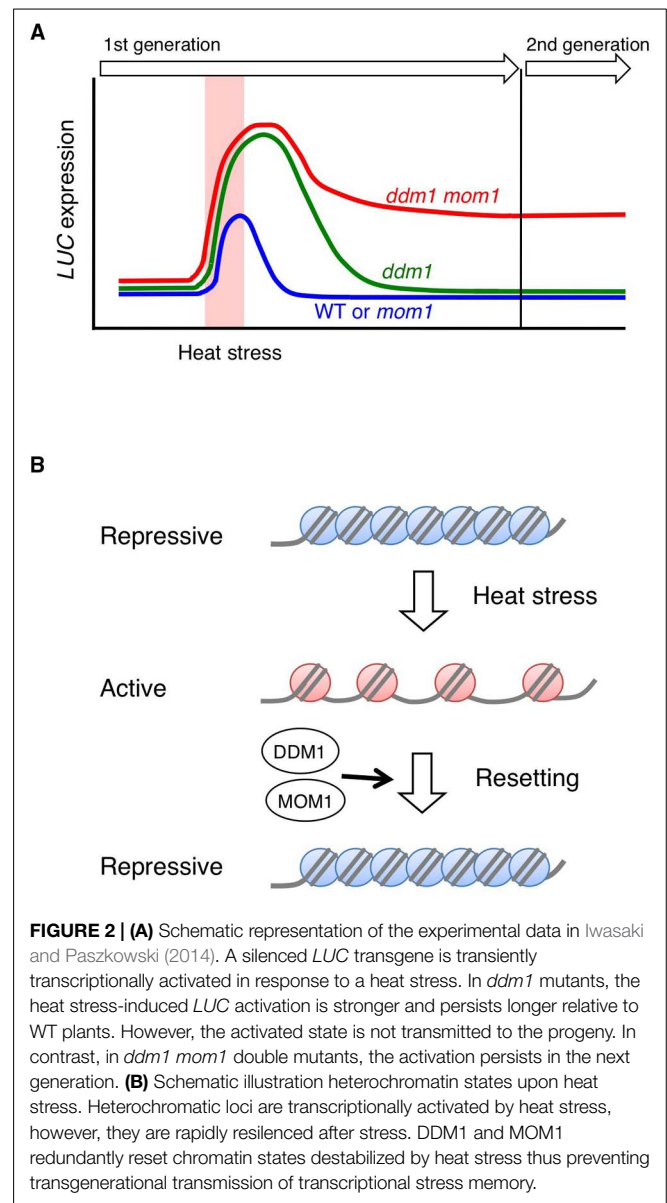


FIGURE 2 | (A) Schematic representation of the experimental data in Iwasaki and Paszkowski (2014). A silenced *LUC* transgene is transiently transcriptionally activated in response to a heat stress. In *ddm1* mutants, the heat stress-induced *LUC* activation is stronger and persists longer relative to WT plants. However, the activated state is not transmitted to the progeny. In contrast, in *ddm1 mom1* double mutants, the activation persists in the next generation. **(B)** Schematic illustration heterochromatin states upon heat stress. Heterochromatic loci are transcriptionally activated by heat stress, however, they are rapidly resiled after stress. DDM1 and MOM1 redundantly reset chromatin states destabilized by heat stress thus preventing transgenerational transmission of transcriptional stress memory.

Recently, Iwasaki and Paszkowski (2014) identified factors preventing transgenerational transmission of stress-induced chromatin changes by forward genetic screen in *Arabidopsis*. A silenced luciferase (*LUC*) reporter gene, whose transcription is transiently activated by heat stress, was used to isolate mutants that retain high or prolonged *LUC* activity after heat stress. This led to the identification of the epigenetic regulators DDM1 and MORPHEUS' MOLECULE1 (MOM1) as components of a mechanism resetting stress-induced chromatin changes. In the *ddm1* mutant, the heat stress-induced *LUC* activation is stronger and persists longer than WT, but the activated state is not transmitted to the progeny. In the *mom1* mutant, stress-induced activation and subsequent extinction is similar to that of WT. However, and remarkably, in *ddm1 mom1* double mutants, the activation persists in the next generation (Figure 2A). Genome-wide transcriptional profiles revealed that stress-induced transcriptional alterations at

various heterochromatic loci were transmitted to next generation in *ddm1 mom1* double mutants. These results indicate that DDM1 and MOM1 redundantly reset chromatin states destabilized by heat stress in order to prevent transgenerational propagation of transcriptional stress memory (Figure 2B).

Both *DDM1* and *MOM1* are required to maintain transcriptional gene silencing (TGS) since mutations in either genes cause release of silencing of heterochromatic loci (Jeddeloh et al., 1998; Amedeo et al., 2000; Steimer et al., 2000). Although *DDM1* and *MOM1* share a number of common target loci for silencing, the regulation mechanism seems to be different for each gene.

DDM1, which is conserved between plants and animals, is a chromatin remodeling factor of the SWI2/SNF2 family (Jeddeloh et al., 1999; Bourc'his and Bestor, 2002; Tao et al., 2011). *ddm1* mutants show progressive global loss of DNA methylation during inbreeding (Kakutani et al., 1996; Jeddeloh et al., 1998). It has been suggested that DDM1 facilitates access of DNA methyltransferases to histone H1-containing heterochromatin (Zemach et al., 2013).

MOM1 is a plant-specific protein with limited homology to the SWI2/SNF2 family whose function remains poorly understood. Mutations in *MOM1* cause release of TGS without major changes in DNA methylation levels, suggesting that MOM1 exerts its silencing function through pathways that are either independent or downstream of those of DNA methylation (Amedeo et al., 2000; Vaillant et al., 2006). Structural and genetic studies indicate that a conserved domain of MOM1 forms a homodimer, which may provide a binding platform for additional silencing factors (Yokthongwattana et al., 2010; Nishimura et al., 2012).

The transient release of transcriptional silencing induced by heat stress is not associated with significant changes in DNA methylation or histone modifications (Pecinka et al., 2010; Tittel-Elmer et al., 2010). Likewise, high levels of DNA methylation were maintained on the promoter of the *LUC* reporter gene in the progeny of heat stressed *ddm1 mom1* mutants despite the occurrence of high *LUC* expression (Iwasaki and Paszkowski, 2014). This strongly suggests that epigenetic marks other than DNA methylation are transmitted to the next generation in the *ddm1 mom1* mutants. The nature of these epigenetic marks necessary for the persistence of stress induced activation of heterochromatic transcription remains to be elucidated.

In summary, this study revealed a previously unidentified function of DDM1 and MOM1 to reset stress-induced chromatin changes. Future studies should address whether similar mechanisms occur in other species given that DDM1 is conserved in yeast and animals.

Concluding Remarks

The recent progress in our understanding of the mechanisms preventing transgenerational transmission of environmentally-induced epigenetic states opens new avenues for the study of epigenetic inheritance while raising new questions such as that of redundancy of the system. It was reported that DDM1 and MOM1 act redundantly to reset chromatin destabilized by heat stress. Furthermore, although about 3,000 loci on the *Arabidopsis* genome are activated by heat stress (Tittel-Elmer et al., 2010), only about one-tenth remain active in the progeny of heat stressed *ddm1 mom1* mutants, suggesting that other factors act in parallel in the same silencing pathway (Iwasaki and Paszkowski, 2014). Similarly, in the *elf6* hypomorphic mutants, the vernalized state of *FLC* is partially restored. *FLC* expression in the progeny of vernalized *elf6* hypomorphic mutants is lower than in the non-vernalized plants, but still higher than in fully vernalized plants (Crevillén et al., 2014). Thus these observations suggest that other factors act redundantly to reset *FLC*.

These redundancies, essential to confer robustness to the system, would be crucial to ensure erasure of parental memory in order to permit progeny to respond appropriately to current environmental conditions. They could also account for the difficulty in documenting the occurrence of transgenerational transmission of environmentally induced epigenetic traits.

It remains possible that certain environmentally induced epigenetic changes could be inherited and become adaptive as in the case of some TEs which contributed to genome evolution. Further investigations would clarify the issue.

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Composition of the SAGA complex in plants and its role in controlling gene expression in response to abiotic stresses

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Protein complexes involved in epigenetic regulation of transcription have evolved as molecular strategies to face environmental stress in plants. SAGA (Spt-Ada-Gcn5 Acetyltransferase) is a transcriptional co-activator complex that regulates numerous cellular processes through the coordination of multiple post-translational histone modifications, including acetylation, deubiquitination, and chromatin recognition. The diverse functions of the SAGA complex involve distinct modules that are highly conserved between yeast, flies, and mammals. In this review, the composition of the SAGA complex in plants is described and its role in gene expression regulation under stress conditions summarized. Some of these proteins are likely involved in the regulation of the inducible expression of genes under light, cold, drought, salt, and iron stress, although the functions of several of its components remain unknown.

Keywords: SAGA complex, chromatin remodeling, transcriptional coactivator, abiotic stress, protein complex, histone acetyltransferase

INTRODUCTION

Transcriptional coactivators are multi-protein complexes that can recognize histone markers, modify chromatin, and recruit the transcriptional machinery to control gene expression (Näär et al., 2001). In general, these complexes regulate eukaryotic gene expression by interacting with transcription factors and/or other regulatory components of the basal transcription machinery. SAGA (Spt-Ada-Gcn5-Acetyl transferase) is a transcriptional coactivator complex involved in the regulation of numerous cellular processes through the coordination of the post-translational modification of various histones. The yeast SAGA complex is thought to control transcription of approximately 10% of genes, particularly stress-related genes (Lee et al., 2000; Huisinga and Pugh, 2004). This complex is generally regarded as a coactivator complex (Kuo et al., 1998), but also has a negative role in gene expression (Belotserkovskaya et al., 2000; Ricci et al., 2002). The SAGA complex is involved in histone acetylation (HAT) (Grant et al., 1997), histone deubiquitination (Daniel et al., 2004), mRNA export (Rodríguez-Navarro et al., 2004), transcription elongation (Govind et al., 2007), chromatin recognition (Pray-Grant et al., 2005), and regulation of the basal transcription machinery (Sterner et al., 1999). Unraveling the modular composition of the SAGA complex has enabled interpretation of its multifunctional role (Wu et al., 2004), principally in regulating the transcription of many stress-inducible (Huisinga and Pugh, 2004) and developmentally regulated genes (reviewed in Wang and Dent, 2014). The diverse functions

of SAGA involve the participation of modules that are highly conserved between yeast, flies, and mammals. The SAGA complex is composed of more than 20 polypeptide subunits, grouped in four modules: the deubiquitinating module, the histone acetyltransferase module, and the SPT and TAF modules, which are implicated in the recruitment and SAGA architecture, respectively (Reviewed in Daniel and Grant, 2007 and Koutelou et al., 2010). Despite the abundance of genetic information available for plants, little is known about the presence and role of SAGA in photosynthetic organisms. Recently, a study determined the genes encoding subunits of the SAGA complex across a number of plants species (Srivastava et al., 2015), suggesting conservation of the SAGA complex throughout evolution. The yeast SAGA is particularly important for stress-induced transcription, and this function seems to be conserved during evolution (Spedale et al., 2012). In this review, the composition and our current knowledge of the role of the SAGA complex in the control of gene expression under stress conditions in plants is summarized.

HISTONE ACETYLATION MODULE

The histone acetylation (HAT) module contains the General Control Non-depressible 5 (GCN5) acetyltransferase in complex with ADA2, ADA3, and SGF29. This module is completely conserved in several photosynthetic organisms (**Table 1**). The GCN5 protein, which harbors a HAT domain has been identified in *Arabidopsis thaliana* (Pandey et al., 2002), *Vitis vinifera* (Aquea et al., 2010), and rice (Liu et al., 2012). The GCN5 protein mainly modifies Lys residue 14 in histone H3 in yeast (Kuo et al., 1996; Grant et al., 1999) and *Arabidopsis* (Benhamed et al., 2006; Earley et al., 2007). HAT by GCN5 has been shown to displace promoter nucleosomes (Barbaric et al., 2001), recruit RNA Polymerase II and coactivators to yeast promoter regions (Qiu et al., 2004; Govind et al., 2005), and increase the efficiency of trimethylation of H3-Lysine 4 in transcribed coding sequences (Govind et al., 2007). In *Arabidopsis*, GCN5 acetylates not only histones but also other proteins, as ADA2 (Mao et al., 2006), and appears to be a phosphorylated given that a phosphatase physically interacts and dephosphorylates GCN5 *in vitro* (Servet et al., 2008). In addition, GCN5 has a BROMO domain that recognizes acetylated lysine residues and increases the retention of the SAGA complex, promoting its HAT, and other functions (Mujtaba et al., 2007). The presence of the HAT and BROMO domain makes GCN5 a “reader” and “writer” of epigenetic marks. ADA2 (alteration/deficiency in activation 2) is an adaptor protein that physically associates with GCN5 (Grant et al., 1997). In *Arabidopsis*, two related ADA2 factors (ADA2a and ADA2b) have been identified (Stockinger et al., 2001), but only ADA2b is considered a member of the SAGA complex (Srivastava et al., 2015). Both proteins can bind directly to GCN5 through their N-terminal regions (Mao et al., 2006). This interaction enhances the ability of GCN5 to acetylate histones *in vitro* and enables GCN5 to acetylate nucleosomal histones (Mao et al., 2006). Maize homologs of GCN5 and ADA2 also interact with each other *in vitro* and *in vivo* (Bhat et al., 2003, 2004). SGF29 (SaGa associated Factor 29) is another

component of the HAT module. In *Arabidopsis*, two homologous proteins of yeast SGF29 have been identified (Kaldis et al., 2011). In humans, SGF29 interacts with GCN5 but not with ADA2 (Nguyen-Huynh et al., 2015). Deletion of yeast SGF29 does not affect SAGA integrity or composition of the HAT module, indicating that SGF29 is a peripheral subunit in this complex (Shukla et al., 2012). In addition, SGF29 binds H3K4me2/3 via its double TUDOR domain (Bian et al., 2011), suggesting a critical role in mediating transcriptional regulation through subsequent chromatin modifications. In Humans, ADA3 is associated with GCN5 and ADA2 to form the catalytic module of the SAGA complex and cooperates to stimulate GCN5-mediated HAT of nucleosomal templates (Gamper et al., 2009). There is no evidence of a role for ADA3 in plants.

RECRUITING MODULE

This module contains the proteins SPT8, SPT20, SPT7, SPT3, ADA1, and TRA1 and is conserved in several photosynthetic organisms with the exception of SPT8 (Srivastava et al., 2015, **Table 1**). Notably, orthologs of the *SPT8* gene are also absent in the genomes of metazoans (Spedale et al., 2012). The SPT3 subunit recruits the TATA Binding-Protein (TBP) and contributes to the formation of the preinitiation transcription complex (Dudley et al., 1999). In plants, a homologous protein of SPT3/TAF13 has been described in *Arabidopsis* (Lago et al., 2004) and pepper (Wen et al., 2013). In *Arabidopsis*, TAF13 interacts physically with other TAFs (TBP-associated factor) proteins (Lawit et al., 2007) and with MEDEA and SWINGER, both members of a plant variant of Polycomb Repressive Complex 2 (PRC2; Lindner et al., 2013). PRC2 is involved in transcriptional repression through tri-methylation of lys27 of histone H3, suggesting a possible link between SAGA and other complexes involved in chromatin remodeling. SPT20 has a primordial function in the assembly of the SAGA complex, as no intact SAGA could be purified in *spt20* yeast mutant strains (Sterner et al., 1999). In plants, an SPT20 domain containing protein has been reported by Endo et al. (2013) and is an interactor that bridges PHYTOCHROME B (phyB) and CONSTANS (CO) proteins involved in the photoperiodic regulation of flowering (Endo et al., 2013). There is no evidence of such a molecular mechanism of SPT20 in plants.

On the other hand, the SPT7 protein works as a scaffold element that maintains and stabilizes the SAGA complex also (Wu et al., 2004). In *Arabidopsis*, their homologous proteins are HAF1 and HAF2, putative proteins that harbor a histone acetyltransferase, and BROMO domain that can interact with acetylated lysine (Jacobson et al., 2000). The SPT7 BROMO domain interacts weakly with individually acetylated lysine residues (Hassan et al., 2007), suggesting that the BROMO domain within GCN5 is perhaps more important for recognition and binding to acetylated lysine residues in the histone tails, whereas the SPT7 BROMO domain may have another function such as recognition of acetylated transcription factors or multiple lysine residues (Hassan et al., 2007). In *Arabidopsis*, genetic analysis has shown that HAF2 interacts with GCN5 to integrate

TABLE 1 | Composition of SAGA complex in plants.

Saga Module	Yeast	Human	Physcomitrella	Arabidopsis	Rice	Grapevine
HAT	GCN5/ADA4	GCN5/PCAF	XP_001766378	GCN5/HAG1	Os10g28040	XP_002275146
	ADA2	ADA2b	XP_001755499	ADA2b (At4g16420)	Os03g53960	XP_002262737
			XP_001784968			XP_002268970
	ADA3	ADA3	XP_001782560	ADA3 (At4g29790)	Os05g28300	XP_002265763
	SGF29	SGF29/STAF36	XP_001755688	SGF29a (At3g27460)	Os12g19350	XP_003633806
			XP_001785583	SGF29b (At5g40550)		XP_003633807
SPT	SPT8	ND	ND	ND	ND	ND
	SPT20/ADA5	SPT20/FAM48A	XP_001762074	SPT20 (At1g72390)	Os01g02860	XP_002272317
	SPT7	STAF65/STAF65 γ	XP_001767625	HAF1 (At1g32750)	Os06g43790	XP_010656962
			XP_001779301	HAF2 (At3g19040)		
	SPT3	SPT3	XP_001759999	TAF13 (At1g02680)	Os01g23630	XP_002275358
			XP_001758422			XP_003632409
	ADA1	ADA1/STAF42	XP_001769204	ADA1a (At2g14850)	Os12g39090	XP_002279502
				ADA1b (At5g67410)	Os03g55450	XP_002280562
						XP_002263494
	TRA1	TRRAP	XP_001764071	TRA1a (At2g17930)	Os07g45064	XP_003631895
				TRA1b (At4g36080)		
TAF	TAF5	TAF5L	XP_001769775	TAF5 (At5g25150)	Os06g44030	XP_003631761
						XP_002285276
	TAF6	TAF6L	XP_001762306	TAF6 (At1g04950)	Os01g32750	XP_002276969
				TAF6b (At1g54360)		XP_002264290
	TAF9	TAF9	XP_001785776	TAF9 (At1g54140)	TAF9 (Os03g29470)	XP_002273931
		TAF9b			TAF9b (Os07g42150)	
	TAF10	TAF10	XP_001781637	TAF10 (At4g31720)	Os09g26180	XP_002266754
						XP_002267115
	TAF12	TAF12	XP_001781440	TAF12 (At3g10070)	Os01g63940	XP_002277150
				TAF12b (At1g17440)	Os01g62820	
DUBm	UBP8	USP22	XP_001765324	UBP22 (At5g10790)	Os04g55360	XP_002283376
						XP_003633155
	SGF11	ATXN7L3	XP_001779739	SGF11 (At5g58575)	Os05g28370	XP_003632167
			XP_001754483			
	SUS1	ENY2	XP_001759104	SUS1 (At3g27100)	Os01g69110	XP_002269535
			XP_001764723			
	SGF73	ATXN7	XP_001760795	ND	ND	ND
Other subunits	CHD1	ND	XP_001767461	CHR5 (At2g13370)	OsJ_25446	XP_002275100
			XP_001782004			

ND, Not defined.

light signals, regulating gene expression and growth (Bertrand et al., 2005; Benhamed et al., 2006). Both genes are required for H3K9, H3K27, and H4K12 acetylation on the target promoters (Benhamed et al., 2006).

The SAGA complex is recruited to gene loci by the interaction of yeast TRA1 protein or its mammalian ortholog TRRAP (Transformation/Transcription domain-Associated Protein) with specific transcriptional activators (Brown et al., 2001). These proteins are large and represent almost one quarter of the mass of the entire SAGA complex, suggesting that TRA1 may serve as a scaffold for complex assembly or for recruitment to chromatin in SAGA (Grant et al., 1998; Murr et al., 2007)

or other complex (Allard et al., 1999; Knutson and Hahn, 2011). TRA1 and TRRAP proteins show a striking sequence similarity to the family of phosphatidylinositol-3-kinase. There are two genes homologous to TRA1 in Arabidopsis. The genes At2g17930 and At4g36080 encode for a 3858 and 3834 amino acid protein, respectively, with a FAT domain and predicted phosphatidylinositol 3-kinase activity. The recruitment of TRRAP precedes that of GCN5, suggesting that TRA1 and TRRAP function in targeting co-activator complexes to specific promoters during transcription (Memudula and Belmont, 2003). The function of At2g17930, At4g36080 or its homologous proteins in other species of plants has not yet been described.

COACTIVATOR ARCHITECTURE MODULE

The coactivator architecture of the TAF module contains the TBP-associated factors TAF5, TAF6, TAF9, TAF10, and TAF12. This module is completely conserved in plants (Table 1), and these proteins are shared with the general transcription factor TFIID (Lee et al., 2011). The amino acid sequences of TAFs are conserved from yeast to humans (Struhl and Moqtaderi, 1998; Albright and Tjian, 2000). Initial studies on *in vitro* transcription suggested that TAFs might act as general co-activators that mediate the transcriptional activation of different activators (Goodrich et al., 1996). However, several TAFs have shown tissue- and/or developmental stage-specific expression and are required for the expression of only a subset of genes (Hiller et al., 2001). The endogenous expression of *TAF10* was monitored in transgenic Arabidopsis plants (*pTAF10:GUS*), yielding mostly vascular tissue preferential expression (Tamada et al., 2007). This expression pattern is closely similar to a *TAF10* homologous gene in *Flaveria trinervia*, as been observed by *in situ* hybridization (Furumoto et al., 2005). The *A. thaliana* *TAF6* gene is expressed in different tissues (Lago et al., 2005). A morphological analysis showed that T-DNA insertion in *TAF6* specifically affects pollen tube growth, indicating that this TAF protein regulates the transcription of only a specific subset of genes in plants (Lago et al., 2005). In addition, *TAF12* is required for proper hormone response, by negatively regulating cytokinin sensitivity (Kubo et al., 2011) and ethylene response in Arabidopsis (Robles et al., 2007) and *TAF5* is an essential gene, required for male gametogenesis, pollen tube growth, and required in transcriptional mechanisms involved in the maintenance of indeterminate inflorescence (Mougiou et al., 2012).

DEUBIQUITINATION MODULE

The Deubiquitination (DUB) module comprises four proteins: UBP8, SGF11, SUS1, and SGF73. This module is conserved in plants with the exception of SGF73 (Table 1). In yeast, the central domain of SGF73 tethers the DUB module to the rest of the SAGA complex while the N-terminal domain forms an integral part of the DUB module (Lee et al., 2009). A homologous protein of SGF73 has only been identified in *Physcomitrella* (Table 1), suggesting that other protein(s) could be involved in this function in higher plants. The homologous protein of UBP8 in Arabidopsis is UBP22 (At5g10790), a member of a family of Ubiquitin-specific proteases highly conserved in eukaryotes. The function of UBP22 has not been described in plants. UBP8 has been described as an ubiquitin protease that specifically removes monoubiquitin from lysine 123 of the H2B C-terminal tail (Henry et al., 2003; Daniel et al., 2004). In humans, biochemical analysis of the substrate specificity of USP22 reveals that it deubiquitylates histone H2A in addition to H2B (Zhang et al., 2008). Although UBP8 contains an ubiquitin-specific hydrolase domain, the protein is inactive unless in complex with the other three DUB module proteins (Weake et al., 2008; Lee et al., 2009). The loci At3g27100 and At5g58575 are the homologous genes of *SUS1* and *SGF11*, respectively. It has been demonstrated that the interaction of *SUS1* with the SAGA complex requires UBP88 and

SGF11, suggesting that SGF11 could be the direct binding partner of *SUS1* (Köhler et al., 2006). Interestingly, although there is no evidence for the role of both proteins in Arabidopsis, the physical interaction between At3g27100 and At5g58575 has been reported (Arabidopsis Interactome Mapping, 2011), suggesting a conserved role inside the SAGA complex in plants.

OTHER SUBUNITS

The protein CHD1 has been identified as a component of the SAGA complex in yeast (Pray-Grant et al., 2005). This protein is involved in ATP-dependent chromatin remodeling and contains a CHROMO domain that binds methylated H3K4. In Arabidopsis, CHR5 is the homologous protein of CHD1. This gene is expressed during embryo development and seed maturation and is directly involved in the activation of *ABI3* and *FUS3* expression, key transcriptional regulators of zygotic embryo development (Shen et al., 2015). This protein might recruit SAGA to chromatin and coordinates different complexes implicated in chromatin remodeling, like the COMPASS complex involved in tri-methyl marks on histone 3 lysine4.

ROLE OF THE SAGA COMPLEX IN CONTROL OF GENE EXPRESSION UNDER ABIOTIC STRESS

Plant growth is significantly affected by environmental stresses such as cold, salinity, drought, light quality, temperature, and excess or deficiency of nutrients (reviewed in Mahajan and Tuteja, 2005 and Hänsch and Mendel, 2009). Therefore, plants have developed diverse strategies to adapt their growth in response to environmental changes and ensure reproductive success (Franklin et al., 2005; Bäurle and Dean, 2006). Epigenetic mechanisms have been implicated in regulating the expression of stress related genes (Chinnusamy and Zhu, 2009). Dynamic and reversible HAT under abiotic stress enables the switch between permissive and repressive chromatin that regulates transcription. Different members of the SAGA complex play pivotal roles in the environmental stress response and in many developmental transitions in plants (Figure 1; Chen and Tian, 2007; Vlachonasios et al., 2011; Kim et al., 2015, reviewed in Kim et al., 2010 and Servet et al., 2010). Additionally, the gene expression of some components of the SAGA complex is induced under elevated salt concentration and high temperature, add weight to a potentially significant role of SAGA components gene expression in plants during abiotic stresses (Srivastava et al., 2015).

SALINITY AND DROUGHT STRESS

Plants have developed sophisticated signaling pathways that act in concert to counteract salinity and drought stress conditions through the action of transcription factors and histone modifications, thereby promoting the induction of many stress responsive genes and ultimately increasing stress tolerance (Reviewed in Chinnusamy and Zhu, 2009; Huang et al., 2012; Yuan et al., 2013; Gollidack et al., 2014). The

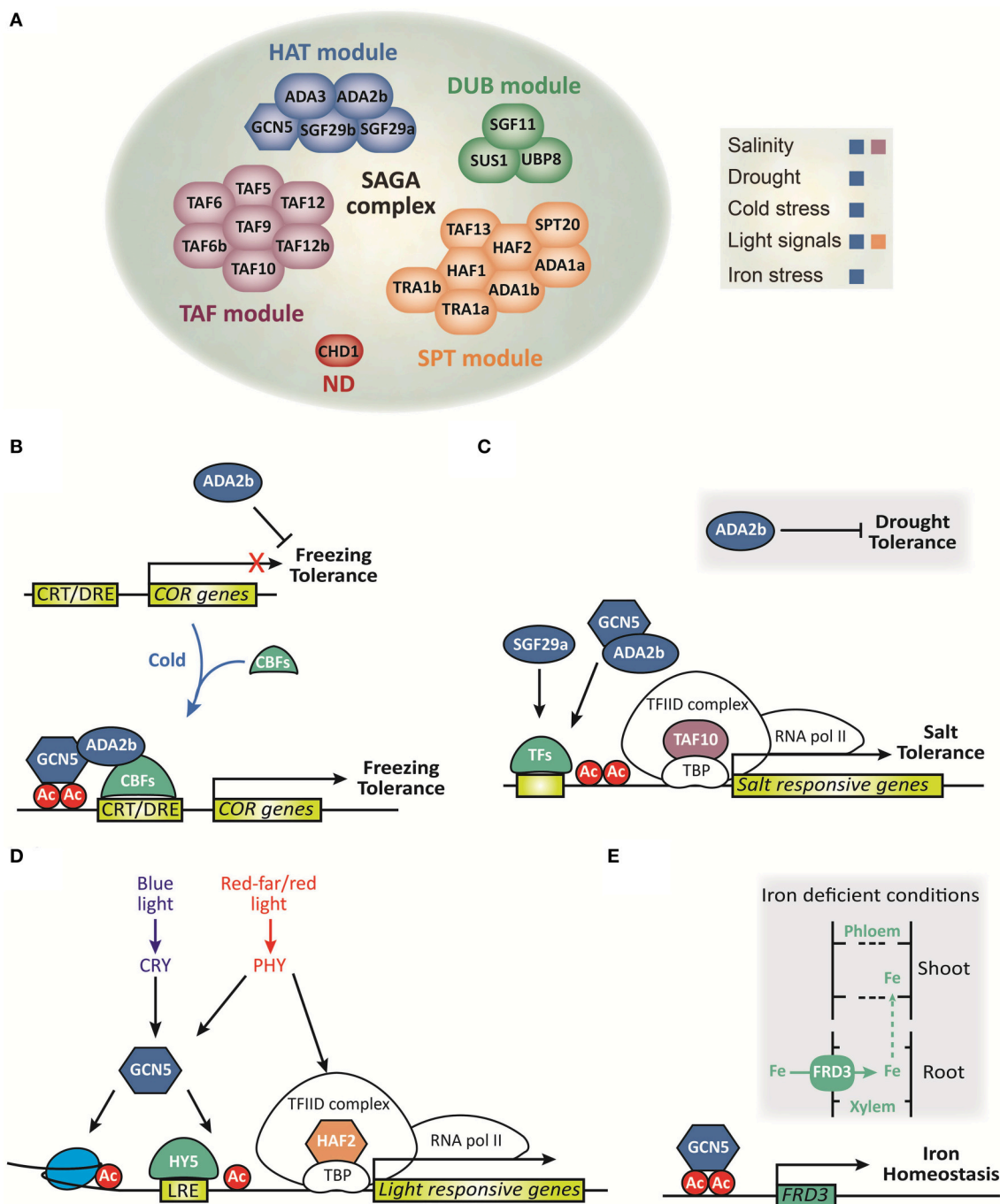


FIGURE 1 | Composition and function of the SAGA complex in plants. (A) Schematic representation of each module that integrates into the SAGA complex and its role in abiotic stress response. ND, not defined. **(B–E)** Schematic representation of molecular functions of the SAGA complex under abiotic stress. **(B)** ADA2b represses freezing tolerance before cold exposure. During cold exposure, CBFs are induced and together with ADA2b and GCN5 promotes COR genes induction and consequently freezing tolerance. **(C)** ADA2b represses drought tolerance whereas it promotes histone acetylation of salt stress responsive genes and confers salt tolerance. TAF10 promotes salt tolerance during seed germination, while SGF29a plays a modest role in the expression of salt stress responsive genes in Arabidopsis. TFs, transcription factors. **(D)** GCN5 integrates both blue and red/far-red light signals to induce histone acetylation and HY5-dependent gene activation of light responsive genes. TAF1 integrates red/far-red light signals to induce histone acetylation and gene activation of light-responsive genes (adapted from Servet et al., 2010). **(E)** Under deficient iron conditions GCN5 promotes histone acetylation of FRD3, which is involved in the transport of Fe into the xylem, to regulate iron homeostasis.

finding that *Arabidopsis* have homologs of both *GCN5* and *ADA2* genes (Stockinger et al., 2001) warrant additional study of how HAT-containing complexes related to SAGA complexes activate gene expression under abiotic stress conditions in plants. In *Arabidopsis*, *ada2b* and *gcn5* mutants, but not *ada2a* mutants, demonstrate pleiotropic effects on plant growth, and development (Vlachonasios et al., 2003). Moreover, both mutants exhibit an altered response to low temperatures and hypersensitivity to salt and abscisic acid (Vlachonasios et al., 2003; Hark et al., 2009). In addition, the whole plant transpiration rate in *ada2b* mutants is lower in comparison to wild-type plants after water starvation, suggesting that drought tolerance arises from a reduction in transpiration water loss that probably occurs through stomata closure (Vlachonasios et al., 2011). Recently SGF29a has been identified as another component of the SAGA complex that is involved in stress response (Kaldis et al., 2011). While in root growth and seed germination assays the *sgf29a-1* mutant plants are more resistant to salt stress, the reduction in transcript levels of salt stress responsive genes compared to wild-type plants suggests that SGF29a plays a modest role in the expression of salt-inducible genes (Kaldis et al., 2011). In contrast, the levels of salt stress responsive genes are dramatically reduced under salinity conditions in *ada2b* mutants. Interestingly, the reduction in transcript levels and the pattern of locus-specific acetylation of histones H3 and H4 of salt stress responsive genes in the *ada2b* mutant plants support the hypothesis that some transcription factors involved in salt stress response are capable of recruiting the SAGA complex to their target promoters (Kaldis et al., 2011).

On the other hand, a mutant screen from a chemical-inducible activation tagging allowed the identification of one mutant, designated *stg1* (salt tolerance during germination1), which demonstrates an increased tolerance to salt and osmotic stress in comparison to wild-type plants during seed germination (Gao et al., 2006). *STG1* encodes a putative *Arabidopsis* TBP-associated factor 10 (TAF10), which constitutes the TFIID complex involved in PIC assembly. The constitutive expression of *TAF10* enhances seed tolerance to salt stress during germination, and the knocked-down mutant is more sensitive to salt stress (Gao et al., 2006). Together, this evidence suggests that TAF10 plays a role in mediating an adaptive response under adverse environmental conditions, but its direct interaction with SAGA complex has not yet been determined.

COLD STRESS

The plant adaptive response to cold temperatures involves extensive physiological and biochemical changes such as stabilization of the integrity of cellular membranes and gene expression of inducible cold regulated (COR) genes (Reviewed in Thomashow, 1999; Lissarre et al., 2010). The inducible expression of COR genes is mediated mainly by a family of transcriptional activator proteins known as CBF/DREB1 which recognize the DNA regulatory element CRT/DRE present in the promoters of many COR and dehydration inducible genes (Yamaguchi-Shinozaki and Shinozaki, 1994; Park et al., 2015). The CBF transcription factors alter the expression of

more than 100 genes that contribute to enhanced freezing tolerance (Fowler and Thomashow, 2002; Vogel et al., 2005). In *Arabidopsis*, protein interaction assays revealed that the DNA-binding domain of CBF1 binds directly to ADA2b-containing SAGA complexes (Mao et al., 2006). Additionally, the evidence that the transcriptional activity of *Arabidopsis* CBF1 in yeasts requires ADA2, ADA3, and GCN5 to activate the transcription of reporter genes carrying the CRT/DRE regulatory element (Stockinger et al., 2001), paired with the observation that the expression of CBFs are induced and COR genes are reduced in *gcn5* and *ada2b* cold-acclimated mutant *Arabidopsis* plants, supports the notion that CBFs stimulate transcription through recruitment of SAGA transcriptional adaptor complexes to the promoters of COR genes (Vlachonasios et al., 2003). Remarkably, non-acclimated *ada2b* mutant plants are more tolerant to freezing temperatures than wild-type plants, indicating that freezing tolerance in non-acclimated *ada2b* mutant is achieved by a novel, undefined pathway that does not require the expression of CBF or COR genes (Vlachonasios et al., 2003). Thus, ADA2b and GCN5 proteins have similar yet distinct functions in gene expression and may be also components of separate co-activator complexes with different biological activities.

LIGHT SIGNALS

Plants perceive light by a set of wavelength-specific photoreceptors such as phytochromes (PHY) and cryptochromes (CRY) that direct adaptive changes in gene expression in response to environmental signals. Ultimately, these light signals are integrated by downstream DNA-binding transcription factors, which bind to several light responsive elements (LRE) present in the promoters of light-inducible genes (Reviewed in Casal and Yanovsky, 2005; Franklin et al., 2005; Jiao et al., 2007). It has been determined that HAF2 functions in concert with GCN5 to integrate light signals and acetylate the core promoter regions of light-inducible genes (Bertrand et al., 2005; Benhamed et al., 2006). Indeed, double mutations of *HAF2* and *HY5*, a bZIP transcription factor that promotes the expression of light-inducible genes, have a synergic effect on hypocotyl length (a photomorphogenesis trait) and light-activated gene expression under different light wavelengths (Bertrand et al., 2005; Benhamed et al., 2006). This suggests that HAF2 is involved in the signaling pathways of both red/far-red and blue signals, and interacts with HY5 to rapidly activate the expression of light-responsive genes. Moreover, *gcn5/taf1* double mutations result in a further loss of light-responsive genes and exert a cumulative effect on both plant growth and H3K9 acetylation (Benhamed et al., 2006). This evidence, together with the observation that GCN5 and HY5 share many genomic targets (Benhamed et al., 2008), indicates that GCN5 and HY5 might act cooperatively to activate the expression of light-inducible genes. Thus, HAF2 is presumably recruited to its target promoters by interacting with the TBPs, while GCN5 may be recruited to the target promoters by interacting either with specific DNA-binding transcription factors such as HY5 and/or with acetylated histone lysine residues of nearby nucleosomes. Recently, it has been reported that the expression of light-activated genes is considerably reduced in six

SAGA subunits in *Arabidopsis* mutants (Srivastava et al., 2015), indicating that other components of SAGA are involved in the expression of light-inducible genes as well.

NUTRITIONAL STRESS

Recently a report demonstrated that a mutation in GCN5 resulted in accumulation of manganese, zinc, and iron in the roots (Xing et al., 2015). Specifically, this mutant exhibited impaired iron translocation from the root to the shoot, and this retention was rescued by TSA treatment, a chemical inhibitor of histone deacetylase (Xing et al., 2015). These results suggest that HAT via GCN5 is an important mechanism for iron distribution in *Arabidopsis*. In addition, GCN5 is necessary for the expression of hundreds of genes involved in iron homeostasis (Xing et al., 2015). These observations, together with the fact that GCN5 directly binds to the promoters of *FRD3*, a key gene in iron homeostasis, and modulates the H3K9/14 global acetylation levels under iron deficient conditions, suggest that GCN5 plays a critical role in iron homeostasis through the regulation of target genes (Xing et al., 2015). There is no evidence for the role of other members of the SAGA complex in the regulation of nutrients homeostasis.

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CONCLUDING REMARKS

The protein complexes involved in chromatin remodeling and epigenetic modifications are highly conserved in eukaryotes. The SAGA complex is no exception, and although highly conserved in plants, the physical and functional relationships between its different modules remain to be elucidated. Additional study is needed to identify the target genes of the SAGA complex in different environmental conditions and developmental stages, as well as which transcription factors interact with these complexes. Further characterization of the SAGA complex presents the opportunity to identify new actors that participate in the control of gene expression in plants.

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Elongator and its epigenetic role in plant development and responses to abiotic and biotic stresses

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Elongator, a six-subunit protein complex, was initially isolated as an interactor of hyperphosphorylated RNA polymerase II in yeast, and was subsequently identified in animals and plants. Elongator has been implicated in multiple cellular activities or biological processes including tRNA modification, histone modification, DNA demethylation or methylation, tubulin acetylation, and exocytosis. Studies in the model plant *Arabidopsis thaliana* suggest that the structure of Elongator and its functions are highly conserved between plants and yeast. Disruption of the Elongator complex in plants leads to aberrant growth and development, resistance to abiotic stresses, and susceptibility to plant pathogens. The morphological and physiological phenotypes of *Arabidopsis* Elongator mutants are associated with decreased histone acetylation and/or altered DNA methylation. This review summarizes recent findings related to the epigenetic function of Elongator in plant development and responses to abiotic and biotic stresses.

Keywords: Elongator, tRNA modification, histone acetylation, DNA methylation, plant development, abiotic stress, plant immunity

Introduction

Elongator was first identified as an elongating RNA polymerase II (RNAP II)-associated protein complex in yeast (Otero et al., 1999; Wittschleben et al., 1999), and was later found to be highly conserved in eukaryotes (Versées et al., 2010). This complex consists of six subunits (ELP1–ELP6) with ELP1–3 forming the core subcomplex and ELP4–ELP6 the accessory subcomplex (Li et al., 2001; Winkler et al., 2001). Deletion of any of the six subunits results in almost identical phenotypes, suggesting that all six subunits are required for Elongator's cellular functions (Krogan and Greenblatt, 2001; Nelissen et al., 2005, 2010; Mehlgarten et al., 2010; Glatt et al., 2012). ELP1 has a nuclear localization sequence essential for Elongator function and WD40 repeats that possibly function together with the WD40-containing subunit ELP2 as scaffolds for complex assembly (Fichtner et al., 2003). ELP3 is the catalytic subunit containing a C-terminal GNAT-type histone acetyltransferase (HAT) domain and an N-terminal iron–sulfur (Fe–S) radical S-adenosylmethionine (SAM) domain (Wittschleben et al., 1999; Chinenov, 2002). ELP4, ELP5, and ELP6 each form a RecA-ATPase-like fold and together assemble into a hexameric ring-shaped structure (Glatt et al., 2012; Lin et al., 2012).

The presence of a conserved HAT domain in ELP3 and the co-purification of Elongator with elongating RNAP II led to the initial assumption that Elongator might facilitate transcription elongation via histone acetylation (Otero et al., 1999; Wittschleben et al., 1999). Indeed, ELP3 is capable of acetylating all four core histones *in vitro* (Wittschleben et al., 1999), and highly

purified holo-Elongator has a dominant preference for lysine-14 of histone H3 and to a small extent for lysine-8 of Histone H4 (Winkler et al., 2002; Li et al., 2009). Consistently, yeast, human, and plant Elongator mutants contain reduced levels of acetylated histone H3 and H4 (Kim et al., 2002; Winkler et al., 2002; Close et al., 2006; Nelissen et al., 2010). Although chromatin immunoprecipitation (ChIP) failed to detect enrichment of Elongator at actively transcribed genomic regions in yeast (Pokholok et al., 2002), RNA immunoprecipitation (RIP) showed that Elongator interacts with nascent mRNA during transcription elongation (Gilbert et al., 2004). Conversely, in humans and plants, ChIP experiments detected association of Elongator with gene promoters and/or coding regions (Kim et al., 2002; Close et al., 2006; Wang et al., 2013). Furthermore, it was recently reported that the ELP4–ELP6 accessory subcomplex assembles into a hexameric ring-shaped structure that is important for recognizing histone H3 (Lin et al., 2012). These results, together with the finding that Elongator facilitates RNAP II transcription through chromatin in an acetyl-CoA-dependent manner (Kim et al., 2002), support that Elongator assists RNAP II during transcription elongation via chromatin remodeling.

ELP3 also contains a putative SAM-binding domain, which was hypothesized to function catalytically in histone demethylation (Chinenov, 2002). In yeast, the radical SAM domain of ELP3 was shown to be a motif required for the structural integrity of Elongator (Greenwood et al., 2009). In contrast, the archaea *Methanocaldococcus jannaschii* ELP3 SAM-binding motif might have a catalytic role, since it binds and cleaves SAM (Paraskevopoulou et al., 2006). Interestingly, a recent study in mouse indicated that Elongator is required for zygotic paternal genome demethylation, which is mediated by the ELP3 radical SAM domain rather than the HAT domain (Okada et al., 2010).

Accumulating evidence suggests that Elongator also plays a role in formation of the 5-methoxycarbonylmethyl (mcm⁵) and 5-carbamoylmethyl (ncm⁵) side chains on uridines at the wobble position in tRNAs (Karlsborn et al., 2015). Yeast Elongator mutants lack tRNA modifications at wobble uridines or thioridines at position 34 of the anticodon (Huang et al., 2005). Interestingly, elevated levels of two tRNA species rescue the defects of transcription and exocytosis in yeast Elongator mutants (Esberg et al., 2006), and overexpression of tRNA^{Lys}_{UUU} complements the stress-related phenotypes of the yeast *sin3/elp3* mutant cells (Fernández-Vázquez et al., 2013). These results are in line with the recent finding that the hexameric ELP456 accessory subcomplex specifically recognizes tRNA (Glatt et al., 2012). Therefore, it was proposed that the effects of *elp* mutations on transcription and secretion might be indirect consequences of inappropriate tRNA modifications (Esberg et al., 2006).

In addition to its functions in histone acetylation and tRNA modification, Elongator has also been implicated in multiple kingdom-specific activities, such as exocytosis in yeast and neuronal development in animals (Rahl et al., 2005; Close et al., 2006). Recent studies performed in the model plant *Arabidopsis thaliana* have revealed that the structure and function of Elongator are conserved in plants (Figure 1; Table 1; Nelissen et al., 2010; DeFraia and Mou, 2011; Van Lijsebettens

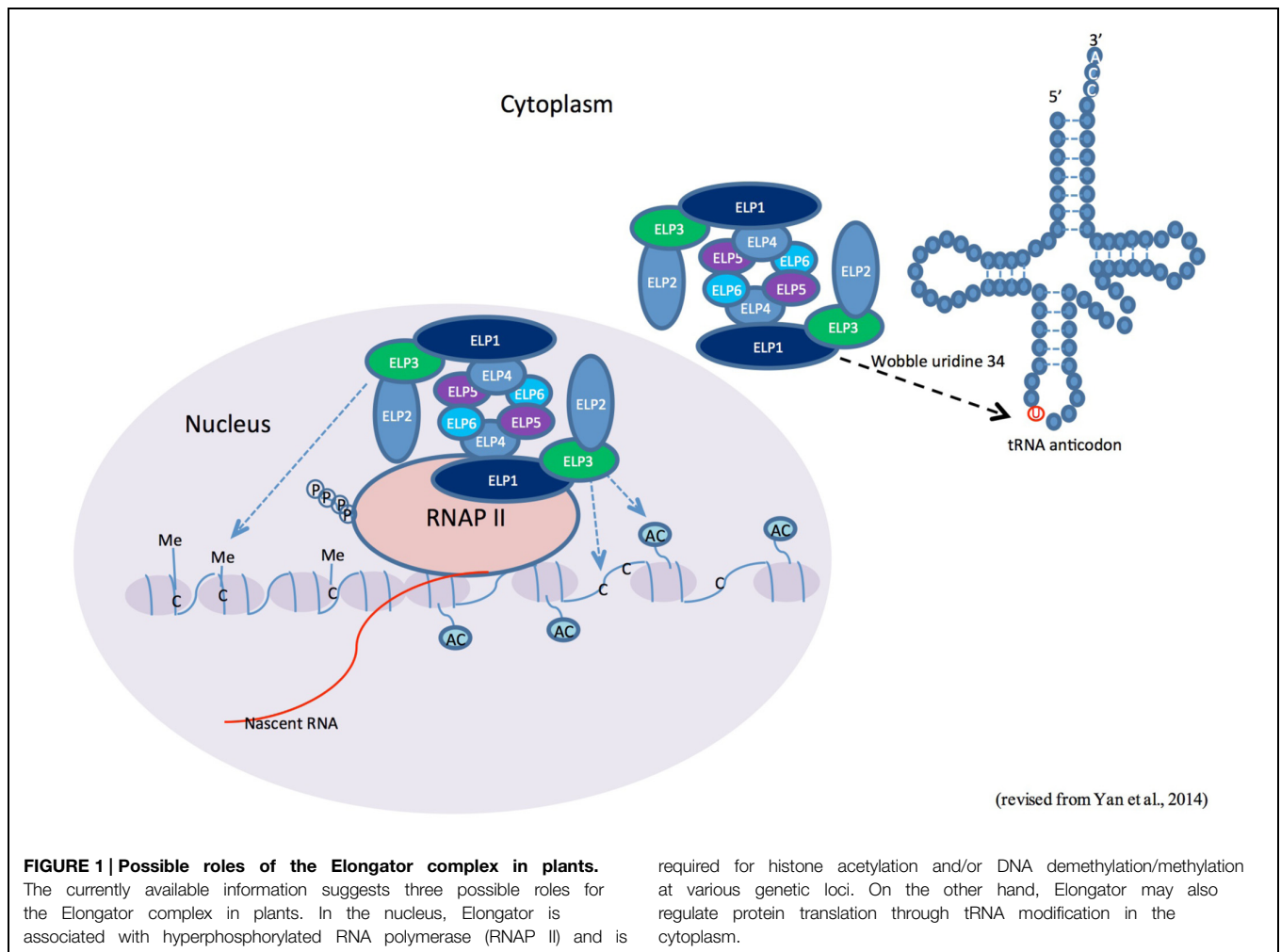
et al., 2014; Yan et al., 2014). This review focuses on recent advances in the study of the epigenetic function of Elongator in plant development and responses to biotic and abiotic stresses.

Identification and Characterization of the Elongator Complex in Plants

In a genetic screen for mutants with abnormal shaped leaves in *Arabidopsis*, four *elongata* (*elo*) mutants, *elo1*, *elo2*, *elo3*, and *elo4*, were isolated (Berná et al., 1999). The *elo4* mutant was later found to be allelic to the *drl1-2* (*deformed roots and leaves1*) mutant, which carries a transposed *Ds* element in the *Arabidopsis* homolog of the yeast Elongator-associated protein KTI12 (KILLER TOXIN INSENSITIVE12; Fichtner et al., 2002; Nelissen et al., 2003). Further BLAST search indicated that homologs of all six subunits of the yeast Elongator are also present in the *Arabidopsis* genome (Nelissen et al., 2003). Indeed, the *elo1*, *elo2*, and *elo3* mutations were identified in the *A. thaliana* (*At*) Elongator subunits *AtELP4*, *AtELP1*, and *AtELP3*, respectively, (Nelissen et al., 2005).

To verify the existence and composition of an Elongator complex in plants, tandem affinity purification (TAP) was performed using *Arabidopsis* cell suspension cultures overexpressing TAP-tagged ELO3/AtELP3, ELO1/AtELP4, or AtELP5 (Nelissen et al., 2010). All six subunits (ELO2/AtELP1, AtELP2, ELO3/AtELP3, ELO1/AtELP4, AtELP5, and AtELP6) were purified in the TAPs with ELO3/AtELP3, ELO1/AtELP4, or AtELP5 as bait, confirming that the Elongator composition is conserved in plants. Moreover, stoichiometric concentrations of ELO2/AtELP1, AtELP2, and ELO3/AtELP3 were found on gels when ELO2/AtELP1 was used as bait, whereas only stoichiometric concentrations of ELO1/AtELP4, AtELP5, and AtELP6 were detected when using ELO1/AtELP4 or AtELP5 as bait, suggesting that the *Arabidopsis* holo-Elongator is also composed of two distinct subcomplexes. The structural conservation was further supported by the fact that AtELP6 interacts with both ELO1/AtELP4 and AtELP5 in yeast two-hybrid assays (Nelissen et al., 2010).

ELO3/AtELP3 was shown to colocalize with euchromatin and the phosphorylated form of RNAP II, indicating that plant Elongator is also involved in the process of RNAP II transcription elongation (Nelissen et al., 2010). The functional conservation was further corroborated by results from heterologous complementation experiments. Ectopic expression of *ELO2/AtELP1* in a yeast *elp1* mutant restores sensitivity toward zymocin, a yeast killer toxin complex (Chen et al., 2006), and *ELO2/AtELP1* and *ELO3/AtELP3* are able to structurally replace the respective yeast Elongator subunits and functionally restore ochre suppression and γ -toxin sensitivity of a yeast *elp1 elp3* double mutant by reconstituting U34 tRNA modifications (Mehlgarten et al., 2010). Moreover, tRNA wobble uridine modifications are compromised in the *Arabidopsis elo2/Atelp1*, *elo3/Atelp3*, and *drl1* mutants (Chen et al., 2010; Mehlgarten et al., 2010). Therefore, the structure of Elongator and its functions in RNAP II transcription elongation as well as tRNA modification are conserved between yeast and plants.



The Epigenetic Function of Elongator in Plant Growth and Development

As *elo/Atelp* mutants exhibit abnormal shaped leaves (Berná et al., 1999; Nelissen et al., 2003, 2005), Elongator clearly plays an important role in plant growth and development. At the macroscopic level, *elo/Atelp* mutants have narrow and elongated leaves, reduced primary roots and lateral root density, abnormal inflorescence phyllotaxis, delayed seedling growth, and reduced apical dominance (Nelissen et al., 2003, 2005, 2010). At the cellular and ultrastructural levels, leaves of *elo/Atelp* mutants have larger and fewer cells, which show less stacked grana in the chloroplasts, a hypotonic vacuole, and massive presence of Golgi vesicles in the cytoplasm (Falcone et al., 2007).

Since the phytohormone auxin plays a leading role in regulating cell proliferation (Wang and Ruan, 2013), the abnormal phenotypes of *elo/Atelp* mutants might be due to defects in auxin signaling or distribution. Indeed, a group of auxin-related genes are down-regulated in *elo/Atelp* mutants (Nelissen et al., 2010). Interestingly, decreased expression of two auxin-related genes, *SHY2/IAA3* and *LAX2*, in the *elo3/Atelp3* mutant is correlated with reduced histone H3K14 acetylation at the coding regions

and 3'-UTRs (Table 2), suggesting that these two genes might be direct targets for Elongator's HAT activity during RNAP II transcription elongation. On the other hand, H3K14 acetylation levels in several other auxin-related genes, which are also down-regulated in *elo3/Atelp3*, are not changed, indicating that Elongator likely targets specific genes for histone acetylation rather than affecting overall histone acetylation levels (Nelissen et al., 2010). Additionally, ethylene (ET) and jasmonic acid (JA) signaling and abiotic stress responses are up-regulated in *elo/Atelp* plants, which might also contribute to their pleiotropic phenotypes (Chen et al., 2006; Zhou et al., 2009; Nelissen et al., 2010).

It is well known that normal cell proliferation is required for the leaf adaxial–abaxial polarity establishment in *Arabidopsis* (Yuan et al., 2010). The abnormal leaf polarity formation in *elo/Atelp* mutants is likely due to the defective cell cycle progression caused by aberrant DNA replication and increased DNA damage (Xu et al., 2012). In yeast, Elongator associates with PCNA (proliferating cell nuclear antigen) and functions in maintenance of genome stability (Li et al., 2009). Elongator also interacts with PCNA in *Arabidopsis*, and the interaction is required for DNA replication and repair. Moreover, *ELO3/AtELP3* is

TABLE 1 | A timeline for the establishment of Elongator's function in plants.

Elongator mutant/subunit	Topic	Reference
<i>elo1, elo2, elo3, elo4</i>	Mutants with abnormal shaped leaves	Berná et al. (1999)
ELO4/DRL1	Identification of ELO4 as DRL1, a homolog of the yeast Elongator-associated protein KTI12	Nelissen et al. (2003)
ELO1/AtELP4, ELO2/AtELP1, ELO3/AtELP3	Identification of three Elongator subunits and their function in cell proliferation during organ growth in <i>Arabidopsis</i>	Nelissen et al. (2005)
<i>abo1/elo2/Atelp1</i>	Role of ABO1/ELO2/AtELP1 in modulating ABA and drought responses; functional conservation between AtELP1 and yeast ELP1	Chen et al. (2006)
ELO1/AtELP4	Cytological investigation of the <i>elo1/Atelp4</i> mutant for ELO4/AtELP1's function in leaf lateral growth	Falcone et al. (2007)
HAG3/AtELP3	Involvement of HAG3/AtELP3 in <i>Agrobacterium</i> -mediated root transformation	Crane and Gelvin (2007)
ELO1/AtELP4, ABO1/ELO2/AtELP1, AtELP2, AtELP6	Role of four Elongator subunits in ABA response, oxidative stress, and anthocyanin biosynthesis; different functions of two subcomplexes in ABA-mediated stomatal movement	Zhou et al. (2009)
All six subunits	Purification of the <i>Arabidopsis</i> Elongator complex; epigenetic function of ELO3/AtELP3 in auxin signaling	Nelissen et al. (2010)
ELO2/AtELP1, ELO3/AtELP3	Functional conservation of tRNA modifications between plants and yeast	Mehlgarten et al. (2010)
AtELP1, ELO4/DRL1	Role of AtELP1 and ELO4/DRL1 in ncm ⁵ uridine modifications of tRNA	Chen et al. (2010)
<i>gns1/Atelp2</i>	Role of GNS1/AtELP2 in plant immunity	DeFraia et al. (2010)
ELO3/AtELP3	Function of ELO3/AtELP3 in the establishment of leaf adaxial-abaxial polarity	Kojima et al. (2011)
ELO1/AtELP4, ELO2/AtELP1, ELO3/AtELP3, ELO4/DRL1	Function of three Elongator subunits in regulating mitotic cell cycle and leaf patterning in <i>Arabidopsis</i>	Xu et al. (2012)
AtELP2	Epigenetic function of AtELP2 in plant immunity	Wang et al. (2013)
<i>gns2/Atelp3</i>	Role of AtELP3 and its histone acetyltransferase (HAT) and S-adenosylmethionine (SAM) domains in plant immunity	DeFraia et al. (2013)
SIELP2L, a tomato Elongator complex protein 2-like protein	Function of SIELP2L in tomato growth and development	Zhu et al. (2015)

required for cellular histone H3 and H4 acetylation and DNA replication-coupled H3K56 and H4K5 acetylation (**Table 2**), which are important for DNA replication-coupled chromatin assembly (Xu et al., 2012). Thus, Elongator likely modulates

TABLE 2 | Epigenetic changes in *Arabidopsis* Elongator mutants.

Epigenetic change	Target gene/region	Biological process	Reference
Histone acetylation	Histone H3K14 at the coding regions and/or 3'-UTRs of <i>SHY2/IAA3</i> and <i>LAX2</i> in <i>elo3</i>	Auxin signaling/cell proliferation	Nelissen et al. (2010)
	Histone H3K9/14 at the coding regions of <i>NPR1</i> , <i>PR2</i> , <i>PR5</i> , <i>EDS1</i> , and <i>PAD4</i> in <i>Atelp2</i>	Salicylic acid (SA) signaling/plant immunity	Wang et al. (2013)
	Cellular levels of acetylated histone H3 and H4 and chromatin-bound H3K56 and H4K5 within replicons in <i>elo3</i>	Mitotic cell cycle	Xu et al. (2012)
	Histone H3K9/14 at the coding regions of <i>WRKY33</i> , <i>ORA59</i> , and <i>PDF1.2</i> in <i>Atelp2</i>	JA/ET signaling/plant immunity	Wang and Mou (unpublished data)
DNA methylation	DNA methylation landscape and pathogen-induced DNA methylation changes at the <i>NPR1</i> promoter region and the <i>PAD4</i> coding region in <i>Atelp2</i>	SA signaling/plant immunity	Wang et al. (2013)

mitotic cell cycle through interacting with PCNA and functioning in histone acetylation.

Elongator may also mediate the establishment of leaf adaxial-abaxial polarity in *Arabidopsis* by repressing transcription of abaxial-determinant genes and class 1 *KNOX* genes (Kojima et al., 2011). This negative role of Elongator in gene expression may be an indirect consequence of positively regulated genes.

Recently, the function of a tomato *AtELP2*-like gene, *SIELP2L*, was characterized (Zhu et al., 2015). Similar to *Atelp* mutants, *SIELP2L*-RNAi transgenic tomato plants display pleiotropic phenotypes, such as delayed seedling development, reduced leaf growth, rapidly senescing leaves and sepals, and dark-green fruits. A number of ET- and ripening-related genes are down-regulated in *SIELP2L*-silencing plants, whereas several DNA methyltransferase genes are up-regulated. It was therefore proposed that the tomato *SIELP2L* might regulate plant growth and development by modulating DNA methylation. Additionally, levels of GA and IAA, which have profound effects on plant growth and development (Martí et al., 2006; Zhao, 2010), are reduced in *SIELP2L*-RNAi plants. Interestingly, some phenotypes of the *SIELP2L*-RNAi tomato plants are in marked contrast to those of *elo/Atelp* mutants. For instance, ET signaling is upregulated in *elo/Atelp* mutants (Nelissen et al., 2010), but down-regulated in *SIELP2L*-silencing plants (Zhu et al., 2015). Moreover, *elo/Atelp* mutants accumulate high levels of auxin, whereas *SIELP2L*-RNAi tomato plants exhibit reduced levels of auxin. These differences suggest that the function of Elongator in different plant species may not be exactly the same.

The Role of Elongator in Plant Responses to Abiotic Stresses

In a genetic screen for *Arabidopsis* mutants with altered drought sensitivity, a drought-resistant mutant, *abo1-1* (ABA-overly sensitive), was identified (Chen et al., 2006). The *abo1-1* mutant is hypersensitive to ABA in stomatal closure and seedling growth and carries a mutation in the *ELO2/AtELP1* gene. In a separate genetic screen for mutants hypersensitive to ABA in root growth, *Atelp2* and *Atelp6* were identified (Zhou et al., 2009). It was found that *abo1/elo2/Atelp1*, *Atelp2*, *elo1/Atelp4*, and *Atelp6* are all hypersensitive to ABA in seed germination and seedling growth. Similarly, the tomato *SlELP2L*-silencing plants exhibit an obvious increase in ABA sensitivity during seedling growth (Zhu et al., 2015). On the other hand, only the core subcomplex mutants *abo1/elo2/Atelp1* and *Atelp2*, but not the accessory subcomplex mutants *elo1/Atelp4* and *Atelp6*, exhibit ABA hypersensitivity in stomatal closure (Zhou et al., 2009). The different functions of the two subcomplexes in ABA-mediated stomatal movement appear to conflict with the notion that both core and accessory subcomplexes are essential for the function of the holo-Elongator described to date (Svejstrup, 2007; Van Lijsebettens et al., 2014; Karlsborn et al., 2015).

Disruption of the *Arabidopsis* Elongator complex results in increased resistance to the oxidative stress caused by CsCl and methyl viologen under light, indicating that Elongator functions as a negative modulator of oxidative stress (Zhou et al., 2009). Consistently, expression of *CAT3* (CATALASE 3), which encodes a catalase decomposing hydrogen peroxide in reactive species homeostasis, is up-regulated in *elo/Atelp* mutants. Elevated expression of *CAT3* may contribute to the increased oxidative stress resistance. The *elo/Atelp* mutants also accumulate high levels of basal and light-induced anthocyanins (Zhou et al., 2009). Since anthocyanins function as antioxidants to protect plants from oxidative stress caused by diverse stressors such as drought, salt, and light (Gould, 2004), the elevated levels of anthocyanins in *elo/Atelp* mutants might also contribute to the enhanced resistance to oxidative stress.

The Epigenetic Function of Elongator in Plant Responses to Biotic Stresses

Salicylic acid (SA) is a key defense signal molecule against biotrophic and hemibiotrophic pathogens in plants (Vlot et al., 2009). SA accumulation occurs after pathogen infection, which is essential for activation of both local and systemic acquired resistance (SAR; Durrant and Dong, 2004). The transcription coactivator NPR1 [NONEXPRESSOR OF PATHOGENESIS-RELATED (PR) GENES] is a master regulator of SA-mediated defense responses (Durrant and Dong, 2004). Mutations in the *NPR1* gene significantly compromise basal immunity, effector-triggered immunity (ETI), and SAR (Durrant and Dong, 2004). These mutations also lead to SA hyperaccumulation during pathogen infection (Shah et al., 1997; Wildermuth et al., 2001) and failure of seedling development on Murashige and

Skoog (MS) medium containing high concentrations of SA (Cao et al., 1997). In a genetic screen for *gns* (green *npr1* seedling on SA medium) mutants, we found that mutations in *AtELP2* and *ELO3/AtELP3* restore SA tolerance to *npr1* on half-strength MS medium containing 0.5 mM SA and also suppress *npr1*-mediated SA hyperaccumulation (DeFraia et al., 2010, 2013). Since high levels of SA trigger production of reactive oxygen species and subsequent cellular damage (Rao et al., 1997), Elongator may facilitate SA toxicity by suppressing the expression of antioxidant genes such as *CAT3* (Zhou et al., 2009).

Mutations in *AtELP2* and *ELO3/AtELP3* also compromise plant immunity (DeFraia et al., 2010, 2013). While *gns1/Atelp2* and *gns2/Atelp3* are more susceptible to both virulent and avirulent *Pseudomonas syringae*, SAR induction in these mutants is normal. Interestingly, simultaneous removal of *AtELP2* and *NPR1* completely abolishes resistance to two different ETI-inducing pathogens, *P. syringae* pv. *tomato* (Pst) DC3000/*avrRpt2* and *Pst* DC3000/*avrRps4*. Microarray analysis revealed that the *gns1/Atelp2* mutation has a broader impact than *npr1* on *Pst* DC3000/*avrRpt2*-induced transcriptome changes, indicating that *AtELP2* is a more general modulator of transcription than *NPR1*. Furthermore, *Pst* DC3000/*avrRpt2*-induced expression of a group of SA pathway defense genes is delayed and/or decreased in the *gns1/Atelp2* mutant compared to wild type. Similarly, both *gns1/Atelp2* and *gns2/Atelp3* are significantly more susceptible than wild type to the necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola* and *B. cinerea*-induced expression of the JA/ET pathway defense genes *OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59* (*ORA59*) and *PLANT DEFENSIN1.2* (*PDF1.2*) is delayed and/or decreased in *gns1/Atelp2* plants (Wang and Mou, unpublished data). These results together suggest that *AtELP2* is an accelerator of plant defense responses (DeFraia et al., 2010).

Genome-wide DNA methylation analysis revealed that the *gns1/Atelp2* mutation increases total number of methylcytosines, decreases average methylation levels of methylcytosines, and alters methylation levels of specific cytosines (Wang et al., 2013).

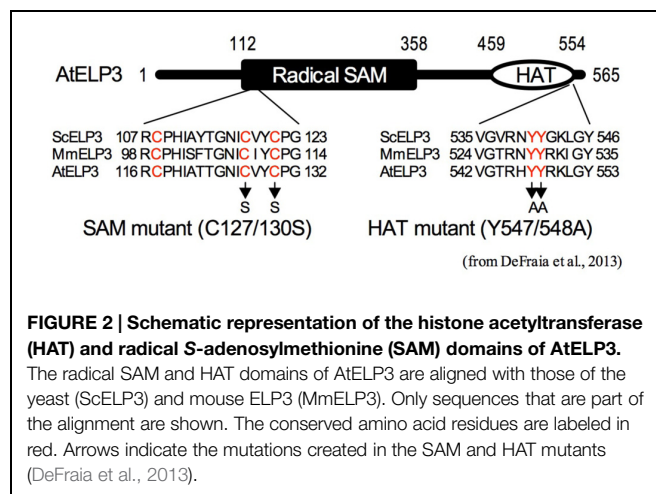


FIGURE 2 | Schematic representation of the histone acetyltransferase (HAT) and radical S-adenosylmethionine (SAM) domains of AtELP3.

The radical SAM and HAT domains of AtELP3 are aligned with those of the yeast (ScELP3) and mouse ELP3 (MmELP3). Only sequences that are part of the alignment are shown. The conserved amino acid residues are labeled in red. Arrows indicate the mutations created in the SAM and HAT mutants (DeFraia et al., 2013).

Further analysis showed that AtELP2 is required for pathogen-induced dynamic changes in DNA methylation levels of two major defense genes *NPR1* and *PAD4* (Table 2). On the other hand, histone acetylation assay indicated that histone H3K9/14ac levels in the coding regions of several defense genes, including *NPR1*, *PAD4*, *EDS1*, *PR2*, *PR5*, *WRK33*, *ORA59*, and *PDF1.2*, are significantly reduced in *gns1/Atelp2* (Table 2). The reduced histone H3K9/14ac levels are correlated with delayed and/or decreased induction of these defense genes in *gns1/Atelp2*, suggesting a role for AtELP2 in histone acetylation (Wang et al., 2013; Wang and Mou, unpublished data). Consistently, mutants of the *Arabidopsis* Elongator catalytic subunit (ELO3/AtELP3) lacking the conserved residues in either the HAT domain or the radical SAM domain (Figure 2), fail to complement *gns2/Atelp3* mutant phenotypes (DeFraia et al., 2013), indicating that both domains are required for Elongator to function in *Arabidopsis*. Therefore, Elongator likely plays an epigenetic role in response to pathogen infections.

Conclusions and Perspectives

Studies in *Arabidopsis* indicate that the structure of the Elongator complex and its functions are highly conserved between plants and yeast (Otero et al., 1999; Winkler et al., 2002; Nelissen et al., 2010). Disruption of the Elongator complex in *Arabidopsis* leads to pleiotropic growth and defense phenotypes, which could be attributed to delayed and/or decreased expression of some genes involved in these processes (Nelissen et al., 2005; Zhou et al., 2009; DeFraia et al., 2010; Xu et al., 2012). The delayed and/or decreased gene expression in *Atelp* mutants is associated with reduced histone acetylation and/or altered DNA methylation (Nelissen et al., 2010; Wang et al., 2013). Although the intrinsic relationship between histone acetylation and DNA demethylation/methylation in *Arabidopsis* Elongator mutants remains to be determined, these results suggest that Elongator may epigenetically modulate plant development and responses to abiotic and biotic stresses.

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- However, a direct role of Elongator in histone acetylation and DNA demethylation or methylation in plants has not been established. Moreover, the enzymatic activities (HAT and DNA demethylase or methyltransferase) of the catalytic subunit ELP3 in plants have not been tested. Without such information, it would be difficult to fully appreciate the epigenetic function of Elongator in plants. Additionally, recent evidence suggests that herbivore and pathogen attack of plants generates defense phenotypes across generations and such transgenerational memory appears to be associated with DNA methylation, histone modifications, and small RNAs (Holeski et al., 2012; Luna et al., 2012; Rasmann et al., 2012; Slaughter et al., 2012). Since Elongator modulates histone acetylation and DNA methylation (Nelissen et al., 2010; Xu et al., 2012; Wang et al., 2013), it would be interesting to test whether Elongator is also involved in transgenerational defense induction and epigenetic inheritance in plants.
- Accumulating evidence suggests that the yeast Elongator may primarily function in tRNA modification (Karlsborn et al., 2015). Studies in *Arabidopsis* also indicate that Elongator may play a role in tRNA modification in plants (Chen et al., 2010; Mehlgarten et al., 2010). However, the connection between Elongator's function in tRNA modification and plant development and responses to abiotic and biotic stresses still remains elusive. It would be very interesting to test whether overexpression of certain types of hypomodified tRNAs (tRNA^{Lys}_{UUU}, tRNA^{Gln}_{UUG}, and tRNA^{Glu}_{UUC}) could rescue some of the plant Elongator mutant phenotypes. Such experiments would help resolve the mystery of the multitasking role of Elongator in plants.

Author Contributions

YD and ZM wrote the manuscript.

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Heterologous Overexpression of Poplar *SnRK2* Genes Enhanced Salt Stress Tolerance in *Arabidopsis thaliana*

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Subfamily 2 of SNF1-related protein kinase (*SnRK2*) plays important roles in plant abiotic stress responses as a global positive regulator of abscisic acid signaling. In the genome of the model tree *Populus trichocarpa*, 12 *SnRK2* genes have been identified, and some are upregulated by abiotic stresses. In this study, we heterologously overexpressed the *PtSnRK2* genes in *Arabidopsis thaliana* and found that overexpression of *PtSnRK2.5* and *PtSnRK2.7* genes enhanced stress tolerance. In the *PtSnRK2.5* and *PtSnRK2.7* overexpressors, chlorophyll content, and root elongation were maintained under salt stress conditions, leading to higher survival rates under salt stress compared with those in the wild type. Transcriptomic analysis revealed that *PtSnRK2.7* overexpression affected stress-related metabolic genes, including lipid metabolism and flavonoid metabolism, even under normal growth conditions. However, the stress response genes reported to be upregulated in *Arabidopsis SRK2C/SnRK2.6* and wheat *SnRK2.8* overexpressors were not changed by *PtSnRK2.7* overexpression. Furthermore, *PtSnRK2.7* overexpression widely and largely influenced the transcriptome in response to salt stress; genes related to transport activity, including anion transport-related genes, were characteristically upregulated, and a variety of metabolic genes were specifically downregulated. We also found that the salt stress response genes were greatly upregulated in the *PtSnRK2.7* overexpressor. Taken together, poplar subclass 2 *PtSnRK2* genes can modulate salt stress tolerance in *Arabidopsis*, through the activation of cellular signaling pathways in a different manner from that by herbal subclass 2 *SnRK2* genes.

Keywords: *SnRK2*, overexpression, salt stress, transport, metabolism, salt tolerance, poplar

INTRODUCTION

Plants face various environmental stresses including drought, high salinity, and extreme temperatures. Such adverse circumstances can often lead to severe agricultural and industrial losses, so it is important to understand the molecular and physiological mechanisms that plants use to cope with abiotic stresses for further stable production of crops and biomass feedstock.

Abbreviations: *SnRK2*, SNF1-related protein kinases 2; ABA, abscisic acid.

Many studies have indicated that regulatory factors of protein phosphorylation play essential roles in response to environmental stimuli (Sopory and Munshi, 1998; Umezawa et al., 2013). One of the well-characterized protein kinases involved in stress responses is the group of sucrose non-fermenting 1 (SNF1)-related protein kinases (SnRKs; Halford and Hey, 2009). SnRKs are grouped into three subfamilies, SnRK1, SnRK2, and SnRK3 (Halford and Hey, 2009), and recent studies have indicated pivotal roles of plant-specific subgroups of SnRK2 and SnRK3 in the link between abiotic stress and abscisic acid (ABA) signaling to regulate metabolic pathways (Hrabak et al., 2003; Halford and Hey, 2009). Increasing evidence shows that SnRK2 proteins function as positive regulators of ABA signaling for stress responses, as well as development, in plants (Umezawa et al., 2013). In *Arabidopsis thaliana* (Arabidopsis) and rice, the SnRK2 family includes 10 members, such as SRK2A-SRK2J or SnRK2.1–2.10 in Arabidopsis and SAPK1-10 in rice (Yoshida et al., 2002; Hrabak et al., 2003; Kobayashi et al., 2004), and they are further classified into three subclasses based on their domain structures (Kobayashi et al., 2004). Most SnRK2 proteins are activated by abiotic stresses, while the members of subclasses 2 and 3 are also activated by ABA (Boudsocq et al., 2004, 2007; Kobayashi et al., 2004). In the current model, ABA-induced activation is largely explained by the interaction between SnRK2s and protein phosphatase type 2C (PP2C) proteins in the ABA signaling pathway (Leung et al., 1994, 1997; Meyer et al., 1994; Saez et al., 2004; Nishimura et al., 2007; Umezawa et al., 2009; Cutler et al., 2010; Ng et al., 2014). In the absence of ABA, group A PP2Cs physically bind to SnRK2s to dephosphorylate SnRK2s, resulting in the inhibition of ABA signal transduction, while in the presence of ABA, SnRK2 will be released from such inhibitory regulation by PP2C, because the soluble ABA receptor PYR/PYL/RCAR inhibits PP2C activity (Umezawa et al., 2009; Vlad et al., 2009).

In Arabidopsis, detailed analyses of subclass 2 (SRK2F/SnRK2.7 and SRK2C/SnRK2.8) and subclass 3 (SRK2D/SnRK2.2, SRK2I/SnRK2.3, and SRK2E/SnRK2.6) have revealed their redundant functions in ABA signaling for abiotic stress responses and developmental controls (Yoshida et al., 2002; Fujii et al., 2007; Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009; Mizoguchi et al., 2010). Importantly, overexpression of *SnRK2* genes resulted in enhanced abiotic stress tolerance in Arabidopsis (AtSRK2C/SnRK2.8, Umezawa et al., 2004; TaSnRK2.3, TaSnRK2.4, TaSnRK2.7, and TaSnRK2.8, Mao et al., 2010; Zhang et al., 2010, 2011; Tian et al., 2013) and in rice (SAPK4; Diédhiou et al., 2008). Overexpression of *SnRK2* genes in Arabidopsis induced the upregulation of several important stress responsive genes, including RD29A and DREB1A/CBF3, and ABA biosynthetic genes, such as ABA1, under normal conditions (Umezawa et al., 2004; Zhang et al., 2011), suggesting that early and quick stress responses supported by the expression of such key genes may enhance stress tolerance in Arabidopsis. The rice *SAPK4* overexpressor showed increased salt tolerance, and major aspects of its tolerance were explained by changes in the expression of genes related to ion homeostasis and oxidative stress responses (Diédhiou et al., 2008). In the cases of SnRK2 overexpressors, the results clearly indicated

that SnRK2 can function in abiotic stress responses in plant cells, through the modulation of stress response-related gene expression.

Comparative genomics studies have demonstrated that the core components of ABA signaling, PYR/PYL/RCAR, SnRK2, and PP2C, are well-conserved in land plant species (Umezawa et al., 2010), suggesting the evolutionary conservation of a molecular system involving these proteins in land plants. Indeed, the conserved molecular characteristics of SnRK2, such as transcriptional induction by abiotic stresses and activation by stress and/or ABA, have been reported for SnRK2 genes not only in Arabidopsis and rice, but also in other crop plants: maize (Huai et al., 2008; Vilela et al., 2012) and wheat (Holappa and Walker-Simmons, 1995; Gómez-Cadenas et al., 1999; Mao et al., 2010; Zhang et al., 2010, 2011; Tian et al., 2013). Additionally, we recently confirmed the physical interaction between SnRK2 and PP2C in a model tree *Populus trichocarpa* (poplar), and proposed the possibility that a similar molecular module containing SnRK2 and PP2C is involved in the ABA signaling pathway in trees (Song et al., 2015). However, information about the molecular functions of the poplar SnRK2 proteins is still limited. Recently, we evaluated the transcriptional regulation of *PtSnRK2* genes and found that some of them are upregulated by abiotic stresses in organ-specific manners, suggesting the involvement of *PtSnRK2* in ABA-dependent and/or ABA-independent regulation of stress responses (Yu et al., unpublished data.).

In this study, to obtain further clues as to the molecular functions of *PtSnRK2* proteins, we heterologously overexpressed the *PtSnRK2* genes in Arabidopsis. Our data indicated that poplar subclass II *PtSnRK2* genes can enhance the salt stress tolerance of Arabidopsis, and that poplar *PtSnRK2* overexpression would activate cellular signaling and stress response pathways in Arabidopsis in a different manner than that by the herbal subclass II *SnRK2* genes.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Young shoots of black cottonwood, *P. trichocarpa* Torr. & A. Gray (poplar), grown in 15-cm-high plant pots, were used for the cloning of *PtSnRK2* cDNA. For the overexpression analysis, *A. thaliana* (Arabidopsis) plants (Columbia strain) were used. The growth conditions were described in Ohtani et al. (2011) for poplar and in Ohtani et al. (2013) for Arabidopsis.

Plasmid Construction and Transformation

The coding sequences of *PtSnRK2* genes were cloned into the Gateway entry vector pENTR/D-TOPO or pCR8/GW/TOPO (Invitrogen), as described by Song et al. (2015), and were transferred to the destination vector pH35GS (Kubo et al., 2005) by the LR reaction using LR clonase II (Invitrogen). In the resulting plasmids, the *PtSnRK2* cDNA was expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The plasmids were electroporated into the *Agrobacterium tumefaciens* strain GV3101 (pMP90). A simplified version of the floral dip method was used for the transformation of Arabidopsis plants (Clough and Bent, 1998).

Establishment of Transgenic Lines

For the screening of transgenic lines carrying the empty vector (vector controls) and 35S::PtSnRK2 (PtSnRK2 overexpressors), T₁ seedlings were grown in germination Murashige and Skoog (MS) medium containing 50 µg mL⁻¹ hygromycin for 2 weeks, and the positive plants were transferred to soil for further growth. Although we failed to generate overexpressors of PtSnRK2.2, PtSnRK2.6, and PtSnRK2.8, more than 16 independent T₂ lines were established for every other PtSnRK2 genes.

To evaluate the expression levels of the introduced PtSnRK2, total RNAs were prepared from 7-day-old seedlings of the wild type, vector controls, and PtSnRK2 overexpressors at the T₂ generation using the RNeasy Mini kit (Qiagen). The first-strand cDNAs were synthesized using SuperScript III (Invitrogen) and subjected to RT-PCR analysis. The RT reaction was performed on a 20-µL scale, with 1 µL first-strand cDNA as a template for PCR, along with 0.5 µM of each gene-specific primer (see Table S2) and Ex Taq polymerase (TaKaRa). The PCR conditions were as follows: for the internal control gene *Ubp10*, 95°C for 5 min, followed by 35 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 15 s, followed by 72°C for 7 min; for PtSnRK2 genes (target genes), 95°C for 5 min, followed by 35 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 1 min 15 s, and followed by 72°C for 7 min. The amplified PCR products were electrophoresed in 3 and 2% (w/v) agarose gels for the *Ubp10* and PtSnRK2 genes, respectively, and gel images were analyzed using the AE-9020 E-shot II (ATTO; Figure S1). Information about the primer sets is provided in Table S11.

Salt Stress Treatment

It is generally known that the expression level of 35S promoter-driven genes would be decreased in T₃ homozygous lines, so we decided to use T₂ lines that were confirmed to show high expression levels of inserted PtSnRK genes as described above, for the salt stress treatment. Wild-type and transgenic T₂ plants were grown at 22°C under LD conditions (16 h light/8 h dark) on 1/2-strength MS medium, after incubation at 4°C for 3 days. Then, 7-day-old plants were transferred to 1/2-MS medium plates with or without NaCl. For the survival rates and chlorophyll contents, 200 mM NaCl was included in the medium, and for the root growth phenotype, 100 mM NaCl was used. After an additional 4-day incubation, the numbers of surviving seedlings were counted to obtain survival rates ($n = 20$), and chlorophyll contents were measured as described below ($n = 10$). The primary root length was measured before and after a 5-day incubation on the NaCl plate, to calculate primary root elongation during salt stress treatment ($n = 10$). The treatments were repeated three times for survival rates and root length and six times for chlorophyll contents.

Chlorophyll Quantitation

Ten seedlings treated with 200 mM NaCl for 4 days were sampled in 3 mL (*N,N*-dimethylformamide, DMF). After incubation in DMF overnight at 4°C in the dark, A_{646.8}, A_{663.8}, and A₇₅₀ were measured using the iMark Microplate Absorbance Reader (Bio-Rad). The chlorophyll quantitation was calculated by the formula:

$$[8.05 \times (A_{663.8} - A_{750}) + 19.43 \times (A_{646.8} - A_{750}) (\mu\text{M})] / \text{the quantity of seedlings (mg)} (\mu\text{M/mg}) \text{ (Porra et al., 1989).}$$

In silico Prediction of the Three-Dimensional Structures of PtSnRK2 Proteins

The amino acid sequences of PtSnRK2.5, PtSnRK2.7, and PtSnRK2.9 were submitted to the web-based SWISS-MODEL service (<http://swissmodel.expasy.org/workspace/>; Arnold et al., 2006), to build protein structure homology models using information on the crystal structure of the recombinant AtSnRK2.6 protein as a template (Ng et al., 2011).

Microarray Analysis

Seedlings of the wild-type and PtSnRK2.7 overexpressor line 20 treated with or without 200 mM NaCl for 2 days were sampled for total RNA extraction. Microarray analysis was performed using ATH1 GeneChips (Affymetrix) according to the manufacturer's instructions on three independent biological replicates. Subsequent procedures of quality control, statistical analysis, and filtering were carried out using GeneSpring GX software (ver. 13.1; Agilent Technologies). Then, p -values were calculated for each probe using Welch's t -test ($n = 3$) for differences between the treated seedlings and the control seedlings, as well as between the wild-type and PtSnRK2.7 overexpressor incubated without salt treatment. We used the Benjamin-Hochberg FDR method to control for false positives. A p -value cut-off of 0.01 was used to select genes whose expression changed with salt treatment. Fold-change values were also computed using GeneSpring GX, and we targeted those probes in which the change was upregulated or downregulated by more than 3-fold. Microarray data presented in this study were submitted to NCBI GEO (www.ncbi.nlm.nih.gov/geo/) and can be retrieved via accession number GSE79997.

Gene Ontology (GO) Term Analysis

Gene ontology (GO) term analysis was performed using the PANTHER classification system (Overrepresentation Test, release 20150430; Mi et al., 2013).

Quantitative RT-PCR Analysis

To evaluate the enhanced upregulation of salt stress response genes in PtSnRK2.7 overexpressors, quantitative RT-PCR analysis was performed. Total RNAs were isolated from the seedlings treated with or without 200 mM NaCl for 2 days using Plant RNA Isolation Reagent (Invitrogen) and then purified using the RNeasy Mini Kit (QIAGEN). The first-strand cDNAs were synthesized as described above, and aliquots of the cDNA solution (0.5 µL for each gene) were used as templates for subsequent PCR amplification. The quantitative PCR analysis was performed using the LightCycler 480 System II (Roche) and LightCycler 480 SYBR Green I Master reagents (Roche). As an internal control, the *Ubc9* gene was used. Information on the primer sets is provided in Table S11.

RESULTS AND DISCUSSION

Overexpression of *PtSnRK2.5* and *PtSnRK2.7* Improved Salt Stress Tolerance in Arabidopsis

In the genome of *P. trichocarpa* (poplar), 12 *PtSnRK2* genes have been identified (Song et al., 2015). For molecular functional analysis of *PtSnRK2*, we generated transgenic plants of *A. thaliana* (Arabidopsis) carrying the chimeric gene 35S::*PtSnRK2*, in which the cDNA regions of *PtSnRK2* genes were regulated by the CaMV 35S promoter sequence, to overexpress poplar *PtSnRK2* genes in Arabidopsis. Unfortunately we could not obtain transgenic plants for *PtSnRK2.2*, *PtSnRK2.6*, and *PtSnRK2.8* overexpression; however, overexpressors of the other nine *PtSnRK2* genes were established successfully. It has been reported that overexpression of *SnRK2* genes enhances abiotic stress tolerance (Umezawa et al., 2004; Diédhiou et al., 2008; Mao et al., 2010; Zhang et al., 2010, 2011; Tian et al., 2013). Thus, we examined salt stress tolerance in the *PtSnRK2* overexpressors.

First, 7-day-old seedlings from 16 independent T₂ lines for each *PtSnRK2* overexpressor were transferred to medium containing 200 mM NaCl, and incubated for 4 days. The *prp9-11 prr7-10 prr5-10* triple mutant (d975) plant, which showed high salinity tolerance because of high expression of salt stress response genes (Nakamichi et al., 2009), was used as a positive control (Figure S2). The survival rates of seedlings after salt treatment demonstrated that the overexpressors of *PtSnRK2.5* and *PtSnRK2.7* showed relatively high survival rates among the *PtSnRK2* overexpressors. These two genes encode the subclass 2 *PtSnRK2* proteins (Song et al., 2015). We further performed a detailed analysis of salt stress tolerance in the *PtSnRK2.5* and *PtSnRK2.7* overexpressors, using the wild-type and vector control plants as negative controls (Figures 2, 3). For the *PtSnRK2.5* and *PtSnRK2.7* overexpressors, three independent lines were selected based on the expression levels of the introduced *PtSnRK2* genes (Figure S1). In the case of the negative controls, the seedling survival rates after 200 mM NaCl treatment were less than 20%, and the living seedlings exhibited yellowed leaves (Figure 2). In contrast, the *PtSnRK2.5* and *PtSnRK2.7* transgenic seedlings showed significantly higher survival rates (~55%) than those of the controls (Figure 2). All three independent *PtSnRK2.7* lines showed increased survival rates, whereas the *PtSnRK2.5* line 6 did not show a significantly enhanced survival rate (Figure 2B).

Next, the chlorophyll contents of 10 seedlings treated with salt stress were analyzed. The salt treatment greatly decreased chlorophyll contents in all plants; however, the chlorophyll contents of *PtSnRK2.5* lines 16 and 20, and all of the *PtSnRK2.7* lines were significantly higher than those of the wild-type and vector control (Figure 2C), in accordance with the survival rates. These results suggested that salt stress tolerance was more stable in the *PtSnRK2.7* overexpressors. Moreover, it was notable that although the chlorophyll contents in the absence of salt stress were at almost the same levels among the wild-type, vector control, and *PtSnRK2.7* lines, but the *PtSnRK2.5* lines 16 and 20 showed lower amounts of chlorophyll under normal growth

conditions (Figure 2C). Thus, *PtSnRK2.5* overexpression may have affected chlorophyll biosynthesis continuously.

In addition to the increased survival rates and chlorophyll contents, the living seedlings of the *PtSnRK2.5* and *PtSnRK2.7* transgenic lines seemed to be larger than those of the negative controls under salt stress conditions (Figure 2A). To clarify the effects of *PtSnRK2* overexpression on seedling growth, we checked the growth of primary roots after salt treatment. Because the 200 mM NaCl treatment almost completely inhibited primary root elongation in both the overexpressors and negative controls, we used 100 mM NaCl conditions to observe primary root elongation. Our data demonstrated no difference in primary root elongation in the mock-treated seedlings (Figure 3), indicating that these two *PtSnRK2* genes are not involved in root elongation regulation, unlike the Arabidopsis subclass II *AtSRK2C/SnRK2.8* gene, overexpression of which was reported to enhance seedling root growth (Shin et al., 2007). However, after a 5-day incubation with 100 mM NaCl, the primary roots of *PtSnRK2.5* line 20 and all of the *PtSnRK2.7* lines elongated significantly more than did those of the negative controls; the negative controls showed elongation of less than 20 mm, whereas the elongated lengths of *PtSnRK2.5* line 20 and all of the *PtSnRK2.7* lines were ~25 mm ($p < 0.01$, t -test). These observations indicated that overexpression of *PtSnRK2.7*, and possibly also *PtSnRK2.5*, can suppress the inhibition of root elongation by high salinity stress.

Our data on the *PtSnRK2.5* and *PtSnRK2.7* transgenic lines demonstrated different phenotypic characteristics between the *PtSnRK2.5* and *PtSnRK2.7* lines. Thus, it was suspected that the molecular basis for the enhancement of salt stress tolerance would be different between the *PtSnRK2.5* and *PtSnRK2.7* transgenic lines. In particular, the *PtSnRK2.5* lines showed unstable salt stress tolerance (Figures 2, 3) and defects in chlorophyll content even under normal growth condition (Figure 2C). The subclass 2 *PtSnRK2* proteins have highly similar amino acid sequences (Figure 1A), and the amino acid sequences of *PtSnRK2.5* (unstable, but significantly enhanced salinity tolerance), *PtSnRK2.7* (stable and high tolerance), and *PtSnRK2.9* (no obviously increased tolerance) did not show major differences between them (Figure 1A). *In silico* modeling of the three-dimensional structures of these proteins, based on the crystal structure of *AtSnRK2.6* (Ng et al., 2011), revealed that some of the differences in amino acid sequences among these *PtSnRK2* proteins could correspond to the molecular surface regions of these proteins (Figure 1B), possibly leading to changes in the molecular activities of the *SnRK2* proteins. The different *SnRK2* protein activities due to subtle substitutions of amino acids could result in the different strengths of salt stress tolerance among the overexpressors.

PtSnRK2.7 Overexpression Widely and Largely Influenced the Transcriptome in Response to Salt Stress

We next performed transcriptomic analyses of the wild-type and overexpressors during salt stress treatment. Based on the data described above (Figures 2, 3), we selected *PtSnRK2.7* line 20, which showed stable and strong salt stress tolerance

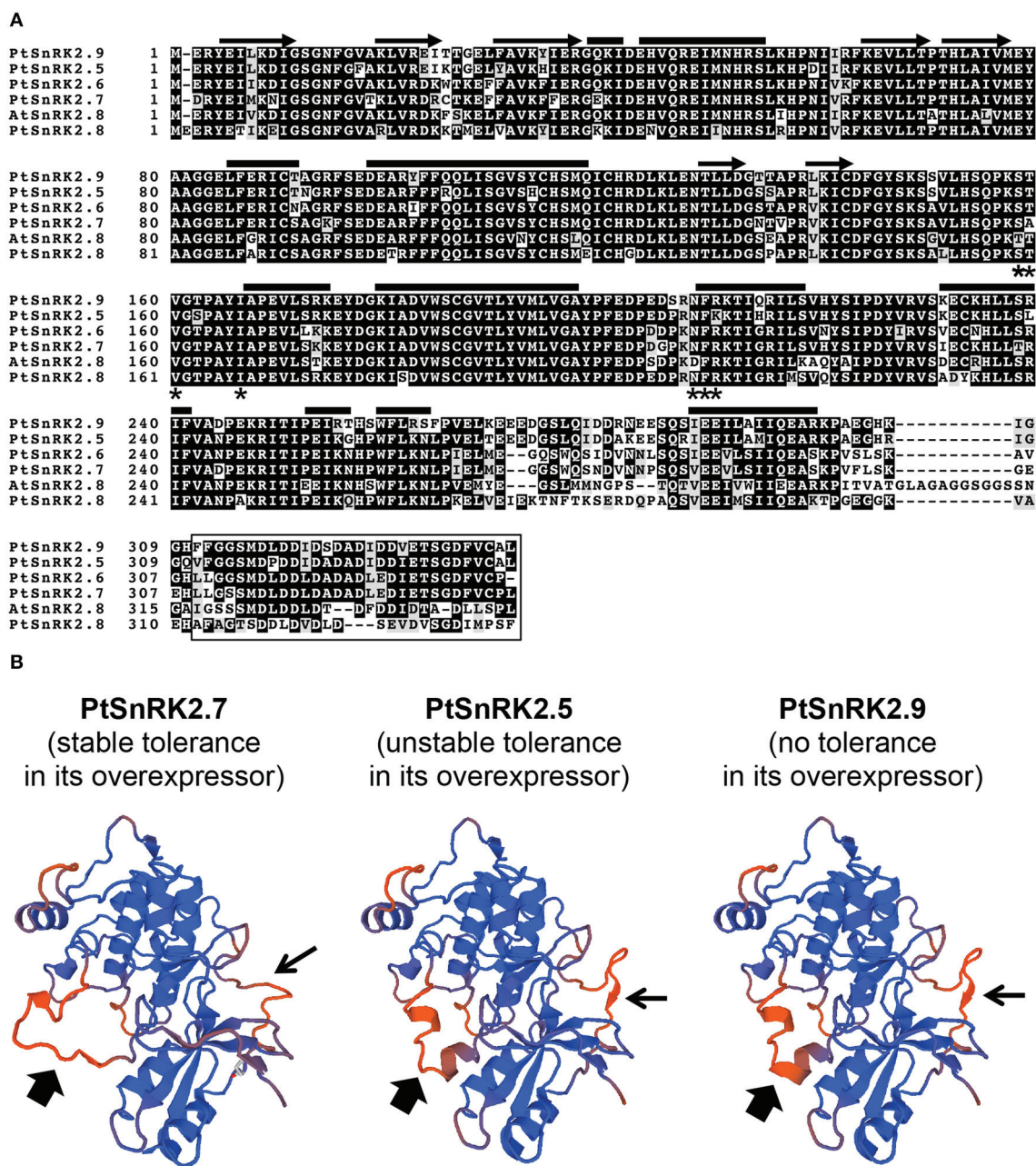


FIGURE 1 | SnRK2 proteins of *Populus trichocarpa* (poplar, Pt), and *Arabidopsis thaliana* (Arabidopsis, At). **(A)** Amino acid sequence alignment of subclass 2 PtSnRK2 proteins with AtSnRK2.8. The residues putatively corresponding to the α -helix, β -sheet, PP2C-interaction residues, and the entire ABA box are marked by bars, arrows, asterisks, and the box, respectively. **(B)** *In silico* modeling of the three-dimensional structures of PtSnRK2.5, PtSnRK2.7, and PtSnRK2.9 proteins. Homology modeling was performed based on the crystal structure of AtSnRK2.6 using the web-based SWISS-MODEL service (<http://swissmodel.expasy.org/workspace/>; Arnold et al., 2006). Thick and narrow arrows indicate regions presumed to differ in their three-dimensional architecture among the PtSnRK2 proteins because of differences in amino acid sequences.

among the transgenic lines, for the gene chip analysis. Then, 7-day-old seedlings of the wild-type and *PtSnRK2.7* line 20 were treated with or without 200 mM NaCl for 2 days and then sampled to extract total RNA. The extracted total RNA samples were subjected to microarray analysis using Affymetrix ATH1 GeneChips (Figure 4A). First, we compared

transcriptomic data between the wild-type and *PtSnRK2.7* overexpressor, which were mock-treated, to examine the effects of *PtSnRK2.7* overexpression on gene expression under normal growth conditions. Thirty and 79 genes were shown to be upregulated and downregulated in the *PtSnRK2.7* overexpressor, respectively ($FC > 2$, $p < 0.05$; Table S1). It has been reported

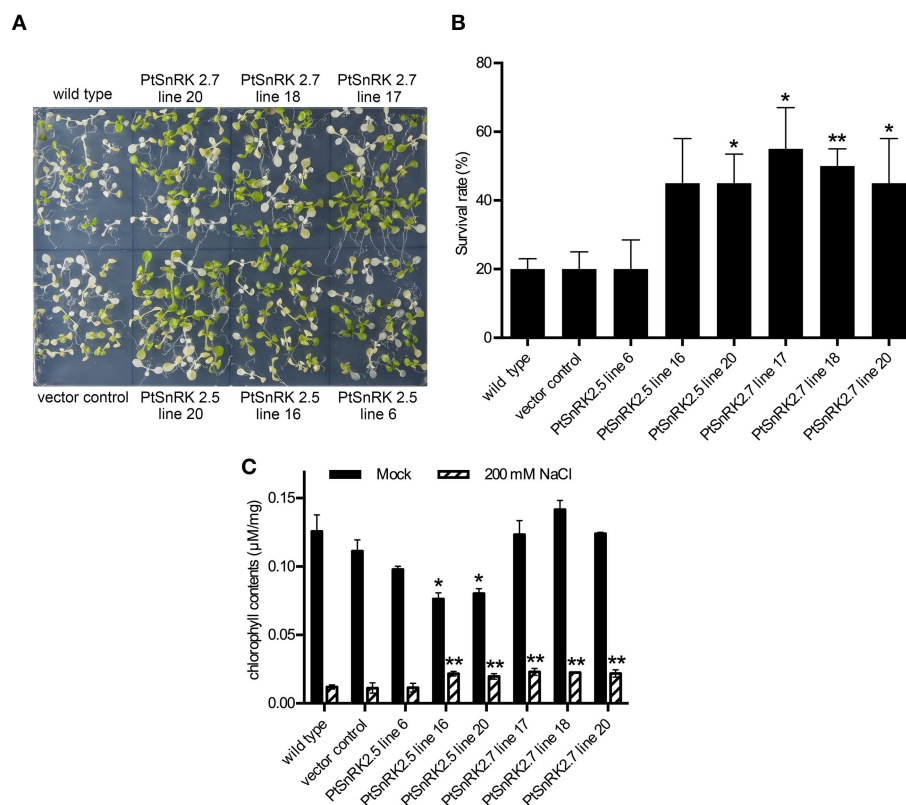


FIGURE 2 | High salinity tolerance phenotype of transgenic Arabidopsis overexpressing *PtSnRK2.5* and *PtSnRK2.7*. (A) Seedlings of the wild-type, vector control, and overexpressors of *PtSnRK2.5* and *PtSnRK2.7* treated with 200 mM NaCl for 4 days. (B) Survival rates determined by observations after a 4-day salt stress treatment. The green seedlings were counted as living seedlings, and the percentages of live seedlings were calculated using 20 seedlings for each line. (C) Chlorophyll contents determined from 20 seedlings after a 4-day salt stress treatment. Results are means \pm SE ($n = 3$). Asterisks indicate statistically significant differences between transgenic and wild-type plants (Student's *t*-test; * $p < 0.05$; ** $p < 0.01$).

that in AtSRK2C/SnRK2.6 and TaSnRK2.8 overexpressors, stress response-related genes including RD29A and DREB1A/CBF3, and ABA biosynthetic genes are upregulated continuously (Umezawa et al., 2004; Zhang et al., 2011). However, these genes were not changed by *PtSnRK2.7* overexpression (Table S1). The gene ontology (GO) term analysis revealed that stress-related metabolic genes, including lipid metabolism and flavonoid metabolism, were significantly downregulated in the *PtSnRK2.7* overexpressor (Table 1). These results suggest that *PtSnRK2.7* overexpression could continuously affect specific ranges of gene expression regulation, which do not overlap with the primary targets of AtSRK2C/SnRK2.6 and TaSnRK2.8 in Arabidopsis.

To examine the impact of *PtSnRK2.7* overexpression on the salt stress response, the genes upregulated or downregulated by salt treatment were compared between the wild-type and *PtSnRK2.7* overexpressor. In the wild-type, 337 and 120 genes were upregulated and downregulated by salt treatment, respectively ($FC > 3$, $p < 0.01$; Figure 4B and Tables S2, S3). Notably, the *PtSnRK2.7* overexpressor showed greater numbers of genes whose expression was changed by salt treatment compared with the wild-type; 631 and 698 genes were upregulated and downregulated in the *PtSnRK2.7* overexpressor,

respectively ($FC > 3$, $p < 0.01$; Figure 4B and Tables S4, S5), suggesting that *PtSnRK2.7* overexpression affected the expression of a wide range of genes during salt stress responses in Arabidopsis. The GO analysis indicated that the genes functioning in stress responses, including the signaling pathway of stress-related phytohormones (ABA, ethylene, jasmonic acid, and salicylic acid) and in signal transduction by protein phosphorylation, were commonly upregulated between the wild-type and *PtSnRK2.7* overexpressor (Figure 4B and Tables S6–S8). Additionally, developmental process-related genes were commonly downregulated in the wild-type and *PtSnRK2.7* overexpressor (Figure 4B and Table S10).

We also found that some GO terms were enriched in the upregulated genes in genotype-dependent manners, such as “respiratory burst” and “ethylene biosynthetic process” found only in the wild-type (Figure 4B and Table S6) and “purine nucleoside transmembrane transport,” “amino acid transport,” “anion transmembrane transport,” and “nucleic acid metabolic process” found only in the *PtSnRK2.7* overexpressor (Figure 4B and Table S7). The overrepresentation of the GO terms related to “anion transmembrane transport” in the *PtSnRK2.7* overexpressor would suggest the enhancement of ion

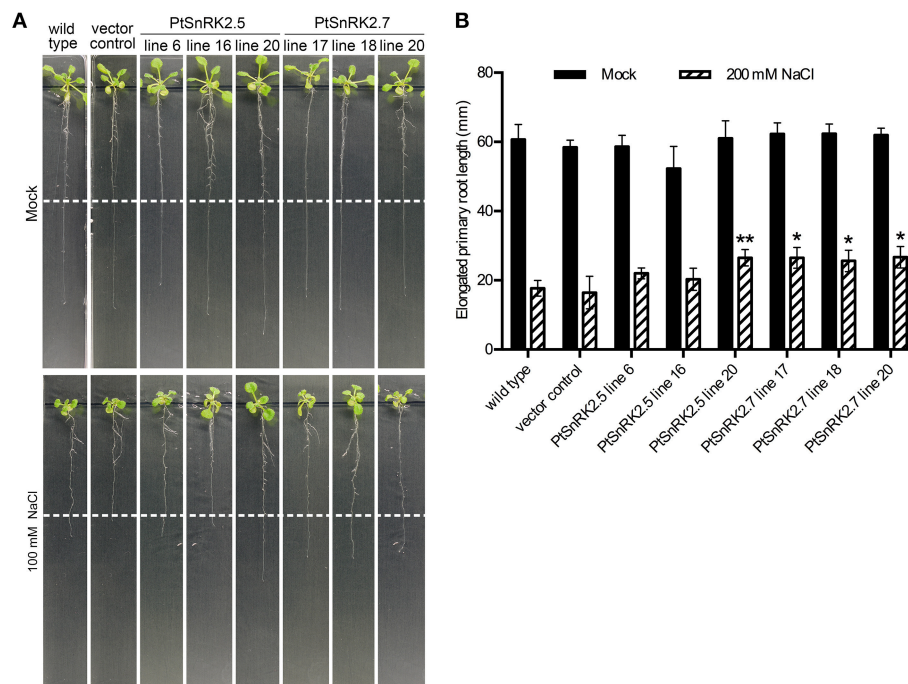


FIGURE 3 | Primary root elongation of transgenic Arabidopsis overexpressing *PtSnRK2.5* and *PtSnRK2.7* during salt treatment. (A) Seedlings of the wild-type, vector control, and overexpressors of *PtSnRK2.5* and *PtSnRK2.7* treated with 100 mM NaCl for 5 days. The positions of the edges of the root tips before salt treatment are indicated by the white dotted lines. Bar = 20 mm. **(B)** Increase in root length after salt stress treatment. Results are means \pm SE ($n = 15$). Asterisks indicate statistically significant differences between transgenic plants and the wild-type (Student's *t*-test; * $p < 0.05$; ** $p < 0.01$).

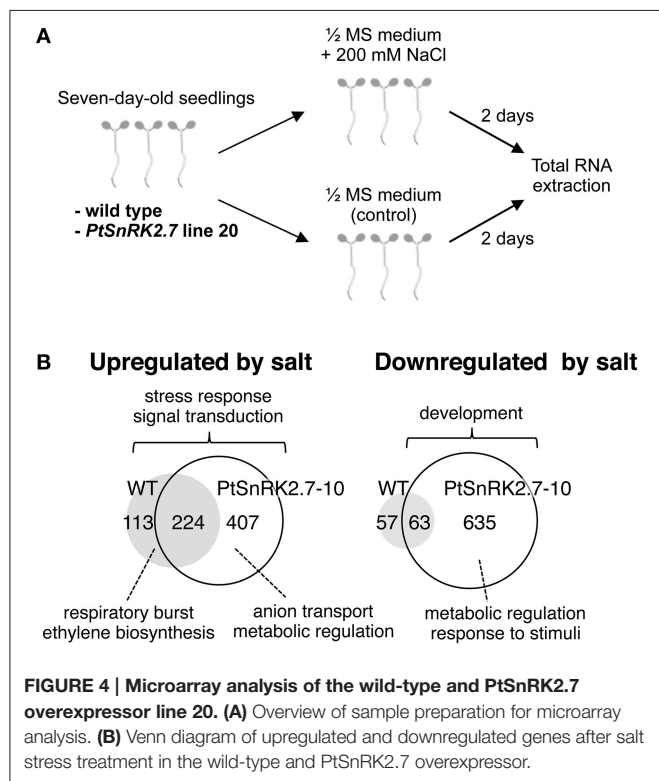


FIGURE 4 | Microarray analysis of the wild-type and *PtSnRK2.7* overexpressor line 20. (A) Overview of sample preparation for microarray analysis. **(B)** Venn diagram of upregulated and downregulated genes after salt stress treatment in the wild-type and *PtSnRK2.7* overexpressor.

homeostasis activity as a result of *PtSnRK2.7* overexpression, possibly leading to higher salt stress tolerance. Our results also showed wild-type-specific enrichment of the term “ethylene biosynthetic process” in upregulated genes (Figure 4B and Table S6). It has been reported that crosstalk between ABA and ethylene is a critical factor in determining salt stress tolerance in Arabidopsis (Dong et al., 2011); thus, *PtSnRK2.7* overexpression may influence the phytohormonal modulating system of abiotic stress responses. Moreover, in the *PtSnRK2.7* overexpressor, more than 5-fold more genes were downregulated by salt treatment than those in the wild type. These downregulated genes are involved in a wide range of molecular functions, including metabolic regulation and responses to stimuli (Figure 4B and Table S9), indicating that the reason for the high salt stress tolerance in the *PtSnRK2.7* overexpressor may be, at least partially, attributed to its significant impact on metabolic regulation.

Comparison of transcriptomic data also suggested that the altered expression levels of upregulated genes were greater in the *PtSnRK2.7* overexpressor than in the wild-type (Tables S2, S4). To confirm this, we selected six genes from the functional categories of stress response (*COR15A*, Artus et al., 1996), phytohormonal signaling (*GASA3*, Herzog et al., 1995), cell wall-related proteins (*AT1G52690* and *AtCWINV5*, Sherson et al., 2003), and lipid metabolism (*AT4G33550*), based on the transcriptomic data, and subjected them to quantitative

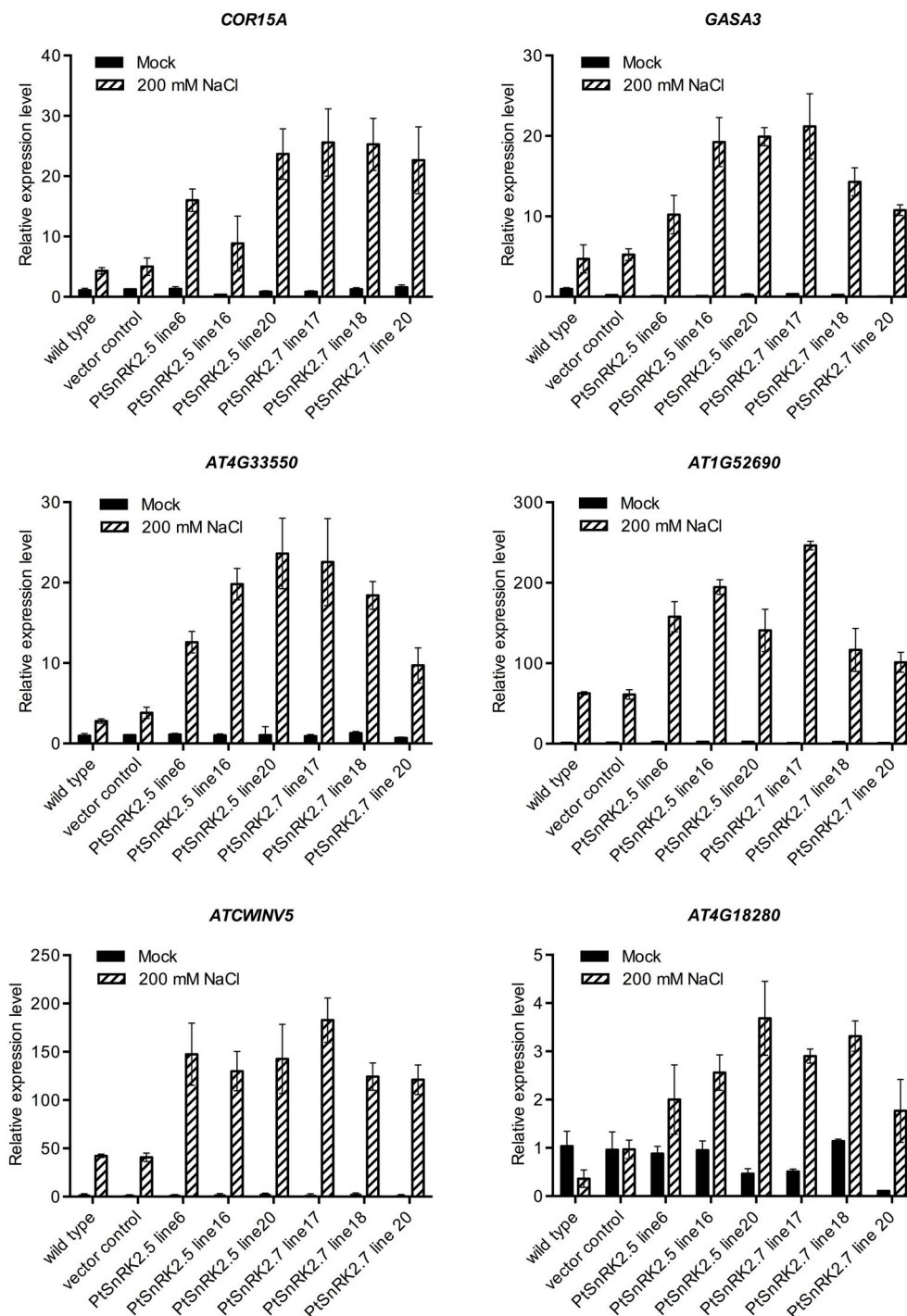


FIGURE 5 | Quantitative RT-PCR analysis of transcript levels of *COR15A*, *GASA3*, *AT4G33550*, *AtCWINV5*, *At1G52690*, and *At4G18280* in the wild-type, vector control, and overexpressors of *PtSnRK2.5* and *PtSnRK2.7*. Seedlings treated with or without 200 mM NaCl for 2 days were analyzed. Results are means \pm SE ($n = 3$).

RT-PCR analysis. *AT4G18280*, which was upregulated only in the *PtSnRK2.7* overexpressor, was also tested to evaluate the consistency of the results between the gene chip analysis and quantitative RT-PCR analysis. Indeed, *AT4G18280* expression

was not induced by salt stress in the wild-type or vector control, whereas it was highly upregulated in the *PtSnRK2.7* line 20 in accordance to the results of gene chip analysis (Figure 5 and Table S4). The results showed that the tested genes were actively

TABLE 1 | GO term analysis of the differentially expressed genes between the wild-type and *PtSnRK2.7* overexpressor grown in the absence of salt stress treatment.

GO biological process complete	Fold Enrichment	p-value
Anthocyanin-containing compound biosynthetic process	44.44	4.52E-03
Anthocyanin-containing compound metabolic process	40.00	7.29E-03
Cellular response to phosphate starvation	33.33	1.12E-09
Galactolipid biosynthetic process	33.33	2.61E-07
Galactolipid metabolic process	33.33	2.61E-07
Glycolipid biosynthetic process	28.57	1.06E-06
Flavonoid biosynthetic process	26.09	2.69E-04
Flavonoid metabolic process	24.00	4.11E-04
Liposaccharide metabolic process	23.53	4.26E-06
Glycolipid metabolic process	23.53	4.26E-06
Membrane lipid biosynthetic process	22.22	6.69E-06
Response to UV-B	20.83	9.49E-03
Cellular response to starvation	18.18	6.92E-09
Cellular response to nutrient levels	17.65	8.89E-09
Membrane lipid metabolic process	17.39	4.15E-05
Response to starvation	17.39	1.08E-08
Cellular response to extracellular stimulus	16.88	1.88E-09
Cellular response to external stimulus	16.67	2.26E-09
Response to nutrient levels	16.67	1.82E-08
Response to extracellular stimulus	16.05	3.73E-09
Pigment biosynthetic process	13.73	1.65E-03
Pigment metabolic process	10.94	7.65E-03
Negative regulation of transcription, DNA-templated	8.43	3.85E-02
Negative regulation of RNA biosynthetic process	8.43	3.94E-02
Negative regulation of nucleic acid-templated transcription	8.43	3.94E-02
Negative regulation of RNA metabolic process	8.43	4.03E-02
Cellular response to stress	5.62	1.09E-04
Response to external stimulus	3.65	2.57E-03
Single-organism biosynthetic process	2.99	6.83E-03

upregulated by salt treatment in the *PtSnRK2.7* overexpressors compared with the wild-type and vector control (Figure 5). Our data also demonstrated that *PtSnRK2.5* overexpression had similar effects on the expression of these genes. Thus, it is supposed that *PtSnRK2.5* overexpression affected the transcriptome in a similar fashion to *PtSnRK2.7* overexpression.

It has been reported that the overexpression of Arabidopsis *AtSnRK2.8* enhanced the stress tolerance of Arabidopsis, probably through the continuous upregulation of key genes for stress responses, such as *RD29A*, *COR15A*, *AtGolS3*, *DREB1A*, and *PKS18* (Umezawa et al., 2004). However, our

results showed that overexpression of poplar *SnRK2* genes did not constitutively induce these well-known stress responsive genes. Rather, the high salt stress tolerance of the *PtSnRK2* overexpressors may be explained by changes in transcriptomic regulation for a wide range of metabolic regulatory genes (Figures 4, 5; Table 1 and Tables S1–S10). It is notable that the genes related to anion transport activity were upregulated in the *PtSnRK2.7* overexpressor specifically, because similar effects on ion homeostasis-related genes were reported in the case of overexpression of rice *SAPK4* (Diédhiou et al., 2008). Moreover, a comparative transcriptomic analysis using salt-tolerant and non-tolerant species of poplar indicated that prominent factors for high salt tolerance were not overexpression of the stress responsive pathway, but rather enhanced activities for osmotic adjustment, ion compartmentalization, and detoxification of reactive oxygen species in poplar (Chen and Polle, 2010). Thus, our results may reflect differences in the regulatory targets of *SnRK2* proteins between *AtSnRK2* and *PtSnRK2*, which could be related to diversified molecular strategies of stress adaption in each plant species, as suggested by Zhang et al. (2014). Future comparative analyses on the mode of actions of *SnRK2* proteins derived from different plant species may provide important information on novel strategies to improve stress tolerance of crops and other useful plants.

AUTHOR CONTRIBUTIONS

XS designed and performed the experiments, and drafted the manuscript. MO designed the experiments, analyzed the data, and wrote the manuscript. XY and CH contributed to the data analysis. QZ and TD drafted the manuscript. All authors contributed to and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00612>

Figure S1 | RT-PCR analysis of seedlings of the wild-type, vector control, and overexpressors of *PtSnRK2.5* and *PtSnRK2.7*.

Figure S2 | Primary screening of transgenic Arabidopsis overexpressing *PtSnRK2* genes for the salt tolerance. Seedlings of the d975 (positive control), vector control, and overexpressors of *PtSnRK2* genes were treated with 200 mM NaCl, and checked their survival rates after 3, 4, and 5 days of NaCl treatment. A

part of results for the transgenic lines of *PtSnRK2.5* (A) and *PtSnRK2.9* (B) treated with 200 mM NaCl for 4 days were shown.

Table S1 | Differentially expressed genes between the wild type and PtSnRK2.7 overexpressor (FC>2, $p < 0.05$).

Table S2 | Upregulated 337 genes in the wild type by the salt treatment (FC>3, $p < 0.01$).

Table S3 | Downregulated 120 genes in the wild type by the salt treatment (FC>3, $p < 0.01$).

Table S4 | Upregulated 631 genes in the PtSnRK2.7 overexpressor by the salt treatment (FC>3, $p < 0.01$).

Table S5 | Downregulated 698 genes in the PtSnRK2.7 overexpressor by the salt treatment (FC>3, $p < 0.01$).

Table S6 | GO term analysis on wild-type-specific upregulated genes by salt treatment.

Table S7 | GO term analysis on PrSnRK2.7 overexpressor-specific upregulated genes by salt treatment.

Table S8 | GO term analysis on commonly upregulated genes between the wild type and PtSnRK2.7 overexpressor by salt treatment.

Table S9 | GO term analysis on PrSnRK2.7 overexpressor-specific downregulated genes by salt treatment.

Table S10 | GO term analysis on commonly downregulated genes between the wild type and PtSnRK2.7 overexpressor by salt treatment.

Table S11 | Oligonucleotides used in this paper.

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The Effect of Exogenous Spermidine Concentration on Polyamine Metabolism and Salt Tolerance in Zoysiagrass (*Zoysia japonica* Steud) Subjected to Short-Term Salinity Stress

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Salt stress, particularly short-term salt stress, is among the most serious abiotic factors limiting plant survival and growth in China. It has been established that exogenous spermidine (Spd) stimulates plant tolerance to salt stress. The present study utilized two zoysiagrass cultivars commonly grown in China that exhibit either sensitive (cv. Z081) or tolerant (cv. Z057) adaptation capacity to salt stress. The two cultivars were subjected to 200 mM salt stress and treated with different exogenous Spd concentrations for 8 days. Polyamine [diamine putrescine (Put), tetraamine spermine (Spm), and Spd], H_2O_2 and malondialdehyde (MDA) contents and polyamine metabolic (ADC, ODC, SAMDC, PAO, and DAO) and antioxidant (superoxide dismutase, catalase, and peroxidase) enzyme activities were measured. The results showed that salt stress induced increases in Spd and Spm contents and ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC), and diamine oxidase (DAO) activities in both cultivars. Exogenous Spd application did not alter polyamine contents via regulation of polyamine-degrading enzymes, and an increase in polyamine biosynthetic enzyme levels was observed during the experiment. Increasing the concentration of exogenous Spd resulted in a tendency of the Spd and Spm contents and ODC, SAMDC, DAO, and antioxidant enzyme activities to first increase and then decrease in both cultivars. H_2O_2 and MDA levels significantly decreased in both cultivars treated with Spd. Additionally, in both cultivars, positive correlations between polyamine biosynthetic enzymes (ADC, SAMDC), DAO, and antioxidant enzymes (SOD, POD, CAT), but negative correlations with H_2O_2 and MDA levels, and the Spd + Spm content were observed with an increase in the concentration of exogenous Spd.

Keywords: Zoysiagrass, polyamine metabolism, salinity stress, exogenous spermidine, antioxidant enzyme

INTRODUCTION

Due to the generation of a hyperosmotic effect by reducing the soil water potential, salt stress, particularly short-term salt stress, is one of the most serious abiotic factors limiting productivity in turf grass (Alshammary et al., 2004; Ahn et al., 2015). Furthermore, salt stress induces a hypertonic effect, and these ions are directly toxic to plant metabolism and nutrition. Additionally, free radicals induce structural damage in plant cells, causing a loss of turgidity and thereby weakening the organism. However, plants have evolved various defense mechanisms to mitigate salt damage, including accumulation of osmolytes such as sugars, glycine betaine, and proline and adaptations to salt stress such as Na^+/H^+ antiporters (Qi et al., 2014). Salinity stress also increases the levels of polyamines (Chattopadhyay et al., 2002).

Polyamines (PAs) are ubiquitous compounds in plant cells that are essential for growth and development. PAs also play an important role in the response of plants to adverse environmental conditions due to their polycationic nature (Puyang et al., 2015; Pál et al., 2015). These compounds mainly exist in three forms in plant cells, diamine putrescine (Put), triamine spermidine (Spd), and tetraamine spermine (Spm), each of which may be present in a free, soluble conjugated or insoluble bound form. Soluble conjugated forms, such as phenolic compounds, are covalently conjugated to small molecules, whereas insoluble bound forms are covalently bound to macromolecules, such as nucleic acids and proteins (Gill and Tuteja, 2010). In plants, arginine decarboxylase (ADC), ornithine decarboxylase (ODC), and S-adenosylmethionine decarboxylase (SAMDC) are the key enzymes responsible for synthesizing PAs. Arginase and ODC convert ornithine to Put, which is also synthesized via

agmatine through three sequential reactions catalyzed by ADC, agmatine iminohydrolase (AIH), and N-carbamoylputrescine amidohydrolase (CPA). Spd and Spm are produced from Put in plants through successive addition of aminopropyl groups from decarboxylated S-adenosylmethionine (dc-SAM), which is generated from SAM by SAMDC. Conversely, PAs are degraded by diamine oxidase (DAO) and polyamine oxidase (PAO; Kusano et al., 2008; Tavladoraki et al., 2012; **Figure 1**).

Much evidence to date has shown that exogenous application of PAs (di- and tri- and tetra-amines) enhances tolerance to salinity stress in plants by stabilizing membrane and cellular structures, scavenging free radicals, modulating ion channels, maintaining the cation-anion balance, and energizing cells via stimulation of ATP synthesis (Hartung et al., 2002; Nuttall et al., 2003; Shi and Sheng, 2005; Yang et al., 2007). Indeed, application of exogenous PAs can effectively augment plant salinity tolerance and ultimately improve plant productivity under high-salinity conditions (Ndayiragije and Lutts, 2006), and studies in various species have indicated that such enhance salinity tolerance occurs through the synergy of a number of mechanisms (Ndayiragije and Lutts, 2006; Roychoudhury et al., 2011; Saleethong et al., 2011; Velarde-Buendía et al., 2012; Shu et al., 2013; Zhang et al., 2014). Among the three major PAs, Spd is most closely associated with stress tolerance in plants (Shen et al., 2000).

To date, there have been substantial efforts toward improving salinity tolerance in plants through transgenic techniques, several of which have been widely applied in *Arabidopsis thaliana* and transgenic rice overexpressing genes for PA biosynthetic enzymes (Roy and Wu, 2001; Kasukabe et al., 2004, 2006). In addition, exogenous application of Spd dramatically reversed the observed cinnamic acid (CA)-induced effects on Spd + Spm and partially restored the PA ratio and RuBPC activity in leaves,

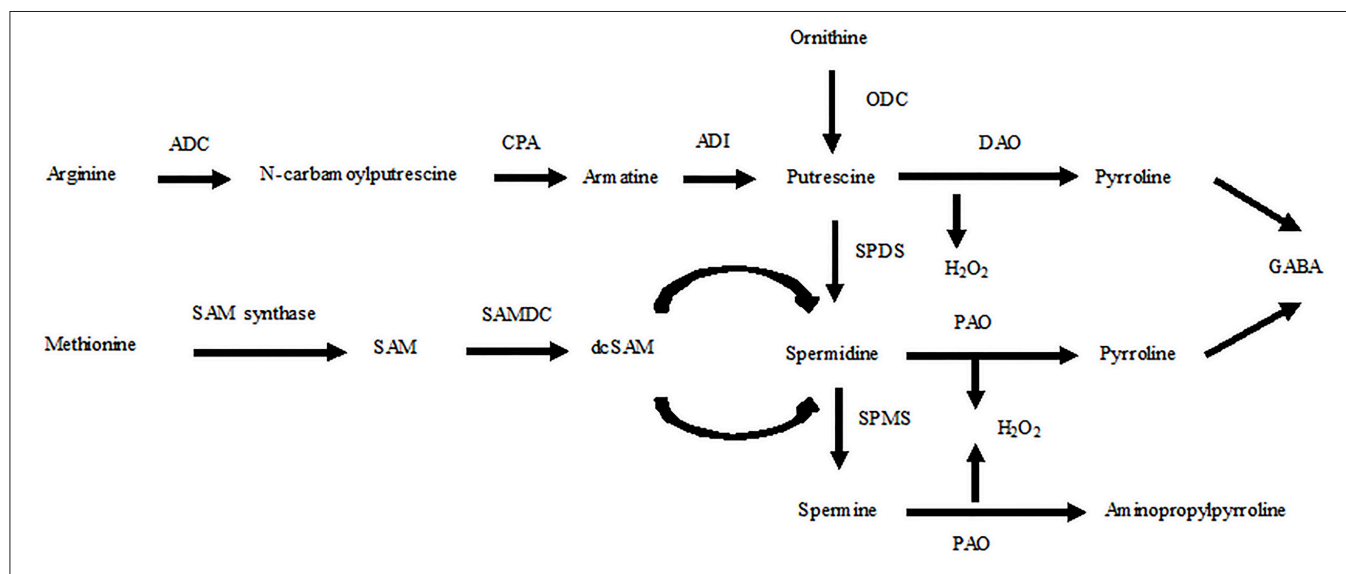


FIGURE 1 | Schematic presentation of the PA biosynthetic pathways for Put, Spd, and Spm in plants and relationships with ethylene biosynthesis. ADC, arginine decarboxylase; CPA, N-carbamoylputrescine amidohydrolase; ADI, agmatine deaminase; DAO, diamine oxidase; PAO, polyamine oxidase; SAMDC, S-adenosylmethionine decarboxylase; dc-SAM, decarboxylated S-adenosylmethionine; SPDS, spermidine synthase; SPMS, spermine synthase; GABA, c-aminobutyric acid.

whereas methylglyoxal-bis, an inhibitor of SAMDC, exacerbated the negative effects induced by CA (Huang and Bie, 2010). Exogenous application of Spd was also found to affect systemic glucosylsalicylic acid levels and ADC gene expression in tobacco leaves (Lazzarato et al., 2009). It has been shown that exogenous Spd alters the activities of polyamine degradation enzymes (PAO, DAO) in many species under salt stress (Hu et al., 2014).

Previous studies have indicated that exogenous Spd application reverses the increases in MDA content and electrolyte leakage caused by chilling. Moreover, high endogenous free PA contents have been observed (Zhang et al., 2009). Shoeb et al. (2001) reported that modulation of cellular PA levels and the Put:Spd ratio by exogenous PA (Put, Spd) application or treatment with difluoromethylarginine, a PA biosynthesis inhibitor, led to plant regeneration in poorly responding genotypes. Li et al. (2015) also reported that exogenous Spd confers short-term salinity tolerance in creeping bentgrass, likely by inducing antioxidant enzymes and affecting the activities of enzymes involved in PA metabolism. Some authors have observed that exogenous application of 0.1 mM Spd affects PA metabolism (Duan et al., 2008), and others have observed that 0.20 or 0.25 mM exogenous Spd has similar effects on PA metabolism in plants subjected to salt stress (Li et al., 2015). Such differences are related to the concentration of exogenous Spd applied and the cultivars examined (Zrig et al., 2011).

The objective of this study was to understand the effect of exogenous Spd concentrations on PA metabolism and the physiological and biochemical responses involved in salinity tolerance, especially their correlations, in a salinity-sensitive cultivar (cv. Z081) and a salinity-tolerant cultivar (cv. Z057). Parameters such as the contents of PAs (Put, Spd, and Spm) and activities of PA biosynthetic enzymes (ADC, ODC, SAMDC, PAO, and DAO) and antioxidant enzymes (SOD, POD, and CAT), as well as the degree of lipid peroxidation, were measured. We hypothesized that different concentrations of exogenous Spd would contribute to salt tolerance in both cultivars. Our second hypothesis was that correlations between Spd + Spm contents and H₂O₂ and MDA accumulation exist with changes in the concentration of exogenous Spd. Our third hypothesis was that correlations also exist between PA metabolism, PA biosynthetic enzymes and antioxidant enzymes with increases in exogenous Spd in the two cultivars analyzed.

MATERIALS AND METHODS

Chemicals

NaOH (AR, > 96%), HCl (AR, 36%), K₂HPO₄ (AR, ≥ 99.0%), KH₂PO₄ (AR, ≥ 99.5%), NaCl (AR, ≥ 99.5%), benzoyl chloride (AR, 99%), perchloric acid (AR, 99%), 1, 6-hexanediamine (AR, ≥ 99.8%), pyridoxal phosphate (AR, 98%), EDTA (AR, ≥ 99.5%), phenylmethylsulfonyl fluoride (GC, > 98%), ascorbic acid (AR, ≥ 99.7%), polyvinylpyrrolidone (AR, > 95%), L-ornithine (BR, 99%), L-arginine (BR, ≥ 99.0%), S-adenosyl methionine (BR, ≥ 98.0%), 4-aminoantipyrine/N,N-dimethylaniline (AR, 99%), horseradish peroxidase (250 units ml⁻¹), acetone (AR, 99.9%), H₂SO₄ (AR, 98%), thiobarbituric acid (AR, ≥ 99%), trichloroacetic acid (AR, ≥ 99%), nitroblue

tetrazolium (AR, 98%), methionine (BR, 99.0%), riboflavin (BR, 99%), guaiacol (GC, > 99%), spermidine (GC, > 99%), putrescine (CP, 99%), spermine (AR, ≥ 97%).

Plant Material and Treatments

Two zoysiagrass (*Zoysia japonica* Steud) cultivars, “Z081” and “Z057,” were used in this study (Table 1). Z081 is sensitive to saline conditions, whereas Z057 is tolerant to saline conditions (Li et al., 2012). The plants were cultivated in tanks containing 1/2 Hoagland solution (pH 6.6 ± 0.1, EC 1.8–2.0 dsm⁻¹) in the greenhouse of China Agricultural University throughout the year and mowed once a week to maintain a height of 10 cm. Zoysiagrass plants of consistent size were transplanted into tanks containing 20 l of 1/2 Hoagland solution (pH 6.6 ± 0.1, EC 1.8–2.0 dsm⁻¹). The roots were clipped to a length of 5 cm before initiating the six treatments: (1) control—1/2 Hoagland solution alone; (2) salt stress control—1/2 Hoagland solution + 200 mM NaCl; (3) 1/2 Hoagland solution + 200 mM NaCl + 0.15 mM Spd; (4) 1/2 Hoagland solution + 200 mM NaCl + 0.30 mM Spd; (5) 1/2 Hoagland solution + 200 mM NaCl + 0.45 mM Spd; (6) 1/2 Hoagland solution + 200 mM NaCl + 0.60 mM Spd. The NaCl concentration was gradually increased in 50 mM increments every day to avoid salinity shock. Spd (Sigma Chemical Co., St. Louis, MO, USA) was added after the salt level reached 200 mM. The solutions were constantly aerated using pumps. The day and night air temperatures were 25–28°C and 17–20°C, respectively, and the relative humidity of the greenhouse was 60–70% (China Agricultural University, Haidian, Beijing, China).

Root samples were collected in triplicate 8 days after initiation of the salinity treatments.

Determination of Root Growth

Root length was measured using a ruler, and dry weight was assessed by weighing roots after drying at 75°C in an oven for 72 h. The relative water content (RWC) was calculated according to the following formula:

$$\text{RWC} = (\text{fresh weight} - \text{dry weight}) / (\text{saturation weight} - \text{dry weight})$$

Polyamine Analysis

Standard Sample and Standard Curve

First, 2 ml 2 N NaOH and 15 μl benzoyl chloride were added to 100 μl 1 mM Put, Spd, and Spm standards. The samples were then vortexed vigorously and incubated for 30 min at 37°C. Next,

TABLE 1 | *Zoysia japonica* cultivars used in the study, the growth conditions and the source of the plants.

Cultivar	Salinity tolerance	Species	Source sponsor	Source location
Z081	150 mM	<i>Z. japonica</i>	Qingdao, Shandong	36°05'N, 120°20'E
Z057	340 mM	<i>Z. japonica</i>	HuaguoShan, Lianyungang	34°36'N, 119°12'E

a 4 ml saturated NaCl solution was added; 1.5 ml of the ether phase was dried and redissolved in 1 ml methanol.

Put, Spd, and Spm standards were prepared in 1 mM benzoylated solution, and a standard curve was generated using standards of different densities (0.03, 0.06, 0.12, 0.15, 0.25, 0.50, 0.75, 1.0 nmol).

Polyamine Analysis

PAs were extracted according to the methods of Sharma and Rajam (1995), with some modifications. Fresh root samples (0.3 g) were homogenized in cold perchloric acid (PCA, 4 ml, 5% v/v), followed by incubation at 4°C for 1 h. Next, 1, 6-hexanediamine was added to the homogenate as an internal standard, and the mixture was centrifuged at $12,000 \times g$ for 30 min at 4°C. The supernatant was subsequently used for determination of free and soluble conjugated PAs, and the pellet was used for determination of insoluble bound PAs. To obtain soluble conjugated PAs, 1 ml PCA extract was blended with 5 ml 6 N HCl and hydrolyzed at 110°C for 18 h in flame-sealed glass ampules. The HCl was evaporated by heating at 70°C, and the residue was suspended in 2 ml 5% PCA after acid hydrolysis, followed by centrifugation at $12,000 \times g$ for 30 min at 4°C. The acid-soluble polyamine solution contained free PAs and conjugates liberated from PAs. To obtain insoluble bound PAs, the pellet was rinsed four times with 5% PCA to remove any trace of soluble PAs and resuspended in 5 ml 6 N HCl. This solution was hydrolyzed using the same procedure as described above.

PAs were recovered from the pellet, and the hydrolyzed supernatant and non-hydrolyzed supernatant were benzoylated as follows. An aliquot of the supernatant containing 2 ml 2 N NaOH and 15 μ l benzoyl chloride was vortexed vigorously and then incubated for 30 min at 37°C. Next, 4 ml saturated NaCl solution was added, and 1.5 ml of the ether phase was dried and redissolved in 1 ml methanol (60% w/v). The solution was stored at -20°C under air-tight conditions.

PAs were assayed via high-performance liquid chromatography (HPLC). A 10 μ l aliquot of a methanol solution of benzoyl polyamines was injected into a 20 ml loop and loaded onto a 5 μ m particle size C18 reverse-phase, 4.6 \times 250 mm column (Eka Chemicals, Bohus, Sweden). The temperature of the column was maintained at 25°C. The samples were eluted with 64% methanol at a flow rate of 0.8 ml min⁻¹ that was maintained by a Dionex P680 Pump. The PA peaks were detected with a UV detector at 254 nm. The concentrations of soluble conjugated forms were calculated by subtracting the free PA concentration from acid-soluble PA concentration.

Analysis of Polyamine Biosynthetic Enzyme Activity

Fresh root samples (0.3 g) were homogenized in 100 mM potassium phosphate buffer (pH 8.0) containing 0.1 mM phenylmethylsulfonyl fluoride, 1 mM pyridoxal phosphate (PLP), 5 mM EDTA, 25 mM ascorbic acid, and 0.1% polyvinylpyrrolidone. The solution was then centrifuged at $12,000 \times g$ for 40 min at 4°C. The supernatant was dialyzed at

4°C against 3 ml 100 mM potassium phosphate buffer (pH 8.0) containing 0.05 mM PLP, 0.1 mM DTT, and 0.1 mM EDTA in darkness for 24 h. The dialyzed extract was used for enzyme assays.

Enzyme activity was determined according to a previously described procedure (Matsuda, 1984), with some modifications. The activities of ODC, ADC, and SAMDC were measured using reaction mixtures prepared with 0.3 ml of the dialyzed enzyme extract and 100 mM Tris-HCl buffer (pH 8.0), 50 μ M pyridoxal phosphate, 5 mM EDTA, and 5 mM DTT. The reactions were incubated at 37°C for 2 min, and 0.2 ml 25 mM L-ornithine, 0.2 ml 25 mM L-arginine (pH 7.5) or 0.2 ml 25 mM SAM was then added. The reaction mixtures were incubated at 37°C for 30 min, and PCA was added to a final concentration of 5%. The reaction mixtures were centrifuged at $3000 \times g$ for 10 min, and the supernatants (0.5 ml) were mixed with 1 ml 2 mM NaOH and 10 μ l benzoyl chloride. The mixture was stirred for 20 s, and 2 ml NaCl solution and 3 ml ether were added and incubated at 37°C for 30 min while stirring thoroughly. The reaction was then centrifuged at $1500 \times g$ for 5 min and extracted with 3.0 ml ether; the ether phase (1.5 ml) was evaporated to dryness and redissolved in 3 ml 60% methyl alcohol. Finally, the solution was subjected to UV light at a wavelength of 254 nm.

Assay for Diamine and Polyamine Oxidase Activities

The activities of DAO and PAO were determined by measuring the generation of H₂O₂, a product of PA oxidation, as described previously (Su et al., 2005), with some modifications. Fresh samples were homogenized in 100 mM potassium phosphate buffer (pH 6.5), and the homogenate was centrifuged at $10,000 \times g$ for 20 min at 4°C. The supernatant was used for the enzyme assay. The reaction mixtures contained 25 ml potassium phosphate buffer (100 mM, pH 6.5), 0.2 ml 4-aminoantipyrine/N,N-dimethylaniline reaction solution, 0.1 ml horseradish peroxidase (250 units ml⁻¹), and 0.2 ml of the enzyme extract. The reactions were initiated by adding 15 μ l 20 mM Put to determine DAO activity or 20 mM Spd + Spm to determine PAO activity. One unit of enzyme activity was defined as a change in absorbance of 0.001 units.

Evaluation of Free Radical Production

Fresh root samples (0.3 g) were homogenized in cold acetone (5 ml) and centrifuged at $10,000 \times g$ for 15 min at 4°C. The supernatant was added to concentrated hydrochloric acid solution containing 20% TiCl₄ (0.1 ml) and concentrated ammonia (0.2 ml). The reaction mixture was centrifuged for 10 min at $8000 \times g$ and 4°C after a 5 min reaction at 25°C. After washing twice with cold acetone, 3 ml 1 M H₂SO₄ was added to the pellet. We measured absorption at 410 nm, and the H₂O₂ concentration was calculated using a standard curve.

Lipid Peroxidation Assay

The malondialdehyde (MDA) content was determined using the thiobarbituric acid (TBA) method (Dhindsa et al., 1981). Fresh root samples (0.3 g) were homogenized in 5 ml of 5% trichloroacetic acid (TCA) centrifuged at $15,000 \times g$ for 20 min.

A 0.5 ml aliquot of the supernatant was added to 1 ml 20% (w/v) TCA containing 0.5% (w/v) TBA. The reaction was placed in boiling water for 30 min, quickly cooled and centrifuged at $10,000 \times g$ for 10 min. Absorbance measurements were obtained at 532 and 600 nm.

Antioxidant Enzyme Activities

Fresh root samples (0.1 g) were homogenized with 1 ml ice-cold phosphate buffer (50 mM, pH 7.8) containing 1 mM EDTA and 4% PVP. The homogenate was centrifuged at 10,000 rpm at 4°C for 15 min, and the supernatant was used for determining the activities of POD, SOD, and CAT at 4°C.

Superoxide dismutase (SOD, EC1.15.1.1) activity was determined by observing the inhibition of NBT reduction. The 3 ml reaction mixture contained phosphate buffer (50 mM, pH 7.8), EDTA (0.1 mM), methionine (130 mM), NBT (0.75 mM), riboflavin (0.02 mM), and enzyme extract (0.1 mM), with the riboflavin added last. The reaction mixture was illuminated for 15 min. The calibration standards consisted of non-illuminated and illuminated reactions without supernatant. One unit of activity was defined as the amount of enzyme causing 50% inhibition of the reduction of nitroblue tetrazolium chloride, as assessed at 560 nm.

Peroxidase (POD, EC 1.11.1.7) activity was determined using guaiacol. The reaction mixture contained guaiacol solution (0.02 ml), hydrogen peroxide solution (0.01 ml), phosphate buffer (3 ml, pH 7.0), and enzyme extract (0.02 ml). The reaction was initiated by addition of the enzyme extract. One unit of activity was equal to an increase in 1 absorbance unit per minute at 470 nm.

Catalase (CAT, EC 1.11.1.6) activity was determined by monitoring the initial H_2O_2 disappearance rate. Phosphate buffer (50 mM, pH 7.0), H_2O_2 (20 mM), and enzyme extract (0.1 ml) were added to the reaction solution (3 ml). The reaction was initiated by addition of the enzyme extract. The reduction of H_2O_2 was observed for at least 3 min at 240 nm.

Statistical Analysis

Root growth measurements were replicated 40 times. Each experimental treatment was completely random and was designed to be replicated at least three times. The results are expressed as the mean \pm standard errors (SE). One-way analysis of variance (ANOVA) with an LSD test was used to determine the significance of the observed differences between treatments.

RESULTS

Plant Growth

Biometric analysis indicated that 8 days of salt treatment significantly reduced root growth in both cultivars ($p < 0.05$). Under salt stress, the fresh root weight, root length, and relative root water content increased initially but later declined in both cultivars as the Spd concentration increased (Table 2). To some extent, exogenous Spd alleviated the salinity-induced reduction in growth, with a greater effect in cv. Z081 than in cv. Z057. For example, compared with untreated plants grown under salt stress, 0.3 mM Spd enhanced the fresh root weight, root length, and relative root water content of cv. Z081 and cv. Z057 by 26, 13, and 16% and 18.7, 12.6, and 13.2%, respectively. However, a high concentration of Spd repressed the growth of cv. Z081 under salt stress.

Polyamine Levels

The biosynthetic pathways of the major PAs Put, Spd, and Spm are shown in Figure 2. These three main PAs differ in the positive changes observed in the physiology of treated cells. In previous reports, Spd and Spm levels and the (Spd + Spm)/Put ratio increased with salinity in all species showing increased salinity tolerance (Figure 2, Table 3). In the present study, we measured PA (Put, Spd, and Spm) levels (Figure 2) and the ratio of (Spd + Spm)/Put in both cultivars (Table 3). Salt stress increased the total PA (Put, Spd, and Spm) content in both cultivars (Figure 2); however, little change in the Spd and Spm contents in the roots

TABLE 2 | Effects of the addition of different concentrations of exogenous spermidine on the growth and water content of zoysiagrass roots exposed to 200 mM NaCl for 8 days.

Cultivar	Treatment	Root fresh weight (g /cm ²)	Root length (cm)	Root relative water content (%)
Z081	Control	0.395 \pm 0.03a	6.67 \pm 0.06a	93.1 \pm 3.7a
	NaCl	0.271 \pm 0.04d	5.49 \pm 0.08e	74.2 \pm 1.4d
	NaCl + 0.15 mM Spd	0.339 \pm 0.02b	6.21 \pm 0.07b	86.4 \pm 2.3b
	NaCl + 0.3 mM Spd	0.341 \pm 0.04b	6.19 \pm 0.08b	86.2 \pm 2.4b
	NaCl + 0.45 mM Spd	0.326 \pm 0.01c	5.94 \pm 0.10c	81.3 \pm 1.2c
	NaCl + 0.6 mM Spd	0.241 \pm 0.04e	5.27 \pm 0.09e	71.5 \pm 2.7e
Z057	Control	0.423 \pm 0.04a	6.99 \pm 0.04a	93.9 \pm 2.9a
	NaCl	0.331 \pm 0.03d	5.97 \pm 0.06e	78.3 \pm 2.2d
	NaCl + 0.15 mM Spd	0.357 \pm 0.02c	6.51 \pm 0.06c	88.5 \pm 1.9b
	NaCl + 0.3 mM Spd	0.383 \pm 0.02b	6.69 \pm 0.02b	88.6 \pm 2.1b
	NaCl + 0.45 mM Spd	0.361 \pm 0.02c	6.54 \pm 0.09c	84.2 \pm 1.5c
	NaCl + 0.6 mM Spd	0.332 \pm 0.03d	6.27 \pm 0.08d	79.0 \pm 2.4d

Data represent the mean \pm SE of three independent experiments. Values in the table sharing the same letters are not significantly different ($p < 0.05$; Duncan's multiple range test).

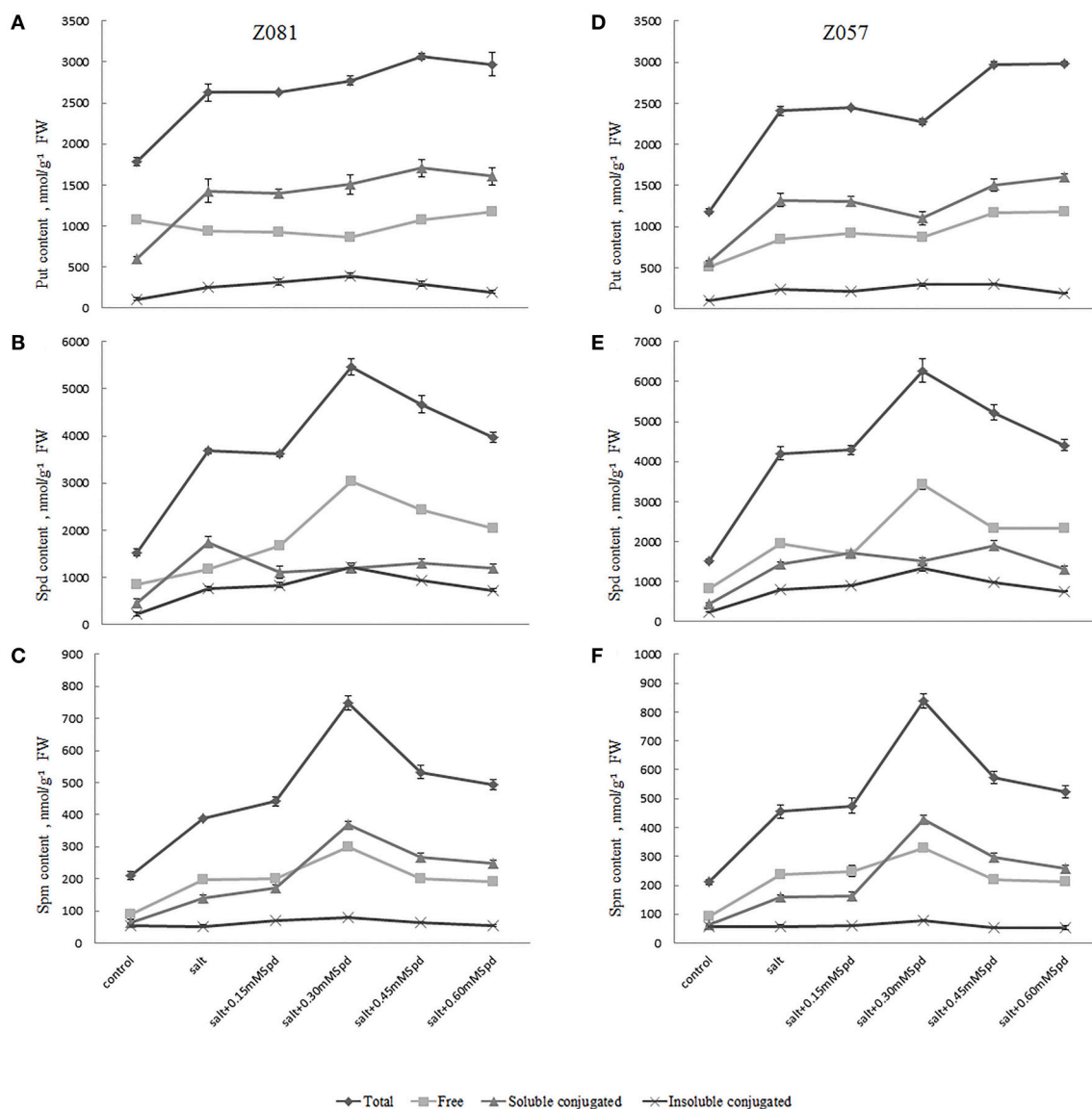


FIGURE 2 | Effects of exogenous spermidine (0, 0.15, 0.30, 0.45, 0.60 mM) on PA contents in the roots of cv. Z081 (A–C) and cv. Z057 (D–F) grown under 200 mM NaCl stress. (A,D) Put (total, free, soluble conjugated, insoluble conjugated) content; **(B,E)** Spd (total, free, soluble conjugated, insoluble conjugated) content; **(C,F)** Spm (total, free, soluble conjugated, insoluble conjugated) content. Data represent the means \pm SE of three replicates. Values in a single column sharing the same letters are not significantly different ($p < 0.05$) (Duncan's multiple range test).

of cv. Z081 was observed under salinity stress. In general, the PA contents of both cultivars first increased and then declined with increasing concentrations of exogenous Spd, whereas the contents, except for Put, increased dramatically and then peaked under 0.3 mM Spd treatment (Figure 2).

Salt stress also caused an increase in the three forms (free form, soluble conjugated form, and insoluble bound form) of Put, Spd, and Spm. The levels of Put (insoluble bound), Spd (free, insoluble bound), and Spm (free, soluble conjugated, insoluble bound) initially increased and then declined with increasing concentrations of exogenous Spd in cv. Z081 (Figures 2A–C). Similar results were observed in cv. Z057, except for free and

soluble conjugated Put (Figures 2D–F). Conversely, the levels of free Put first decreased and then rose slightly in the two cultivars with increasing exogenous Spd concentrations.

Additionally, the ratio of (Spd + Spm)/Put and the Spd + Spm contents, which are related to increased tolerance to salt stress, first increased and then decreased, peaking in both cultivars with 0.3 mM exogenous Spd (Table 3).

Polyamine Biosynthetic Enzyme Activities

The activities of several representative enzymes, including ODC and ADC (Figure 1), were measured in roots to determine the effects of exogenous Spd application on PA synthesis under salt

TABLE 3 | Changes in polyamine content in zoysiagrass under salt stress.

Endogenous polyamines content (nmol/g ⁻¹ fw)				
Cultivar	Treatment	Put	Spd + Spm	(spd + spm)/Put
Z081	Control	1781 ± 49d	1744 ± 83d	0.98 ± 0.03c
	NaCl	2626 ± 101c	4070 ± 60c	1.55 ± 0.06b
	NaCl + 0.15 mM Spd	2631 ± 17c	4066 ± 41c	1.55 ± 0.03b
	NaCl + 0.3 mM Spd	2772 ± 56bc	6220 ± 198a	2.24 ± 0.12a
	NaCl + 0.45 mM Spd	3072 ± 39a	5204 ± 201b	1.69 ± 0.09b
	NaCl + 0.6 mM Spd	2972 ± 142ab	4466 ± 164c	1.50 ± 0.02b
Z057	Control	1186 ± 30d	1715 ± 57d	1.45 ± 0.01c
	NaCl	2407 ± 51b	4664 ± 188c	1.94 ± 0.05b
	NaCl + 0.15 mM Spd	2444 ± 21b	4762 ± 146c	1.95 ± 0.08b
	NaCl + 0.3 mM Spd	2772 ± 36c	7112 ± 317a	3.1 ± 0.11a
	NaCl + 0.45 mM Spd	2976 ± 28a	5803 ± 205b	1.95 ± 0.08b
	NaCl + 0.6 mM Spd	2982 ± 21a	4933 ± 167c	1.65 ± 0.06c

The data represent the mean values ± standard error (SE) from at least three independent experiments. Means with different letters are significantly different at $p < 0.05$.

stress. As shown in **Figures 3A,D**, ODC activity in the roots of both cultivars was enhanced under salt stress. The exogenous Spd-induced increase in ODC activity was greater in cv. Z057 than in cv. Z081, and the enhancement first increased and then decreased with increasing concentration of exogenous Spd. However, compared with the control roots under salt stress, ODC activity was inhibited in cv. Z081 by treatment with 0.6 mM Spd. In contrast, ADC activity did not respond to salt stress, and exogenous Spd had no significant effect on ADC activity in either cultivar (**Figures 3B,E**).

The activity of SAMDC increased slightly when the roots were exposed to salt stress. As the concentration of exogenously added Spd increased, SAMDC activity was first augmented and then diminished. Spd treatment at 0.3 mM resulted in the greatest SAMDC activity in both cultivars, with a greater effect in cv. Z057 than in cv. Z081 (**Figures 3C,F**).

Polyamine-Degrading Enzyme Activities

To elucidate the polyamine metabolism underlying the salt tolerance induced by exogenous Spd, we measured two PA-degrading enzymes. The activity of DAO in roots increased rapidly in both cultivars under salinity stress (**Figures 4A,C**). The DAO activity in roots treated with exogenous Spd initially increased and then decreased in a dose-dependent manner in both cultivars. With increasing exogenous Spd, DAO activity showed a faster downward trend in Z081 than in Z057 (**Figures 4A,C**).

Salinity stress only induced a slight increase in PAO activity. Moreover, compared with the corresponding control, Spd application did not have a significant effect on root PAO activity in either cultivar (**Figures 4B,D**).

H₂O₂ Concentration and Lipid Peroxidation

Compared with the controls, salinity stress led to greater increases in H₂O₂ and MDA levels in cv. Z081 than in cv. Z057, and treatment with exogenous Spd reduced the levels of H₂O₂

and malondialdehyde (MDA) in both cultivars. However, this decrease was much smaller in cv. Z057 than in cv. Z081. The levels of H₂O₂ and MDA initially increased and then decreased, with the minimum values in both cultivars observed in the roots of plants treated with 0.3 mM Spd (**Figure 5**).

Antioxidant Enzyme Activities

The activities of several representative antioxidant enzymes, including SOD, POD, and CAT, were measured in zoysiagrass to determine the physiological effect of exogenous Spd on these antioxidant enzymes within the context of salt stress. During salinity treatment (200 mM NaCl), SOD activity was determined to be 54.1 U min⁻¹ g⁻¹ FW but was found to be as high as 89.2 U min⁻¹ g⁻¹ FW in cv. Z081 treated with 0.3 mM exogenous Spd (**Figure 6A**). Similar results were observed in cv. Z057, and the extent of increase in the roots due to Spd application was much greater in cv. Z057 than in cv. Z081 (**Figure 6D**). Regarding POD and CAT activities, treatment of salinity-stressed plants with Spd resulted in a tendency of increase followed by decrease in both cultivars. For example, treatment with 0.3 mM Spd increased the activities of POD and CAT by 30.1 and 26.8% and 43.4 and 34.7% in cv. Z081 and cv. Z057, respectively, compared with treated, salinity-stressed plants (**Figures 6B,C,E,F**).

Correlation Analysis

The correlation coefficients between exogenous Spd level indexes, as analyzed by Pearson's correlation, are listed in **Tables 4, 5**. The exogenous Spd indexes related to several parameters showed significant correlations. In both cultivars, the Spd + Spm contents displayed positive correlations with PA biosynthetic enzymes and antioxidant enzymes, though the Spd + Spm contents showed a negative correlation with MDA and H₂O₂ levels (**Tables 4, 5**). DAO activity also showed a positive correlation with Spd + Spm contents in both cultivars (**Tables 4, 5**).

DISCUSSION

In plants, salt stress causes reductions in fresh root weight, root length and relative root water contents, with severe damage to the organism, and in our study, such reductions were greater in cv. Z081 than in cv. Z057, indicating the salt tolerance of the latter (**Table 2**).

Exogenous Spd has been shown to act as a stimulant in a variety of organisms, and recent work indicates that exogenous Spd treatment enhances salt tolerance in plants (Liu et al., 2004). The plant PA metabolism response to salt stress varies with different exogenous Spd concentrations, plant species, and interactions among other stress factors (Gill and Tuteja, 2010). Indeed, our results showed different PA responses to salt stress in cv. Z081 and cv. Z057 in relation to different concentrations of exogenous Spd.

PA levels changed under salt stress, with Put decreasing and Spd and/or Spm increasing in most cases. It has been reported that with an increase in the (Spd + Spm)/Put ratio, salinity tolerance increased in all species examined (Zapata et al., 2004) and that Spd and Spm facilitate the osmotic stress tolerance of wheat seedlings (Liu et al., 2004). We investigated PA metabolism

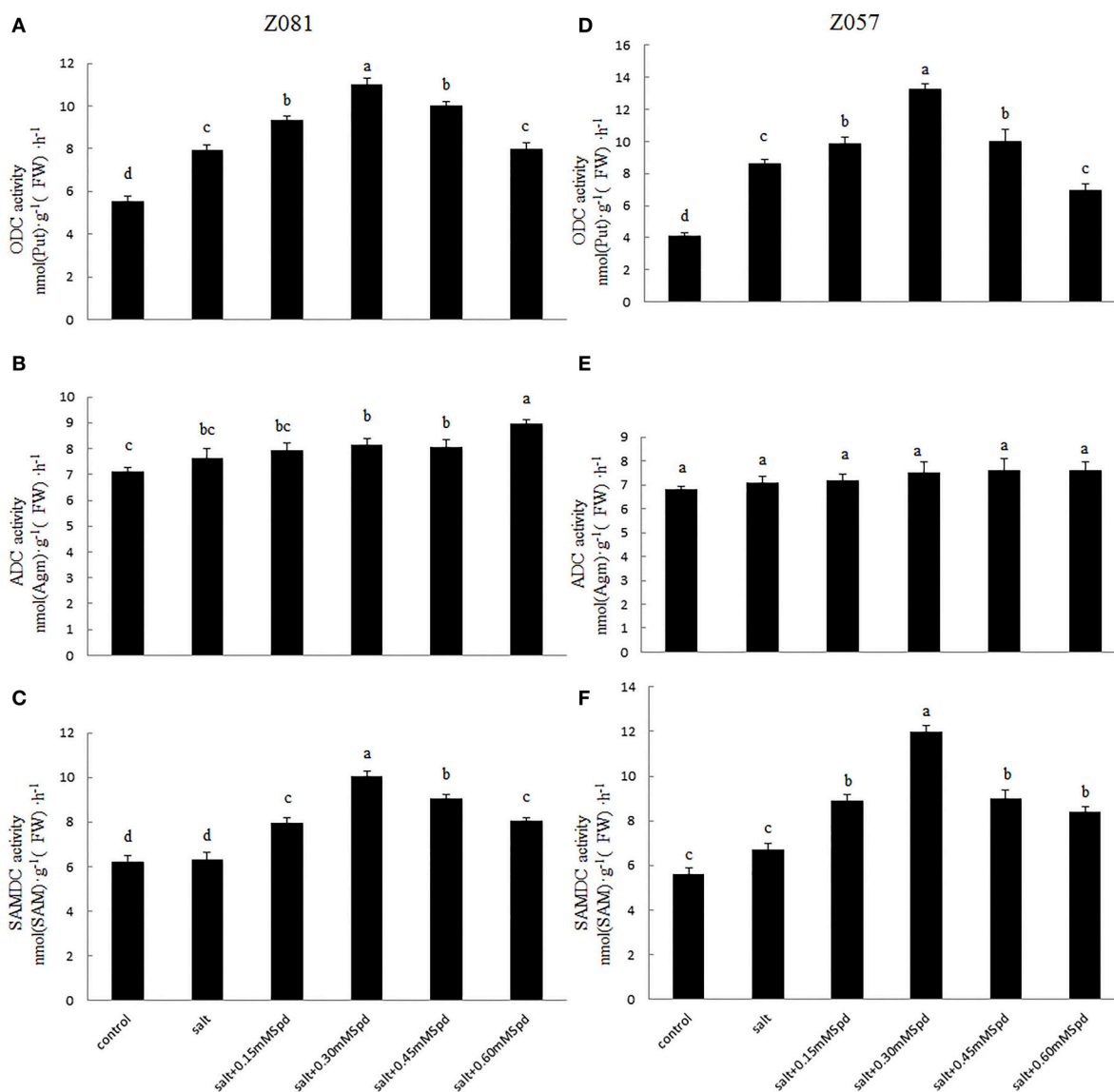


FIGURE 3 | Effects of exogenous spermidine (0, 0.15, 0.30, 0.45, 0.60 mM) on activities of ODC, ADC, and SAMDC in the roots of cv. Z081 (A–C) and cv. Z057 (D–F) grown under 200 mM NaCl stress. (A,D) ODC activity; (B,E) ADC activity; (C,F) SAMDC activity. Data represent the means \pm SE of three replicates. Values in a single column sharing the same letters are not significantly different ($p < 0.05$) (Duncan's multiple range test).

and the physiological responses of two cultivars of salt-stressed *Z. japonica* treated with different concentrations of exogenous Spd. Both cultivars first showed an upward trend followed by a downward trend in total Spd and Spm contents under salt stress and the significant changes in the three forms of PAs under different treatment with concentrations of exogenous Spd suggest an efficient PA adaptive mechanism (Figure 2). Exogenous Spd inhibited accumulation of free Put and promoted accumulation of free Spd and Spm as well as both soluble conjugated and insoluble bound Put, Spd, and Spm (Figure 2). The importance of soluble conjugated and insoluble bound PAs has also been illustrated in previous work, and overexpression of the SAMDC gene in tobacco significantly increased the concentrations of soluble conjugated PAs (Jia et al., 2010).

Expression of several genes encoding enzymes of PA metabolism, such as ADC, ODC, or SAMDC, were found to improve environmental stress tolerance in most plant species, revealing a useful tool for gaining new insight into the regulation of PA metabolism (Bagni and Tassoni, 2001; Liu et al., 2007). Synthesis of the diamine Put proceeds through either ADC via agmatine (Agm) or ODC, whereas the triamine Spd is synthesized by SPDS via addition to Put of an aminopropyl moiety donated by decarboxylated S-adenosylmethionine (dcSAM) formed by SAMDC (Franceschetti et al., 2004). The results of the present study show a significant positive correlation between the Spd + Spm content and the activities of ADC and SAMDC with exogenous Spd application in both cultivars, proving that such variation in exogenous Spd

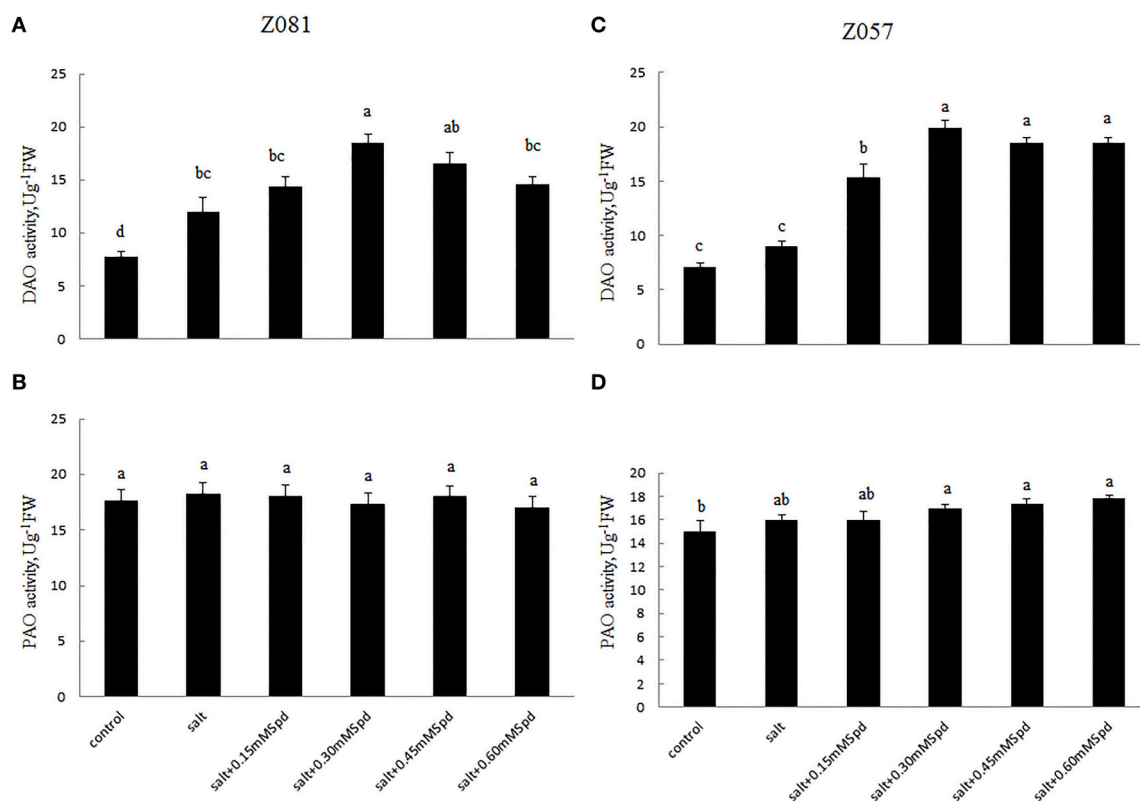


FIGURE 4 | Effects of exogenous spermidine (0, 0.15, 0.30, 0.45, 0.60 mM) on the activities of DAO and PAO in the roots of cv. Z081 (A,B) and cv. Z057 (C,D) grown under 200 mM NaCl stress. (A,C) DAO activity; (B,D) PAO activity. Data represent the means \pm SE of three replicates. Values in a single column sharing the same letters are not significantly different ($p < 0.05$) (Duncan's multiple range test).

TABLE 4 | Pearson's correlation coefficients among PA contents and physiological and biochemical parameters in cv. Z081 exposed to salt stress and treated with exogenous Spd (0, 0.15, 0.30, 0.45, 0.60 mM).

	Put	Spd + Spm	ADC	ODC	SAMDC	PAO	DAO	H ₂ O ₂	MDA	SOD	POD	CAT
Put	—	0.299	0.390	0.143	0.377	−0.089	0.316	−0.472	−0.235	0.197	0.406	0.511
Spd + Spm	0.299	—	0.233	0.851**	0.888**	0.076	0.875**	−0.573*	−0.493	0.935**	0.569*	0.746**
ADC	0.390	0.233	—	0.051	0.396	0.336	0.511	−0.137	−0.005	0.392	0.109	0.161
ODC	0.143	0.851**	0.051	—	0.891**	0.224	0.874**	−0.572*	−0.554*	0.873**	0.441	0.679**
SAMDC	0.377	0.888**	0.396	0.891**	—	0.067	0.947**	−0.762**	−0.678**	0.946**	0.666**	0.852**
PAO	−0.089	0.076	0.336	0.224	0.067	—	0.345	0.490	0.552*	0.151	−0.600*	−0.407
DAO	0.316	0.875**	0.511	0.874**	0.947**	0.345	—	−0.532*	−0.432	0.953**	0.435	0.662**
H ₂ O ₂	−0.472	−0.573*	−0.137	−0.572*	−0.762**	0.490	−0.532*	—	0.930**	−0.574*	−0.932**	−0.944**
MDA	−0.235	−0.493	−0.005	−0.554*	−0.678**	0.552*	−0.432	0.930**	—	−0.517*	−0.826**	−0.830**
SOD	0.197	0.935**	0.392	0.873**	0.946**	0.151	0.953**	−0.574*	−0.517*	—	0.530*	0.725**
POD	0.406	0.569*	0.109	0.441	0.666**	−0.600*	0.435	−0.932**	−0.826**	0.530*	—	0.921**
CAT	0.511	0.746**	0.161	0.679**	0.852**	−0.407	0.662**	−0.944**	−0.830**	0.725**	0.921**	—

Each square indicates the Pearson's correlation coefficient of a pair of parameters. Put, diamine putrescine ($\text{nmol/g}^{-1}\text{FW}$); Spd + Spm, triamine spermidine + tetraamine spermine ($\text{nmol/g}^{-1}\text{FW}$); ADC, arginine decarboxylase ($\text{nmol(Agm)}^{-1}\text{g}^{-1}\text{FW}\cdot\text{h}^{-1}$); ODC, ornithine decarboxylase ($\text{nmol(Put)}^{-1}\text{g}^{-1}\text{FW}\cdot\text{h}^{-1}$); SAMDC, S-adenosylmethionine decarboxylase ($\text{nmol(SAM)}^{-1}\text{g}^{-1}\text{FW}\cdot\text{h}^{-1}$); PAO, polyamine oxidase ($\text{U g}^{-1}\text{FW}$); DAO, diamine oxidase ($\text{U g}^{-1}\text{FW}$); H₂O₂, hydrogen peroxide ($\text{nmol g}^{-1}\text{FW}$); MDA, malondialdehyde ($\text{nmol g}^{-1}\text{FW}$); SOD, superoxide dismutase ($\text{U min}^{-1}\text{g}^{-1}\text{FW}$); POD, peroxidase ($\text{U min}^{-1}\text{g}^{-1}\text{FW}$); CAT, catalase ($\text{U min}^{-1}\text{g}^{-1}\text{FW}$). Correlations are significant at * $p < 0.05$; ** $p < 0.01$.

concentration affected PA metabolism by altering the activity of ADC and SAMDC (Figure 3, Tables 3, 4). Previous studies have reported that exogenous Put is quickly absorbed and

converted to Spd and Spm and that synthetic PAs accumulate in stems and roots and alter endogenous PA contents (Ohe et al., 2005).

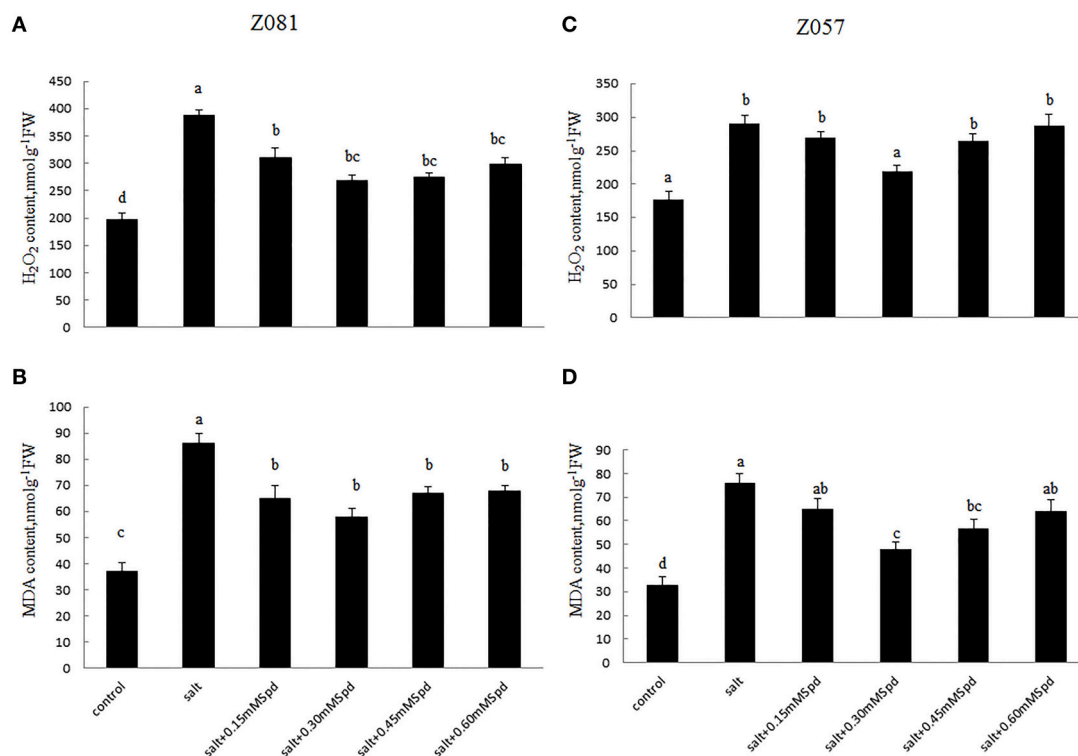


FIGURE 5 | Effects of exogenous spermidine (0, 0.15, 0.30, 0.45, 0.60 mM) on the levels of H₂O₂ and MDA in the roots of cv. Z081 (A,B) and cv. Z057 (C,D) grown under 200 mM NaCl stress. (A,C) H₂O₂ content; (B,D) MDA content. Data represent the means \pm SE of three replicates. Values in a single column sharing the same letters are not significantly different ($p < 0.05$) (Duncan's multiple range test).

TABLE 5 | Pearson's correlation coefficients among PA contents and physiological and biochemical parameters in cv. Z057 exposed to salt stress and treated with exogenous Spd (0, 0.15, 0.30, 0.45, 0.60 mM).

	Put	Spd + Spm	ADC	ODC	SAMDC	PAO	DAO	H ₂ O ₂	MDA	SOD	POD	CAT
Put	—	-0.268	0.163	-0.586*	-0.303	0.475	0.308	0.422	0.043	-0.277	-0.104	0.173
Spd + Spm	-0.268	—	0.221	0.888**	0.903**	0.340	0.669**	-0.894**	-0.819**	0.956**	0.928**	0.839**
ADC	0.163	0.221	—	0.129	0.172	0.381	0.311	-0.071	-0.263	0.382	0.387	0.399
ODC	-0.586*	0.888**	0.129	—	0.886**	0.035	0.474	-0.900**	-0.726**	0.876**	0.761**	0.625*
SAMDC	-0.303	0.903**	0.172	0.886**	—	0.287	0.790**	-0.886**	-0.871**	0.922**	0.884**	0.849**
PAO	0.475	0.340	0.381	0.035	0.287	—	0.658**	-0.218	-0.405	0.290	0.448	0.595*
DAO	0.308	0.669**	0.311	0.474	0.790**	0.658**	—	-0.599*	-0.801**	0.700**	0.769**	0.925**
H ₂ O ₂	0.422	-0.894**	-0.071	-0.900**	-0.886**	-0.218	-0.599*	—	0.818**	-0.815**	-0.814**	-0.682**
MDA	0.043	-0.819**	-0.263	-0.726**	-0.871**	-0.405	-0.801**	0.818**	—	-0.798**	-0.848**	-0.823**
SOD	-0.277	0.956**	0.382	0.876**	0.922**	0.290	0.700**	-0.815**	-0.798**	—	0.919**	0.860**
POD	-0.104	0.928**	0.387	0.761**	0.884**	0.448	0.769**	-0.814**	-0.848**	0.919**	—	0.885**
CAT	0.173	0.839**	0.399	0.625*	0.849**	0.595*	0.925**	-0.682**	-0.823**	0.860*	0.885**	—

Each square indicates the Pearson's correlation coefficient of a pair of parameters. Put, diamine putrescine (nmol g⁻¹ FW); Spd + Spm, triamine spermidine + tetraamine spermine (nmol g⁻¹ FW); ADC, arginine decarboxylase (nmol(Agm)-g⁻¹(FW)-h⁻¹); ODC, ornithine decarboxylase (nmol(Put)-g⁻¹(FW)-h⁻¹); SAMDC, S-adenosylmethionine decarboxylase (nmol(SAM)-g⁻¹(FW)-h⁻¹); PAO, polyamine oxidase (U g⁻¹ FW); DAO, diamine oxidase (U g⁻¹ FW); H₂O₂, hydrogen peroxide (nmol g⁻¹ FW); MDA, malondialdehyde (nmol g⁻¹ FW); SOD, superoxide dismutase (U min⁻¹ g⁻¹ FW); POD, peroxidase (U min⁻¹ g⁻¹ FW); CAT, catalase (U min⁻¹ g⁻¹ FW). Correlations are significant at * $p < 0.05$; ** $p < 0.01$.

PAs are catabolized into ammonia and H₂O₂ by DAO and PAO (Figure 1). These enzymes are localized in the plant cell wall, and hydrogen peroxide resulting from Put catabolism may be important in cross-linking reactions under both normal and

stress conditions (Eller et al., 2006). In both cultivars, we observed a possible connection between the PA content and degradation enzyme with different exogenous Spd concentrations. Our results showed a positive correlation between increases in PA contents

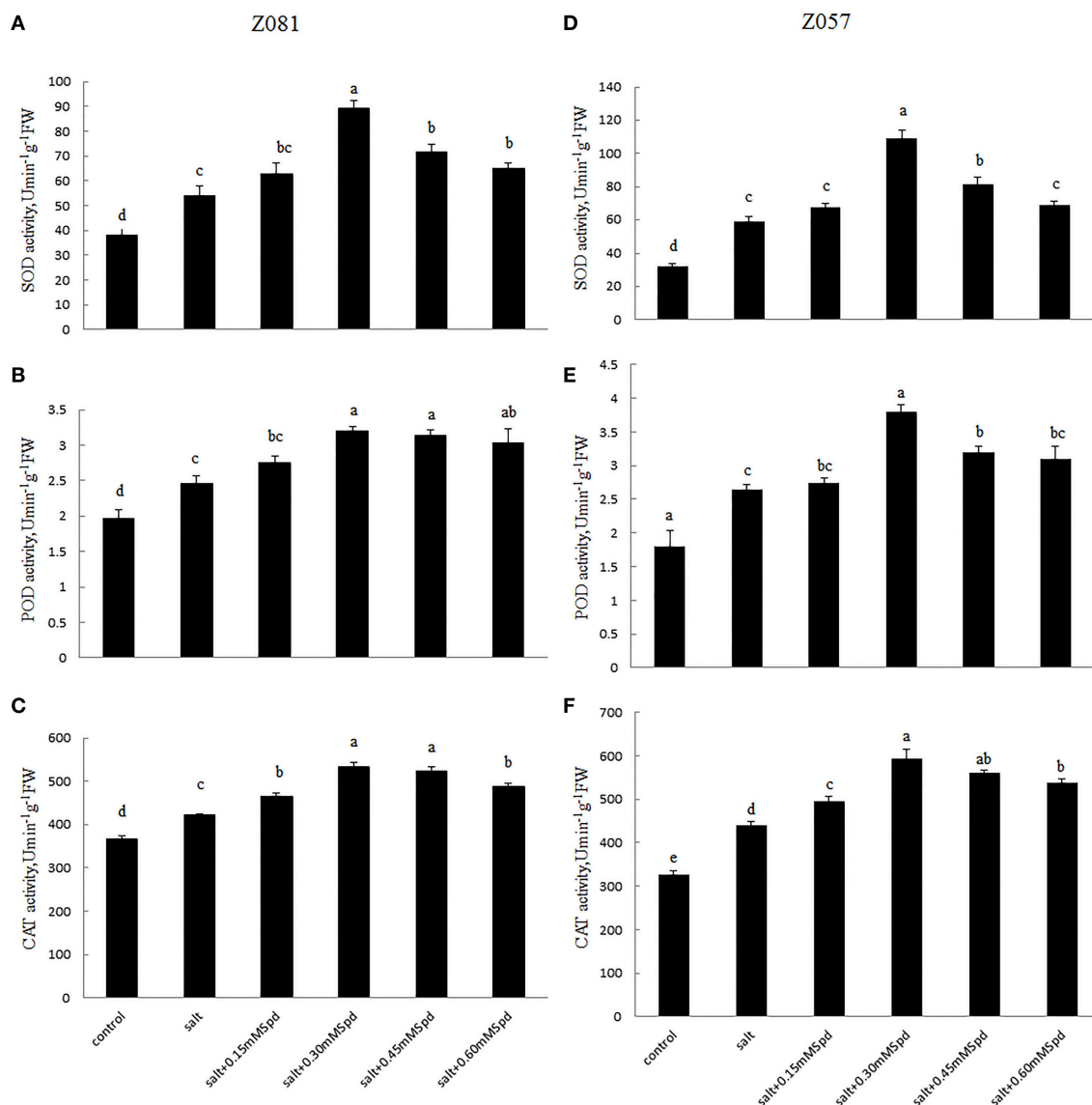


FIGURE 6 | Effects of exogenous spermidine (0, 0.15, 0.30, 0.45, 0.60 mM) on the activities of SOD, POD, and MDA in the roots of cv. Z081 (A–C) and cv. Z057 (D–F) grown under 200 mM NaCl stress. (A,D) SOD activity; (B,E) POD activity; (C,F) CAT activity. Data represent the means \pm SE of three replicates. Values in a single column sharing the same letters are not significantly different ($p < 0.05$) (Duncan's multiple range test).

and DAO in both cultivars, which indicated that the increase in PA contents was due to the activity of DAO rather than PAO (Figure 4).

Salt stress leads to the generation of reactive oxygen species, such as H_2O_2 , which cause lipid peroxidation and disturb normal cellular metabolism. PAs also reversed salinity-induced reductions in seedling growth and biomass accumulation and increased O_2^- , H_2O_2 , and MDA levels and the activity of antioxidant enzymes and carotenoids in salt-stressed *Brassica juncea* seedlings (Verma and Mishra, 2005). Our study shows that as exogenous Spd increased, the concentrations of H_2O_2 and MDA were augmented and then diminished in the roots of both cultivars, and this defense response was most likely

due to the increase in antioxidant enzyme activity (Matsuda, 1984). Our research showed that SOD, POD, and CAT activities increased significantly in salt-stressed roots when treated with different concentrations of exogenous Spd (Figure 6). With changing exogenous Spd concentration, we found a positive correlation between the Spd + Spm content and antioxidant enzyme activities but a negative correlation between the Spd + Spm content and H_2O_2 and MDA levels in response to salt stress (Figure 5).

In summary, although exhibiting different responses, the two zoysiagrass cultivars shared similar PA metabolism and physiological and biochemical mechanisms in response to salt stress with increasing exogenous Spd concentration. By

enhancing the activities of ODC, DAO, and SAMDC, cv. Z057 showed a better salt stress adaptation ability with exogenous Spd application, which promoted the conversion of Put into Spd and Spm. The addition of exogenous Spd further induced antioxidant enzyme activities, reduced H₂O₂ and MDA levels, and improved the tolerance of zoysiagrass to salinity stress.

Although our findings are important for academic research and cultivation, more research is needed to determine the number and frequency of exogenous Spd applications required to achieve optimal zoysiagrass growth.

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AUTHOR CONTRIBUTIONS

SL designed research; SL performed research; HJ contributed new reagents/analytic tools; SL and HJ analyzed data; and SL, HJ, and QZ wrote the paper.

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