Modification of DNA methylation marks in plants

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the works of others.

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Abstract

Phenotypic variation in higher eukaryotes has been found to be associated with different epigenetic states, some of which are associated with defined DNA methylation patterns. In this study, over-expressions of different forms of *DNA METHYLTRANSFERASE1 (MET1)*, with and without catalytic sites and SAM binding domains, were used to alter the epigenetic states of potential target genes and therefore cause epigenetic variation in plants. The DNA methylation is mediated by the addition of a methyl group obtained from S-adenosylmethionone (SAM) to the carbon 5 of a cytosine residue. In plants, it involves *de novo* and maintenance of methylation in CG, CHG and CHH (H representing A, C or T) contexts catalysed by three classes of methyltransferases, namely METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLTRANSFERASE 3 (CMT3), and DOMAIN-REARRANGED METHYLTRANSFERASE 2 (DRM2). *De novo* methylation in all sequence contexts is carried out via DRM2 as part of the RNA-directed DNA methylation (RdDM) pathway, while maintenance of CG and CHG methylation are catalysed by MET1 and CMT3, respectively. Phenotypic analysis of the *Arabidopsis* transformants of *MET1* with and without catalytic sites (namely, the *METo A1* and *METo I1* lines) revealed a reduction in the primary root length in all lines and delayed germination in some of the line. In contrast, *Arabidopsis* transformants of *MET1* without catalytic sites and SAM-binding domains (namely, the *MSM* line) lack the phenotypes that were observed in the *METo* lines. Root transcriptome analysis revealed increased expression of genes encoding transposable elements, non-coding RNAs and proteins. These findings in this study may form a foundation for future research to identify epigenetic control of specific target gene responsible for the root phenotype.

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Abbreviations

ABA	Abscisic acid
Ago	Agonoute
BAH	Bromo-Adjacent Homology
Bp	Base pairs
C	cytosine
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary Deoxyribonucleic Acid
CHD	Chromo-Domain
CK	Cytokinin
CKX	cytokinin dehydrogenase
CMT2	Chromomethyltransferase 2
CMT3	Chromomethyltransferase 3
Col	Arabidopsis Columbia
DDM1	Decrease in DNA Methylation 1
DME	Demeter
DML	Demeter-Like
DNMT1	DNA Methyltransferase 1
DNMT3A	DNA Methyltransferase 3A
DNMT3B	DNA Methyltransferase 3B
DRM2	Domains Re-Arranged Methyltransferase 2
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EpiRILs	Epigenetic Recombinant Inbred Lines
FWA	Flowering Wageningen
HDA6	Histone Deacetylase 6
HOG1	Homology-Dependent Gene Silencing 1
GA	Giberellin
IPT	isopentenyl transferase
LB	Lysogeny broth
LC-MS	Liquid chromatography with mass spectrometric detection
mC	5-methyl-cytosine
MET1	Methyltransferase 1

mRNA	Messenger Ribonucleic Acid
MS	Murashige and Skoog
NASC	Nottingham Arabidopsis Stock Centre
nc	Non-coding
NCBI	National Center for Biotechnology Information
NLS	Nuclear Localisation Sequence
nt	Nucleotide
NOS	Nopaline synthase
NPT	Neomycin PhosphoTransferase
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
POLII	RNA Polymerase II
POLIV	RNA Polymerase IV
PTGS	Post-Transcriptional Gene Silencing
RdDM	RNA-directed DNA Methylation
rDNA	Ribosomal DNA
RDR2	RNA-Dependent RNA polymerase 2
RDR6	RNA-Dependent RNA polymerase 6
RILs	Recombinant inbred lines
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Repressor of Silencing
SAH	S-adenosyl methionine
SAM	S-adenosyl methionine
SDS	Sodium Dodecyl Sulphate
si	Small Interfering
SNP	Single Nucleotide Polymorphism
SRA	Set and Ring Associated
Sup	Superman
SUVH4	Suppressor of Variegation 3-9 Homologue 4
T	Thymine
TAE	Tris-acetate-EDTA
T-DNA	Transfer-DNA
TE	Transposable Element

TGS	Transcriptional Gene Silencing
Ws	Arabidopsis Wassilewskija
WT	Wild-type

Author's declaration

The experiments and their analyses detailed herein were performed by the author under the supervision of Professor Peter Meyer, except where specified in Chapter 6. Specifically, the RNA-seq was performed by the Next Generation Sequencing Facility, Leeds Institute of Biomedical & Clinical Sciences, St James's University Hospital, Leeds, United Kingdom. The quantification of endogenous plant hormones was performed by the Laboratory of Hormonal Regulations in Plants, Institute of Experimental Botany, Prague, Czech Republic.

Chapter 1

Introduction

1.1 General introduction to epigenetics

Epigenetics is the study of heritable changes in chromatin states that regulate gene expression and phenotypic traits. These changes are independent of genetic variation and do not involve changes in DNA sequence (Berger et al., 2009). Epigenetic mechanisms regulate chromatin conformation through covalent modifications of histones and DNA or through the effects of chromatin modulating factors and non-coding RNAs (Roudier et al., 2011). The term 'epigenetics' was coined by Conrad Waddington and was defined as 'the interactions between genes and their products which bring phenotype into being' (Waddington, 1968).

Gene expression is regulated by dynamic changes in chromatin structure, which are mediated through the interactions of nucleosomal DNA, histone proteins, and various protein complexes. DNA strands of approximately 146 bp wrap twice around a complex composed of two copies of each core histone (H2A, H2B, H3 and H4), forming a histone monomer called a nucleosome. The histone complexes are subject to posttranslational modification, which occurs primarily at their N-terminal tails. The amino acids at the N-terminal can be modified via acetylation, methylation, phosphorylation, ubiquitination and sumoylation. These modifications cause changes in chromatin structure and packaging, which consequently affects DNA accessibility (Berr et al., 2011). The term heterochromatin refers to the 'silent' or 'closed' chromatin state in which repetitive DNA sequences, such as transposons, are transcriptionally inactive. In contrast, in euchromatin, the 'active' or 'open' chromatin state, genes are transcriptionally active and comprise limited repetitive elements (Andrews and Luger, 2011).

1.2 DNA methylation

DNA methylation is a covalent modification of DNA in which a methyl group is added to either the fifth carbon residue, the fourth nitrogen of cytosine or the sixth nitrogen of adenine (Zhou et al., 2018). In general, DNA methylation occurs in fungi, insects, mammals, and plants but not in nematodes, budding yeast or fission yeast. In prokaryotes, the primary function of cytosine methylation is to protect DNA from restriction enzymes (Casadesús and Low, 2006). The most common and well-studied modification of DNA in higher eukaryotes is the addition of a methyl group to carbon 5 in cytosine residues (**Figure 1.1**). Methylation levels range from 3 to 8% in vertebrates and 3.9 to 30% in plants (Niederhuth et al., 2016). The DNA methylation landscapes vary between mammals and plants. In mammals, DNA methylation occurs globally except for short unmethylated regions termed “CpG islands”, whereas in fungi and plants, mosaic patterns are observed in which methylated DNA is interspersed with stretches of unmethylated DNA (Ramsahoye et al., 2000; Mirouze et al., 2009).

DNA methylation is categorized into three different sequence contexts: the symmetrical CG and CHG sequences and the asymmetrical CHH (where H = A, C, or T) (Henderson and Jacobsen, 2007). Plants have a high degree of cytosine methylation, as the methylation patterns occur in all contexts: CG, CHG and CHH. A genome-wide study of DNA methylation in *Arabidopsis* leaves revealed a distribution of 30.5% in the CG context, 10.0% in CHG and 3.9% in CHH (Niederhuth et al., 2016). In contrast, cytosine methylation in mammals occurs primarily at CG sequences with the exception of certain cell types, such as embryonic stem cells and brain cells, which are associated with non-CG methylation (Ramsahoye et al., 2000; Varley et al., 2013).

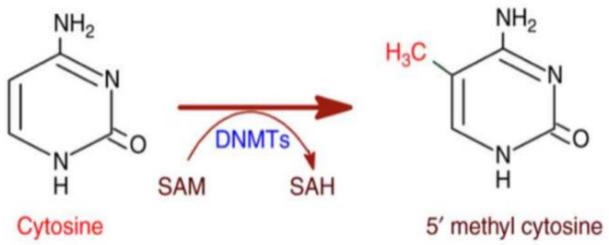


Figure 1. 1. DNA methyltransferases catalyse the transfer of methyl groups to the fifth carbon of cytosine to form 5' methyl cytosine. The DNA methyltransferases require SAM as the source of the methyl group, producing SAH. SAM, S-adenosyl methionine; SAH, S-adenosylhomocysteine (Thankam et al., 2019).

1.2.1 Maintenance of DNA methylation

In plants, the maintenance of DNA methylation depends on the cytosine sequence contexts. For maintenance of CG and CHG methylation after replication, the process is catalysed by maintenance methyltransferases, namely DNA METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) (Lindroth et al., 2001; Cao and Jacobsen, 2002). MET1, which is homologous to mammalian DNMT1, predominantly controls CG methylation. The hemi-methylated CG sites generated after replication are recognized by proteins in the VARIANT IN METHYLATION (VIM) family, which then recruit MET1 to fully catalyse the CG state for maintaining CG methylation (Woo et al., 2008).

Maintenance of CHG methylation is catalysed by CMT3. CMT3 is a member of the chromomethyltransferase class, which contains an additional chromodomain as part of the catalytic domain found exclusively in the plant kingdom (Lindroth et al., 2001). CHG methylation is maintained through a feedback loop associated with histone H3 lysine 9 dimethylation (H3K9me2) (Johnson et al., 2002). CMT3 binds to H3K9me2 and methylates DNA near the CHG sites and recruits SU(VAR) HOMOLOGUE 4 (SUVH4). Consequently, SUVH4 mediates the deposition of H3K9me2 marks on the nucleosome around the methylated CHG DNA to produce a CHG-H3K9me2 positive feedback loop (Du et al., 2012).

Depending on the genomic region, the CHH methylation is maintained by either DRM2 or CMT2. For RdDM target regions, such as transposons and repeat sequences, the CHH methylation is maintained by DRM2 through the RdDM pathway. In contrast, a pathway independent of RdDM is required for maintenance of CHH methylation at heterochromatic loci, which is mediated through CMT2 and DDM1. The DDM1 plays a role in displacing the linker histone H1 in heterochromatic loci, which allows CMT2 to access the DNA, thereby enabling it for methylation of cytosines in the CHG and CHH contexts (Zemach et al., 2013; Matzke et al., 2015).

1.2.2 Establishment of DNA methylation

DNA methylation is carried out by specific DNA methyltransferases classified as either *de novo* or maintenance type. *De novo* methylation refers to the synthesis of newly methylated DNA, while maintenance methylation is the process of maintaining pre-existing methylated DNA after replication. In mammals, DNA methylation patterns are established and maintained by the DNA methyltransferases DNMT3a/3b and DNMT1 (Goll and Bestor, 2005). In plants, the establishment of DNA methylation in all sequence contexts (CG, CHG and CHH) occurs through the RNA-directed DNA methylation (RdDM) pathway with the activity of the *de novo* DOMAINS REARRANGED METHLTRANSFERASE (DRM) proteins, which are homologs of mammalian DNMT3a/3b (He et al., 2011; Zhang et al., 2018) (**Figure 1.2**).

1.2.3 RNA-directed DNA methylation

RNA-directed DNA methylation (RdDM) was first discovered in tobacco infected with plant pathogens known as viroids (Wassenegger et al., 1994). Viroids are circular noncoding RNA several hundred nucleotides in length. Replicating viroids were found to trigger *de novo* methylation of viroid cDNAs that had been integrated as transgenes into the tobacco genome. RdDM was then established as a general transcriptional silencing mechanism in plants, including transgene

silencing, transposon silencing and gene imprinting (Law and Jacobsen, 2010). The RdDM methylation machinery consists of various proteins that process and generate siRNAs, modify histones, remodel chromatin and methylate cytosines. RdDM includes the action of the plant-specific RNA polymerases IV and V (Pol IV and Pol V), with components of, or related to, RNA interference (RNAi), such as DICER-LIKE (DCL) and ARGONAUTE (AGO) proteins (Matzke et al., 2015). RdDM can add DNA methylation from all sequence contexts of CG, CHG, and CHH, where H is any nucleotide except G. However, CHH is the hallmark of RdDM because continuation of CHH methylation at many silenced loci involves continuous, RNA-guided *de novo* methylation in every cycle (Law and Jacobsen, 2010).

1.2.4 Mechanisms of RNA-directed DNA methylation

The initial part of the RNA-directed DNA methylation (RdDM) pathway involves the biogenesis of small RNAs (sRNAs). First, RNA POLYMERASE IV (POL IV) which is a plant-specific RNA polymerase complex, is recruited to silent heterochromatin via its interaction with CLASSY (CLSY) proteins and SAWADEE homeodomain homolog 1 (SHH1) (Matzke and Mosher, 2014; Cuerda-Gil and Slotkin, 2016a; Wendte and Pikaard, 2017). POL IV transcribes these regions and approximately 30 to 45 nucleotides of short single-stranded RNAs (ssRNAs) are produced, each of which is the precursor for a single sRNA (Blevins et al., 2015; Zhai et al., 2015; Singh et al., 2019). RNA- directed RNA polymerase 2 (RDR2), which physically associates with POL IV then converts these ssRNAs into double-stranded RNAs (dsRNAs) (Blevins et al., 2015). The endoribonuclease DICER-LIKE 3 (DCL3), which is partially redundant with other dicer-like enzymes DCL2 and DCL4, cleaves the dsRNA into 24 nucleotide siRNAs (Singh et al., 2019).

Subsequently, the RdDM DNA methylation machinery is guided to DNA sequences complementary to the sRNAs generated in the initial part of the pathway. One strand from each 24 nucleotide double-stranded sRNA is loaded

into ARGONAUTE (AGO) proteins AGO4, AGO6, or AGO9 (Matzke and Mosher, 2014). Once formed, the AGO-sRNA duplex finds and binds complementary sequences along an RNA ‘scaffold’ produced by RNA POLYMERASE V (POL V), with the help of interactions with SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5L), the INVOLVED IN DE NOVO 2- IDN2 PARALOG (IDN2- IDP) complex, and the POL V subunit NRPE1 (Wierzbicki et al., 2008). DRM2 is then recruited and methylates nearby DNA (Cao and Jacobsen, 2002; Matzke and Mosher, 2014; Cuerda-Gil and Slotkin, 2016b).

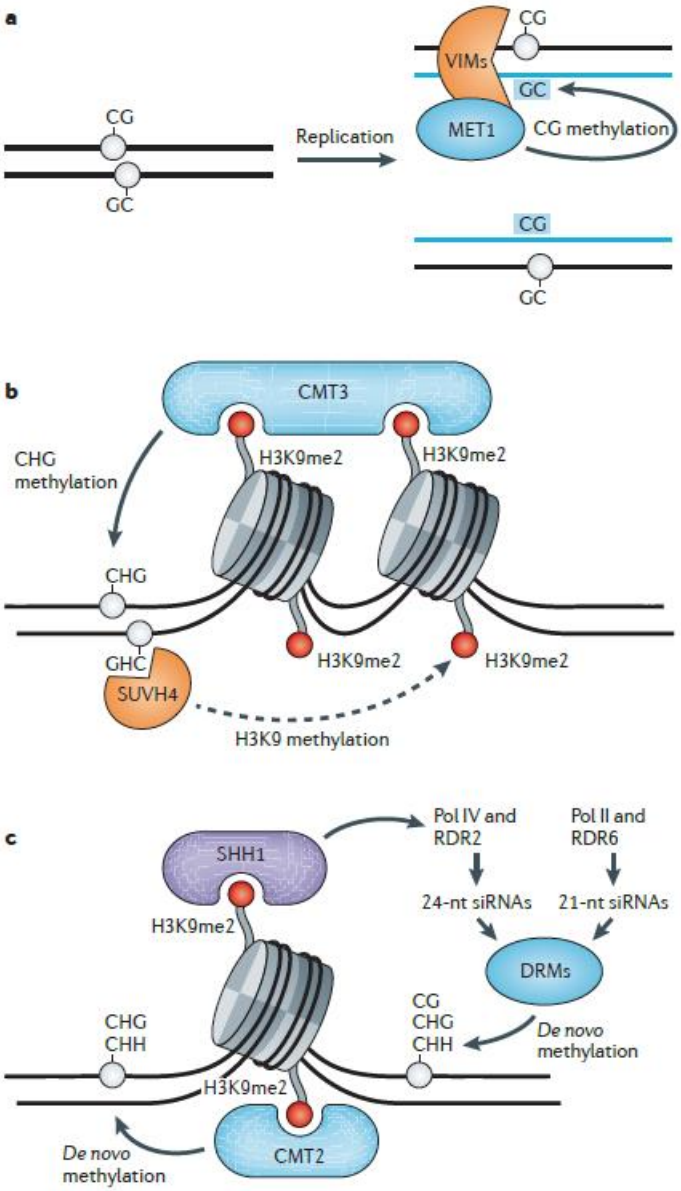


Figure 1.2 Models of CG, CHG and CHH DNA methylation. A. MET1 is recruited by VIM to the hemi-methylated CG sites to maintain CG methylation patterns following DNA replication. B. CHG methylation occurs through a self-reinforced loop, in which CMT3 mediates CHG methylation by binding to H3K9me2, and methylated CHG recruits SUVH4 binding for methylating H3K9. C. For *de novo* methylation, SHH1 involved in siRNA biogenesis through POLIV with RDR2, or POLII with RDR6 pathways before recruitment of DRMs complex for methylating all sequence contexts. CMT2 methylates CHH and CHG via *de novo* methylation by binding to H3K9me2 (Kawashima and Berger, 2014).

1.2.5 Non- canonical RNA-directed DNA methylation

The 24- nucleotide small interfering RNAs (siRNAs) are produced in the canonical POL IV-RDR2-DCL3 pathways. In addition, there are several small RNA pathways that direct RdDM, known as non-canonical RdDM pathways. Most of these pathways differ primarily in the source and entry points for small RNAs into the canonical RdDM pathway (Nuthikattu et al., 2013; McCue et al., 2015). The non-canonical pathway usually functions to establish initial DNA methylation at new target loci such as novel transposable elements (TEs) insertions, rather than maintaining existing heterochromatin, as occurs in canonical RdDM (Cuerda-Gil and Slotkin, 2016a). One example is the initiation of TE silencing through a non-canonical RdDM mechanism, namely Pol II-DCL3 RdDM (Panda et al., 2016). This is exemplified in maize, in which a long Pol-II-derived inverted repeat (IR) of a Mutator family TE is cleaved into 24-nt siRNAs and directs *trans*-RdDM and epigenetic transcriptional silencing to the rest of this TE family (Slotkin et al., 2005).

There is also an alternative to the RdDM pathway involving 21- to 22-nt sRNA and RDR6, known as the RDR6-RdDM pathway (Nuthikattu et al., 2013; Bond and Baulcombe, 2015). This pathway is not reliant on the canonical RdDM components Pol IV, RDR2 or DCL3, but the 21-22 nt siRNAs generated from Pol II-RDR6-derived TE mRNAs are directly incorporated into AGO6 (McCue et al., 2015). Pol V and DRM2 are required for the downstream targeting complex of RDR6 RdDM, similar to canonical RdDM (Panda et al., 2016). Additionally, in RDR6-DCL3 RdDM, DCL3 cleaves the RDR6-derived dsRNA into 24-nt siRNA and loads into Pol V-mediated RdDM (Marí-Ordóñez et al., 2013). There is also a dicer-independent pathway in which a non-diced dsRNA from either Pol-II-RDR6 or Pol IV-RDR2 is directly loaded on to AGO4. Subsequently, the RNA is trimmed by exonucleases from the exosome core complex then used in Pol V-mediated RdDM (Ye et al., 2016).

1.3 DNA demethylation

In plants, DNA demethylation can take place passively, during replication, or actively through the activities of DNA glycosylases (via a base excision repair pathway) (Gong et al., 2002). Passive DNA demethylation occurs through the inactivation or failure of maintenance methyltransferase, or the shortage of a methyl donor after DNA replication, which causes the reduction of methylation (Saze et al., 2003; Rocha et al., 2005; Zhang et al., 2012; Zhou et al., 2013). Active demethylation involves enzymatic removal of DNA methylation. There are four DNA glycosylases in *Arabidopsis,* including REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-LIKE 2 (DML2), and DEMETER-LIKE 3 (DML3) that remove DNA methylation in all contexts (Penterman et al., 2007). These enzymes remove methylcytosine from the DNA backbone and, subsequently, the resulting single nucleotide gap is filled with unmethylated cytosine through the base excision repair (BER) pathway (Gong et al., 2002; Gehring et al., 2006; Ortega-Galisteo et al., 2008). ROS1, DML2 and DML3 are expressed in all vegetative tissues (Penterman et al., 2007). Meanwhile, DME is preferentially expressed in the central cell of the female gametophyte and in the vegetative cell of the male gametophyte (Schoft et al., 2011).

ROS1 is the first DNA demethylase that has been genetically characterized. Mutations in ROS1 results in DNA hypermethylation and transcriptional gene silencing of the *RD29A::LUCIFERASE (RD29A::LUC)* reporter gene and the endogenous *RD29A* gene (Gong et al., 2002). ROS1 functions to repress silencing at several endogenous loci including transposons (Zhu et al., 2007). ROS1 preferentially targets protein coding genes close to highly methylated TEs and therefore prevents the spreading of DNA methylation established by RdDM to avoid hypermethylation and adverse gene silencing (Tang et al., 2016). In *ros1* mutants, decreased expression of TEs were observed due to increased DNA methylation. Several genes are also silenced in *ros1* mutants as a result of DNA hypermethylation of nearby TEs (Zhu et al., 2007; Le et al., 2014; Lang et al., 2015). ROS1 is targeted to specific genomic regions through an anti-silencing

protein complex, (MBD7-IDM complex), comprised of IDM1, IDM2, IDM3, METHYL-CPG-BINDING DOMAIN-CONTAINING PROTEIN 7 (MBD7), HARBINGER TRANSPOSON-DERIVED PROTEIN 1 (HDP1) and HDP2 (Li et al., 2012; Qian et al., 2014; Lang et al., 2015; Duan et al., 2017).

DME is mainly required for genomic imprinting during female gametophyte development (Huh et al., 2008). DME targets *MEDEA (MEA), FLOWERING WAGENINGEN (FWA)* and *FERTILIZATION INDEPENDENT SEED 2 (FIS2).* These genes are methylated in vegetative tissues. Nevertheless, they are hypomethylated and transcriptionally active in the reproductive central cell (Xiao et al., 2003; Kinoshita et al., 2004; Jullien et al., 2006). DME is involved in seed development and controls the expression of the maternally imprinted *MEA* gene in the endosperm tissue. Mutation of the maternal copy of the *DME* gene causes silencing of the *MEA* gene in the endosperm and results in seed abortion (Choi et al., 2002; Bauer and Fischer, 2011).

DML2 and DML3 function in the distribution of DNA methylation within the genome (Penterman et al., 2007). DNA demethylation is crucial to protect genes from RdDM spreading leading to transcriptional silencing. The *ros1dml2dml3* triple mutants gain methylation in all sequence contexts in proximal gene regions, primarily around TEs and TE-derived sequences (Penterman et al., 2007; Lister et al., 2008). In flowering plants, active DNA demethylation is required for gene imprinting during reproduction and for sustaining normal methylation patterns throughout the plant (Gehring and Henikoff, 2008; Calarco et al., 2012). After fertilization, DNA demethylation in the female gametophyte is important for establishing gene imprinting in the endosperm (Calarco et al., 2012). ROS1 controls imprinted gene expression in the endosperm and controls seed dormancy in *Arabidopsis*. In the endosperm, *DOGL4* is highly expressed from the maternal allele and only expressed at low levels from the paternal allele. Dysfunction of ROS1 results in full silencing of *DOGL4* expression from the paternal allele (Zhu et al., 2018).

1.3.1 Coordination between DNA methylation and demethylation

In plants, DNA methylation patterns are dynamically regulated by DNA methylation and active DNA demethylation in response to developmental or environmental stimuli (Zhu, 2009). Changes in expression for proteins involved in DNA methylation and demethylation cause genome-wide changes in DNA methylation. Over 2000 genomic regions were hypermethylated in *ros1-4* mutants (Li et al., 2012; Lei et al., 2015; Williams et al., 2015). At specific loci, ROS1 counteracts the RdDM pathway to prevent DNA hypermethylation (Gong et al., 2002). Reduction in *ROS1* gene expression was observed in the RdDM mutants, which suggests a feedback mechanism for the regulation of DNA methylation. The hypomethylation status of some genomic loci in RdDM mutants causes the reduction of ROS1 transcript levels to prevent further demethylation on the genome (Penterman et al., 2007; Zhong et al., 2014).

The levels and activities of the demethylases are tightly regulated to adjust to the levels and activities of methyltransferases. *ROS1* gene expression was reduced in *met1* mutants with significant reduction in DNA methylation (Huettel et al., 2006). In *met1-3* plants with decreased *ROS1* transcript levels, CG hypomethylation at 5S ribosomal DNA sequences is counteracted by a rapid increase in CHH methylation levels in successive generations, leading to re-establishment of transcription silencing (Mathieu et al., 2007).

A complex balance between DNA methylation and active demethylation is essential to transcriptionally regulate the expression of *ROS1*. There is a 39 bp sequence in the *ROS1* promoter region called “DNA methylation monitoring sequence” (MEMS), that functions as a methyl-rheostat. Expression of *ROS1* is activated by DNA methylation of MEMS and conversely repressed by active DNA demethylation of MEMS (Lei et al., 2015; Williams et al., 2015). The *ROS1* promoter contains a helitron transposon that negatively controls *ROS1* expression. DNA methylation of an RdDM target sequence between the *ROS1* 5’UTR and the promoter TE region antagonizes the helitron to regulate *ROS1* expression (Lei et al., 2015).

It was also discovered that ROS3 is a component of a small RNA-directed demethylation system that prevents DNA hypermethylation (Zheng et al., 2008). ROS3 co-localises with ROS1 in discrete foci dispersed throughout the nucleus. ROS3 contains RNA recognition motifs and is able to bind to small RNAs, which therefore might act as regulators of DNA demethylation (Zheng et al., 2008). Elevated *ROS3* expression was observed in *ros1* mutants and *ROS1* expression was elevated in *ros3* mutants due to enhanced methylation levels at some target loci in *ros1* and *ros3* mutants (Zheng et al., 2008).

1.4 Molecular functions of DNA methylation

1.4.1 Gene regulation

DNA methylation has distinctive functions based on genomic features. In plants, DNA methylation maintains genome stability by repressing the transcription of mobile DNA elements such as transgenes, transposons and retro-elements (Ito, 2012). Transposable elements pose threats to genome stability by transposing into introns, therefore disrupting an active host gene or modulating the regulated expression of the host genome (Slotkin and Martienssen, 2007). Genome-wide analysis of DNA methylation using the model plant *Arabidopsis* revealed that within the promoter region, approximately 5% of genes are methylated, and the methylated genes exhibit tissue-specific expression (Zhang et al., 2006; Cokus et al., 2008).

DNA methylation regulates gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA. When DNA methylation occurs within gene promoters, it can impede the transcriptional machinery from accessing DNA and initiating transcription, which represents the most clear and direct mechanism by which gene expression can be regulated by this epigenetic mechanism. However, not all transcription factors are sensitive to DNA methylation occurring within their binding sites. On the other

hand, methyl-CpG-binding proteins have been implicated in the DNA-methylation-mediated regulation of gene expression. These proteins bind to methylated DNA and mediate interactions between DNA methylation and histone modifications, producing a repressive chromatin structure. Thus, DNA methylation can also regulate gene expression by altering chromatin structure. Examples include repressing transcription by promoting repressive histone modifications such as H3K9me2 and inhibiting permissive histone modifications such as histone acetylation (Zhu et al., 2016).

Promoter DNA methylation usually inhibits gene transcription. For example, methylation at the *FWA* gene, restricted to the promoter and 5' untranslated region which contain siRNA-generating tandem repeats, resulted in transcriptional silencing, which is released following demethylation of the maternal allele in the endosperm (Kinoshita et al., 2007). In *met1* and *ddm1*, endogenous *FWA* is demethylated and deactivated, which indicates that CG methylation caused complete silencing of endogenous *FWA* (Kakutani et al., 1996; Kinoshita et al., 2007; Johannes et al., 2009).

Gene body methylation (GbM) is CG methylation found within the exons of the transcribed genes and it is associated with intermediate levels of expression (Zilberman, 2017). In *Arabidopsis*, CG methylation was found in more than 20% of the expressed genes in central regions excluding the promoter and 3' regions (Zhang et al., 2006; Cokus et al., 2008). It has been suggested that GbM may play a role in preventing transcription from internal cryptic promoters, thereby assisting in gene expression (Zilberman et al., 2007). As CG methylation is more abundant in exons, it is also proposed that GbM influences splicing efficiency (Takuno and Gaut, 2012).

1.4.2 Transposon silencing

Transposons can threaten genome integrity through the relocation of DNA transposons or the insertion of new copies of retrotransposons. Therefore, gene silencing is essential to prevent the activation of transposons. Pericentromeric heterochromatin and some transposon-containing or repeat-containing euchromatin regions are heavily methylated in all cytosine contexts in *Arabidopsis* (Zhang et al., 2006; Cokus et al., 2008). In double mutations of *MET1* and *CMT3*, or dysfunction of *DDM1*, strong DNA hypomethylation in both CG and CHG contexts and increased levels of transposition were observed. Transposition of *CACTA1* was found in *met1cmt3* double mutants (Kato et al., 2003). In *ddm1*, a member the of long-terminal repeat (LTR) retrotransposon class, *AtGP3-1* was mobilised (Mirouze et al., 2009; Tsukahara et al., 2009).

1.5 DNA methylation in plant development

DNA methylation influences biological functions during plant development including embryogenesis and seed viability, imprinting, floral organ identity, leaf morphology and flowering time (Jacobsen et al., 2000; W. Xiao et al., 2006). Gene imprinting is a parent-of-origin-specific gene expression observed in the endosperm during seed development. Imprinted genes such as *FWA, MEA, FIS2* and *PHERES1* are expressed from the maternal genome of the endosperm whereas the alleles from the paternal genome are silenced (Kinoshita et al., 2004; Köhler et al., 2005; Jullien et al., 2006; Gehring et al., 2006). The DNA repeat region in the *FWA* promoter is hypomethylated for normal expression of FWA in which FWA is expressed in the endosperm but not in other tissue. The *FWA* imprint is controlled by DME (Kinoshita et al., 2004). During seed development, the expression of *DME* is concentrated in the central cells and endosperm in which it specifically demethylates the maternal genome in central cells (progenitors of endosperm), and therefore causes the expression of imprinted genes (Gehring et al., 2009; Hsieh et al., 2009). Ectopic *FWA* expression, which

causes late flowering, was observed during loss of MET1 activity. This suggests that the maintenance of endosperm-specific and parent-of-origin-specific *FWA* expression depends on MET1 (Soppe et al., 2000; Kinoshita et al., 2007). DME and MET1 work antagonistically to regulate the expression of maternal alleles in the *Arabidopsis* central cell and endosperm (Jullien et al., 2006).

Genome-wide reduction of DNA methylation causes various developmental phenotypes. The *MET1* missense mutations result in genome-wide hypomethylation and delayed flowering (Finnegan et al., 1996; Ronemus et al., 1996; Kankel et al., 2003). The *met1cmt3* double mutant shows a decrease in seed size and viability as compared to wild-type plants (W. Xiao et al., 2006). In the *ddm1* mutants, a reduction of global DNA methylation by approximately 70% and widespread increased expression of transposable elements (TEs) were observed. The *ddm1* mutant plants showed mild phenotypic changes; however, the severity and the aberrant phenotype increased across generations after repeated selfing (Vongs et al., 1993; Kakutani et al., 1996).

In the *Arabidopsis* root meristem, the highest level of methylation, particularly in the CHH context, was identified in the columella root cap (Kawakatsu et al., 2016). Meanwhile in the shoot apical meristem, the highest level of CHH methylation was observed during early vegetative growth, accompanied by increased RdDM factors to reinforce TE silencing (Baubec et al., 2014). In the *Arabidopsis* endosperm, the level of DNA methylation was reduced in all sequence contexts compared to the embryo, which is the same in rice (Hsieh et al., 2009; Zemach et al., 2010). The distinctive DNA methylation levels in different tissue types is likely due to the connection of different DNA methylation pathways that take place.

1.5.1 Interactions between hormones and epigenetic modifications in plants

Most epigenetic mutants show defects in development and morphology, such as late flowering and stunted vegetative growth. Plant hormones regulate numerous aspects of plant development and response to the environment. There has been an emerging focus on the links between epigenetic mechanisms and plant hormone regulations. For instance, the transcription factor *WUSCHEL (WUS)* has been shown to be essential during shoot regeneration. In the *Arabidopsis met1* mutant, DNA methylation was lost at the regulatory region of *WUS* resulting in elevated *WUS* expression. Additionally, increased expression of *AUXIN RESPONSE FACTOR 3 (ARF3)* was observed, which suggests that *MET1* is involved in shoot regeneration by repressing *WUS* expression and auxin signalling (Li et al., 2011). A study by Liu et al. (2018) revealed that *WUS* expression is also modulated by the local balance between *MET1* and cytokinin cell cycle factor *CYCD3-EF2A*. Both of these studies showed a direct effect of *MET1* during shoot meristem initiation through the interaction between auxin and cytokinin to modulate *WUS* expression.

Nevertheless, there are several candidate genes that showed changes in the transcript level during shoot regeneration in the *met1* mutant but displayed no methylation, such as *CYTOKININ OXIDASE 1 (CKX1), ARABIDOPSIS REGULATOR15 (ARR15)* and *INDOLE-3-ACETIC ACID27 (IAA27),* suggesting an indirect effect of *MET1* (Li et al., 2011). In *met1-6* mutants, a late flowering phenotype was observed, accompanied by mis-regulation of *PIN1* promoter activity and lack of a proper auxin gradient. However, *MET1* showed indirect regulation in *PIN1* expression based on the absence of DNA methylation at the *PIN1* locus in both wild-type and *met1* null allele plants (W. Xiao et al., 2006).

Several studies have revealed that certain hormones could change epigenetic modifications. For instance, tobacco plants treated with GA_3 showed a reduction in DNA methyltransferase activity accompanied by a decline in global DNA

methylation (Manoharlal et al., 2018). In *Azalea,* the initiation of flowering is regulated through the cytokinin components, zeatin riboside and isopentenyladenine and shows a correlation with decreased DNA methylation. Further treatment with GA biosynthesis inhibitors caused changes in the cytokinin levels and affected flowering (Meijón et al., 2011). In trifoliate orange (*Poncirus trifoliata*), treatment with DNA methylation inhibitor, 5-azacytidine, resulted in aberrations in flowering time and increased expression of the *LEAFY* homologue gene (*CiLFY*) accompanied by DNA demethylation at a specific locus. The regulation of *CiLFY* during the flowering process was also previously shown to be linked with GA, which therefore suggests the interconnection of epigenetic modification and GA and that it controls *CiLFY* transcription (Zhang et al., 2014).

1.5.2 The interaction between methyl donor S-adenosylmethionine and epigenetic modifications

S-adenosyl methionine (SAM) is a universal methyl donor that is essential during the methylation process. SAM is derived from methionine, which depends on the folate that provides the 5-methyl-tetrahydrofolate (5-CH3-THF) moiety for methionine synthesis. Unlike mammals, which obtain folate from external sources, plants synthesize folate *de novo* from pterin, para-aminobenzoic (PABA) and Glu precursors (Roje, 2007; Hanson and Gregory, 2011). One-carbon (1C) metabolism is an essential molecular process, as it is the source of one-carbon units for proteins and nucleic acids and it is inter-connected with the methionine and SAM cycle (**Figure 1.4**). DNA methyltransferases require SAM to transfer a methyl group to the cytosine residue in DNA and, during the reaction, SAM is converted into S-adenosyl homocysteine (SAH), which is a competitive inhibitor of DNA methyltransferases. The SAM cycle, also known as the activated methyl cycle (AMC), is therefore important to recycle the by-product, SAH, to maintain the proper SAM:SAH ratio.

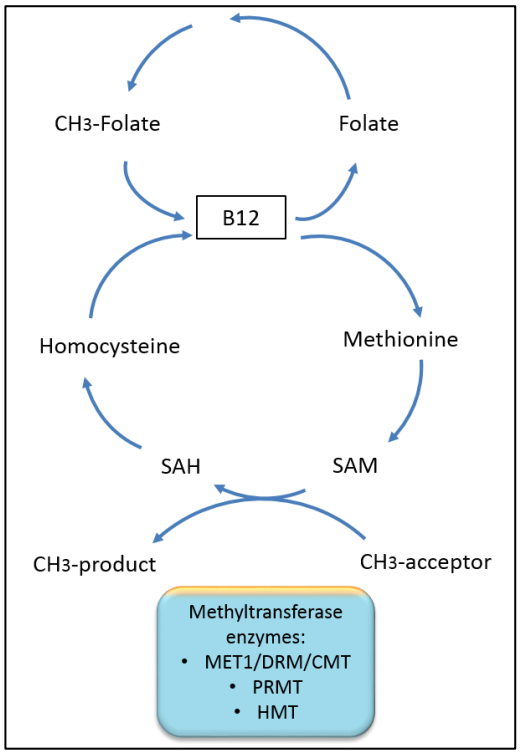


Figure 1. 3. One-carbon (1C) metabolic pathways, which consist of the folate cycle, methionine and SAM. Key methyltransferase enzymes; MET1/DRM/CMT, *de novo* and maintenance DNA methyltransferases; HMT, histone methyltransferase; PRMT, protein arginine methyltransferase. Enzyme cofactor B12.

The *HOMOLOGY-DEPENDENT GENE SILENCING1 (HOG1)* gene encodes for S-ADENOSYL HOMOCYSTEINE HYDROLASE (SAHH), an enzyme that functions to hydrolyse SAH into homocysteine (Hyc) and adenosine (Ado). In *Arabidopsis hog1-1*, the mutation in the *HOG1* gene causes genome-wide hypomethylation and induces many developmental defects (Rocha et al., 2005). This suggests that maintenance of a proper ratio of SAM and SAH is crucial for the methyltransferase's activity. In addition, decreased SAM flux into plastids also induced a growth-retarded phenotype in *Arabidopsis*, accompanied by hypomethylation and decreased histone methylation (Bouvier et al., 2006). Similarly, in rice, it has been demonstrated that decreased SAM supply leads to suppression of DNA and H3K4me3 transmethylations at key flowering genes, resulting in a late-flowering phenotype (Li et al., 2011). This suggests that the methionine cycle and SAM level could influence the feedback mechanisms between DNA and histone methylation.

The association of the methyl donor SAM, methionine and folate with epigenetic regulation such as DNA methylation has been studied in animals. For instance, in the yellow agouti (A^{vy}) mouse model, constant supplementation with folate resulted in an increased level of DNA methylation at the A^{vy} gene. The correlation of the coat colour variation with the epigenetic marks established during early development provides an advantage for studying the effects of nutritional and environmental factors on the epigenome in the fetus (Dolinoy, 2008). In plants, there has been increasing interest in studying the association of DNA methylation with the methyl group supply derived from methionine and folate metabolism as it becomes more evident that they are interdependent. A missense mutation in *METHYLENE TETRA HYDROFOLATE DEHYDROGENASE 1 (MTHD1)* caused a disruption in folate metabolism. In the *mthfd1-1* mutant, high levels of homocysteine and SAH were observed, accompanied by a reduction of DNA methylation, particularly involving CMT3 and CMT2, loss of H3K9me and reactivation of transposons (Groth et al., 2016). Reduction of the DNA methylation level was observed in *Arabidopsis* treated with sulphametazine, an inhibitor of folate biosynthesis that caused methionine and SAM deficiencies due to a reduction in the folate pool size (Zhang et al., 2012).

1.5.3 Epigenetic variation contributes to phenotypic variation

The characterization of numerous epialleles has provided evidence of how epigenetic variation contributes to phenotypic variation in plants. Examples of mechanisms and phenotypes controlled by DNA methylation include floral symmetry (peloria) in *Linaria vulgaris* (Cubas et al., 1999) and fruit pigmentation (colourless non-ripening, *cnr*) in *Solanum lycopersicum* (Manning et al., 2006) and *Zea mays* (B') (Stem et al., 2002). In a natural mutant of *Linaria vulgaris,* the change in floral symmetry from bilateral to radial is due to the *LCYC* gene, that controls dorsoventral asymmetry, being silenced by DNA methylation (Cubas et al., 1999). The hypermethylation of the promoter region of the *COLOURLESS NON RIPENING (CNR)* gene caused a defective ripening phenotype in the agronomically important crop *Solanum lycopersicum* (Manning et al., 2006). Examples of epialleles in the model plant *Arabidopsis* include *FLOWERING*

WAGENINGEN (FWA), which is silenced in wild type plants (Soppe et al., 2000), and *BONSAI (BSN*) in the SWI/SNF-like ATP-dependent chromatin remodeller mutant, *ddm1* (Saze and Kakutani, 2007).

The impact of DNA methylation on plant phenotypes was further exemplified in a study using epigenetic Recombinant Inbred Lines (epiRILs) derived from a cross between *Col-0* and *ddm1* (Kakutani et al., 1996; Johannes et al., 2009). The epiRILs contain highly similar DNA sequences but differ in their DNA methylation patterns and demonstrate phenotypic variation in complex traits such as plant height, biomass, flowering time and NaCl stress tolerance (Johannes et al., 2009; Reinders et al., 2009). Evidence indicates that epigenetics can induce heritable variation in plant traits that is not limited only to complex traits but also observed in response to defence hormones such as jasmonic acid (JA) and salicylic acid (SA). A study by Latzel et al. (2012) using the epiRILs showed a potential epigenetic variation underlying the response to JA and SA treatments, as reflected by the variation in plant growth rates.

1.6 DNA METHYLTRANSFERASE 1

DNA METHYLTRANSFERASE 1 (MET1) recognizes hemi-methylated CG dinucleotides following DNA replication and catalyses the transfer of a methyl group from the cofactor S-adenosylmethionine (SAM) to carbon 5 in cytosine. The first gene encoding *MET1* was isolated from *Arabidopsis,* in which it is expressed in vegetative and floral tissues and shows the highest expression in meristematic cells (Finnegan and Dennis, 1993; Ronemus et al., 1996). In *Arabidopsis*, *MET1* is a member of a small multigene family, with four characterized genes which possibly arose by duplication of an ancestral gene. Apart from *MET1*, it also includes *MET2a, MET2b* and *MET3*. The *MET2a* and *MET2b* are expressed at lower levels in vegetative and floral organs as compared to *MET1,* however their function is unclear (Jullien et al., 2012). Whereas *MET3*

encodes a truncated protein and is exclusively expressed in the endosperm (Genger et al., 1999; Jullien et al., 2012).

1.6.1 The structure and function of MET1

MET1 consists of 1534 amino acids and shares significant homology with the mammalian methyltransferase DNMT1. MET1 and DNMT1 share 50% identity in the C-terminal domain involved in methylation and 24% identity in the N-terminal. However, in contrast with mammalian DNMT1, MET1 lacks cysteine-rich regions known as the CXXC domains, which are involved in binding to unmethylated cytosines but contains an acidic region of unknown function (Song et al., 2011). This acidic region consists of at least 50% glutamic acid plus aspartic acid residues which are conserved at the same location within the amino acid terminus of all plant methyltransferases, therefore suggesting that it is important for enzyme function (Finnegan and Kovac, 2000; Pavlopoulou and Kossida, 2007).

The N-terminal domain which is comprised of amino acids 1 to 1093, includes nuclear localization signal (NLS), replication foci targeting sequence (RFTS) and bromo-adjacent homology (BAH) (Pavlopoulou and Kossida, 2007). NLS acts as a nuclear targeting sequence in tobacco (Van Der Krol and Chua, 1991). Several clusters of basic amino acids, for instance lysine and arginine, are presumed to be a part of NLS (Hicks and Raikhel, 1993). Interaction of the RFTS domains results in DNMT1 dimerization based on protein interaction assays and mutational analysis. However, there is no direct evidence of whether MET1 acts as a dimer via its RFTS (Fellinger et al., 2009). Two BAH domains exist in MET1 which are proposed to serve as a protein-protein interaction module and therefore may provide interconnection between DNA methylation, replication and transcriptional regulation (Callebaut et al., 1999).

The N-terminal domain and C-terminal domains are joined via Glycine-Lysine (GK) repeats which also known as KG linker. The C-terminal domain of MET1

contains ten amino acid motifs, designated I to X, of which eight are highly conserved among plants, mammals and prokaryotes. The functional active site has been identified in motif IV (Kumar et al., 1994). Motif VI functions in the binding of the methyltransferase domain to the targeted cytosine (Hermann et al., 2004). Between motifs VIII and IX is a variable region that functions as a target recognition domain (TRD) that identifies both the target sequence and the base to be methylated (Klimasauskas et al., 1991). Motif VIII possibly assists in negating the negative charge of the DNA backbone through nonspecific association with cytosine residues while motif IX is involved in the organization of the TRD (Kumar et al., 1994; Lee et al., 2002).

The SAM binding site is notably conserved in all DNA methyltransferases and has been implicated with motifs I and motif X (Figure 1.5) (Kumar et al., 1994; Kozbial and Mushegian, 2005). Previous mutagenesis experiments have confirmed the roles of these conserved amino acid residues, as exemplified by mutagenesis studies done by conversion of the conserved glycine residue of motif I to an alanine, which resulted in a lost capacity for SAM binding for the enzyme guanidinoacetate methyltransferase (Hamahata et al., 1996). This observation therefore supported the association of motif I with SAM binding. This enzyme functions in the metabolic conversion of SAM to SAH in mammalian liver by catalysing the SAM-dependent methylation of guanidinoacetate to form creatine (Takata et al., 1994).

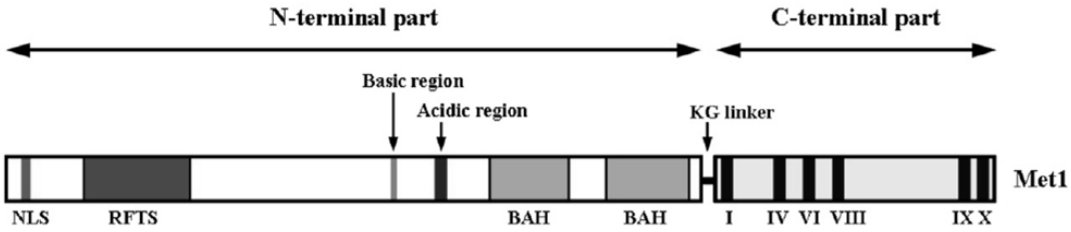


Figure 1. 4 Domain architecture of the MET1 protein. The N-terminal domain of MET1 contains nuclear localization signal (NLS), replication foci targeting sequence (RFTS), basic and acidic regions and two bromo-adjacent homology (BAH) domains. The C-terminal domain of MET1 comprises the catalytic domains as indicated by roman numerals. The N-and C-terminal domains are joined via a stretch of alternating glycine-lycine (GK) residues known as KG linker (Pavlopoulou and Kossida, 2007).

The function of MET1 in plants is reflected in studies using partial and complete loss-of-function *met1* mutations as well as *MET1* antisense transgenic plants, which resulted in aberrant plant phenotypes and global DNA hypomethylation. A decrease in DNA methylation of approximately 90 %, particularly in CG contexts, was observed in the Arabidopsis *met1* antisense and resulted in changes to plant floral organs (Finnegan et al., 1996; Kankel et al., 2003; Saze et al., 2003). In the *met1-1* mutant with a point mutation that causes single amino acid change at the catalytic site (a P to S mutation at amino acid 1300), the methylation level at TCGA sites decreased by 70 %. A smaller reduction in methylation level (50%) was observed in the *met1-2* mutant, which has a point mutation in the SAM binding site (a G to S mutation at amino acid 1101) (Kankel et al., 2003). This shows that the efficiency of MET1 can be altered by point mutations in a target-specific way, reflecting a direct role for MET1 in target selection, or interaction with target-specific factors. The *met1-2* mutant suggests that SAM binding activity in MET1 that can influence DNA methylation through SAM as the methyl donor, which gives further insight into another aspect of modification of DNA methylation through quantitative changes in the SAM level. Additionally, a null allele, *met1-3*, causes almost complete loss of CG methylation (Saze et al., 2003; Mathieu et al., 2007a). The *met1-6* null allele has a loss of catalytic activity caused by a premature translation stop codon and has a late flowering phenotype, which correlates with a reduction in DNA methylation in the *MEDEA* gene promoter (Xiao et al., 2006).

MET1 is responsible for maintaining CG methylation after DNA replication (Law and Jacobsen, 2010). However, there is evidence that MET1 is also essential for *de novo* methylation of CG sites (Aufsatz et al., 2004). At selected loci, the *de novo* methylation of CG of a transgene system could not be established in the *met1* mutant. This suggests that MET1 is necessary for *de novo* CG methylation. In addition, the *de novo* methylation activity of MET1 was observed during partial restoration of gene body CG methylation when MET1 was reintroduced in the *met1* mutant (Zubko et al., 2012). MET1 exhibits a non-CG methylation function based on a decrease in DNA methylation at the CHG and CHH contexts in the *met1* mutant (Cokus et al., 2008). It is hypothesized that MET1 is involved in a multiprotein complex together with CMT2 and/ CMT3 and is required for CG, CHG and CHH methylation.

1.6.2 MET1 interactions with other proteins

MET1 requires the VARIANT IN METHYLATION (VIM) protein to be recruited to the hemimethylated CG sites produced after DNA replication (Shook and Richards, 2014). VIM contains a methylcytosine-binding SET- and RING-associates (SRA) domain that can identify methylated cytosine in any sequence context, specifically including hemi-methylated CG sites, a feature lacking in MET1 (Yao et al., 2012). There is also a connection between all of these pathways during DNA methylation and chromatin remodelling. An SWI/SNF chromatin-remodeling factor, DECREASE IN DNA METHYLATION (DDM1), assists MET1 in accessing heterochromatin to silence TEs and repeats (Kakutani et al., 1996; Zemach et al., 2013).

DNA methylation is associated with transcriptional silencing at heterochromatin sites. Histone deacetylation is also linked to repressive chromatin marks, and a histone deacetylase, HISTONE DEACETYLASE 6 (HDA6), has been found to interact with MET1 (To et al., 2011). Similar to the *met1* mutant, the *hda6* mutant also shows a reduction in CG methylation (Stroud et al., 2013; Blevins et al., 2014). Additionally, for certain loci, reductions in H3K9me2 methylation and non-

CG methylation, including CHG and CHH, were also observed due to mutations of the *HDA6* and the *MET1* genes. This suggests an interplay between all of these pathways during interconversion between permissive and repressive chromatin structures (Deleris et al., 2012).

The co-operation between MET1, DDM1 and HDA6 is exemplified by silencing of members of the *Sadhu* family of non-autonomous non-LTR retrotransposons. *Sadhu 3-1* is located in a repeat-rich pericentromeric region, and joint activities of MET1, DDM1 and HDA6 are required to maintain high DNA methylation and H3K9me2 levels (Rangwala and Richards, 2007). For selected target loci, MET1 may have joint activity with other methyltransferases, including CMT3 and DRM2. In the *RPS* transgene, co-operative activity of MET1, CMT3 and DRM2 is required for establishment of the DNA methylation pattern (Singh et al., 2008). RPS is a repetitive hypermethylated DNA fragment from *Petunia hybrida* that attracts DNA methylation when transferred into *Petunia* or other species (Müller et al., 2002).

1.7 Research aims

DNA METHYLTRANSFERASE 1 (MET1) has a primary role in maintaining cytosine methylation after DNA replication in which it is recruited to hemi-methylated cytosines, specifically in the symmetrical CG context to add a methyl group to the newly synthesized strand (Law and Jacobsen, 2010). Various approaches have been used to study MET1 including *Arabidopsis* mutants generated by *MET1* knockdowns and knockouts (Finnegan et al., 1996; Kankel et al., 2003). Recently, a study was carried out by Brocklehurst et al. (2018) to assess the effect of increasing the MET1 level. It is predicted that over-expression of MET1 will cause an increase in CG methylation, based on MET1 function. Additionally, an increase in DNA methylation may cause instability of the DNA methylation-demethylation equilibrium, thereby altering DNA methylation

patterns across the genome (Li et al., 2012). Over-expression of MET1 could cause upregulation of demethylases and result in phenotypic changes.

Apart from the enzymatic activity of MET1, there could be effects of MET1 over-expression that are independent of enzymatic activity (after loss of catalytic function), which therefore may reflect effects based on physical interaction of MET1 with other proteins. It is hypothesized that MET1 has a coordinating role in forming a methylation complex that associates with DNA methyltransferases, chromatin remodeling factors and/or histone modifiers. The stability of the methylation complex may be influenced by the increase in MET1 concentration. It is hypothesized that besides the catalytic function of MET1, the SAM-binding domain of MET1 is also required, in which increasing the MET1 with a SAM-binding domain could lead to a competition for SAM for the methyl group required during the methylation process. The aim of this research project was to investigate how overexpression of different forms of MET1, with and without a catalytic site and/or SAM binding domain, can be employed to alter epigenetic states at target genes caused by DNA methylation modifications. Phenotypes arising from mutants were observed to identify possible targets that had altered DNA methylation status or expression. Hormone analysis and transcriptome profiling were performed to find any correlation between the global changes in gene expression and the phenotype observed.

Chapter 2

Phenotypic analysis of Arabidopsis MET1 overexpression lines

2.1 Introduction

DNA methylation is a covalent modification, found in the genomes of both plants and animals, that has major roles in the regulation of gene expression and silencing of transposable elements (TEs) and repetitive sequences (He et al., 2011; Zhang et al., 2018). In plants, patterns of DNA methylation are stably transmitted over several sexual generations and influence heritable phenotypes (Schmitz et al., 2011; Becker et al., 2011). Some epigenetic alterations may persevere even after the conditions that generated them have been reversed, and some may be passed down to next generations as epigenetic alleles (epialleles). These heritable epialleles are being evaluated as a potential source of increased variation in breeding programs (Zhang et al., 2013). Several traits such as root morphology, resistance to pathogens and nutrient absorption are associated with phenotypic variation that can arise through epigenetic variation (Zhang et al., 2013; Cortijo et al., 2014). Furthermore, plant agronomic traits including vernalisation (Sung and Amasino, 2005), seed development (Kawakatsu et al., 2017), plant height (Johannes et al., 2009) were also documented. Changes in DNA methylation may also play a role in adaptive response to environmental stimuli, which therefore serve as a mechanism to adapt to biotic and abiotic stress (Dowen et al., 2012; López Sánchez et al., 2016; Hewezi et al., 2018).

Comprehensive maps of DNA methylation have contributed to our comprehension of potential tissue-specific epigenetic changes and activities in plants (Cokus et al., 2008; Lister et al., 2008). Comparison in DNA methylation, nucleosome distributions and transcriptional levels between shoots and roots

revealed organ-specific changes in gene activity in response to distinct epigenetic profiles (Widman et al., 2014). Meristems, which are centres for histogenesis and organogenesis in plants, are also important locations for epigenetic regulation of developmental plasticity (Baubec et al., 2014).

In *Arabidopsis*, the symmetric CG methylation is faithfully maintained by DNA METHYLTRANSFERASE1 (MET1), whereas methylation of CHG and CHH sites (H representing C, T or A) is established and maintained by plant specific CHROMOMETHYLASE3 (CMT3) and CHROMOMETHYLASE2 (CMT2) respectively (Stroud et al., 2014). All sequence contexts (CG, CHG and CHH) are methylated *de novo* by DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2), which is guided to DNA by RNA-directed DNA methylation (RdDM) pathway (Matzke and Mosher, 2014). In *Arabidopsis*, DNA methylation is maintained by MET1, that recognizes hemi-methylated CG following DNA replication and methylates the cytosine in the daughter strand (Kankel et al., 2003; Law and Jacobsen, 2010). The *MET1* gene has been extensively studied in the model plant *Arabidopsis thaliana* using the *met1* mutants which result in plant developmental abnormalities and an accompanying genome-wide reduction in DNA methylation (Finnegan et al., 1996; Kankel et al., 2003).

Recently, over-expression studies of the *MET1* gene, that induced epigenetic variation in *Arabidopsis*, have been reported by Brocklehurst et al. (2018). Two-types of *MET1* gene were over-expressed, the endogenous *MET1* gene and a mutated *MET1* gene (inactivated methyltransferase activity), resulting in phenotypic variation. The *MET1* lines displayed specific shoot and root phenotypes. Reduction in primary root length was observed in all lines, whereas delay in bolting and an increase in secondary roots was observed in a subset of lines (Brocklehurst et al., 2018). Previous studies by Virdi et al. (2015) showed that treatment with the DNA methylation inhibitor 5-azacytidine resulted in shorter primary roots in *Arabidopsis*, which implicates cytosine hypomethylation as the cause of the observed phenotypes. Therefore, the aim of this chapter is to investigate the effects of *MET1* overexpression in inducing phenotypic changes, particularly the primary root length and seed germination. The hormone analyses were carried out to identify factors that may contribute to the phenotypes.

2.1.1 Description of the *MET1* over-expression lines

The *Arabidopsis* homozygous lines of *METo A1+, A1-, I1+* and *I1-* were generated by Brocklehurst et al. (2018) as summarized in the Table 2.1. The *METo A1+* contains 35S:MET1 whereas the METo *I1-* contains 35:MET1mut. The *METo A1-* and *I1-* are lines that had lost the transgene (35S:MET1 and 35S:MET1mut respectively). The 35S:MET1 and 35S:MET1mut constructs are using plant transformation vector pGreen II 0179 35S-*NOS.* Full information on the constructs and generation of the lines are described as in Brocklehurst et al. (2018). MET1 transcript levels were increased approximately 3-fold in *A1+* and 15-fold in *I1+* in 4-week old seedlings. Meanwhile, *MET1* transcript levels were restored to wildtype in lines that had lost the transgene. The insertion site and copy number were unknown (Brocklehurst et al., 2018).

The *Arabidopsis METo* lines were summarized in the following Table 2.1.

Lines	Description
METo A1+	Contain *MET1* cDNA construct under 35 promoter (35S:MET1)
METo I1+	Contain *MET1* cDNA construct under 35 promoter with no catalytic function (35S:MET1mut). The catalytic function was removed by exchanging the cysteine residue in the active site loop region in MET1 by serine residue according to Hsieh, (1999).
METo A1-	Line that had lost the *MET1* transgene (35S:MET1) through genetic segregation
METo I1-	Line that had lost the *MET1* transgene with no catalytic function (35S:MET1mut) through genetic segregation

Table 2.1. Description of *Arabidopsis MET1* over-expression (METo) lines.

2.2 Results

2.2.1 Phenotypic analysis of *Arabidopsis METo* lines: primary root length

Analysis of root assay carried out with 7-day-old seedlings revealed that the fifth generation of *Arabidopsis METo* lines showed significant reduction in the primary root length as compared with *Col-0* (Figure 2.1). To investigate further if there are any differences in the root meristem between the *METo* lines and *Col-0*, the root tips of 7-day-old seedlings were observed using laser scanning confocal microscopy. For propidium iodide (PI) staining, the root tips were treated with 10 µg/ml PI and then lmounted on glass slides. PI fluorescence was observed using an LSM700 laser scanning confocal microscope (Zeiss) with 559 nm excitation and 575–675 nm emission. The number of cells in the root meristem was assessed. The meristematic zone is defined as the region of cells from the quiescent centre (QC) to the cell that is twice the length of the immediately preceding cell. From the results obtained, all of the *METo* lines showed a significant reduction in meristem cell number as compared with *Col-0* (**Figure 2.1**).

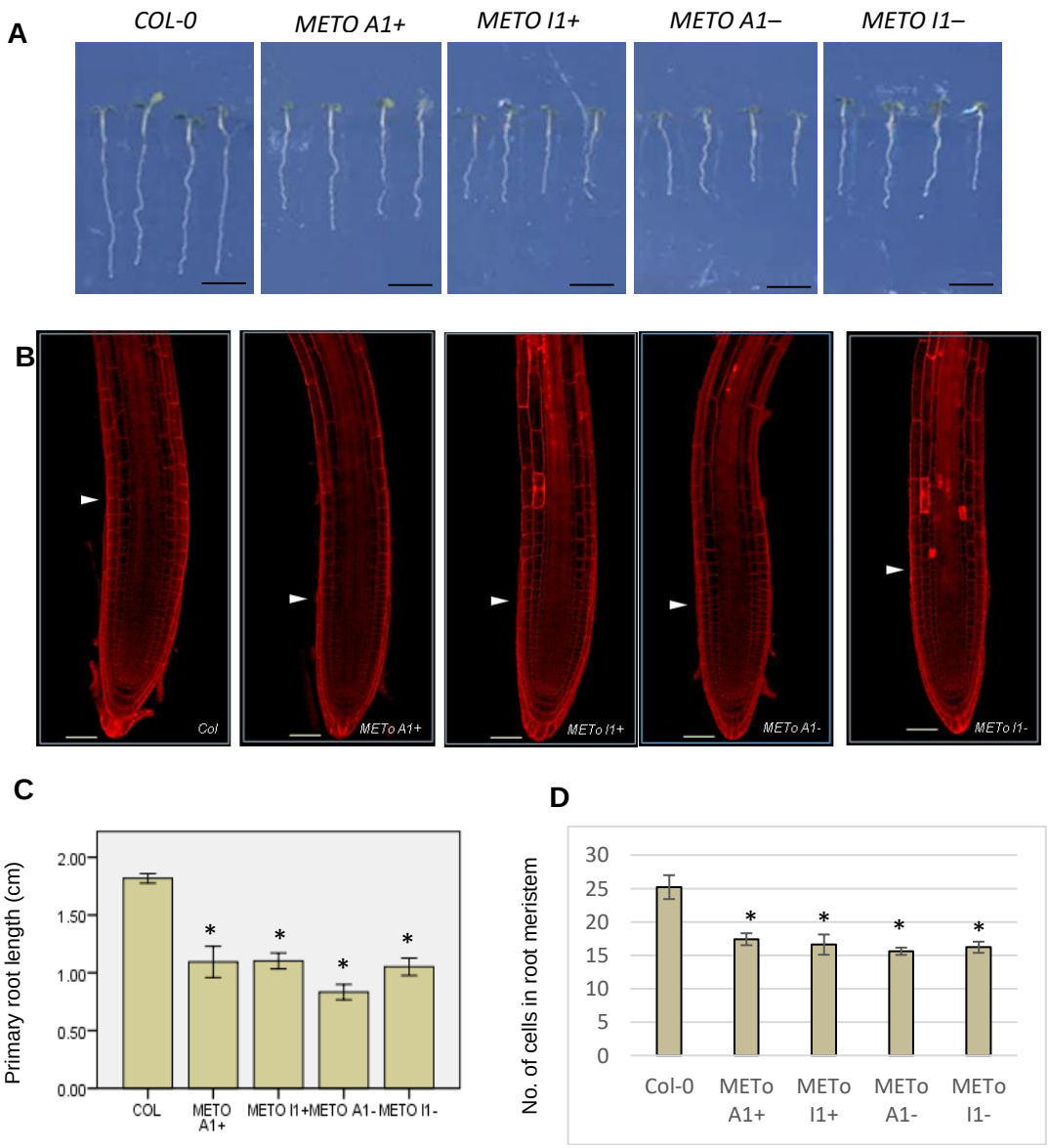


Figure 2.1 (A) Phenotype of 7-day wild type (*Col-0*) and *METo* lines (*A1+*, *I1+*, *A1–* and *I1–*). Scale bars: 5 mm. (B) Confocal images of 7-day-old *METo* lines (*A1+*, *I1+*, *A1–* and *I1–*) roots stained with propidium iodide showing a reduction in meristem size with respect to *Col-0*. Arrows indicate the boundary between the meristem and the elongation zone of the roots. Scale bars: 50 µm. (C) Primary root length of the *METo* lines as compared with the *Col-0* at 7 days after germination (DAG). A star (*) indicates statistically significant differences in primary root length between the *METo* lines and *Col-0* ($p < 0.05$, One-way ANOVA). Values are means ± SE, $n = 20$ per genotype. (D) Number of cells in root meristem in the *METo* lines as compared with the *Col-0* at 7 DAG. A star (*) indicates statistically significant differences in number of cells in root meristem between the *METo* lines and *Col-0* ($p < 0.05$, One-way ANOVA). Values are means ± SE, $n = 10$ per genotype.

2.2.1.1 Analysis of endogenous gibberellins

Hormones have been identified as a major factor in the regulation and maintenance of the meristematic activity in primary roots (Takatsuka and Umeda, 2014). The requirement of gibberellin (GA) for root growth has been exemplified in GA-deficient mutants with short root phenotype. The regulation of GA in meristem size and root growth elucidates the role of GA signalling in root growth and development (Ubeda-Tomás et al., 2009). There are many GA derivatives in plants but very few GAs are biologically active, including GA_3 which is highly present in growing tissues ((Yamaguchi, 2008). To determine if the reduced primary root length in the *METo* lines is associated with endogenous GA level, liquid chromatography (LC-MS) was performed. As shown in previous section, all *METo* lines displayed reduced primary root lengths. Therefore, as representative, *METo A1+* and *A1-* were chosen for GA_3 level quantification. To measure the endogenous GA_3, the primary roots of 10-day-old seedlings grown in MS media under control conditions were harvested and analysed using LC-MS. The roots' fresh weights were measured, snap-frozen in liquid nitrogen and then freeze-dried. The LC-MS analysis was performed by Dr. Jiri Malbeck (Laboratory of Hormonal Regulations in Plants, Institute of Experimental Botany, Prague, Czech Republic). From the results obtained, there was a significant decrease in the endogenous GA_3 level in the *METo A1+* and *A1–* lines as compared with *Col-0* (**Figure 2.2**).

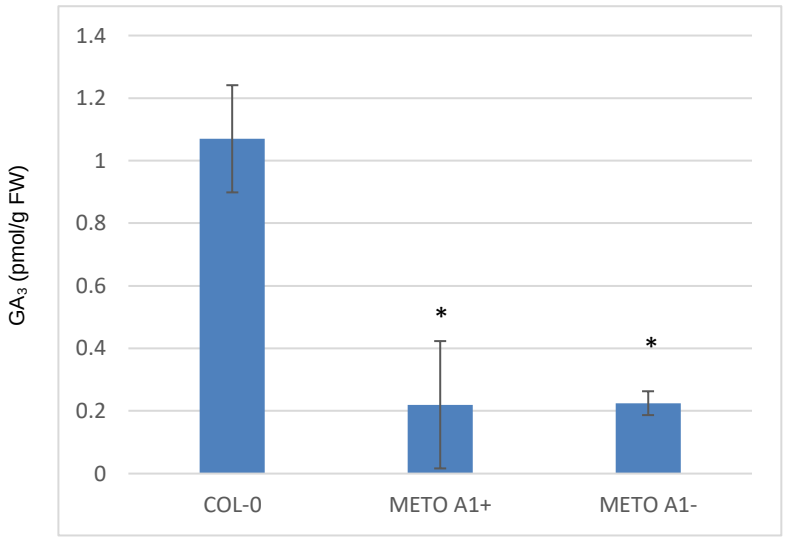


Figure 2.2. The concentration of GA_3 as determined by LC-MS in primary roots of *Col-0*, *METo A1+* and *A1–* lines. Statistical analysis using ANOVA with log transformation showed that the GA_3 in the *METo A1+* and *A1–* lines were significantly decreased compared with wild type *Col-0*. Significant differences are marked with an asterisk ($p < 0.05$) and error bars represent SE of three biological replicates. FW, fresh weight.

2.2.1.2 Treatments with exogenous GA hormone

To investigate whether the reduced primary roots observed in the *METo* lines could be due to defects in GA biosynthesis, the seedlings were treated with GA or the GA inhibitor, paclobutrazol. After 7 days of growing on normal MS media, the seedlings were transferred to new MS media with different treatments; 1 µM GA, 10 µM GA and 5 µM paclobutrazol (PAC). The seedlings were grown vertically and root elongation was measured after 7 days of treatment. Under the control condition, all *METo* lines showed significantly shorter primary root as compared with *Col-0*. When treated with 1 µM GA, the *METo I1+, A1-* and *I1-* lines showed significantly shorter primary roots as compared with *Col-0*, but not for *METo A1+* line. When added with higher GA level (10 µM GA), no significant difference was observed in the root length between *METo* lines and *Col*-0 which suggested that addition of 10 µM GA inhibited *Col-0* root growth. Treating the WT root with 5 µM PAC resulting in reduced primary root length, mimicking the phenotype in the *METo* lines (Figure 2.3).

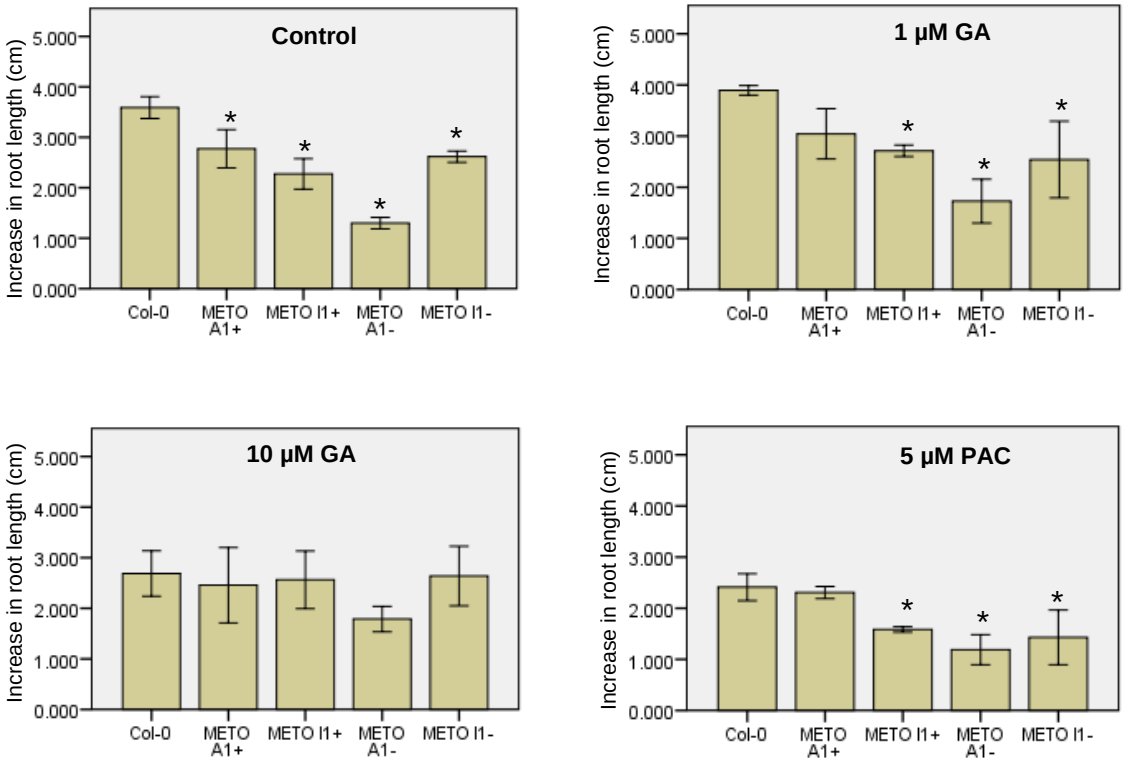


Figure 2.3. One-week-old seedlings germinated on MS plates were transferred to new MS media; (1 µM GA, 10 µM GA and 5 µM PAC) and allowed to grow vertically for 7 days before determination of primary root elongation. The star (*) indicates statistically significant differences in primary root length between treatments and control condition ($p < 0.05$, One-way ANOVA). Values are means ± SE, $n = 20$ per genotype.

2.2.1.3 Analysis of endogenous cytokinin metabolites

Plant hormones play important roles during the growth and development of plants. Cytokinin (CK) has not only been shown to be involved in the elongation and differentiation zone of the root, but also plays a role in modulating cell division in root apical meristem (Ioio et al., 2007; Ruzicka et al., 2009). All four *METo* lines (*A1+, I1+, A1*– and *I1*–) showed similar phenotypes, that is reduced primary root growth, therefore, the *METo A1+* line was chosen as representative to measure the concentration of CK metabolites in comparison to *Col-0*.

To examine the CK endogenous level, 26 CKs were monitored using liquid chromatography-mass spectrometry (LC-MS). From the 26 endogenous CK investigated (Table 2.1), 5 CK metabolites were found with significant changes. LC-MS analysis of the concentration of CK metabolites revealed that three highly active trans-zeatin (*t*Z-type) CKs, namely, *trans*-zeatin-7-glucoside, *trans*-zeatin-9-riboside and *trans*-zeatin-9-riboside(ph) and one dihydrozeatin (DHZ-type CK), dihydrozeatin-7-glucoside (active CK) were significantly reduced in the *METo A1+* line as compared with *Col-0*. Meanwhile, *cis*-zeatin-9-riboside-O-glucoside, which is a cis-zeatin (*c*Z-type) CK (inactive CK), showed a significant increase in the *METo A1+* line as compared with *Col-0* (**Figure 2.4**).

Individual cytokinin metabolites	Abbreviation
isopentenyladenine (iP)	
isopentenyladenine-7-glucoside	IP7G
isopentenyladenine-9-glucoside	IP9G
isopentenyladenine	IP
isopentenyladenine-9-riboside	IP9R
isopentenyladenine-9-riboside-ph	IP9R-ph
Cis-zeatin type (cZ)	
cis-zeatin-9-glucoside	c-Z9G
cis-zeatin-O-glucoside	c-ZOG
cis-zeatin	c-Z
***cis*-zeatin-9-riboside-O-glucoside**	**c-Z9ROG**
cis-zeatin-9-riboside	c-Z9R
cis-zeatin-7-glucoside	c-Z7G
Transzeatin type (tZ)	
***trans*-zeatin-7-glucoside**	**Z7G**
trans-zeatin-9-glucoside	Z9G
trans-zeatin-O-glucoside	ZOG
trans-zeatin	Z
trans-zeatin-9-riboside-O-glucoside	Z9ROG
***trans*-zeatin-9-riboside**	**Z9R**
***trans*-zeatin-9-riboside-ph**	**Z9R-ph**
Dihydrozeatin type (DZ)	
dihydrozeatin-7-glucoside (1)	**DHZ7G_1**
dihydrozeatin-7-glucoside (2)	DHZ7G_2
dihydrozeatin-9-glucoside	DHZ9G
dihydrozeatin-O-glucoside	DHZOG
dihydrozeatin-9-riboside-O-glucoside	DHZ9ROG
dihydrozeatin-9-riboside-ph	DHZ9R-ph
dihydrozeatin-9-riboside-ph	c-Z9R-ph
meta-topolin	m-OH-BAP

Table 2.2. List of cytokinin metabolites analysed by liquid chromatography-mass spectrometry (LC-MS) analysis. (Bold indicate significant change in the metabolite level).

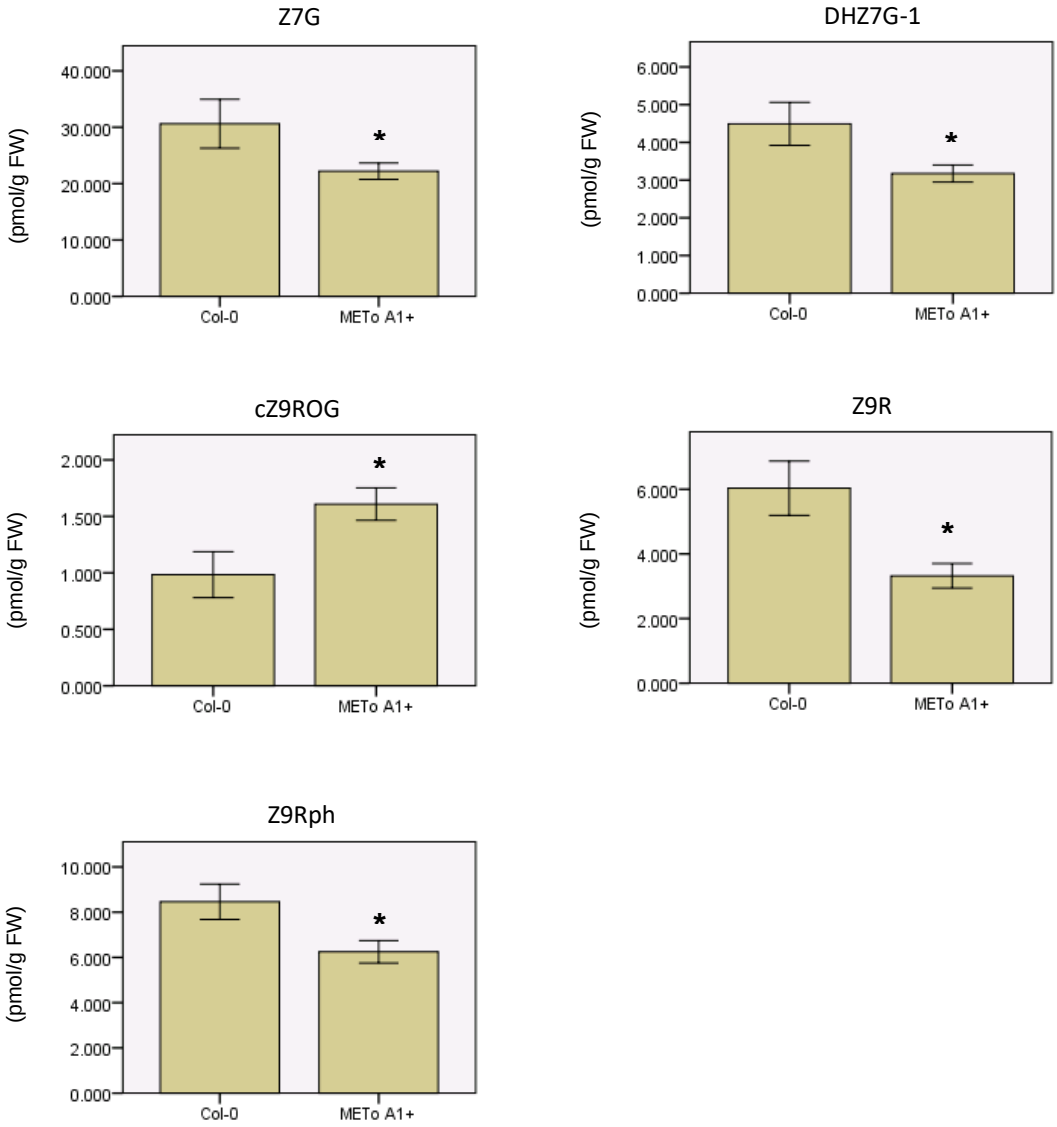


Figure 2.4. The concentration of cytokinin derivatives (pmol/g) as determined by LC-MS in the roots of 4-week-old *Arabidopsis Col-0 and METo A1+* lines. Z7G, *trans*-zeatin-7-glucoside; DHZ7G-1, dihydrozeatin-7-glucoside (1,2); cZ9ROG, *cis*-zeatin-9-riboside-O-glucoside; Z9R, *trans*-zeatin-9-riboside; Z9Rph, *trans*-zeatin-9-riboside(ph). Fifty milligrams of *Arabidopsis* roots per genotype were pooled, and three biological samples were taken for each genotype. Significant differences are marked with an asterisk ($p < 0.05$) and error bars represent SE of three biological replicates. FW, fresh weight.

2.2.2 Phenotypic analysis of *Arabidopsis METo* lines: germination response under control condition and treatment with hormones and inhibitor

When growing the plants to investigate the short root phenotype, I noticed that the *METo A1-* line germinates later as compared to other lines (lack of uniformity during germination). There has been increasing evidence that various mutants with abnormalities during seed maturation, dormancy and germination are associated with genes involved in DNA methylation and chromatin structure which suggested that epigenetic regulation is important during these stages of development (Kawakatsu et al., 2017; Narsai et al., 2017). Therefore, seed germination assays were carried out for further investigation. Seed germination is an important stage of plant growth and development, which is followed by the post germinative growth of the seedling (Penfield et al., 2007). For this experiment, the criterion used to score that a seed germination has been successful is the developmental stage when elongating radicle protrudes from micropilar endosperm tissue (Bewley, 1997).

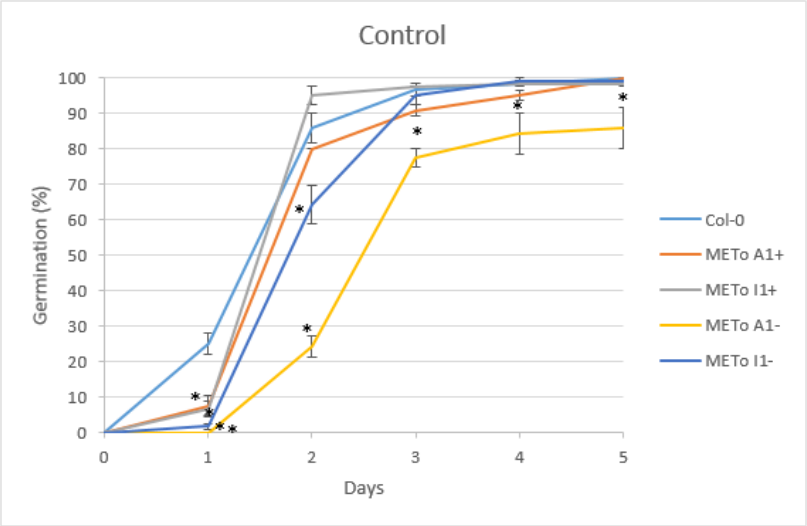
The seed germination assay revealed that all of the *METo* lines showed normal germination under control conditions except for the *METo A1–* line that exhibited delayed germination as compared with *Col-0* (**Figure 2.5A**). GA and ABA are hormones involved in the germination process, predominantly from the aspect of regulating a balanced ratio of GA and ABA in the seeds (Vittorioso et al., 2018). GA plays role during initiation of radicle protrusion by weakening the tissue that encapsulates the embryo including the aleurone and testa. This is shown when the surrounding tissue of the embryos of nongerminating GA-deficient mutants are removed mechanically, allowing the mutants to grow into dwarf plants (Silverstone et al., 1997). Additionally, GA elevates the growth potential of the embryo, as shown by decreased growth rate of embryos with reduced GA level (Groot and Karssen, 1987). ABA maintains seed dormancy and inhibits seed germination (Nambara et al., 2010; Yan and Chen, 2017).

Therefore, to identify whether there is any defect in the hormone balance between the GA and ABA that contributes to the seed phenotype, the *Col-0* and *METo* lines seeds were sown and grown in MS media supplemented with ABA, GA and GA biosynthesis inhibitor, paclobutrazol (PAC).

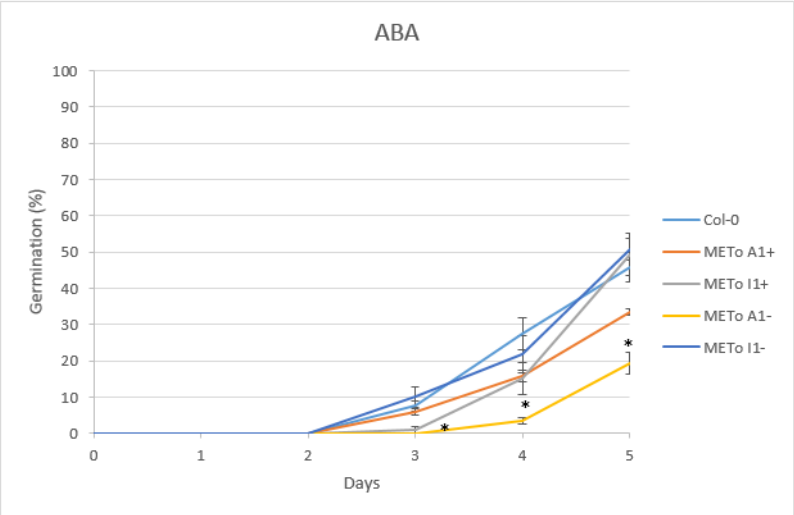
ABA is an effective germination inhibitor (Kermode, 2005). The sensitivity to ABA of the METo lines were evaluated by applying ABA to the germination medium. Treatment with 5 µM ABA inhibited germination in all the *METo* lines, with the *METo A1*– line showing the highest sensitivity to ABA (Figure 2.5B). In contrast, treatment with 5 µM GA improved germination in the *METo A1*–, which showed normal germination levels by day 4 post stratification (Figure 2.5C). When the GA treatment was increased to 10 µM, the *METo A1*– line showed similar germination as *Col-0* during the five consecutive days post stratification. Interestingly, germination in the *METo A1*+ line, however, was inhibited by high GA level (Figure 2.5D).

To test for the phenotypic changes linked with decreased GA level, the ability of seeds to germinate in the presence of paclobutrazol was assessed. Paclobutrazol is an inhibitor of GA biosynthesis which causes germination inhibition (Zhu et al., 2004). Treatment with paclobutrazol causes inhibition in germination in all lines, with the *METo A1*– showing the highest sensitivity to this GA inhibitor (Figure 2.5E).

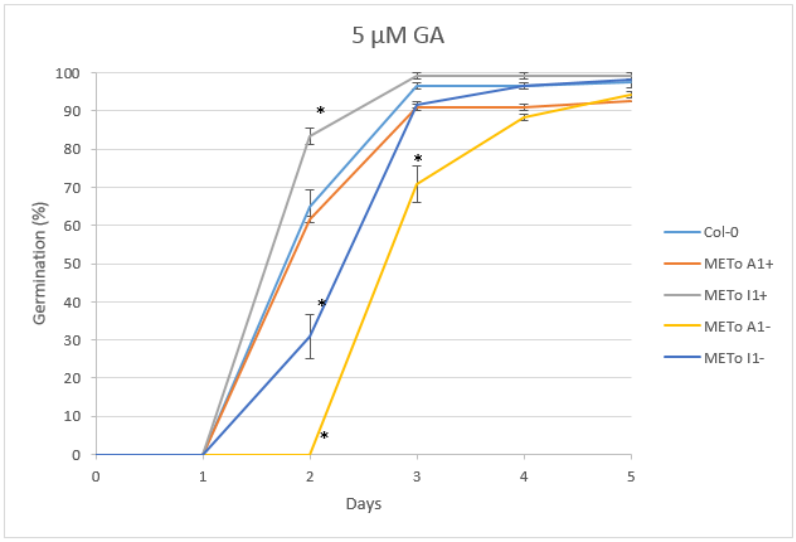
A


Control
Germination (%)
Days
Col-0
METo A1+
METo I1+
METo A1-
METo I1-

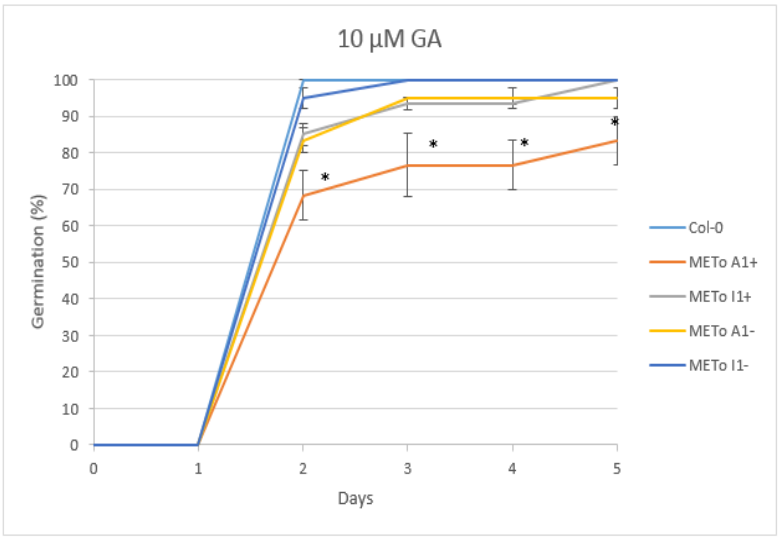
B


ABA
Germination (%)
Days
Col-0
METo A1+
METo I1+
METo A1-
METo I1-

C


5 µM GA
Germination (%)
Days
Col-0
METo A1+
METo I1+
METo A1-
METo I1-

D


10 µM GA
Germination (%)
Days
Col-0
METo A1+
METo I1+
METo A1-
METo I1-

E

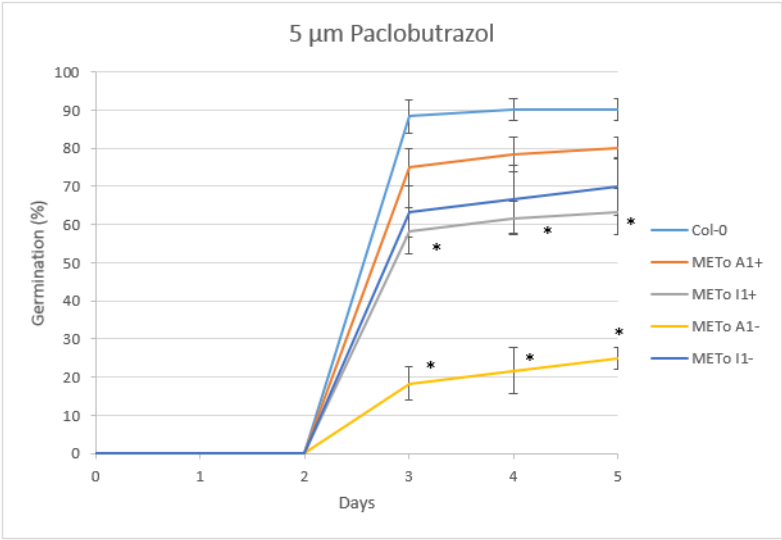
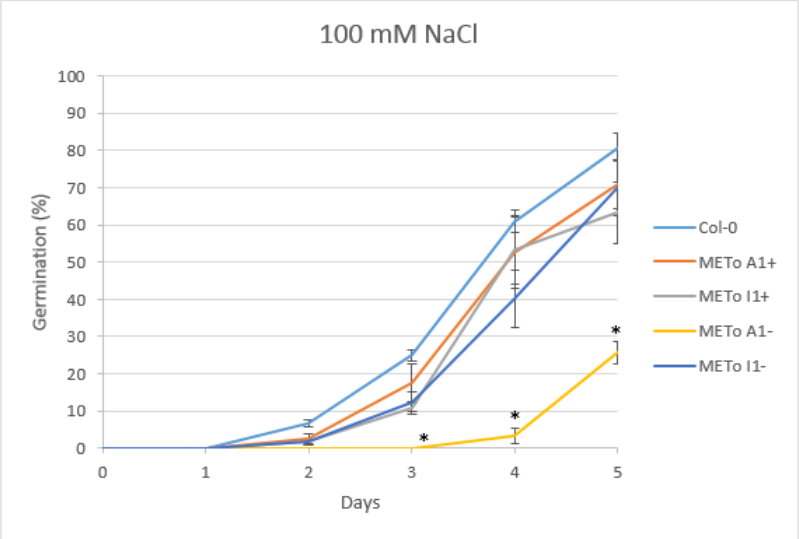


Figure 2.5. Germination (%) of *Arabidopsis METo* lines under (A) control conditions, (B) 5 µM ABA, (C) 5 µM GA, (D) 10 µM GA and (E) 5 µM paclobutrazol. Seed germination was evaluated for 5 days after stratification based on radicle emergence from the seed coat. The star (*) indicates statistically significant differences in germination (%) between the *METo* lines as compared with *Col-0*. One-way ANOVA was used to analyse the differences through Tukey's test ($p < 0.05$). Values represent the means ± SE ($n = 40$) of three replicates.

2.2.2.1 Germination response of *Arabidopsis METo* lines under salinity stress

Regulation of hormones in seeds is also essential during abiotic stress such as salinity stress (Vittorioso et al., 2018). High-salinity is one of the main abiotic stresses that poses a great challenge to plant survival, especially at the early stage of development (Wang et al., 2003). Because ABA is associated with abiotic stress, the germination assay was further carried out under salinity stress. When treated with 100 mM NaCl, the *METo A1–* line showed hypersensitivity in which the germination was greatly inhibited (**Figure 2.6A**). However, the addition of 10 µM GA improved germination so that the *METo A1–* line showed similar germination as *Col-0* starting day 4 post stratification (**Figure 2.6B**).

A



B

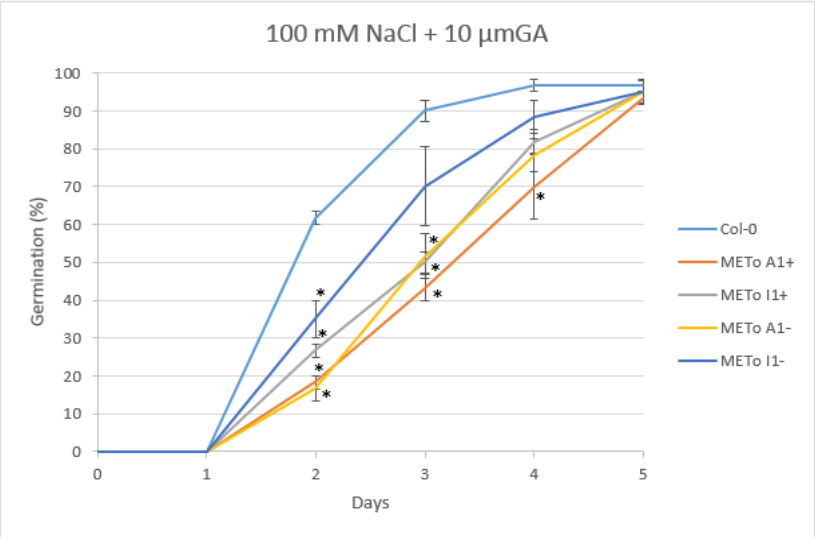
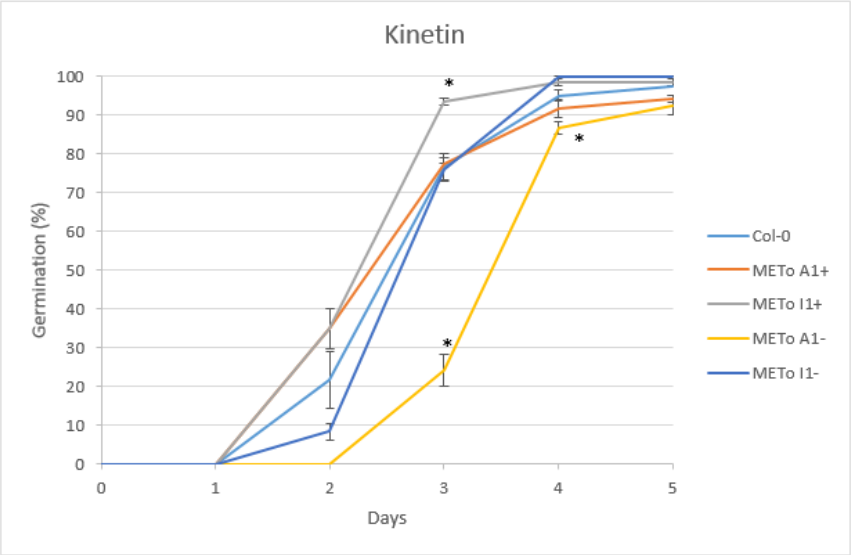


Figure 2.6. Germination (%) of *Arabidopsis METo* lines under (A) 100 mM NaCl, (B) 100 mM NaCl and 10 µM GA. Seed germination was evaluated for 5 days after stratification based on radicle emergence from seed coat. The star (*) indicates statistically significant differences in germination (%) between the *METo* lines as compared with *Col-0*. One-way ANOVA was used to analyse the differences through Tukey's test ($p < 0.05$). Values represent the means ± SE ($n = 40$) of three replicates.

2.2.2.2 Germination response of *METo* lines to auxin and cytokinin

Apart from the pivotal roles of GA and ABA during seed germination, growing evidence has also shown that hormones such as auxin and cytokinin possess an interconnected hormonal regulation during seed germination (Liu et al., 2007; Day et al., 2008). Thus, further investigations were performed to determine whether the germination phenotype in the *METo* lines was also influenced by these hormones. From the germination assay, treatment with 5 µM kinetin improved germination in the *METo A1–* line at day 5 post stratification, although that was at a slightly later stage as compared with treatment with GA (**Figure 2.7A**). Similar results were observed when treated with 5 µM IAA in which the *METo A1–* line showed similar germination as *Col-0* at day 5 post stratification. Interestingly, the *METo A1+* line showed inhibited germination when treated with 5 µM IAA as in the previous response when treated with 10 µM GA (**Figure 2.7B**).

A



B

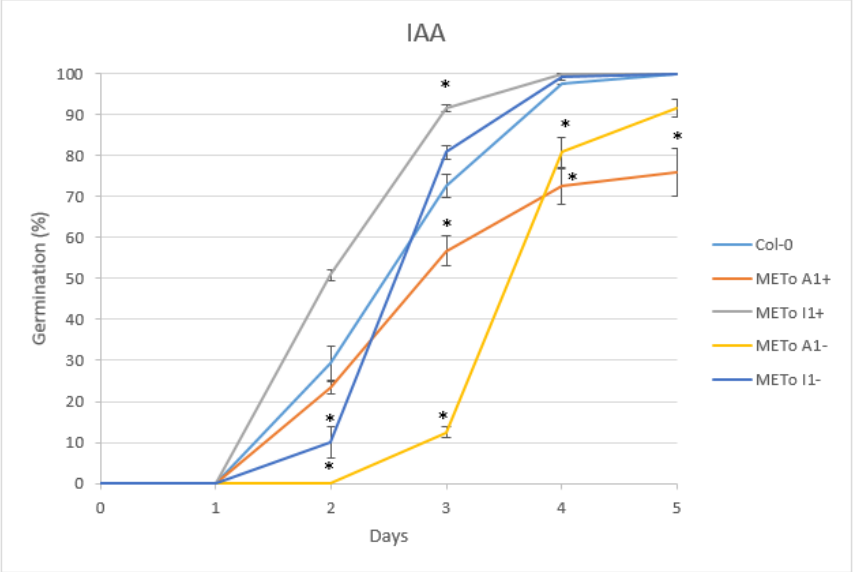


Figure 2.7. Germination (%) of *Arabidopsis METo* lines under (A) 5 µM kinetin, (B) 5 µM IAA. Seed germination was evaluated for 5 days after stratification based on radicle emergence from the seed coat. The star (*) indicates statistically significant differences in germination (%) between the *METo* lines as compared with *Col-0*. One-way ANOVA was used to analyse the differences through Tukey's test ($p < 0.05$). The values represent the means ± SE ($n = 40$) of three replicates.

2.3 Discussion

Modification of DNA methylation can produce phenotypic changes at the molecular, cellular, tissue and organism levels. DNA methylation variation is mostly caused by the inability to sustain methylation states. For instance, *met1* mutants with the loss of CG methylation resulted in developmental defects including reduced fertility, short primary roots, altered flowering time and narrow leaves (Kankel et al., 2003). The aim for *MET1* over-expression is to induce epigenetic variation as a source for trait variation, which will lead to identification of novel epialleles. This requires the identification of stable phenotypes prior to identification of novel epi-alleles. The root is one of the important tissues that define plant productivity, especially under unfavourable environmental conditions such as abiotic stress (Comas et al., 2013). Upon germination, the primary root is the first to emerge, which reflects the critical turning point of post-embryonic development (Dolan et al., 1993).

Over-expression of *MET1* resulted in phenotypic variations in which all the *METo* lines showed a reduction in the primary root length. This phenotype was observed in all lines either containing the *MET1* transgene with or without catalytic activity (*METo A1*+ and *I1*+ line, respectively) and also in the lines that had lost both types of *MET1* transgene (*METo A1*– and *I1*– lines). Further analysis of root meristem size was carried out, which revealed that all the *METo* lines exhibit a reduction in meristem size as measured by the number of cells in the root meristem.

The *Arabidopsis* roots, as shown in Figure 2.8, is made up of four distinct zones including the stem cell niche (SCN), the meristematic zone (MZ), the transition zone (TZ), and the elongation/differentiation zone (EDZ) (Ubeda-Tomás et al., 2012). The SCN is comprised of a quiescent centre (QC) and surrounding stem cells. Each cell forms a lineage which initiates from the QC, that produces daughter stem cells initial during embryogenesis. The QC is comprised of four cells that divide infrequently and regulate the undifferentiated state of the neighbouring initials. The repeated division of the initial daughter cells for the epidermis, cortex, endodermis and stele take place in the MZ, prior to differentiation in the transition zone and expansion in the elongation zone (Figure 2.8) (Petricka et al., 2012). In the TZ, the transition from cell division to cell elongation occurs with mitosis switching to endoreplication, causing polyploidization. In the EDZ, rapid cell elongation continues, but not in the transverse direction (Takatsuka and Umeda, 2014). These processes of division, differentiation and expansion are regulated by the interplay of several hormones during the root growth and development (Benková and Hejátko, 2009). From the outside to inside at the root tip, comprise of lateral root cap, epidermis, cortex, endodermis, pericycle and stele (Figure 2.9).

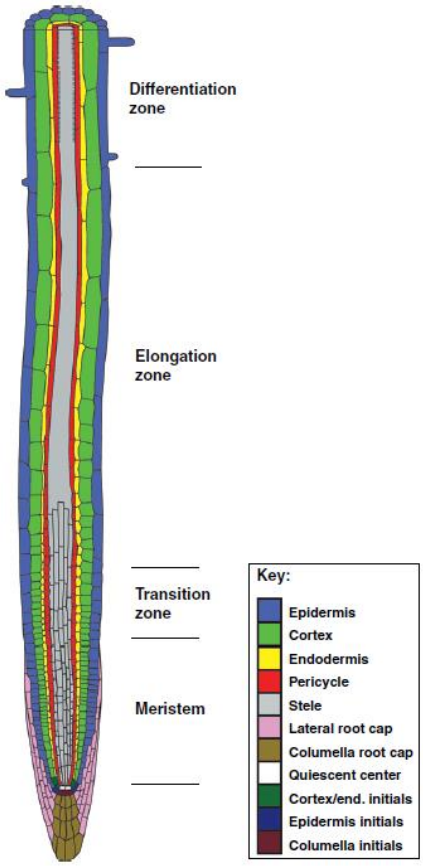


Figure 2.8. The zones and cell types of the Arabidopsis primary root .The root consists of a highly organized pattern of cell types along radial and longitudinal axes (Ubeda-Tomás et al., 2012).

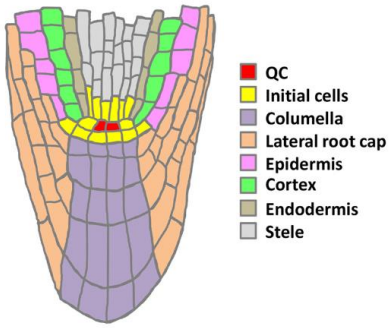


Figure 2.9. Stem cell organisation in the *Arabidopsis* primary root apex. The stem cells surrounding the quiescent centre (QC) cells give rise to each cell lineage, such as columella, lateral root cap, epidermis, cortex, endodermis, and provascular tissues/ stele (Takatsuka and Umeda, 2015).

Primary root growth is influenced by several key plant hormones in which individual hormones act specifically in the particular root tissues. Gibberellin (GA) has been shown to control root elongation primarily via the endodermis, which in turn also controls root apical meristem size (Ubeda-Tomás et al., 2008; Ubeda-Tomás et al., 2009). It was demonstrated that the endodermis represents the primary target tissue for GA in regulating the root elongation. By blocking the GA response in the endodermis, it was observed that the morphology of expanding cells in adjacent tissue layers are disrupted. The site of GA response was identified in a mutant version of the GAI DELLA growth repressor (*gai*) in which degradation could no longer be triggered by GA. This is reflected by the expression of the GA-resistant DELLA mutant in the endodermis that inhibits root elongation through GA-induced DELLA degradation (Ubeda-Tomás et al., 2008). In the transition zone, cytokinin is predominantly required for root cell differentiation and meristem size, with increased cytokinin levels causing a reduction in meristem size (Ioio et al., 2007). In the cytokinin-deficient plants, increased root meristem size and enhanced growth was observed, whereas increased cytokinin showed the opposite effect (Werner et al., 2001; Ioio et al., 2007).

The hormone GA is important in many aspects of plant development including seed germination, organ elongation and expansion, trichome development, vegetative to reproductive growth transition, development of flower, seed and fruit (Davière and Achard, 2013; Hedden and Sponsel, 2015). Hundreds of GAs have been identified, but only a few are bioactive, including GA_1, GA_3, GA_4 and GA_7 (Hedden and Phillips, 2000). GA_3 was the first GA to be structurally characterized and has been acknowledged to be involved in important developmental processes such as pollen-tube growth, seed germination, stem elongation and flowering (Hauvermale et al., 2012). The expression of GA biosynthesis genes vary according to different tissues, cell types and developmental stages (Yamaguchi et al., 2001; Kaneko et al., 2003).

In this study, analysis of endogenous GA hormone by LC-MS further revealed that GA_3 was significantly decreased in the *METo A1+* and *A1–* lines. This is in

agreement with previous report in which in the GA-deficient mutants *ga1-3* and *3ox1/ga3ox2*, a reduction in meristem size was observed (Ubeda-Tomás et al., 2009). Changes in root growth are correlated with experimentally altered GA level due to, for instance, addition of GAs to the root system, application of GA-biosynthesis inhibitors, changes of genes in GA pathways, or mutations of genes involved in GA biosynthesis (Inada and Shimmen, 2000; Yaxley et al., 2001; Gou et al., 2010). In rice, loss-of-function of a CHD3 chromatin remodeling factor (namely CHR729) resulted in morphological and growth defects including late seed germination, low germination rate, dwarfism and inhibited root growth. This *t483* mutant also has decreased bioactive GA_3 in which the shorth root length phenotypes were partially rescued by application of exogenous GA_3. Therefore, the CHR729 in rice is suggested to control the root growth via gibberellin pathway (Ma et al., 2015).

Cytokinin (CK) is involved in numerous developmental processes such as shoot growth, senescence and root growth (Gan and Amasino, 1995; Kurakawa et al., 2007). CKs are adenine derivatives, having an isoprenoid or aromatic side chain replaced at the N6-position in nature (Frebort et al., 2011). The CKs are categorised by their N6 side chains, either isoprenoid CKs or aromatic CKs (Sakakibara, 2006). Isoprenoid CKs are divided into four classes including trans-Zeatin (tZ), isopentenyladenine (iP), cis-Zeatin (cZ) and dihydrozeatin (DZ). Aromatic CKs include 6-benzyladenine, *ortho*-topolin (oT) and *meta*-topolin. Endogenous CK levels are determined by the balance between synthesis, conjugation and degradation (Sakakibara, 2006; Oslovsky et al., 2019).

Analysis of the concentration of CK metabolites revealed that three highly active CK; trans-zeatin (*t*Z-type) CKs, namely, *trans*-zeatin-7-glucoside, *trans*-zeatin-9-riboside and *trans*-zeatin-9-riboside(ph) and one dihydrozeatin (DHZ-type CK), dihydrozeatin-7-glucoside (active CK) were significantly reduced in the *METo A1+* line as compared with *Col-0*. This results were in contrast to previous studies, which showed that active CKs have inhibitory effect on root elongation (Werner et al., 2001; Ioio et al., 2007). Meanwhile, *cis*-zeatin-9-riboside-O-glucoside (cZ9ROG), showed a significant increase in the *METo A1+* line as compared with *Col-0.* However, cZ9ROG is a cis-zeatin (*c*Z-type) CK which is biologically

inactive (Schäfer et al., 2015). These suggested that the reduction in the meristem size in the *METo A1+* line was not linked with the changes in the CK levels. The LC-MS analysis of the CK metabolite was performed only in the *METo A1+* line as a representative of the reduced primary root phenotype observed in all *METo* lines including the METo *A1-, I1+* and *1-* lines. This was due to technical limitation in doing the LC-MS. Therefore the consistency of the CK metabolite analysis of the *METo A1+* line could not be confirmed.

Apart from hormones, regulation of stem cell fate and RAM maintenance during root development are associated with a combination of transcription factors, phytohormones, small signalling molecules, and miRNAs (Drisch and Stahl, 2015). Knowledge regarding function of DNA methylation in root development is still limited. A study by Kawakatsu et al. (2016) identified that columella root cap contained the highest level of DNA methylation compared to other cell types in RAM. Histone acetylation functions in the regulation of the cellular patterning in the root epidermis. Mutants of histone deacetylases (HDAC) and histone acetyltransferases (HAT) show altered cellular patterns in the root epidermis (Chen et al., 2016). Primary root elongation and lateral root emergence are inhibited by HDAC inhibitors via regulation of 26S proteosome-mediated degradation of PIN1 and changes in auxin distribution in Arabidopsis (Nguyen et al., 2013).

Generally, CHD3 chromatin remodeling factors control gene expression via promotion of trimethylation of lysine 27 of histone H3 (H3K27me3) in plants. In *Arabidopsis*, the CHD3 protein PICKLE (PKL) was discovered to be essential to maintain root meristems, since the *pkl* mutant exhibits low root meristem activity and a shorter root (Aichinger et al., 2011). In the *pkl* mutant, a reduced level of H3K27me3 is observed in normally H3K27me3-enriched genes, suggesting a role for PKL in the modification of repressed chromatin (Zhang et al., 2008). There is also an association between GA and the CHD3 chromatin remodeler, PKL, because PKL is essential is essential for controlling gene expression in numerous developmental processes regulated by GA. The *pkl* mutant resembles GA-response mutant in which it exhibits a ‘pickle root’ phenotype, with a thick green primary root that retains embryonic traits (Ogas et al., 1997). GA deficiency

conditions further enhance the '*pkl* root' phenotype in *pkl* mutants (Park et al., 2017).

2.3.1 Seed dormancy and germination

Seeds receive external cues, including temperature, light, water and nutrients, which stimulate molecular responses and enable the process of seed dormancy to germination (Ogawa et al., 2003). Germination starts when seeds take up water, followed by elongation of the embryonic axis, and is completed with the emergence of the radicle (Figure 2.10). There are three phases during germination. Phase I is initiated by an imbibition process, or water uptake for cellular rehydration. During phase II, germination *sensu stricto* (prior to radicle emergence) involves initial mobilization of seed proteins and lipid reserves and increased metabolic activity until initiation of growth, which corresponds to radicle emergence (phase III). Phase III also includes subsequent growth such as completion of nutrient mobilization, cell division and seedling growth (Figure 2.11) (Nonogaki et al., 2010).

Endogenous growth-regulating hormones that control germination are comprised of abscisic acid (ABA) and gibberellic acid (GA), which interact antagonistically (Figure 2.12). While ABA is responsible for maintaining seed dormancy, GA, in contrast, stimulates seed germination. Accordingly, the ABA:GA ratio has been is the central mechanism that controls dormancy and germination (Cao et al., 2006; Carrera et al., 2008).

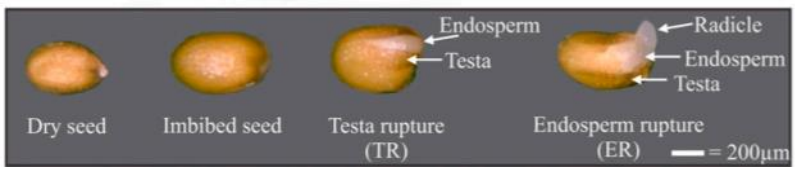


Figure 2.10. Seed germination process in *Arabidopsis*. The germination initiates with water uptake by imbibition by the dry seed, followed by expansion of the embryo. The embryo is enclosed by two covering layers called endosperm and testa. As the embryo axis elongates, it breaks through the covering layers until radicle emergence, which marks the completion of germination (Dekkers et al., 2013).

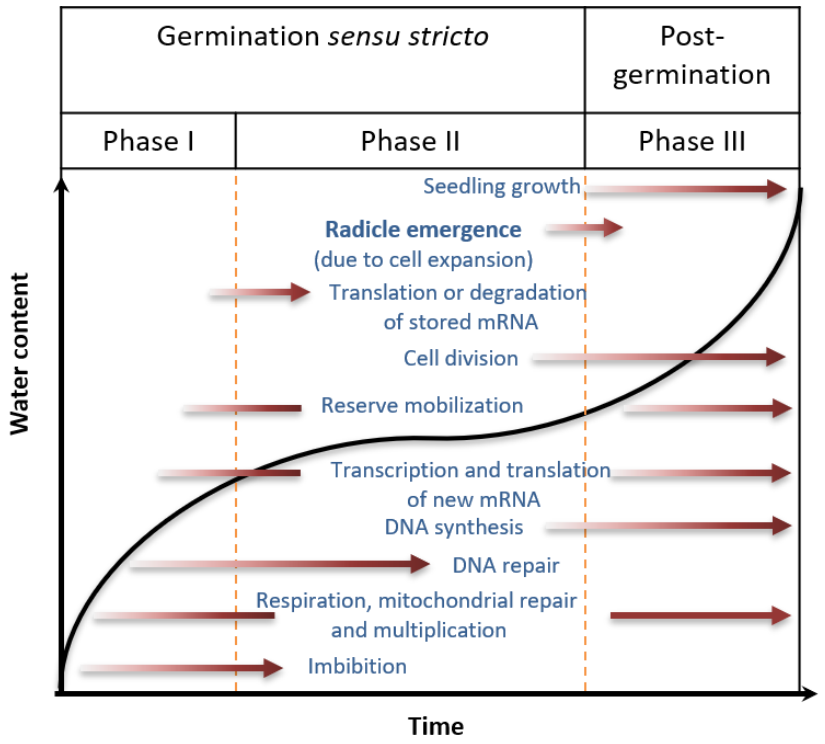


Figure 2.11. The phase of germination as defined into three stages. Time course of physical and metabolic events that occur during germination (Phases I and II) and early seedling growth (Phase III). The time taken for these events to occur varies between species and is influenced by germination conditions. The curve shows a stylized time course of water uptake. Image adapted from Nonogaki et al. (2010).

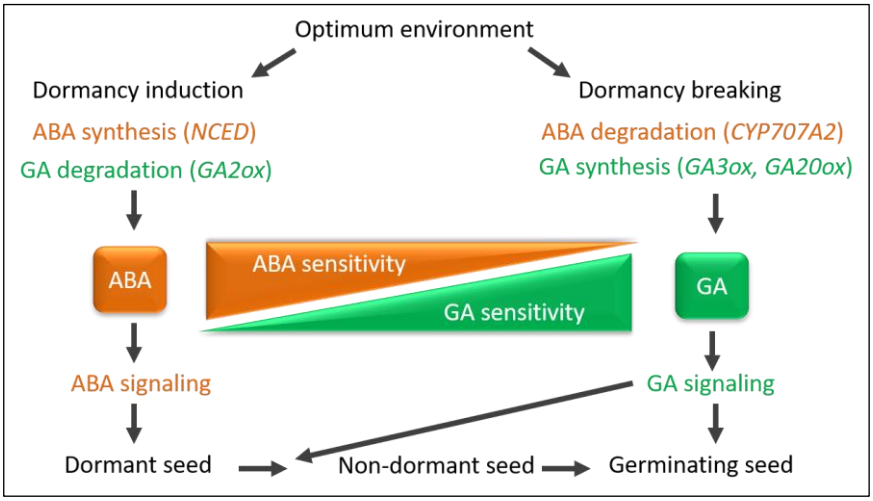


Figure 2.12. Regulation of seed dormancy and germination by ABA and GA. The ABA dominates dormant seed as reflected by ABA synthesis and GA degradation while germinating seed is dominated by GA due to ABA degradation and GA synthesis. As the seed loses dormancy on the transition to germination, the seed has reduced sensitivity to ABA and increased sensitivity to GA. Key target genes involved are in parentheses. Image adapted from Finch-Savage and Leubner-Metzger (2006).

The *METo A1*+, *I1*+ and *I1*– lines showed normal germination under control conditions except for the *METo A1*– line. Two major hormones, ABA and GA, have been shown to play important roles during seed germination (Shu et al., 2015). Reduction of seed dormancy can be caused by a transition from a high to low ABA:GA ratio, through elevated GA synthesis and ABA degradation (Cadman et al., 2006; Wang et al., 2018). Germination on different concentrations of GA and a GA inhibitor, PAC, revealed different phenotypes. Exogenous application of 5 µM GA improved germination at 4 days post-stratification in the *METo A1*– line, whereas application of a higher GA level (10 µM), resulted in the *METo A1*– line having similar germination as *Col-0* for the 5 consecutive days post-stratification. This showed that GA application enhances germination in the *METo A1*- line. This is in agreement with the previous study that demonstrated the role of GA during germination in which exogenous GA application helped the GA biosynthesis mutant, *ga1-3* to germinate (Karssen et al., 1989). The *GA1* gene encodes copalyl synthase, which is a preliminary enzyme required for GA biosynthesis (Hedden and Kamiya, 1997). Mutation in the GA biosynthesis gene such as *GA1* results in very low levels of endogenous GA and fail to germinate unless exogenously supplied with bioactive GA (Karssen et al., 1989). This suggests that delayed germination in *METo A1*- line could be due to low levels of GA in the seeds. However, this requires further experiment to measure the endogenous GA levels in the seed using liquid chromatography-mass spectrometry (LC-MS).

Application of kinetin and IAA managed to increase germination in the *METo A1*- line at day 5 post stratification. Recent reports have suggested that cytokinin and auxin are also involved in the control of seed dormancy and germination (Huang et al., 2017; Hussain et al., 2020). The auxin signalling repressor Aux/IAA8 accumulates and promotes seed germination by suppressing transcription of *ABSCISIC ACID INSENSITIVE3 ABI3*, which is a negative regulator of seed germination (Hussain et al., 2020). Accumulation of IAA8 inhibits AUXIN RESPONSE FACTOR (ARF) activity, which is coupled with downregulation of *ABI3* gene expression. *iaa8-1*, an IAA8 loss-of-function mutant, showed delayed seed germination (Hussain et al., 2020). Cytokinin may also have a permissive role in seed germination that act antagonistically with ABA (Riefler et al., 2006). At the beginning of the germination, the inhibitory role of ABA may be

counteracted by cytokinin by reducing the expression of *the ABA INSENSITIVE (ABI5)* gene (Wang et al., 2011). Kinetin possibly increases cell division rates and acts as priming agents to enhance seed germination and seedling robustness (Sawan et al., 2000; Tahaei et al., 2016).

During salinity stress and ABA treatment, the *METo A1*– line displayed hypersensitivity in both conditions, with germination being greatly inhibited as compared with *Col-0*. High-salinity conditions caused a delay in germination by stimulating endogenous ABA biosynthesis (Xiong et al., 2001). Hypersensitivity to ABA and NaCl was also observed in a HISTONE DEACETYLASE6 (HDAC6), the *hda6* mutant and *HDA6*-RNAi plants which suggested the involvement of *HDA6* under the ABA and abiotic stress response (Chen et al., 2010).

Among all the *METo* lines, only *METo A1*- showed reduced germination. One possible explanation is this could be due to secondary effect of *MET1* over-expression in which various stochastic epi-mutations and phenotypes are produced as a manifestation of a random established epigenetic marks. For instance, the *ddm1* mutant develops growth defects merely after numerous rounds of self-pollination (Kakutani, 1997). The dynamic reconfiguration of DNA methylation occurs during seed development and germination (Xiao et al., 2006; Kawakatsu et al., 2017). From the early to the late phases of seed development, methylation of CHH dramatically increases, then progressively decreases after germination. In developing seeds, the methylation of CHH is controlled by RdDM and CMT2 as observed in Arabidopsis and soybean (Kawakatsu and Ecker, 2019). During the initial phase of seed development, CHH methylation is 6% before increasing to 11% during the later stages (onset of dormancy) (Lin et al., 2017; An et al., 2017). During the subsequent seed maturation phase, the levels of CHH methylation in transposable elements (TEs) rapidly decreased (Kawakatsu and Ecker, 2019). This is in contrast with CG and CHG methylation which are maintained over the course of seed development (Bouyer et al., 2017; Narsai et al., 2017; Chen et al., 2018).

Germination-associated gene expression may be partially connected to increased methylation level during seed development and reduced methylation during germination (Kawakatsu et al., 2017). In parallel, both seed dormancy and germination genetic pathways are also under control of local demethylation. For instance, *DOG4L* stimulates germination while suppressing dormancy and ABA sensitivity. This gene which is paralog of *DOG1* is maternally expressed and showed differential promoter methylation between alleles. ROS1 which a DNA demethylase, functions to prevent promoter hypermethylation at the paternal alleles, therefore allowing *DOG4L* expression (Zhu et al., 2018).

2.3.2 Conclusions

In this study, I showed that the *Arabidopsis* over-expression lines (fifth generation) exhibit reduced primary root length as compared to wild-type (WT) plants. The reduction in the primary root length was accompanied with reduction in meristem cell numbers. These phenotypes correlate with a reduction in endogenous GA_3 levels in the *METo A1+* and *A1-* lines. However, the increment in endogenous cytokinin was not correlated with the phenotypes as observed in the *METo A1+* line. Ideally, the hormone analysis should be conducted in all *METo* line which therefore could confirm the consistency of the results. As for the delayed germination phenotype observed in the *METo A1-* line, reduced GA levels may contribute to delayed germination, however further experiments should be carried out including the endogenous hormone analysis and transcript analysis during seed germination. This could contribute in the discovery of new seed quality markers as a way to optimize crop yields.

Chapter 3
Investigating gene expression in Arabidopsis mutants with different S-adenosyl methionine levels

3.1 Introduction

The methyl-group donor S-adenosyl methionine (SAM) is essential for DNA methylation enzymes. In the *Arabidopsis*, SAM is produced from methionine and adenosine triphosphate (ATP) by the SAM SYNTHETASE (SAMS) enzyme (Li et al., 2011). During methylation, SAM is converted into S-adenosyl homocysteine (SAH). SAH is an inhibitor of methylation reactions, therefore it is converted by SAH HYDROLASE (SAHH) to homocysteine and adenosine (**Figure 3.1**). Methionine, the precursor of SAM, is produced from the one-carbon (1C) metabolic pathway. The 1C metabolic pathway requires folate as a cofactor for it to function in synthesizing purines; amino acids; and various secondary metabolites such as lignin and phytohormones, which are crucial to cellular function (Roje, 2007; Gorelova et al., 2017). The effect of the methyl group supply on epigenetic modification is becoming an interesting aspect to study, that previously has not been extensively explored. A recent study by Wang et al. (2017) revealed the role of folate homeostasis in the epigenetic regulation of *FWA*, a gene involved in flowering. As the methyl group donor, SAM, methionine and folate are interconnected in the 1C metabolic pathway, it is hypothesized that any changes in these elements will influence the level of DNA methylation (Friso et al., 2017). As reported by Zhang et al. (2012), treatment with sulfamethazine, a folate inhibitor, resulted in a reduction in DNA methylation and H3K9 dimethylation. Another example is the fact that impaired folate metabolism leads to an increased level of SAH due to mutation in the *MTHFD1* gene, which causes an imbalance in the SAM:SAH ratio (Groth et al., 2016). A proper level of the SAM:SAH ratio is crucial, as a high level of SAH is inhibitory to DNA methyltransferases (Rocha et al., 2005).

A missense mutation in *SAHH* leads to the accumulation of SAH, which mainly causes a loss of non-CG methylation under the control of CMT3 and H3K9 methylation (Mull et al., 2006). This possibly due to preferential inhibition toward CMT3 by the SAH as compared with other DNA methyltransferases such as MET1 and DRM2. Several reasons proposed for this include different active site architectures, in which CMT3 belongs to the chromomethylase family and contains an additional chromodomain (Goll and Bestor, 2005). Furthermore, the DNA methyltansferases may have different affinities toward SAM and SAH; as such, the enzymes with the least affinity may be most affected by the decreased SAM level and increased SAH level (Meng et al., 2018).

Based on previous work performed by Watson (2013), an uncharacterised SAM-dependent methyltransferase (*AT2G41380*) was identified, with increased expression in some *Arabidopsis* plants expressing a catalytically inactive *MET1* gene under the control of the 35S promoter. In the *met1* mutant, *AT2G41380* showed decreased expression, with no direct changes in DNA methylation. As *AT2G41380* encodes for a protein methyltransferase and requires SAM, similar to MET1, this leads to a hypothesis that *AT2G41380* may be involved in a substrate specific feedback loop with MET1. Feedback regulation related to epigenetic modifiers has been observed in plants. In the *met1* mutant, *ROS1* and *DME*, which encode demethylation functions, were found to have reduced expression. However no direct DNA methylation changes were observed in the *ROS1* promoter, suggesting indirect DNA methylation changes (Mathieu et al., 2007). *AT2G41380* showed increased expression when *MET1* levels increased. Increased MET1 levels could result in reduced SAM, therefore expression of *AT2G41380* may increase to compete for SAM supply.

As a SAM-dependent DNA methyltransferase, MET1 requires SAM to donate a methyl group in order to maintain DNA methylation. This leads to a hypothesis that overexpression of *MET1* could cause changes in the expression of the genes encoding the enzymes involved in the SAM-dependent pathway, as a feedback mechanism to adjust to a reduced SAM supply, caused by MET1-induced titration of SAM (Figure 3.1). Therefore, this study aimed to identify potential target genes

that are influenced by changes in the level of SAM due to a secondary effect of the *MET1* overexpression. The identification of DNA methyltransferases' methylation-independent activities will provide new insight into these epigenetic regulators.

Arabidopsis mutants exhibiting different SAM levels were used to analyse the potential feedback loop between *MET1* and SAM. *mto1* (Inaba et al., 1994), *mto2* (Bartlem et al., 2003), and *mmt* (Kocsis et al., 2003 were used to investigate whether selected target genes are directly regulated by increased levels of SAM (Table 3.1). These mutations interrupt the methionine metabolic pathway (Figure 3.2). The *mto1* (*methionine overaccumulation 1*) mutant has a point mutation in the gene encoding *CYSTATHIONINE GAMMA-SYNTHASE (CGS)* which prevents negative feedback regulation of *CGS* mRNA stability, resulting in a 40-fold increase in the level of methionine in *mto1* (Inaba et al., 1994). In the *mto2* mutant, contains a single mutation in the gene encoding *THREONINE SYNTHASE (TS)* and attains a 20-fold higher methionine levels. The mutation in the *TS* disrupts the conversion of *O*-phosphohomoserine (OPH) substrate to threonine for the production of isoleucine (Bartlem et al., 2000). In the *mmt* mutant, approximately a 1.6- fold increase in SAM level was observed, due to a mutation in the gene encoding *METHIONINE S-METHYLTRANSFERASE (MMT)*, which plays a role in the methyl cycle (Kocsis et al., 2003).

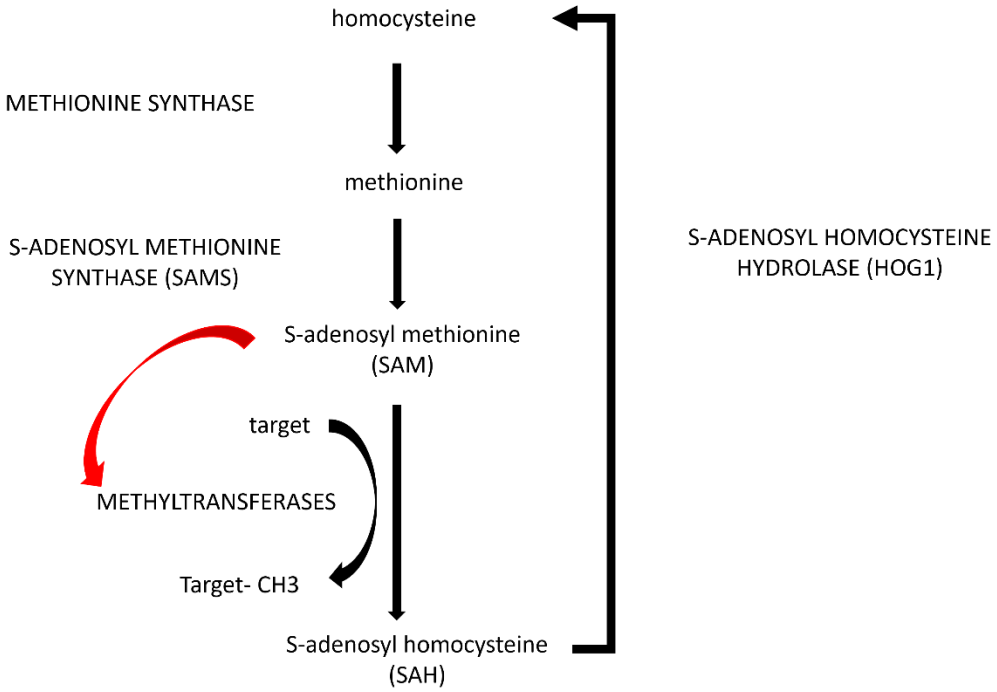


Figure 3.1. A model to illustrate a proposed feedback system between S-adenosylmethionine (SAM) levels and METHYLTRANSFERASE proteins, as shown by the red arrow. A methyl group (-CH_3) is transferred from SAM to target proteins and DNA by METHYLTRANSFERASE. S-adenosylhomocysteine (SAH), a by-product of methylation is metabolised back to SAM via S-ADENOSYL HOMOCYSTEINE HYDROLASE, METHIONINE SYTHASE and S-ADENOSYLMETHIONINE SYNTHASE.

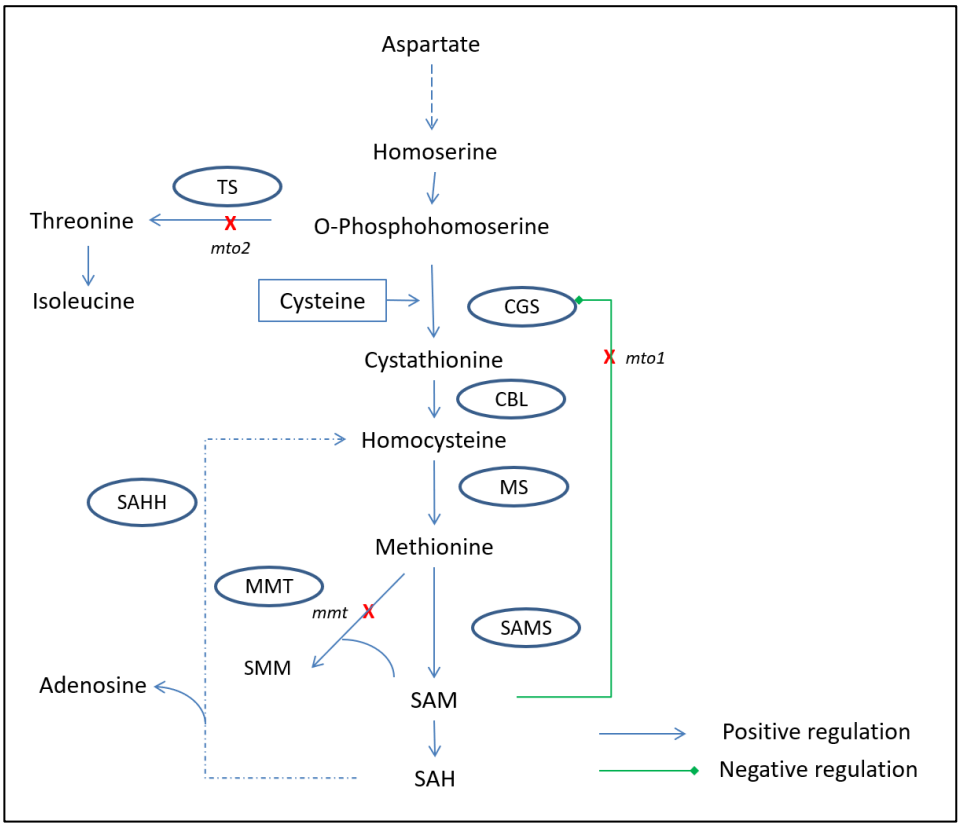


Figure 3.2 Methionine and SAM synthesis pathway. Green arrow; negative feedback of allosteric regulation in response to the overaccumulation of SAM. Abbreviations within ovals represent enzymes responsible for catalysis of the indicated reactions: SAM, S-adenosylmethionie; SAH, S-adenosylhomocysteine; SMM, S-METHYLMETHIONINE; TS, THREONINE SYNTHASE; CGS, CYSTHATHIONINE GAMMA SYNTHASE; CBS, CYSTHATHIONINE BETA LYSE; MS, METHIONINE SYNTHASE; SAMS, SAM SYNTHASE; MMT, METHIONINE S-METHYLTRANSFERASE. Modified from Hanafy et al. (2013). The *mto1* mutants are defective in CGS gene as referred to red cross, while *mto2* and *mmt* mutants have defects in TS gene and MMT gene respectively.

Mutant	Ecotype	Methionine level	SAM level	Reference
mto1	*Columbia (Col-0)*	40-fold increased	3-fold increased	Inaba et la., 1994
mto2	*Wassilewskija (Ws)*	20-fold increased	3-fold increased	Bartlem et al., 2000
mmt	*Wassilewskija (Ws)*	Not altered	1.6-fold increased	Kocsis et al., 2003

Table 3.1. *Arabidopsis* mutants with increased methionine and SAM levels.

3.2 Results

3.2.1 Investigating the effects of SAM levels on the genes involved in SAM-dependent pathways

A total of 14 genes (**Table 3.2**) were screened for changes in gene expression by using semiquantitative PCR. These genes were selected based on the preliminary RNA-seq data (Brocklehurst et al., 2018), in which they are involved in biological pathways requiring S-adenosylmethionine (SAM) as methyl donor such as polyamines, ethylene and transmethylation reaction of DNA and protein (Figure 3.3).

Gene ID	Description	Symbol
AT3G06930	*ARGININE METHYLTRANSFERASE 4B*	*PRMT4B*
AT5G49020	*ARGININE METHYLTRANSFERASE 4A*	*PRMT4A*
AT3G12270	*ARGININE METHYLTRANSFERASE 3*	*PRMT3*
AT2G19670	*ARGININE METHYLTRANSFERASE 1A*	*PRMT1A*
AT4G16570	*ARGININE METHYLTRANSFERASE 7*	*PRMT7*
AT1G76090	*STEROL-C-METHYLTRANSFERASE 3*	*SMT3*
AT1G19640	*JASMONIC ACID CARBOXYL METHYLTRANSFERASE*	*JMT*
AT4G19020	*CHROMOMETHYLASE 2*	*CMT2*
AT1G69770	*CHROMOMETHYLASE 3*	*CMT3*
AT1G01480	*1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE*	*ACCS*
AT3G02470	*S-ADENOSYLMETHIONINE DECARBOXYLASE*	*SAMDC*
AT4G34840	*5'-METHYLTHIOADENOSINE NUCLEOSIDASES 2*	*MTN2*
AT5G62480	*GLUTATHIONE TRANSFERASE*	*GT*
AT5G49810	*METHIONINE S-METHYLTRANSFERASE*	*MMT*

Table 3. 2. List of the 14 potential target genes encoding enzymes that are involved in the pathway requiring SAM for the methyl group.

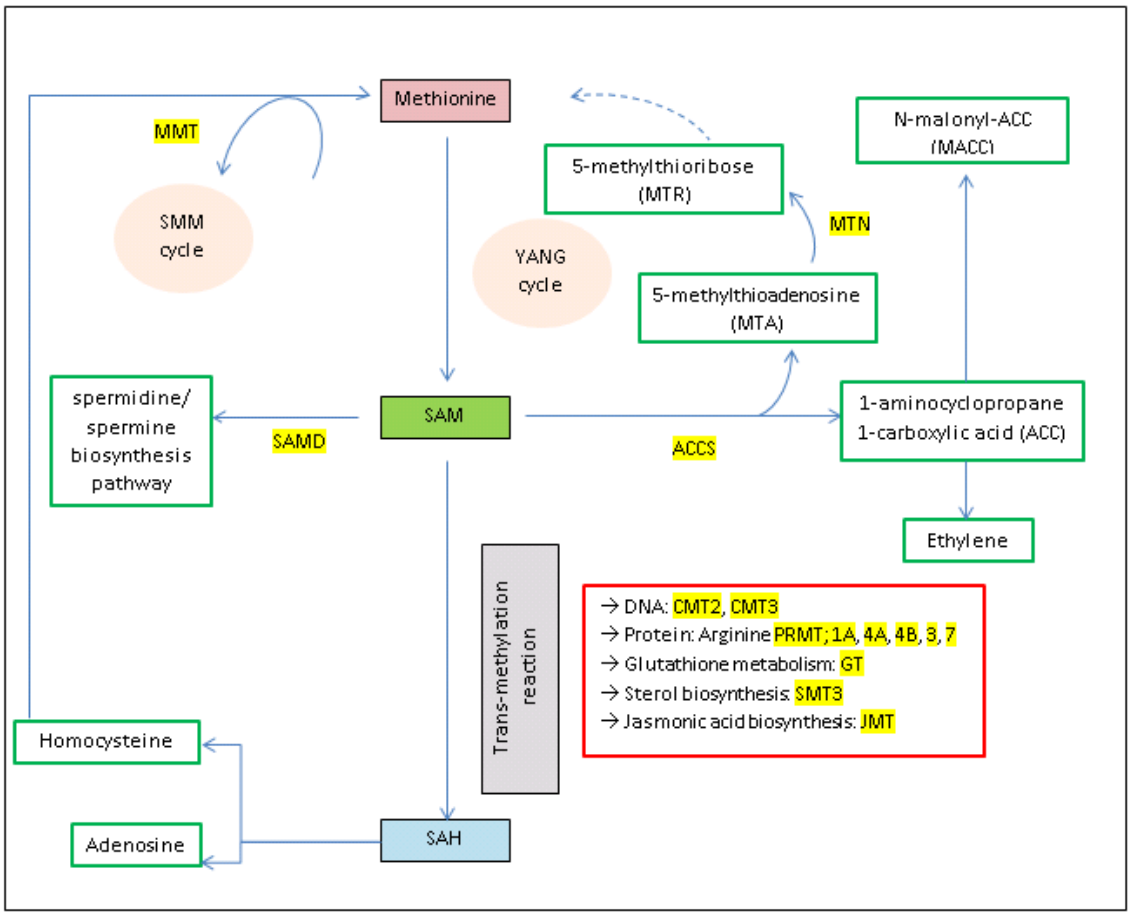
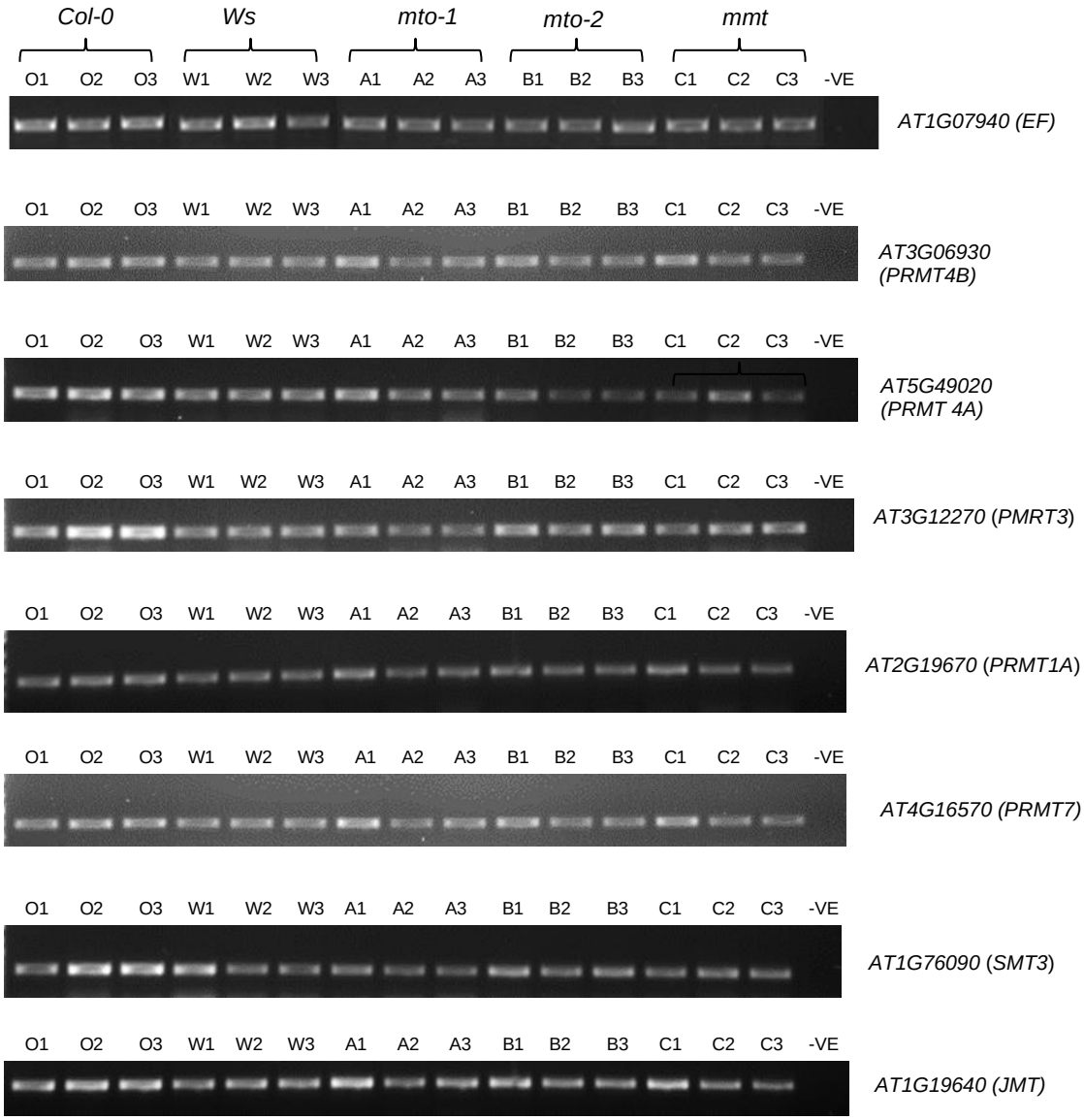


Figure 3.3. S-adenosyl methionine (SAM) metabolic pathways. The selected genes encoding enzymes involved in S-adenosyl methionine (SAM) metabolic pathways were highlighted in yellow; *PRMT4B, ARGININE METHYLTRANSFERASE; PRMT4A ,ARGININE METHYLTRANSFERASE; PRMT3, ARGININE METHYLTRANSFERASE 3; PRMT1A, ARGININE METHYLTRANSFERASE 1A; PRMT7, ARGININE METHYLTRANSFERASE 7; SMT3, STEROL-C-METHYLTRANSFERASE; JMT, JASMONIC ACID CARBOXYL METHYLTRANSFERASE; CMT2, CHROMOMETHYLASE 2; CMT3, CHROMOMETHYLASE 3; ACCS, 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE;SAMDC, S-ADENOSYLMETHIONINE DECARBOXYLASE; MTN2, 5'-METHYLTHIOADENOSINE NUCLEOSIDASES; GT, GLUTATHIONE TRANSFERASE; MMT, METHIONINE S-METHYLTRANSFERASE;*

3.2.1.1 Investigation of selected candidate genes in the *Arabidopsis* mutants with increased SAM levels

To examine changes in gene expression, caused by an elevated SAM level, the selected genes were analysed in the *Arabidopsis mto 1, mto 2,* and *mmt* mutant backgrounds. Using a semiquantitative PCR approach, no significant changes in expression caused by an increase in SAM level were observed for any of the 14 selected genes in the mutants, as compared with in the case of the wild-type (**Figure 3.3**).



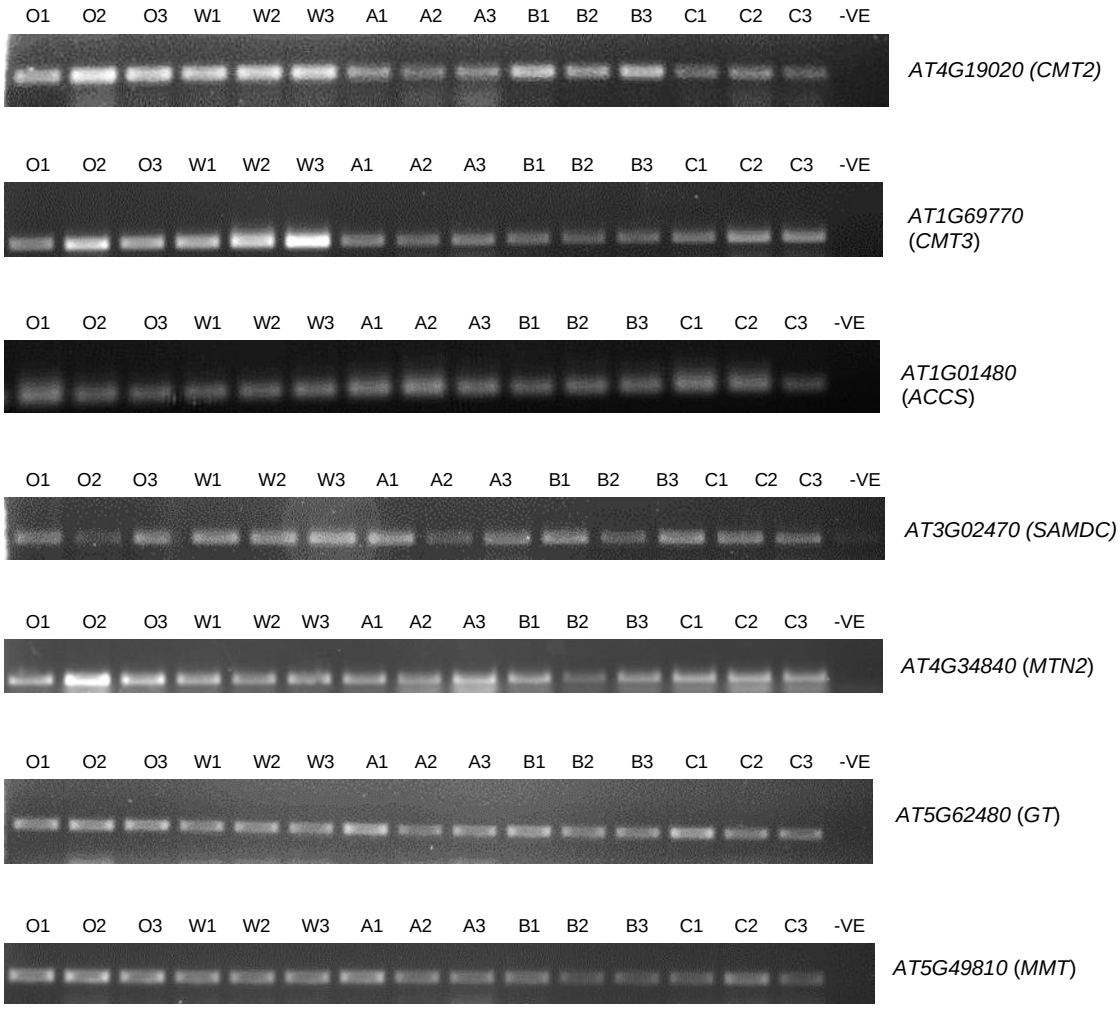


Figure 3.4. Expression of genes encoding enzymes involved in the SAM-dependent pathway in the wild type *Col-0* and *Wassilewskija (Ws)* and the following mutants with increased SAM levels: *mto-1, mto-2,* and *mmt*. The expression analysis of the 14 genes revealed that no genes showed significant changes in transcript levels as compared with the wild-type. The relative band intensity was measured by ImageJ analysis software (appendix). Corresponding gene locus identities (Gene IDs) are provided. *ELONGATION FACTOR (EF)* was used as an expression control.

3.2.2 Generation of *sam1/sam2* double homozygous line to investigate the effect of reduced SAM level

S-adenosyl methionine (SAM) is produced from precursor methionine by SAM SYNTHETASE. In *Arabidopsis,* SAM SYNTHETASE is encoded by four *SAM SYNTHETASE (SAMS)* genes, as follows: *SAMS1 (AT1G02500), SAMS2 (AT4G01850), SAMS3 (AT2G36880),* and *SAMS4 (AT3G17390)* (Goto et al., 2002; Chen et al., 2016). *SAMS1, SAMS2* and *SAMS4* are expressed in almost all tissues meanwhile *SAMS3* is mostly expressed in pollen (Lorraine et al., 2013). *SAMS1* and *SAMS2* share high similarity in sequence and expression pattern and the *sam1/sam2* double mutant showed a low level of ethylene (Mao et al., 2015).

To investigate the effects of SAM deficiency, T-DNA insertion–based knockouts of the two characterised *Arabidopsis SAM SYNTHETASE 1 (SAMS1) (AT1G02500)* and *SAM SYNTHETASE 2 (SAMS2) (AT4G01850)* genes were crossed, with the aim of generating double mutants to knock down SAM SYNTHETASE activity and therefore cause a reduction in the SAM concentration. Crossed plants were from two lines of single mutants, each containing a T-DNA insertion (*SALK_073599c* and *SALK_097197c*), in the protein coding sequence of either *SAM1* or *SAM2* respectively. Heterozygous crosses were self-pollinated and seed stock were pooled. The genetic crosses were performed by Alexander Warne at P. Meyer lab, at the University of Leeds.

To confirm the genotype of the double mutants, PCR analysis that amplified the insertion sequence and the wild-type gene was carried out. *sam1* (*SALK_073599c*) and *sam2* (*SALK_097197c*) mutants were screened according to http://signal.salk.edu/tdnaprimers.html, with the following primer sets: first, PCR was performed with the LP and RP primers of *sam1* and *sam2*, respectively, to amplify the wild-type allele. A 462-bp amplified fragment was expected if the plants were heterozygous of wild-type for *sam1*, while a 593-bp fragment was expected for *sam2*, whereas no band was expected if the plants were homozygous. Second, PCR was designed to separate the heterozygous plants

from wild-type plants using the SALK LBb1.3 and LP primers for *sam1* and *sam2* which produced a 461-bp and a 540-bp fragment respectively (**Figure 3.4**).

Figure 3.5. Genotyping of *sam1/sam2* double homozygous mutants using primer pairs complementary to the T-DNA insertion and its surrounding genomic sequence. Lane 1 amplifies the *sam1-wild-type* allele, lane 2 amplifies the *sam1-mutant* allele, lane 3 amplifies the *sam2-wild-type* allele, and lane 4 amplifies the *sam2-mutant* allele.

From the *sam1/sam2* mutants produced, three lines were further selected for phenotypic changes and gene expression analysis. From the semiquantitative PCR results, none of the 14 potential target genes showed any significant changes in expression, as compared with the wild type (Figure 3.6). No obvious phenotypic differences were observed in the sam1/sam2 mutants as compared to wild type. As for the comparison with the *METo* lines that showed reduced primary root length, analysis of primary root length showed that the *sam1/sam2 mutants* resembles wild type (Figure 3.7).

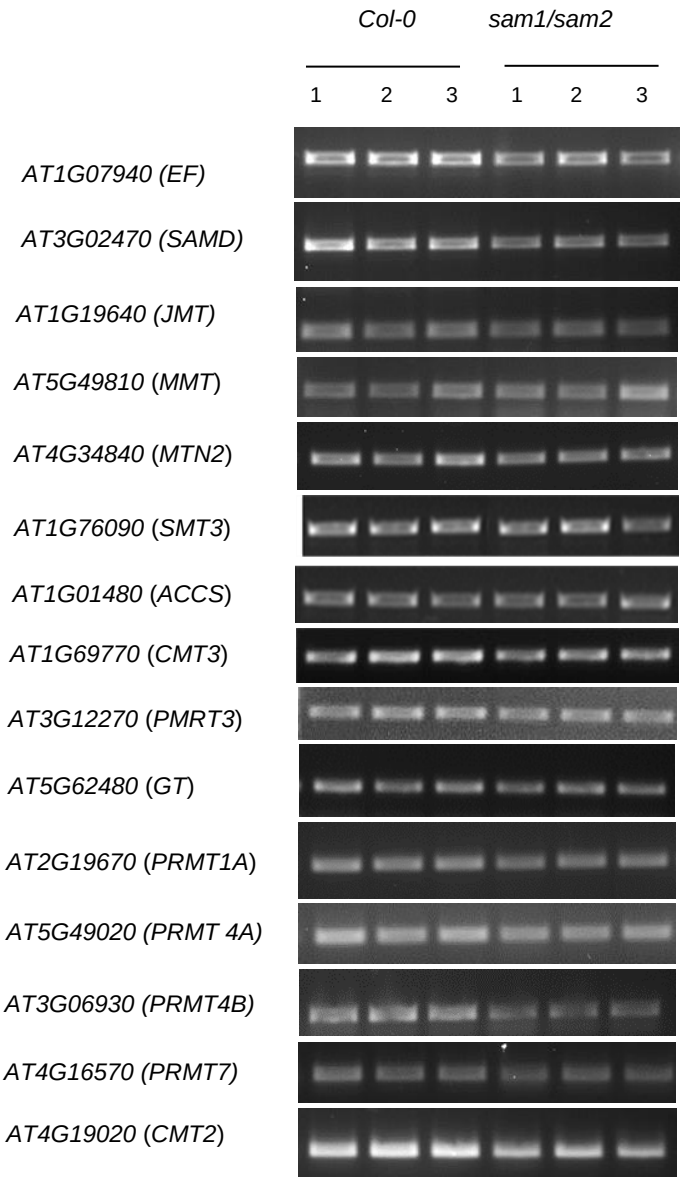


Figure 3.6. Expression of genes encoding enzymes involved in the SAM-dependent pathway in wild-type *Col-0* and *sam1/sam2* double homozygous mutant. The expression analysis of the 14 genes showed that the genes have similar expressions to that of the wild-type. Corresponding gene locus identities (Gene IDs) are provided. *ELONGATION FACTOR (EF)* was used as an expression control.

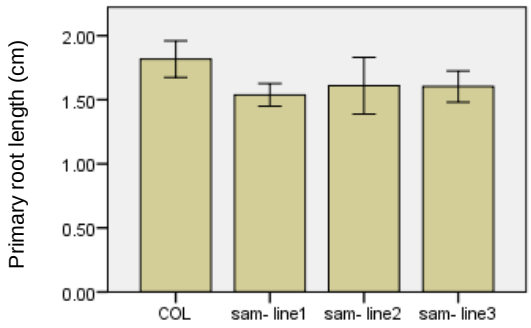


Figure 3.7. Primary root length of the *sam1/sam2* lines at seven days after germination. No significant differences in primary root length were observed in the *sam1/sam2* lines versus *Col-0* ($p > 0.05$, one-way analysis of variance). Values are presented as means ± SE, n = 20 per genotype.

3.2.3 Investigating the effects of increasing *MET1* copies with inactive catalytic and SAM binding sites

The *MET1* overexpression construct was produced to assess the effect of increasing *MET1* level whereas the *MET1* with mutation in the active site was produced to analyse the methylation-independent effects of *MET1* over-expression. However, *MET1* transgene still have SAM function therefore another *MET1* transgene was produced to eliminate both catalytic function and SAM binding function and was named as *METsammut* transgene. Mutations were introduced both at catalytic site and SAM binding domains to distinguish the methylation-independent effects of *MET1* over-expression in Arabidopsis. This was achieved by introducing a mutation at the SAM binding domain, using a similar strategy to remove the catalytic function of MET1 to that used by (Hsieh, 1999). Based on the sequence logo generated by comparing plant DNA methyltransferases, conserved motifs in the SAM binding domain were identified in Motif I and Motif X, while the active site is located in Motif IV (Pavlopoulou and Kossida, 2007), as shown in Figures 3.8 and Figure 3.9.

Motif I
SAM binding

Motif IV
Active site

Motif X
SAM binding

Figure 3.8. The sequence logo above showed the most conserved amino acids in the Motifs I, IV, and X of plant DNA methyltransferases (Pavlopoulou and Kossida, 2007). Point mutations were introduced at the Motif I, Motif IV and Motif X in the METsammut transgene.

To remove the function of SAM binding, amino acid glycine at position 1101 in Motif I is substituted to serine, while, for Motif X amino acid, leucine at position 1516 is substituted to valine and the active site cysteine at position 1197 in Motif IV is replaced with serine, as summarized in Table 3.3. The single amino acid changes in the Motif I was chosen similar to changes in *met1-2* allele which encode a glycine to serine mutation at amino acid 1101 (Kankel et al., 2003).

```
MET1          IVKPVEPPKEIRLATLDIFAGCGGLSHGLKKAGVSDAKWAIEYEEPAGQAFKQNHPESTV   1140
MET1mut       IVKPVEPPKEIRLATLDIFAGCGGLSHGLKKAGVSDAKWAIEYEEPAGQAFKQNHPESTV   1140
MET1sammut    IVKPVEPPKEIRLATLDIFASCGGLSHGLKKAGVSDAKWAIEYEEPAGQAFKQNHPESTV   1140
              ********************.************************************

MET1          FVDNCNVILRAIMEKGGDQDDCVSTTEANELAAKLTEEQKSTLPLPGQVDFINGGPPCQG   1200
MET1mut       FVDNCNVILRAIMEKGGDQDDCVSTTEANELAAKLTEEQKSTLPLPGQVDFINGGPPSQG   1200
MET1sammut    FVDNCNVILRAIMEKGGDQDDCVSTTEANELAAKLTEEQKSTLPLPGQVDFINGGPPSQG   1200
              ************************************************************.**

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MET1          LYGRLDWQGNFPTSVTDPQPMGKVGMCFHPEQHRILTVRECARSQGFPDSYEFAGNINHK   1500
MET1mut       LYGRLDWQGNFPTSVTDPQPMGKVGMCFHPEQHRILTVRECARSQGFPDSYEFAGNINHK   1500
MET1sammut    LYGRLDWQGNFPTSVTDPQPMGKVGMCFHPEQHRILTVRECARSQGFPDSYEFAGNINHK   1500
              ************************************************************

MET1          HRQIGNAVPPPLAFALGRKLKEALHLKKSPQHQP     1534
MET1mut       HRQIGNAVPPPLAFALGRKLKEALHLKKSPQHQP     1534
MET1sammut    HRQIGNAVPPPLAFAVGRKLKEALHLKKSPQHQP     1534
              **************:*****************
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Figure 3.9. Amino acid comparison between three different versions of the *MET1* transgene—namely, *MET1, MET1mut*, and *MET1sammut*—used in the studies. The mutation in the nucleotide sequence that resulted in amino acid changes was highlighted as above. The *MET1mut* transgene contains a mutation at the active site, while the *MET1sammut* transgene contains two SAM binding sites in addition to the active site.

Description	Point mutation	Nucleotide substitution	Amino acid substitution
Motif I (SAM binding site)	A	GGT-AGT	Gly-1101-Ser
Motif IV (active site)	C	TGT-TCT	Cys-1197Ser
Motif X (SAM binding site)	G	CTA-GTA	Leu-1516-Val

Table 3.3. Summary of mutations introduced to remove the function of the active site and SAM binding domain of the *MET1sammut* transgene.

3.2.3.1 Production of *MET1* with mutation in SAM binding domain and active site

In *MET1*, the locations of three point mutations to be introduced are located between the unique restriction enzyme sites *BsuI* and *SpeI*. A 1906 bp fragment containing mutations at SAM binding site and active site was ordered from Invitrogen and cloned in pGEM-T vector. To produce *MET1sammut*, the *MET1* cDNA in pGEMT vector was digested with Bsu36I and SpeI to release the original sequence and replaced with the mutated version (Figure 3.10). After excision from an agarose gel and purification, both fragments were ligated and transformed into *E. coli* M3 competent cells (Figure 3.11 and Figure 3.12). Plasmids from eight putative colonies were extracted and proceed with diagnostic digest. Three out of eight colonies contained positive plasmids (Figure 3.13).

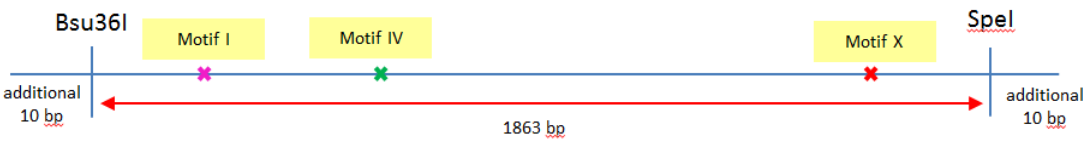


Figure 3.10. The schematic diagram of a fragment containing a point mutation in Motif I, Motif IV, and Motif X. The fragment contains the restriction sites Bsu36I and SpeI to allow for the exchange of the original fragment with the mutated fragment after the restriction of enzyme digestion.

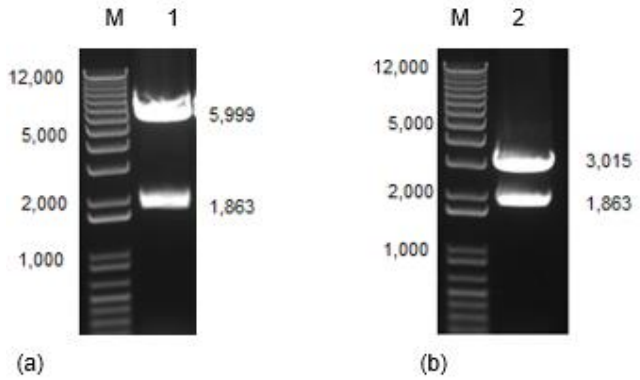


Figure 3.11. Agarose gel analysis of isolated plasmids digested by Bsu36I and SpeI restriction enzymes. (a) *MET1* cDNA in the pGEM-T vector and (b) mutated fragment of *MET1* in the pGEM-T vector. The 5,999 bp fragment in the Lane 1 and the 1,863 bp in the Lane 2 were excised from the agarose gel for purification. M is DNA marker.The original SAM binding domain of *MET1* cDNA was replaced with the SAM binding domain with mutation.

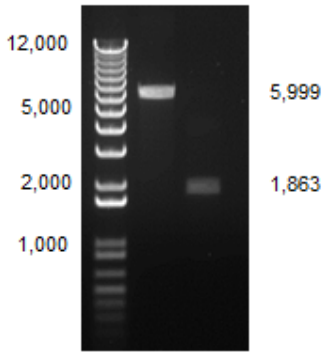


Figure 3.12. Agarose gel analysis of the purified fragments of 5999 bp of original *MET1* and 1863 bp of mutated fragments. Both fragments were ligated and transformed into *E. coli* competent cells.

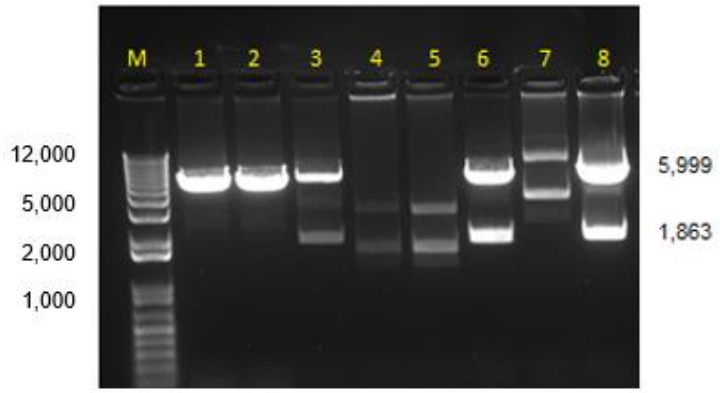


Figure 3.13. Diagnostic digest to confirm the presence of inserts. Plasmids in lanes 3, 6 and 8 produced expected sizes of 1863 and 5999 bp when digested with Bsu36I and SpeI and were selected further.

3.2.3.2 Transfer of *MET1sammut* into to pGreen II with NOS promoter

The *MET1sammut* cDNA sequence from the p-GEM T easy vector was removed and inserted into the polylinker region of the plant transformation vector pGreen II 0179 with the NOS promoter (Figure 3.14). Following ligation and transformation into *E. coli* competent cells, the colonies produced were proceed with miniprep and diagnostic digest. Positive clones were send for sequencing for confirmation (Figure 3.15).

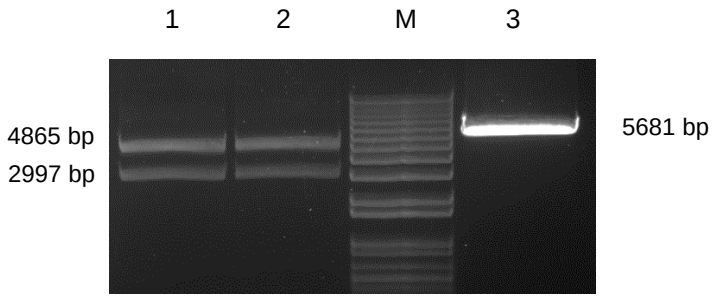


Figure 3.14. Agarose gel analysis of isolated plasmids digested with EcoRI restriction enzyme. Lane 1 and 2; *MET1* cDNA with mutated SAM binding (*MET1sammut*) in pGEM-T vector were digested with EcoRI producing two fragments of 4865 bp and 2997 bp while the plasmid pGreenII containing NOS cassette were linearized into producing single fragment of 5862 bp. The bands were gel excised and purified prior ligation. M is DNA marker.

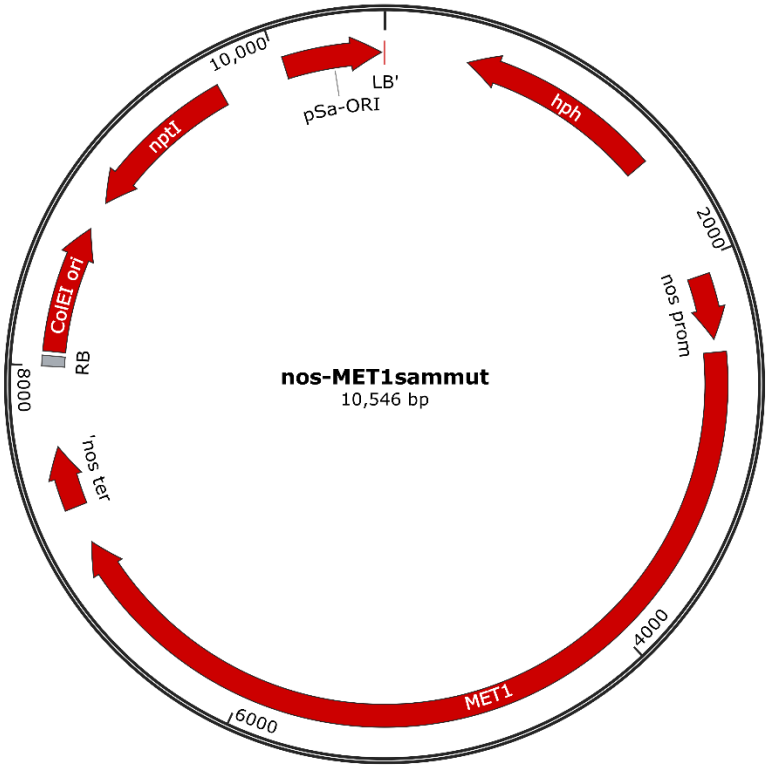


Figure 3.15. A schematic representation of *pNOS-MET1sammut* construct in plant transformation vector pGreen II. LB (left border) and RB (right border) mark the T-DNA region. The T-DNA region contains the plant selectable marker hygromycin (hph) and *MET1sammut* expression cassette. Co1E1 and pSa-ORI mark the replication of origin in *Escherichia coli* and *Agrobacterium*, respectively. Red arrows indicate gene orientation with the arrow head corresponding to the 3' end of the respective gene.

3.2.3.3 The production of *Arabidopsis MSM* lines

Four-week-old *A. thaliana Col* were transformed with *pNOS-MET1sammut* via a floral-dip, *Agrobacterium*-mediated transformation (Clough and Bent, 1998). The seeds were harvested and sown onto MS, supplemented with hygromycin for the selection of transformants. Two-week-old hygromycin-resistant seedlings were transferred to soil for seed collection. The T1 transformants were genotyped for the presence of the construct using primers flanking the NOS promoter and *MET1sammut* cDNA and three transgenic lines were selected that over-express MET1 (**Figure 3.16**)

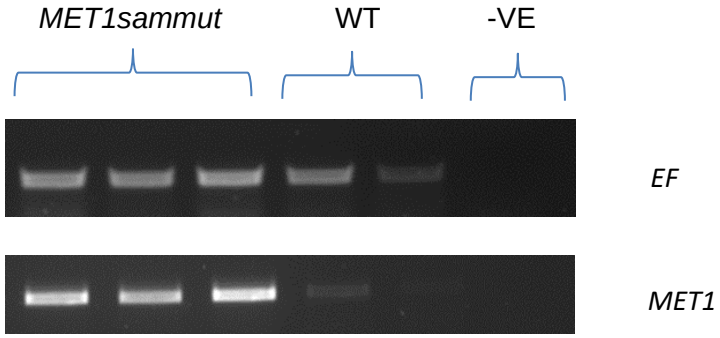
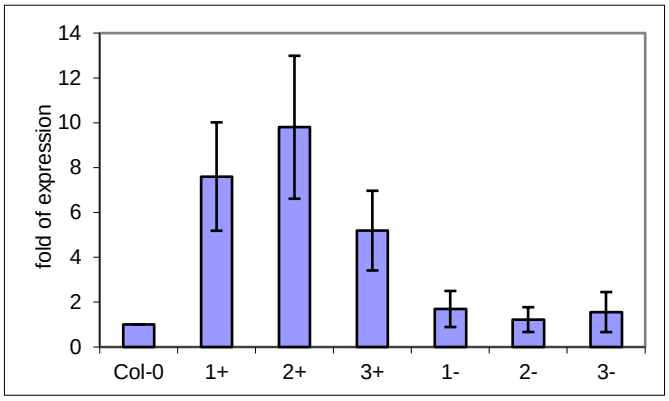


Figure 3.16. Semi-quantitative RT-PCR analysis of *MET1* expression level in *METsammut* transformants. *ELONGATION FACTOR (EF)* was used as internal control to compare transgene expression levels to wild-type (WT). "-ve" refers to no template control.

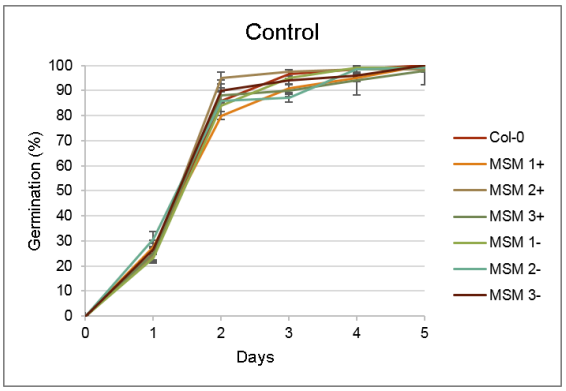
3.2.3.4 Phenotypic analysis of the *Arabidopsis MSM* lines

Three transgenic lines containing the *MET1sammut* transgenes were selected for further analysis—namely, *MSM 1+, 2+,* and *3+*. To investigate heritability, lines that had lost the transgene through segregation were generated and labelled as the *MSM 1-, 2-,* and *3-* lines. From the six *MET1sammut* lines that had been generated, three of which were homozygous for the *MET1sammut* transgene and three of which had lost the transgene, phenotypic analysis was carried out. In plants that had retained the transgene, *MET1* transcripts were approximately seven-fold, 10-fold, and five-fold higher in the *MSM 1+, 2+*, and *3+* lines, respectively. Separately, in lines that had lost the transgene (i.e., the *MSM 1-, 2-*, and *3-* lines), the transcript level had been restored to wild-type level. In the *METo* lines, reduced primary root lengths were observed in all lines and reduced germination was observed in the *METo A1-* line. As for comparison, phenotypic analysis at the germination stage and primary roots showed that the *MSM* lines resembled the wild-type phenotype (Figure 3.17).

A


fold of expression
14
12
10
8
6
4
2
0
Col-0
1+
2+
3+
1-
2-
3-

B


Control
Germination (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Days
Col-0
MSM 1+
MSM 2+
MSM 3+
MSM 1-
MSM 2-
MSM 3-

C

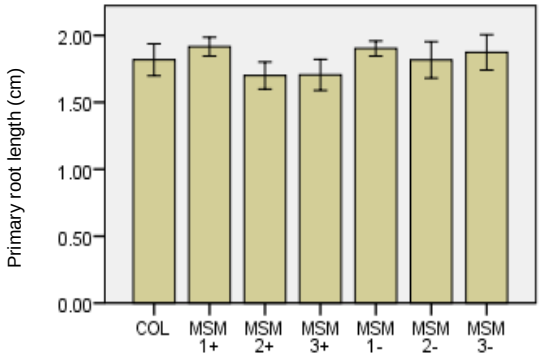

Primary root length (cm)
2.00
1.50
1.00
0.50
0.00
COL
MSM 1+
MSM 2+
MSM 3+
MSM 1-
MSM 2-
MSM 3-

Figure 3. 17. (A) *MET1* expression level in *Arabidopsis MSM* lines by quantitative real-time PCR (qPCR). The mean and the standard error are shown for three biological replicates, each having three technical replicates for each line. Values on the y-axis represent the fold difference as compared with the control line. (B) Germination phenotype of the wild-type *Col-0* and *METo* lines under the control conditions. Seed germination was evaluated for five days after stratification based on radicle emergence from the seed coat. No significant difference in the germination was observed between the *METsammut* lines as compared with the wild type *Col-0.* Values represent the means ± SE (n = 40) of three replicates. (C) Primary root length of the *MSM* lines seedlings at seven days after germination. An analytical software (ImageJ; National Institutes of Health, Bethesda, MD, USA) was used to measure the primary root of all lines. No significant difference in the primary root length was observed between the *METsammut* lines as compared with the wild-type *Col-0* ($p < 0.05$, one-way analysis of variance, Tukey's test). Values are means ± SD, n = 20 per genotype. All statistical analysis was performed using the Statistical Package for the Social Sciences version 22.0 software (IBM Corp., Armonk, NY, USA).

3.3 Discussion

3.3.1 SAM levels

DNA methyltransferase enzymes, including MET1, obtain the methyl group required for cytosine methylation from SAM. As the universal methyl group donor, SAM is widely used in various methylation-dependent pathways not only for the methylation of DNA but also during the methylation of proteins, lipid, and polyamines biosynthesis (Yan et al., 2010; Sauter et al., 2013). Previous work showed over-expression of MET1 induced heritable changes in gene expression (Brocklehurst et al., 2018). We hypothesized that the overexpression of *MET1* could impose a secondary effect that alters the expression of the genes that encode enzymes involved in the methionine and SAM pathways. This could be due to the SAM-binding domain of overexpressed MET1 interfering with the amount of available SAM. If this hypothesis is true, these genes should not experience changes in their expression in the *MET1*-overexpression lines containing transgenes with the SAM binding function removed.

To investigate whether there is a SAM-dependent regulatory network linked with MET1, the following approaches were used. In the first approach, a total of 14 potential target genes were chosen, initially based on their functions and involvement in the SAM-dependent pathways. To see if any of these genes are regulated by quantitative changes of SAM, three *Arabidopsis* mutants with increased SAM levels were used (*mto 1, mto 2* and *mmt)*. These mutants disrupt the methionine metabolic pathway, resulting in an altered SAM level (Inaba et al., 1994; Bartlem et al., 2000; Kocsis et al., 2003).From the semiquantitative PCR results, no significant changes in the gene expression was detected in the *Arabidopsis mto 1, mto 2* and *mmt* mutants with an increased SAM level. All the selected genes were involved in the metabolic process that requires SAM as methyl donor, however there were no evidence that the expression of these genes are sensitive to SAM levels. For instance, PROTEIN ARGININE METHYLTRANSFERASES is belongs to histone methyltransferases (HMTs) which catalyse the transfer of methyl to histone and release S-adenosylhomocysteine (SAH) as a by-product (C. Liu et al., 2010). This post-translational modification affects a wide range of cellular functions through its action on proteins implicated in the regulation of chromatin structure, transcription, RNA processing, signal transduction and cellular differentiation (Blackwell and Ceman, 2012). However, changes of expression of this gene was observed when induced with altered nitrite oxide (NO) level (Lindermayr et al., 2020).

3.3.2 The *sam1/sam2* double mutant

The SAM SYNTHETASE enzyme is involved in the synthesis of S-adenosylmethionine (SAM) from ATP and methionine and, for that reason, this enzyme could be manipulated to alter the SAM level. Therefore, double knockout *sam1/sam2* mutants were generated with the aim of producing an *Arabidopsis* mutant with a reduced SAM level. The semiquantitative PCR analysis of the 14 selected target genes showed no changes in the genes' expression. In *Arabidopsis,* the SAM SYNTHETASE is encoded by four *SAM SYNTHETASE (SAMS)* genes, as follows: *SAMS1 (AT1G02500), SAMS2 (AT4G01850), SAMS3*

(AT2G36880), and *SAMS4 (AT3G17390)* (Goto et al., 2002; Y. Chen et al., 2016). They share 94% amino acid identity, which therefore indicated a redundancy in function (Loraine et al., 2013). This might explained the insufficient effect of the knockout of *sam1/sam2,* as their function might be compensated for by the *SAMS3* and *SAMS4*. It has recently been identified that *SAMS4* is the most crucial among the four *SAM SYNTHETASES* gene, as a knockout of *SAMS4* is embryonically lethal (Meng et al., 2018). Therefore, it could not be confirmed as to whether these 14 genes are affected by a reduction in the SAM level or not, as reduction of the SAM level in *sam1/sam2* was proposed, however it was not measured. Therefore, further experiment is required to measure the SAM level using liquid chromatography-mass spectrometry (LC-MS). Analysis of primary root length of *sam1/sam2* showed normal phenotype. However, this requires further phenotypic analysis such as different development stages and different parts of the plants. In the *msa1-1* mutant with reduced SAM level in the roots, showed high sulphur phenotype without apparent morphological changes (Huang et al., 2016).

Previous studies reported changes in SAM levels resulted in different phenotypic changes at different developmental stages. Reduction of 35% of SAM in *sam4* mutant leads to smaller seedlings. Both *sam1* and *sam2* have 6% reduction in SAM levels whereas no changes in *sam3*. The *sam1sam2* double mutants displayed no abnormal phenotype (Meng et al., 2018).

3.3.3 The *msm* transgenic lines

Reduced primary root length were observed in the four genotypes of *METo* lines, which contain the *MET1* transgene with catalytic function (*METo A1+*), and the *METo* lines, which contain the *MET1* transgene without catalytic function (*MET1mut*), i.e., *METo I1+*. Another two lines (*METo A1-* and *METo I1-*) are lines that have lost the *MET1* and *MET1mut* transgenes, respectively, through genetic segregation. This showed that the reduced primary root length is heritable and consistent as a result of *MET1* overexpression. In the *MSM* line (with mutated

MET1 transgene at catalytic site and SAM binding domains), the lack phenotypic changes in the primary root length could be due to several reasons. The absence of phenotypes in the MSM lines suggests that one or both SAM binding regions are essential for the induction of phenotypes as observed for the *METo* lines, assuming that all transformants' expression and protein levels are comparable. There are at least three possibilities to consider. First, the mutated MET1 protein in the *MSM* lines is less stable than the MET1 in the *METo* lines. The MET1 mutation in the MSM lines results in a conformational change that prevents this MET1 version from interacting with other partner molecules, therefore preventing any effects for its over-expression. Additionally, the SAM binding region(s) is (are) required for the induction of phenotypic effects, which could be triggered by SAM depletion. Therefore, to determine the MET1 protein level in the MSM line, this can be done by producing FLAG-tagged MET1 over-expression transformants and checked by western blot analysis.

3.4 Conclusion

This chapter aims to identify potential candidate genes regulated by methylation-independent functions of DNA methyltransferases particularly through SAM feedback mechanisms. However, no target genes were identified with significant changes in expressions due to altered SAM levels. This general ability of outcomes is limited, as the SAM levels were not measured, not only in the *METo* lines, but also in the *sam1/sam2* double mutants and *MSM* lines. The lack of phenotypes in the *MSM* lines, herein referred to reduced primary root type as observed in the *METo* line, requires measurement of MET1 protein levels for further analysis.

Chapter 4

Root transcriptome analysis in *METo A1+* and *A1-* lines

4.1 Introduction

DNA methylation is crucial for normal plant development and physiology, such as during endosperm generation, vegetative growth and flowering. Previous studies have reported changes in gene expression of endogenous genes in various mutants of epigenetic regulators with severe loss of DNA methylation (Chen et al., 2006; Lister et al., 2008; To et al., 2011; Kim et al., 2014). This highlights the significance of DNA modifications in gene expression and physiological regulation. Changes in the promoter, adjacent regions, and/within the gene body may influence gene expression. In the *Arabidopsis met1-1* mutant, missense mutations in *MET1* caused a significant reduction in CG methylation, which resulted in phenotypic changes such as increased size and number of rosette leaves, increased numbers of cauline leaves, delayed flowering and altered flower morphology (Finnegan and Kovac, 2000; Kankel et al., 2003; Saze et al., 2003). In addition, there has been a report that showed the association of epigenetic regulation during root growth. Treatment with 5-azacytidine, a DNA methylation inhibitor, caused a decrease in primary root length in *Arabidopsis*, accompanied by reduced cytosine methylation level (Virdi et al., 2015).

Silencing of transposable elements (TEs) is crucial to maintain genome integrity. As the progenitor cells, plant stem cells are most susceptible to the TE activities, because TE insertions within the stem cells will be passed to all descendent cells. Plant stem cells include those found in the shoot apical meristem (SAM) and root apical meristem (RAM) (Pierre-Jerome et al., 2018). It was found that increased activities of the RdDM factors, DNA methyltransferases, and DDM1 silenced TE in the SAM during early vegetative growth (Baubec et al., 2014). In the *Arabidopsis* root meristem, CHH methylation is especially prevalent in the

columella. This CHH hypermethylation is caused by decreased expression of DDM1, heterochromatin decondensation and increased production of 24 nt siRNAs, in order to reinforce silencing in the RAM stem cells, which are located above the columella (Kawakatsu et al., 2016). Enhanced TE activity due to loss of DNA methylation was observed in *Arabidopsis ddm1* and *met1* mutants (Mirouze et al., 2009; Tsukahara et al., 2009; Zemach et al., 2013).

In the *Arabidopsis MET1* overexpression *(METo)* line, reduction in the primary root length was observed and this phenotype was heritable until the fifth generation (as discussed in Chapter 2). I am interested in investigating the molecular changes responsible for the reduction in primary root length in the *METo* lines. Therefore, RNA was extracted from primary roots of 10-day-old wild type *Col-0* and *METo* (*A1+* and *A1-* lines) with three biological replicates each. The RNA samples were sent to the Next Generation Sequencing Facility, Leeds Institute of Biomedical & Clinical Sciences, St James's University Hospital, Leeds. Next generation sequencing libraries were created from mRNA using the TruSeq Stranded mRNA kit (Ilumina) and sequenced on a NextSeq 500 to generate 75 bp single end sequence data. The RNAseq analysis was carried out by Dr Ian M Carr (University of Leeds, UK). All further data analysis was conducted by the author.

4.2 Results

A differential expression analysis was performed using DESeq R package (1.10.1), conducted by Dr. Ian Carr. All further data analysis was conducted by the author. The genes with a significant p- value of <0.05, and an absolute log_2 fold change of >1 were assigned as being differentially expressed (Wang et al., 2009). Applying a cut-off of a log_2-fold change of 1, increased expression levels, relative to WT, were observed for 39 genes in *A1+* and 119 genes in *A1-*. Meanwhile, reduced expression was observed in 1 gene in *A1+* and 144 genes in *A1-* line.

4.2.1.1 Genes with increased in expression

To identify the factors responsible for the reduced primary root length exhibited both in *A1+* and *A1-* lines, lists of genes that were mis-regulated in both *A1+* and *A1-* were analysed. The analysis started with the list of genes that show the biggest changes in expression. Table 4.1 presents the most up-regulated genes in the *METo A1+* line. The gene that showed the highest expression change was AT3G01345 which encodes for an unknown protein. This gene was found down-regulated in *sdg4* mutant flowers, which has defective reproductive (Cartagena et al., 2008). The *Arabidopsis* SET domain protein SDG4 is known to be linked to the epigenetic control of gene expression in pollen tube growth which affects fertilization. It has function to maintain methylation of histone H3K4 and K36 in the mature pollen grain. SDG4 is also involved during regulation of root apical meristem growth, in which it forms a functional CSR-SEU-SDG4 transcriptional complex (Cartagena et al., 2008; Kumpf et al., 2014; Zhai et al., 2020). AT2G03965 encodes a hypothetical protein of unknown function. AT1G32010 encodes a myosin heavy chain-like protein, which is up-regulated in the seedling-lethal *dpa1* (deficiency of plastic ATP synthase1) mutants (Dal Bosco et al., 2004). AT1G67105 is a long noncoding RNA that was found up-regulated in the roots of *Arabidopsis* treated with nitrate (Liu et al., 2019). AT5G45570 encodes for *Mutator*-like elements (MULEs), that contain two ubiquitin-like specific protease-like sequences (ULP1) (Van Leeuwen et al., 2007).

Table 4.2 presents the most up-regulated genes in the *METo A1-* line. The gene that showed highest fold expression change was AT2G03965 which encodes a hypothetical protein with unknown function. AT2G15555 is a non-coding RNA. AT3G01345 encodes an expressed protein with unknown function, and was also the most up-regulated gene in the *METo A1+* line. AT2G05914 is antisense long noncoding RNA. AT2G13547 is protein coding with unknown function.

Gene ID	Log2-fold change	pvalue	Gene description
AT3G01345	4.948714704	0	Expressed protein (protein coding)
AT2G03965	4.759638044	0	Hypothetical protein (protein coding)
AT1G32010	3.623900449	1.45E-177	Myosin heavy chain-like protein
AT1G67105	3.547390017	2.87E-200	Long noncoding RNA
AT5G45570	3.286079715	3.67E-140	Ulp1 protease family protein
AT1G43815	3.057527185	6.87E-121	Hypothetical protein (protein coding)
AT4G16215	2.972235981	2.74E-120	Hypothetical protein (protein coding)
AT2G13547	2.943352023	2.01E-111	Hypothetical protein (protein coding)
AT1G23915	2.822400079	4.48E-101	Hypothetical protein (protein coding)
AT2G05914	2.688879966	8.67E-92	Potential natural antisense gene, locus overlaps with AT2G05915
AT5G45095	2.502605971	4.89E-87	Hypothetical protein (protein coding)
AT4G08593	2.40217289	3.91E-73	Hypothetical protein (protein coding)
AT5G03090	2.278032221	6.93E-66	Mto 1 responding down protein
AT1G47660	2.271282935	1.67E-65	Hypothetical protein (protein coding)
AT2G15420	2.212183049	3.11E-62	Myosin heavy chain-like protein
AT1G20400	2.155171279	1.73E-59	Hypothetical protein (protein coding)
AT5G28235	2.089417593	7.65E-56	Ulp1 protease family protein
AT1G62580	2.049410411	2.15E-61	Encodes a flavin monooxygenase that binds NO
AT1G06963	1.967221399	1.36E-49	Long noncoding RNA
AT1G36020	1.907304001	4.03E-47	DEAD/DEAH-box RNA helicase family protein

Table 4.1. List of the top 20 up-regulated genes in primary roots of the *METo A1+* line

Gene ID	Log2-fold change	pvalue	Gene description
AT2G03965	7.73333981	0	Hypothetical protein
AT2G15555	7.45556100	0	RNA
AT3G01345	7.41787230	0	Expressed protein
AT2G05914	6.64877747	2.05E-290	Antisense long noncoding RNA
AT2G13547	5.64250783	3.76E-181	Protein coding
AT5G15360	5.58569378	2.81E-193	Transmembrane protein
AT1G67105	5.15030317	0	RNA
AT4G18150	5.06769911	4.16E-141	Serine/Threonine-kinase
AT4G25530	4.79753850	1.42E-123	FWA
AT3G23060	4.79434441	2.10E-295	RING/U-box superfamily protein

AT2G07750	4.68125240	2.20E-161	DEA(D/H)-box RNA helicase family protein
AT3G44070	4.62740269	4.60E-112	Glycosyl hydrolase family 35 protein
AT2G07213	4.56878242	2.40E-115	Potential natural antisense gene
AT4G03950	4.56868055	3.09E-106	Nucleotide/sugar transporter family protein
AT4G06643	4.46598073	5.41E-102	Hypothetical protein
AT2G19850	4.37383459	1.11E-100	Transcription repressor
AT3G54730	4.33436613	1.17E-94	Transcription repressor
AT3G30770	4.27717188	1.75E-92	Eukaryotic aspartyl protease family protein
AT2G04885	4.25748937	6.47E-92	Long noncoding RNA
AT3G45820	3.64863938	1.65E-61	Protein coding

Table 4. 2. List of the top 20 up-regulated genes in primary roots of the METo A1-line.

4.2.1.2 Genes with decreased in expression

For genes with decreased in expression, only one gene was down-regulated in the *METo A1+* line. AT4G10640 was down-regulated in the *METo A1+* line which encodes for ABNORMAL SHOOT 6 (ABS6) as shown in Table 4.3. The AT4G10640 was also showed reduced expression in the *METo A1-* line. Table 4.4 presents the most down-regulated genes in the *METo A1-* line. The gene that showed highest fold expression change was AT4G10640 which encodes for ABNORMAL SHOOT 6 (ABS6). AT5G08710 encodes for Regulator of chromosome condensation (RCC1) family protein.

Gene ID	**Log2-fold change**	**pvalue**	**Gene description**
AT4G10640	-2.351519712	2.20E-71	ABNORMAL SHOOT 6, ABS6, IQ-DOMAIN, IQD16

Table 4.3. List of the top 20 down-regulated genes in primary roots of the METo A1+ line.

Gene ID	**Log2-fold change**	**pvalue**	**Gene description**
AT4G10640	-4.267367069	8.268E-123	ABNORMAL SHOOT 6, ABS6, IQ-DOMAIN, IQD16
AT5G08710	-3.141267103	3.02694E-74	Regulator of chromosome condensation (RCC1) family protein
AT5G25880	-2.848800777	1.36749E-40	NADP-MALIC ENZYME 3 (NADP-ME3)
AT2G22590	-2.7943023	1.89384E-70	UDP-Glycosyltransferase superfamily protein
AT3G59330	-2.496290141	3.47246E-31	Solute carrier family 35 protein
AT1G63240	-2.494857044	1.46723E-34	ROS1-ASSOCIATED METHYL-DNA BINDING PROTEIN 1 (RMB1)

AT5G26270	-2.320832599	1.28667E-30	Transmembrane protein
AT4G17730	-2.140174474	1.04176E-87	SYNTAXIN OF PLANTS 23 (SYP23)
AT4G17260	-2.059751299	1.29131E-43	Lactate/malate dehydrogenase family protein
AT2G35720	-2.050452025	2.73793E-19	ORIENTATION UNDER VERY LOW FLUENCES OF LIGHT 1 (OWL1)
AT4G16250	-2.033074936	8.89558E-79	PHYTOCHROME D (PHYD)
AT2G09250	-1.999768254	1.06121E-20	Long noncoding RNA
AT2G27535	-1.997682353	8.58175E-19	Ribosomal protein L10A family protein
AT2G37900	-1.912412267	6.77172E-20	Major facilitator superfamily protein
AT1G62540	-1.870122746	2.88946E-25	FLAVIN MONOOXYGENASE GLUCOSINOLATES- OXYGENASE 2 (FMO GS-OX2)
AT1G80340	-1.865280335	1.16155E-24	GIBBERELLIN 3-OXIDASE 2 (GA3OX2)
AT3G55920	-1.864061249	1.4702E-36	CYCLOPHYLIN 12-2 (CYP21-2)
AT5G48850	-1.79706264	7.17349E-19	SULPHUR DEFICIENCY-INDUCED 1 (ATSDI1)
AT4G07820	-1.740940389	4.55708E-38	CAP (Cysteine-rich secretory proteins)
AT4G19865	-1.713704218	5.42199E-19	Galactose oxidase/kelch repeat superfamily protein

Table 4.4. List of the top 20 down-regulated genes in primary roots of the METo A1- line.

4.2.2 Genes that were mis-regulated in both *METo A1+* and *A1-*

The reduced primary root phenotypes are similar in both the *METo A1+* and the *A1-* lines. Therefore genes that were mis-regulated in both *A1+* and *A1-* were checked. 18 genes are upregulated in both lines and these were further investigated, as shown in the Table 4.5. Among these genes includes transposable elements, coding genes and non-coding genes. AT3G30820 encodes for Retrotransposon ORF-1 protein. Increased expression of AT3G30820 suggested that overexpression of *MET1* may cause disruption in DNA methylation that leads to transposon reactivation. Similarly, increased transcripts of retrotransposon was observed in a chromatin-remodeling (DDM1) mutant, *ddm1* (Lippman et al., 2004). AT2G05914 encodes for antisense long non-coding RNA. In plants, long non-coding RNA (lncRNAs) are found to be associated with root development, seedling light response, flowering time and stress response (Amor et al., 2009; Liu et al., 2010; Wang et al., 2014; Ariel et al., 2014).

AT1G62580 encodes for flavin containing monooxygenase FMO GS-OX-like protein which showed higher affinity for nitrate oxide (NO) (Mulaudzi et al., 2011). AT1G67105 is one of the nitrate-responsive lncRNAs, which was expressed in cauline leaves (Liu et al., 2019) and is a candidate gene regulated by RdDM

(Kurihara et al., 2008). AT5G15360 encodes for transmembrane protein which is found expressed during early Arabidopsis seedling development (Meyer et al., 2012). AT5G24240 encodes for phosphatidylinositol 4-kinase gamma-like protein (PI4Kγ3) which is found controlled by DNA demethylation and abiotic stresses (Akhter et al., 2016). The AT5G24240 showed increased expression in a *drm1drm2cmt3* mutant as compared with wild type (Kurihara et al., 2008). Meanwhile, only 1 gene, AT4G10640 which encode for ABNORMAL SHOOT6 (ABS6) was found down-regulated in both *A1+* and *A1-* line (Table 4.6).

Gene ID	line	Log2-fold change	pvalue	Gene description
AT1G62580	A1+	2.04941041	2.15E-61	NITRIC OXIDE-DEPENDENT GUANYLATE CYCLASE 1, NOGC1
	A1-	3.57256853	2.55E-157	
AT1G67105	A1+	3.54739001	2.87E-200	RNA
	A1-	5.15030317	0	
AT2G03965	A1+	4.75963804	0	Hypothetical protein
	A1-	7.73333981	0	
AT2G04885	A1+	1.50477483	2.47E-31	Long non-coding RNA
	A1-	4.25748937	6.47E-92	
AT2G05914	A1+	2.68887996	8.67E-92	Antisense long non-coding RNA
	A1-	6.64877747	2.05E-290	
AT2G13547	A1+	2.94335202	2.01E-111	Protein coding
	A1-	5.64250783	3.76E-181	
AT2G15420	A1+	2.21218304	3.11E-62	Myosin heavy chain-like protein
	A1-	2.92526779	4.14E-38	
AT3G01345	A1+	4.94871470	0	Expressed protein
	A1-	7.41787230	0	
AT3G14670	A1+	1.03896722	4.32E-16	Protein coding
	A1-	3.27708803	9.63E-63	
AT3G30820	A1+	1.00776948	4.88E-17	Retrotransposon ORF-1 protein
	A1-	3.26570509	1.20E-48	
AT3G44070	A1+	1.53960923	2.07E-32	Glycosyl hydrolase family 35 protein
	A1-	4.62740269	4.60E-112	
AT3G45820	A1+	1.08699094	6.43E-19	Protein coding
	A1-	3.64863938	1.65E-61	
AT4G03950	A1+	1.42393681	1.70E-28	Nucleotide/sugar transporter family protein
	A1-	4.56868055	3.09E-106	
AT4G06643	A1+	1.23115779	8.68E-23	Hypothetical protein
	A1-	4.46598073	5.41E-102	
AT4G08593	A1+	2.40217289	3.91E-73	Hypothetical protein
	A1-	2.98894046	1.56E-39	
AT4G16215	A1+	2.97223598	2.74E-120	Hypothetical protein
	A1-	1.08662741	1.92E-06	

AT5G15360	A1+ A1-	1.49120372 5.58569378	9.22E-31 2.81E-193	Transmembrane protein
AT5G24240	A1+ A1-	1.48863260 1.86062042	1.88E-30 1.10E-22	Encodes PI4Kγ3, localizes to the nucleus

Table 4.5. List of genes with increased (positive log2-fold change) transcript level in both *METo A1+* and *A1-* lines.

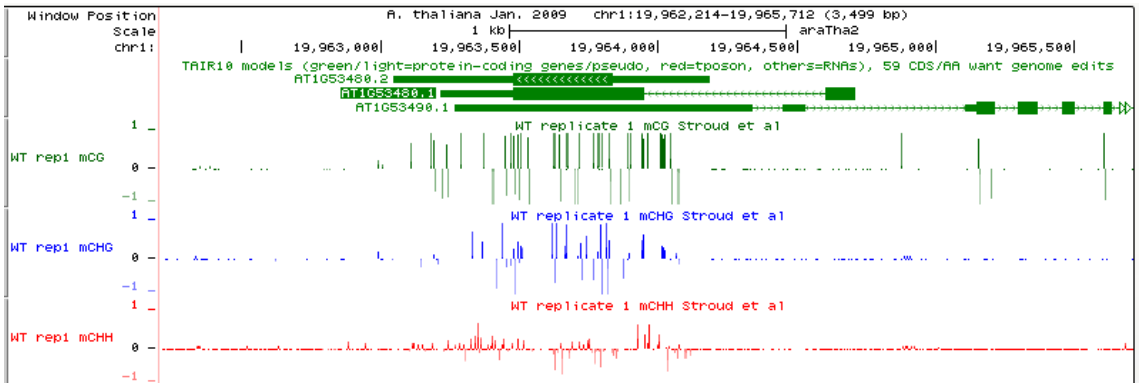
Gene ID	**line**	**Log2-fold change**	**pvalue**	**Gene description**
AT4G10640	A1+ A1-	-2.35152 -4.26737	2.20E-71 8.27E-123	ABNORMAL SHOOT 6, ABS6, IQ-DOMAIN, IQD16 Expression profiling of cytokinin action in Arabidopsis (Rashotte et al., 2003)

Table 4.6. List of genes with decreased (negative log2-fold change) transcript level in both *METo A1+* and *A1-* lines.

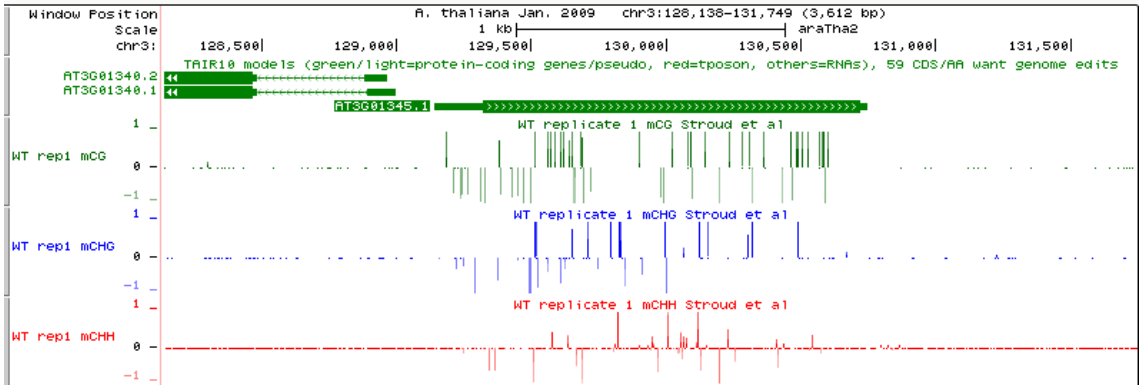
4.2.3 Genes with significant changes in expression with dense methylation

To investigate the direct and indirect effect of *MET1* over-expression, three differentially expressed genes were selected based on the presence of dense methylation in the category of upstream (dense methylation in the promoter or 5' region), genic (gene region) and region (dense methylation that is restricted to a particular exon or intron). These genes with methylation in all three sequence contexts of CG, CHG and CHH were identified using the epigenome browser (http://neomorph.salk.edu) (Figure 4.1). AT1G53480 encodes for METHIONINE RESPONDING DOWN1 (MRD1) in which showed reduced transcript level in the *Arabidopsis* mutant with increased SAM level, *mto1* mutant (Goto et al., 2002). This gene was selected as the methylation status of this gene was reported under the control of MET1 (Havecker et al., 2012).

AT1G53480 METHIONINE RESPONDING DOWN 1 (MRD1)



AT3G01345 HYDROLASE



AT3G44070 GLYCOSYL HYROLASE FAMILY 35 PROTEIN

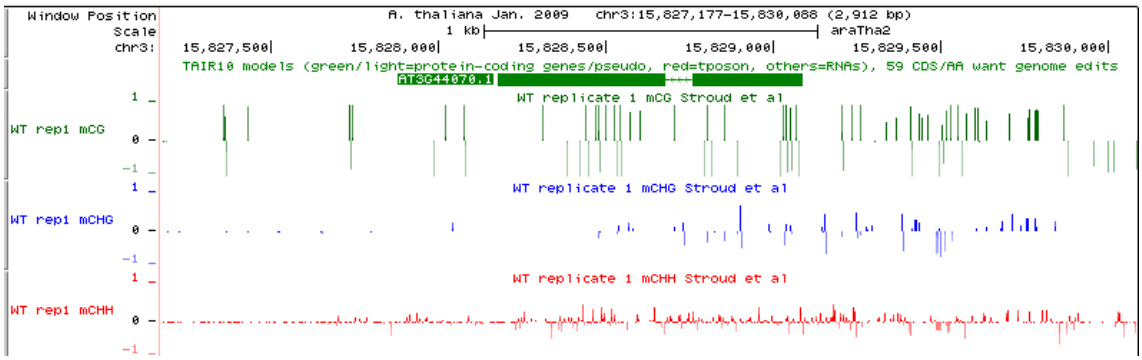


Figure 4.1. The dense methylation patterns of selected loci observed in *Arabidopsis Col-0* background which are lost the *met1* mutant. Methylation types including CG, CHG and CHH are colour coded and represent by different line. The methylation profile is obtained from epigenome browser (http://genomes.mcdb.ucla.edu/AthBSseq/).

MET1 has been shown to interact with other epigenetic regulators, such as the VIM, and HDA6 proteins. Therefore, the genes differentially expressed in the *METo* line were compared with the publicly-available transcriptome data for mutant in genes encoding these factors to look for potential overlaps (Zhang et al., 2006; Lister et al., 2008; To et al., 2011; Kim et al., 2014) (Table 4.7).

Gene ID	line	Log2-fold change	pvalue	Location of dense methylation	**Description**
Common genes in *METo* and *vim1/2/3*					
AT3G44070	A1+ A1-	1.539609231 4.627402691	2.07E-32 4.60E-112	Region	Glycosyl hydrolase family 35 protein
AT4G03950	A1+ A1-	1.423936816 4.568680549		Region	Nucleotide/sugar transporter family protein
Common genes in *METo* and *hda6*					
AT1G67105	A1+ A1-	3.54739002 5.150303	2.87E-200 0		ncRND
AT2G15420	A1+ A1-	2.21218305 2.925268	3.11E-62 4.14E-38		myosin heavy chain-like protein
AT3G44070	A1+ A1-	1.539609231 4.627402691	2.07E-32 4.60E-112	Region	Glycosyl hydrolase family 35 protein
AT1G67105	A1+ A1-	3.547390017 5.150303174	2.87E-200 0	Upstream	HDA6 target locus

Table 4.7. List of genes with increased (positive log2-fold change) transcript level in the *METo A1+* and *A1-* lines. These genes with dense cytosine methylation in all three sequence contexts (CG, CHG and CHH) were also common in the epigenetic mutants.

4.2.4 Confirmation of candidate genes by quantitative real-time PCR (qRT-PCR)

To validate the RNA-seq results reported in the previous section, quantitative reverse transcriptase PCR (qRT-PCR) was performed (with three biological replicates and 3 technical replicates) on a Bio-RAD CFX96. The relative expression level was computed by the method of comparative deltadeltaCT, and *ELONGATION FACTOR* gene was picked to normalize all results. Genes were selected which showed increased expression in the *METo A1+* and *A1-* lines based on RNA-seq data. The qPCR results confirmed the increased expression in these genes in all cases tested (Figure 4.2)

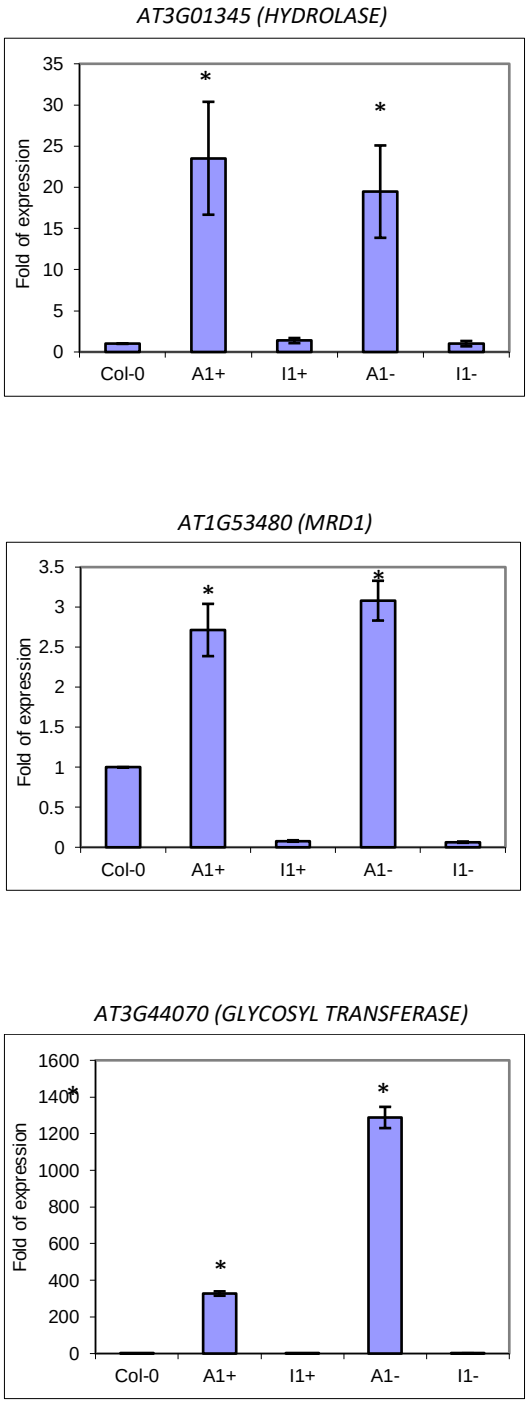
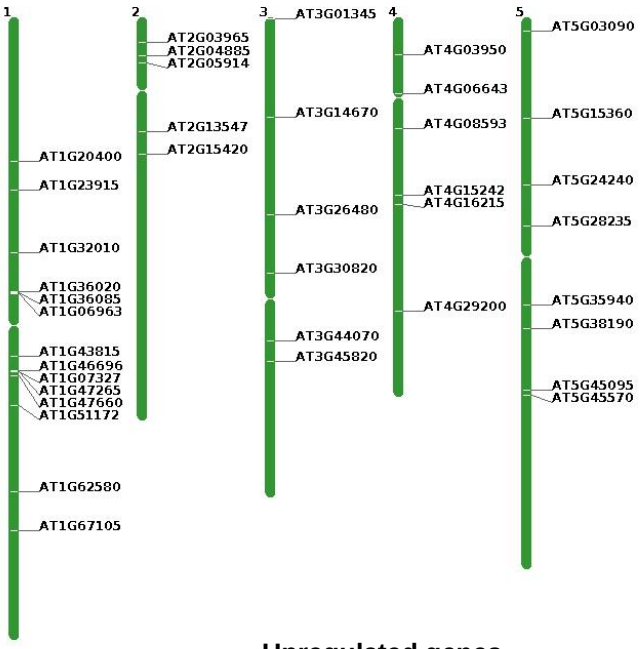


Figure 4.2. Validation of RNA-seq results using qPCR for the selected genes. The analysis was performed in the *METo* line with transgene (+) and a line that had lost the transgene (–). Line *A1* expresses a catalytically active *MET1* transgene and line *I1* expresses a catalytically inactive *MET1* transgene. The mean and standard error are shown for three biological replicates each having three technical replicates for each line. Values on the y-axis represent the fold difference compared with the control line. Statistically significant differences were determined using a one-way ANOVA and Tukey's post hoc test ($p < 0.05$).

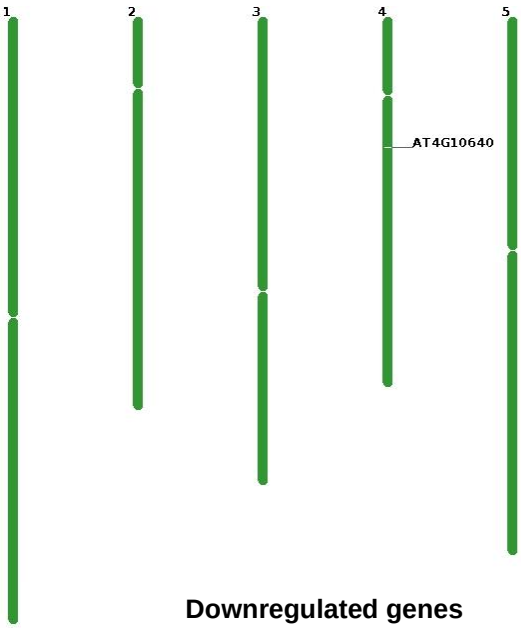
4.2.5 The chromosomal distribution of differentially regulated genes

To examine the chromosomal distribution of the differentially regulated genes in the *METo A1+* and *A1–* lines, the scattering of respective locations was displayed on the according chromosomes using the chromosome Mapping Tool available at TAIR (http://www.Arabidopsis.org/jsp/ChromosomeMap/tool.jsp). In the *METo A1+* line, all up- and downregulated genes exhibited an unbiased distribution among the chromosomes (Figure 4.4A). The genes for *METo A1–* lines also showed an unbiased distribution for both the up- and downregulated genes' categories (Figure 4.4B). No biased distribution occurred clustering near the centromeric and pericentromeric regions.

(A)
METo A1+

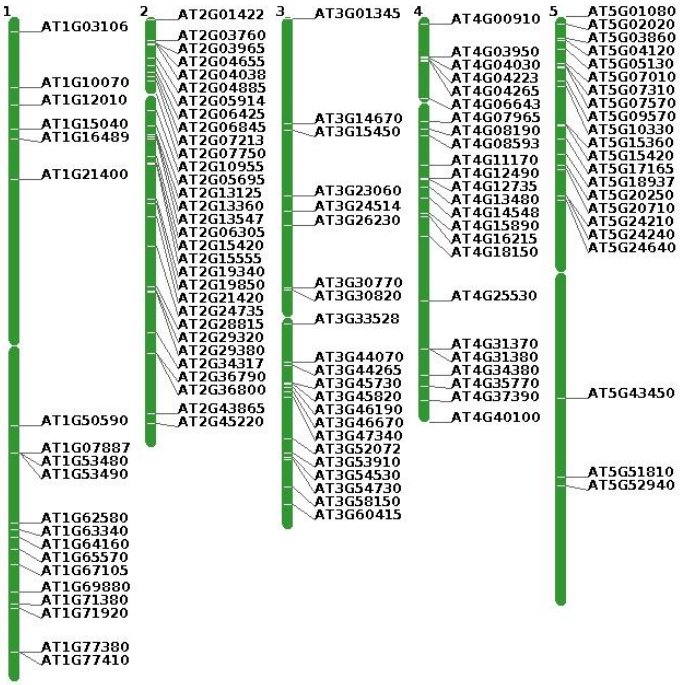


Upregulated genes

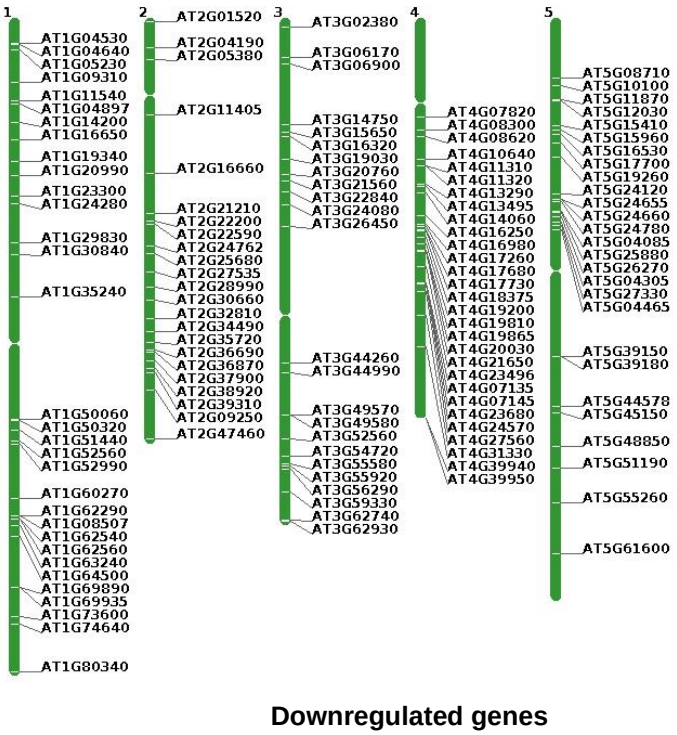


Downregulated genes

(B)
METo A1-



Upregulated genes



Downregulated genes

Figure 4.3. Chromosomal distribution of differentially expressed genes. Upregulated and downregulated genes in either (A) *METo A1+* line, (B) *A1–* line For depicting the chromosomal distribution, the chromosome map tool of the TAIR database was used (http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp).

4.3 Discussion

In order to analyse the effects of the *MET1* overexpression on global changes in gene expression and in association with the reduction in the primary root phenotype, the RNAseq analysis was conducted in a 10-day-old wild type (*Col-0*) and *METo* lines (A1+ and A1-) plants using ILLUMINA sequencing. Thirty nine loci were transcriptionally up-regulated in the *METo A1+* line meanwhile 119 loci were transcriptionally up-regulated in the *METo A1-* line when compared with WT plants (fold change > 1.0 and p-value < 0.05) with differential expression observed in the 1.0- 7.0 -fold range. One gene was found with reduced expression in *METo A1+* whereas 114 genes with reduced expression in *METo A1-* line.

Among the upregulated genes in both *METo A1+* and *A1-* include transposable elements (TEs) such as noncoding RNAs and retrotransposon. Mutants with disrupted epigenetic changes have been found to exhibit developmental defects in plants, some of which are heritable. Early generations of *ddm1* hypomethylated mutants in *Arabidopsis* showed only minor morphological alterations, but morphological abnormalities became more pronounced following repeated self-pollination over several generations (Kakutani et al., 1996). However, several inherited developmental defects were not related to the *DDM1* gene (Kakutani et al., 1999). Some of these mutations are the result of transposons being mobilised due to the loss of DNA methylation from the transposon. For instance, insertion of a *CACTA1* transposon in the *DWF4* gene, which encodes 22-α-hydroxylase in the brassinosteroid biosynthetic pathways, caused lack of expansion in the petioles and shoots (Mlura et al., 2001). Similar transposition was also seen in *met1cmt3* double mutants (Kato et al., 2003). Long noncoding RNAs and retrotransposon were found with increased expression in both *METo A1+* and *A1-* lines which may suggest the association of increased expression in TEs with the short root phenotype in the *METo* lines. Nevertheless, this requires further analysis.

In the Chapter 2, gibberellin (GA) hormone analysis revealed that both *METo A1+* and *A1-* lines showed reduced GA level. GA is a class of tetracyclic diterpenoid carboxylic acids and one of the major hormones that are crucial in plant growth and development from seed germination to vegetative growth (Ogawa et al., 2003). Therefore, having the optimal bioactive GA level tightly controlled during GA biosynthesis, signalling and degradation is important for having normal growth and development in plants. In the GA biosynthesis process, two key enzymes are responsible for the final step used to produce bioactive GA, namely, GA 20-OXIDASE (GA20ox) and GA0 3-OXIDASE (GA3ox) (Hedden and Thomas, 2012). However, from the transcript analysis, no genes encoding enzymes involved in GA biosynthesis showed changes in expression. Therefore, no correlation was observed between the endogenous GA level and changes in the gene expression encoding enzymes in GA biosynthesis pathways.

Previous studies of *Arabidopsis* mutants with short root phenotypes identified three major genetic regulatory pathways involved in the regulation of root apical meristem (RAM) establishment and maintenance. This includes the PLETHORA (PLT) transcription factors, that belong to the AP2/ERF superfamily (Aida et al., 2004; Santuari et al., 2016). *plt1* shows subtle changes of the cell division pattern in the quiescent centre (QC) and root cap cells whereas *plt1 plt2* double mutants exhibit considerably shorter primary roots caused by RAM exhaustion (Galinha et al., 2007). The GRAS transcription cofactors SCARECROW (SCR) and SHORT-ROOT (SHR) are also involved in RAM maintenance. *Arabidopsis scr* and *shr* loss-of-function mutants display changes in QC identity and possesses a shorter primary root as compared with the wild type (Helariutta et al., 2000; Nakajima et al., 2001). Finally, WUSHEL-RELATED HOMEOBOX 5 (WOX5) is also transcription factor which is specifically expressed in the QC and regulates stem cell homeostasis in roots (Sarkar et al., 2007; Nardmann et al., 2009). Nevertheless, based on the RNAseq data in the *METo* lines, none of these genes showed altered transcripts levels which therefore the short root phenotype in the *METo* lines did not associated with a failure to maintain the RAM.

Several genes with increased transcript levels were also found to be activated in the epigenetic mutants, including the *vim1/2/3, hda6* and *ddm1* mutants. These genes include AT4G03950 and AT2G15420, which encode the nucleotide/sugar transporter family protein and myosin heavy chain-like protein, respectively which suggested that they may regulate common loci.

The analysis of the transcriptome profiling revealed the influence of *MET1* over-expression in *Arabidopsis METo* line with the reduced primary root phenotype. *MET1* could be both a transcriptional repressor and activator, as both up- and down-regulated genes were identified in the *METo A1+* line with the *MET1* transgene and the *METo A1-* line which had lost the transgene through segregation. Changes in gene expressions were identified, but further experiments are needed, such as bisulphite sequencing of the selected candidate genes, to identify if the changes in gene expression are directly correlated with changes in DNA methylation.

Chapter 5

General Discussion

Modification of DNA methylation influences changes in gene expression and contributes to epigenetic variation, as manifested in variation in plant phenotypes. Altering the level of DNA METHYLTRANSFERASE 1 (MET1) by over-expression of MET1 provides a strategy to create novel epigenetic variants. The identification of phenotypic changes as a consequence of MET1 over-expression in Arabidopsis, could enable the identification of genetic loci underlying the observed phenotypes, which could have wider implications in improving plant traits.

5.1 Phenotypic changes in the *MET1* overexpression lines

Plant phenotypes are regulated by gene expression, which is controlled by *cis*- and *trans*-acting factors and genetic variation. Another layer of genetics, termed epigenetics, can also cause phenotypic variation through different epigenetic states, some of which are associated with defined DNA methylation patterns (Paszkowski and Grossniklaus, 2011; Becker et al., 2011). Genetic variation is due to changes in gene sequence while epigenetic variation is independent of gene sequence, but differs in the modification of mechanisms that modulate chromatin structure through DNA methylation or histone modifications. Epialleles are heritable through mitosis and/or meiosis via a mechanism such as cytosine methylation in a CG sequence context (Paszkowski and Grossniklaus, 2011). After replication, the CG methylation is maintained by DNA METHYLTRANSFERASE 1 (MET1) which uses the hemimethylated DNA to copy the methylation to the newly synthesized strand (Finnegan et al., 1998). During the cytosine methylation, MET1 obtains a methyl group from S-adenosyl methionine (SAM) (Ravanel et al., 1998).

Disruption of the DNA methylation components, such as MET1, is detrimental to plant growth and development. Late flowering, thick inflorescence stems, development of an aerial rosette and delay in senescence are examples of growth defects observed in the *Arabidopsis met1-1* mutant (Kankel et al., 2003). These phenotypic variations result from changes in global methylation which affecting various genes, implying plants response to global DNA changes.

Several phenotypes under the control of DNA methylation have been documented, including flowering time (Soppe et al., 2000), floral symmetry (Cubas et al., 1999) and stomatal development (Yamamuro et al., 2014). Reduced primary root is another phenotype associated with DNA methylation as exemplified by treatment with the DNA methylation inhibitor, 5-azacytidine, which causes a reduction in the primary root length (Virdi et al., 2015). Distinctive phenotypic changes were observed in *Arabidopsis* overexpressing different forms of *MET1*. Reduced primary root length was observed in the *METo A1+* and *METo I1+* lines, which contain the *MET1* transgene with and without catalytic function, respectively, and this phenotype was maintained in the line that had lost the *MET1* transgene. The observed reduced primary root length was accompanied by a reduction in root meristem size as measured by a reduction in the meristem cell length (Figure 2.1). Hormone analysis of the roots using liquid chromatography-mass spectrometry revealed a reduction in gibberellin (Figure 2.2) and cytokinin (Figure 2.4). A short primary root was also observed in the *ga1-3* mutant which has low GA levels due to impaired GA biosynthesis (Ubeda-Tomás et al., 2012). It seems that dose-dependent application of bioactive GA can enhance the root growth in the *METo* line, suggesting that GA deficiency may contribute to reduced primary root length phenotype in the *METo* lines. Nevertheless, based on the primary root transcriptome analysis described in the Chapter 4, no changes in expression of the genes encoding the enzymes involved in GA metabolism were observed in the *METo* lines with reduced GA levels.

5.2 MET1 over-expression effects

There are a number of examples of epigenomic state feedback regulation in plants. For instance, the DNA demethylase *REPRESSOR OF SILENCING* (*ROS1*) expression is influenced by DNA methylation levels across the genome (Williams et al., 2015). Additionally, expression of the *INCREASE IN BONSAI METHYLATION 1* (*IBM1*) which encodes a H3K9 demethylase is affected by H3K9me2/DNA methylation levels inside an intronic repeat (Rigal et al., 2012). Similarly, an increase in DNA methylation may happen when MET1 is over-expressed. This could influence changes to demethylases, causing alterations in the DNA methylation patterns across the genome.

MET1 overexpression could inhibit other proteins from interacting, thereby disrupting a multiprotein complex. For example, MET1 may form methylation complexes with other methyltransferases including CMT2, CMT3 and DRM2. Changes in the phenotypes were observed in the *METo A1+* line, which contains the *MET1* transgene with catalytic and SAM-binding functions. In addition, in the *METo I+* containing the *MET1* transgene with a mutation at the catalytic site, reduced primary root phenotype is also observed, which supports the protein–protein interaction mechanism, as the overproduction of catalytically inactive enzyme could also give an inhibitory effect to a multiprotein complex.

5.3 The effect of mutated SAM-binding domain in *MET1* overexpression

Another proposed explanation for the phenotypes observed on *MET1* overexpression is a competition-based mechanism, referring to the methyl group aquired from SAM during the methylation process. This would be plausible, since both the *METo A1* line and *METo I1* line contain the *MET1* transgene with SAM-binding function. Therefore, to distinguish the possible effect of a SAM feedback mechanism resulting from the titration by increasing *MET1* copy number, a *MET1* transgene with devoid of SAM-binding function was generated.

In *Arabidopsis* overexpressing the *MET1* transgene with a mutation at the SAM-binding site (*METsammut* transgene), namely the *MSM* lines, the primary root length resembles wild-type phenotype, in contrast to *METo* lines. The *MET1* transcript level was increased approximately 5- to 10-fold in the *MSM* line, suggesting that increased *MET1* solely is insufficient for the induction of gene expression and phenotypic changes. There could be several options that explains this observation. The *MSM* line contains a *MET1* transgene with mutations at motifs I and motif X denoted as SAM-binding domains, which are highly conserved between plants, mammal and prokaryote methyltransferases (Kumar et al., 1994). Therefore, this suggests that one or both SAM binding regions are required for the induction of phenotypes as observed for the *METo* lines.

Alternatively, the MET1 mutation in the *MSM* lines could cause a conformational change that prevents this MET1 version from interacting with other partner molecules thus preventing any effects from its overexpression. Alterations in enzyme conformation due to DNA mutations that change amino acids has been observed in a herbicide resistance mechanism that prevents herbicide binding at the enzyme target site (Kaundun, 2014). For instance, a mutation at the carboxyltransferase domain of the gene encoding ACETYL COA CARBOXYLASE (ACCASE) enzyme that functions during fatty acid biosynthesis causes inhibition of the herbicide binding (Liu et al., 2007).

5.4 Future prospects and outlook

Epigenetic variation offers advantages as a resource for variation in plant traits through mechanisms other than genetic variation. Phenotypic variation in plants has been shown to be induced by epigenetic variation at a higher rate than genetic mutation (Schmitz et al., 2011). The creation of epigenetic recombinant inbred lines (epiRILs) in *Arabidopsis thaliana* has allowed for comparison to RILs which were made from wild type and a mutant defective in DNA methylation, *met1* or *ddm1* (Vongs et al., 1993; Finnegan et al., 1996). EpiRILs have a lot of variation in terms of DNA methylation, but only a little variance in terms of DNA sequence

(Johannes et al., 2009; Reinders et al., 2009). EpiRILs are created in the same way that traditional recombinant inbred lines (RILs) are created: two genetically divergent parents are crossed, and inbred lines are established from the recombinant progeny. Two populations of epiRILs of *Arabidopsis* thaliana were made from the cross between *Col-0* and methylation mutant *ddm1* (Johannes et al., 2009) and *met1* (Reinders et al., 2009) respectively. A study by Zhang et al. (2018) made the comparison between epiRILs, RILs and natural ecotypes that for similar phenotypic variation and found that the epiRILs offer more advantages in which it create stable phenotypic variation. Epigenetic variations are mostly responsible for the phenotypic variances seen among epiRILs. The analysis of epiQTL mapping of epiRIL population studies revealed that the heritability of flowering time and the length of root were linked with changes in DNA methylation rather than a difference in the DNA sequence (Cortijo et al., 2014).

Phenotypic variation in the *Arabidopsis METo* lines was observed during seed germination and root growth, two important traits in plant development and crop improvement. Identification of the factors involved in these traits will help to produce further desirable crop traits. For instance, DNA methylation profiling could be used to identify defective clones in palm oil with the undesirable ‘mantled phenotype’ at an early plant stage (Ong-Abdullah et al., 2015). Detection at an early stage can prevent loss, an important aspect of an efficient agriculture sector.

5.5 Outlook and open questions

5.5.1 DNA methylation analysis of selected target genes

Several parts of this studies need to be investigated further. The candidate genes can be selected based on gene expression analysis and contain MET1 dependent DNA methylation located within or adjacent to its loci, as in the Chapter 4. This is to investigate if the changes in gene expression corresponds to changes in DNA methylation as detected by using bisulphite sequencing.

Candidate genes can be chosen based on three categories. First, genic methylation which is DNA methylation that present throughout the entire body of the gene. Second, 5'methylation that is located within the promoter region of the gene, but not present in the gene body. Third, regional methylation, which is DNA methylation that is localized at a particular exon or intron. This could assist to distinguish target genes under direct and indirect influence of MET1 over-expression.

5.5.2 SAM levels

DNA methyltransferases require S-adenosylmethionine (SAM) for the methyl group donor during methylation process (Fontecave et al., 2004). It is hypothesized that MET1 over-expression may interfere with levels of available SAM. However the SAM levels have never been analysed in *MET1* over-expression lines. Therefore, the SAM level could be determined by liquid chromatography mass spectrometry (LC-MS) (Mao et al., 2015). This is an important consideration as there could be feedback regulation between MET1 and other factors, meaning that MET1 over-expression requires more SAM supply in which may therefore be limiting. Additionally, this could provide insights if changes in SAM have the same consequences as MET1 depletion, which can be manipulated as indirect mechanisms to change DNA methylation patterns.

5.5.3 Phenotypic analysis of MSM lines

In comparison with the *METo* lines which have reduction in primary root length (Figure 2.1 in Chapter 2), the MSM lines show normal primary root length (Figure 3.12c in Chapter 3). As seen in chromomethylase *cmt3* mutants, the plants have wild-type morphology and develop normally even after numerous generations, despite reduced CHG methylation (Bartee et al., 2001). In contrast, over generations of self-crossing, phenotypic variation in particular mutants, such as *met1*, *ddm1* and *ibm1*, grows stronger (Ito et al., 2015). Therefore, phenotypic analysis of the *MSM* lines produced in this study need to be further conducted in

succeeding generations. In addition, although no changes in plant morphology are observed, there could be unnoticed phenotypic changes that occur at cellular level. For example, In hypomethylation mutants including *met1*, changes in epidermal cell morphology were observed (Vassileva et al., 2016). Additionally, the *MSM* lines could be tested by stress treatment which would help in identification of new target genes.

Chapter 6

Materials and Methods

6.1 Materials

6.1.1 Plant materials

The *Arabidopsis thaliana* plants that were used throughout this project derived from the Columbia-0 unless stated otherwise. The *Arabidopsis* mutant lines with higher S-adenosylmethionine (SAM) level; *mto1-1*, *mto2-1* and *mmt* were previously described (Kocsis et al., 2003; Inaba et al., 1994; Bartlem et al., 2000) and originally purchased from Nottingham Arabidopsis Stock Centre (NASC). The *sam1* (SALK_073599c) *sam2* (SALK_097197c) mutant lines were originally obtained from the SALK T-DNA insertion collection (Alonso et al., 2003).

The *Arabidopsis MET1* overexpression lines with and without catalytic function were obtained from Dr Samuel Brocklehurst (P. Meyer lab, Centre for Plant Sciences, University of Leeds). T1 transformants of *Arabidopsis MET1* overexpression were selected on hygromycin medium and were self-pollinated. T2 progeny plants of each line were grown without selections and were genotyped. For genotyping negative transformants (without the *MET1* transgene), primers were designed annealing either side of an intron of the MET1 gene. These primers amplify part of the endogenous MET1 gene yielding a 1161 bp fragment. Meanwhile, for positive transformants, these primers amplify a part of the MET1 cDNA transgene without the intron, producing a 786 bp fragment. Plants with (+) and without (-) the transgene were isolated and selfed. In order to confirm that the transgene had been lost in (-) plants and to identify (+) lines that were homozygous for the transgene, T3 seeds of these plants were placed on hygromycin. For further analysis, one (-) plant and one (+) plant, homozygous for the transgene, were selected for each line.

6.1.2 Bacterial strains

Plasmid cloning was carried out using *Escherichia coli Dh5α* (New England Biolabs). Plant transformation was carried out using *Agrobacterium tumafaciens GV3101:PMP90* (Hellens et al., 2000).

6.1.3 Vectors and DNA sequences

The pGreen0029 containing the 35S-nos cassette was provided by Dr Michael Watson. The Arabidopsis *MET1* cDNA cloned in pGEM-T easy (Promega) was provided by Dr Elena Zubko (P. Meyer lab, Centre for Plant Sciences, University of Leeds).

6.1.4 Primer sequences

	Forward primer	Reverse primer
qPCR analysis		
AT5G03090	GAACATTGGCTCTCGACGACTG	CAAGCTCGAAAACCACGATCC
AT5G26270	CTGTACCCGTTCCGTGTCTT	TGAGGGGTAAAACCAGAAACA
AT2G19850	GAAATTTCACGCAGGATAAAGGA	CTACAATAGCATAGGCGACAAAG
AT3G01345 HYD	CGAGGCCAAAGCTTCCAAAC	ATTGACTTCAAGGGGAGCCG
AT1G53480 MRD1	GTCCGTTTTAGGCTTAGGACCG	GAAAACATTTCTAGCACAAAGAAA
AT5G51810	ACCGAGACTATTTCCGAGGATT	TGTTTGGCATGGAGGATAATG
AT1G07920	CTCTCCTTGAGGCTCTTGACCAG	CCAATACCACCAATCTTGTAGACATCC
AT3G63110 IPT3	CCAAGATGGATGCTAACGTG	CGACACAGTATCTGTGCTTGGT
AT3G23630 IPT7	ACTCCTTTGTCTCAAAACGTGTC	TGAACACTTCTCTTACTTCTTCGAGT
AT4G29740 CKX4	CCCTTCCCATTATTGACCAG	CGAAATACGGAACATCTTGTACG
AT1G75450 CKX5	CCATGGTCCTCAAATTAGTAACG	TCTGAGCATCTCATCACCTCTC
AT3G14440 NCED3	CGGTGGTTTACGACAAGAACAA	CAGAAGCAATCTGGAGCATCAA
AT3G17520 AIL1	GAGAAGTTGGCGGAGACAGT	TTCTCCTTCGCACTCTTACTCTC
AT5G66400 RAB18	TCGGTCGTTGTATTGTGCTTTTT	CCAGATGCTCATTACACACTCATG
AT5G49160 MET1	AGACCTCCGAAGAAGAAACAGA	CTCACGGTGATTGGACGGAA
AT1G07920 (EF1 α)	CTCTCCTTGAGGCTCTTGACCAG	CCAATACCACCAATCTTGTAGACATCC
Semi qPCR analysis		

AT1G19640F-JAS	CGCTCCTTACTATGCTGCGA	GGCTTCGGGTTTGGACCTTA
AT1G69770F-CMT3	GTCCCCAAGAGAAAGGGAGC	TTGGGTGGGCCAAAGAGTTT
AT1G01480F-ACC	GCGACTAACAATCAACACGGA	CGCTCATAACCACCCTCTCC
AT3G02470F-SAMD	GATCTGTTCCGTCGTTGAGGA	TAACCGAGGGGAGCTTCGTA
AT4G34840F-MTN	TTGGCACAGTTCCTGCATCA	GGAAGGTGTTCCGCATACCA
AT5G62480F-GT	AACGTGGAGTAACGGTCCAC	CGTGTGCCTTGTAAGGGCTA
AT3G06930F-4B	CGGTCCAAAGATGGCTCACA	TGTCCTGCTGTGTACTTAGCTC
AT5G49020.1F-4A	AAGGATCAGCCTTGCCGAAC	AGCCAGTGCAGCAAACATTG
AT3G12270F-3	ACCTGGCTACCATGAAACCG	GCAGATGAATGCTTGAGGCG
AT4G16570.1F-7	TGAGGACAATGACCAACCCG	TAAGACTCACACGCCGTCAC
AT2G19670.1F-1	ACCGTCTTGTATGCTCGCAA	CGGTCCTGTTGAGAAACCCA
AT1G76090.1F-STL	CAAGGCCCAAGTCACTGGAA	AGGAAGTGCGTCTCCTCTCT
AT5G49810F-SMM	AGTCTGTGAAGAAGCCGTGG	GGAGGGTATGGAAGGCATCG
AT4G19020.1F-CMT2	ATCTTGCCACTTCCTGGTCG	ACAAGACGGCTCAAAGCGTA
AT1G07940EF1a-F	GCGTGTCATTGAGAGGTTCG	GTCAAGAGCCTCAAGGAGAG
AT1G07920 (EF1 α)	GCGTGTCATTGAGAGGTTCG	GTCAAGAGCCTCAAGGAGAG
Genotyping		
MET1 transgene	TCCAATCACCGTGAGAGACAC	TCATAGTCTATAGACATCATTGCTTG
Sam1	CCTACTCTGTTTCTGTCTTCATTCC	GCCAAAGATCACATTGCCCT
Sam2	TTTTATGTCACTTGAGTGTGG	TTCAGTGAGACGAGCACCAA
SALKLBb1.3	ATTTTGCCGATTTCGGAAC	

6.2 Methods

6.2.1 DNA analysis and Cloning Techniques

6.2.1.1 Isolation of genomic DNA from plants

Isolation of plant genomic DNA was carried out using a modified Vejlupkova and Fowler, (2003) method. 560 µl of extraction buffer (2 M NaCl; 200 mM Tris-HCl, pH 8.0; 0.07 M EDTA pH 8.0; and 20 mM Sodium Bisulphite) and 50 µl of 5 % Sarcosyl was added to 0.5 g of plant tissue ground in liquid nitrogen. The suspension was mixed and incubated at 60^{o}C for 1 hour. An equal volume of phenol:chloroform:isoamyl-alcohol (12:12:1) was added and then centrifuged at 12,000 x g for 10 min at 4^{o}C. The upper phase was transferred to a new Eppendorf tube and the phenol:chlorophorm:isoamyl-alcohol extraction was

repeated. The DNA was precipitated using 300 µL isopropanol and pelleted by centrifugation at 12,000 x g for 10 min at 4ºC. The supernatant was discarded and the pellet washed with 70% ethanol. The DNA was re-pelleted, the supernatant was removed and the pellet allowed to air-dry. The DNA was re-suspended using 100 µl of sterile distilled water. The DNA was stored at -20ºC.

6.2.1.1.1 Polymerase chain reaction (PCR)

PCR for genotyping was performed using MyTaq DNA polymerase (Bioline) according to manufacturer's instruction. The reaction consisted of 0.3 µL MyTaq DNA polymerase, 1X concentration red buffer, 10 µM forward and reverse primers, DNA template and sterile water to make the final volume to 50 µL. The following thermal cycling profile was used: initial denaturation at 95ºC for 5 min, 25-29 cycles of denaturation at 65ºC for 30 sec, annealing temperature (Ta, according to the primer annealing temperature) for 15 sec and extension at 72ºC for 10 sec followed by final extension at 72ºC for 5 min.

6.2.1.2 Isolation of plasmid DNA from *E. coli*

Isolation of plasmid DNA from *E. coli* was carried out using a modified alkaline lysis method (Sambrook et al., 1989). Individual colonies were grown overnight at 37ºC in liquid lysogeny broth (LB) medium (10 g/L bacto-tryptone; 5 g/L bacto-yeast extract; 10 g/L NaCl) supplemented with the required antibiotics. 1 ml of this culture was transferred to a 1.5 mL tube and centrifuged for 5 min at maximum speed to pellet the cells. The supernatant was removed and the pellet resuspended in 100 µl of solution I (50 mM glucose; 25 mM Tris-HCl, pH 8; and 10 mM EDTA, pH 8) and thoroughly mixed. 200 µl of fresh solution II (0.2 M NaOH and 1% SDS) and 150 µl of solution III (3 M Potassium Acetate; pH 8) were added prior to incubation on ice for 10 – 30 minutes. The tube were then centrifuged at 12,000 x g for 10 min at 4ºC. The supernatants were transferred into a new tubes with an equal volume of ice-cold isopropanol. The suspension was centrifuged at 12,000 x g for 10 min at 4ºC to pellet the precipitated plasmid DNA. The

supernatant was discarded, and the pellet was washed with 70% ethanol and allowed to air-dry. The DNA was re-suspended in 50 µl of sterile distilled water and RNase A (20 mg/l).

6.2.1.3 Isolation of plasmid DNA from *Agrobacterium*

Isolation of plasmids from *Agrobacterium* was carried out using a modified alkaline lysis method (Wang, 2006). After overnight LB medium culture with antibiotics, cells were harvested. The cell pellets were lysed with 100 µl of lysis buffer (50 mM glucose; 25 mM Tris-HCl, pH 8; 10 mM EDTA, pH 8; and 4 mg/ml Lysozyme), 200 µl of a freshly prepared solution II (0.2M NaOH and 1% SDS), and 150 µl of 3 M sodium acetate (pH 4.8). Cell debris was pelleted, and the supernatant was extracted with 1x phenol:chloroform:isoamylalcohol (25:24:1) and 1x chloroform. The plasmid DNA was precipitated from the upper layer with isopropanol, and washed with 70% ethanol. The DNA was re-suspended in 50 µl of sterile distilled water and kept at -20^{o}C.

6.2.1.4 Production of *MET1* overexpression with mutated SAM binding domain

A 1863 bp fragment containing the mutations at the SAM binding domain (motif I and motif X) were synthesized from Genewiz (England). The fragment contains the restriction sites Bsu36I and SpeI to allow for the exchange of the original fragment with the mutated fragment after the restriction of enzyme digestion. The *MET1* cDNA with mutated fragment was cloned into pGreenII009, between the Nos promoter and Nos terminator. *Arabidopsis Col-0* was transformed with this construct using floral dip method.

6.2.1.5 Restriction digests

The digestion reactions were carried out using the appropriate restriction enzymes, in a final volume of 20 µL. Approximately 1 µg of DNA was digested according to the manufacturer's instructions.

6.2.1.6 Agarose gel electrophoresis of DNA

DNA molecules were separated electrophoretically using horizontal agarose gel (07-2.0%), containing 0.1 µg/ml ethidium bromide in TAE (Sambrook et al., 1989). DNA was visualised on a UV trans-illuminator and captured by a digital imaging system (Syngene Bio-imager and GeneSnap).

6.2.1.7 Ligation of vectors and insertion of DNA

The ligation reaction was carried out using T4 DNA Ligase (Promega) according to the manufacturer's instructions. An insert to vector ratio of 3:1 was used, and the reaction was incubated at 4^{o}C overnight.

6.2.1.8 *Escherichia coli* transformation

E. coli competent cells were made according to (Sambrook et al, 1989). *E. coli* was grown in a shaking incubator in 500 ml of liquid lysogeny broth (LB) media (10 g/l bacto-tryptone; 5 g/l bacto-yeast extract; 10 g/l NaCl) at 37^{o}C. When an OD600 0.4 was reached cells were pelleted (6000 g) and re-suspended three times using 100 mM $MgCl_2$, 100 mM $CaCl_2$ and 85 mM $CaCl_2$ 15% glycerol. The final re-suspension was aliquoted, frozen using liquid nitrogen and stored at -80^{o}C. For heat shock transformation, 10–50 ng of DNA plasmid, or DNA from ligation reactions was added to thawed competent cells. The mixture was incubated on ice for 10 min followed by incubation at 42^{o}C for 90 seconds and the immediate transfer to ice for 2 min. 900 µl of SOC medium (2% tryptone, 0.5%

yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added to the cells, followed by incubation in a 37^oC shaking incubator for 1 hour. Positive bacteria were selected on LB medium with antibiotics, by growing overnight at 37^oC.

6.2.1.9 *Agrobacterium* plasmid transformation

The electrocompetent *Agrobacterium* was prepared by following the protocol described by Shen and Forde (1989). The fresh overnight cultures were inoculated in 500 ml of LB medium with antibiotic, and grown overnight at 28^oC in a shaking incubator, to OD_{600} = 0.4. The cells were harvested and washed three times with ice-cold sterile distilled H_2O. The cell pellets were re-suspended in a 5 ml solution of ice-cold sterile distilled H_2O and 10% (v/v) sterile glycerol, aliquoted, and stored at -80^oC.

6.2.1.9.1 *Agrobacterium* plasmid electroporation

For *agrobacterium* electroporation, 1 mm gap cuvettes were used to electroporate the plasmid DNA, pSoup and electrocompetent *Agrobacterium* cells. Electroporation was carried out using the BioRAD Gene pulser cell-porator, with the following parameters: C = 25 µF, R = 400 Ω, 8-9 ms delay, and pulsed at V = 1.8 KV. LB medium (950 µl) was added and incubated in 1.5 ml tubes, followed by growth in a shaking incubator at 28^oC, for four hours. Positive cells were selected on LB plates with antibiotics, and incubated at 28^oC for three days.

6.2.2 *Arabidopsis* transformation by floral dip

The floral dip transformation was performed by following methods described by Clough and Bent (1998). The *Arabidopsis* plants were grown until they were flowering. The *Agrobacterium* colony, which contained the construct, was inoculated in 100 ml of LB medium, and incubated overnight at 28^oC with vigorous agitation. The *Agrobacterium* cells were harvested and re-suspended in 5%

sucrose and 0.05% SilwetL-77 surfactant to a final OD_{600} = 0.8. The plants were inverted, and all the above-ground parts were dipped into the solution for 10 seconds with gentle agitation. The dipped plants were covered, and placed in the dark overnight. The plants were watered and grown normally. Dipping was repeated after one week. The plants were grown until seeds could be collected. Primary transformants were selected with MS medium containing selection medium.

6.2.3 Plant growth condition

6.2.3.1 Seed sterilization

Sterilised seeds were sown on Murashige & Skoog basal media (Duchefa Biochemie). Seeds were sterilized by washing with 70% ethanol for 5 minutes followed by 10% bleach for 10 minutes and the 3X 5 minute washes with sterilized water.

6.2.4 Germination

6.2.4.1 Seed preparation

Plants were grown in tissue culture for 2 weeks and moved to soil. The seedlings were carefully removed from the plates and grown in growth chambers in long day conditions (16 hour photoperiod), at 22^{o}C. Following dehiscence, seeds were harvested from plants. Seeds were ready to harvest from the plant when siliques readily opened when touched. Seeds were stored in universal tubes at ambient temperature and humidity.

6.2.4.2 Germination for plant growth

Sterilised seeds were stratified at 4^{o} C for 48 hours before being moved to a tissue culture room and germinated under long day conditions, 16 hour photoperiod (80 µmol.m^{-2}.s^{-1}) at 22^{o}C.

6.2.4.3 Germination assays

Seeds were sown in MS medium (4.4 g/l Murashige and Skoog with vitamins; 10 g/l sucrose; 1% agar; pH 5.8) supplemented with or without NaCl, IAA, kinetin, ABA, GA and paclobutrazol. Seeds were stratified at 4^{o} C for 48 hours before being moved to a tissue culture room and germinated under long day conditions, 16 hour photoperiod (80 µmol.m^{-2}.s^{-1}) at 22^{o}C. Seeds were scored for germination, defined by emergence of the radicle from the seed coat using a stereomicroscope to monitor radicle protrusion (Bewley, 1997). Observations were taken at daily interval. Independent biological replicates were performed for verification of results. The parental plants were grown again and the new batches of seed were harvested.

6.2.5 Root analysis

For the measurement of *Arabidopsis* primary root length, the seeds were sown in 120 mm square plate Petri-dishes containing MS media (1 % agar). Each plate contained 10 seedlings and was grown under long day conditions, 16 hour photoperiod (80 µmol.m^{-2}.s^{-1}) at 22^{o}C. At 7-day-old, root images were captured using a flat-bed scanner at 800 ppi (HP Scanjet G3110) and the root lengths were measured with ImageJ software (http://rsb.info.nih.gov/ij/).

6.2.6 Confocal microscopy of root meristem

Confocal microscopy to image *Arabidopsis* roots was performed using LSM 700 confocal laser scanning microscope. 10-day-old seedlings were dipped with propidium iodide (10 µg/ ml; Sigma) to stain the cell walls. After staining, seedlings were mounted under a coverslip in distilled water. For root meristem analysis, the cell length was measured along single epidermal cell files. The number of epidermal cells was used as measurement of the meristem size. The meristematic zone was defined as the region of isodiametric cells from QC up to the cell that was twice the length of the immediately preceding cell.

6.2.7 Endogenous hormone analysis

Primary roots were harvested from at least 0.5 g of 10-day-old wild type *Col-0* and *METo* (*A1+* and *A1-* lines) with three biological replicates each. The roots' fresh weights were measured, snap-frozen in liquid nitrogen before proceed with freeze drying. The samples were sent to Dr Jiri Malbeck (Laboratory of Hormonal Regulations in Plants, Institute of Experimental Botany, Prague, Czech Republic) for the cytokinin and gibberellin quantification using liquid chromatography mass spectrometry system.

6.2.8 Statistical analysis

Statistical tests were performed using one-way analysis of variance (ANOVA) analysis through Tukey's multiple comparison post-test using SPSS software. The data are presented as the mean ± standard error. Differences at $p < 0.05$ were considered significant.

6.2.9 Software and online tools for data analysis

The Neomorph platform (http://neomorph.salk.edu/epigenome.html) were used to extract the DNA methylation patterns to identify genes with dense DNA methylation patterns. The ThaleMine platform (https://apps.araport.org/thalemine/begin.do) was used to extract the annotation for extracted genes).

6.2.10 RNA analysis

6.2.10.1 RNA Extraction

RNA was isolated using method described in Stam et al. (2000). 750 µL of RNA extraction buffer (100 mM Tris HCl pH 8.5, 100 mM NaCl, 20 mM EDTA pH 8.0, 1% Sarcosyl) was added to 0.5 g of plant tissue ground in liquid nitrogen. The suspension was mixed and equal volume of phenol:chlorophorm:isoamyl-alcohol (12:12:1) was added then centrifuged at 12,000 x g for 10 min at 4^{o}C. The top phase was transferred to a new Eppendorf tube and the phenol:chlorophorm:isoamyl-alcohol extraction was repeated. The aqueous phases were precipitated with 375 µL isopropanol and 375 µL sodium citrate and chilled for 5 min in ice, followed by centrifugation at 12,000 x g for 10 min at 4^{o}C. The supernatant was discarded and centrifugation was repeated for 1 min to remove all isopropanol. The pellet was dissolved in 400 µL Diethylpyrocarbonate (DEPC) water and mixed with 400 µL 4M LiCl prior to overnight incubation on ice. The mixture was centrifuged at 12,000 x g for 15 min at 4^{o}C. The pellet was dissolved in 400 µL DEPC water and added with 40 µL 3M NaAc (pH 7.0) and 1 mL 99% ethanol for final precipitation. After incubation for 20 min at -20^{o}C, the mixture was centrifuged at 12,000 x g for 10 min at 4^{o}C. The pellet was washed with 1 mL 70% ethanol, and centrifuged again for 5 min at 4^{o}C. The supernatant was discarded and the tube was left open to allow remaining ethanol to evaporate. The pellet was dissolved in DEPC water. Extractions were quantified using the NanoDrop ND-1000 Spectrophotometer(Thermo Scientific). DNA was

removed using the Turbo DNase I kit (Ambion Applied Biosystems) according to the manufacturer's instructions. The DEPC water was generated by adding 1 ml DEPC (Diethylpyrocarbonate) per 1000 ml of distilled water (to final concentration 0.1%) and mixed thoroughly. The DEPC-mixed water was incubated for 12 hours at 37ºC before autoclaving for 15 min.

6.2.10.2 cDNA Synthesis and semiquantitative PCR

First strand cDNA was synthesized from 1 µg of extracted RNA using the SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The RNA, 2 µL 10 mM dNTP mix and 1 µL 10 mM oligo-dT were incubated at 65ºC for 5 min and then placed on ice for at least 1 min. The 1x concentration of First Strand Buffer, 0.1 M DTT and 40 units/µL of RNaseOut was added to each reaction. The mix was incubated 25ºC for 2 min and 1 µL SuperScript II Reverse Transcriptase was added. The mix was incubated for a further 10 min, then 42ºC for 50 min then final 70ºC for 15 min.

1 µL of the diluted cDNA solution was added to a standard MyTaq reaction (Bioline). The reaction consisted of 0.3 µL MyTaq DNA polymerase, 1X concentration red buffer, 10 µM forward and reverse primers, cDNA template and sterile water to make the final volume to 50 µL. The reaction was placed into a thermocycler with the following settings: initial denaturation at 95ºC for 5 min, 25-29 cycles of denaturation at 65ºC for 30 sec, annealing temperature (Ta, according to the primer annealing temperature) for 15 sec and extension at 72ºC for 10 sec/kb. When the thermocycler had performed 20 cycles, the reaction was held at 72ºC while 6 µL was removed from the total reaction. This step was repeated twice more every three cycles then run on an agarose gel. Using Elongation Factor 1α, which is ubiquitously expressed, the reaction's exponential phase could be determined and used to standardize for expression analysis.

6.2.10.3 Real-time quantitative PCR (qRT-PCR)

Real-time quantitative PCR was performed using Biorad CFX Real-Time PCR system. All qRT-PCRs were carried out using three biological replicates in a final volume of 25 µL containing 2 µL of cDNA, 1 µL of primers (each primer at 10 µM), 12.5 µL SYBR green PCR master mix and nuclease free water. Reactions were carried out in a sealed ninety-six well plate. The qRT-PCR reactions were carried out with the following cycle conditions; 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 10 s, 60^{o}C for 15 s and 72^{o}C for 15 s. Following cycling, the melting curve was determined in the range of 60-95^{o}C, with a temperature increment of 0.01^{o}C/sec. Each reaction was run in triplicate (technical replicates). A negative control was included in each run where a reaction was conducted in the absence of template (2 µL of nuclease-free water instead of 2 µL of cDNA).

Primer efficiency was determined by serial dilution of the template and the specificity of primer pairs was obtained by the melting curve analysis of the amplicons. *ELONGATION FACTOR (EF)* was used as an internal control for normalization. Quantification of the relative changes in gene expression was performed by using delta delta CT (ddCT) method (Livak and Schmittgen, 2001).

6.2.10.4 RNA-seq expression analysis

RNA was extracted from primary roots of 10-day-old wild type *Col-0* and *METo* (*A1+* and *A1-* lines) with three biological replicates each. The RNA samples were sent to Next Generation Sequencing Facility, Leeds Institute of Biomedical & Clinical Sciences, St James's University Hospital, Leeds. Next generation sequencing libraries were created from mRNA using the TruSeq Stranded mRNA kit (Ilumina) and sequenced on a NextSeq 500 to generate 75 bp single end sequence data.

Data analysis was carried out Dr Ian M Carr (University, Leeds, England). The data was aligned to the Arabidopsis genome (TAIR web site https://www.arabidopsis.org) using STAR aligner (Dobin et al., 2013). Reads mapping to each transcript were determined using the R package rsubRead (Liao et al., 2013) and pairwise comparisons between the wildtype sample and each of the modified samples were performed using the R package DeSeq2 (Love et al, 2014) to identify transcripts whose expression varied markedly between the control and experimental sample for each condition Reads were used to calculate the mean value of read mapping to a transcript in all samples in the analysis (base Mean), the change in expression between the control sample and the test sample given as a Log to the base 2 value (log2FoldChange), the standard error of variation for the log2FoldChange values in the analysis (lfcSE = log fold change Standard Error), the Wald statistic; the log2FoldChange divided by lfcSE, the probability the result is real; the log2FoldChange divided by lfcSE, compared to a standard Normal distribution to generate a two-tailed p-value (pvalue) and the *p*-value adjusted for multiple testing using the Benjamini-Hochberg test (Padj).

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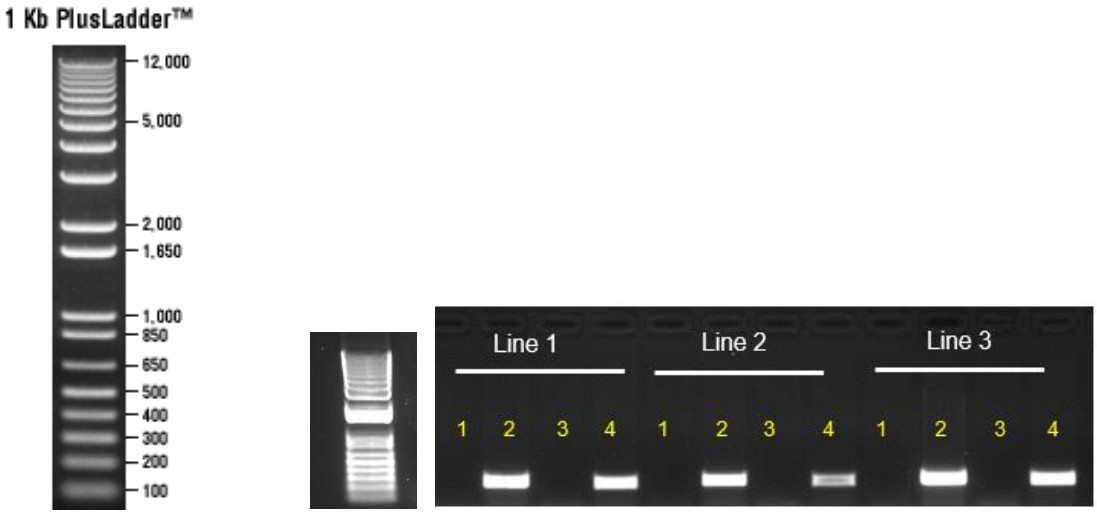
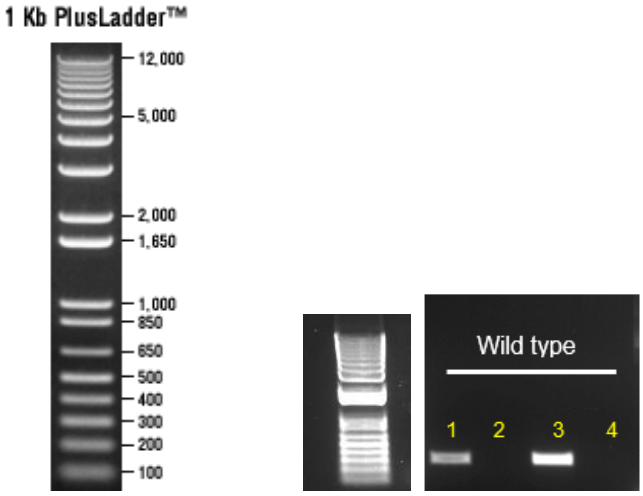
Appendices

Chapter 3





The bands were not really distinguishable because the bands were not distantly separated as shown in the agarose gel electrophoresis picture.

Gel intensity

No	Gene	A (MTO1)	B (MTO2)	C (MMT)
1	PRMT4B	1.242	1.093	0.950
2	PRMT 4A	0.784862	1.111869	0.942105
3	PMRT3	0.740855	1.081152	0.899293
4	PRMT1A	1.024024	1.103768	1.04059
5	PRMT7	1.23099	1.077128	0.851264
6	SMT3	0.707991	0.992542	0.738233
7	JMT	1.093269	1.19458	0.908269
8	CMT2	0.742962	0.7322	0.494074
9	CMT3	0.859668	0.450664	0.571336
10	ACCS	1.452097	1.144411	1.332317
11	SAMD	1.383702	0.906854	0.72243
12	MTN2	1.005221	0.935888	1.26108
13	GT	1.225022	1.161526	1.074008
14	MMT	1.14197	0.834702	0.813683

Relative band intensity measured by ImageJ analysis software as shown in Figure 3.4 in the Chapter 3.