

**Epigenetic mechanisms governing plant growth, development, and responses
to nematode parasitism**

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ABSTRACT

Epigenetic mechanisms, including histone and DNA methylation and microRNAs, play key roles in mediating transcriptional changes during plant development and stress responses. However, how these interconnected epigenetic components regulate gene expression in a spatiotemporal fashion remains partially known. Here, I generated 15 transgenic *Arabidopsis* GUS reporter lines for genes involved in DNA methylation and demethylation pathways. The spatiotemporal expression patterns of these genes were profiled in various plant organs during development, exogenous phytohormone response, and plant-parasitic nematode pathogenesis. The analyses revealed unique and overlapping expression patterns in roots, shoots, and reproductive organs, emphasizing the importance of a DNA methylation—demethylation equilibrium. Additionally, promoter activities suggest hormone-linked methylome regulatory mechanisms facilitate tissue differentiation.

In the syncytia and galls formed by the cyst (*Heterodera schachtii*) and the root-knot (*Meloidogyne incognita*) nematodes, respectively, CG and non-CG methyltransferases exhibited similar and distinct expression patterns at various stage of infection. DNA demethylases were more active in response to *M. incognita* than to *H. schachtii*. Furthermore, hypermethylated mutants defective in active DNA demethylation exhibited opposite responses to infection, which can be partially explained by the opposite regulation of pathogenesis-related genes by *H. schachtii* and *M. incognita*. These results suggest methylation-dependent mechanisms similarly and differentially regulate plant responses to infection by two distinct nematode species.

Finally, the regulatory mechanism through which miR778 induces histone modifications, DNA methylation, and transcriptome reprogramming during *H. schachtii* parasitism of *Arabidopsis* was investigated. miR778 post-transcriptionally silences the histone 3 lysine 9 (H3K9) dimethyltransferases *SUVH5* and *SUVH6* in roots following nematode infection. Genetic manipulation of the expression of miR778 and its target genes via overexpression, target mimicry, and T-DNA insertional mutation altered plant susceptibility to *H. schachtii*. Chromatin immunoprecipitation and sequencing (ChIP-seq) analysis and RNA-seq analysis of *suvh5* and *suvh6* single mutants revealed novel functions for SUVH5 and SUVH6 in affecting many genes

with development- and defense-related functions under nematode-infected conditions. Additionally, SUVH5/6-mediated H3K9 dimethylation associates with CG methylation in response to *H. schachtii*. Taken together, the data presented in this study highlight the importance of epigenetic mechanisms in establishing plant-nematode interactions and open new avenues for developing epigenetic signatures for nematode resistance.

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CHAPTER 1
GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Overview of epigenetics

Epigenetics mechanisms enable organisms to respond to dynamic changes in the environment over the course of development and during stress response (Hewezi, 2020; Hewezi et al, 2018; López Sánchez et al, 2016; Du et al, 2015; Matzke and Mosher, 2014; Zhang et al, 2010; Zilberman et al, 2007; Fransz and Jong, 2002). These modifications are stably inherited, yet reversible under contrasting conditions without altering the underlying nucleotide sequences (Henderson and Jacobsen, 2007). Epigenetic mechanisms regulate gene expression through coordinated modifications of DNA and histones. Non-coding small RNAs (sRNAs) as well as larger portions of chromatin are also redirected or remodeled to command epigenetic changes in gene expression (Henderson and Jacobsen, 2007).

Of these epigenetic mechanisms, DNA methylation is the most common. In addition to its involvement in plant development and stress response, DNA methylation is known specifically for its role in transposable element (TE) silencing, genomic imprinting, intercellular and interallelic communication, and genome interactions (Matzke and Mosher, 2014). During DNA methylation, a methyl group is added to the C5 position of cytosine by DNA methyltransferases. DNA methylation occurs in the CG, CHG, and CHH sequence contexts (where H is any nucleotide except G) (Figure 1.1A) (Law and Jacobsen, 2010; Lister et al, 2008). Methylated C5 (mC5) recruit histone methyltransferases, resulting in chromatin condensation and transcriptional silencing, particularly in the promoter of protein-coding and microRNA (miRNA) genes (Figure 1.1B) (Vaillant and Paszkowshi, 2007; Paszkowski and Whitham, 2001). However, an accumulating amount of evidence suggests that DNA methylation also contributes to transcriptional activation (Rambani et al, 2020a; Huang et al, 2019; Lang et al, 2017; Hewezi et al, 2017; Zilberman et al, 2007). In this case, transcription factors may have specific binding affinity to methylated *Cis*-binding elements leading to gene activation, as reported in human (Yin et al, 2017).

Some studies have associated DNA methylation of transcribed genic regions, i.e., the gene body with the regulation of gene expression and splicing efficiency. However, the functional role of gene body DNA methylation is not well-understood (Niyikiza et al, 2020; Bewick and Schmitz, 2017; Wang et al, 2016). However, recent studies indicated that gene body DNA methylation is of

biological significance (Seymore and Gaut, 2020; Horvath et al, 2019; Muyle and Gaut, 2019; Zilberman, 2017; Hewezi et al, 2017; Takuno et al, 2017; Bewick et al, 2017). DNA methylation was also reported in the primary transcript region of miRNA genes in plants (Rambani et al, 2020a, Piya et al, 2021). DNA methylation changes in primary transcript regions of miRNA genes may impact the processing and biogenesis of the primary transcripts by reducing the elongation rate of RNA polymerase II, thereby giving the needed time for newly synthesized long transcripts to be processed into mature 21-22 nt molecules (Rambani et al, 2020a; Glaich et al, 2019).

1.2 Mechanisms of DNA methylation

In *Arabidopsis*, *de novo* DNA methylation is established through canonical and non-canonical RNA-directed DNA methylation (RdDM) pathways. In the canonical pathway, RNA polymerase (Pol IV) transcribes heterochromatic loci into single-stranded RNA (ssRNA), which is recognized as an aberrant transcript. To eliminate ssRNA, RNA-DEPENDENT RNA POLYMERASE2 (RDR2) is recruited to Pol IV for the synthesis of a complementary RNA strand (Zhai et al, 2015; Belvins et al, 2015; Haag et al, 2012; Law et al, 2011). Double-stranded RNA (dsRNA) is then diced into 24-nucleotide (nt) small interfering RNA (siRNA) by DICER-LIKE3 (DCL3). The cleaved siRNAs are then loaded into ARGONAUTE4 (AGO4) or AGO6 to form an RNA-induced silencing complex (RISC) (Havecker et al, 2010). Downstream at the target loci, Pol V generates a nonfunctional transcript that acts a scaffold for AGO proteins (Wierzbicki et al, 2008). The RISC is directed to the target loci by complementary base pairing of the 24-nt siRNA, to which *de novo* DNA methyltransferases are recruited for the establishment of the methyl mark (Ebbs and Bender, 2006; Jackson et al, 2002). Although their impact is far less significant, there are several non-canonical mechanisms of RdDM that accompany the canonical pathway (Panda et al, 2016; Jackel et al, 2016; McCue et al, 2015; Nuthikattu et al, 2013; Wu et al, 2012). While non-canonical RdDM pathways function similarly, they typically involve homologues of Pol IV, RDR2, DCL3, and AGO4 and 6 (Cuerda-Gil and Slotkin, 2016). Regardless of the enzymes involved in the generation of siRNAs, all known pathways of *de novo* DNA methylation involve the catalyzation of 5mC primarily by DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) and also DRM1

(Figure 1.2A) (Cao and Jacobsen, 2002a; Cao et al, 2002). *DRM1* and 2 compose a class of catalytic domains with sequence similarity to that of the mammalian Dnmt3 methyltransferases, Dnmt3a and Dnmt3b (Cao et al, 2000; Hsieh et al, 1999). Indeed, DRM1 and 2 were found to be required for the *de novo* establishment of 5mC in all sequence contexts (Cao and Jacobsen, 2002a; Cao et al, 2002).

The maintenance of DNA methylation is mediated by specialized systems through DNA replication and cell division (Du et al, 2015; Matzake and Mosher, 2014). Methylation in the CG context is maintained by METHYLTRANSFERASE1 (MET1) (Figure 1.2B) (Law and Jacobsen, 2010). Hypomethylated *met1* mutants are not viable, suggesting has an essential function in plant growth and development (Stroud et al, 2014). Evidence indicates that *MET1* paralogs, *MET2b*, and *MET3* also participate in this process but to a much lesser extent (Quadrana et al, 2016; Stroud et al, 2014; Belmonte et al, 2013; Stroud et al, 2013). The ubiquitin E3 ligases VARIANT IN METHYLATION1 (VIM1), VIM2, and VIM3 also contribute to the maintenance of CG methylation (Kim et al, 2014; Kraft et al, 2008). Additionally, the heterochromatin-remodeling component DECREASE IN DNA METHYLATION (DDM1) is an important factor for CG DNA methylation (Zemach et al, 2013).

Non-CG methylation is maintained by the plant-specific chromo domain-containing CHROMOMETHYLASE (CMT) family proteins CMT2, and CMT3 and through the RdDM pathway (Figure 1.2B) (Du et al, 2015). CMT3 is the main enzyme responsible for CHG methyl maintenance, while CMT2 along with DRM1 and DRM2 are responsible for CHH methyl maintenance (Law and Jacobsen, 2010; Chan et al, 2005; Bartee et al, 2001; Lindroth et al, 2001). Although it usually exists as a truncated protein, CMT1 is also thought to be involved in the maintenance of non-CG methylation (Stroud et al, 2014). For symmetrical CG and CHG DNA methylation maintenance, MET1 and CMT2 and CMT3 are targeted to hemimethylated DNA and copy the methyl mark to the methylation-depleted strand (Schmitz et al, 2011; Becker et al, 2011; Jones et al, 2001). The chromo domain of CMT2 and CMT3 have also been shown to bind methylated histones, resulting in effective gene silencing (Du et al, 2012). Non-symmetric CHH methyl maintenance relies on the RdDM pathway for reestablishment of CHH methylation after meiosis or DNA replication (Law and Jacobsen, 2010; Chan et al, 2005). In addition, the

catalytically mutated paralog of DRM2, DRM3, is required for non-CG methylation maintenance and *de novo* methylation at repetitive sequences (Ashapkin et al, 2016; Costa-Nunes et al, 2014; Henderson et al, 2010).

As with any balanced system, DNA demethylation is an equally important epigenetic process. DNA Demethylation is essential for methylation equilibrium (Ortega-Galisteo et al, 2008; Penterman et al, 2007a; Gong et al, 2002) and maintaining DNA methylation boundaries of hypermethylated regions (Penterman et al, 2007a; Penterman et al, 2007b; Zhu et al, 2007). DNA demethylation can be either passive or active. Passive DNA demethylation occurs as a result of low activity or inhibition of DNA methyltransferase(s) at specific stages of plant growth, development, and responses to biotic and abiotic factors (Zhang and Zhu, 2012; Zhu, 2009). Active DNA demethylation, however, requires the activity of DNA demethylases (or glycosylases). Four DNA demethylases have been identified in Arabidopsis: REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DME-LIKE2 (DML2), and DML3 (Figure 1.2C). ROS1 is directed to loci for demethylation by the RNA-binding protein ROS3 or by the histone acetyltransferase INCREASE DNA METHYLATION1 (IDM1) also known as ROS4 (Li et al, 2012a; Qian et al, 2012; Zheng et al, 2008). METHYL BINDING DOMAIN PROTEIN7 (MBD7) and HARBINGER TRANSPOSON-DERIVED PROTEIN1 (HDP2) dually bind to hypermethylated CG sites and recruit IDM1 to render DNA accessible via histone acetylation (Duan et al, 2017; Lang et al, 2015; Li et al, 2015). Evidence suggests that DNA demethylases are recruited to target loci by gene activating histone modifications such as the acetylation of histone 3 (H3) lysine 18 (K18) (Tang et al, 2016; Rea et al, 2012; Ikeda et al, 2011). DNA demethylases remove a 5mC, creating an apurinic/apyrimidinic site to be filled with an unmethylated cytosine via one of several proposed base excision repair (BER) mechanisms (Zhu, 2009). Several lines of experimental evidence indicate that DNA methylation and active DNA demethylation are highly coordinated (Lei et al, 2015). Specifically, expression of *ROS1* is tightly regulated by an upstream DNA methylation monitoring sequence (MEMS). When global methylation levels are high, this MEMS becomes hypermethylated by MET1, upregulating transcriptional activity of *ROS1* and initiating active DNA demethylation (Lei et al, 2015). Furthermore, ROS1 has been suggested for a role in the maintenance of low methylation levels in promoters of stress-responsive genes to permit rapid

transcriptional changes (Deleris et al, 2016). Furthermore, because RdDM and ROS1 pathways are cyclically regulated, ROS1 is a candidate “fine-tuner” of differential gene expression in response to stress (Zhang and Zhu, 2012; Zhu, 2009; Penterman et al, 2007a; Zhu et al, 2007).

1.3 Involvement of DNA methylation in plant development

DNA methylation and demethylation pathways are important components of plant growth and development for vegetative and reproductive tissues. Comparative analyses of DNA methylation profiles in various vegetative tissues and organs revealed dynamic changes in the levels and patterns of DNA methylation throughout plant development. Roots, leaves, petioles and shoot apical meristems (SAM) generally contain higher levels of DNA methylation compared to cotyledons (Bartels et al, 2018). Specifically, meristematic tissues are hypermethylated compared to differentiated tissues (Baubec et al, 2014). These differences in methylation patterns are predominantly found in the CHH sequence context of young vegetative tissues and peaks in mature leaves (Bartels et al, 2018). Evidence in rice and maize points to a role for gene body methylation in regulating the expression levels of vegetative growth-related genes (Zhou et al, 2016; Candaele et al, 2014). Furthermore, evidence provides a direct link to DNA methyltransferases and demethylases activity in regulating vegetative tissues. For example, tomato MET1 regulates leaf and flower development (Yang et al, 2019). However, the DNA demethylase ROS1 has been shown to regulate stomatal development by upregulating *EPIDERMAL PATTERNING FACTOR 2 (EPF2)*, which activates SPCH for asymmetric cell division in stomatal development (Yamamuro et al, 2014).

The transition from juvenile to adult tissues and subsequent reproductive development is highly dependent on DNA methylation in association with phytohormones and other epigenetic factors. Together, they regulate the major flowering induction factors CONSTANS (CO), FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) (Campos-Rivero et al, 2017), SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) (Xu et al, 2018), and LEAFY (LFY) (Campos-Rivero et al, 2017). The phytohormone-mediated process of

flower initiation has been shown to induce DNA demethylation and reduce local auxin concentrations in pre-floral meristem (Camos-Rivero et al, 2017; Wanda et al, 2010; Achard et al, 2007; Martínez et al, 2004). Once floral organs are established, reproductive organs reportedly become hypermethylated (Camos-Rivero et al, 2017). Consistent with this finding, methylome analyses revealed that floral tissues are hypermethylated in all sequence contexts in comparison with whole shoot tissues (Bartels et al, 2018).

DNA methylation and demethylation also play important roles in regulating various aspects of embryogenesis and seed development. For example, DME demethylates maternal genes in the central cell that are preferentially expressed in the endosperm after fertilization (Gehring et al, 2017; Park et al, 2016; Bauer and Fischer, 2011; Hsieh et al, 2009). Methylome analyses in soybean and Arabidopsis revealed significant changes in non-CG DNA methylation genome wide. Though there is significant non-CG hypermethylation during seed development, non-CG methylation is not required for seed viability (Lin et al, 2017). However, the activity of some endospermic TEs upregulates RdDM-associated siRNAs, which in turn silence homologous TEs in the embryo via DRM2 and DRM3 (Ibarra et al, 2012). These epigenetically activated siRNAs also regulate parental genome dosage and seed viability (Martinez et al, 2018). Evidence in rice reveals that DNA methylation levels peak during embryo cellularization (Xing et al, 2015). DNA demethylation is associated with the accumulation of seed storage proteins in the endosperm of maturing seeds (Van Herpen et al, 2008). At the same time, hypermethylation and chromatin condensation have been associated with gene silencing in dormant seeds for seed dehydration and germination prevention (Bouyer et al, 2017; Kawakatsu et al, 2017). To resume expression of developmental genes during and following germination, DNA demethylases were found to be upregulated, leading to chromatin decondensed and nuclei expansion (Xing et al, 2015).

Despite the findings that DNA methylation plays a critical role in transcriptome reprogramming during various developmental contexts, spatial and temporal expression patterns of genes involved in DNA methylation and demethylation throughout plant growth and development are largely unknown. In the second chapter, I characterized the expression profiles of genes involved in DNA methylation and active demethylation in roots, shoots, and reproductive tissues using GUS reporter lines. The results reveal unique and overlapping expression patterns of these genes in roots, shoots,

inflorescences, siliques, and developing seeds and highlight the importance of DNA methylation—demethylation equilibrium in these tissues.

1.4 Involvement of DNA methylation in hormone signaling

Phytohormones are a centerpiece in the regulation of tissue differentiation, growth, and development (Campos-Rivero et al, 2017). Crosstalk between phytohormone signaling and epigenetic mechanisms has been reported, particularly in meristematic tissues (Maury et al, 2019). Auxin plays key role in cellular differentiation, organogenesis and morphogenesis (Kasahara, 2016). Auxin signaling is controlled by multiple layers of interconnected epigenetic pathways, including microRNAs, histone modifications, and chromatin remodeling. In addition, limited experimental evidence indicate that auxin may contribute to epigenome reprogramming, suggesting that an auxin-epigenetic regulatory mechanism may exist (Yamamuro et al, 2016). In this regard, MET1 has been shown to indirectly influence the auxin efflux carrier PIN1, contributing to many diverse developmental processes, such as embryogenesis and the transition from vegetative to reproductive phase (Xiao et al, 2006; Kankel et al, 2003). In addition, ROS1 and its homologs were found to be involved in activating the master regulatory gene for auxin signaling, PINOID (PID), which controls polarized localization patterns of PINs. Active DNA demethylation opens a regulatory chromatin loop controlling PID, giving auxin response factors (ARFs) the ability to bind to the regulatory region of PID (Ariel et al, 2014).

The ratio of auxin and cytokinin is precisely regulated for cellular differentiation, organ patterning, and apical dominance in meristematic tissues (Schaller et al, 2015). Other phytohormones also play specific roles in plant development. Cytokinin, for example, regulates aspects of fruit and flower development, and gibberellic acid (GA) controls seed development, stem elongation, leaf expansion, and flowering alongside DNA methylation pathways (Zuñiga-Mayo et al, 2018; Campos-Rivero et al, 2017). A role of epigenetic mechanisms in regulating signaling components of cytokinin and gibberellic acid (GA) has also been reported (Manoharlal et al, 2018; Zuñiga-Mayo et al, 2018).

Similarly, DNA methylation and demethylation have been linked to a regulatory function in fruit and seed development by modulating the biosynthesis and signaling of ethylene and ABA (Campos-Rivero et al, 2017). For instance, in tomato, DML2-mediated DNA demethylation contributed to global hypomethylation during fruit ripening through the regulation of ethylene (Guo et al, 2019; Zou et al, 2018; Lang et al, 2017; Liu et al, 2015). During orange and *Brassica rapa* seed development, however, DNA demethylases are downregulated, resulting in hypermethylation and differential expression of key genes involving ABA signaling (Huang et al, 2019; Grover et al, 2018). ABA is also involved in plant growth and development as well as responses to stress. Chromatin remodeling and histone modifications are thought to regulate ABA levels and the induction of ABA-responsive genes in seeds during maturation, dormancy, and inhibition of germination (Trivedi et al, 2016; Chinnusamy et al, 2008). Also known for its involvement in defense responses (Khan et al, 2015; Vlot et al, 2009; Durner et al, 1997), salicylic acid (SA) plays a role in developmental processes such as root initiation and growth and root apical meristem (RAM) patterning via auxin redistribution (Pasternak et al, 2019; Dempsey and Klessig, 2017). DNA methylation negatively regulates SA-mediated plant responses by downregulating the expression of defense-response genes, such as *PATHOGEN RESISTANCE 1 (PR1)* (López Sánchez et al, 2016; Dowen et al, 2012; Agorio and Vera, 2007).

Despite several studies pointing to a role for phytohormone signaling in controlling epigenetic pathways, the nature of the biochemical signals that associate with DNA methylome changes during plant development and stress responses are not understood and direct experimental evidence remains lacking (Zhu, 2010; Yamamuro et al, 2016; Maury et al, 2019; Xiao et al, 2020). With aim to better understand the connection between DNA methylation and phytohormones, chapter 2 also explores the spatiotemporal expression patterns of genes involved in DNA methylation and demethylation in roots and shoots in response to treatments with six phytohormones: auxin, cytokinin, GA, ethylene, ABA, and SA. My results point to a role of phytohormone signaling in establishing plant methylomes associated with differentiation and development of various tissues.

1.5 Involvement of DNA methylation in biotic stress responses

DNA methylation and demethylation pathways control transcriptional responses to plant pathogens such as viruses, bacteria, fungi, and nematodes (Hewezi et al, 2018). Plant viruses utilize host transcriptional, translational, and/or replicative machinery for the production and multiplication of viral proteins. However, plant defense mechanisms work to suppress foreign genetic material through post-transcriptional gene silencing (PTGS) via the RNA silencing pathway and through transcriptional gene silencing (TGS) via DNA and histone methylation (Wieczorek and Obreepalska-Steeplowsha, 2015). For RNA silencing, viral RNAs are processed into small RNAs and loaded into an ARGONAUTE protein to form a RISC that is localized to viral RNA through complementary base pairing (Baulcombe and Dean, 2014). Plants also target viral genetic material for TGS using DNA and histone methylation (Raja et al, 2010). However, plant DNA viruses can overcome TGS by suppressing MET1 and CMT3 (Pooggin, 2013; Rodríguez-Negrete et al, 2013; Paprotka et al, 2011; Zhang et al, 2011; Buchmann et al, 2009; Bian et al, 2006; Wang et al, 2005; 2003).

Bacterial infection also induces changes in plant DNA methylation levels and subsequent gene transcriptional responses (Downen et al, 2012; Pavet et al, 2006). Mutants deficient in DNA methylation were characterized by the misexpression of pathogen-responsive genes (Downen et al, 2012). In addition, hypomethylation is associated with enhanced bacterial resistance, suggesting that DNA methylation represses plant defense mechanisms in the compatible interaction between *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 and Arabidopsis (Downen et al, 2012). Similarly, Arabidopsis hypomethylation mutants display increased resistance to biotrophic and hemibiotrophic plant pathogenic fungi and increased susceptibility was observed for hypermethylation mutants (López Sánchez et al, 2016; Le et al, 2014). Again, these phenotypes were associated with differential expression of stress-responsive genes (Le et al, 2014). Conversely, plant susceptibility to necrotrophic fungi was significantly augmented by the loss of DNA methylation, suggesting that DNA methylation pathways exert common and/or unique responses to different plant pathogens (Hewezi et al, 2018; López Sánchez et al, 2016). DNA

methylation and other epigenetic mechanisms have also been shown to regulate nematode parasitism and subsequent plant defense responses (Hewezi, 2020).

1.6 Agronomic and economic importance of cyst and root-knot nematodes

Plant-parasitic nematodes (PPNs) cause massive yield losses of the world's most valuable crops, such as wheat, rice, maize, potato, cotton, soybean, and vegetable crops, likely accounting for over \$100 billion dollars in economic losses annually (Bernard et al, 2017; Ghareeb et al, 2020). While efforts are being made to control PPN infection, they are an increasing problem worldwide (Bernard et al, 2017; Jones et al, 2013; Nichol et al, 2011). Among the 4100 species of PPNs identified, ten species have been named the most devastating in agriculture (Jones et al, 2013). Root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* and *globodera* spp.) nematodes are the most agronomically and economically important PPNs due to their wide host range, persistence in agricultural soils, and the substantial induction of genetic and epigenetic reprogramming they elicit in host plants (Jones et al, 2013; Meyer, 2003). There are 98 species of root-knot nematodes (RKNs), however the most significant species are *M. incognita*, *M. arenaria*, *M. javanica*, and *M. hapla* (Agrios, 2012). RKNs have a wide host range, parasitizing almost every vascular plant (Jones et al, 2013). Although cyst nematodes (CNs) have a narrower host range, their agronomic impact on crops like soybean, and potato accounts for over \$1.5 billion annual losses in the US alone (Nichol et al, 2011; Chen et al, 2001). Contributing to the difficulty of controlling root-knot and cyst nematodes is the unique and complex interaction between the nematodes and their host plants.

Both cyst and root-knot nematodes are sedentary endoparasites, but there are distinct differences in their life cycles. Parasitic second-stage juveniles (J2s) hatch from the eggs and penetrate host roots, physically breaking through root cell walls using their needle-like stylet (Sobczak and Golinowski, 2009). These PPNs also secrete various effector proteins to partially digest cell walls (Hewezi and Baum, 2013; Hewezi, 2015). RKNs preferentially enter host root systems in the elongation zone and migrate intercellularly to the vascular tissue (Sobczak and Golinowski, 2009).

CNs, on the other hand, penetrate root systems unbiased in location and then migrate intracellularly to the vascular cylinder (Sobczak and Golinowski, 2009). Once compatible cells are selected, the nematodes establish their permanent feeding sites, galls (RKN) or syncytia (CN). Through the release of a suite of effectors, infected cells become multinucleated and metabolically hyperactive, such that plant nutrition is redirected to the feeding sites. Compatible cells selected by RKN are transformed into giant cells through repeated cycles of mitosis without cytokinesis. This results in the enlargement of target and surrounding cells by hundreds of times, creating knot-like galls on host roots (Sobczak and Golinowski, 2009). During syncytia formation, CNs induce partial cellular fusion of over a hundred cells surrounding the initial feeding cells. Cyst and root-knot nematode feeding site development occurs in three distinct stages: initiation, development, and maintenance (Sobczak and Golinowski, 2009). As feeding structures are developed, cyst and root-knot nematodes use their stylet to uptake nutrients from the host to support nematode development into the third-stage juvenile (J3), fourth stage juvenile (J4) and finally the adult nematode (Sobczak and Golinowski, 2009). During this process, thousands of protein-coding genes and TEs are transcriptionally altered as infected cells undergo massive reprogramming for cellular redifferentiation in syncytia (Hewezi et al, 2017; Szakasits et al, 2009; Fuller et al, 2007) and giant cells (Yamaguchi et al, 2017; Barcala et al, 2010; Fuller et al, 2007; Jammes et al, 2005). The life cycle of RKN is completed by mitotic parthenogenesis and by outcrossing for cyst nematode (Sobczak and Golinowski, 2009).

1.7 Involvement of DNA methylation in cyst and root-knot nematode feeding site development and plant defenses

In-depth methylome profiling of Arabidopsis and soybean reveals distinct trends in DNA methylation patterning induced by CNs (Rambani et al, 2020a; Hewezi et al, 2017; Rambani et al, 2015). In Arabidopsis, global hypomethylation is significantly induced in response to infection by the beet cyst nematode (BCN, *H. schachtii*) (Hewezi et al, 2017). Specific signature methylome profiles are also evident for syncytia in developmental and maintenance stages, where developing

syncytia had almost one and a half times the number of hypomethylated DMRs as syncytia in the maintenance stage (Hewezi et al, 2017). In both developmental stages, CG hypomethylation of protein-coding genes and TEs represent the majority of the DMRs (Hewezi et al, 2017). Genome-wide hypomethylation is also induced in soybean roots in response to the soybean cyst nematode (SCN, *H. glycines*), the majority of which occur as CG context DRMs in the gene body (Rambani et al, 2015). These reports point towards a significant role for gene body hypomethylation in CN parasitism. Additionally, it has been reported elsewhere that active DNA demethylation is induced in response to various other plant pathogens, such as bacteria and fungi (Downen et al, 2012; López Sánchez et al, 2016).

Recently, the methylation profiles of susceptible and resistant near-isogenic lines (NIL) of soybean were assessed in response to SCN infection (Rambani et al, 2020b). During the compatible interaction, DNA methylation levels decreased in both protein-coding genes and TEs, whereas the resistant line showed increased DNA methylation levels (Rambani et al, 2020b). A similar trend was observed for the resistant NIL over miRNA genes in response to SCN (Rambani et al, 2020a). Although the susceptible NIL also exhibited global hypermethylation of miRNA genes, the majority of the differentially methylated regions (DMRs) were hypomethylated (Rambani et al, 2020a). Taken together, there is a strong correlation between genome hypomethylation and CN syncytia development in plants.

In the susceptible interaction of BCN and Arabidopsis, 262 genes were identified as differentially methylated and significantly differentially expressed, nearly 90% of which were hypomethylated and upregulated (Hewezi et al, 2017). The majority of the differentially expressed genes (DEGs) hosted a DMR in the gene body and were differentially methylated in the CG context. Additionally, 136 of 189 TEs near protein-coding genes were also differentially expressed, most of which were hypomethylated in the CG context (Hewezi et al, 2017). Further investigation of these differentially expressed TEs revealed that about 70% were activated in response to BCN infection (Piya et al, 2017a). Thus, the trends connecting DNA methylation and transcriptional response are dependent on methylation sequence context and location within the genic region, as was previously established (Rambani et al, 2015; Yang et al, 2015; Gent et al, 2013; Schmitz et al, 2013). Of 24 randomly selected soybean genes containing DRMs induced in the promoter or gene body during

the compatible interaction with SCN, 21 were also differentially expressed upon SCN infection (Rambani et al, 2015). Among the tested genes containing DMRs, there is a general trend of downregulation for hypermethylated promoters and upregulation for hypomethylated promoters. DEGs in the gene body, however, exhibit the opposite transcriptional responses (Rambani et al, 2015).

The mechanism by which hypomethylation subjugates plant defenses can be broadly deduced by transcriptome profiling. Over 750 differentially methylated genes, differentially methylated either in the gene body or promoter, induced by SCN in the compatible interaction were identified to be differentially expressed in the syncytium (Rambani et al, 2020b). Of which, an *ADAPTIN* family protein gene, likely involved in redirecting resources to the syncytium, was found to be severely depleted of DNA methylation in the promoter and gene body in various sequence contexts and was highly upregulated in response to SCN. In addition, *CYCLIN DOF FACTOR 2 (CDF2)*, which has been reported to control the expression of numerous miRNA genes, was found to be hypomethylated in the gene body and hypermethylated in the promoter. This differential methylation pattern resulted in the downregulation of *CDF2*, likely giving rise to the observed changes in miRNA gene methylation and expression in response to SCN (Rambani et al, 2020a; 2020b). Furthermore, these studies provide evidence that DNA methylation serves as a key regulatory mechanism for subsequent epigenomic and transcriptomic reprogramming (Rambani et al, 2020b; Piya et al, 2017a; Hewezi et al, 2017; Rambani et al, 2015).

Pathogen-induced hypomethylation and transcriptional gene silencing (TGS), particularly of defense-response genes, have been largely but not exclusively reported for a wide variety of pathogens (Tirnaz and Batley, 2019). In a recent study, the methylation status of susceptible and resistant tomato cultivars was assessed under the infection of two species of RKN, *M. incognita* and *M. javanica* (Leonetti and Molinari, 2020). In both pathosystems, resistant plants were hypomethylated and susceptible plants were hypermethylated. Tomato *DRM5* was significantly downregulated in the incompatible interaction, likely leading to the hypomethylation state (Leonetti and Molinari, 2020).

DNA methylation regulates the activation of PR proteins and phytohormone-driven immunity pathways (Gheysen and Mitchum, 2019). SA, in particular, is known to be activated against cyst and root-knot nematode infection (Molinari, 2015; Kammerhofer et al, 2015; Molinari et al, 2013; Wubben et al, 2008). Other phytohormones, including ethylene and ABA have been shown to act as positive regulators of BCN and RKN pathology, respectively (Piya et al, 2019; Kyndy et al, 2017; Moosavi, 2017; Kammerhofer et al, 2015). In the second chapter, the temporal and spatial expression patterns of genes involved in *de novo* DNA methylation, DNA methylation maintenance, and DNA demethylation in response to exogenous application of various phytohormones were characterized. It was observed that DNA methyltransferases and demethylases were differentially expressed in response to exogenous applications of SA, ABA, and ethylene. This and other recent compelling studies implicate DNA methylation in the involvement of biotic stress response (Hewezi et al, 2018; Deleris et al, 2016; Kim and Zilberman, 2014). However, the direct impact of DNA methylation and demethylation-related genes on the compatibility of the interactions between parasitic nematodes and host plants remains unknown. Thus, in chapter 3 I investigated the temporal and spatial expression patterns of DNA methyltransferases and demethylases in response to BCN and the RKN. In addition, the susceptibility levels of various DNA methylation and demethylation mutants were assessed in response to BCN and RKN in order to better understand the role of specific DNA methylation pathways in plant-nematode interactions.

1.8 Involvement of histone methylation in plant defense

Not only does DNA methylation work in concert with other epigenetic marks, histone modifications, for example, are directly correlated to changes in gene expression. Histone modifications, such as acetylation and methylation have been observed to impact plant epigenome during development and stress responses (Ramirez-Prado et al, 2018; Yuan et al, 2013; Berr et al, 2011). Such histone marks correspond to different effects on gene expression. For example, histone acetylation tends to be a permissive mark (Berger, 2007; Shahbazian and Grustein, 2007), whereas methylation of histones at specific lysine residues are repressive (Xiao et al, 2016).

Broadly, histone acetylation and methylation of histone 3 (H3) at lysines 4 (K4) and 36 (K36) are associated with transcriptional activation. Loss of the H3K4 methyltransferase, ARABIDOPSIS TRITHORAX1 (ATX1) increases susceptibility to *Pst* DV3000 and results in the downregulation of *PR1* and *WRKY70*, the link between JA- and SA-mediated defense pathways (Alvarez-Venegas et al, 2007; 2003). Likewise, the H3K36 methyltransferase SET DOMAIN GROUP8 (SDG8) displayed increased susceptibility to the plant-pathogenic fungi *A. brassicicola* and *Botrytis cinerea* and misregulation of pathogen- and JA-responsive genes (Dóczi et al, 2007; Berr et al, 2010; Rasmussen et al, 2012). Euchromatic regions of the genome are also highly enriched in acetylated histones (Berger, 2007; Shahbazian and Grustein, 2007). However, stress stimuli can alter the activity of histone deacetylases and specific methyltransferases resulting in local chromatin compaction and inaccessibility of transcriptional machinery (Zhou et al, 2020; Wu et al, 2017; Lee and Shilatifard, 2007; Fuks, 2005).

HISTONE DEACETYLASE19 (HDA19), which deacetylates lysines 9, 14,18, and 23 of histone 3 and 5, 8, 12, and 16 of histone 4 (Lusser et al, 2001), has been shown to play a role in immunity to bacterial and fungal plant pathogens (Ramirez-Prado et al, 2018). HDA19 was found to be a positive regulator of defense against the fungi *Alternaria brassicicola*, whereas HDA19 and its homolog HDA6 were negative regulators of *Pst* DC3000 (Choi et al, 2012; Zhou et al, 2010; Kim et al, 2008; Wu et al, 2008; Zhou et al, 2005). Additionally, HISTONE DEACETYLASE2b (HD2B), which specifically removes H3K9 acetylation marks, regulates plant susceptibility to plant-pathogenic bacteria and it is thought to fine-tune the expression of defense-related genes (Ramirez-Prado et al, 2018; Latrasse et al, 2017). Other repressive histone marks include H3K27 and H3K9 methylation. Histone methylation can occur as mono- (me), di- (me₂), and tri- (me₃) methylations evidentially have unique and overlapping functions (Xiao et al, 2016). H3K27me and H3K27me₃ are both repressive marks; however, H3K27me is located in heterochromatic regions, while H3K27me₃ is targeted to protein-coding genes. H3K27me₃ has been linked to the regulation of defense-response when challenged by *Pst* DC3000 (SA-mediated defense) and the fungi *Sclerotinia sclerotiorum* (JA-mediated defense) (Le Roux et al, 2014). H3K9 demethylation has recently been identified as a positive regulator of plant defense-response to *Pst* DC3000 (Dutta et al, 2017). However, investigation of the role of H3K9 methylation in plant immunity is lacking.

1.9 Impact of H3K9 methylation on the plant epigenome

Heterochromatin is generally characterized by heavy mono- and dimethylation of H3K9 (Jackson et al, 2004; Fuchs et al, 2006). H3K9me₂, in particular, corresponds to the repression of detrimental TEs and repetitive sequences (Lippman et al, 2004). However, H3K9me₃, though far less abundant, is generally located in euchromatic protein-coding genes (Mathieu et al, 2005; Turck et al, 2007). These chromatin modifications provide the platform for additional machinery to reinforce the repressive conformation. Interestingly, a positive correlation between H3K9me₂ and non-CG DNA methylation has been reported. Further investigation revealed a positive feedback loop between these two pathways (Liu et al, 2010; Vaillant and Paszkowski, 2007; Mathieu et al, 2005; Tariq et al, 2003; Jackson et al, 2002; Johnson et al, 2002; Malagnac et al, 2002; Soppe et al, 2002).

SU(VAR)3-9 HOMOLOG5 (SUVH5) and SUVH6 catalyze the dimethylation of H3K9me₂ using a distinct 5-methyl-dC (5mC) base-flipping mechanism and are particularly important for transcriptional regulation (Rajakumara et al, 2011; Deal and Henikoff, 2011; Jackson et al, 2004; Ebbs and Bender, 2006). DNA methyltransferases CMT2 and CMT3 are recruited by the SET domain of SUVH5 and 6 (Bernatavichute et al, 2008; Stroud et al, 2014). CMT2 and 3 recognize H3K9me₂ via their bromo-adjacent homology (BAH) and chromo domains, thus are targeted to H3K9 dimethylated nucleosomes for gene silencing reinforcement through non-CG DNA methylation (Du et al, 2015; Stroud et al, 2014). More recently, it was shown that SUVH5 and SUVH6 are also targeted to methylated DNA and showed distinct binding preferences for 5mC in different sequence contexts (Li et al, 2018). While SUVH5 binds strongly to mCG, SUVH6 preferentially binds to mCHG sites. Both SUVH5 and SUVH6 showed similar preference for mCHH sites (Li et al, 2018). In this way, H3K9me₂ and DNA methylation positively regulate each other through a feedback loop. Through this pathway, chromatin condenses and becomes inaccessible to transcriptional machinery, ultimately resulting in gene silencing (Rajakumara et al, 2011; Stroud et al, 2014; Du et al, 2015; Li et al, 2018).

Genome-wide genetic and chromatin dynamics are controlled by complex, integrated epigenetic systems regulating the epigenome and subsequent transcriptional reprogramming (Niederhuth and

Schmitz, 2017; Law and Jacobsen, 2010; Vaillant and Paszkowski, 2007). An increasing number of studies are showing how histone modifications coupled with DNA methylation impact many cellular and physiological responses to stress (Dutta et al, 2017; Kim et al, 2015; Bilichak et al, 2012; Song et al, 2012; Karan et al, 2012). In addition, recent studies have shown complete metabolic rewiring required for nematode feeding site development is achieved through widespread methylome and transcriptome reprogramming (Szakasits et al, 2009; Rambani et al, 2015; Hewezi et al, 2017). However, the role of the repressive H3K9 methyl mark has not been explored in response to nematode infection. In chapter 3, I show that mutations of various components of DNA methylation pathways result in the misregulation of defense-responsive genes and altered plant susceptibility. Thus, chapter 4 investigates the impact of SUVH5 and SUVH6 on histone methylation, transcriptome profiling, and the interaction between histone and DNA methylation during BCN parasitism of Arabidopsis.

1.10 Involvement of microRNAs in plant-nematode interactions

The transcriptome is also greatly influenced by the activity of miRNAs. In eukaryotes, miRNAs are primarily responsible for post-transcriptional gene silencing (PTGS) (Sun, 2012; Friedman et al, 2009; Bartel, 2004). Plant miRNA genes can be integrated into their host gene body (Yang et al, 2012) or encoded by TEs (Piriyapongsa and Jordan, 2008), but are most commonly in intergenic regions (Nozawa et al, 2012; Xie et al, 2005). The miRNA genes are transcribed much like protein coding genes, except the RNA transcript produced is the end product (Szarzynska et al, 2009; Xie et al, 2005). The result of transcription is the primary miRNA (pri-miRNA) transcript, which contains 5'- and 3'- sequences to be trimmed. Pri-miRNA folds into a stem-loop structure and is processed into mature 21-24 nt miRNA duplexes by DICER-LIKE1 (DCL1) (Kurihara et al, 2004; Reinhart et al, 2002; Park et al, 2002). Mature miRNAs are methylated and transported out of the nucleus to be loaded into AGO1, forming a RISC in the cytoplasm (Meister and Tuschl, 2004; Baumberger and Baulcombe, 2005; Chandradoss et al, 2015). The miRNA-RISC is then localized to target mRNA(s) via near-perfect complementary sequence binding for mRNA degradation or

translational repression, leading to post-transcriptional gene silencing (Sun, 2012; Xie et al, 2015). Through this mechanism, miRNAs are responsible for maintaining a diverse array of rigid developmental processes as well as regulating dynamic stress responses (Hewezi, 2020; Barrera-Figueroa et al, 2013; Kruszka et al, 2013; Sun, 2012; Nonogaki et al, 2010; Liu et al, 2009).

A significant number of miRNA genes have been identified as differentially expressed in response to cyst nematode infection, suggesting their involvement in mediating plant-nematode interactions (Tian et al, 2017; Xu et al, 2014; Li et al, 2012b; Hewezi et al, 2008). Few, however, have been functionally characterized. *miR396*, for example, which targets seven of the nine Arabidopsis growth-regulating transcription factors, is downregulated during syncytium initiation but highly induced in the maintenance stage of syncytium development (Hewezi et al, 2012). Of the seven growth-regulating factors, *GROWTH REGULATING FACTOR1 (GRF1)* and *GRF3* are post-transcriptionally upregulated in syncytium throughout development (Hewezi et al, 2012). In addition, overexpression of *miR396*, or *miR396*-resistant versions of *GRF1* or *GRF3*, and the *grf1/2/3* triple mutant all showed a significant reduction in plant susceptibility, suggesting *miR396* mediates plant susceptibility to BCN largely through the combined action of *GRF1* and 3 (Hewezi et al, 2012). Further investigation of syncytial sizes and nematode development over the course of parasitism revealed that increased plant susceptibility in overexpression lines is likely due to arrested nematode development during the early stages of parasitism (Hewezi et al, 2012). Finally, microarray was used to determine downstream gene targets of *GRF1* and *GRF3*. Gene targets reported in this study represented diverse groups of biological processes, such as metabolism, biotic and abiotic stress responses, and other developmental processes (Hewezi et al, 2012). One interesting observation was that overexpression of *GRF1* and *GRF3* resulted in a reduction of *miR396* abundance and *miR396* expression levels were significantly increased in the *grf1/2/3* triple knockout mutant, indicating that *GRF1* and 3 negatively regulate the abundance of *miR396* (Hewezi and Baum, 2012). Thus, the expression of *miR396* and its targets *GROWTH REGULATING FACTOR1 (GRF1)* and *GRF3* operate in a positive feedback loop which serves as a vital component of syncytium stage transition (Hewezi and Baum, 2012; Hewezi et al, 2012). In this context, *GRF1* and 3 have been further shown to coordinate growth-stress antagonism (Piya et al, 2020; Noon et al, 2019; Liu et al, 2014).

miR858 is known to target several members of the large family of MYB transcription factors (Sharma et al, 2016; Addi-Quaye et al, 2008; Fahlgren et al, 2007). In the early stages of BCN parasitism, miR858 is highly expressed but is downregulated specifically in syncytia in the later formation and maintenance stages of syncytium development (Piya et al, 2017b). One MYB transcription factor post-transcriptionally targeted by miR858, MYB83, has been known for its role in cell wall modification and secondary cell wall biosynthesis, which are fundamental cellular processes involved in syncytium development (Hewezi, 2015; Bohlmann and Sobczak, 2014). Time-course spatiotemporal expression assays and quantitative PCR (qPCR) determined that *MYB83* was strongly induced in the syncytium throughout development but is post-transcriptionally controlled by miR858 in the early stages of syncytium development (Piya et al, 2017b). In addition, suppression of *MYB83* via miR858 facilitates BCN infection, given that transgenic plants overexpressing miR858 and a loss-of-function *myb83* mutant resulted in reduced plant susceptibility, while overexpression of a miR858 mimic sequence and overexpression of miR858-resistant *MYB83* resulted in increased plant susceptibility (Piya et al, 2017b). RNA-seq analysis of miR858-resistant *MYB83* and miR858 overexpression lines revealed that the majority of the DEGs identified were common to both overexpression constructs, indicating that *MYB83* is the main target of miR858 in roots (Piya et al, 2017b). Additionally, almost 40% of the DEGs identified in the miR858-resistant *MYB83* overexpression line contained at least three MYB83 cis-binding elements in the promoter region, many of which are involved in transport, transcription, metabolism, defense, and stress responses (Piya et al, 2017b). About 45% of the MYB83-regulated genes overlapped with syncytium DEG identified by Szakasits et al (2009), indicating that 16.6% of the syncytium transcriptome is regulated by MYB83, 6% of which are directly targeted by MYB83 (Piya et al, 2017b).

The ubiquitin E3 ligase *NITROGEN LIMITATION ADAPTATION (NLA)*, which positively regulates the expression of defense-response genes when challenged with various plant stressors (Hewezi et al, 2016; Park et al, 2014; Lin et al, 2013; Kant et al, 2011; Peng et al, 2007), is post-transcriptionally regulated by miR827 (Hewezi et al, 2016; Hsieh et al, 2009; Addo-Quaye et al, 2008; Fahlgren et al, 2007). In response to BCN, a miR827-*GUS* fusion and an *NLA-GUS* fusion excluding the miR827 binding site are highly active throughout syncytium development. However,

an *NLA-GUS* fusion containing the miR827 binding site is downregulated specifically in the syncytium throughout development, suggesting *NLA* is post-transcriptionally regulated by miR827 during BCN pathogenesis (Hewezi et al, 2016). Additionally, overexpression of miR827 or a mutation in *NLA* resulted in increased plant susceptibility, whereas inhibition of miR827 via target mimicry or overexpression of a miR827-resistant *NLA* sequence resulted in reduced plant susceptibility (Hewezi et al, 2016). To examine the specific role of *NLA* in syncytium development, yeast two-hybrid (Y2H) screens were used to identify 51 protein substrates targeted by *NLA*, 31 of which were also differentially expressed in microdissected syncytia (Hewezi et al, 2016; Szakasits et al, 2009). Among the 31 syncytia differentially expressed *NLA* protein substrates are defense-related proteins PR4, thaumatin superfamily protein, and β -1,3-endoglucanase, which is targeted by the CN effector 30C02 (Hamamouch et al, 2012) as well as several transcription factors (Hewezi et al, 2016). Thus, miR827 serves as an upstream regulator of key transcription factors and defense response induced by the BCN. These examples provide evidence of a role for miRNA PTGS in the mediation of plant-nematode interactions. However, more research is required to explore the impact of other differentially expressed miRNAs.

miR778 has been identified to specifically target the histone methyltransferases SUVH5 and SUVH6 (Lu et al, 2006; Fahlgren et al, 2007). Recently, SUVH5 was identified as a negative regulator of hormone signaling and developmental pathways during light-mediated seed germination (Gu et al, 2019). Furthermore, miR778 was recently found to be employed for *SUVH6* PTGS in response to phosphate starvation, which induces several stress-responsive genes (Sega et al, 2020; Wang et al, 2015). Chapter 4 goes on to reveal miR778 as a positive regulator of BCN pathology through PTGS of *SUVH5* and *SUVH6* and demonstrate that miR778 acts as an upstream switch to control gene silencing through H3K9me2 and non-CG DNA methylation.

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Appendix

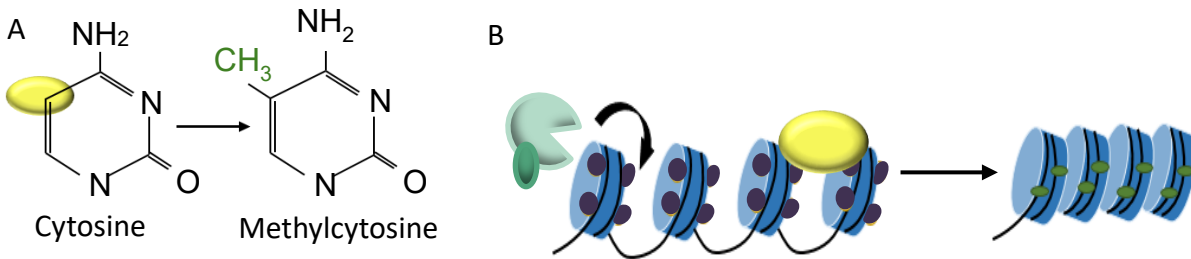


Figure 1.1. Schematic view of the molecular process of DNA methylation. (A) DNA methylation occurs on cytosine by a DNA methyltransferase (yellow oval) covalently binding a methyl group in the C5 position. (B) Chromatin alterations from epigenetic mechanisms induce gene silencing. Unmethylated, active DNA undergoes histone deacetylation (acetylation depicted as brown circles) along with histone and DNA methylation, which causes chromatin condensation. Chromatin condensation denies the transcriptional machinery (teal) access to the DNA. DNA methylation (depicted as green ovals) and other chromatin modifications renders the DNA transcriptionally inactive.

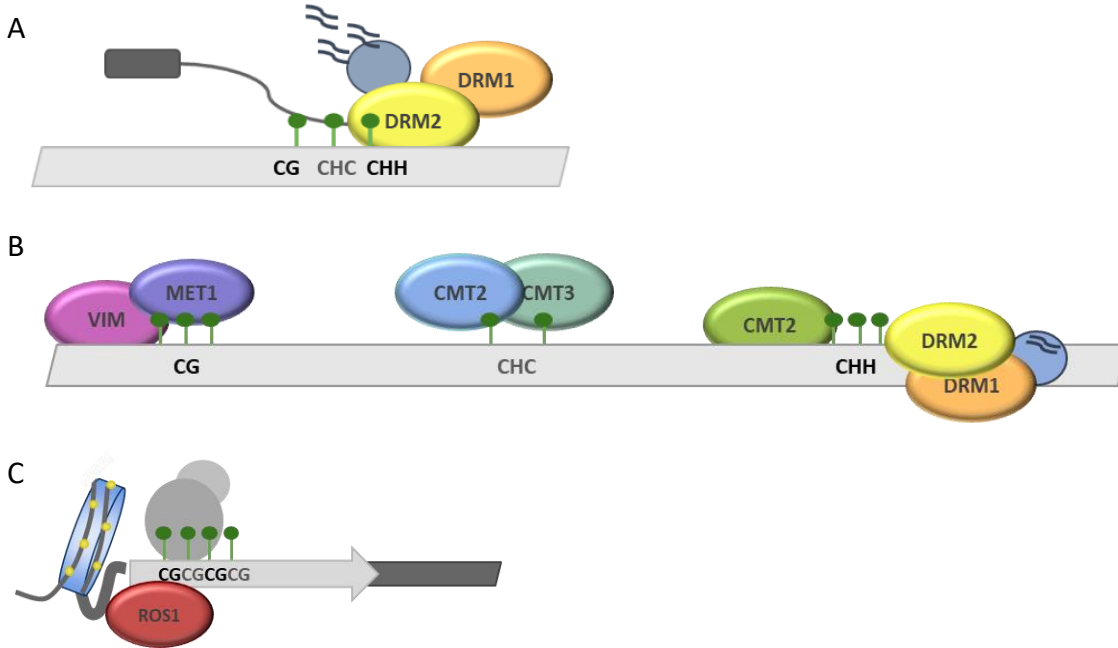


Figure 1.2. DNA methylation and demethylation pathways. (A) *De novo* methylation marks are established through the action of DRM2 and DRM1 at targeted loci in all sequences contexts through RNA-directed DNA methylation (RdDM). RdDM methyltransferases are recruited by a RISC complex (blue grey) that is guided to the target loci by Pol V-generated complementary transcripts (dark grey). (B) DNA methylation is maintained by specific methyltransferases, depending on the sequence context of methylation. For CHH methylation, methyl marks are conserved via RdDM together with CMT2. (C) ROS1--specific demethylation is activated via the recruitment of H3K18ac and H3K23ac (blue cylinder) which interact with IDM1 and MBP7 (grey circles) at dense CG regions.

CHAPTER 2

**EXPRESSION PATTERNS OF DNA METHYLATION AND
DEMETHYLATION GENES DURING ARABIDOPSIS DEVELOPMENT
AND IN RESPONSE TO PHYTOHORMONES**

This chapter is based on the submitted paper:

“Expression patterns of DNA methylation and demethylation genes during plant development and in response to phytohormones” by Morgan Bennett, Kailyn Cleaves, and Tarek Hewezi. MB was responsible for carrying out the experiments and contributed to data analysis, interpretation of results, and writing the manuscript.

2.1 Abstract

DNA methylation and demethylation precisely and effectively modulate gene expression during plant growth and development and in response to stress. However, expression profiles of genes involved in DNA methylation and demethylation during plant development and their responses to phytohormone treatments remain largely unknown. The spatiotemporal expression patterns of genes involved in *de novo* methylation, methyl maintenance, and active demethylation in roots, shoots, and reproductive organs were characterized using β -glucuronidase (GUS) reporter lines. In general, promoters of DNA demethylases were more highly active at the mature root tissues, whereas promoter of genes involved in DNA methylation were more highly active at fast-growing root tissues. The promoter activity also implies that methylation status in shoot apical meristems, leaf primordia, floral organs, and developing embryos is under tight equilibrium through the activity of genes involved in DNA methylation and demethylation. The promoter activity of DNA methylation and demethylation-related genes in response to various phytohormone treatments revealed that phytohormones can alter DNA methylation status in specific and redundant ways. Overall, the results illustrate that DNA methylation and demethylation pathways act synergistically and antagonistically in various tissues and in response to phytohormone treatments and point to the existence of hormone-linked methylome regulation mechanisms that may contribute to tissue differentiation and development.

Keywords: Arabidopsis, DNA demethylases, DNA methyltransferases, embryogenesis, GUS activity, plant growth and development, phytohormones

Abbreviations: abscisic acid (ABA), auxin (AUX), cytokinin (CK), ethylene (ET), gibberellic acid (GA), RNA-directed DNA methylation (RdDM), root apical meristem (RAM), salicylic acid (SA), shoot apical meristem (SAM)

2.2 Introduction

Epigenetic mechanisms coordinate the expression of thousands of genes in a heritable, yet flexible manner without changing the underlying DNA sequence (Henderson and Jacobsen, 2007). DNA methylation, along with histone modifications, chromatin remodeling, and non-coding sRNAs constitute the highly dynamic and interconnected epigenome, which contributes to the regulation of gene expression during plant development and stress response (Fransz and Jong, 2002; Zilberman et al, 2007; Zhang et al, 2010; Matzke and Mosher, 2014; Du et al, 2015; López Sánchez et al, 2016; Hewezi et al, 2018; Hewezi, 2020). DNA methylation is also involved in transposable element (TE) regulation, reproduction, genomic imprinting, intercellular communication, interallelic communication, and genome interactions (Matzke and Mosher, 2014). DNA methylation is generally regarded as a repressive mark of gene expression. DNA methylation in the promoter of protein coding and microRNA genes has been generally shown to cause transcriptional repression despite recent reports point to a possible role of DNA methylation in gene transcriptional activation (Zilberman et al, 2007; Hewezi et al, 2017; Lang et al, 2011; Huang et al, 2019; Rambani et al, 2020). The functional role of DNA methylation in gene body regions, however, remains largely unknown even though recent studies associate DNA methylation levels and patterns with gene expression and splicing efficiency (Wang et al, 2016; Bewick and Schmitz; 2017; Niyikiza et al, 2020).

DNA methylation is the addition of a methyl group to the C5 position of cytosine and occurs in the CG, CHG, and CHH sequence contexts (where H is any nucleotide except G) (Lister et al, 2008). In *Arabidopsis*, the *de novo* formation of 5-methylcytosine (5mC) is catalyzed by DNA methyltransferases DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) and DRM3 through the RNA-directed DNA methylation (RdDM) pathway (Cao and Jacobsen, 2002;

Cao et al, 2003; Henderson et al, 2010).

DNA methylation is maintained by distinct sequence context-dependent enzymes following DNA replication and cell division (Saze et al, 2003). Methylation in the CG context is maintained by METHYLTRANSFERASE1 (MET1) (Kankel et al, 2003). There is also evidence that MET1 paralogs MET2b and MET3 contribute to the maintenance of CG methylation (Quadrana et al, 2016). VARIANT IN METHYLATION1 (VIM1), VIM2, and VIM3 and the heterochromatin-remodeling component DECREASE IN DNA METHYLATION (DDM1) are also considered important factors for CG DNA methylation (Zemach et al, 2013; Kim et al, 2014; Long et al, 2018). CHG methylation is maintained by CHROMOMETHYLASE2 (CMT2) and CMT3 (Stroud et al, 2014). For CHH methylation, CMT2 maintains DNA methylation within pericentromeric long TEs, whereas the RdDM pathway is employed for the maintenance of CHH methylation at other loci (Law and Jacobsen, 2010; Zemach et al, 2013). The catalytically mutated paralog DRM3 is required for *de novo* methylation and maintenance of methylated CHH loci at repetitive sequences (Henderson et al, 2010; Costa-Nues et al, 2014). In addition, CMT1 often exists as a truncated CMT protein and is thought to be involved in the maintenance of non-CG methylation (Stroud et al, 2014).

As with any balanced system, active DNA demethylation is an equally important epigenetic process for controlling genome-wide methylation levels (Gong et al, 2002; Penterman et al, 2007a; Penterman et al, 2007b; Zhu et al, 2007; Ortega-Galisteo et al, 2008). In Arabidopsis, four DNA demethylase paralogs have been identified for active DNA demethylation: REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DME-LIKE2 (DML2) and DML3 (Choi et al, 2002; Gong et al, 2002; Agius et al, 2006; Gehring et al, 2006; Morales-Ruiz et al, 2006; Penterman et al, 2007b). These DNA demethylases remove methylated cytosines through the base excision repair (BER) mechanism and replace them with unmethylated cytosine (Zhang and Zhu, 2012). Experimental evidence indicate that DNA methylation and active DNA demethylation are highly synchronized (Lei et al, 2015). ROS1 is tightly controlled by the methylation level of an RdDM target sequence upstream of the transcriptional start site, called a DNA methylation monitoring sequence (MEMS). MEMS is hypermethylated when cellular RdDM increases or DNA demethylation decreases. MEMS methylation upregulates ROS1, thereby activating DNA

demethylation (Lei et al, 2015).

DNA methylation-mediated transcriptional reprogramming has been reported in various plant developmental processes, from gametogenesis to fruit ripening. Local and global DNA methylation and demethylation are also heavily involved in plant responses to environmental stimuli, including biotic (Dowen et al, 2012; Le et al, 2014; López Sánchez et al, 2016; Ramirez-Prado et al, 2018) and abiotic (Secco et al, 2015; Zhang et al, 2016; Hewezi et al, 2018; Guo et al, 2019; Hewezi, 2020;) stresses. However, the nature of the biochemical signals that associate with DNA methylome changes during plant development and stress responses are not understood. Despite several studies pointing to a role for phytohormone signaling in controlling epigenetic pathways, direct experimental evidence remains lacking (Zhu, 2010; Yamamuro et al, 2016; Maury et al, 2019; Xiao et al, 2020).

Many orthologous DNA methyltransferases and demethylases have been identified in non-model plant species as well as evolutionary unrelated organisms including invertebrates, humans, and other mammals. Thus, DNA methylation pathways are thought to be conserved and functionally significant. DNA methylation has long been recognized as a critical component of gene regulation, biochemical and hormonal balance, and environmental plasticity (Vanyushin, 2005; Kooke et al, 2015; Herman and Sultan, 2016; Yamamuro et al, 2016). However, the spatial and temporal expression patterns of genes involved in DNA methylation and demethylation throughout growth and development are largely unknown. In this study, the expression profiles of 15 genes involved in DNA methylation and active DNA demethylation in roots, shoots, and reproductive tissues was established using GUS reporter lines. In order to better understand the connection between DNA methylation and phytohormones, the spatiotemporal expression patterns of genes involved in DNA methylation and demethylation in roots and shoots was also assessed in response to treatment of six phytohormones: auxin (AUX), cytokinin (CK), gibberellic acid (GA), ethylene (ET), abscisic acid (ABA), and salicylic acid (SA). These results reveal unique and overlapping expression patterns of these genes in roots, shoots, inflorescences, siliques, and developing seeds and highlight the importance of DNA methylation—demethylation equilibrium in these tissues. Additionally, these results point to a role of phytohormone signaling in establishing plant methylomes associated with differentiation and development of various tissues.

2.3 Materials and methods

Plant materials and growth conditions

All transgenic *Arabidopsis* lines were generated in the Col-0 ecotype background. Plants were grown in controlled growth chambers at 22 °C under long day conditions, 16 hours of light and 8 hours of dark.

Generation of GUS reporter lines

Promoter regions (up to 2 kb region upstream of the translation start codon ATG) were amplified from Col-0 genomic DNA using primers containing restriction enzyme sites (Table 1). In some cases, the distance between the ATG and the 3'untranslated region (3'UTR) of the neighboring gene is less than 2 kb. Therefore, the largest possible promoter region without including the 3'UTR of the adjacent genes was used. Amplified PCR products were digested, purified, and cloned upstream of the β -glucuronidase (GUS) gene in the binary vector pBI101. Constructs were confirmed by sequencing before transferring into *Agrobacterium tumefaciens* (strain C58). The functionality of these constructs was validated using an *A. tumefaciens* infiltration assay (Li et al, 2009). Four-day-old seedlings were infiltrated with transformed *A. tumefaciens* for 36 hours and seedlings were analyzed for GUS activity. Within 0.5 to 24 hours of staining, colorimetric changes were visually observed for all infiltrated seedlings, confirming the function of all gene promoters.

Agrobacteria containing the binary vectors were then used to transform *Arabidopsis* (Col-0) using the floral dip method (Clough and Bent, 1998). Transgenic T1 plants were screened on MS medium containing 50 mg/L kanamycin and 100 mg/L cefotaxime. Transgenic T1 lines were self-fertilized to generate T2 lines. T2 and subsequent generations were used for GUS assays.

Histochemical analysis of GUS activity

At least two independent transgenic lines were assayed for promoter activity, with at least six biological replicates. Seeds were grown in 12-well plates containing MS medium for root and

shoot assays. For GUS assays in reproductive tissues, transgenic plants were grown in soil at 22 °C under long day conditions. Roots and shoots or reproductive tissues, including buds, open flowers, emerging siliques, fully developed siliques, and mature siliques from fourteen-day-old plants were assayed for GUS activity (Jefferson et al, 1987) using the X-Gluc substrate (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) at 37°C and checked every 30 minutes for colorimetric changes. GUS staining time ranged from 30 minutes to 12 hours based on the promoter activity. Once the staining is clearly visible the reaction was terminated, to avoid overstaining, by replacing the GUS solution with 70% ethanol. The samples were then stored in ethanol at room temperature. Developing seeds were also precleared using 1:1 acetic acid ethanol for 4-6 hours and then cleared for 5 days using 75% strength Hoyer's medium without gum arabic (Stangeland and Salehian, 2002).

Phytohormone treatments

Seeds of the transgenic GUS reporter lines were surface-sterilized and planted in 12-well plates containing MS medium in controlled growth chambers at 22 °C and 16 h light/8 h dark cycle. At 13 day after planting, phytohormone and control treatments were applied to the transgenic seedlings. Excess liquids were removed using autoclaved filter papers. Phytohormone and control treatments were maintained for 24 hours before determining GUS activity by adding GUS solution directly to the 12-well plates. The following phytohormones were used at the designated concentrations: 5 nM indole-3-acetic acid (IAA, AUX), 15 nM 6-benzylaminopurine (BAP, CK), 75 µM gibberellic acid (GA), 10 µM 1-aminocyclopropane-1-carboxylic acid (ACC, ET), 10 µM abscisic acid (ABA), and 2 mM salicylic acid (SA) as previously described (Smalle et al, 1997; Fujii and Zhu, 2009; Bidadi et al, 2010; Khokon et al, 2011; Piya et al, 2019). Stock solutions of these phytohormones were prepared in 100% ethyl alcohol (IAA, ABA and SA), 1 N NaOH (BAP), or distilled water (GA and ACC). Mock treatments for control plants were carried out using distilled water (for GA and ACC treatments), 1.63×10^{-9} mol/L, 0.224 mol/L, and 0.08003 mol/L ethyl alcohol for IAA, ABA and SA treatments, respectively. Mock-treated control plants were included for each experiment and compared to treated plants. Phytohormone treatments were performed with two independent transgenic lines per construct.

Quantitative reverse transcription PCR (qRT-PCR) analysis

The expression levels of phytohormone marker genes for auxin *AUXIN RESPONSE FACTOR19* (*ARF19*, *AT1G19220*), cytokinin [*ARABIDOPSIS RESOPNSE REGULATOR7* (*ARR7*), *AT1G19050*], gibberellic acid [*RGA-LIKE 1* (*RGL1*)], ethylene [*ETHYLENE RESPONSE 2* (*ETR2*), *AT3G23150*], abscisic aid (*MYB96*, *AT3G22830*) and salicylic acid [*PATHOGENESIS-RELATED GENE 1* (*PR1*), *AT2G14610*] were quantified in phytohormone-treated roots and shoots. Seeds of Arabidopsis wild-type ecotype Col-0 were planted in 12-well plates containing MS medium, and 13-day-old seedlings were subjected to various phytohormone and control treatments as described above. Twenty-four hours after the treatments, leaves and roots tissues were collected in three biological samples and used for RNA extraction and *qRT-PCR* quantification. Total RNA was extracted using the Zymo Direct-zol RNA mimiprep kit (R2050). *qRT-PCR* quantification was performed using 30 ng of DNase-treated RNA was used per reaction with Verso SYBR green 1-step *qRT-PCR* (Thermo Scientific, USA) according to the manufacturer's instructions. *qRT-PCR* was performed in the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, USA) using the following program: 50°C for 15 min, 95°C for 15 min followed by 40 cycles of 95°C for 15 s, 60°C for 90 s and 72°C for 30 s. The PCR products were then subjected to 95°C for 15 s and 60°C for 75 s, followed by a slow gradient from 60°C to 95°C to generate the dissociation curves. Gene expression levels were normalized using two different reference genes: *Actin8* (*ACT8*, *AT1G49240*) and *PROTEIN PHOSPHATASE 2A SUBUNIT A3* (*PP2AA3*, *AT1G13320*) (Piya et al, 2020). Phytohormone-treated roots and shoots were then compared to mock-treated roots and shoots, respectively. Primer sequences of phytohormone marker genes are provided in Table S1.

Light microscopy and imaging

Roots were imaged immediately because of their rapid degradation, even preserved in ethanol. Images of media-grown roots were captured from the bottom of the plate using an EVOS M7000

microscope. Shoots and reproductive tissues, on the other hand were incubated in 70% ethanol for a few days to remove chlorophyll pigmentation, allowing GUS expression patterns to be clearly visible. These tissues were then imaged using a Zeiss Stemi 2000-CS dissecting microscope.

RNA-seq data analysis

Gene expression data of DNA methylation and demethylation-related genes in roots, root tips, leaf, petiole, buds, open flower and mature silique were downloaded from TraVA (Transcriptome Variation Analysis, <http://travadb.org/>) (Klepikova et al, 2016). Absolute RNA-seq read counts normalized by trimmed mean of M samples (TMM) were log10 transformed and used to generate a heatmap using the R package gplots and heatmap.2 function.

2.4 Results

2.4.1 Generation of GUS reporter lines of 15 DNA methylation and demethylation-related genes

In order to assess the spatial and temporal expression patterns of genes involved in DNA methylation and demethylation in various plant tissues and organs, a number of transgenic GUS reporter lines were generated. The 15 selected genes include known cytosine methyltransferases, DNA demethylases (or glycosylases), and other genes required for DNA methylation, all of which are listed in Table S1. Each reporter construct expresses GUS under the control of the gene's respective native promoter (i.e., up to approximately 2 kb upstream of the translation start codon). GUS expression assays were performed and analyzed using at least two independent transgenic lines per construct in 2-week-old roots, shoots, and in various reproductive tissues and organs.

2.4.2 Expression patterns of DNA methylation and demethylation-related genes in roots

Several genes involved in DNA methylation and demethylation, including *MET1*, *CMT1*, *DRM2*, *DRM3*, *DME*, *DML2*, and *DML3* are highly expressed throughout root tissues (Figure 2.1). The CG methyltransferase *MET1* is highly expressed toward the newly grown parts of the root and into the root tips as well as in developing lateral roots (Figure 2.1A). However, other genes involved

in CG methylation, *MET2b* and *DDM1*, have very limited expression in roots (Figure 2.1A). *VIM1* expression is observed in the tips of main and lateral roots and in the vascular tissues of the newly grown parts of the root (Figure 2.1A). *DDM1* is uniquely expressed in the root cap of primary root and lateral root primordia (Figure 2.1A).

Promoter of the *de novo* methyltransferases *DRM2* and *DRM3* are strongly active in most of the root tissues, particularly in the newly grown parts of the root and in the root tip, similarly to *MET1* (Figure 2.1B). Thus, it seems likely that *DRM2* and *DRM3* participate in *de novo* CG methylation and *MET1* maintains those methylated cytosines following DNA replication in rapidly driving root tissues. While *CMT1* is expressed in the majority of root tissues, its expression is mostly towards the mature end of the roots (i.e., towards the shoot) as well as at the base of the lateral roots (Figure 2.1B). Similar to *CMT1*, *CMT3* is expressed at the mature end of the root, but to a lesser extent (Figure 2.1B). Therefore, it appears that *CMT1* and *CMT3* have more specific roles in non-CG methyl maintenance at the mature end of the root tissues.

The DNA demethylase *ROS1* is expressed exclusively in the root apical meristem (RAM) and in root caps of the main and auxiliary roots (Figure 2.1C). *DME*, *DML2*, and *DML3*, on the other hand, are expressed throughout the root, with *DME* being the most highly expressed (Figure 2.1C). *DML2* and *DML3* have similar expression patterns, predominantly towards the mature end. These data support the conclusion that *DME*, *DML2* and *DML3* have overlapping functions in active DNA demethylation in most parts of the root tissues.

2.4.3 Expression patterns of DNA methylation and demethylation-related genes in shoots

GUS staining shows that all tested DNA methyltransferases and demethylases are expressed in the two-week-old shoot tissues (Figure 2.2). Interestingly, all these genes are transcriptionally active in leaf primordia, suggesting a role for DNA methylation-demethylation equilibrium in leaf morphogenesis. Also, the promoter of *MET1*, *MET2b*, *VIM2*, and *DDM1* showed activity in the shoot apical meristem (SAM) (Figure 2.2A). Notably, *ROS1*, *DME*, and *DML2* appear to be the most highly active genes in leaf primordia (Figure 2.2A, C). Together, these results suggest that DNA demethylation plays an important role during early stages of leaf differentiation and development.

The DNA demethylases *ROS1*, *DME*, and *DML2* are highly expressed in shoots (Figure 2.2C). *ROS1* and *DML2* are strongly expressed particularly in young leaves. *DME*, on the other hand, is highly expressed in leaves, cotyledons, and trichomes, irrespective of leaf developmental stage. These data indicate that *DME* is driving active DNA demethylation throughout leaf and cotyledon development and that *ROS1* and *DML2* contribute to this process merely during early stages of leaf development. The localized expression patterns of *DML3* at the tips of leaves and cotyledons suggest its contribution to active DNA demethylation, specifically in these regions.

Of the genes involved in DNA methylation, *MET1*, *VIM1*, *VIM2*, *CMT1*, *DRM2*, and *DRM3* were highly expressed in leaf tissues (Figure 2.2A-B). *VIM1* and *VIM2* showed stage-specific expression patterns in leaves, where *VIM1* is expressed in mature leaves and *VIM2* is expressed in younger tissues (Figure 2.2A). *CMT1* and *DRM2* showed distinct expression in leaves. *CMT1* is highly expressed in leaf petiole and vascular tissues, whereas *DRM2* is expressed in leaf blades but not in petioles or leaf vascular tissue (Figure 2.2B). To the contrary, *MET1* and *DRM3* display expression patterns similar to each other. Both are expressed in leaf blades and in cotyledon vascular tissues (Figure 2.2A-B), suggesting a coordinated methylation of CG and CHH loci in these tissues.

2.4.4 DNA methylation and demethylation expression patterns in reproductive tissues

2.4.4.1 Buds and open flowers

This analysis reveals that several genes involved in DNA methylation and demethylation are expressed in open flowers compared and buds (Figure 2.3). Of the tested genes, only the CG methyltransferase *MET1* was expressed in unopened, pre-anthesis flowers. *MET1*, *VIM1*, *DRM2*, *DRM3*, *DME*, and *ROS1* are expressed in various tissues of open flowers. Remarkably, *DRM2* and *DME* showed strong expression in petals, anthers, and stigma, suggesting a role for these two enzymes in DNA methylation fine-tuning during flower development (Figure 2.3B-C). *ROS1* is expressed in filaments, suggesting a unique role for DNA demethylation in this structure (Figure 2.3C). In addition to *MET1*, *DRM2*, *DRM3*, and *DME* are highly expressed in pollen grains (Figure 2.3), reinforcing the hypothesis that *DRM2* functions in parallel with *DRM3* in RdDM pathway (Zhong et al, 2015; Costa-Nunes et al, 2014; Henderson et al, 2010). Taken together, these results

demonstrate that DNA methylation and demethylation pathways are involved in flowering and fertilization.

2.4.4.2 Siliques

DNA methylation and demethylation pathways are particularly active in the junction between the silique and the pedicle (or internode) (Figure 2.3). This region is composed of the gynophore, nectaries, and abscission zone and functions in attaching the silique to the pedicle until mature seeds are ready for dispersal (Roeder and Yanofsky, 2006). In the emerging siliques, *VIM1* promoter is active in the pedicle, internode junction, and style (Figure 2.3A). *VIM2* expression is predominant in the pedicle, but distinctly absent from the internode junction (Figure 2.3A). Based on these results, *VIM1* could be involved in the early stages of fruit ripening and pedicle development in association with *VIM2*.

It was also evident from GUS activity that genes involved in CG methylation are mostly expressed in early stages of silique development, whereas genes involved in *de novo* methylation, non-CG methylation, and DNA demethylation were expressed throughout silique development (Figure 2.3). *DME* is the highest DNA demethylase expressed in mature silique tissues (Figure 2.3C). Notably, *DRM2* and *DMR3* appear to have roles in non-CG context methylation of the distal and proximal ends of mature siliques, respectively. In addition, *CMT1* and *DRM2* show overlapping expression patterns in the style of mature siliques (Figure 2.3B).

2.4.4.3 Seeds

These results demonstrate that promoters of many genes involved in DNA methylation and demethylation are also active in the developing seeds from the globular to mature stages of embryogenesis. The CG methyltransferases *MET1* and *MET2b* are highly expressed both in the endosperm and embryo throughout all stages of embryogenesis, whereas *MET3* is highly expressed during early embryo developmental stages and decreases with advancement in development (Figure 2.4A). In contrast to *MET3*, *VIM2* showed increased expression in endosperm and embryo with advancement in development (Figure 2.4A). *VIM1* is expressed exclusively at the globular and heart stages of embryogenesis. However, during the walking-stick and bent-cotyledons embryo stages, the expression of *VIM1* was observed both in endosperm and

embryonic tissues (Figure 2.4A). *DDM1* is specifically expressed at the tip of the radical in later stages of embryo development (Figure 2.4A).

Of the non-GC methyltransferases expressed, *DRM2* is the most highly expressed genes, showing expression both in endosperm and embryo issues throughout all embryo developmental stages (Figure 2.4B). *CMT1* and *CMT2* seem to be expressed in a sequential manner during embryogenesis. While *CMT1* is expressed at the globular and heart stages, *CMT2* is expressed at the heart, torpedo and walking-stick stages (Figure 2.4B). *DRM3* exhibits specific expression in the elaiosome at all embryo stages. Weak expression of *DRM3* was also detected in the embryo and endosperm at the walking-stick and bent-cotyledon stages (Figure 2.4B).

The DNA demethylases *DME* and *DML3* exhibit strong GUS staining in endosperm and embryo during all embryo developmental stages (Figure 2.4C). High activity of *DML2* promoter was observed during early stages of embryogenesis (globular and heart), and then declined in more advanced embryonic stages (torpedo and walking-stick) (Figure 2.4C). *ROS1* showed the lowest expression among the four DNA demethylases, as its expression was barely detected only at the bent-cotyledon stage (Figure 2.4C). Together, these results indicate that embryogenesis and seed development involve dynamic reprogramming DNA methylation and active demethylation. GUS lines that did not exhibit a colorimetric change upon GUS staining are documented in Figure 2.5.

2.4.5 RNA-seq data support promoter activity of DNA methylation and demethylation-related genes

To provide confirmation of these promoter activity data, GUS activity of DNA methylation and demethylation-related genes was compared with gene expression levels in root, root tips, leaf, petiole, bud, open flower and mature silique using TraVA RNA-seq database (Klepikova et al, 2016). The gene expression levels are generally in agreement with the observed GUS activity of DNA methylation and demethylation-related genes in various tissues. For example, high expression levels of *MET1*, *DRM3*, and *DME* in root and root tips are in agreement with these data showing strong GUS activity of these three genes in these tissues (Figure 2.6). Similarly, the high expression levels of *ROS1* and *DME* in leaf and leaf petiole; *MET1*, *VIM1*, *DRM2*, *DRM3*, and

DME and in buds and open flower; and DME in mature silique are consistent with GUS activity presented here (Figure 2.6). Also, the relative low GUS activity of *MET2b*, *MET3*, *CMT1*, and *DML3* in various tissues are congruent with RNA-seq data (Figure 2.6).

2.4.6 Promoter activity of DNA methylation and demethylation-related genes in roots following phytohormone treatments

Next, GUS activity in the roots of the transgenic lines was assessed in response to phytohormone treatments, including auxin (AUX), cytokinin (CK), gibberellic acid (GA), ethylene (ET), abscisic acid (ABA), and salicylic acid (SA). The effectiveness of phytohormone treatments in triggering local and systemic responses in leaves and roots, respectively, was examined by quantifying the expression levels of marker genes for AUX (*AUXIN RESPONSE FACTOR19*, *ARF19*), CK (*ARABIDOPSIS RESPONSE REGULATOR7*, *ARR7*), GA (*RGL1*), ET (*ETHYLENE RESPONSE 2*, *ETR2*), ABA (*MYB96*), and SA (*PATHOGENESIS-RELATED GENE 1*, *PR1*) responses (Wen and Chang, 2002; Nemhauser et al, 2006; Mathur et al, 2013) in phytohormone-treated plants and the corresponding controls 24 hours after phytohormone application using *qRT-PCR*. Data from three biological replicates revealed significant induction of all these marker genes both in shoot and root tissues as compared with the corresponding mock-treated controls (Figure 2.7), confirming the effectiveness of phytohormone treatments in inducing local and systemic responses.

SA treatments inhibited the expression of *MET1*, *VIM1*, *DDM1*, *DRM2*, and *DRM3*. Interestingly, the expression of all four DNA demethylases is also inhibited in response to SA application. SA treatment, however, enhanced the expression of *CMT1*. Thus, SA seems to inhibit DNA methylation and demethylation pathways alike, with the exception of *CMT* family genes (Figure 2.8). ABA treatment enhanced the expression of *VIM1* and *CMT1* but reduced the expression of *DRM2* and *DML3* (Figure 2.8). AUX treatment induced a reduction in the expression of *DRM2* and *DML3* and an increase in the expression of *CMT3* (Figure 2.8B-C). Notably, GA treatment elicited an increase in the expression of *DML2* and *DML3* (Figure 2.8C). CK and ET treatments negatively regulated the expression of *CMT2* and *CMT3*, respectively (Figure 2.8B). The upregulation of *DML2* and *DML3* upon GA treatment and their downregulation following SA

treatment points to a possible role of active DNA demethylation in establishing the equilibrium between plant growth and defense responses mediated by GA and SA. A summary description of changes in promoter activity of DNA methylation and demethylation-related genes roots in response to various phytohormone treatments is provided in Table 2.2.

2.4.7 Promote activity of DNA methylation and demethylation-related genes in shoots following phytohormone treatments

As shown in Figure 2.9, the promoter activity of genes involved in DNA methylation and demethylation were altered in phytohormone-treated shoots. Exogenous applications of AUX, CK, GA, ET, and ABA inhibited the expression of *MET1*, *VIM2*, and *DDM1*. Notably, GA treatment oppositely affected *VIM1* and *VIM2*; positively regulating *VIM1* and negatively regulating *VIM2* (Figure 2.9A). In contrast, ABA treatment upregulated *VIM1* and completely suppressed *VIM2* (Figure 2.9A). The expression patterns observed for *VIM1* and *VIM2* in response to ABA and GA treatments suggest that they have complementary roles in CG methylation both during development and in response to stress. Overall, genes involved in CG methylation are largely negatively regulated by the phytohormones tested (Figure 2.9A).

SA treatment negatively impacted *MET1* and *DDM1* expression, but positively influenced *VIM2* expression. Specifically, SA treatment inhibited *MET1* expression in older leaves, producing a similar expression pattern to that of untreated *VIM2*. SA-treated *VIM2* shoots, however, are upregulated in older leaves. The same pattern is evident for *DRM2* and *DRM3* in response to SA treatment. Thus, SA evidentially modulates DNA methylation in mature leaves (Figure 2.9A-B). SA and ABA treatments yield distinctly opposite expression patterns for *DRM3* (Figure 2.9B). In addition to total suppression of *VIM2*, ABA treatment also inhibited the expression of *CMT3*, suggesting ABA also regulates components of DNA methylation in two-week old shoots (Figure 2.9A-B). Furthermore, GA completely suppressed expression of *CMT3*, but enhanced expression of *DRM2*, giving opposite and complementary expression patterns of these two genes following GA treatment (Figure 2.9B).

Both ET and AUX treatments reduced *DME* expression in fast-growing tissues but enhanced its expression in mature leaves (Figure 2.9C). Interestingly, the expression of *DML2* or *DML3* was

completely suppressed by ET or AUX, respectively (Figure 2.9C). In addition, ET treatment resulted in an increase in *ROS1* expression (Figure 2.9C). Therefore, in response to ET or AUX treatments, *ROS1* appears to be the most responsive DNA demethylase in leaf primordia and young leaves, whereas DME is the most responsive DNA demethylase in more mature shoot tissues (Figure 2.9C). SA treatment also impacted DNA demethylation. *DML3* and *DME* expression was suppressed by SA. In contrast, the expression of *ROS1* and *DML2* was enhanced in young leaves by SA treatment (Figure 2.9C). Thus, in general, SA treatment not only inhibits DNA methylation in older leaves, but it also enhances DNA demethylation in fast-growing tissues. The expression patterns of *MET2b*, *MET3*, and *CMT1*, and *CMT3* did not alter in response to any of the applied hormone treatments (Figure 2.10B). A summary description of changes in promoter activity of DNA methylation and demethylation-related genes in response to various phytohormone treatments is provided in Table 2.3. The expression patterns of *MET2b*, *MET3*, and *VIM2* did not alter in response to any of the applied hormone treatments (Figure 2.10A).

2.5 Discussion

2.5.1 Expression patterns of DNA methylation and demethylation-related genes in various plant tissues and organs

This analysis revealed that DNA demethylases were generally more highly expressed at the mature end of the root, whereas genes involved in DNA methylation were more highly expressed in the newly grown regions of the roots (Figure 1). The spatial expression patterns of these reporter lines together with the broader distinction between mature and newly grown parts of the root are congruent with previous DNA methylation profiles reported in root tissues (Bartels et al, 2018). These results also substantiate previous conclusions that DNA methylation is important for the development of young fast-growing tissues (Baubec et al, 2014). In addition, high expression levels of non-CG methyltransferases *DRM2* and *DRM3* at the root tip (Figure 1B) is consistent with the findings that hypermethylation in root tips occurs primarily in the CHH context (Bartels et al, 2018). These finding that several of the tested genes exhibit differential expression patterns

in cotyledons, leaf primordia, young and old leaves establish DNA methylation as part of the regulatory mechanism controlling shoot differentiation. This is consistent with the finding that DNA methylation plays a key role in cell identity (Maury et al, 2019).

Transition from juvenile to adult tissues and subsequent reproductive development is highly dependent on DNA methylation alongside other regulatory factors and exogenous signals (Yang et al, 2015; Yamamuro et al, 2016; Campos-Rivero et al, 2017). It has been shown that pre-anthesis buds are hypomethylated directly following the vegetative to reproductive transition, but the mechanism behind this loss of DNA methylation has not been determined (Campos-Rivero et al, 2017). The absence of DNA methyltransferase and demethylase expression in pre-anthesis buds (Figure 3) supports the notion that hypomethylation of pre-anthesis floral organs is due to passive DNA demethylation. In contrast, reproductive tissues are hypermethylated during floral establishment and in subsequent stages of development (Campos-Rivero et al, 2017; Bartels et al, 2018). These results point to *MET1*, *VIM1*, *DRM2*, and *DRM3* as key contributors to modulating the methylome status for the initial hypermethylation in early floral development (Figure 3A-B). In addition, *VIM2* and *DDM1* appear to play a role in later stages of flower development at the receptacle (Figure 3A). This analysis also suggests that methylation status in various floral organs is under tight equilibrium through the activity of genes involved in DNA methylation and demethylation. For example, the high expression of *DME* in carpel, stamen, and pollen was accompanied by high expression of *MET1*, *DRM2*, and *DRM3* (Figure 3A-C).

The high expression of several genes involved in DNA methylation and demethylation during various stages of seed development implies that the methylomes of developing seeds are established through coordinated functions of methyltransferases and demethylases. *MET2b* and *MET3* were highly expressed during embryogenesis, despite their expression being barely detectable in other tissues (Figure 4A). *MET1* homologs have been reported to express exclusively during embryogenesis (Jullien et al, 2012; Xu et al, 2016). This finding suggests that *MET2b* and *MET3* acquired tissue-specific function to support the activity of *MET1* in maintaining CG methylation during seed development (Figure 4A). The embryo-specific expression of *VIM1* suggests a coordinated function with *MET1* to maintain CG methylation at specific loci, as suggested by Kim et al, (2014).

The transcriptional activity of *CMT1*, *CMT2*, and *DRM2* during embryogenesis is consistent with the programmed increase in CHH methylation during seed development reported in Arabidopsis and other plants (Hsieh et al, 2009; Zemach et al, 2010; Jullien et al, 2012; Zhang et al, 2014; Lin et al, 2017). This points to their implication in maintenance and *de novo* CHH methylation, respectively. These data also suggest that *CMT1* and *CMT2* sequentially support *DRM2* in maintaining CHH methylation, particularly in TEs during early and late stages of seed development, respectively (Figure 4B). In addition, the expression of *CMT2* is in line with the significant increase of global CHH-context methylation within TEs located in pericentromeric region during seed development (Lin et al, 2017).

During embryogenesis and seed development, the endosperm tissues are hypomethylated particularly at short TEs located nearby genes (Gehring et al, 2009; Hsieh et al, 2009; Ibarra et al, 2012). *DME* was established as the main demethylase responsible for genome-wide hypomethylation of Arabidopsis endosperm (Gehring et al, 2009; Hsieh et al, 2009; Gehring and Satyaki, 2017). In addition to the role of *DME*, these data suggest a key role for *DML2* and *DML3* in this process and their role in establishing a hypomethylated endosperm merits further analysis (Figure 4C).

Tomato DML2-mediated DNA demethylation contributes to global hypomethylation for fruit ripening (Guo et al, 2019; Zuo et al, 2018; Lang et al, 2017; Liu et al, 2015). These assays show that *ROS1* and/or *DME* were expressed at silique internodes throughout development and that *DME* is highly expressed in the replum and septum, indicating their role in silique maturation (Figure 3C). Together with the expression of genes involved in DNA methylation in internodes, replum, stigma, and style (Figure 3A-B), these results suggest that DNA methylation and demethylation have a role in silique development and fruit ripening as recently reported (Xing et al, 2015; Kawakatsu et al, 2017; Lang et al, 2017; Huang et al, 2019).

2.5.2 Phytohormone-induced changes in the expression patterns of DNA methylation and demethylation-related genes

These results suggest that phytohormones can alter DNA methylation dynamics in a specific and coordinated manner. In root tissues, SA treatment inhibited the expression of all tested genes, with

the exception of the *CMT* gene family (Figure 5). Thus, the role of salicylic acid in root initiation and root growth (Dempsey and Klessig, 2017) may be brought about in part through inactivation of DNA methylation pathways. SA-induced *CMT1* expression in lateral roots implies a unique function of this enzyme in salicylic acid-mediated methylome changes in lateral roots (Figure 5B). Consistent with the role of salicylic acid in root apical meristem (RAM) patterning via auxin redistribution (Pasternak et al, 2019), these results point into a possible role of salicylic acid-dependent induction of *CMT2* in this process, since *CMT2* was the only gene expressed in the RAM of both AUX- and SA-treated roots (Figure 5B).

MET1 and the RdDM pathway have been identified to regulate active DNA demethylation through the methylation of a regulatory sequence in the *ROS1* promoter (Lei et al, 2015). These GUS assays in roots and shoots support this regulation and implicate salicylic acid in this regulatory mechanism. In roots, the expression of *MET1*, *DRM2*, and *DRM3* in root tips, and *ROS1* in root cap was strongly repressed in response to SA treatment (Fig 5). Similarly, in shoots, SA treatments reduced or eliminated the expression of *MET1*, *DRM2*, *DRM3*, and *ROS1* in older leaves and/or cotyledons (Figure 6). ABA maybe also involved in this regulatory mechanism particularly in younger leaves as the expression of *MET1*, *DRM3*, and *ROS1* was reduced or eliminated in these tissues upon ABA application (Figure 5).

AUX, CK, ET and ABA treatments also impacted the expression of certain DNA methylation and demethylation-related genes in various root tissues, suggesting that a hormone-linked methylome regulation mechanism may contribute to root growth and development. In many cases, phytohormone treatments resulted in obvious impact on GUS expression in the shoots. Some genes showed similar responses to various hormone treatments as in the case of *MET1* and *DDM1*, whose expression was inhibited in response to all hormone applications (Figure 6A). Other genes showed opposite responses to certain hormone treatments. For example, *VIM2* was positively regulated by SA and GA treatments in older leaves but its expression was strongly suppressed by ABA (Figure 6A). Interestingly, *ROS1* expression is enhanced in response to GA and ET treatments, but repressed by CK (Figure 6C), suggesting that *ROS1*-mediated DNA demethylation can be modulated by phytohormone signaling. Also, some hormone applications can differentially influence the expression of gene family members. For instance, *VIM1* was upregulated in leaves

following ABA treatment, whereas *VIM2* expression was almost completely abolished. A similar effect was also observed in response to GA treatment (Figure 6A). Additionally, hormone applications triggered tissue-specific expression patterns. In this context, *DRM3* was found to be downregulated in younger leaves and upregulated in older leaves in response to ABA treatment, whereas the opposite effect was seen in response to SA treatment (Figure 6B). Also, *MET1* expression is reduced following ABA treatment in shoots, but not in roots (Figure 5A, 6A), supporting previous gene expression data showing *MET1* is downregulated in a concentration-dependent manner in response to ABA treatments (Arıkan et al, 2018). Taken together, these findings suggest that hormone signaling may contribute to the dynamic changes of DNA methylation associated with tissues development.

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Appendix

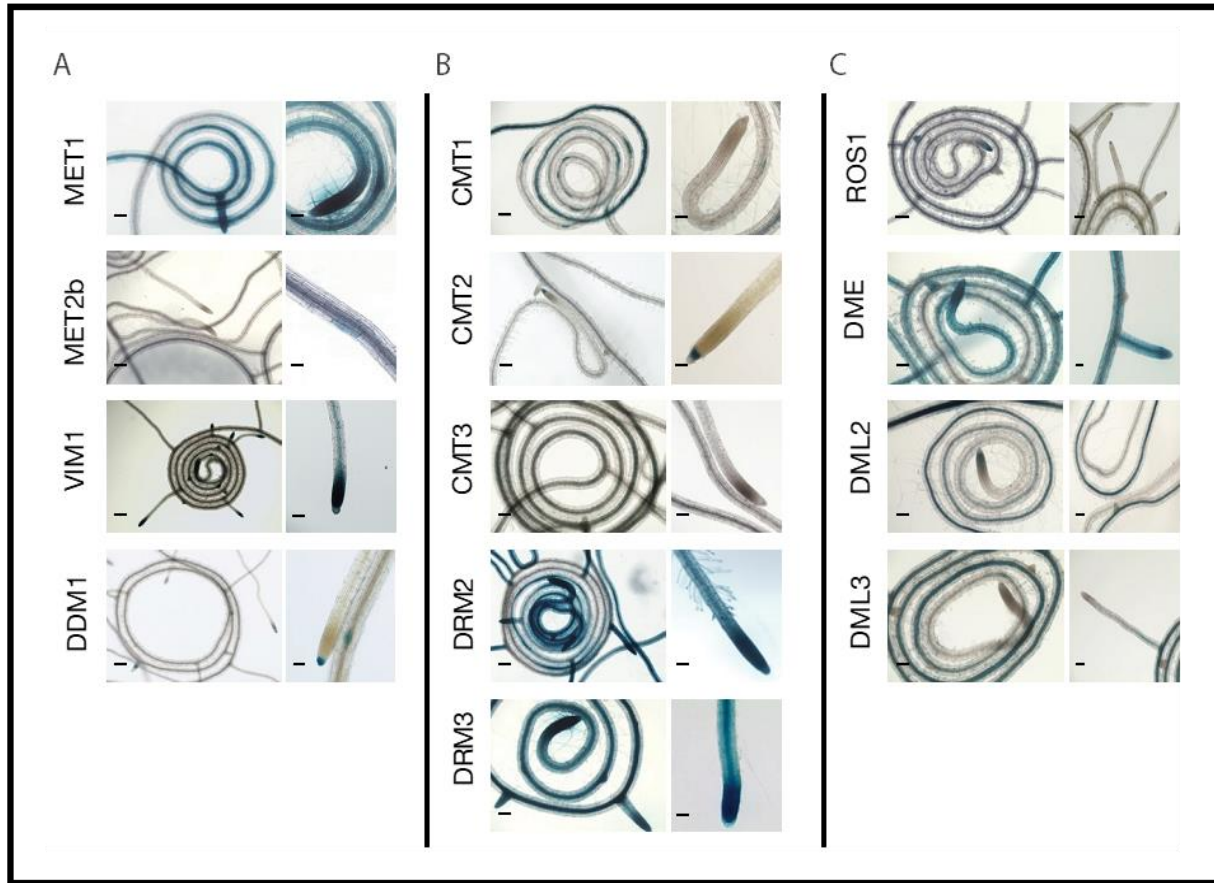


Figure 2.1. Promoter activity of DNA methylation and demethylation-related genes in two-week-old roots. A-C: Histochemical localization of GUS activity directed by promoters of the CG methylation-related genes *MET1*, *MET2b*, *VIM1*, and *DDM1* (A), the non-CG and RdDM-related methyltransferases *CMT1*, *CMT2*, *CMT3*, *DRM2*, and *DRM3* (B), and the demethylases *ROS1*, *DME*, *DML2*, and *DML3* (C). Scale bar = 5 mm.

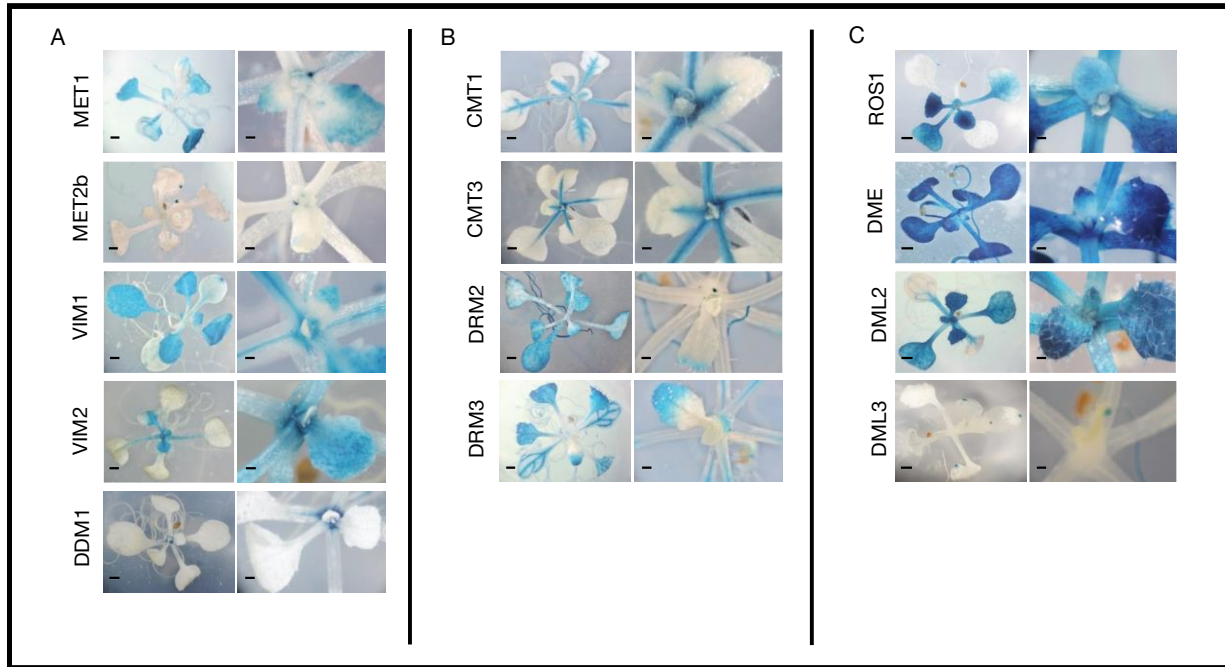


Figure 2.2. Promoter activity of DNA methylation and demethylation-related genes in two-week-old shoots. A-C: Histochemical localization of GUS activity directed by the promoters of the CG methylation-related genes *MET1*, *MET2b*, *VIM1*, *VIM2*, and *DDM1* (A), the non-CG and RdDM-related methyltransferases *CMT1*, *CMT3*, *DRM2*, and *DRM3* (B), and the demethylases *ROS1*, *DME*, *DML2*, and *DML3* (C). Scale bar = 5 mm.

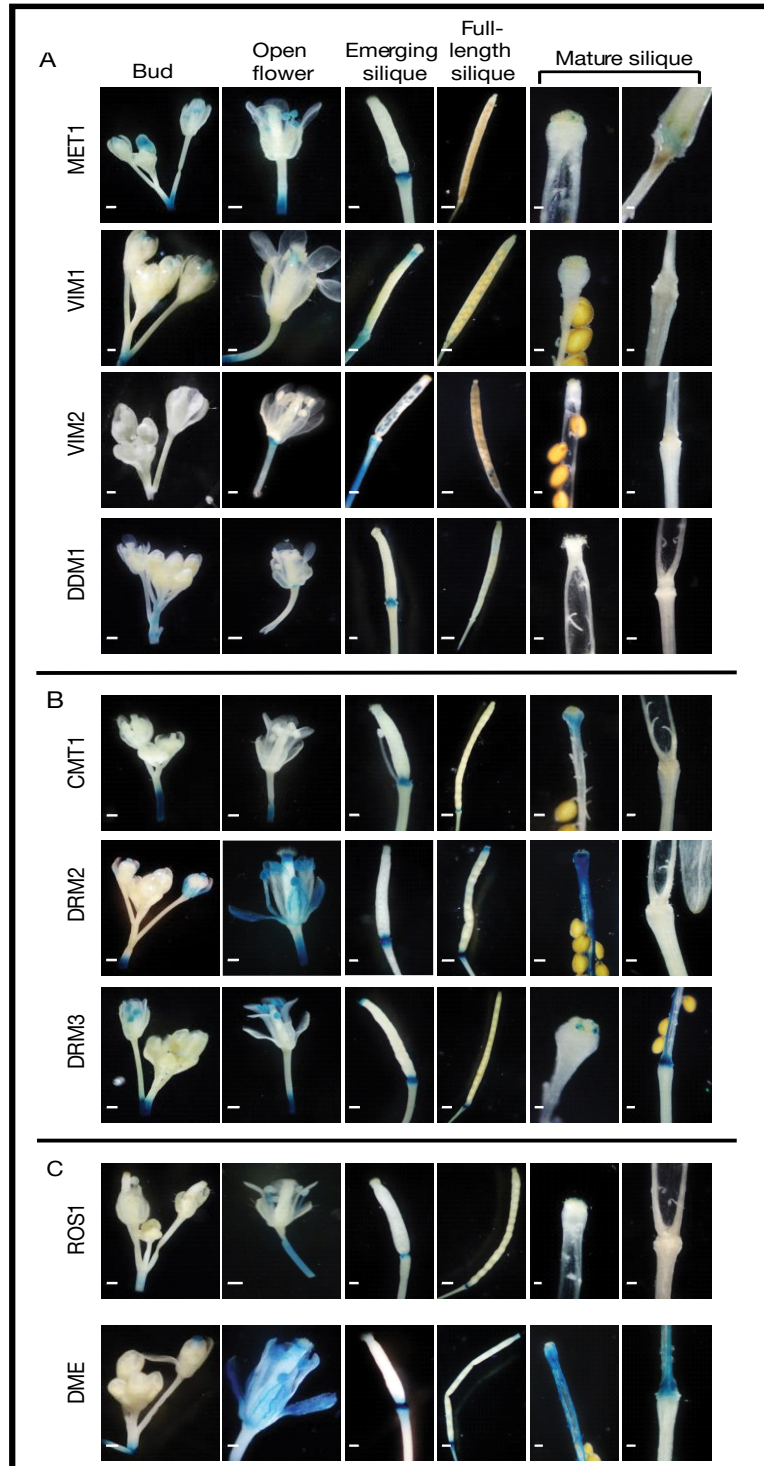


Figure 2.3. Promoter activity of DNA methylation and demethylation-related genes in floral tissues and organs.

A-C: Histochemical localization of GUS activity directed by promoters of the CG methylation-related genes *MET1*, *VIM1*, *VIM2* and *DDM1* (A), the non-CG and RdDM-related *CMT1*, *DRM2*, and *DRM3* methyltransferases (B), and the demethylases *ROS1* and *DME* demethylases (C) in inflorescence buds, open flowers, emerging siliques, distal end of mature siliques, and proximal end of mature siliques. Scale bar = 5 mm.

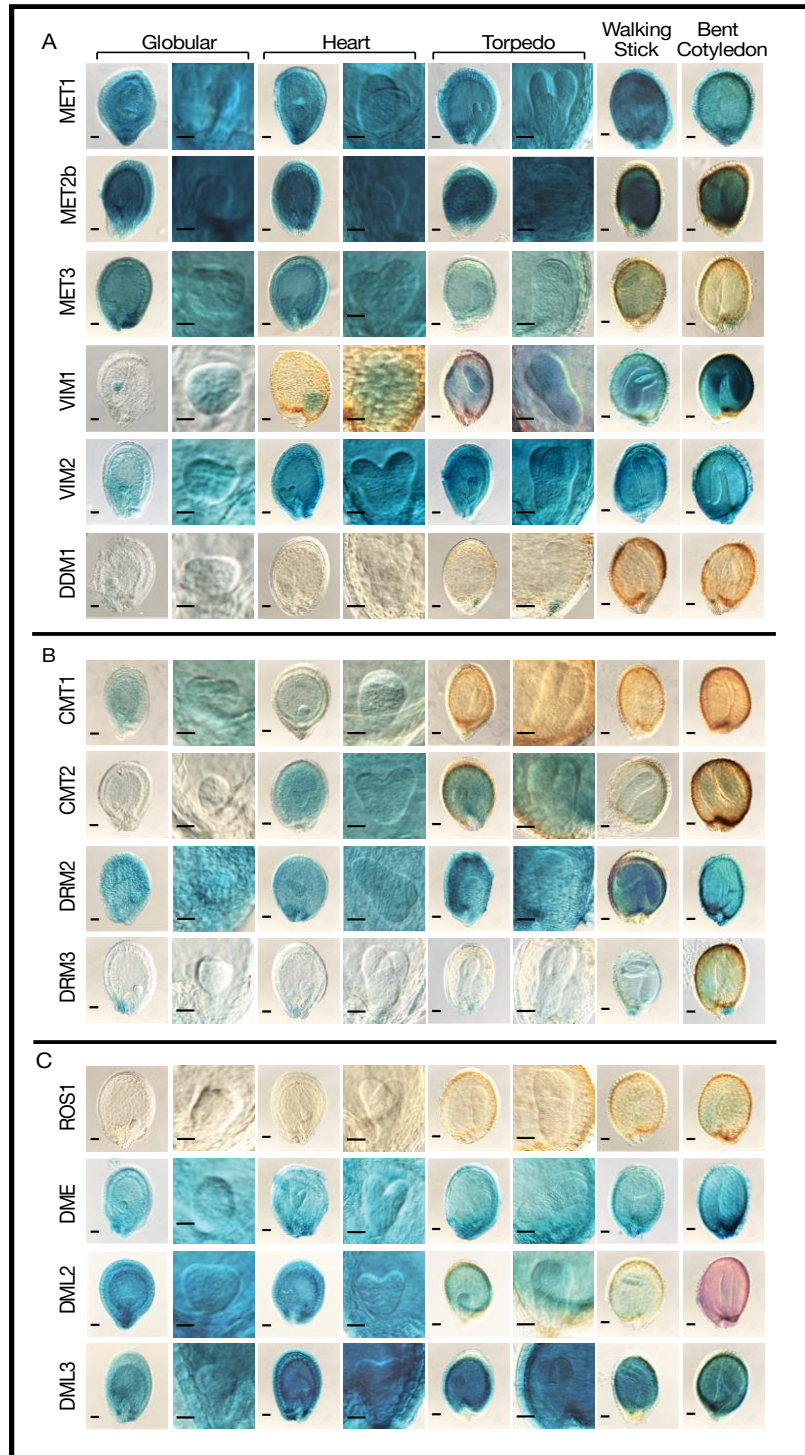


Figure 2.4. Promoter activity of DNA methylation and demethylation-related genes during various stages of embryogenesis.

A-C: Histochemical localization of GUS activity directed by promoters of the CG methylation-related genes *MET1*, *MET2b*, *MET3*, *VIM1*, *VIM2*, and *DDM1* (A), the non-CG and RdDM-related methyltransferases *CMT1*, *CMT2*, *DRM2*, and *DRM3* (B), and the demethylases *ROS1*, *DME*, *DML2*, and *DML3* (C) at globular, heart, torpedo, walking stick, and bent cotyledon embryo stages. Scale bar = 5 mm.

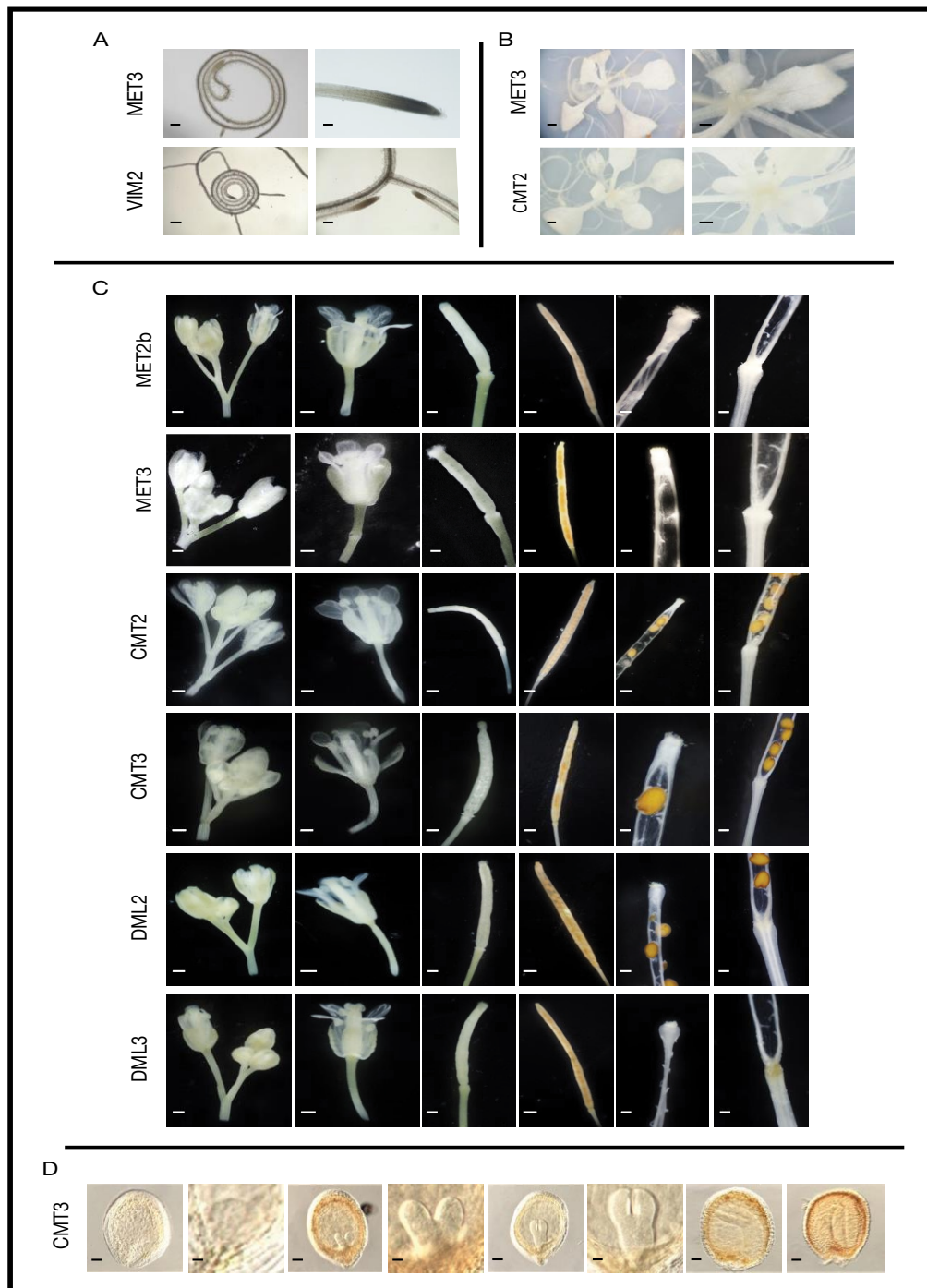


Figure 2.5. No promoter activity detected for the indicated genes. A-D: GUS staining assays of transgenic reporter lines showing absence of expression of the indicated DNA methylation and demethylation-related genes in roots (A), shoots (B), floral tissues and organ (C), and various stages of embryogenesis (D). Scale bar = 5 mm.

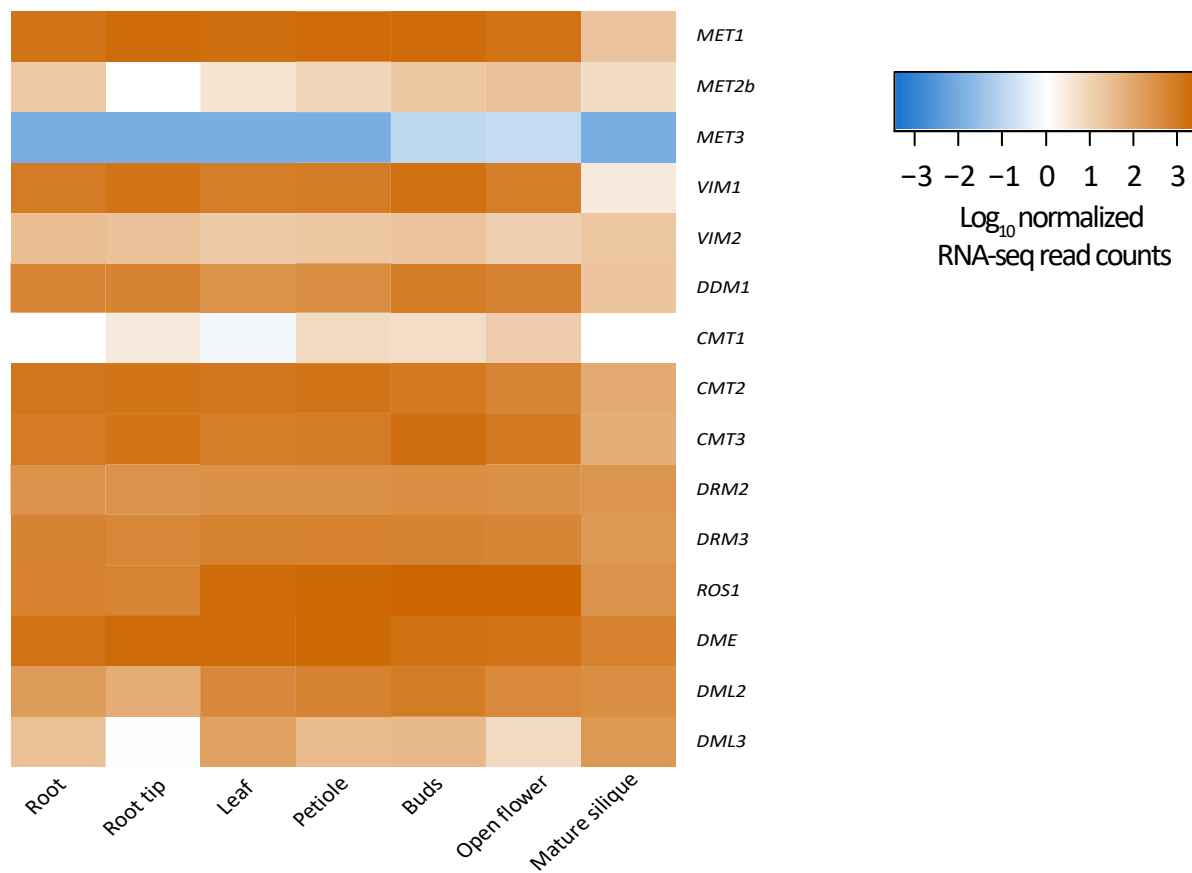


Figure 2.6. Heatmap representation of gene expression levels of DNA methylation and demethylation-related genes across different tissues and organs. Values are represented as $\log_{10}$ -normalized RNA-seq read counts.

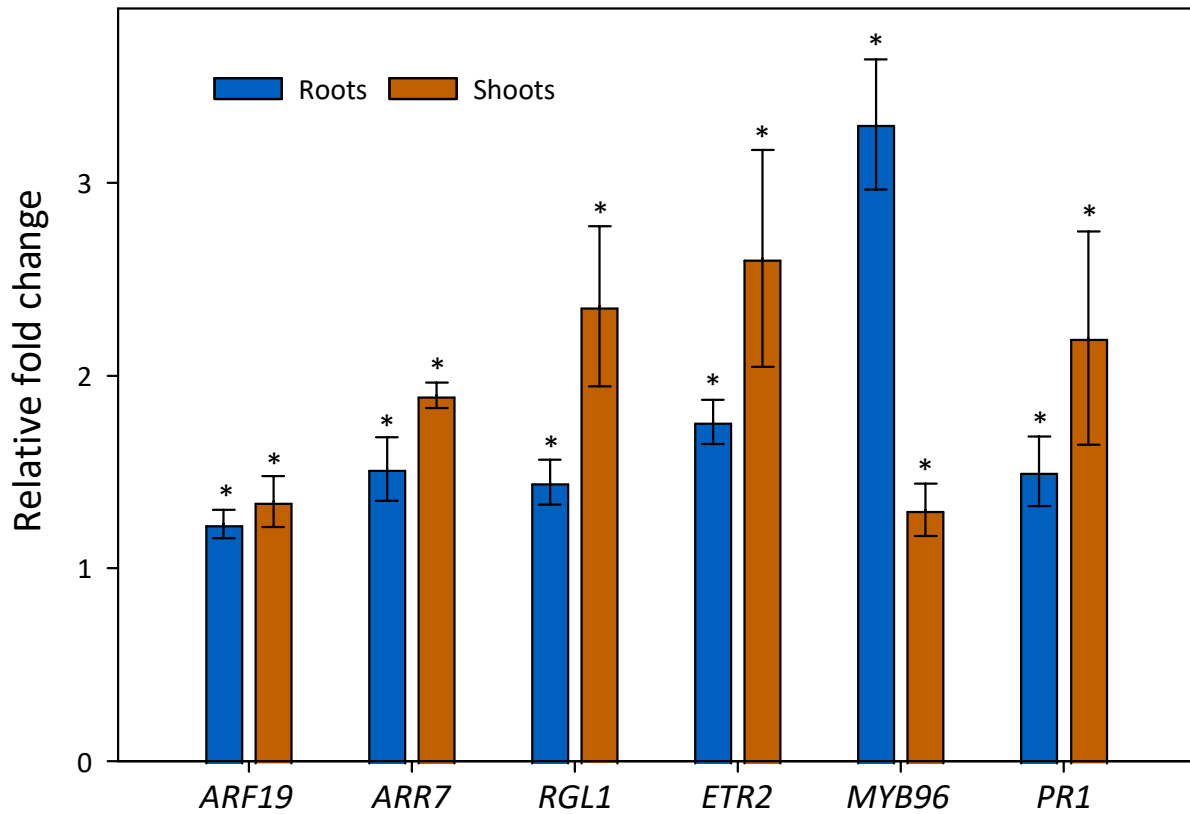


Figure 2.7. Upregulation of phytohormone marker genes after hormone application. The expression levels of marker genes for auxin (*ARF19*), cytokinin (*ARR7*), gibberellic acid (*RGL1*), ethylene (*ETR2*), abscisic acid (*MYB96*), and salicylic acid (*PR1*) response were quantified in roots and shoots of treated and control wild-type Col-0 plants 24 hours after hormone application. Relative fold change values were calculated from three biological samples and represent expression in hormone-treated plants relative to mock-treated plants. *ACTIN8* and *PP2AA3* were used as internal controls to normalize gene expression values. Asterisks indicate statistically significance differences between hormone-treated and mock-treated plants at $P < 0.05$ according to t tests.

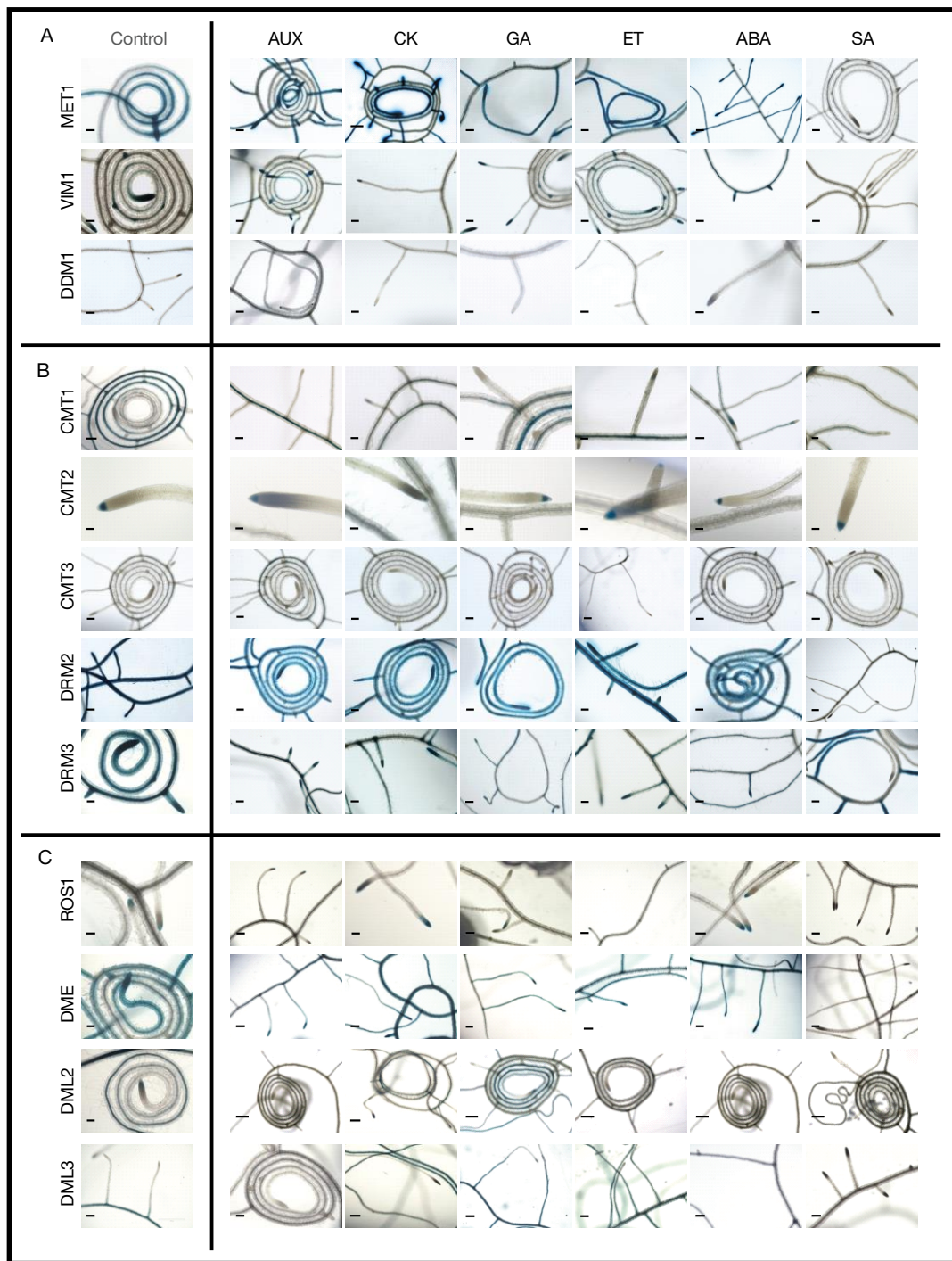


Figure 2.8. Promoter activity of DNA methylation and demethylation-related genes in two-week-old roots in response to phytohormone treatments.

A-C: Histochemical localization of GUS activity directed by promoters of the CG methylation-related genes *MET1*, *VIM1*, and *DDM* (A), the non-CG and RdDM-related methyltransferases *CMT1*, *CMT2*, *CMT3*, *DRM2*, and *DRM3* (B), and the demethylases *ROS1*, *DME*, *DML2*, and *DML3* (C) in two-week-old roots in response to exogenous application of auxin (AUX), cytokinin (CK), gibberellic acid (GA), ethylene (ET), abscisic acid (ABA), and salicylic acid (SA). Scale bar = 5 mm.

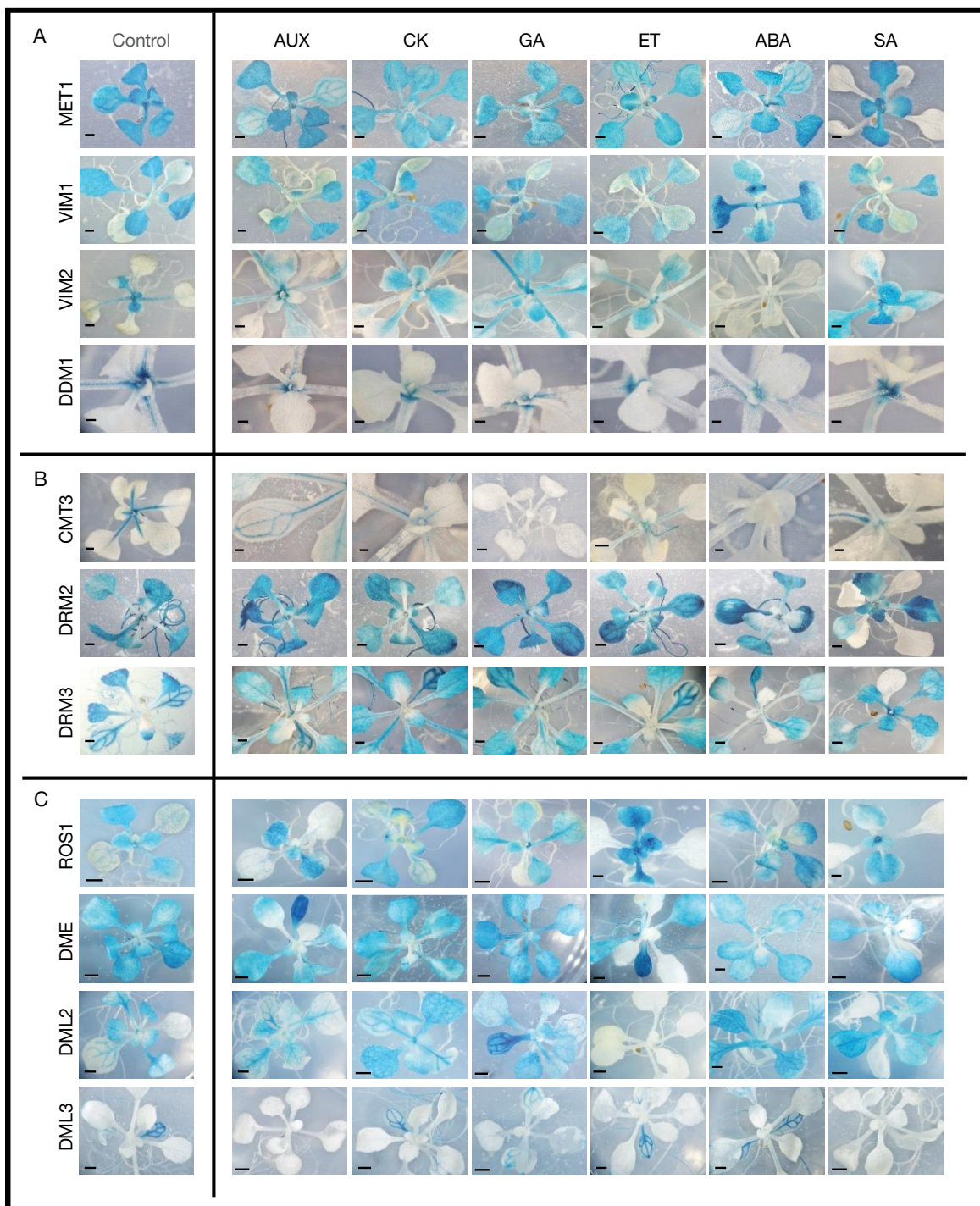


Figure 2.9. Promoter activity of DNA methylation and demethylation-related genes in two-week-old shoots in response to phytohormone treatments.

A-C: Histochemical localization of GUS activity directed by promoters of the CG methylation-related genes *MET1*, *VIM1*, *VIM2*, and *DDM1* (A), the non-CG and RdDM-related methyltransferases *CMT3*, *DRM2*, and *DRM3* (B), and the demethylases *ROS1*, *DME*, *DML2*, and *DML3* (C) in two-week-old shoots in response to exogenous application of auxin (AUX), cytokinin (CK), gibberellic acid (GA), ethylene (ET), abscisic acid (ABA), and salicylic acid (SA). Scale bar = 5 mm.

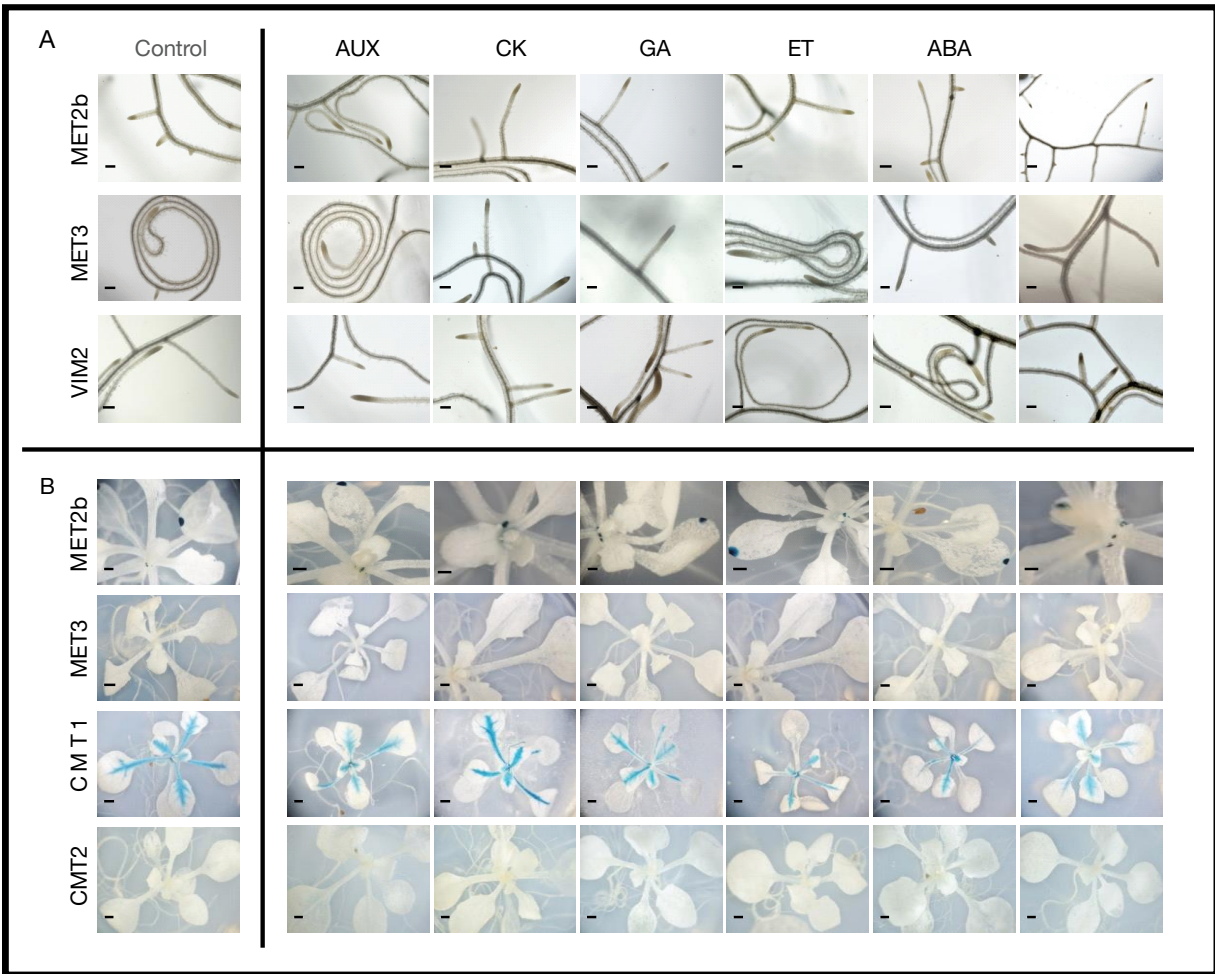


Figure 2.10. No changes in promoter activity detected for the indicated genes following phytohormone treatment. GUS staining assays of transgenic reporter lines showing no changes in expression of the indicated DNA methylation-related genes in roots (A) or shoots (B) following treatment with auxin (AUX), cytokinin (CK), gibberellic acid (GA), ethylene (ET), abscisic acid (ABA), or salicylic acid (SA). Scale bar = 5 mm.

Table 2.1. Gene identification numbers and primers used in this study.

Gene name	Gene ID	5' primer	3' primer	Fragment length (bp)	Restriction enzymes
MET1	AT5G49160	TATAAAGCTTGTGGACCTAATTGGGGCTAT	TATAGGATCCTTTCAAAATCCCTAGTTTCAAAATC	1959	HindIII/BamHI
MET2b	AT4G08990	TATAAAGCTTTTGTGGTGGGCCAGACCTAA	TATAGGATCCGAAAAGACCTAATTTTGAAACAAA	1789	HindIII/BamHI
MET3	AT4G13610	TATAGTCGACGCTCTATATCGCGGTCTATG	TATACCCGGGTTCCAAGAAAAACCTAATTTTGAAA	1923	Sall/SmaI
VIM1	AT1G57820	TATAAAGCTTACGACCTTCGGAAGATGCAAG	TATAGGATCCTTTTGGGGAAAAAGATCAGAGAG	1978	HindIII/BamHI
VIM2	AT1G66050	TATAAAGCTTATTCTTAATCCTTAGGTTTAT	TATAGGATCCTGTTGAACGAGTGAGTGAGAGAG	583	HindIII/BamHI
DDM1	AT5G66750	TATATCTAGACAACACACTCGTCAACTGAG	TATACCCGGGCGAAGGAGAAGGAAATCCGGTAA	423	XbaI/SmaI
CMT1	AT1G80740	TATAAAGCTTCTGACCTAGAGCTATAGAGCCC	TATACCCGGGTTTCGACAGAAAGACGAGCA	2016	HindIII/SmaI
CMT2	AT4G19020	TATATCTAGACTGAATCAAATGACATGAGG	TATACCCGGGCGAGAAATATAAGAATCTAAAAGG	1372	HindIII/Sall
CMT3	AT1G69770	TATAAAGCTTGTCCCTCCTCGTGTACAC	TATAGGATCCGATTGAGTCAGAGAATCGAAGG	1933	HindIII/BamHI
DRM2	AT5G14620	TATAGTCGACGTGTGTGTGTGTATGTACAC	TATAGGATCCGAGTAATACCCAGAGAATTTTAG	771	Sall/BamHI
DRM3	AT3G17310	TATAGTCGACTTGGCAGGAGGTGCTCTCT	TATAGGATCCTTGAATTAATAATTCACACAAAAAT	1984	Sall/BamHI
ROS1	AT2G36490	TATAGTCGACTTGTATGTTTGGAGTGATGCG	TATACCCGGGTTCTGACTATTTTTACTTTTCT	2018	Sall/SmaI
DME	AT5G04560	TATAGTCGACCTAGATTCAAACGTAGACCA	TATAGGATCCAACACACTTGATGAATCACTCCCC	2038	Sall/BamHI
DML2	AT3G10010	TATAGTCGACAAGGGGGGTGATTGACGGCG	TATAGTCGACAAGGGGGGTGATTGACGGCG	1979	Sall/BamHI
DML3	AT4G34060	TATAGTCGACTTGACTCCTCCTACTCTGAG	TATAGGATCCGAAAAAAAAAATTGAATTAACGA	2029	Sall/BamHI

CHAPTER 3
ESTABLISHMENT AND MAINTENANCE OF DNA METHYLATION IN NEMATODE
FEEDING SITES

3.1 Abstract

A growing body of evidence indicates that epigenetic mechanisms, particularly DNA methylation, play key regulatory roles in plant-nematode interactions. Nevertheless, the transcriptional activity of key genes mediating DNA methylation and active demethylation in the nematode feeding sites remains largely unknown. Here, we profiled the promoter activity of 12 genes involved in maintenance and *de novo* establishment of DNA methylation and active demethylation in the syncytia and galls induced respectively by the cyst nematode *Heterodera schachtii* and the root-knot nematode *Meloidogyne incognita* in Arabidopsis roots. The promoter activity assays revealed that expression of the CG-context *METHYLTRANSFERASEs* is restricted to feeding site formation and development stages. *CHROMOMETHYLASE1* (*CMT1*), *CMT2*, and *CMT3* and *DOMAINS REARRANGED METHYLTRANSFERASE2* (*DRM2*) and *DRM3*, which mediate non-CG methylation, showed similar and distinct expression patterns in the syncytia and galls at various time points. Notably, the promoters of various DNA demethylases were more active in galls as compared with the syncytia, particularly during the early stage of infection. Mutants impaired in CG or CHH methylation similarly enhanced plant susceptibility to *H. schachtii* and *M. incognita*, whereas mutants impaired in CHG methylation reduced plant susceptibility only to *M. incognita*. Interestingly, hypermethylated mutants defective in active DNA demethylation exhibited contrasting responses to infection by *H. schachtii* and *M. incognita*, a finding most likely associated with differential regulation of defense-related genes in these mutants upon nematode infection. These results point to shared and distinct methylation-dependent mechanisms regulating plant response to infection by cyst and root-knot nematodes.

Key words: Arabidopsis, *Heterodera schachtii*, *Meloidogyne incognita*, DNA methylation, active DNA demethylation, promoter activity, syncytium, gall

Abbreviations: 5-methylcytosine (5mC), beet cyst nematode (BCN), RNA-directed DNA methylation (RdDM), root-knot nematode (RKN)

3.2 Introduction

DNA methylation and demethylation pathways regulate many aspects of plant development and stress responses through reversible, non-genetic modification of cytosine to 5-methylcytosine (5mC) (Fransz and Jong 2002; Zilberman et al, 2007; Henderson and Jacobsen 2007; Zhang et al, 2010; Matzke and Moshier 2014; Du et al, 2015; López Sánchez et al, 2016; Hewezi et al, 2018; Hewezi 2020). In plants, this occurs in the CG, CHG, and CHH sequence contexts (where H is any nucleotide except G) (Lister et al, 2008). The *de novo* formation of 5mC is catalyzed by DNA methyltransferases DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) and DRM3 through the RNA-directed DNA methylation (RdDM) pathway (Cao and Jacobsen 2002a, b; Cao et al, 2003; Henderson et al, 2010). Following DNA replication and cell division, DNA methylation is maintained in a sequence context-specific manner (Saze et al, 2003). CG methylation is maintained by METHYLTRANSFERASE1 (MET1), likely with aid from its paralogs MET2b and MET3 (Kankel et al, 2003; Quadrana et al, 2016). CHROMOMETHYLASE1 (CMT1), CMT2, CMT3, and the RdDM pathway are responsible for maintaining non-CG methylation (Henderson et al, 2010; Law and Jacobsen 2010; Costa-Nues et al, 2014; Stroud et al, 2014). 5mC is removed and replaced by cytosine via base excision repair (BER) in active DNA demethylation by paralogous DNA demethylases REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DME-LIKE2 (DML2) and DML3 (Choi et al, 2002; Gong et al, 2002; Agius et al, 2006; Gehring et al, 2006; Morales-Ruiz et al, 2006; Penterman et al, 2007b; Zhang and Zhu 2012). Together, DNA methylation and demethylation fine-tune genome-wide methylation levels and subsequent transcriptional reprogramming (Gong et al, 2002; Penterman et al, 2007a, b; Zhu et al, 2007; Ortega-Galisteo et al, 2008; Lei et al, 2015).

Recent studies have provided intriguing evidence for the involvement of DNA methylation and active demethylation pathways in modulating defense response against various phytopathogens (Hewezi et al, 2018). Genome-wide methylation profiling of *Arabidopsis* leaves inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) revealed widespread changes in plant methylomes, particularly in gene-rich regions (Downen et al, 2012). *Pst*-induced DNA methylation changes were found to be associated with differential transcript abundances of a significant number

of stress-responsive genes (Downen et al, 2012). Similarly, infection of canola (*Brassica napus*) by the fungal pathogen *Leptosphaeria maculans* triggered differential DNA methylation in the promoters of thousands of genes both in resistant and susceptible cultivars, of which numerous genes are associated with defense response (Tirnaz et al, 2020). The correlation of signature genome-wide DNA methylation patterns and gene expression changes were also reported in rice and tobacco in response to infection by rice black-streaked dwarf virus or cucumber mosaic virus, respectively (Wang et al, 2018; Li et al, 2020).

Changes in DNA methylation levels seem to impact plant interactions with fungal pathogens and oomycetes. For example, hypomethylated mutants defective in DNA methylation establishment increased plant resistance to *Hyaloperonospora arabidopsidis* (*Hpa*), whereas hypermethylated mutants impaired in active DNA demethylation increased plant susceptibility to both *Hpa* and *Fusarium oxysporum* (Le et al, 2014; Lopez Sanchez et al, 2016). Gene expression analyses of these mutants provided strong evidence for the involvement of DNA methylation in the regulation of a significant number of genes with defense- and stress-related functions. However, modulation of defense- and stress responsive genes in hyper- or hypomethylated mutants may exhibit opposite response to various phytopathogens, as documented by López Sánchez et al, (2016).

Cyst nematodes (*Globodera* and *Heterodera* spp.) and root-knot nematodes (*Meloidogyne* spp.) are sedentary obligate biotrophs that infect the root systems of a broad-spectrum of host plants, including the model plant *Arabidopsis*. Nematode parasitism of host plants is characterized by the formation of syncytia and giant cells as permanent feeding sites for cyst and root-knot nematodes, respectively. The formation of giant cells by root-knot nematode is accompanied by the formation of galls at the site of infection as a result of increasing cell division of the neighboring cells. The parasitic second-stage juveniles (J2) feed from these metabolically hyperactive feeding cells and develop into adult females and complete the life cycle. Several experimental evidences indicate that epigenetic mechanisms, particularly DNA methylation, play important role in plant-nematode interactions (Hewezi et al, 2020). For example, mutants partially impaired in non-GC DNA methylation exhibited reduced susceptibility to cyst and root-knot nematodes (Hewezi et al, 2008; Ruiz-Ferrer et al, 2018; Atighi et al, 2020). Genome-wide DNA methylation analysis of soybean roots exposed to soybean cyst nematode (SCN, *H. glycines*) revealed widespread DNA

hypomethylation in the promoter and body of a significant number of genes that were previously reported to be differentially expressed in the syncytium (Rambani et al, 2015). Similarly, beet cyst nematode (BCN, *H. schachtii*) was found to induce dynamic changes in Arabidopsis root methylomes concurrent with considerable increases in hypomethylation levels of protein-coding genes and transposable elements (TEs) (Hewezi et al, 2017). Further analysis indicated that BCN-induced DNA methylation changes may directly impact the transcript abundance of more than one-fourth of syncytium differentially expressed genes (Hewezi et al, 2017). Methylome analysis of two near isogenic lines of soybean differing in their response to SCN revealed distinct and specific methylation profiles over protein-coding and miRNA genes as well as TEs, where the susceptible line exhibited global hypomethylation patterns and the resistant line showed global hypermethylation (Rambani et al, 2020a, b). Global DNA hypomethylation patterns, predominantly in the CHH context, were also observed in the early developing galls induced by the root-knot nematode *M. graminicola* in rice (Atighi et al, 2020). The biological significance of DNA methylation changes induced by parasitic nematodes was established using various functional assays of overexpression and mutation analyses (Hewezi et al, 2017; Atighi et al, 2020; Rambani et al, 2020a, b). However, the transcriptional activity of key genes involved in DNA methylation and active demethylation in nematode feeding sites remains mostly unknown. This issue was addressed by profiling the spatiotemporal expression patterns of genes involved in maintenance and *de novo* establishment of DNA methylation as well as those involved in active DNA demethylation in the syncytia and galls induced respectively by *H. schachtii* and *M. incognita* in Arabidopsis roots. Also, mutants of these genes were assayed for susceptibility to *H. schachtii* and *M. incognita*. These analyses revealed a unique and similar role of DNA methylation in mediating plant susceptibility to two evolutionary distant nematode species.

3.3 Results

3.3.1 Promoter activity of DNA methyltransferases and demethylases in the syncytia induced by the beet cyst nematode H. schachtii

Seeds of transgenic *Arabidopsis* lines (T2 generation) expressing the β -glucuronidase (GUS) reporter, guided by the promoter of 12 different DNA methyltransferase and demethylase genes were planted. Ten-day-old seedlings were inoculated with second-stage juvenile (J2) nematodes of *H. schachtii* or *M. incognita*. Promoter activity was determined by staining the plants at various time points post infection. In response to *H. schachtii* infection, the major CG methyltransferase *MET1* was highly expressed in the syncytium induced by *H. schachtii* at 3- and 7-days post infection (dpi), as indicated by strong GUS staining in the syncytium. At later time points, however, its expression was strongly downregulated in the syncytium (Figure 3.1A). The *MET1* homolog *MET3*, whose expression was not detected in roots tissues under noninfected conditions, is strongly and specifically upregulated in the syncytium at 3 dpi. The expression of *MET2b* was not detected in the syncytium at any time points (Figure 3.1A). These expression patterns indicate that maintenance of the CG-context methylation in the syncytia is restricted to early formation and development stages.

Promoter activity of *CMT3*, the main CHG methyltransferase, and its homolog *CMT1*, revealed similar patterns of high expression in the syncytium at all time points (Figure 3.1B), suggesting that CHG-context methylation is actively maintained in the syncytium during early and late stages of *H. schachtii* infection. In contrast, the expression of *CMT2* was not activated in the syncytium at any time points. The RdDM methyltransferases *DRM2* and *DRM3* showed similar expression patterns of upregulation in the syncytia at 3 dpi and downregulation at 14 dpi (Figure 3.1B). However, at 7 and 10 dpi *DRM2* and *DRM3* exhibited opposite expression patterns, i.e., *DRM2* was downregulated and *DRM3* was upregulated in the syncytia (Figure 3.1B). These results suggest that *de novo* DNA methylation is actively established in the syncytia.

The DNA demethylases *ROS1*, *DME*, and *DML3* were downregulated in the syncytia during early and/or late infection stages. Unlike these three DNA demethylases, *DML2* is upregulated in the syncytium at 3 and 7 dpi (Figure 3.1C), suggesting active DNA demethylation in the syncytia is mediated solely through the activity of *DML2*.

3.3.2 Mutations of DNA methylation and demethylation-related genes compromise plant susceptibility to *H. schachtii*

Next, the impacts of mutations in these DNA methylation-related genes on plant response to nematode infection were investigated. Homozygous T-DNA insertional mutants for 10 genes were identified and phenotypically analyzed. This included mutants of *MET2b* (*SALK_093835C* and *SALK_102231C*), *MET3* (*SALK_099592C* and *SALK_024049C*), *CMT1* (*SALK_138685C* and *SALK_030404C*), *CMT2* (*SALK_012874C* and *CS879822*), *CMT3* (*CS6365*), *DRM2* (*CS16386* and *SALK_129477C*), *DRM3* (*SALK_024820C* and *SALK_136439C*), *ROS1* (*SALK_045303C* and *CS66099*) *DML2* (*SALK_131712C*) and *DML3* (*SALK_056440C*). Loss-of-function mutants of *MET1* and *DME* are embryonic lethal (Choi et al, 2002; Mathieu et al, 2007), and therefore were not included in the susceptibility assays. No morphological irregularities in the shoots of mutants assessed in this study were detected when compared with the wild-type plants (Figure 3.7). With the exception of the short root phenotypes observed in the *ROS1* mutants (*SALK_045303C* and *CS66099*) no noticeable morphological changes in root structure and length were noticed in these mutants as compared with wild-type plants (Figure 3.8).

Seeds of the mutant lines along with the wild types were planted in 12-well plates and inoculated with freshly hatched J2s of *H. schachtii*. The numbers of nematode J4 females per root system were counted three weeks after inoculation and used to evaluate plant susceptibility. As shown in Figure 3.2, both mutant alleles of *MET2b* (*SALK_102231C* and *SALK_102231C*) showed susceptibility levels similar to the wild-type Col-0, consistent with the absence of *MET2b:GUS* expression in the syncytium. In contrast, the mutant allele of *MET3* *SALK_024049C* was significantly more susceptible to *H. schachtii* in comparison with the wild-type Col-0, suggesting that CG hypomethylation is associated with increased plant susceptibility to *H. schachtii*. All mutants of the three *CMT* genes did not show any significant impact on plant susceptibility (Figure 3.2B and C). Interestingly, both mutant alleles of *DRM2* and *DRM3* showed statistically significant increases in plant susceptibility (Figure 3.2D). These results suggest that non-CG hypomethylation is also associated with increased plant susceptibility to *H. schachtii*. All tested mutants of the DNA demethylases *ROS1*, *DML2*, and *DML3* were more susceptible as compared

with the corresponding wild-type Col-0 or C24 (Figure 3.2E and F), signifying that inhibition of active DNA methylation promotes *H. schachtii* parasitism of Arabidopsis.

3.3.3 Promoter activity of DNA methyltransferases and demethylases in the galls induced by the root-knot nematode *M. incognita*

The CG methyltransferases *MET1*, *MET2b*, and *MET3* were expressed in the galls induced by the root-knot nematode *M. incognita* at 4 dpi (Figure 3.3A). At 7 dpi, only the promoter activity of *MET1* was detected in the galls. At later time points, none of these three genes was activated in the galls, suggesting that CG methylation is maintained in the gall tissues during the early stage of infection.

The expression of the *CMT* gene family members showed varied expression patterns in the galls (Figure 3.3B). While the promoter activity of *CMT1* can be seen in the galls at 4, 7 and 14 dpi, *CMT3* promoter was active in the galls only at 7 dpi. *CMT2* expression was not detected in the galls at any time points (Figure 3.3B). These data suggest variable activities of the enzymes mediating CHG methylation in the galls induced by *M. incognita*. The *de novo* methyltransferases *DRM2* and *DRM3* exhibited high expression in the galls at all time points, implying a role for *de novo* DNA methylation in establishing the gall methylome during the formation and development stages.

The expression of the four DNA demethylases *ROS1*, *DME*, *DML2*, and *DML3* was observed in the galls at the 4 dpi, signifying a role for active DNA demethylation during early stage of *M. incognita* infection. However, at 7 dpi, only *DML2* showed strong GUS staining in the galls. *ROS1* and *DML3* showed weak but visible staining at 7 dpi. At 14 dpi, none of the four DNA demethylases showed detectable GUS staining, suggesting that active DNA methylation occurs primarily during the early stage of *M. incognita* infection.

3.3.4 Mutations of DNA methylation and demethylation-related genes alter plant susceptibility to *M. incognita*

The T-DNA insertional mutants mentioned above were also evaluated for their susceptibility to *M. incognita*. Three-week-old Arabidopsis growing in soil were inoculated with freshly hatched

J2s of *M. incognita* and the number of galls per root system were counted three weeks after inoculation and used to evaluate susceptibility levels of the mutants in comparison with the corresponding wild-type plants. Of the genes involved in CG methylation, mutant alleles of *MET2b* (*SALK_093835C*) and *MET3* (*SALK_099592C* and *SALK_024049C*) were significantly more susceptible as compared with wild-type Col-0 plants (Figure 3.4A). In contrast, mutants of *CMT1* (*SALK_138685C*) or *CMT2* (*SALK_012874C* and *CS879822*) were statistically less susceptible to *M. incognita* compared with Col-0 (Figure 3.4B). These results suggest that genes involved in the CG and non-CG methylation may exert opposite effects on plant susceptibility to *M. incognita*. Mutant alleles of *DRM2* (*CS16386* and *SALK_129477C*) and *DRM3* (*SALK_136439C*) showed statistically significant increases in plant susceptibility (Figure 3.4B). Interestingly, all examined mutants of the DNA demethylases *ROS1* (*SALK_045303C* and *CS66099*), *DML2* (*SALK_131712C*), and *DML3* (*SALK_056440C*) were statistically significantly less susceptible to *M. incognita* as compared with the corresponding wild-type Col-0 or C24 (Figure 3.4E and F), implying the importance of active DNA demethylation for *M. incognita* parasitism of Arabidopsis.

3.3.5 Defense response genes are oppositely regulated in DNA demethylase mutants in response to infection by cyst and root-knot nematodes

Several reports have indicated that DNA hypomethylation contributes to the regulatory mechanism of plant-induced defense response (Yu et al, 2013; Le et al, 2014; Lopez Sanchez et al, 2016). Therefore, it was examined whether the expression levels of pathogenesis related (*PR*) genes in the DNA demethylase mutants correspond to the contrasting susceptibility phenotypes observed in response to infection by cyst and root-knot nematodes. The expression of *PR1*, *PR5*, and *PLANT DEFENSIN1.2* (*PDF1.2*) in *ros1* (*CS66099* and *SALK_045303C*), *dml2* (*SALK_131712C*) and *dml3* (*SALK_056440C*) root tissues under noninfected conditions were first quantified. As shown in Figure 3.5A-C, *PR1*, *PR5* and *PDF1.2* expression was comparable to that observed in the wild-type plants Col-0 or C24.

The expression of *PR1*, *PR5*, and *PDF1.2* in these four DNA demethylase mutants as well as the wild-type plants Col-0 and C24 in roots tissues infected with *H. schachtii* or *M. incognita* at 4 dpi

was then quantified. In response to *H. schachtii* infection, the expression of *PR1*, *PR5*, and *PDF1.2* showed significant downregulation in the infected wild-type roots compared with noninfected roots (Figure 3.5D-F). Similarly, the expression levels of *PR1*, *PR5*, and *PDF1.2* were significantly downregulated in almost all *H. schachtii*-infected mutant lines when compared with the noninfected mutants (Figure 3.5D-F). However, the levels of downregulation of *PR5* and *PDF1.2* were much higher in the infected mutants than the infected wild types (Figure 3.5E and F).

In response to *M. incognita* infection, the expression of *PR1*, *PR5*, and *PDF1.2* was downregulated in the infected wild-type roots compared with noninfected roots (Figure 3.5G-I). In the mutant lines, however, there was a general trend observed of upregulation of *PR1*, *PR5*, and *PDF1.2* transcripts in *M. incognita*-infected roots as compared with noninfected roots (Figure 3.5G-I). Together, these results suggest that opposite regulation of defense-related genes in the DNA demethylation mutants in response to infection by cyst and root-knot nematodes may have contributed to the observed opposite susceptibility phenotypes.

3.4 Discussion

This analysis revealed an important role of DNA methylation and active demethylation pathways in mediating Arabidopsis interactions with *H. schachtii* and *M. incognita*. Promoter activity and mutant analyses of 12 key genes involved in DNA methylation or active demethylation unveiled important similarity and distinct differences between these two pathosystems in terms of expression patterns and plant susceptibility. For example, the promoter activity of the CG methyltransferase *MET1* and its homolog *MET3* were both detected in the syncytia and galls during the early stage of infection. In contrast, the expression of *MET2b* was detected only in galls at 4 dpi (Figures 3.1A and 3A), a finding that is consistent with increased susceptibility of *met2b* mutant plants (*SALK_093835C*) to *M. incognita* but not *H. schachtii* (Figures 3.2B and 3.4B). Strikingly, no such activity of *MET3* promoter was detected in the root tissues under noninfected conditions, but this promoter was activated specifically in the syncytia and galls during feeding site initiation

(Figures 3.1A and 3.3A). Consistent with this finding, mutant alleles of *MET3* were more susceptible to both *H. schachtii* and *M. incognita* (Figures 3.2B and 3.4B). The finding that *MET3* expression is restricted to the developing embryo (Jullien et al, 2012) suggests a unique role of the encoding enzyme in reprogramming the CG methylation context of syncytia and galls in a way similar to the developing embryos, which undergo extensive DNA methylation changes (Xiao et al, 2006; Gehring et al, 2009; Jullien et al, 2012; Wang et al, 2015; Satyaki and Gehring 2017).

Notably, none of the examined mutants of the *CMT* genes were found to alter plant susceptibility to *H. schachtii* despite the strong activation of *CMT1* and *CMT3* in the syncytia during both early and late stages of infection. While this finding could be attributed to the possible functional redundancy between *CMT1* and *CMT3*, whose expression was detected in the syncytia (Figure 3.1B), this explanation doesn't support the results showing that single mutants of this gene family were altered in their susceptibility to *M. incognita* (Figure 3.4B-C). One possible explanation is that global loss of CHG methylation in *cmt1* and *cmt3* (Stroud et al, 2013) mutants have little or no effect on syncytium formation and function, despite the non-significant increase in plant susceptibility to *H. schachtii* seen in the *cmt3* mutant (CS6365) (Figure 3.2C). Alternatively, global decrease in CHG methylation can be compensated with an increase of CG and CHH methylation in the syncytium as recently reported (Rambani et al, 2020). In support of this hypothesis, the expression of the CG methyltransferases *MET1* and *MET2b* as well as the RdDM methyltransferases *DRM2* and *DRM3*, which establish CHH methylation *de novo*, are strongly induced in the syncytia (Figure 3.1A-B). Although DRMs are established as CHH methyltransferases, they were found to regulate CHG methylation as well (Cao and Jacobsen 2002; Stroud et al, 2013).

The intense GUS staining of the *DRM2* and *DRM3* promoters in the syncytia and galls (Figures 3.1B and 3.3B) along with the results showing that mutant alleles of *DRM2* and *DRM3* were more susceptible to both *M. incognita* and *H. schachtii* point into the importance of establishing DNA methylation *de novo* in syncytial and gall cells as a defense mechanism to control cyst and root-knot nematode infection. Similarly, RdDM-defective mutants were found to be more susceptible to *Botrytis cinerea* and *Plectosphaerella cucumerina* (Lopez et al, 2011). More recently, it was shown that tomato *DRM5* expression is significantly upregulated in the roots of a resistant tomato

cultivar upon infection with a virulent root-knot nematode field population (Leonetti and Molinari 2020). Together, these findings suggest that the RdDM pathway positively regulates plant resistance against various phytopathogens including cyst and root-knot nematodes.

Remarkably, the promoter activity of the four DNA demethylases *ROS1*, *DME*, *DML2*, and *DML3* exhibited opposite expression patterns in the syncytia and galls (Figures 3.1C and 3.3C). With the exception of *DML2* upregulation in the syncytium at 3 and 7 dpi, all the DNA demethylases were either downregulated or not expressed at a detectable level in the syncytia (Figure 3.1C). In contrast, strong GUS activity was observed for all DNA demethylases, particularly in young galls at 4 dpi (Figure 3.3C). Consistent with the contrasting expression patterns, the mutant alleles of *ROS1*, *DML2*, and *DML3* showed opposite responses to infection by cyst and root-knot nematodes, where the DNA demethylase mutants exhibited increased susceptibility to *H. schachtii* and decreased susceptibility to *M. incognita* (Figures 3.2C and 3.3C). The involvement of Arabidopsis active DNA demethylases in disease resistance has been reported in few studies. For example, a loss-of-function mutation in the Arabidopsis *ROS1* gene enhanced plant susceptibility to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Yu et al, 2013). In addition, Arabidopsis *ros1/dml2/dml3* (*rdd*) triple mutant displayed enhanced disease susceptibility to the fungal pathogen *Fusarium oxysporum* (Le et al, 2014). The opposite response of Arabidopsis hypermethylated mutants to various plant pathogens has also been reported. For instance, the hypermethylated mutant *ros1* was less resistant to *Hpa*, but more resistant to the necrotrophic fungi *Alternaria brassicicola* and *P. cucumerina* (López Sánchez et al, 2016).

This quantitative reverse transcription PCR (qRT-PCR) analysis of *PR1*, *PR2*, and *PDF1.2* expression levels point to opposite regulation of defense-related genes in the DNA demethylase mutants upon infection by cyst and root-knot nematodes (Figure 3.5B-C). This may partially explain the opposite susceptibility phenotypes of these mutants in response to infection by *H. schachtii* and *M. incognita* (Figures 3.2C and 3.4C). These results are consistent with the finding that active DNA demethylases function mainly in regulating the expression of defense and stress-related genes (Yu et al, 2013; Le et al, 2014; Lopez Sanchez

et al, 2016) and point to distinct methylation-dependent mechanisms regulating defense gene expression upon infection by cyst and root-knot nematodes.

3.5 Materials and methods

Plant materials and growth conditions

All transgenic *Arabidopsis* (*Arabidopsis thaliana*) lines expressing the GUS reporter gene under the control of various DNA methylation and demethylation related-genes were generated in the Columbia-0 (Col-0) background as described in chapter 2. All mutant lines were generated in the Col-0 background, except *ros1* (CS66099) is in the C24 background. The mutant lines *cmt3-7* (CS6365, Lindroth et al, 2001), *drm2-2* (CS16386, Cao and Jacobsen 2002), and *ros1-1* (CS66099, Gong et al, 2002) have been previously characterized. T-DNA insertional mutants for *met2* (SALK_102231C and SALK_093835C), *met3* (SALK_024049C and SALK_099592C), *cmt1* (SALK_138685C and SALK_030404C), *cmt2* (SALK_012874C and CS879822), *cmt3* (CS6365) *drm2* (CS16386 and SALK_129477C), *drm3* (SALK_136439C and SALK_024820C), *ros1* (CS66099 and SALK_045303C), *dml2* (SALK_131712C) and *dml3* (SALK_056440C) were obtained from the Arabidopsis Biological Resource Center (ABRC) (Figure 3.6A). mRNA expression levels of these genes were quantified in uncharacterized mutant lines and wild-type plants using qRT-PCR (Figure 3.6B). Ten-day-old plants grown in plates containing MS medium were used for RNA extraction and gene expression quantification as described below using primers provided in Table 3.1. In addition, shoot and root phenotypes of all mutant lines along with wild-type plants were assessed in four-week-old soil-grown plants. Length of the main root of 15 two-week-old plants per line was measured and statistically significant differences from wild-type plants were calculated using *t* tests ($P < 0.05$).

RNA isolation and qRT-PCR

Total RNA was isolated from mutant lines and the corresponding wild-type plants using the method previously described by Verwoerd et al (1989), and then treated with DNase I (Invitrogen).

DNase-treated RNA samples were diluted to a concentration of 50 ng μL^{-1} and used as a template for qRT-PCR reactions. The reactions were performed using Verso 1-step qRT-PCR (Thermo Fisher Scientific) in QuantStudio 6 Flex (Applied Biosystems) under the following conditions: 50 °C for 15 min, 95 °C for 15 min, and 40 cycles of 95 °C for 15 s and 55 °C for 30 s. Gene expression levels were quantified using 3 biological and 2 technical replicates. Quantification of expression levels was conducted using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001). *PROTEIN PHOSPHATASE 2A SUBUNIT A3* (*PP2AA3*, AT1G13320) was used as internal control to normalize gene expression levels (Piya et al, 2020).

Histochemical analysis of GUS activity

Seeds of the transgenic GUS reporter lines were sterilized using a 2.8% bleach solution for 5 min followed by several washes with sterilized distilled water. The sterilized seeds were randomly planted in culture plates (BD Biosciences) containing modified Knop's medium solidified with 0.8% Daishin agar (Brunschwig Chemie). Ten-day-old plants were inoculated with about 100 surface-sterilized J2 of *H. schachtii* or *M. incognita*. Histochemical GUS staining was performed at 3, 7, 10, and 14 days post inoculation (dpi) for BCN and 4, 7, and 14 dpi for RKN, according to Jefferson et al, (1987). Samples were incubated with the X-Gluc substrate (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) at 37°C and checked every 30 minutes for colorimetric changes. After staining, the GUS solution was replaced with 70% ethanol to terminate the reaction and samples were stored in ethanol at room temperature. At least two independent transgenic lines were assayed for each reporter, with at least 24 biological replicates per line. Nematode-infected roots were imaged immediately following GUS staining. Images were captured using an EVOS M7000 microscope with a 4x lens for 3 and 4 dpi images and a 2x lens for all other time points.

Nematode susceptibility assays

H. schachtii infection assays were determined in 12-well plates containing modified Knop's medium (Sijmons et al, 1991) solidified with 0.8% Daishin agar. Seeds of the mutants and corresponding wild type were surface-sterilized and planted in a randomized block design. Ten

days after planting, each seedling was inoculated with approximately 200 surface-sterilized J2 *H. schachtii*. Following inoculation, the plates were incubated in the dark at 24 °C for 3 days to facilitate nematode infection. The plants were then grown in 16-h-light/8-h-dark cycles at 24°C for three weeks before counting the number of J4 females per root system. Two independent experiments were performed with 20 replicates per genotype for each mutant and their corresponding wild-type controls.

For *M. incognita* infection assays, seeds of the mutants and wild type were surface-sterilized and planted on modified Knop's-containing plates. Ten-day-old seedlings were then transplanted to pots containing steam-sterilized sand mixed with topsoil at a 3 to 1 ratio and organized in a randomized complete block design. A week after transplantation, each plant was inoculated with approximately 500 J2 *M. incognita* and grown in 16-h-light/8-h-dark cycles at 24°C. Whole root systems were collected five weeks after inoculation and the number of galls per root system was counted using light microscope. Statistically significant differences between the mutant lines and wild-type controls were calculated using a modified *t* test in the statistical software package SAS, where *P* values less than 5% were considered statistically significant.

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Appendix

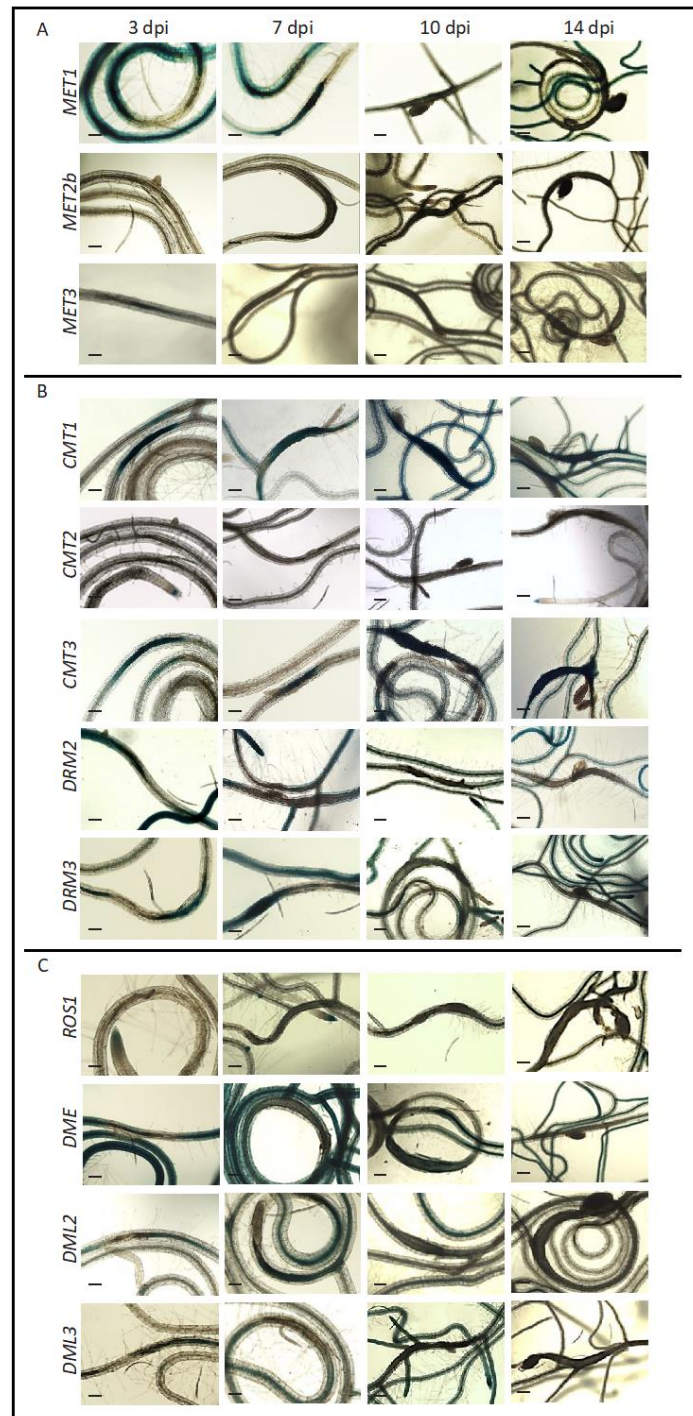


Figure 3.1. Promoter activity of DNA methyltransferases and demethylases in the syncytia induced by *H. schachtii* on Arabidopsis roots.

A-C: Time-course experiments showing GUS activity controlled by the CG methyltransferases *MET1*, *METb*, and *MET3* (A), the non-CG methyltransferases *CMT1*, *CMT2*, *CMT3*, *DRM2*, and *DRM3* (B), and the demethylases *ROS1*, *DME*, *DML2*, and *DML3* (C) at 3, 7, 10, and 14 dpi. Scale bar = 5 mm.

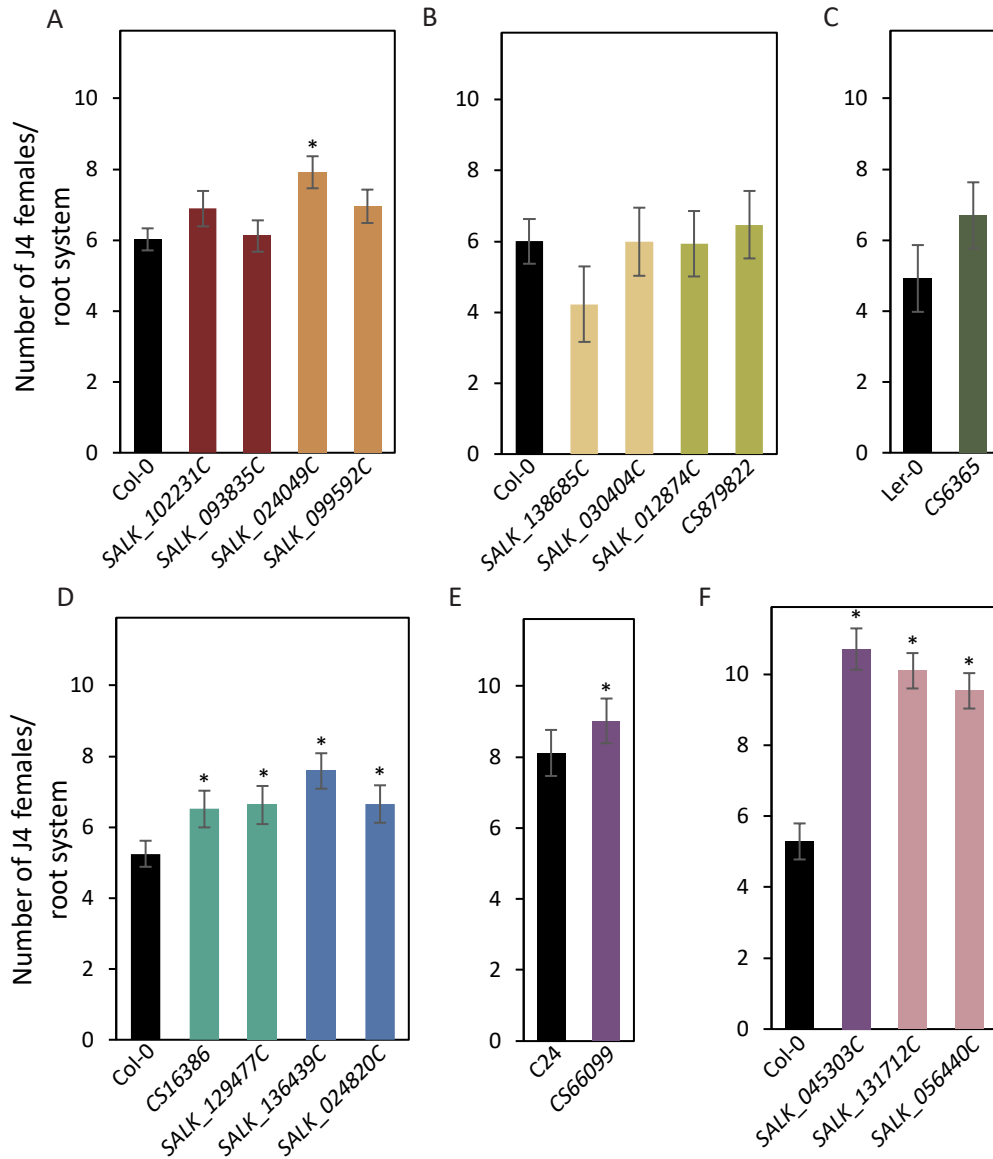


Figure 3.2. Mutations of DNA methyltransferases and demethylases enhance plant susceptibility to *H. schachtii*. A-F: *H. schachtii* infection assays of mutant alleles of *MET2b* (SALK_102231C and SALK_093835C), *MET3* (SALK_024049C and SALK_099592C) (A), *CMT1* (SALK_138685C and SALK_030404C), *CMT2* (SALK_012874C and CS879822), *CMT3* (CS6365) (B), *DRM2* (CS16386 and SALK_129477C), *DRM3* (SALK_136439C and SALK_024820C) (C), *ROS1* (CS66099) (D), *ROS1* (SALK_045303C) and *DML2* (SALK_131712C) and *DML3* (SALK_056440C) (E). The number of J4 female nematodes per root system was counted 3 weeks post inoculation. Data are presented as means $\pm$ SE (n = 20). Asterisks denote statistically significant differences from the wild-type Col-0 or C24 as determined by unadjusted *t* tests ($P < 0.05$).

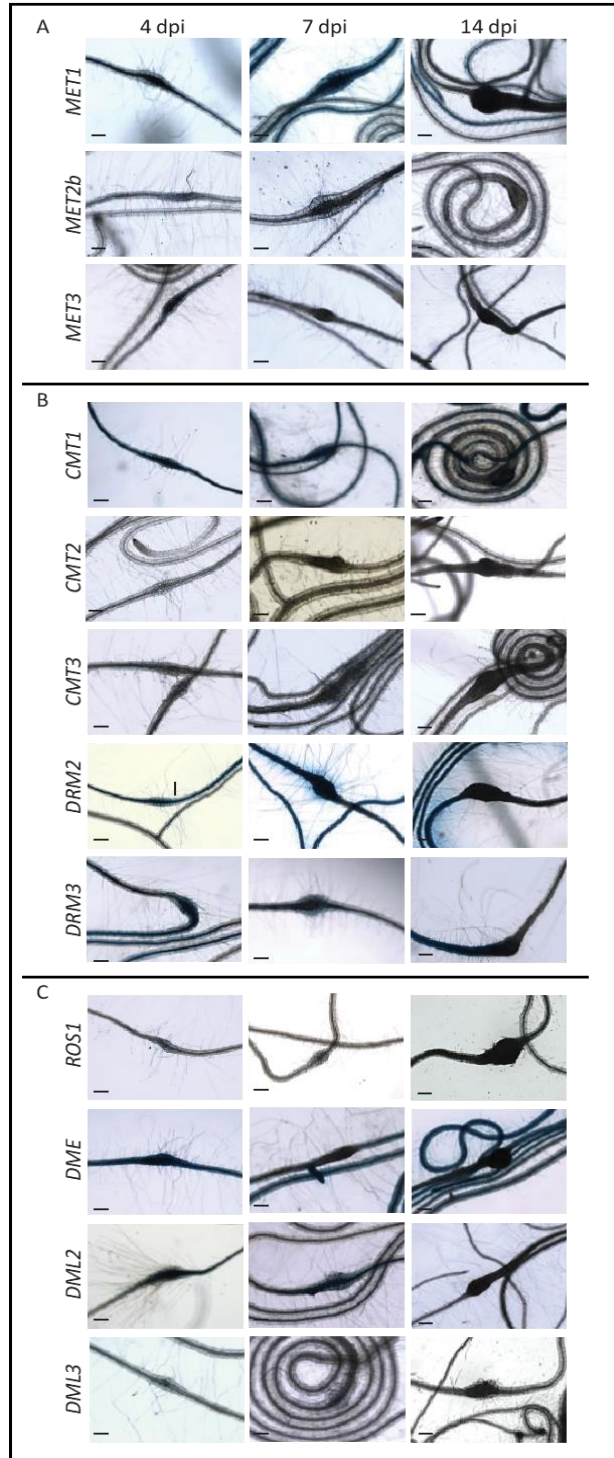


Figure 3.3. Promoter activity of DNA methyltransferases and demethylases in the galls induced by *M. incognita* on *Arabidopsis* roots.

A-C: Time-course experiments showing GUS activity controlled by the CG methyltransferases *MET1*, *MET2b*, and *MET3* (A), the non-CG methyltransferases *CMT1*, *CMT2*, *CMT3*, *DRM2*, and *DRM3* (B), and the demethylases *ROS1*, *DME*, *DML2*, and *DML3* (C) in the galls formed by *M. incognita* at 3, 7, and 14 dpi. Scale bar = 5 mm.

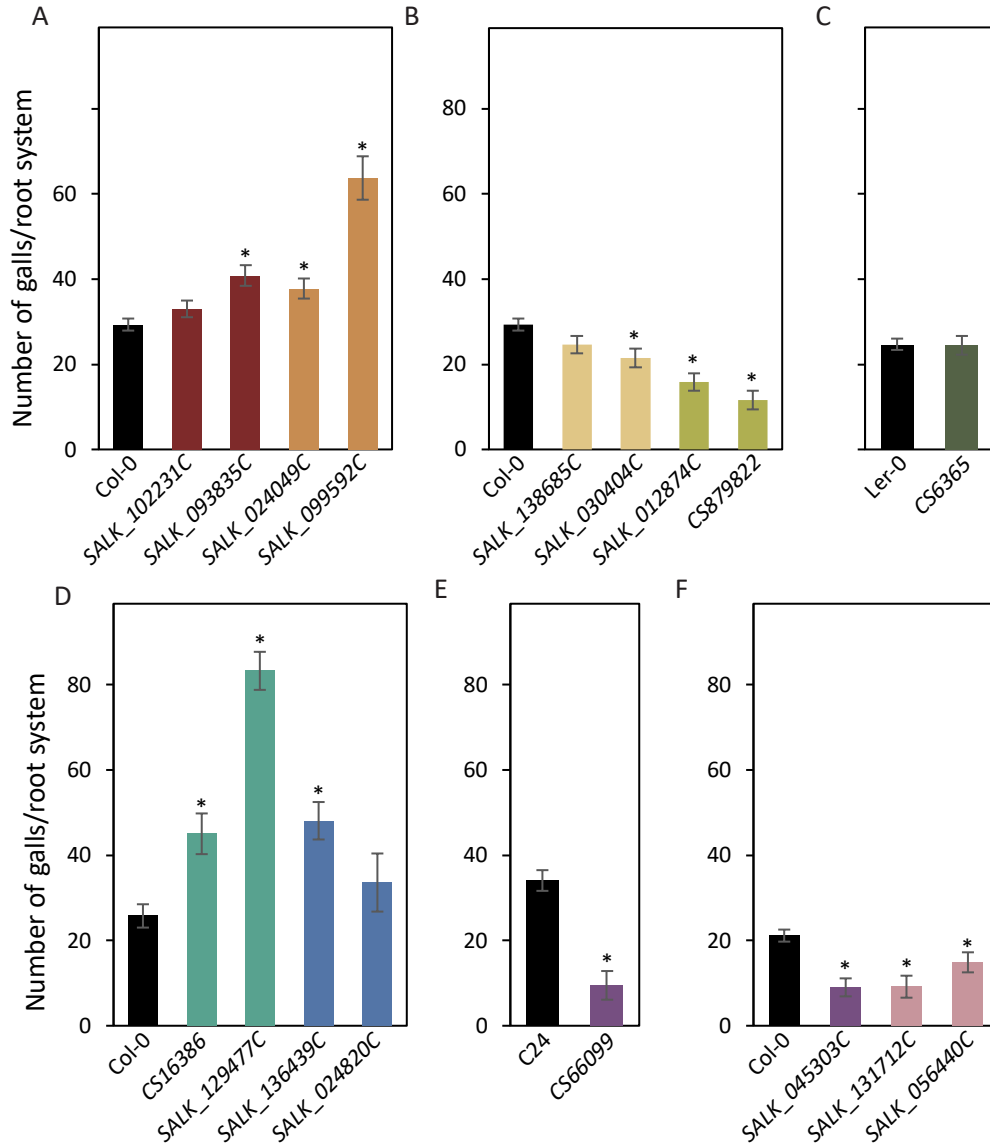


Figure 3.4. Mutations of DNA methyltransferases and demethylases alter plant susceptibility to *M. incognita*. A-F: *M. incognita* infection assays of mutant alleles of *MET2b* (SALK_102231C and SALK_093835C), *MET3* (SALK_024049C and SALK_099592C) (A), *CMT1* (SALK_138685C and SALK_030404C), *CMT2* (SALK_012874C and CS879822), *CMT3* (CS6365) (B), *DRM2* (CS16386 and SALK_129477C), *DRM3* (SALK_136439C and SALK_024820C) (C), *ROS1* (CS66099) (D), *ROS1* (SALK_045303C) and *DML2* (SALK_131712C) and *DML3* (SALK_056440C) (E). The number of galls per root system was counted 3 weeks post inoculation. Data are presented as means $\pm$ SE (n = 20). Asterisks denote statistically significant differences from the wild-type Col-0 or C24 as determined by unadjusted *t* tests ($P < 0.05$).

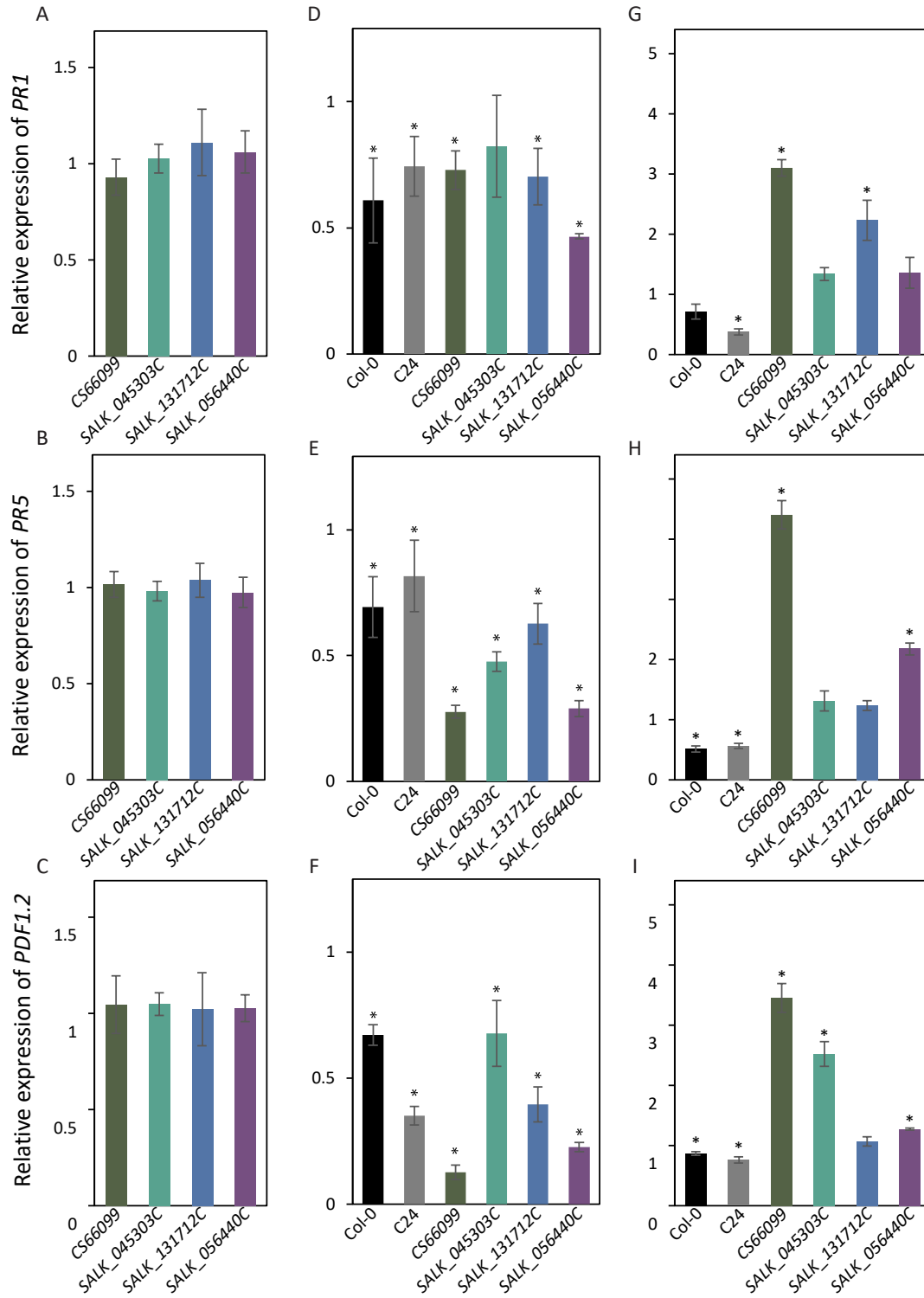


Figure 3.5. Expression levels of defense-related genes in demethylase mutants in response to infection by cyst and root-knot nematodes.

A-C Expression of *PR1* (A), *PR5* (B) and *PDF1.2* (C) in roots tissues of the mutant alleles of *ROS1* (*CS66099* and *SALK_045303C*), *DML2* (*SALK_131712C*), and *DML3* (*SALK_056440C*) under noninfected conditions relative to wild-type plants. D-F: Expression of *PR1* (D), *PR5*(E) and *PDF1.2* (F) in the *H. schachtii*-infected roots tissues of wild-type (Col-0 and C24) and the mutant alleles of *ROS1* (*CS66099* and *SALK_045303C*), *DML2* (*SALK_131712C*), and *DML3* (*SALK_056440C*) relative to noninfected root tissues. G-H: Expression of *PR1* (G), *PR5* (H) and *PDF1.2* (I) in the *M. incognita*-infected roots tissues of wild-type (Col-0 and C24) and the mutant alleles of *ROS1* (*CS66099* and *SALK_045303C*), *DML2* (*SALK_131712C*), and *DML3* (*SALK_056440C*) relative to noninfected root tissues. Relative gene expression levels were obtained from three biological samples and presented as mean $\pm$ SE. *PP2AA3* was used as internal reference genes to normalize gene expression. Asterisks indicate statistically significance differences from control treatments at $P < 0.05$.

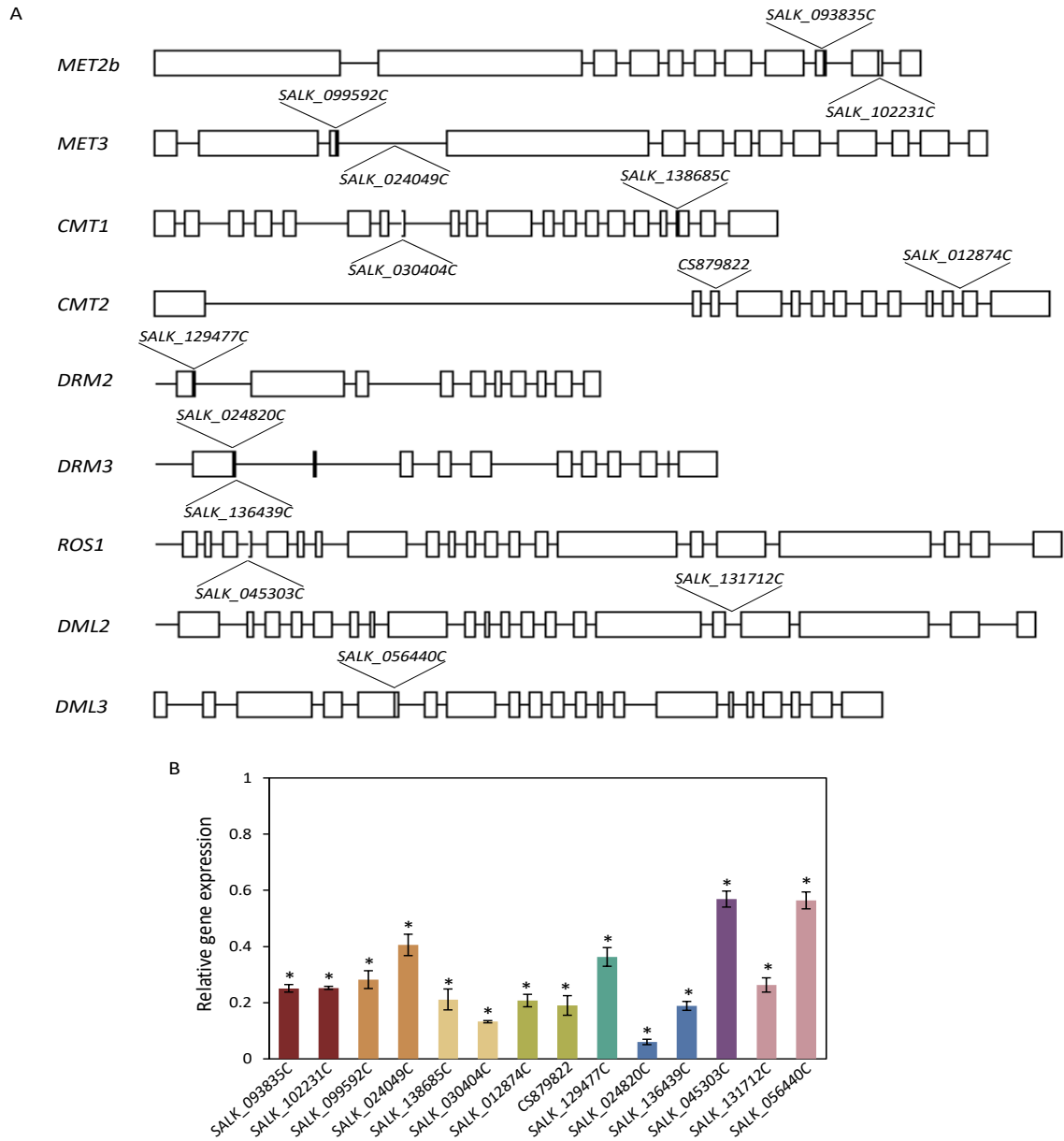


Figure 3.6. Characterization of Arabidopsis DNA methyltransferase and demethylase mutants. A: Schematic representation of various DNA methyltransferase and demethylase mutant alleles showing locations of the T-DNA insertions. B: mRNA expression levels in various Arabidopsis DNA methyltransferase and demethylase mutants. mRNA expression levels were determined in 10-day-old plants using qRT-PCR. *PP2AA3* was used as internal reference genes to normalize gene expression. Relative fold-change values reflect changes in mRNA abundance in the mutant lines relative to wild-type plants. Shown are averages of three biologically independent samples $\pm$ SE. Asterisks indicate statistically significant differences from the wild type at $P < 0.05$.

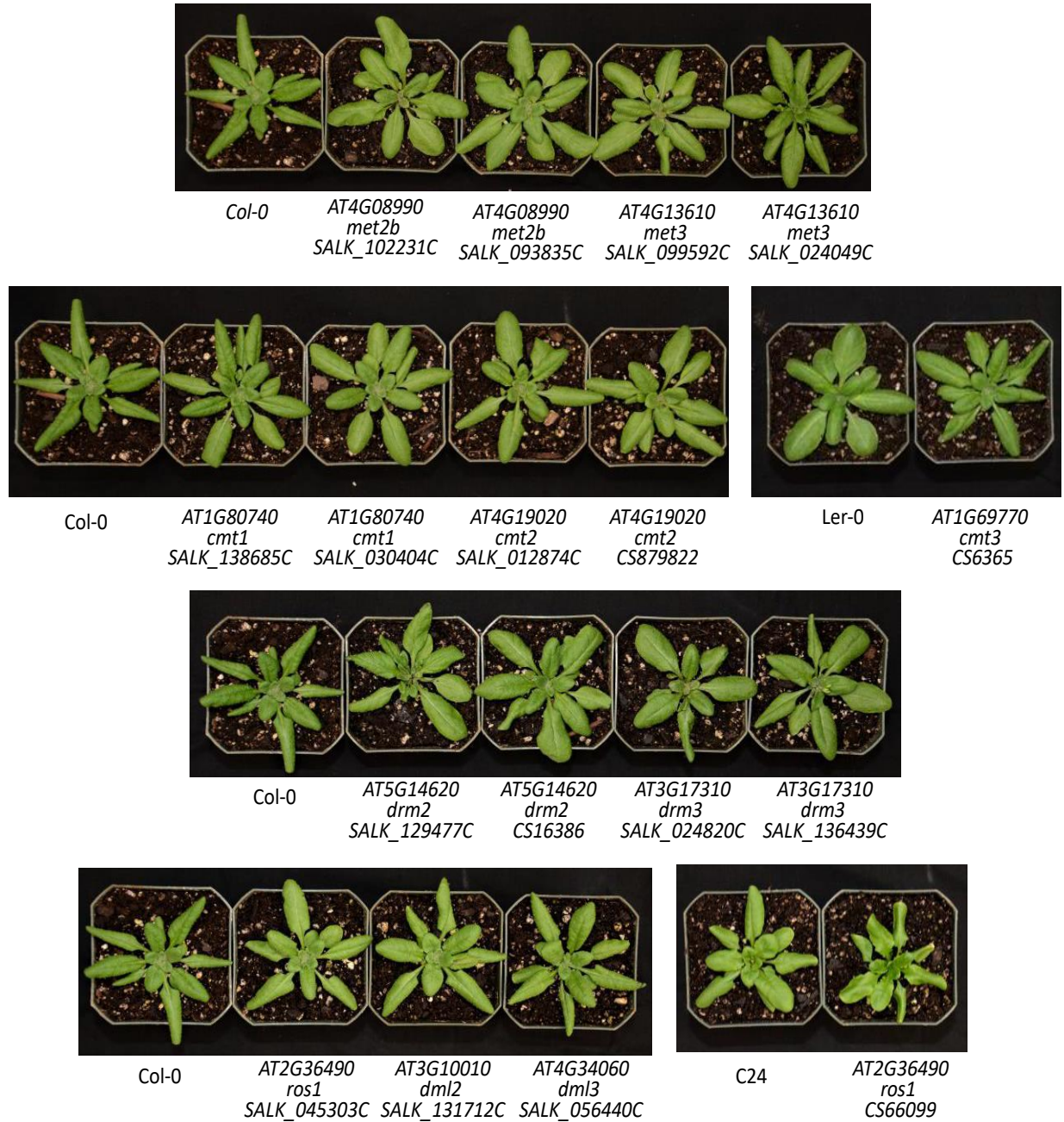


Figure 3.7. Shoot phenotypes of four-week-old *Arabidopsis* DNA methyltransferase and demethylase mutants used in the current study. Seeds of the indicated mutant lines and corresponding wild types were planted in soil and shoot phenotypes of four-week-old plants were photographed.

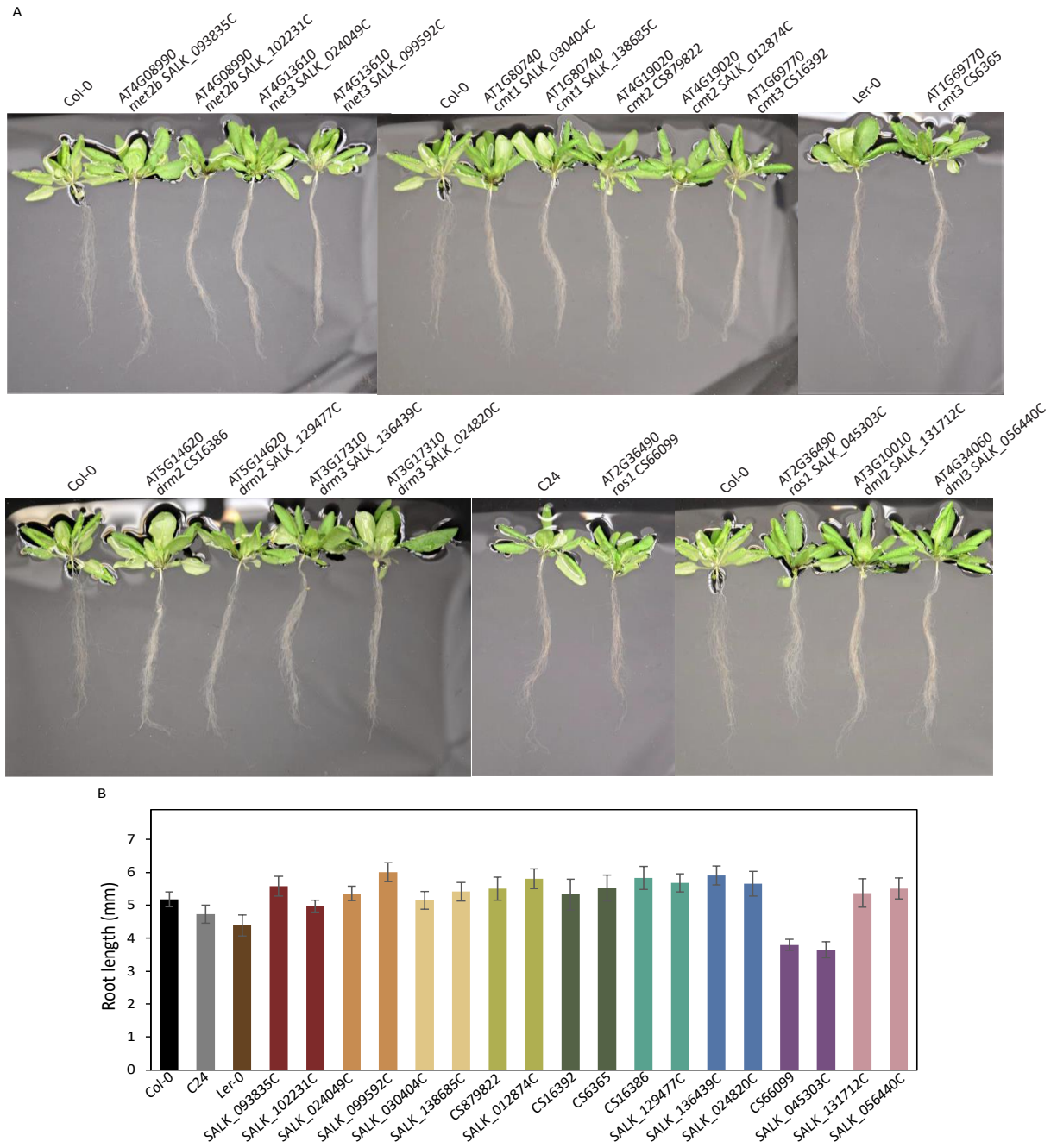


Figure 3.8. Root phenotypes of four-week-old *Arabidopsis* DNA methyltransferase and demethylase mutants used in the current study. A: Root phenotypes of the indicated four-week-old *Arabidopsis* DNA methyltransferase and demethylase mutants and the corresponding wild types. B: Root lengths of the indicated *Arabidopsis* DNA methyltransferase and demethylase mutants and the corresponding wild types. Root lengths of at least 15 plants were measured two weeks post planting. Data are shown as means $\pm$ SE. No statistical differences were found ($P < 0.05$) for root lengths of mutant lines compared to wild-type plants.

Table 3.1. Primer sequences used in the current study.

Gene	Gene ID	5' primer	3' primer
<i>METHYLTRANSFERASE2b (MET2b)</i>	AT4G08990	GCTTGTGCTTGTGTGGAGGTG	GGCTGTCTCATCCAGACCAGC
<i>METHYLTRANSFERASE 3 (MET3)</i>	AT4G13610	GCTAAACTACTGCAAGAGGAAG	CCTCGCATCAACCGCATAGCC
<i>CHROMOMETHYLASE 1 (CMT1)</i>	AT1G80740	TCCTCTCACCTGCATCTGGTC	GCAATTCAAGGCAGAGTCACTT
<i>CHROMOMETHYLASE 2 (CMT2)</i>	AT4G19020	TCAGCTTCGGAGGTCGCCGAG	CCATCTCCAGGCTCTTGGCAA
<i>DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2)</i>	AT5G14620	CATGGAGAAGACAATATGGAAGC	GGATCTTTCTCATCATCTGAGT
<i>DOMAINS REARRANGED METHYLTRANSFERASE 3 (DRM3)</i>	AT3G17310	TCACTTGATGGTGCTGAGATC	CACCAGTAACAATTGACTCAGC
<i>REPRESSOR OF SILENCING 1 (ROS1)</i>	AT2G36490	GAGGAAATATGTGCGGAAGAAGG	CCATCTCACCTGCTTCTCTAATG
<i>DEMETER-LIKE 2 (DML2)</i>	AT3G10010	GACCTTCAAGAAGAAGATGAGG	CTTCTGGTCCGGTATTGACAA
<i>DEMETER-LIKE 3 (DML3)</i>	AT4G34060	AGAGGGAAAAAGAGGACCACC	GCATCTGCTTGTCCATTAATAG
<i>PATHOGENESIS-RELATED GENE 1 (PR1)</i>	AT2G14610	TGGACAACCAGGCACGAGGAG	GCCAGACAAGTCACCGCTACC
<i>PATHOGENESIS-RELATED GENE 5 (PR5)</i>	AT1G75040	CAATTGCCCTACCACGTCTGG	CTTAGACCGCCACAGTCTCCG
<i>PLANT DEFENSIN1.2 (PDF1.2)</i>	AT5G44420	CGCACCGGCAATGGTGGAAGC	GCATGATCCATGTTTGGCTCCTTC
<i>PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2AA3)</i>	AT1G13220	AAGCGGTTGTGGAGAACATGATACG	TGGAGAGCTTGATTGCGAAATACGC

CHAPTER 4
INTERPLAY BETWEEN GENE EXPRESSION, HISTONE
MODIFICATIONS, AND DNA METHYLATION MEDIATED BY MIR778
DURING CYST NEMATODE PARASITISM

4.1 Abstract

Coordinated epigenetic mechanisms, such as DNA methylation, histone modifications, and microRNAs (miRNAs), are known to regulate many aspects of plant growth and stress responses. miR778 post-transcriptionally silences the histone 3 lysine 9 (H3K9) dimethyltransferases *SUVH5* and *SUVH6* during plant development and in response to stress stimuli. However, the epigenomic and transcriptomic effects of the miR778-*SUVH5/6* regulatory mechanism remain unknown. It is shown here that *SUVH5* and *SUVH6* are targeted for post-transcriptional silencing by miR778 in the syncytia formed by the beet cyst nematode *H. schachtii* in *Arabidopsis* roots. Manipulating the expression of miR778 and its two target genes through overexpression, target mimicry, and T-DNA insertional mutation significantly altered plant susceptibility to *H. schachtii*. RNA-seq analysis of *suvh5* and *suvh6* single mutants and wild-type plants under both infected and noninfected condition revealed a key role of *SUVH5* and *SUVH6* in establishing the transcriptome of *Arabidopsis* roots upon *H. schachtii* infection through redundant regulation of the expression of thousands of genes involved in a wide range of biological processes and molecular functions. In addition, ChIP-seq analysis revealed that under noninfected conditions *SUVH5* and *SUVH6* establish H3K9me2 mainly at transposable elements (TEs). However, under *H. schachtii* infected conditions, *SUVH6* and to a much lesser extent *SUVH5* mediate H3K9me2 deposition mainly at protein-coding genes both in promoter and transcribed regions. Further analysis revealed that *SUVH5* and *SUVH6* are the major enzymes mediating H3K9me2 deposition in response to *H. schachtii* infection, contribute to gene expression changes in nematode feeding sites, and associate with CG methylation. Together, these results uncovered multi-layered epigenetic regulatory mechanisms that contribute to transcriptome reprogramming during cyst nematode parasitism of *Arabidopsis*.

Keywords: *Arabidopsis*, ChIP-seq, DNA methylation, *Heterodera schachtii*, histone 3 lysine 9 dimethylation (H3K9me2), miR778, RNA-seq, *SUVH5*, *SUVH6*, syncytium

4.2 Introduction

Cyst nematodes (*Heterodera* spp.) are among the most agronomically and economically devastating plant pests, accounting for over a billion dollars in annual yield losses (Bernard et al, 2017). The difficulty in controlling cyst nematodes lies in their unique and complex interaction with host plants. Second-stage juveniles (J2) penetrate host roots and migrate intracellularly to establish their permanent feeding site, called syncytia (Sobczak and Golinowski, 2009). During syncytia development, individual cells undergo cell wall dissolution and cell-to-cell fusion, the disappearance of large vacuoles, and an increase in metabolic organelles, i.e., mitochondria and nuclei (Sobczak and Golinowski, 2009). Syncytium development occurs in three distinct stages: initiation, formation, and maintenance, in which the nematode progresses through its life cycle concordantly (Sobczak and Golinowski, 2009).

Syncytium development is underlined by metabolic rewiring and the differential expression of thousands of genes (Fuller et al, 2007; Szakasits et al, 2009; Hewezi et al, 2017; Rambani et al, 2020a). The induction of such massive cellular and molecular responses requires transcriptional, post-transcriptional, and post-translational modifications (Hewezi, 2015). DNA methylation in particular has been linked to transcriptome reprogramming induced by cyst nematodes (Rambani et al, 2015; Hewezi et al, 2017; Piya et al, 2017; Rambani et al, 2020a; 2020b). Plant DNA methylation occurs in three sequence contexts: CG, CHG, and CHH, where H is any nucleotide except G (Lister et al, 2008). Evidence provides that DNA methylation closely associates with other epigenetic mechanisms, such as histone methylation and microRNA (miRNA)-mediated post-transcriptional gene silencing (PTGS) to give rise to genome-wide changes in gene expression (Du et al, 2015; Rambani et al, 2020a). PTGS mostly happens through the action of miRNA in eukaryotes (Bartel, 2004; Friedman et al, 2009; Sun, 2012). Plant miRNA genes are transcribed much like protein coding genes, but the primary transcripts are processed into mature 21- 22-nt molecules. Mature miRNA molecules are mobilized by the RNA-induced silencing complex (RISC) in the cytoplasm for near-perfect complementary binding at target sites. Target mRNAs are cleaved by RISC and degraded for effective gene silencing (Sun, 2012; Xie et al, 2015). Through this mechanism, miRNAs are responsible for regulating various developmental processes

and stress responses (Liu et al, 2009; Nonogaki et al, 2010; Nonogaki et al, 2010; Sun, 2012; Barrera-Figueroa et al, 2013; Kruszka et al, 2013).

A significant number of miRNAs have been identified as differentially expressed in response to cyst nematode infection, suggesting their involvement in mediating plant-nematode interactions (Hewezi et al, 2008; Li et al, 2012; Xu et al, 2014; Tian et al, 2017). Few, however, have been functionally characterized. Of the miRNA that have been characterized, the activity of miR396 and its targets *GROWTH REGULATING FACTOR1* (*GRF1*) and *GRF3* were found to serve as a vital components of syncytium stage transition and the coordination of growth-stress antagonism in the syncytium (Hewezi and Baum, 2012; Hewezi et al, 2012; Liu et al, 2014; Noon et al, 2019; Piya et al, 2020). Another miRNA that can target transcription factors, amplifying its effect on post-transcriptional reprogramming is miR858. During the early stages of syncytium development, miR858 is highly active in targeting *MYB83*, which is involved in the regulation of hormone signaling, cell wall modification, sucrose transport, and other processes crucial for syncytium development (Piya et al, 2017). miR827 specifically targets the ubiquitin E3 ligase *NITROGEN LIMITATION ADAPTATION* (*NLA*), which positively regulates the expression of defense-response genes when challenged with various plant stressors (Peng et al, 2007; Kant et al, 2011; Lin et al, 2013; Park et al, 2014; Hewezi et al, 2016). In response to the beet cyst nematode (*H. schachtii*, BCN), miR827 is highly active throughout syncytium development and *NLA* was found to physically associate with proteins involved in defense response and key transcription factors (Hewezi et al, 2016). These examples provide evidence for the key roles that miRNAs play in mediating plant-nematode interactions. However, more research is required to explore the impact of other syncytium differentially expressed miRNAs and their target genes.

Histone methylation is another important epigenetic mark for controlling gene expression and regulating biological processes (Berr et al, 2011; Yuan et al, 2013; Zhao et al, 2019; Ueda and Seki, 2020). Histone tail modifications directly alter nucleosome structure and trigger downstream transcriptional changes, either for gene activation or gene silencing. Broadly, euchromatic regions of the genome are highly enriched in acetylated histones (Berger, 2007; Shahbazian and Grustein, 2007) and DNA and histone methylation, particularly histone 3 lysine 9 methylation (H3K9me), are both highly enriched in heterochromatic regions, where they tend to

be repressive epigenetic marks (Tariq and Paszkowski, 2004; Xiao et al, 2016). However, stress stimuli can redirect histone deacetylases and methyltransferases for local chromatic and transcriptomic changes that can induce stress-specific responses (Pfluger and Wagner, 2007). For example, HISTONE DEACETYLASE 19 (HDA19), HDA6, and HDA2B, which remove H3K9 acetylation marks, regulate plant susceptibility to plant pathogenic fungi and bacteria, respectively, and are thought to fine-tune the expression of defense-related genes (Wu et al, 2000; Zhou et al, 2005; Kim et al, 2008; Wu et al, 2008; Zhou et al, 2010; Choi et al, 2012; Latrasse et al, 2017; Ramirez-Prado et al, 2018). For transcriptional repression, nucleosomes are often methylated at H3K9 (Pfluger and Wagner, 2007). Histone methylation can occur as mono- (me), di- (me₂), and tri- (me₃) methylations evidentially have unique and overlapping functions (Xiao et al, 2016). Although heterochromatin is generally characterized by heavy mono- and dimethylation of H3K9 (Jackson et al, 2004; Fuchs et al, 2006), investigation of the role of H3K9 methylation in plant immunity is lacking. H3K9 demethylation has recently been identified as a positive regulator of plant defense response to *Pst DC3000* (Dutta et al, 2017), but more research is needed to determine the broader impact of H3K9 methylation in plant defense responses.

SU(VAR)3-9 HOMOLOG5 (SUVH5) and SUVH6 catalyze the dimethylation of H3K9 (H3K9me₂), which is the most prominent H3K9 mark, using a distinct 5-methyl-dC base-flipping mechanism and is particularly important for transcriptional regulation (Jackson et al, 2004; Ebbs and Bender, 2006; Deal and Henikoff, 2011; Rajakumara et al, 2011). It is known that SUVH5 and SUVH6 have a strong functional overlap with their homolog, SUVH4 (Malagnac et al, 2002; Jackson et al, 2004; Ebbs et al, 2005; Ebbs and Bender, 2006; Johnson et al, 2007; Du et al, 2014; Li et al, 2018). The SET domain of SUVH4, 5, and 6 recruits DNA methyltransferases CMT2 and CMT3 (Bernatavichute et al, 2008; Stroud et al, 2014), which recognize H3K9me₂ using their bromo-adjacent homology (BAH) and chromo domains (Du et al, 2014; Stroud et al, 2014; Du et al, 2015). Based on the correlation of predominately SUVH4-driven H3K9me₂ and non-CG methylation, a self-reinforcing feedback loop has been proposed to associate specifically with non-CG methylation (non-mCG) sites (Jackson et al, 2002; Malagnac et al, 2002; Bernatavichute et al, 2008). Other lines of evidence indicate that H3K9me₂ also interacts with mCG sites, but to a lesser degree in unchallenged conditions (Soppe et al, 2002; Tariq et al, 2003; Mathieu et al, 2005; Kim

et al, 2014; Stroud et al, 2014). The known function of SUVH5 and SUVH6 in a developmental context has primarily extended to H3K9me2 deposition at TEs and repetitive sequences (Ebbs et al, 2005; Ebbs and Bender, 2006). However, the role of SUVH5- and SUVH6-mediated H3K9me2 in response to stress stimuli has little been explored. Interestingly, *SUVH5* and *SUVH6* mRNA transcripts contain a near-perfect complementary binding site for miR778. miR778 (AT2G41616) is encoded by a gene on chromosome 2 and the mature miR778 molecule post-transcriptionally silences expression of *SUVH5* and 6 (Lu et al, 2006; Fahlgren et al, 2007).

Here, the regulatory module miR778—*SUVH5/6* is assessed for its role in *H. schachtii* parasitism of Arabidopsis roots. Functional redundancy and transcriptional feedback regulation of *SUVH5* and *SUVH6* was also explored under *H. schachtii*-infected and noninfected conditions. Furthermore, a novel role for SUVH5- and SUVH6-established H3K9me2 was investigated in response to *H. schachtii* infection. Further investigation of this novel mechanism suggests a regulatory mechanism including multiple epigenetic modifications is employed for plant responses to *H. schachtii* infection.

4.3 Results

4.3.1 miR778 *regulates SUVH5 and 6 during syncytium development*

To examine the potential implication of miR778 and its target genes (Figure 4.1A) in the plant—nematode interaction, transgenic lines expressing the β -glucuronidase (GUS) reporter gene under the control of miR778, *SUVH5*, and *SUVH6* promoters were generated. Promoter activity of at least three transgenic lines (T2 generation) was determined for each construct under noninfected conditions and in the syncytium induced by the beet cyst nematode *H. schachtii*. Under *H. schachtii*-infected conditions, histochemical GUS assays of the miR778 promoter revealed high expression in the developing syncytium formed by the second-stage juveniles (J2) at 3 days post infection (dpi) as well as in the fully developed syncytia of third-stage juveniles (J3) at 7 and 10 dpi (Figure 4.1B). GUS activity of miR778 promoter was also detected in the syncytium of J4

nematodes at 14 dpi (Figure 4.1B). GUS activity directed by the *SUVH5* promoter was also observed in the developing syncytium formed by J2 *H. schachtii* at 5, 7, and 10 dpi (Figure 4.1C) and GUS activity directed by the *SUVH6* promoter was induced in the developing syncytium of early J3 *H. schachtii* specifically at 5 dpi (Figure 4.1D). *SUVH5* and *SUVH6* were also reported as differentially expressed in the syncytial cells induced by *H. schachtii* in Arabidopsis (Szakasits et al, 2009). The transcriptional activity of miR778 and its *SUVH5* and *SUVH6* targets in the syncytium implies a posttranscriptional regulation of *SUVH5* and *SUVH6* by miR778 upon nematode infection.

To test the possibility of PTGS of *SUVH5* and 6 by miR778, the expression levels of primary and mature miR778 transcripts as well as uncleaved transcript levels of *SUVH5* and *SUVH6* were quantified in root tissues collected from *H. schachtii*-infected and noninfected Col-0 plants at 4, 7, 10, and 14 dpi. Uncleaved transcript levels of *SUVH5* and *SUVH6* were measured using two primer pairs flanking the miR778 binding site. Quantitative PCR (qPCR) data of three biological samples revealed significant ($P < 0.05$) upregulation of primary and mature miR778 transcripts in the infected roots at 7, 10, and 14 dpi as compared with noninfected control roots (Figure 4.2). Concurrently, the levels of uncleaved *SUVH5* and *SUVH6* transcripts were significantly downregulated in the infected roots compared with noninfected roots at the same time points (Figure 4.2), which were measured using quantitative reverse transcription PCR (qRT-PCR). Together, these data indicate the *SUVH5* and *SUVH6* are subjected to PTGS by miR778 following nematode infection.

4.3.2 Altered expression of miR778 impacts plant susceptibility to *H. schachtii*

The role of miR778 in modulating plant response to nematode infection was examined by generating transgenic Arabidopsis lines with increased and reduced miR778 expression using overexpression and target mimicry approaches, respectively. The overexpression lines were generated by overexpressing the primary miR778 sequence using the 35S promoter (35S:miR778). Several transgenic T3 lines were phenotypically characterized. miR778 overexpression impacted leaf morphology but no noticeable effects on root morphology and length were detected (Figure 4.3A-C). Consistent with PTGS of *SUVH5* and *SUVH6* by miR778, the increased expression of

miR778 in overexpression lines was associated with significant reduction of *SUVH5* and *SUVH6* transcript levels (Figure 4.3D). The miR778 overexpression lines along with wild-type Col-0 plants were then assayed for *H. schachtii* susceptibility. The numbers of J4 female nematodes per root system were counted and used to determine plant susceptibility relative to wild-type Col-0 plants. Interestingly, the overexpression lines showed statistically significant increases in susceptibility levels as compared with Col-0 plants (Figure 4.3E).

The transgenic lines overexpression an artificial mimic sequence for mature miR778 (MIM778) were similarly analyzed (Figure 4.4). The artificial mimic sequence was generated by overexpressing a modified version of the non-protein coding gene *IPS1* (INDUCED BY PHOSPHATE STARVATION1) in which the native miR399 binding site was replaced by a mimic sequence for miR778. The mimic sequence (MIM778) includes a three-nucleotide (nt) bulge (TTC) in the miRNA binding site that would impede transcript degradation without affecting miR778 binding ability (Figure 4.4A). Transgenic T3 plants overexpressing MIM778 under control of the 35S promoter (35S:MIM778), showed no morphological irregularity in roots, however, the MIM778 overexpression lines showed altered leaf morphology similar to that of miR778 overexpression (Figure 4.4B-D). The expression levels of miR778 in three MIM778 overexpression lines were significantly downregulated (Figure 4.4E), confirming the efficiency of the artificial non-cleavable binding sequence in inhibiting the activity of miR778. miR778 downregulation in the MIM778 lines was also associated with significant increases in the expression of its *SUVH5* and *SUVH6* target genes (Figure 4.4E). Importantly, when the MIM778 overexpression lines were assayed for nematode susceptibility, these lines showed the opposite effect of miR778 overexpression, with significant decrease in plant susceptibility in comparison with Col-0 plants (Figure 4.4F). Together, these data indicate that *H. schachtii*-induced upregulation of miR778 in the feeding cells promotes nematode parasitism of Arabidopsis plants.

4.3.3 Mutations of *SUVH5* and *SUVH6* alter plant susceptibility to *H. schachtii*

To directly reveal the functional role of *SUVH5* and *SUVH6* in plant-nematode interactions, the single mutants *suvh5* (GK-263C05, Zheng et al, 2012 and *FLAG_280C12*) and *suvh6* (*SAIL_1244_F04*, Wang et al, 2015, and *SALK_071138C*) were evaluated for *H. schachtii*

susceptibility (Figure 4.5). For mutants not previously characterized (*FLAG_280C12* and *SALK_071138C*), the expression of *SUVH5* and *SUVH6* was determined. Relative expression of *SUVH5* or *SUVH6* was significantly downregulated in the *FLAG_280C12* and *SALK_071138C* mutants, respectively (Figure 4.5A and B). While it was expected that the mutant lines will phenocopy *miR778* overexpression of increased susceptibility, unexpectedly these mutants exhibited statistically significant decreases in susceptibility to *H. schachtii* as compared with the corresponding Col-0 or C24 wild-type plants (Figure 4.5C-F).

4.3.4 Mutations of SUVH5 and SUVH6 modulate the transcriptome of Arabidopsis roots upon H. schachtii infection

Next, RNA-seq analysis of *suvh5* (*GK-263C05*) and *suvh6* (*SAIL_1244_F04*) mutants and Col-0 plants was performed under both noninfected and *H. schachtii*-infected conditions to better understand how *SUVH5* and *SUVH6* altered plant susceptibility. A total of 18 biological samples were collected from root tissues of infected and the corresponding noninfected control plants at 5 dpi. Under noninfected condition, 1057 and 3390 differentially expressed genes (DEGs) were identified respectively in the *suvh5* and *suvh6* mutants compared with Col-0 plants using an adjusted *P* value cutoff of 0.05 (Tables 4.1 and 4.2). Of those DEGs, a similar number were up- and downregulated (Figure 4.13A). However, Gene Ontology (GO) term analysis of DEGs enriched in *suvh5* and *suvh6* revealed distinct differential expression patterns for a significant number of genes involved in a wide range of biological processes. For example, genes involved in responses to stimuli and stress were downregulated (Figure 4.13B and Table 4.3). Notably, 491 (46.5%) of the DEGs identified in *suvh5* were also differentially expressed in *suvh6*, 487 (99%) of which were differentially expressed in the same direction in *suvh5* and *suvh6*, supporting previous reports of their redundant function (Ebbs and Bender, 2006; Johnson et al, 2007; Li et al, 2018) (Figure 4.13C).

Root transcriptomes of *suvh5* and *suvh6* single mutants were then compared with that of Col-0 in response to *H. schachtii* infection at 5 dpi. There were 2487 and 846 DEGs identified respectively in infected *suvh5* and *suvh6* when compared with infected Col-0 (adjusted *P* < 0.05) (Tables 4.4 and 4.5). Consistent with the redundant function of *SUVH5* and *SUVH6* observed under

noninfected conditions, about half of the DEGs identified in *suvh6* were also differentially expressed in *suvh5*. There were also 4566 DEGs in *H. schachtii*-infected Col-0 roots identified when compared with noninfected roots (Table 4.6). The proportion of genes that were up- and downregulated were similar for infected Col-0, *suvh5*, and *suvh6* (Figure 4.6A). Nonetheless, GO terms analysis revealed distinct differential expression patterns for many of the DEGs enriched in infected Col-0, *suvh5* or *suvh6* (Figure 4.6B and Table 4.7). Among these are terms related to innate immune response, response to biotic stimuli, and toxin metabolism, which are downregulated in *H. schachtii*-infected Col-0 and upregulated in infected *suvh5*. Whereas other terms associated with metabolic processes, photosynthetic reactions, cellular component organization and biosynthesis, and responses to cytokinin are upregulated in infected Col-0 and downregulated in *suvh5* and/or *suvh6*. Consistent with the redundant function of *SUVH5* and *SUVH6* observed under noninfected conditions, GO term analysis also revealed differential expression patterns common to *suvh5* and *suvh6* (Figure 4.6B). These terms include genes that are upregulated in *H. schachtii*-infected *suvh5* and *suvh6* root and are associated with terms related to secondary metabolism, response to toxic substances, and cell communication and signaling. Whereas genes that are downregulated are associated with terms related to cell cycling, cell division, and cytokinesis. Furthermore, about half of the DEGs identified in *suvh6* were also differentially expressed in *suvh5*. Notably, 51% (1277 genes) and 70% (592 genes) of the DEGs identified respectively in the infected *suvh5* and *suvh6* single mutants overlapped with DEGs identified in infected Col-0 plants (Figure 4.6C). These data suggest that *SUVH5* and *SUVH6* are involved in shaping the transcriptome of Arabidopsis roots upon *H. schachtii* infection.

4.3.5 SUVH5 and SUVH6 expression is controlled through a feedback mechanism

Careful examination of the RNA-seq data revealed remarkable upregulation (4.3- $\log_2$ fold) of *SUVH6* in the knockout *suvh5* mutant (Table 4.1). Similarly, *SUVH5* was significantly upregulated (5.1- $\log_2$ fold) in the knockout *suvh6* mutant (Table 4.2). The expression of *SUVH5* and *SUVH6* was then quantified in the other mutant alleles of *suvh6* (*SAIK_071138C*) and *suvh5* (*FLAG_280C12*), respectively, using qRT-PCR. *SUVH6* showed 1.22-fold upregulation in the *suvh5* mutant *FLAG_280C12* relative to wild-type C24, whereas *SUVH5* showed 1.85-fold upregulation in the *suvh6* mutant *SAIK_071138C* relative to wild-type Col-0 (Figure 4.5A and B).

While these gene expression data imply that a feedback regulatory mechanism connecting the expression of *SUVH5* and *SUVH6* may exist, they also explain the unexpected decreases in nematode susceptibility observed in *suvh5* and *suvh6* single mutants (Figure 4.5C-F).

4.3.6 *SUVH5* and *SUVH6* establish *H3K9me2* mainly in TEs under noninfected conditions

ChIP-seq was performed in order to investigate *H3K9me2* deposition by *SUVH5* and *SUVH6* under both noninfected and *H. schachtii*-infected conditions. To this end, 10-day old Col-0 and the single knockout mutants *suvh5* (GK-263C05, Zheng et al, 2012) and *suvh6* (SALK_1244_F04, Wang et al, 2015) were inoculated. At 5 dpi, root tissues were collected from both infected and noninfected plants in triplicate and used for library preparation and sequencing. Peak-calling analysis identified 511 and 143 peaks that were depleted in noninfected *suvh5* and *suvh6*, respectively, when compared with noninfected Col-0 plants (Tables 4.8 and 4.9). Annotation of the 511 peak regions depleted in *suvh5* were associated with 106 protein-coding genes, 6 miRNAs, and 727 TEs (Figure 4.14A). Similarly, the 143 peak regions depleted in *suvh6* were associated with 35 protein-coding genes, 2 miRNAs, and 197 TEs (Figure 4.14B). Investigation of the TEs commonly depleted in *suvh5* and *suvh6* revealed that 94% of TE-associated peaks that were absent in *suvh5* compared to Col-0 were also depleted in *suvh6* (Tables 4.8 and 4.9). The peak regions enriched in Col-0 compared with the mutants were found in the promoter and gene body of protein-coding genes, particularly next to the transcriptional start site (TSS) (Figure 4.4C and D). The low number of peak regions depleted in *suvh5* and *suvh6* can be attributed to the redundant function of the SUVH family members (Ebbs and Bender, 2006; Johnson et al, 2007; Li et al, 2018).

4.3.7 *SUVH5* and *SUVH6* establish *H3K9me2* mainly in protein-coding genes under *H. schachtii*-infected conditions

It was identified 353 and 3106 peaks that were depleted in *H. schachtii*-infected *suvh5* and *suvh6*, respectively, when compared with infected Col-0 plants (Tables 4.10 and 4.11). The 353 peak regions that were enriched in infected Col-0 compared with infected *suvh5* were associated with 352 protein-coding genes, 1 miRNA, and 247 TEs (Figure 4.7A). The 3106 peak regions that were enriched in infected Col-0 compared with infected *suvh6* were associated with 4594 protein-coding

genes, 11 miRNA, and 279 TEs (Figure 4.7B). The H3K9me2 deposition associated with protein-coding genes depleted in *suvh5* were detected in the promoter (102) and gene body (250), particularly near the TSS (Table 4.10; Figure 4.7C). The H3K9me2 deposition associated with protein-coding genes depleted in *suvh6* were also found in the promoter (1816), gene body (3366), or in both the promoter and gene body (558) and were largely located near the TSS (Table 4.11; Figure 4.7D). These results indicate that, under infected conditions, SUVH6 and to a much lesser extent SUVH5 mediate H3K9me2 deposition mainly in protein-coding genes both in promoter and transcribed regions.

H3K9me2 deposition was analyzed in the roots of wild-type Col-0 plants in response to nematode infection. There were 791 peak regions enriched and 306 peaks depleted in infected Col-0 roots in comparison with noninfected roots (Tables 4.12 and 4.13). The 791 peak regions mapped to 1180 protein-coding genes, 3 miRNAs, and 159 TEs (Figure 4.8A). Interestingly, the large majority of the peaks depleted in infected Col-0 overlapped with TEs and to a much lesser extent with protein-coding genes. Specifically, these 306 peaks overlapped with 37 protein-coding genes, 7 miRNAs, and 503 TEs (Figure 4.8B). The H3K9me2 marks enriched in infected Col-0 roots that were located in protein-coding genes were detected in the promoter (468), gene body (858), or in both the promoter and gene body (146), primarily close to the TSS (Table 4.12; Figure 4.8C). Similarly, the few H3K9me2 marks depleted in infected Col-0 roots that were located in protein-coding genes were identified in the promoter (26), gene body (22), or in both the promoter and gene body (11), particularly close to the TSS (Table 4.13; Figure 4.8D).

4.3.8 H3K9me2 marks established by SUVH5 and/or SUVH6 are associated with gene expression changes upon H. schachtii infection

Furthermore, the extent to which SUVH5 and 6 contribute to the deposition of H3K9me2 following *H. schachtii* infection was investigated. For this, the list of protein-coding genes enriched in H3K9me2 in infected Col-0 were compared with those showing H3K9me2 depletion in the mutants under infected conditions. Notably, of the 1180 genes exhibiting H3K9me2 deposition in Col-0, 1129 genes (95.7%) were exhibiting H3K9me2 depletion uniquely in *suvh5* (5 genes) or *suvh6* (892 genes), or in both mutants (232 genes) (Figure 4.9A). These intriguing

data indicate that SUVH5 and SUVH6 are the key enzymes mediating H3K9me2 deposition in wild-type plants upon nematode infection. Additionally, 94% (330/352) of the genes with H3K9me2 depletion in *suvh5* mutant were also detected in *suvh6* (Figure 4.9A), a finding that further confirms the redundant function of SUVH5 and SUVH6.

The expression levels of the 1129 genes showing H3K9me2 deposition in infected wild-type plants and established by SUVH5 or SUVH6 were then examined in the mutant lines. Of these 1129 genes, 122 were differentially expressed in infected *suvh5* or *suvh6* roots (Figure 4.9B). These 1129 genes were also examined for possible overlaps with the previously reported syncytium DEGs (Szakasits et al, 2009). Interestingly, 290 of syncytium-specific DEGs were common to DEGs identified in *suvh5* or *suvh6* (Figure 4.9C). Together, these results indicate that H3K9me2 deposition mediated by SUVH5 and SUVH6 following nematode infection contributes to gene expression changes both in roots and nematode feeding sites.

4.3.9 SUVH5- and SUVH6-mediated H3K9me2 associate with CG methylation in H. schachtii-infection roots

Finally, the association between H3K9me2 and DNA methylation was investigated using the list of differentially methylated genes (DMGs) in *H. schachtii*-infected Col-0 compared to noninfected Col-0 generated by Hewezi et al, (2017). Because H3K9me2 and DNA methylation can operate in a positive feedback loop (Rajakumara et al, 2011; Stroud et al, 2014; Du et al, 2015; Li et al, 2018), DMGs from 5 and 10 dpi roots were considered for this analysis. Of the 1180 protein-coding genes enriched in H3K9me2 in infected Col-0, 257 genes were also found to be differentially methylated, 87.9% of which were hypomethylated upon *H. schachtii* infection (Table 4.10; Figure 4.10A). This reveals that H3K9me2 is associated with the hypomethylated profile observed following cyst nematode infection (Hewezi et al, 2017). Similarly, 91% and 90 % of the DMGs that were depleted in H3K9me2 in *suvh5* (81/89) and *suvh6* (1028/1137) were hypomethylated in *H. schachtii*-infected Col-0 roots. This indicates a redundant function between SUVH5 and SUVH6 for establishing the hypomethylation patterns induced in roots upon *H. schachtii* infection (Figure 4.10A).

To determine the contribution of *suvh5* and *suvh6* in driving the connection between H3K9me2 and DNA methylation during *H. schachtii* infection, the list of 257 differentially methylated and H3K9me2 enriched genes in infected Col-0 were overlapped with the lists of differentially methylated and H3K9me2 depleted genes in *suvh5* and *suvh6*. Interestingly, 71.2% (183/257) of the genes enriched in H3K9me2 and differentially methylated in infected Col-0 were also depleted in H3K9me2 and differentially methylated uniquely in *suvh5* (1 gene) or *suvh6* (147 genes), or in both mutants (35 genes) (Figure 4.10B). These results reveal a noteworthy connection between SUVH5- and SUVH6-mediated H3K9me2 deposition and DNA methylation that is elicited during *H. Schachtii* pathology.

The association between H3K9me2 and DNA methylation was further investigated in various sequence contexts for the 183 DMGs that are established in H3K9me2 by SUVH5 and/or SUVH6. Previous studies report that H3K9me2 is predominately associated with non-CG methylation (Stroud et al, 2013; 2014). However, of the 172 hypomethylated genes, surprisingly, 160 genes (93%) were differentially methylated in the CG context, while only 12 genes (6.98%) were differentially methylated in the non-CG context (Figure 4.10C). The other 11 genes that were differentially methylated were all hypermethylated in the CG context (Figure 4.10C), revealing a strong connection between CG hypomethylation and SUVH5- and SUVH6-mediated H3K9me2 in *H. schachtii*-infected roots.

4.4 Discussion

It is well known that various epigenetic mechanisms, such as miRNA, histone methylation, and DNA methylation are highly coordinated to regulate gene expression in a variety of developmental and stress response contexts (Chuang and Jones, 2007; Sato et al, 2011; Hewezi, 2020). miR778 post-transcriptionally regulates two H3K9 methyltransferases, SUVH5 and SUVH6 (Lu et al, 2006; Fahlgren et al, 2007). Recently, activity of miR778 was described in response to phosphate starvation, which induces the transcriptional activation of stress-responsive genes (Wang et al,

2015; Segal et al, 2020). Here, it was shown that miR778 acts as an upstream switch controlling a self-reinforcing circuit of H3K9me2 and DNA methylation in *H. schachtii* parasitism.

In response to *H. schachtii* infection, miR778 and its target genes are all strongly induced in developing syncytia. PTGS of *SUVH5* and *SUVH6* by miR778 was apparent by coincidental increases of primary and mature miR778 transcripts and depletion of *SUVH5* and *SUVH6* uncleaved mRNA at 7, 10, and 14 dpi. Furthermore, *SUVH5* and *SUVH6* were downregulated when miR778 was overexpressed, whereas target genes were upregulated when miR778 expression was reduced via target mimicry. This regulatory role of miR778 over *SUVH5* and 6 has a significant impact on plant susceptibility to *H. schachtii*. These data show that increased miR778 expression resulted in increased plant susceptibility and reduced expression of miR778 conferred reduced plant susceptibility as compared with wild-type plants. Interestingly, however, when either *SUVH5* or *SUVH6* was independently mutated, plants exhibited increased resistance compared to wild-type plants, similar to the MIM778 overexpression lines. This decrease in plant susceptibility was evidently due to the significant induction of *SUVH5* in the *suvh6* mutant and *SUVH6* in the *suvh5* mutant. Thus, the regulation of *SUVH5* and *SUVH6* are coordinated in a reciprocal manner to convey resistance to *H. schachtii*, but in wild-type plants this effect is subjugated by miR778-driven PTGS, which contributes to the susceptible phenotype observed in wild-type plants.

SUVH5 and 6 are known to function redundantly to *SUVH4*, predominately at TEs and repetitive sequences (Ebbs et al, 2005; Ebbs and Bender, 2006; Li et al, 2018). Evidence of coordinated *SUVH5* and *SUVH6* activity was further revealed by their redundant localization to H3K9 for dimethylation. Relatively few H3K9me2 peaks were identified in the roots of *suvh5* and *suvh6* single knockout mutants compared to Col-0, most of which were associated with TEs, similar to the results of Li et al (2018) who profiled H3K9me2 in two-week-old *suvh5* and *suvh6* seedlings. This implies that, under noninfected conditions, H3K9 associated with TEs can be dimethylated by multiple H3K9 dimethyltransferases, i.e., other SUVH family members. The vast majority of TE-associated peaks that were absent in *suvh5* compared to Col-0 were also depleted in *suvh6*, indicating a strong functional overlap between *SUVH5* and *SUVH6* under noninfected conditions. *SUVH4* has been considered the main H3K9 dimethyltransferase in unchallenged plants (Jackson

et al, 2002; 2004; Malagnac et al, 2002; Stroud et al, 2013; 2014; Li et al, 2018). However, *SUVH5* and 6 are differentially expressed in syncytia, while the expression of *SUVH4* remains unchanged in syncytial cells (Szakasits et al, 2009). It was also noted of RNA-seq data presented here that, of the *SUVH* family genes, *SUVH5* was the most highly upregulated in *H. schachtii*-infected roots. This apparent shift in the roles of *SUVH4*, 5, and 6 reveals that a novel regulatory mechanism controlling the expression these genes is induced by *H. schachtii* infection. Indeed, *H. schachtii* parasitism induces H3K9me2 by *SUVH5* and *SUVH6* primarily targeted to protein-coding genes, 94% of which are redundantly methylated by *SUVH5* in the *suvh6* mutant. This regulatory overlap between *SUVH5* and *SUVH6* also effects gene expression changes in the *suvh5* and *suvh6* mutants. Under both noninfected and *H. schachtii*-infected conditions, about half of the DEGs overlapped between *suvh5* and *suvh6*.

The novel role of *SUVH5* and *SUVH6* in predominantly targeting H3K9 associated with protein-coding genes in response to *H. schachtii* infection was further illustrated by examining genes with H3K9me2 attributed to *SUVH5* and/or *SUVH6*. *SUVH5* and *SUVH6* account for over 95% of the 1180 differentially H3K9 dimethylated genes upon *H. schachtii* infection, a significant number of which were also found to be differentially expressed in syncytial cells (Szakasits et al, 2009). *SUVH5*- and *SUVH6*-driven H3K9me2 deposition is likely responsible the transcriptional suppression of various genes, including *PINOID* (*PID*, AT2G3465), *SUCROSE-PROTON SYMPORTER 2* (*SUC2*, AT1G22710), and *THAUMATIN-LIKE PROTEIN 1* (*TLP1*, AT4G24180), the roles of which were further investigated in syncytium formation or plant defense response. The Ser/Thr protein kinase, *PID*, is responsible for temporal and spatial auxin distribution and is therefore involved in many biological processes involving auxin localization (Friml et al, 2004; Michniewicz et al, 2007). Indeed, auxin redistribution has been identified as an important part of syncytium development (Karczmarek et al, 2004; Grunewald et al, 2009; Hewezi et al, 2014). These RNA-seq data show that *PID* is downregulated (-0.873 log₂ fold change, adjusted *P* = 0.033) in *H. schachtii*-infected *suvh6* roots as compared with infected Col-0, suggesting it could play a role in aberrant cell proliferation characteristic of syncytial cells. Similarly, *SUC2*, which is involved unloading sucrose from the phloem, also seems to be downregulated (-0.899 log₂ fold change, adjusted *P* = 0.015) in *H. schachtii*-infected *suvh6* roots

compared to infected Col-0, which could be a result of increased resistance conferred by loss of *SUVH6*. *TLP1*, which is in the same gene family as *PATHOGENESIS-RELATED 5 (PR5)* has been positively associated with defense response in various pathosystems (El-kereamy et al, 2011; Misra et al, 2016). Interestingly, *TLP1* is significantly downregulated (-1.31 log₂ fold change, adjusted *P* = 0.006) in *H. schachtii*-infected *suvh6* roots as compared with infected Col-0, indicating that the increase in resistance to *H. schachtii* observed for *suvh6* is achieved independent of *TLP1*. Taken together, *SUVH5* and *SUVH6* exhibit unique and overlapping H3K9me2 deposition, resulting in the suppression of genes likely involved in syncytium development and plant responses.

In the case of cyst nematode infection, as well as in many other phytopathosystems, plant susceptibility is strongly associated with global hypomethylation (Rambani et al, 2015; Hewezi et al, 2017; Hewezi, 2020). In fact, about 88% of the genes that were differentially H3K9 dimethylated and differentially expressed in Col-0 were hypomethylated in a dataset generated by Hewezi et al (2017) of *H. schachtii*-infected Col-0 roots compared to noninfected Col-0 roots. A similar trend was found for DEGs with differentially dimethylated H3K9 in *suvh5* and *suvh6* mutants. These findings reiterate the importance of *SUVH5* and *SUVH6* and their establishing plant responses for *H. schachtii* infection.

A positive feedback loop has been identified that cyclically drives H3K9me2 and DNA methylation (Stroud et al; 2013; 2014). This finding that the vast majority of the genes enriched in H3K9me2 and differentially methylated in infected Col-0 were also depleted in H3K9me2 and differentially methylated in *suvh5* and/or *suvh6* reveals the imminent interplay between DNA methylation and *SUVH5/6*-mediated H3K9me2 deposition under *H. schachtii* infection. Using the *suvh4/5/6* triple mutant, Stroud et al (2013; 2014) showed that the majority of depleted H3K9me2 sites overlapped with differentially methylated CHG and CHH, implying the presence of a positive feedback mechanism that pertains specifically to H3K9me2 and non-CG methylation. Even in the *suvh4/5/6* triple mutant, however, they report that some H3K9me2 overlapped with DRM2 target sites (Stroud et al, 2014), leaving open the possibility that H3K9me2 and DNA methylation are also coordinated at mCG sites. Indeed, CG methylation and H3K9me2 have been directly connected in their synergistic role of heterochromatin assembly (Soppe et al, 2002). Additionally,

impedance of CG methylation in the *met1* and *vim1/2/3* mutants leads to a significant reduction in H3K9me2 (Tariq et al, 2003; Kim et al, 2014; Stroud et al, 2014). This analysis indicates that SUVH5/6-driven H3K9me2 are predominately associated with CG methylation. Furthermore, SUVH5 and SUVH6 are significantly hypomethylated and significantly differentially expressed to a greater degree than SUVH4 in wild-type roots in response to *H. schachtii* (Szakasits et al, 2009; Hewezi et al, 2017). Taken together, these results suggest that the connection between H3K9me2 and non-CG DNA methylation observed under normal developmental conditions is mainly driven by SUVH4. However, under *H. schachtii* infected conditions, SUVH5 and SUVH6 become the main enzymes mediating H3K9me2 deposition in protein-coding genes that are also associated with CG methylation.

Careful examination of the list of DMGs generated by Hewezi et al (2017) revealed that *SUVH4*, 5, and 6 are all hypomethylated in response to *H. schachtii* infection in roots. *SUVH5* and *SUVH6*, however contain more hypomethylated sites in their promoter and coding regions than *SUVH4*, all of which are in the CG context (Hewezi et al, 2017). Interestingly, *VARIANT IN METHYLATION 1* (*VIM1*, AT1G57820), the methylcytosine-binding E3 ubiquitin ligase with a strong preference for mCG, was both hypomethylated in the CG context in infected Col-0 and contained H3K9me2 contributed to by SUVH6. *VIM1* was also found to be significantly upregulated in RNA-seq data of *H. schachtii*-infected Col-0 roots as well as in syncytial cells (Szakasits et al, 2009). These results therefore suggest that SUVH5- and SUVH6-driven H3K9me2 participate in a novel multi-level epigenetic mechanism involving CG DNA methylation via *VIM1*. This mechanism could explain the significant degree of genome-wide hypomethylation, and at some loci, could explain changes in gene expression induced upon *H. schachtii* infection. A number of other genes were observed to be CG hypomethylated, downregulated, and targeted by SUVH5 and/or SUVH6 for H3K9me2 deposition under infected conditions. For example, the transcription factor *ETHYLENE RESPONSE FACTOR 74* (*ERF74*, AT1G53910) is known to facilitate the early stages of abiotic stress responses by enhancing the ROS burst and controlling a respiratory burst oxidase (Yao et al, 2017). Another gene, *KINESIN-12B* (AT3G23670), which has recently been identified to regulate spindle dynamics during cell division (Herrmann et al, 2021), could be an important factor in syncytium development. Nonetheless, SUVH5 and SUVH6 H3K9me2 also seems to influence

gene expression independent of DNA methylation. Therefore, *H. schachtii* infection induces multiple novel mechanisms whereby SUVH5 and SUVH6 take a leading role in regulating H3K9me2 and subsequent transcriptional reprogramming required for *H. schachtii* pathology.

4.5 Materials and methods

Plant materials and growth conditions

All transgenic *Arabidopsis thaliana* lines were generated in the Col-0 ecotype background. T-DNA insertional mutants for *suvh5* (*GK-263C05* and *FLAG_280C12*), *suvh6* (*SALK_1244_F04* and *SALK_071138C*) were obtained from Arabidopsis Biological Resource Center (ABRC) (Figure 4.12A). All mutant lines were in the Col-0 background, except *FLAG_280C12*, which is in the Wassilewskija (WS) background. The mutant lines *GK-263C05* (Zheng et al, 2011) and *SALK_1244_F04* (Wang et al, 2015) have been previously characterized as knockout mutants. *SUVH5* and *SUVH6* expression levels of these genes were quantified in *FLAG_280C12* and *SALK_071138C* lines and wild-type plants using qRT-PCR as described below (Figure 4.5A and B). In addition, shoot and root phenotypes of all mutant lines along with wild-type plants were assessed in four-week-old plants (Figure 4.12B and C). Length of the main root of at least 15 two-week-old plants per line was measured and statistically significant differences from wild-type plants were calculated using *t* tests ($P < 0.05$) (Figure 4.12D). Plants were grown in controlled growth chambers between 22- 24 °C under long day conditions, 16 hours of light and 8 hours of dark.

Generation of transgenic overexpression and GUS reporter lines

For the generation of GUS reporter lines, the 2 kb promoter regions upstream of the translation start codon were amplified from Col-0 genomic DNA. Primers containing restriction enzyme sites used for PCR amplification are provided in Table 4.14. The amplified PCR fragments were digested, purified, and cloned upstream of the β -glucuronidase (GUS) gene in the binary vector pBI101. The binary vector of miR778 overexpression was constructed by amplifying the miR778

precursor (200 bp) from Col-0 genomic DNA using primers containing *Bam*HI and *Sac*I restriction enzyme sites. The amplified PCR product was digested, purified, and cloned into the pBI121 vector downstream of the 35S promoter. For the MIM778 overexpression, the 22-nucleotide region of the noncoding Arabidopsis *INDUCED BY PHOSPHATE STARVATION1 (IPS1)* gene that is complementary to miR339 was replaced by a mimic sequence for the mature miR778 as previously described by Hewezi et al (2016). This mimic sequence (MIM778) contains a three-nucleotide bulge (TCC) at nucleotide number 12 through 14 as well as two additional mismatches at nucleotide numbers 2 and 11. The MIM778-containing *IPS1* gene was then cloned into the pBI121 vector under the control of the 35S promoter using *Bam*HI and *Sac*I restriction enzyme sites. The primers used for cloning are listed in Table 4.14.

All constructs were confirmed by sequencing before transferring into *Agrobacterium tumefaciens* (strain C58). Agrobacteria containing the binary vectors were then used to transform Arabidopsis (Col-0) using the floral dip method (Clough and Bent, 1998). Transgenic T1 plants were screened on MS medium containing 50 mg/L kanamycin and 100 mg/L cefotaxime. Transgenic T1 lines were self-fertilized to generate T2 lines. T2 and subsequent generations were used for GUS assays.

Histochemical analysis of GUS activity

The roots of transgenic GUS reporter lines were assayed for GUS activity at several time points after *H. schachtii* infection. Seeds were surface sterilized using a 2.8% bleach solution for 5 min followed by several washes with sterilized distilled water. Sterilized seeds were germinated on modified Knops's medium solidified with 0.8% Daishin agar. Ten-day-old plants were inoculated with 100 surface-sterilized second-stage juvenile (J2) *H. schachtii*. GUS staining was performed according to Jefferson et al (1987) at different time points post inoculation. Briefly, samples were incubated with the X-Gluc substrate (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) at 37°C and checked every 30 minutes for colorimetric changes. Once staining was evident, the GUS solution was removed, and the samples were stored in 70% ethanol at room temperature to terminate the GUS reaction. Three independent reporter lines were assayed, each with at least 24 biological replicates per line. Immediately following staining, GUS-stained

syncytia were imaged using the EVOS M7000 microscope. GUS activity in shoot and root tissues under noninfected conditions are provided in Figure 4.11.

Quantitative PCR (qPCR) analysis

For quantifying the expression levels of primary and mature miR778, total RNA was extracted using Trizol (Invitrogen) following the manufacturer's protocol. Total RNA was then polyadenylated and reverse transcribed using the Mir-X miRNA First-Strand Synthesis Kit (Clontech). Approximately 50 ng of the synthesized cDNA was used as a template in the qPCR reaction containing TB green Advantage qPCR mix (Takara) according to the manufacturer's instructions. For both primary and mature miR778 amplification, the universal mRQ 3' primer was used as the reverse primer. The mature *miR788* forward primer consisted of the mature miR778 sequence with two adenines on the 3' end was used as forward primer sequence to ensure correct binding of the primer to the poly(T) region of the mature miR858 cDNA and preclude potential binding to the miR858 precursor. The primary miR778 transcript was quantified using a miR778-specific precursor sequence as the forward primer. These primers were applied to both control and treatment RNA samples. Both the U6 small nuclear RNA included in the Mir-X miRNA First-Strand Synthesis Kit (Clontech) as well as the 60S ribosomal (AT2G44860) genes were used as reference genes to normalize miRNA gene expression. The PCR reactions were performed using the following program: 95°C for 3 min followed by 40 cycles of 95°C for 30 s and 60°C for 30 s in QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The PCR products were then exposed to a temperature ramp to generate the dissociation curves and determine amplification specificity. The dissociation program was 95°C for 15 s and 50°C for 15 s, followed by a slow gradient from 50°C to 95°C.

Total RNA used to quantify the expression of MIM778, *SUVH5*, and *SUVH6* in various overexpression and mutant lines was extracted using to the method described by Verwoerd et al (1989). Total RNA was then treated with DNase I (Invitrogen) and approximately 50 ng of DNase-treated RNA was used as a template for qRT-PCR with Verso SYBR green One-Step qRT-PCR mix (Thermo Scientific) according to the manufacturer's instructions. The forward primer used to amplify MIM778 consisted of the MIM778 sequence as described above and the reverse primer

was selected from a sequence in the wild-type *IPS1* gene, downstream of the *miR339* binding sequence. Expression of MIM778 was compared to wild-type *IPS1* in 10-day-old Col-0 seedling DNase-treated RNA. qRT-PCR reactions were performed in the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, USA) and the qRT-PCR program was 50°C for 15 min, 95°C for 15 min followed by 40 cycles of 95°C for 15 s, 60°C for 90 s and 72°C for 30 s. Gene expression levels were normalized using *Actin8* (*ACT8*, AT1G49240) and *PROTEIN PHOSPHATASE 2A SUBUNIT A3* (*PP2AA3*, AT1G13320) as reference genes. Primers used in this study are listed in Table 4.14.

Nematode infection assay

Sterilized seeds of wild-type and various overexpression and mutant lines were germinated in a randomized block design in 12-well plates as previously stated. Ten-day-old seedlings were then inoculated with approximately 200 surface-sterilized J2 *H. schachtii*. Plates were incubated in the dark at 24 °C for 3 days directly following inoculation to facilitate nematode infection before grown for three weeks in light conditions. J4 female nematode were counted 3-week post infection plants. Two independent experiments were conducted with 20 replicates per genotype. Statistically significant differences between the mutants or overexpression lines and their wild-type controls were calculated using a modified *t* test in the statistical software package SAS, where *P* values less than 5% were considered statistically significant.

RNA-seq analysis

Seeds of *suvh5* (*GK-263C05*) and *suvh6* (*SALK_1244_F04*) and wild-type Col-0 were germinated in large petri plates containing modified Knop's medium. Several plates of each genotype were inoculated with 100 J2 *H. schachtii* per plant at 10-days-old. Inoculated replicates were incubated in the dark at 26°C for 24 hours to facilitate nematode infection. Following incubation, inoculated replicates were grown in light conditions as previously described until 5 dpi. At the same time, several plates of each genotype remained un-inoculated and continued growing in light until 15 days old. Root tissues of 15-day-old (noninfected) or 5 dpi (*H. schachtii*-infected) plants were collected and frozen immediately in liquid nitrogen. Total RNA was extracted using Trizol reagent

(Invitrogen) using the Zymo Direct-zol RNA Miniprep Plus kit (R2071) from three replicates of each genotype for noninfected and *H. schachtii*-infected samples. RNA quality was assessed using an RNA nano bioanalyzer chip (Agilent 2100). mRNAs were processed and captured using oligo(dT) beads following the addition of a 3' poly(A) tail for the construction of a non-stranded, non-directional mRNA library. The 18 RNA-seq libraries were then multiplexed and sequenced using the NovaSeq 6000 (Illumina), outputting 150-bp paired-end reads. Quality assessment was performed on fastq raw data files using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). High quality paired-end RNA-seq reads were then mapped to the *Arabidopsis thaliana* reference genome (TAIR10.28) with STAR (v2.5) (Dobin et al, 2013) using default settings. Uniquely mapped paired-end reads were counted using Htseq (v0.10.0) (Anders et al, 2015). Then, read counts of uniquely mapped reads were used to identify DEGs using DESeq package in R (Love et al, 2014) with an adjusted *P* value cutoff of 0.05. GO terms enrichment analysis of the DEGs was performed using agriGO database (Du et al, 2010) with Fisher's exact test and Bonferroni multi-test adjustment with a significance cutoff *P* value of 0.05. The web server REVIGO was used to summarize the list of GO terms for visualization purposes using the semantic similarity metric SimRel set to 0.5 or 0.4 (Supek et al, 2011). The full list of GO terms are available in Table 4.3.

Chromatin immunoprecipitation (ChIP)- seq analysis

Seeds of *suvh5* (GK-263C05) and *suvh6* (SALK_1244_F04) and wild-type Col-0 plants were planted on modified Knop's medium and 10-day-old plants were inoculated with 100 J2 *H. schachtii* per plant and incubated in the dark at 26°C for 24 hours and then transferred back to light conditions. At 5 dpi, root tissues were collected from both noninfected and *H. schachtii*-infected samples) and ChIP-DNA was extracted in triplicate for each genotype per condition using the Anti-Histone H3 (di methyl K9) ChIP Grade antibody (ab1220) following the manufacturer's protocol (Abcam). Libraries were prepared according to the manufacturer's instructions using the NEBNext Ultra II DNA Library Prep Kit (NEB E7645). The 18 ChIP-seq libraries were multiplexed and sequenced using the NovaSeq 6000 (Illumina) to produce 150-bp paired-end reads. Quality assessment was performed on fastq raw data files using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Trimmomatic (v0.39) (Bolger et al,

2014) was used to trim adapter sequences, poor quality reads, and overrepresented sequences from raw data followed by additional quality assessments for trimmed data using FastQC. Paired-end ChIP-seq reads were mapped to the *Arabidopsis thaliana* reference genome (TAIR10.28) with Bowtie2 (v2.2.9) (Langmead and Salzberg, 2012) using the very sensitive mapping parameter. Mapping depth and coverage were determined using Samtools (v1.3.1) (Li et al, 2009) and IGV (v2.7.2) (Robinson et al, 2017), respectively. The mapping output files of the three replicates for each genotype per condition were merged to call H3K9me2 peaks using the broad peak function of MACS2 (v2.1.1.2) (Zhang et al, 2008) for paired-end reads. Peaks were then annotated using the closest function of Bedtools2 (v2.29.2) (Quinlan and Hall, 2010). For noninfected samples, peaks were identified to be depleted in *suvh5* and *suvh6* by comparing Col-0 to each mutant. Peaks enriched in *H. schachtii*-infected Col-0 were called by comparing infected Col-0 to noninfected Col-0. Peaks depleted in *H. schachtii*-infected *suvh5* and *suvh6* were called by comparing infected Col-0 to infected *suvh5* and *suvh6*, respectively. The H3K9me2 peak signal was quantified using the log2 of RPKM (reads per kilobase per million mapped reads) values for reads associated with peaks using deepTools2 (3.5.0) (Ramírez et al, 2016) to generate heatmaps.

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Appendix

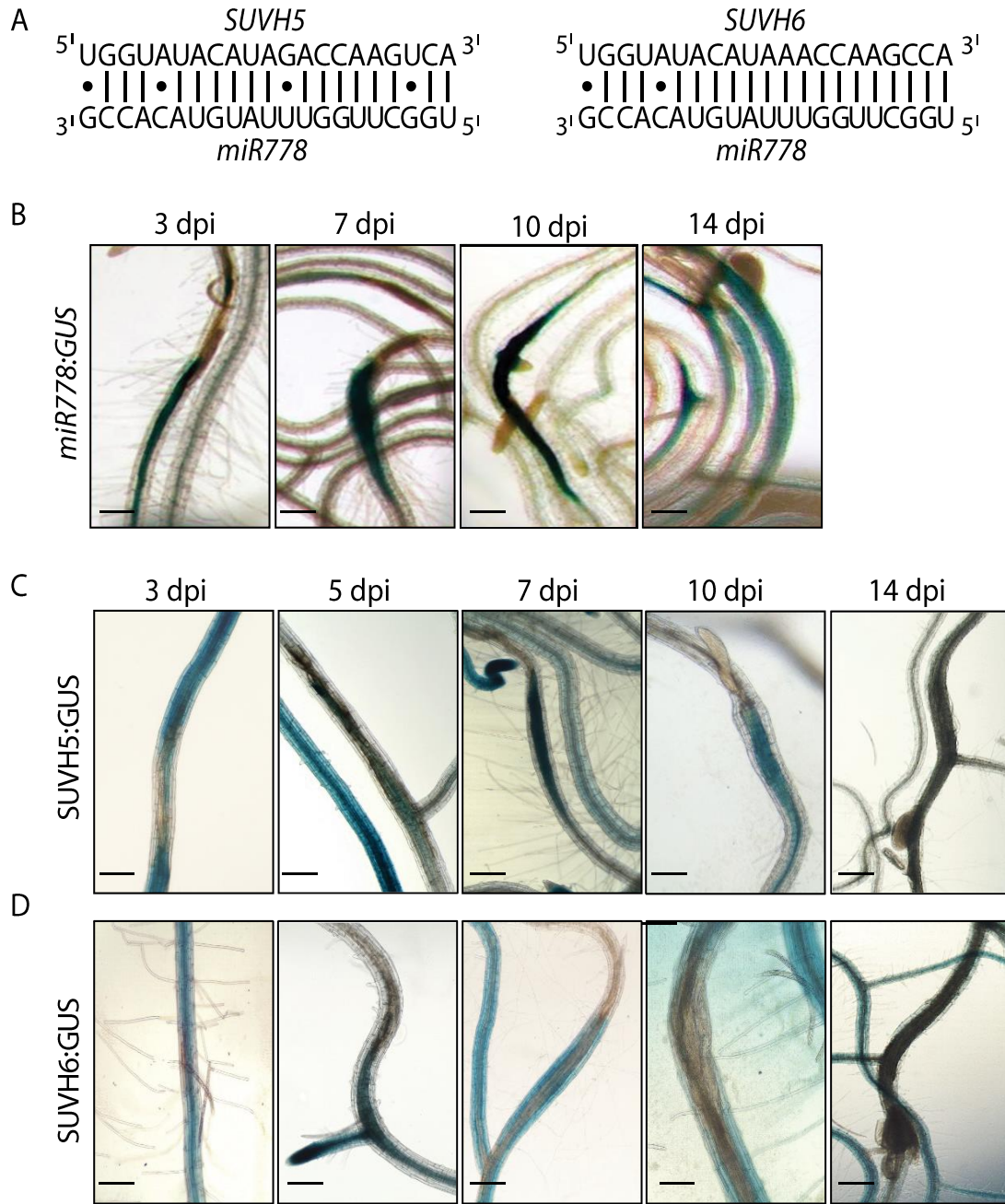


Figure 4.1. miR778 and its target genes *SUVH5* and *SUVH6* are expressed in *H. schachtii*-induced syncytium. A: Mature sequence of miR778 and its base pairing with its target genes. B-D: Histochemical localization of GUS activity directed by the promoters of miR778 (B) and its target genes *SUVH5* (C) and *SUVH6* (D). Scale bar = 5 mm.

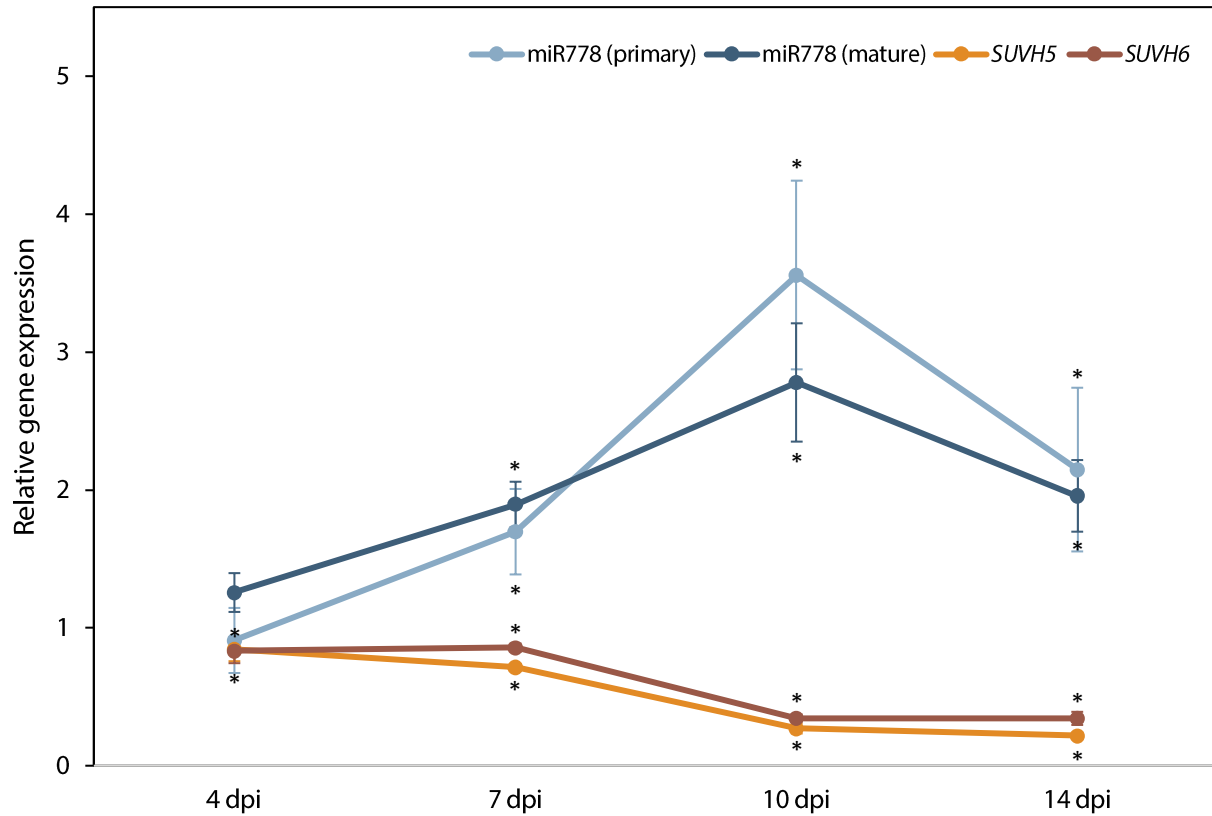


Figure 4.2. miR778 post-transcriptionally regulates *SUVH5* and *SUVH6* in *H. schachtii*-infected roots. Expression levels of primary and mature miR778, *SUVH5*, and *SUVH6* in Col-0 roots infected with *H. schachtii* at 4, 7, 10, and 14 dpi relative to noninfected Col-0 roots at the same time points. Relative gene expression values were quantified for *H. schachtii*-infected versus noninfected Arabidopsis roots by qPCR for miR778 transcripts and qRT-PCR for *SUVH* transcripts using three biological samples. Shown are the averages of the biological samples $\pm$ SE. Statistically significant differences were determined using two-tailed *t* tests ($P < 0.05$) and are indicated by asterisks.

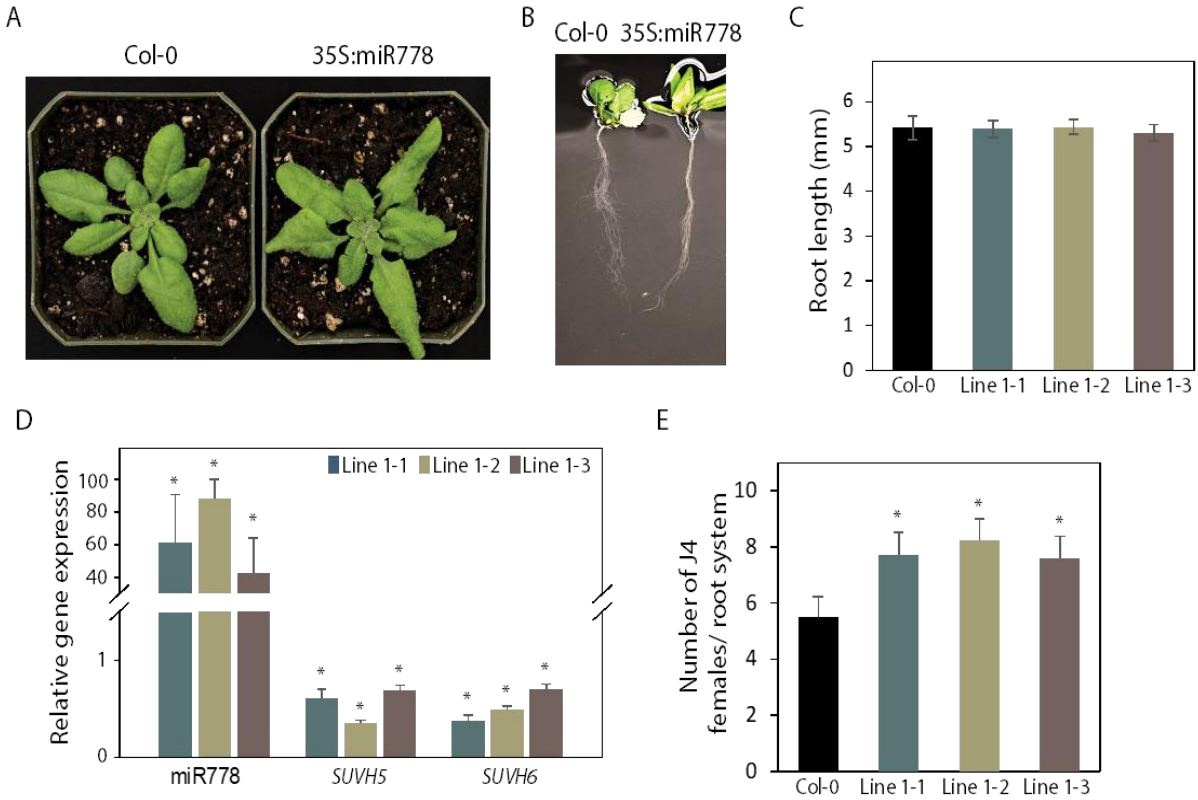


Figure 4.3. Overexpression of miR778 increases plant susceptibility to *H. schachtii*. A-C: Shoot (A) and root (B-C) phenotype of miR778 overexpression lines compared to Col-0. D: Relative gene expression of the mature miR778 transcript and its target genes *SUVH5* and *SUVH6* compared to Col-0 as determined by qPCR and qRT-PCR, respectively. Data are the average of three independent biological samples $\pm$ SE. Asterisks indicate statistically significant differences determined using two-tailed *t* tests ($P < 0.05$). E: Nematode susceptibility levels of miR778 overexpression lines compared to Col-0. Data are the presented as means $\pm$ SE ($n = 20$). Mean values significantly different from Col-0 were determined by an unadjusted paired *t* test ($P < 0.05$) and are indicated by an asterisk. Similar results were obtained from at least two independent experiments.

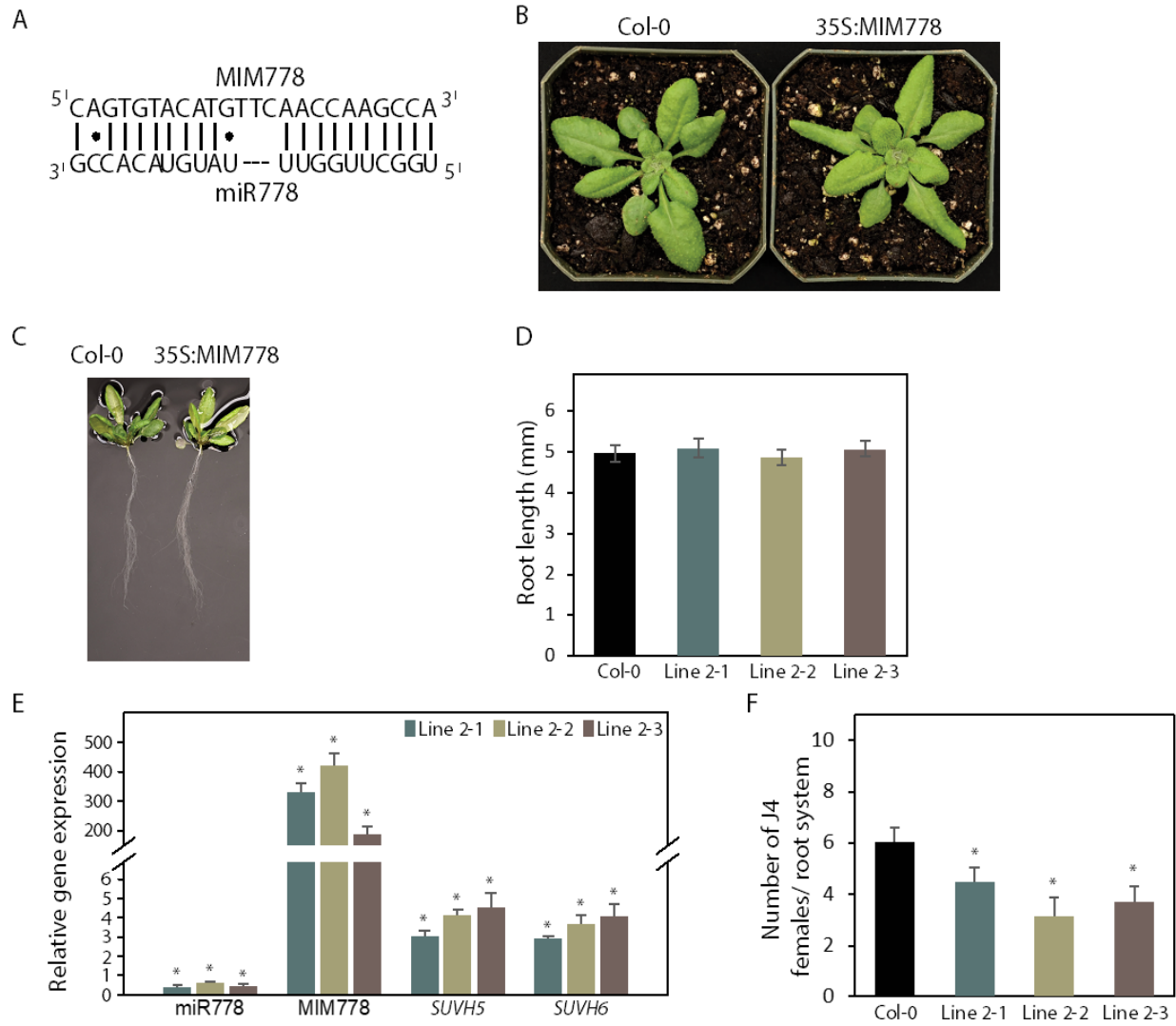


Figure 4.4. Suppression of miR778 via target mimicry decreases plant susceptibility to *H. schachtii*. A: Mature sequence of miR778 and its base pairing with the MIM778 sequence. B-C: Shoot (B) and root (C-D) phenotype of MIM778 overexpression lines compared to Col-0. E: Relative gene expression values of the indicated genes were determined by Relative gene expression values were quantified by qPCR for miR778 and MIM778 transcripts and qRT-PCR for SUVH transcripts. Data are the average of three independent biological samples $\pm$ SE. Asterisks indicate statistically significant differences determined using two-tailed *t* tests ($P < 0.05$). F: Nematode susceptibility levels of MIM778 overexpression lines compared to Col-0. Data are presented as means $\pm$ SE ($n = 20$). Mean values significantly different from Col-0 were determined by an unadjusted paired *t* test ($P < 0.05$) and are indicated by asterisks. Similar results were obtained from at least two independent experiments.

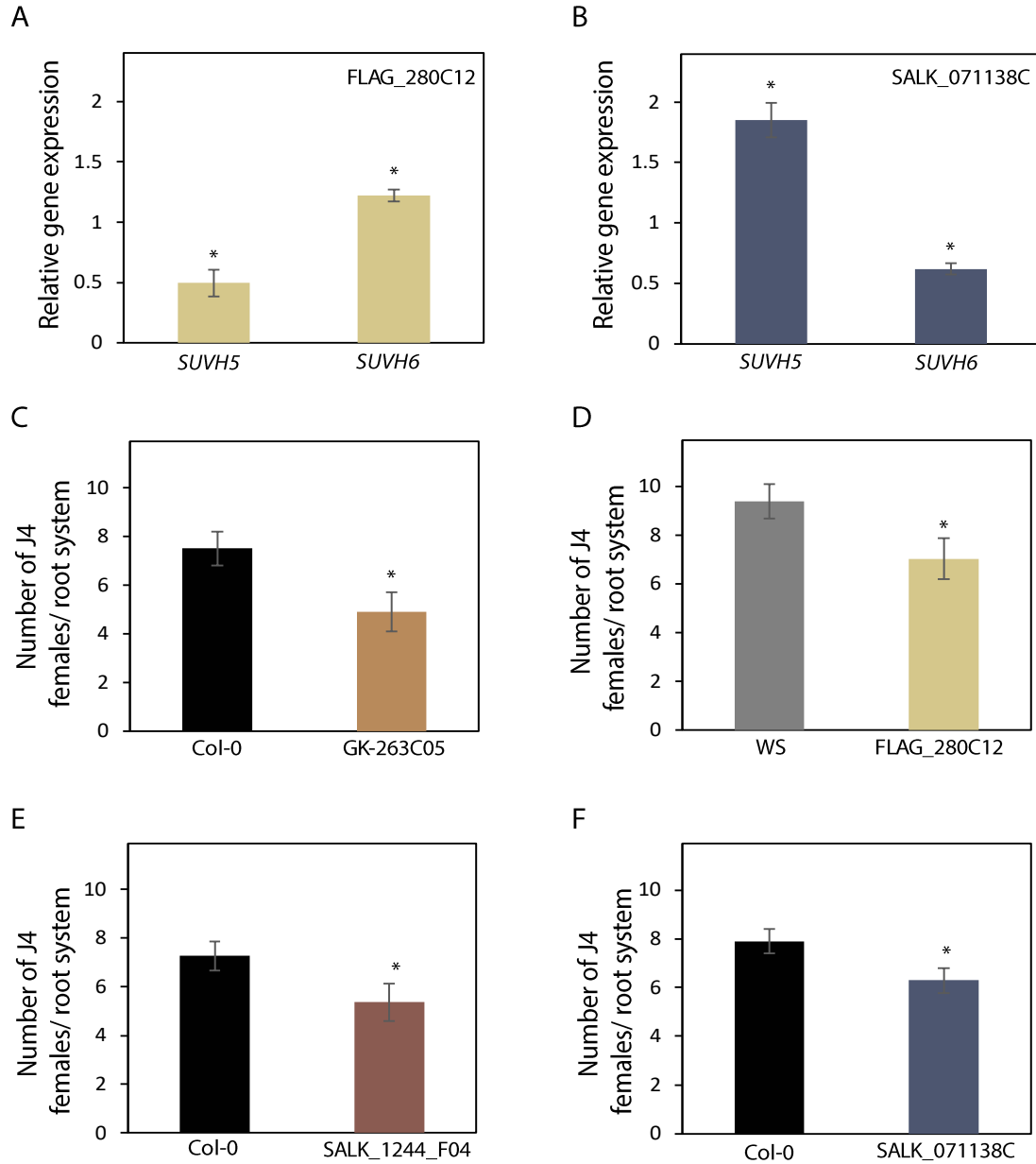


Figure 4.5. Mutations of *SUVH5* and *SUVH6* affect decrease plant susceptibility to *H. schachtii*. A-B: Mutations of *SUVH5* increase expression of *SUVH6* (A) and vice versa (B). Relative gene expression was determined by qRT-PCR from three biological replicates $\pm$ SE. Asterisks indicate statistically significant differences determined using two-tailed *t* tests ($P < 0.05$). C-F: J4 female index of *suvh5* (GK-263C05 and *FLAG_280C12*) and *suvh6* (SALK_1244_F04 and *SALK_071138C*) mutant lines compared to the wild type. Data are the presented as means $\pm$ SE ($n = 20$). Mean values significantly different from the wild type were determined by an unadjusted paired *t* test ($P < 0.05$) and are indicated by an asterisk. Similar results were obtained from two independent experiments.

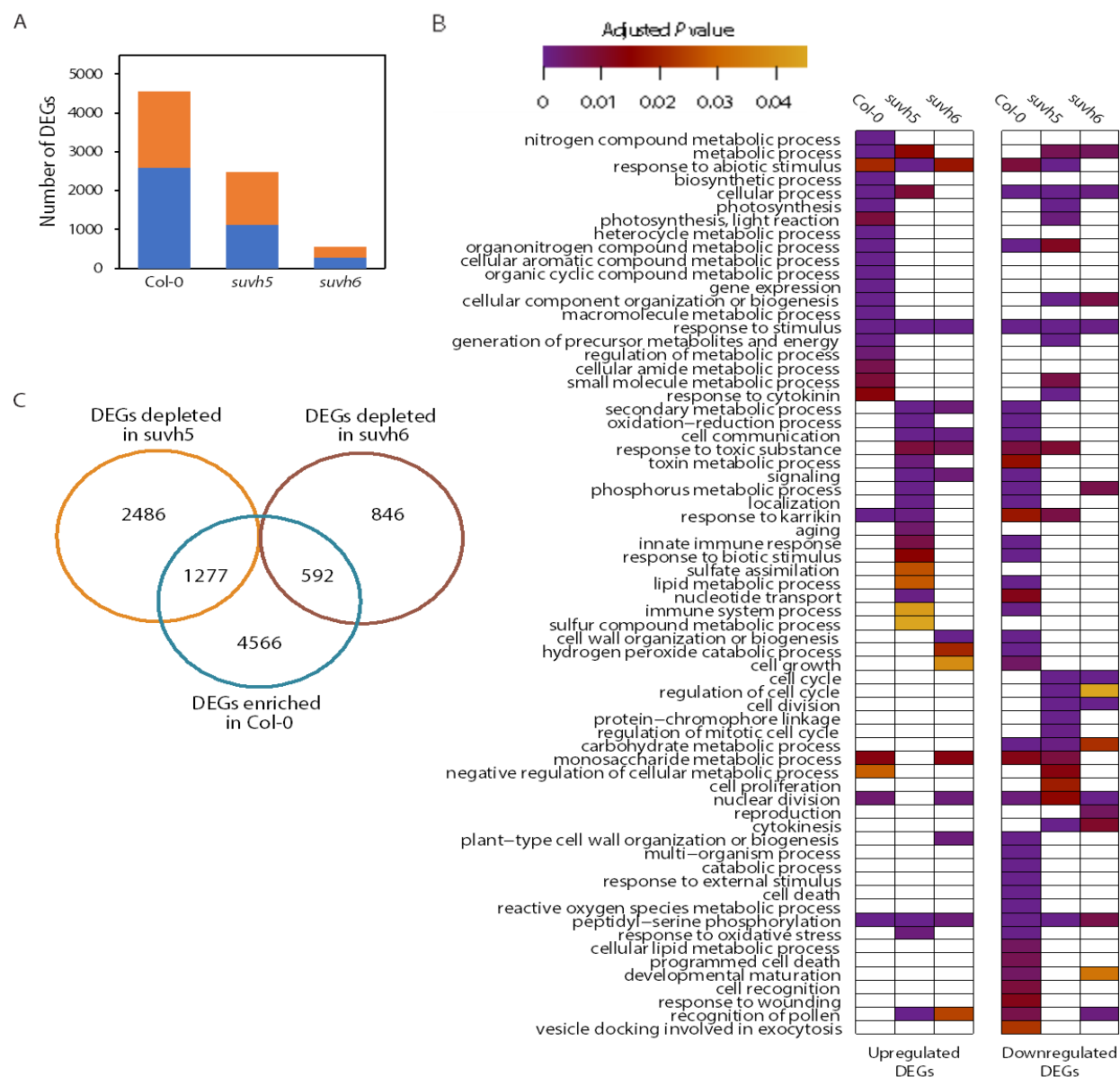


Figure 4.6. SUVH5 and SUVH6 regulate the expression of a significant number of genes in *H. schachtii*-infected root tissues. A: Number of DEGs identified in infected *suvh5* and *suvh6* mutant roots that are up- (orange) and downregulated (blue) in comparison to infected Col-0. B: GO term classification and enrichment analysis of up- and downregulated DEGs in infected Col-0 versus infected *suvh5* and *suvh6*. Enrichment analyses of upregulated and downregulated genes were performed separately and were performed using Fisher's exact test and Bonferroni multi-test correction with a significance cutoff $P < 0.05$. GO term categories represented are a condensed list generated by REVIGO using the semantic similarity measure SimRel = 0.5. The full list of GO terms is available in Table 4.7. C: Venn diagram showing the overlap between the indicated lists of DEGs.

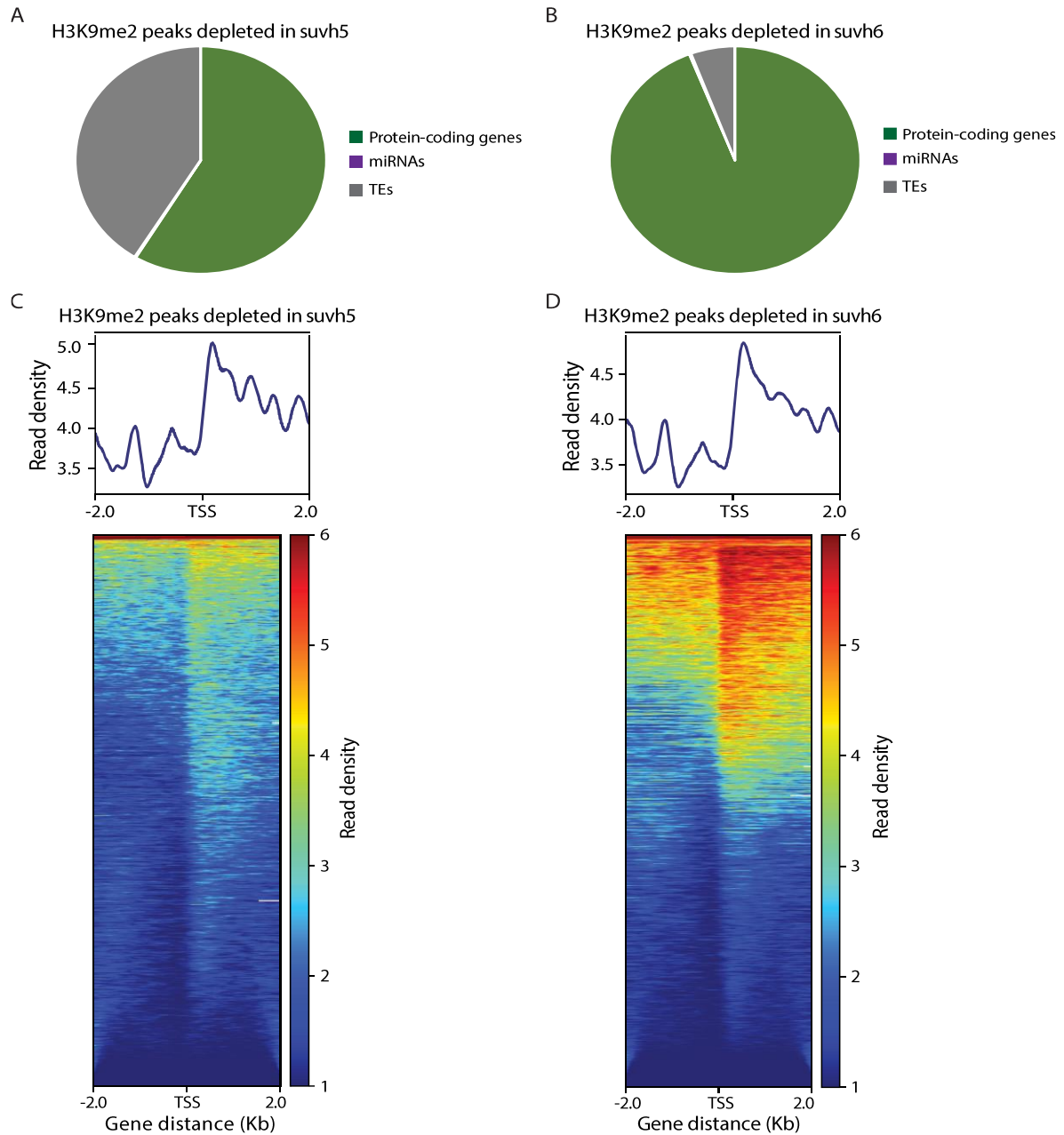


Figure 4.7. H3K9me2 deposition mediated by SUVH5 and SUVH6 in *H. schachtii*-infected roots. A-B: Distribution of H3K9me2 peaks depleted in infected *suvh5* (A) and infected *suvh6* (B) compared to infected Col-0. Shown are peaks overlapping with protein-coding genes (green), miRNAs (purple), and TEs (grey). C-D: Heatmaps of ChIP-seq signal densities for peaks associated with SUVH5 (C) and SUVH6 (D) across 2 kb windows showing location of genic H3K9me2 peak enrichment. The numbers in the bars on the right indicate signal H3K9me2 intensity of infected Col-0 relative to infected mutants. Target sequences are ordered based on signal strength.

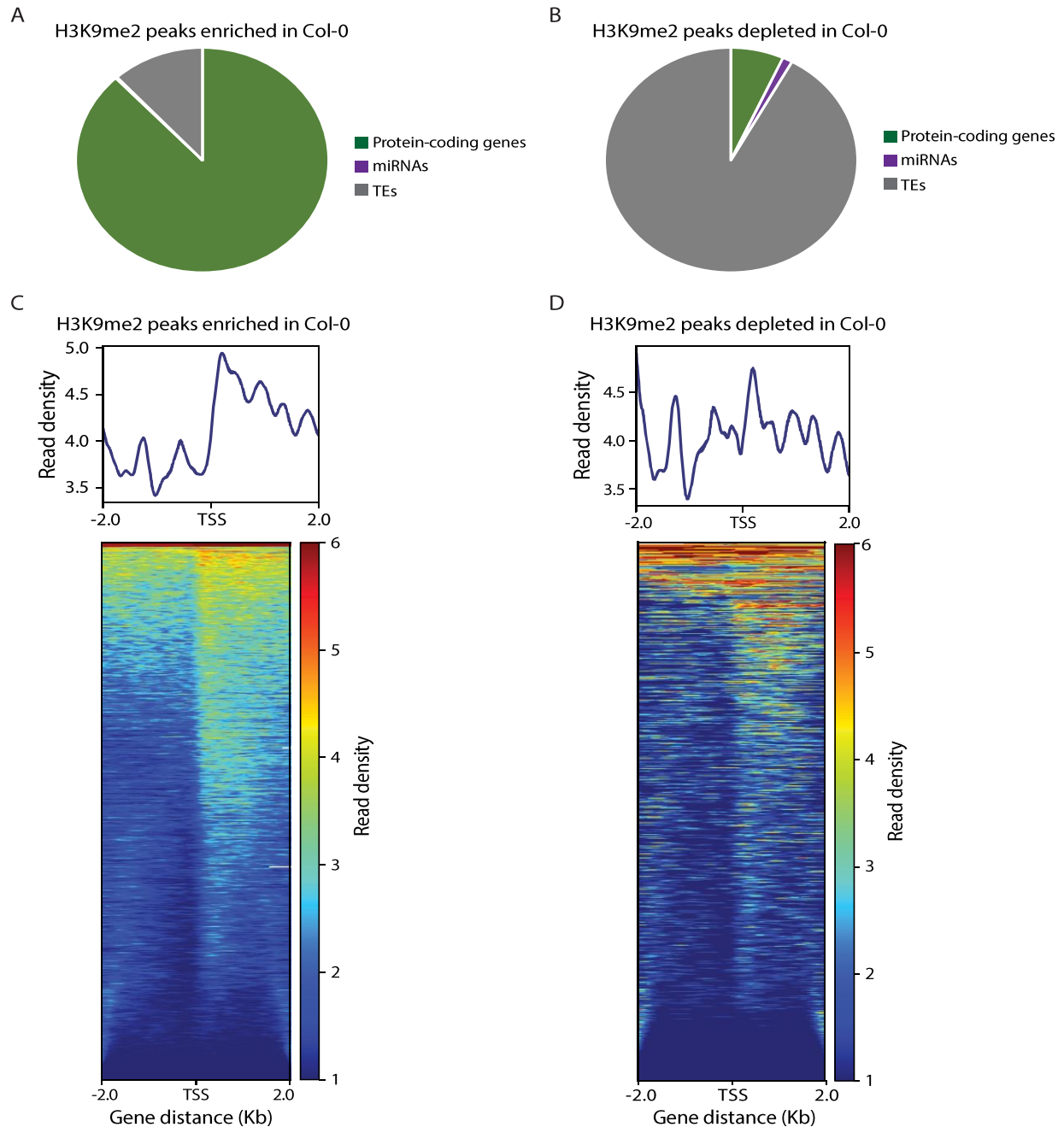


Figure 4.8. H3K9me2 deposition induced by *H. schachtii* in Col-0 roots. A-B: Distribution of H3K9me2 peaks overlapping with protein-coding genes (green), miRNAs (purple), and TEs (grey) enriched (A) or depleted (B) in infected Col-0 compared to noninfected roots. C-D: Heatmaps of ChIP-seq signal densities for peaks enriched (C) or depleted (D) in *H. schachtii*-infected Col-0 roots compared to noninfected roots across 2 kb windows showing location of genic H3K9me2 peak enrichment. The numbers in the bars on the right indicate H3K9me2 signal intensity. Target sequences are ordered based on signal strength.

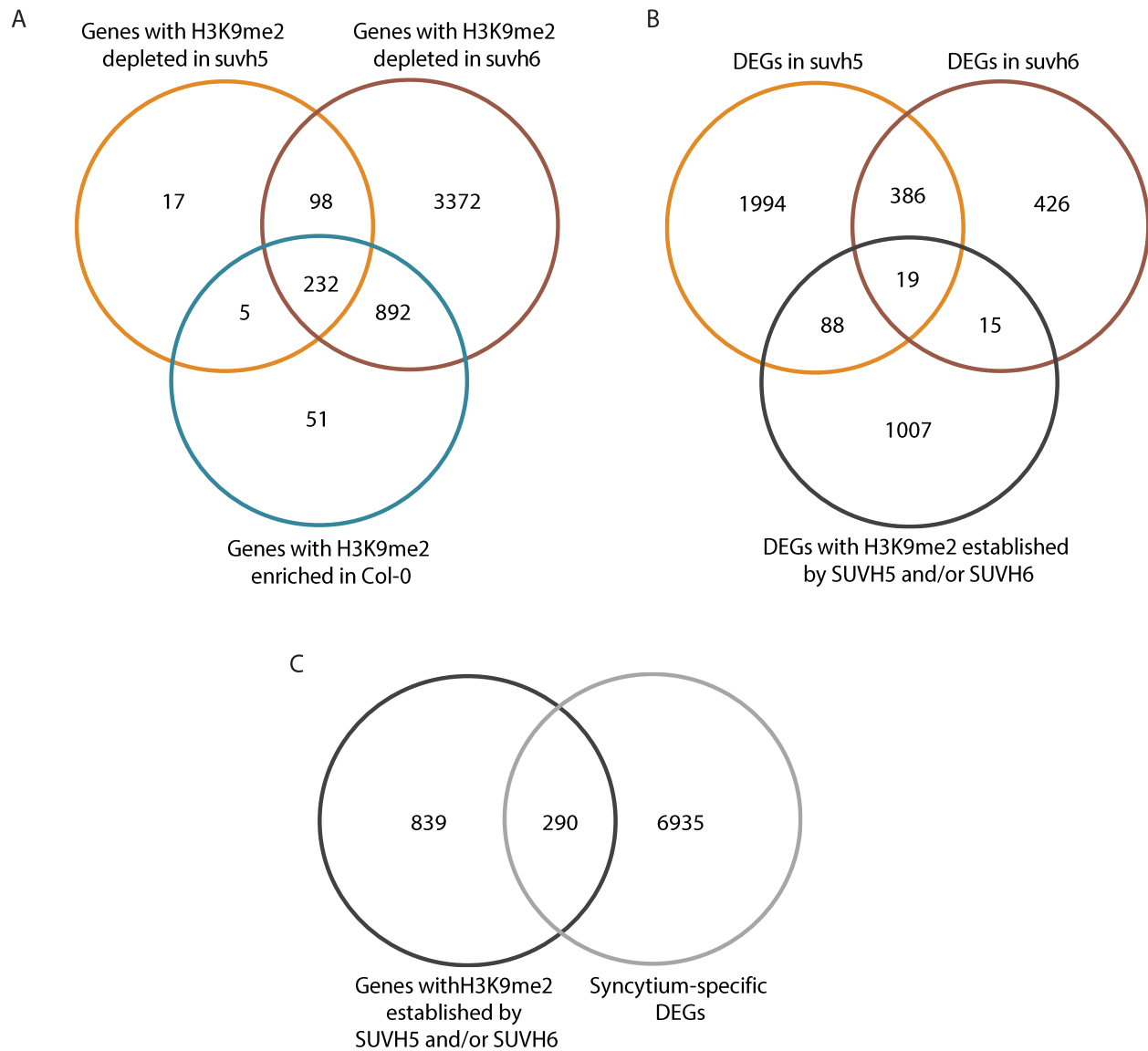


Figure 4.9. H3K9me2 marks established by SUVH5 and/or SUVH6 contribute to gene expression changes upon *H. schachtii* infection. A: Venn diagram showing the overlap of genes with H3K9me2 deposition upon *H. schachtii* infection enriched in Col-0 and depleted in *suvh5* and/or *suvh6* mutants. B: Venn diagram showing the overlap of *suvh5* and *suvh6* DEGs with the 1129 genes with H3K9me2 deposition established by SUVH5 and/or SUVH6. C: Venn diagram showing the overlap of syncytia DEGs (Szakasits et al, 2009) with the 1129 genes with H3K9me2 deposition established by SUVH5 and/or SUVH6.

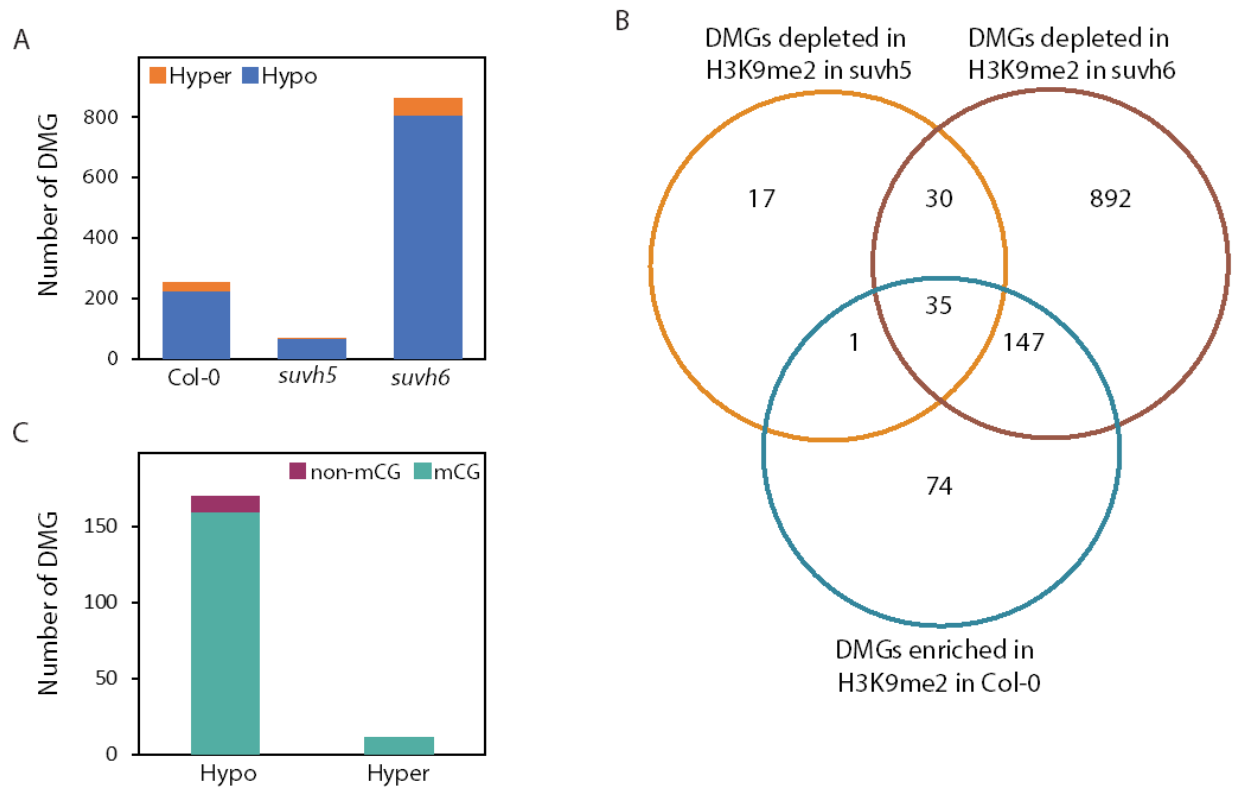


Figure 4.10. SUVH5/6-mediated H3K9me2 associates with CG methylation in *H. schachtii*-infected roots. A: Number of DMGs that are hyper- (orange) and hypomethylated (blue) in Col, *suvh5*, and *suvh6* upon *H. schachtii* infection. B: Venn diagram showing the overlap of DMG with *H. schachtii*-induced H3K9me2 deposition enriched in Col-0 and depleted in *suvh5* and/or *suvh6*. C: Number of DMGs that are methylated in the non-CG (fuchsia) and CG (teal) context in Col, *suvh5*, and *suvh6* upon *H. schachtii* infection.

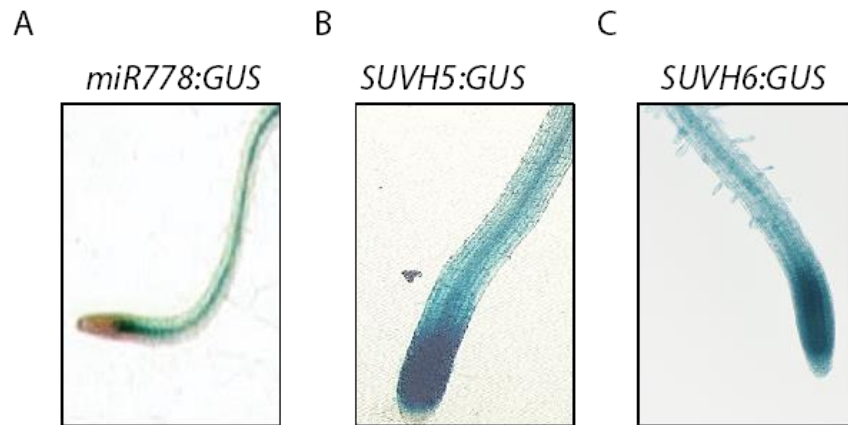


Figure 4.11. miR778 and its target genes *SUVH5* and *SUVH6* are expressed in two-week-old noninfected roots. A-C: Histochemical localization of GUS activity directed by promoters of miR778 (A) and its target genes *SUVH5* (B) and *SUVH6* (C).

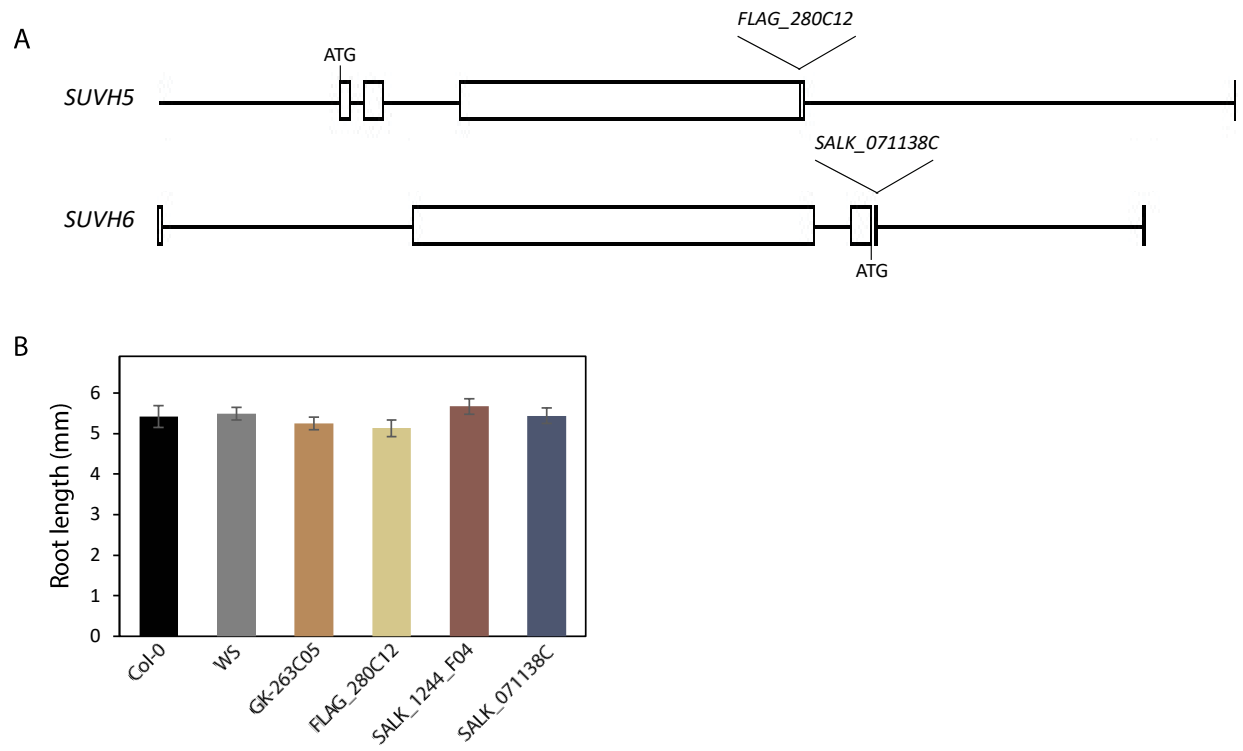


Figure 4.12. Characterization of *FLAG_280C12* and *SALK_071138C* mutants. A: Schematic diagram of gene structures and insertion sites for *FLAG_280C12* (exon 3) and *SALK_071138C* (5' UTR). B: Root lengths of two-week-old mutant and wild-type plants. No mean differences were found using *t* tests, $n \geq 15$.

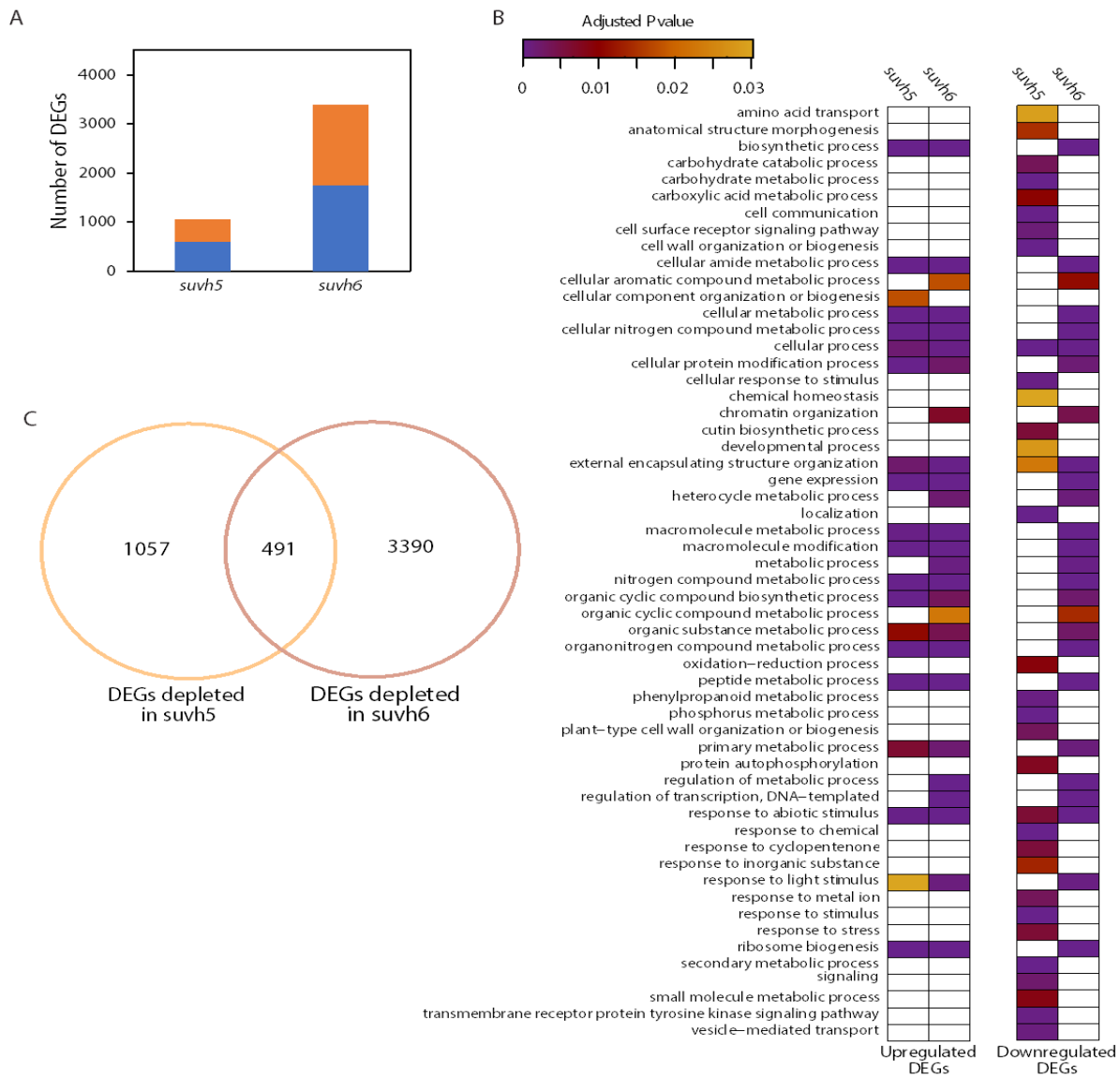


Figure 4.13. SUVH5 and SUVH6 regulate the expression of a significant number of genes in root tissues under noninfected conditions. A: Number of DEGs identified in the roots of *suvh5* and *suvh6* mutant that are up- (orange) and downregulated (blue) in comparison to Col-0 under noninfected conditions. B: GO term classification and enrichment analysis of up- and downregulated DEGs in *suvh5* and *suvh6* in comparison with Col-0. Enrichment analyses of upregulated and downregulated genes were performed separately and were performed using Fisher's exact test and Bonferroni multi-test correction with a significance cutoff $P < 0.05$. GO term categories represented are a condensed list generated by REVIGO using the semantic similarity measure SimRel = 0.5. The full list of GO terms is available in Table 4.3. C: Venn diagram showing the number of DEGs common to *suvh5* and *suvh6*.

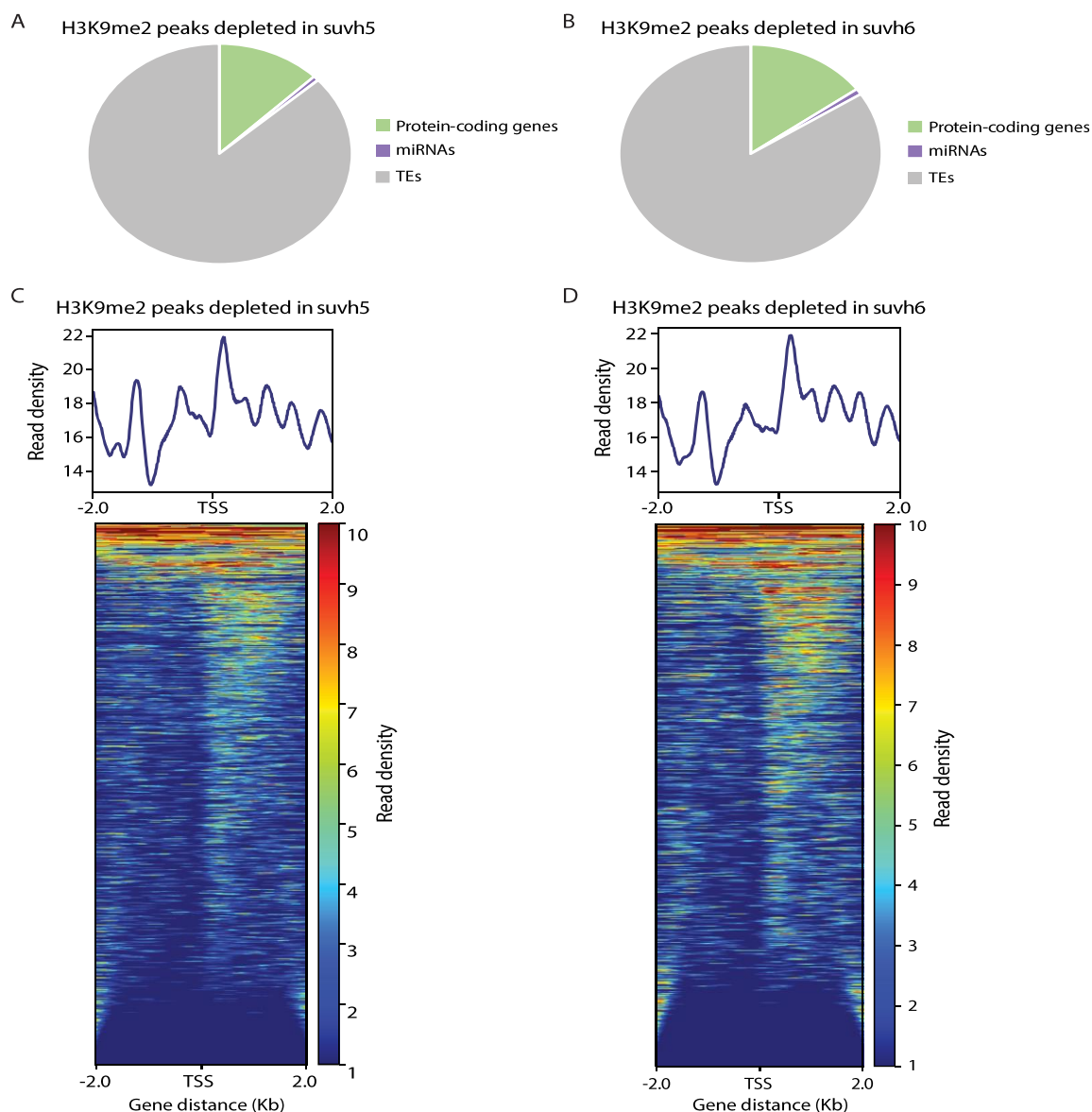


Figure 4.14. H3K9me2 deposition mediated by SUVH5 and SUVH6 in roots under noninfected conditions. A-B: Distribution of H3K9me2 peaks overlapping with protein-coding genes (green), miRNA (purple), and TEs (grey) depleted in *suvh5* (A) or *suvh6* (B) in comparison with Col-0 under noninfected conditions C-D: Heatmaps of ChIP-seq signal densities for *suvh5* (C) and *suvh6* (D) across 2 kb windows showing location of genic H3K9me2 peak enrichment. The numbers in the bars on the right indicate H3K9me2 signal intensity of Col-0 relative to mutant plants. Target sequences are ordered based on signal strength.

Table 4.1. Gene identification numbers and primers used in this study.

Gene	Gene ID	5' primer	3' primer
<i>SU(VAR)3-9 HOMOLOG 5 (SUVH5)</i>	AT2G35160	AATAATGAACCACCCAAGACCAG	TTCCACCAGATACAATCCATCGT
<i>SU(VAR)3-9 HOMOLOG 6 (SUVH6)</i>	AT2G22740	TGGAGAAACGAGGAGTGTCAG	CTTCAACAACATCCATTCTCAC
Mature <i>MICRORNA778 (miR778)</i>	AT2G41616	TGGGCTTGGTTTATGTACACGGAA	Universal mRQ 3' primer provided by the Mir-X miRNA First-Strand Synthesis Kit (Clontech)
Primary <i>MICRORNA778 (miR778)</i>	AT2G41616	GTTTGGTCTCATGTACACCAG	Universal mRQ 3' primer provided by the Mir-X miRNA First-Strand Synthesis Kit (Clontech)
<i>U6</i>	-	Universal primer provided by the Mir-X miRNA First-Strand Synthesis Kit (Clontech)	Universal primer provided by the Mir-X miRNA First-Strand Synthesis Kit (Clontech)
<i>60S</i>	AT2G44860	GTAGGTCGAAGTGTCACAAGAA	GTCGTATCTCTCAGGTCTGTTTC
<i>MIMIC778 (MIM778)</i>	-	CAGTGATCATGTTCAACCAAGCCA	<i>IPS1</i> 3' primer
<i>INDUCED BY PHOSPHATE STARVATION 1 (IPS1)</i>	AT3G09922	AAGGCTGATTGACTGCGAG	CCTCACAAAGAACACACAA
<i>ACTIN 8 (ACT8)</i>	AT1G49240	AGTGGTCGTACAACCGGTATTGT	GAGGATAGCATGTGGACCTGAGAA
<i>PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2AA3)</i>	AT1G13220	AAGCGGTTGTGGAGAACATGATACG	TGGAGAGCTTGATTGCGAAATACGC

CHAPTER 5

CONCLUSIONS AND FUTURE OUTLOOK

5.1 Conclusions

The development of tools to investigate epigenetic mechanisms, such as DNA methylation, have led to a wide-spread understanding of the importance of epigenetics (Foerster and Scheid, 2010; Zhong, 2016; Gallego-Bartolomé, 2020). A surmounting collection of evidence shows that DNA methylation plays essential roles in regulating numerous molecular and biological processes involved in virtually every aspect of plant growth and development (Finnegan et al, 2000; He et al, 2011; Zhang et al, 2018). The primary purpose of this dissertation research was to investigate the activity of DNA methylation pathways in various plant tissues throughout development as well as its functional significance in plant stress responses. To this end, a novel GUS reporter system was created for visualizing the activity of all known DNA methyltransferases and demethylases, as well as some auxiliary enzymes involved in these pathways. These GUS reporter lines, which can be applied to numerous developmental and stress-response contexts of *Arabidopsis*, enables real-time temporal and spatial monitoring of GUS expression driven by the promoters of 15 genes involved in DNA methylation and demethylation. Here, this GUS reporter system was used to characterize the expression profiles of these genes in roots, shoots, and reproductive tissues at various stages of development. Results concur with previous reports that DNA methylation is particularly important for fast-growing root tissues, shoot differentiation, and vegetative cell identity (Baubec et al, 2014; Bartels et al, 2018; Maury et al., 2019). Reproductive tissues were marked by the strong upregulation of DNA methyltransferases and demethylases alike, suggesting a tightly controlled DNA methylation equilibrium of developing flowers and seeds. MET1, VIM1, DRM2, and DRM3 in particular, are likely key contributors to the influx of global hypermethylation for floral establishment and subsequent floral development (Campos-Rivero et al., 2017; Bartels et al, 2018), whereas ROS1 and/or DME participate in active DNA demethylation for silique development (Xing et al, 2015; Kawakatsu et al, 2017; Lang et al, 2017; Huang et al, 2019). The collaboration of VIM1 and MET1 or MET1 homologues were also implied to facilitate early embryogenesis, which probably maintains CG methylation at specific loci in the embryo (Jullien et al, 2012; Kim et al, 2014; Xu et al, 2016). Results proceeding from chapter 2 of this dissertation not only reveal the involvement of specific DNA methylation pathways during *Arabidopsis* growth and development but can be extrapolated to inform of DNA methylation

pathway activity in crops due to the highly conserved nature of DNA methylation pathways across plant species.

Another important function of these data are that they serve as a basis of comparison, not only among developing tissues, but also relative to DNA methyltransferase and demethylase expression profiles in response to external stimuli. DNA methylation and phytohormone signaling pathways cooperate throughout plant development for coordinating cellular, molecular, and biological processes (Vanyushin, 2005; Zhu, 2010), particularly in response to stress stimuli (Downen, et al, 2012; Vidhyasekaran, 2015). Therefore, GUS reporter lines were used to observe DNA methylation and demethylation promoter activities of roots and shoots in response to applications of six phytohormones: auxin (AUX), cytokinin (CK), gibberellic acid (GA), ethylene (ET), abscisic acid (ABA), and salicylic acid (SA). The results of these experiments were compared to DNA methylation pathway expression profiles obtained from developing *Arabidopsis* tissues. Results yield that phytohormones can alter DNA methylation dynamics in a specific and coordinated manner, particularly SA. Data presented here suggest a specialized role for CMT in root development and implicate SA in the regulation of active DNA demethylation via MET1 and RNA-directed DNA methylation (RdDM) (Lei et al, 2015). Other phytohormone treatments also impacted the expression profiles of DNA methylation- and demethylation-related genes, suggesting that *Arabidopsis* root and shoot methylomes are regulated by a hormone-linked mechanism. By giving the scientific community access to these GUS reporter lines via the *Arabidopsis* Biological Resource Center (ABRC), this GUS reporter system can help advance our understand of DNA methylation and demethylation in many different developmental and stress contexts, as it has been illustrated here.

Recent studies have revealed a role for DNA methylation in regulating the compatible interaction of plant pathogenic nematodes (PPNs) and *Arabidopsis*, particularly for cyst (*Heterodera* spp.) and root-knot nematodes (*Meloidogyne* spp.) (Hewezi, 2020). Because cyst and root-knot nematodes account for the vast majority of the massive yield losses world-wide due to PPNs, stable and effective sources of genetic resistance to these sedentary endoparasites is in high demand (Bernard et al., 2017; Ghareeb et al, 2020). Understanding feeding site development is a key avenue to engineering resistant plants by exploiting feeding site vulnerabilities. Thus, the GUS

reporter system was utilized for observing DNA methylation and demethylation gene expression patterns in the feeding sites of the beet cyst nematode (*H. schachtii*, BCN), which forms syncytia and of the root-knot nematode (*M. incognita*, RKN), which forms galls in the sedentary phase of its life cycle. Results indicate that these pathosystems utilize various DNA methylation and demethylation pathways in similar and distinct ways for the formation of syncytia and galls. For example, MET1 and MET3 both appear to be driving CG methylation in the early stages of BCN and RKN feeding site development, however, *MET2b* was only detected in early-stage galls. This approach was complemented by susceptibility assays for DNA methyltransferase and demethylase mutant lines, which revealed that *met2b* exhibited increased plant susceptibility to RKN, but not to BCN. Compared to the expression profile of MET3 under noninfected conditions, it seems that this gene has a specialized function in early feeding site establishment that is also operational in developing embryos, which undergo extensive changes in DNA methylation (Xiao et al, 2006; Gehring et al, 2009; Jullien et al, 2012; Wang et al, 2015; Satyaki and Gehring 2017). RdDM methyltransferases DRM2 and DMR3 are apparently important for establishing plant defenses against BCN and RKN, similar to previous reports for other plant pathogens (Lopez et al, 2011). However, largely contrasting reports for DNA demethylases indicate distinct activities of DNA demethylation pathways are exploited by BCN and RKN for their pathology, which is also the case for biotrophic versus necrotrophic fungi (Le et al, 2014; López Sánchez et al, 2016). Chapter 3 demonstrates the use of these GUS reporter lines to address two important plant-PPN interactions. The results described here perpetuate our understanding of the role of DNA methylation pathways, which can help us to create signature crop genotypes which undermine feeding site stability and allow for plant resistances to these agronomically devastating nematodes.

In addition to exploiting feeding site vulnerabilities, plant defense mechanisms can be enhanced and directed specifically towards overcoming plant susceptibility. Targeting specialized defense responses through the modulation of epigenetic mechanisms, such as such as DNA methylation, histone modifications, and microRNAs (miRNAs), provides a promising opportunity for generating new sources of resistance. It was shown here that *SUVH5* and *SUVH6* are targeted for post-transcriptional silencing by miR778 in the syncytia formed by *H. schachtii* in *Arabidopsis* roots. The effects of miR778-*SUVH5/6* was also evident on plant susceptibility, where

overexpression of miR778 leads to increased plant susceptibility and miR778 suppression resulted in increased plant resistance. However, the redundant function of SUVH5 and SUVH6 caused contrasting transcriptional profiles of these genes in single *suvh5* and *suvh6* mutants, which also exhibited increases in plant resistance. The redundant function of SUVH5 and 6 in response to *H. schachtii* was further illustrated by the strong overlap of H3K9me2 sites and differentially expressed genes in ChIP- and RNA-seq experiments, respectively. Not only do SUVH5/6 act as the primary H3K9 dimethyltransferases under *H. schachtii*-infected conditions, but their activity leads to the deposition of H3K9me2 and differential expression for thousands of genes implicated in syncytium development and plant defense, revealing a novel function for these genes. Further investigation of recent DNA methylation data for *H. schachtii*-infected roots reveals that SUVH5/6-diven H3K9me2 preferentially associate with mCG sites, suggesting a multi-layered epigenetic regulatory mechanism that is employed in response to *H. schachtii* infection. Importantly, transcriptome analyses of *suvh5* and *suvh6* single knockout mutants revealed that SUVH5 and SUVH6 play a key role in redundantly regulating the expression of thousands of genes involved a wide range of biological processes and molecular functions, many of which are involved in syncytium development and plant defense.

Chapter 4 explores how the regulatory module miR778-SUVH5/6 can be manipulated to control *H. schachtii* feeding site development. This and other similar studies create a broader illustration of how regulatory elements, particularly including epigenetic mechanisms, trans-acting regulatory elements, and hub genes can be utilized for the generation of resistant plant genotypes. For example, master regulator genes, such as miR778, SUVH5, and SUVH6, can be mis-expressed specifically in feeding site cells via CRISPR-Cas9 to mitigate plant injury and arrest nematode development. Taken together, this dissertation provides many novel insights into the activity and function of DNA methylation and other associated regulatory pathways and reveals potential opportunities of future investigation.

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