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Research Paper

Genome-wide analysis of cytosine DNA Methylation revealed salicylic acid promotes defense pathways over seedling development in pearl millet

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Abstract

Cytosine DNA methylation is an epigenetic regulatory system used by plants to control gene expression. Methylation pattern always changes after abiotic stresses, pathogens and pest infections or after a treatment with salicylic acid (SA). The latter is a key player in plant development and defense against insect herbivores, pathogens, and abiotic stresses. The roles of SA on the methylation patterns and the plant development were carried out in four pearl millet

(*Pennisetum glaucum*) varieties. Seedlings of four early-flowering photosensitive genotypes (PMS3, PMI8, PMG, and PMT2) were grown on MS medium supplemented with null or different doses of SA. Root growth was used as a parameter to evaluate the effects of SA at early stage development. DNA from these seedlings was extracted and Methylation-Sensitive Amplified Polymorphism (MSAP) was measured to assess the effects of SA on methylome. The methylation analysis revealed that SA treatment decreased the methylation, while inhibiting the root growth for all varieties tested, except in PMG at 0.5mM, indicating a dose and a genotype response-dependence. The methylation level was positively correlated with the root growth. This suggests that SA influences both the methylome by demethylation activities and the root growth by interfering with the root development-responsive genes. The demethylation process, induced by the REPRESSOR OF SILCENCING 1 (ROS1) may activate R genes, or GH3.5 and downregulate the hormonal pathway under root development. These findings showed the pearl millet metabolism prioritized and promoted the defense pathways over vegetative development during stress.

Key words

Cytosine DNA Methylation; Salicylic acid; demethylation; root growth; MSAP; Pearl millet

Introduction

Pearl millet, *Pennisetum glaucum* (L.) R. Br., is a major rural grown cereal in Sahelian region in Africa and in the dry areas of tropical, India, China, the United States, Russia, etc.[1,2]. However, this cereal is faced with numerous biotic and abiotic constraints[3-7]. These are pausing as potential source of food insecurity in light of high human population growth and reduction in food diversity and land degradation. To address these issues, the plant defense and development must be studied. DNA methylation is one the most important regulatory systems that control the plant development and defense-responsive genes in plant. The Cytosine base may be methylated by DNA methyltransferases (DNMTs)[8,9], or demethylated by REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), and DEMETER like-proteins (DME 2,-3)[10]. Cytosine DNA methylation controls gene expression either at transcription[11] or through posttranslational gene silencing (PTGS)[12,13]. Despite being heritable mechanisms[14], DNA methylation pattern may be altered by multiple factors such as abiotic[15,16] and biotic elicitors such as environmental factors, pathogen and pest attacks[17,18]. Plants have evolved innate immune systems that recognize elicitors. These mechanisms are pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI)[19-21]. PTI is conferred by pattern recognition receptors (PRRs) that recognize salicylic acid (SA), PAMPs or endogenous elicitors[22].

Like elicitors, SA, a regulatory plant hormone[23], is seen capable of triggering plant defense mechanisms and affecting plant development. SA treatments have many effects on plant development, particularly in root growth[23-25]. The root growth is also considered to be under the control of epigenetic regulation for optimal growth under variable environments[26]. In

Arabidopsis, DNMTs except the DRM1 are strongly expressed in the root tip[27]. Furthermore, a genome-wide methylation analysis of *Arabidopsis* revealed that root methylation level is more than 22% at CG sites[28]. Other studies showed the implication of SA in plant defense. SA-responsive genes may induce oxidative stress, increase level of hydrogen peroxide (H_2O_2)[29], inhibit catalase activity, affect activation of pathogenesis-related genes[30], hypersensitive response (HR) and the systemic acquired resistance (SAR) during pest attacks and pathogen infection[31]. This molecular level interference was also observed when SA increase was blocked through the expression of a bacterial salicylate hydroxylase gene in transgenic nahG tobacco plants that compromised TMV-induced HR and abolishes SAR[32]. Whatever SA plays in plant cells, particularly in plant defense and root growth, the epigenetic mechanism behind these pathways remain unclear. However, few works have been conducted on the possible interaction between SA and cytosine DNA Methylation. This research focused on evaluating the possibility of the SA influencing the genomic methylation patterns, and its incidence in plant and defense development pathways in pearl millet using Methylation-Sensitive Amplified Polymorphism (MSAP) technique.

Results

Effects of salicylic acid on plant development

The SA effects were screened and measurements on root length were determined after 48hrs of growth under optimal conditions. In addition, the results showed a significant difference between the SA doses and between the varieties ($p< 0.05$), PMT2 and PMG being more sensitive to SA (0.5mM) than PMS3. The root growth decreased when the concentrations of SA increased. At high SA concentration (3mM), the root growth was completely inhibited, except for PMG

variety (Figure 1). SA also delayed the germination, particularly at 0.5mM dose ($p<0.05$) (Table 1).

Effects of salicylic acid the DNA methylation level

Genome-wide methylation analysis was carried out for 0.5mM SA dose. The effects of SA treatment on the pearl millet methylome using MSAP polymorphism showed, 63% of epiloci are polymorphic. The level of methylation decreased following the application of 0.5mM of SA for all varieties, except PMG ($p<0.05$). This result was positively correlated to the root growth (Figure 2).

Furthermore, all the varieties tested including the controls were external cytosine hypermethylated (hemimethylation, mCCGG), except PMT2 where the hypermethylation was internal. In addition, the hypomethylation state mostly occurred in the internal cytosine methylation (PMS3, PMT2 Treated plants, PMG control plants). The varieties are highly hyper-hemimethylated (mCCGG), while the methylation level is low at the internal cytosine (CmCGG) for PMS3 (Hypomethylation) (Figure3).

Discussion

Salicylic acid inhibits plant development

A significant difference ($p< 0.05$) between the SA doses and between the varieties suggests a genotype-dependence response to SA. Root growth decreased when the concentrations of SA increased. The SA screening tests revealed the seedlings did not tolerate SA concentrations that inhibited root growth or delayed germination. These results were similar to many studies[23-25]. In pepper, this SA inhibiting effect is found for high doses[33]. In addition, high SA is seen inhibiting

the growth in *Arabidopsis*[34]. Like root growth, all the physiological aspects are affected by SA. The percentage of germination is also reduced by SA treatments in *Arabidopsis*[25]. This showed SA may have detrimental effects on early plant development in pearl millet either by disturbing the molecular root growth network or by involving in the posttranslational regulation pathways. However, others studies showed contradictory results. Low SA doses (10-50µM) improved the germination rate and the root length after 7 days, and 100--500µM of SA inhibited the plant development[35]. In wheat, SA increased the root length of the two varieties drought tolerant and drought susceptible[36], as well as in faba bean[37]. This demonstrated a plant genotype-dependence response to SA.

Salicylic acid decreases the methylation level

The methylation-sensitive amplified polymorphism analysis revealed a positive correlation between the level of methylation and the root growth of the seedlings. In fact, the root growth decreased with the treatment of SA, as well as the methylation level for PMS3, PMI8 and PMT2 varieties. In contrast, when the root growth increased after SA application, the methylation rate also increased for PMG variety. This indicated that SA and the DNA methylation may participate to downregulate the root growth genes. The expression of demethylase increased after SA treatment in Vitis amurensis[38], suggesting a decrease of methylation level. The non-methylated CCGG regions are lowered down in all varieties. The CCGG sites are variably hypermethylated either at external or internal cytosine bases. Hypomethylation often followed SA treatments, particularly for PMT2, indicating the activation and overexpression of certain R genes. These SA-demethylation targeted regions may play a dynamic role in plant development and defense.

Regulatory Model of Salicylic acid on pearl millet DNA methylation

SA shows playing different roles in many pathways, such as growth and defense. SA decreases methylation rate while inhibiting or minimizing the plant development. Indeed, two hypotheses may be stated (Figure 4). The first hypothesis is the Salicylic Acid Repressing Pathway 1 (SARP). In this case, SA may downregulate the RNA-directed DNA Methylation (RdDM) pathway through repression of Pol IV and AGO4, inducing the Repressor of Silencing 1 (ROS1) demethylase activity. The exogenous SA application is detected by the Flagellin Sensitive 2 (FLS2), one of the PRRs, that initiates the demethylation pathway[39]. Therefore, a mutated NRPD2 gene encoding DNA-directed RNA polymerases IV and V subunit 2 and responsible for the overexpression of an SA-Inducible gene procured a functional relationship between stresses signaling and the RNA-directed DNA methylation (RdDM) pathway[40]. Moreover, the same results were obtained in mutants partially defective in CG and non-CG methylation, and applications of demethylating agents, such as 5-azadeoxycytidine, reduces plant defense[41]. Enhancing RdDM in ROS1-4 plants leads to lowered resistance to Pst DC3000[42].

The second hypothesis is about the inhibition of plant development, particularly the root growth through SARP2. This SARP2 could be deployed to minimize the vegetative development like root growth and prioritizes the plant defense responses. Some of the R genes after activation by demethylation via SARP1 may control some genes implicated in root growth by downregulating its expression. Auxin is the main hormone regulating root and plant development[43-45]. Emerging evidence indicates that auxin is involved in plant disease susceptibility. GH3.5, a member of the GH3 family of early auxin-responsive genes in *Arabidopsis,* acts as a bifunctional modulator in both SA and auxin signaling during pathogen infection. Studies showed an upregulation of the

GH3.5 gene in an activation-tagged mutant gh3.5-1D led to an elevated accumulation of SA and increased expression of PR-1 in local and systemic tissues in response to virulent pathogens. Furthermore, two T-DNA insertional mutations of GH3.5 partially compromised the SAR with downregulation of PR-1 in systemic tissues[46]. The SA pathway is amplified by GH3.5 through inducing SA-responsive genes and basal defense components, whereas the auxin pathway is depressed through up-regulating indole-3-acetic acid (IAA) biosynthesis and down-regulating auxin repressor genes[46], causing root growth and plant development inhibition (Figure 4).

This finding offers a comprehensive insight into root development regulation by epigenetic and hormonal controls. SA pathway plays an important role in both plant development, as well as plant defense by initiating demethylation activities that inhibit or minimize root growth. Thus, plant metabolism prioritizes defense pathway above vegetative development during stress. However, the MSAP technique despite being able to detect methylation changes[17,47,48], do not include smallest fragments. The demethylated genes involved in the control of root growth-responsive genes should be further identified and characterized, as well as GH3 family and its interaction with R genes are genuine candidates to decipher the molecular basis of SA response in dryland crops. This can serve as a breeding entry point for selecting using priority genes in gene engineering programs to trigger biotic and abiotic tolerances amongst crops in light of the prevailing climate change phenomena.

Materials and methods

Plant materials

Four genotypes of pearl millet used: Souna 3 (PMS3), IBV 8004 (PMI8), Gawane (PMG), and Thialack 2 (PMT2). There are early-flowering photosensitive varieties with a growth cycle between 85--95 days. The seeds were obtained from the Senegalese Agricultural Research Institute of Bambey (Senegal).

Experimental design and growth conditions

A completely randomized block design was adopted for of SA treatments. The seeds were sown on MS medium (Sigma-Aldrich, M9274), with sucrose. The medium was supplemented with different doses of SA (0mM, 0.5mM, 1mM, 2mM and 3mM). The control treatment comprised of the medium with no SA added. The pH was adjusted to about 5.7 before autoclaving to ensure good plant development conditions. The media were autoclaved, cooled down to room temperature for 48hrs to be sure that the media was not contaminated. Prior to germination, the 15 seeds per treatment for each block were soaked in 1% calcium hypochlorite for 10 minutes and then washed with sterile deionized water to remove any remaining chlorine. The experiment was replicated three times. In total, 180 seedlings for each variety were used in the experiment. After sowing, the media were incubated at the darkness for 48hrs at room temperature to allow germination. After that time elapsed, the delay germination and the root length were recorded. The seedlings which had root lengths measuring less than 1mm were not considered as germinated but were recorded as seedlings with delayed germination. The media with the germinated seedlings were kept in a growth chamber set at 8hrs of darkness and a 16hrs of light,

and a temperature of 25°C. After the SA screening germination tests, the optimal dose of SA was chosen for molecular analysis according to the screening results. The choice depended on the capacity of the seeds of each variety to tolerate the SA applied.

MSAP epigenotyping

After one week in the culture chamber, five seedling leaves were harvested from each treatment including the control. DNA was extracted from the leaves of 20 seedlings (4 lines, 5 SA doses) with four replicates. DNA samples from SA treatments (20 samples) were considered individually and analyzed using ZR plant/seed DNA miniprep (Zymo Research, Cat No. D6020) following the company protocol and an alcohol isoamyl-chloroform step was included to increase DNA yield and quality. The extracted DNA was subjected to a Methylation-Sensitive Amplified Polymorphism (MSAP) analysis using two isoschizomeres MspI and HpaII targeting the CCGG motifs, and EcoRI targeting GAATTC sites. This method is based primarily on Amplified Fragments Length Polymorphism (AFLP) analysis[49]. It uses two different reactions with MspI/EcoRI, and HpaII/EcoRI. The isoschizomeres recognize the same DNA site 5'-CCGG-3' with different sensitivity to methylation. 100ng DNA samples were digested with NEB EcoRI-10 U at 37°C for 2hrs, before deactivation by heating at 65°C for 20mn. Then, the digested DNA fragments were subjected to NEB HpaII-10 U and NEB MspI-10 U digestion into two separated series at 37°C overnight. The restriction enzymes were deactivated by heating at 80°C for 15 min. Then, each MSAP series was subjected to ligation reactions (NEB T4 DNA ligase-10U) with EcoRI adaptors 5'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTCTAC-3' (10mM), and MspI/HpaII adaptor 5'-CGAGCAGGACTCATGA-3' (10mM). The adaptors were renatured by heating at 98°C for

5min, cooled down at room temperature in a polystyrene box for 2h, and held at 4°C. The ligation mix was incubated overnight at room temperature. Pre-selective amplification was performed in a 50 µL reaction volume with EcoRI primer 5'-GACTGCGTACCAATTC-3' (10mM), and MspI/HpaII primer 5'-ATCATGAGTCCTGCTCGG-3' (10mM), diluted restriction-ligation DNA and One Taq standard buffer. The pre-selective amplification was realized with the following temperature cycling conditions: one cycle at 94°C for 30 s; 30 cycles at 94°C for 30 s, 51°C for 30 s, and 72°C for 60 s, and finally one cycle at 72°C for 2 min. A 10-µL aliquot of the pre-selective amplification products was run on a 1.5% agarose gel with a 1kb DNA ladder to validate the pre-amplification step. Finally, a second amplification was done by selectively amplifying methylated DNA fragments using different primer combinations to generate an MSAP fingerprint. The PCR conditions were as follows: 94°C for 30s, 12 cycles at 94°C for 30s, 65°C for 30s, and 72°C for 60s, 23 cycles at 94°C for 30s, 51°C for 30s and 72°C for 60s, and finally one cycle at 72°C for 60s. The PCR products were then run on a gel, and the MSAP profile was used for data scoring.

Data scoring and analysis

The MSAP profile was then transformed into a binary matrix, with 1 as a presence of loci and 0 the absence of loci[50]. Only 50-bp or longer PCR products were considered for analysis. The internal cytosine methylation and the external cytosine methylation (hemimethylation) were considered in this study. The raw data from the MSAP profile were analyzed using Rmsap 1.1.8 to determine the percentage of methylated and unmethylated fragments and the types of methylation[51,52]. Analysis of variance was performed to compare the treatments and least significance difference (LSD) for mean separation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Table 1: Germination delay (%) of Pearl millet varieties under different levels of Salicylic acid. Four different Pearl varieties have been used. Same letter means significant difference at 0.05 level.

Varieties	SA doses (mM)				
	0	0.5	1	2	3
PMS3	0	33	7	0	0
PMG	13	67	10.25	17	7
PMI8	0	7	10.25	10.25	3
PMT2	0	17	7	7	7
	a	b	a	a	a

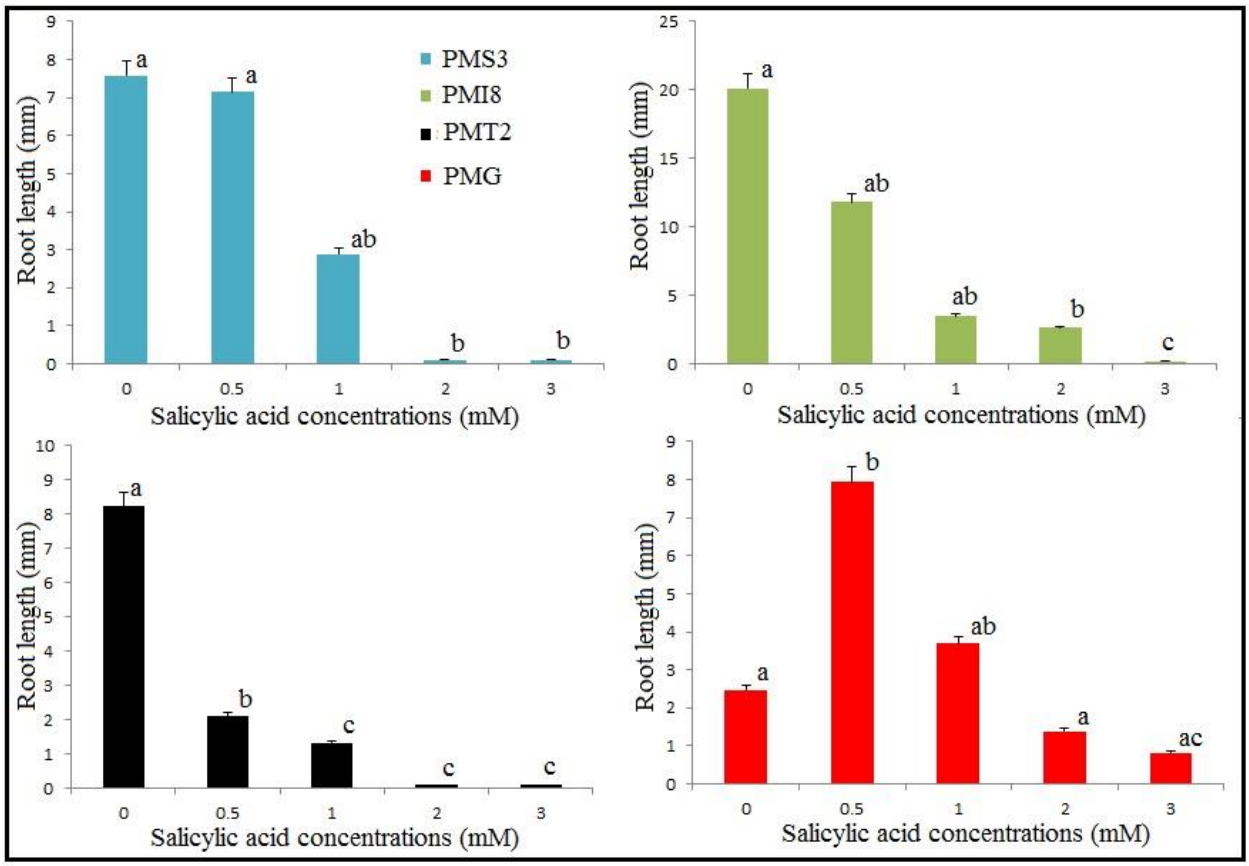

Figure 1: Root length after SA treatment. High SA concentrations inhibited root growth. Error bars indicate s.d. The results are a representative of three biological repetitions with 180 seedlings for each line. Same letter means no significant difference at 0.05 level. SA: salicylic acid.

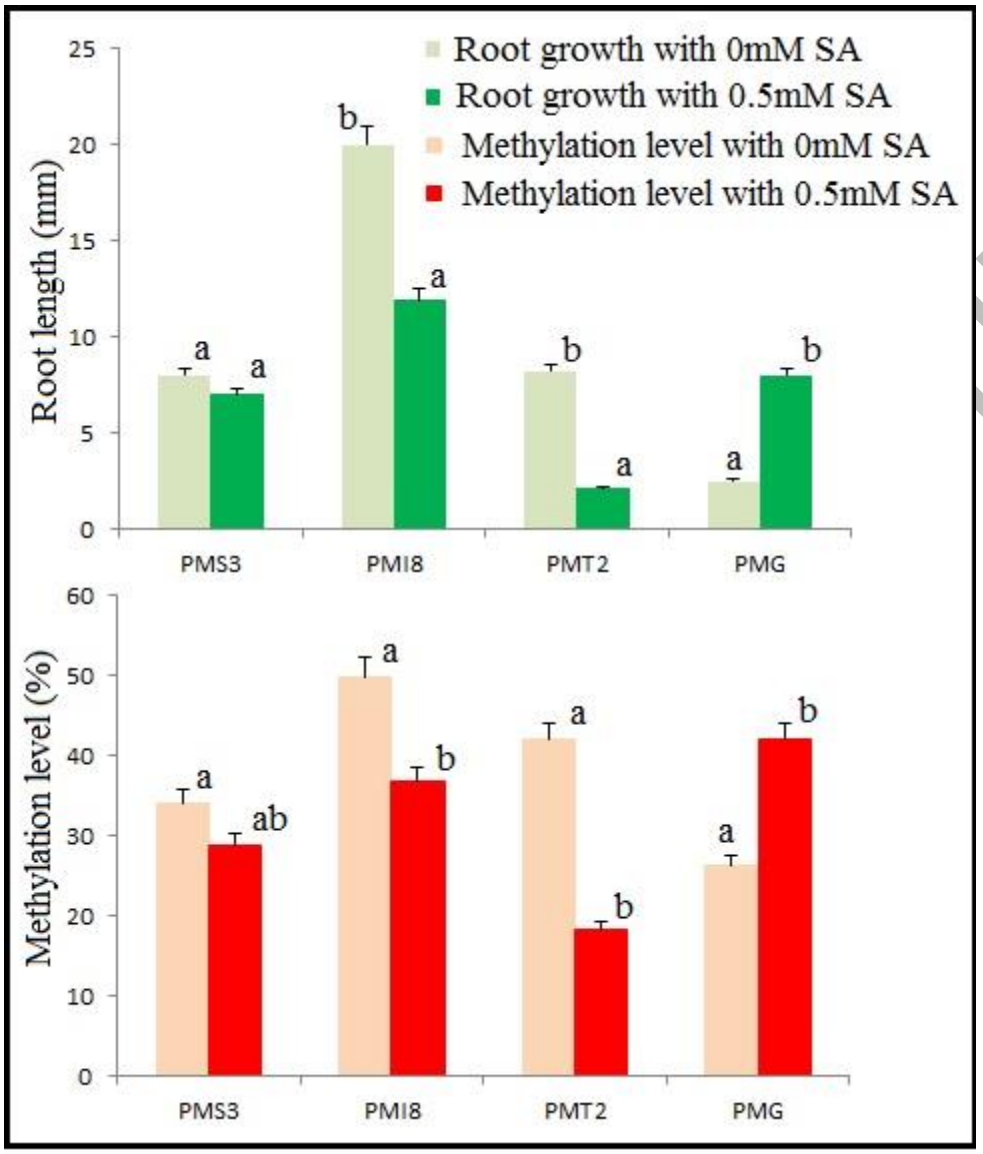

Figure 2: Dynamic correlation between methylation level and root growth under SA treatment. This showed a positive correlation. The methylation level decreased with the root length for PMS3, PMI8 and PMT2, in contrary for PMG. Same letter means no significant difference at 0.05 level. SA: salicylic acid.

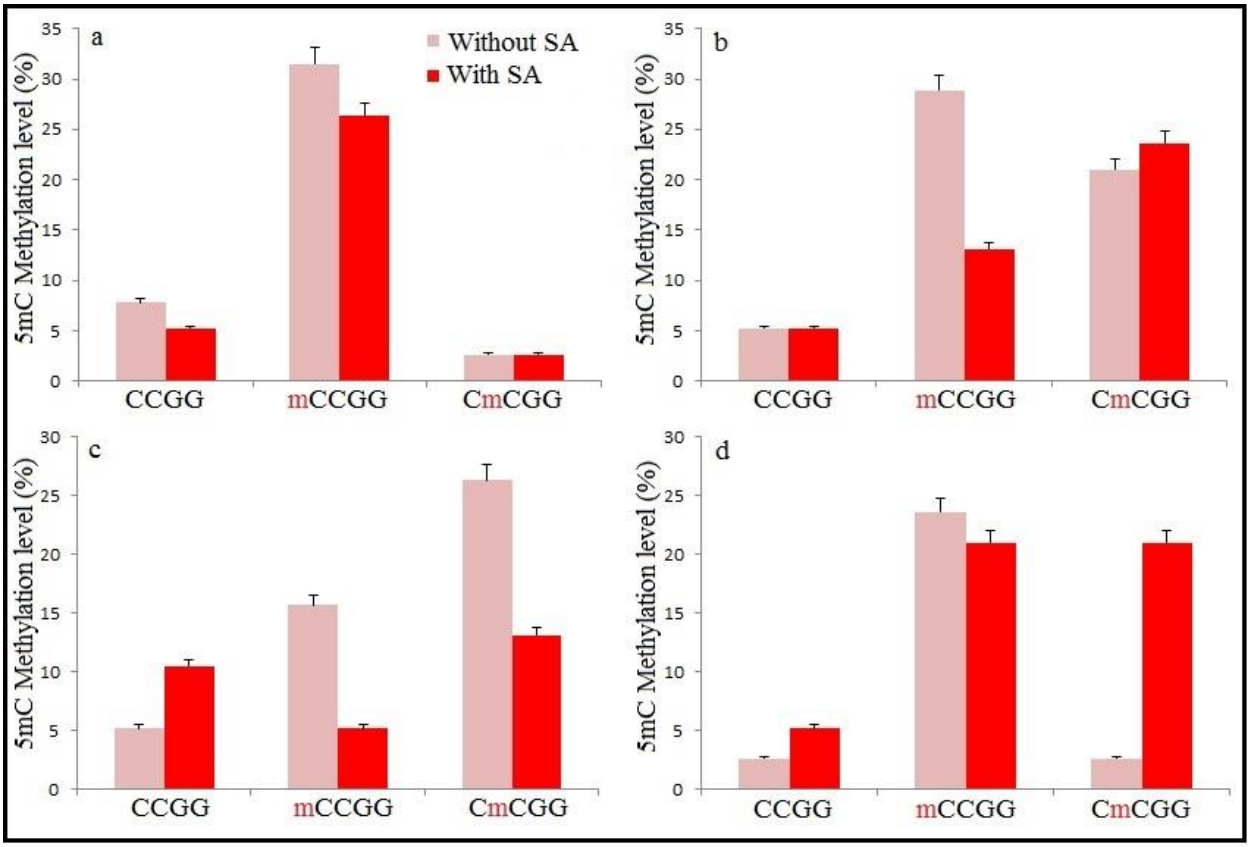

Figure 3: External and Internal cytosine methylation level. Hypermethylation occurred mostly at mCCGG for all varieties with and no SA treatment. Data are shown as percentage ± s.d. a: PMS3, b: PMI8, c: PMT2, and d: PMG. SA: salicylic acid.

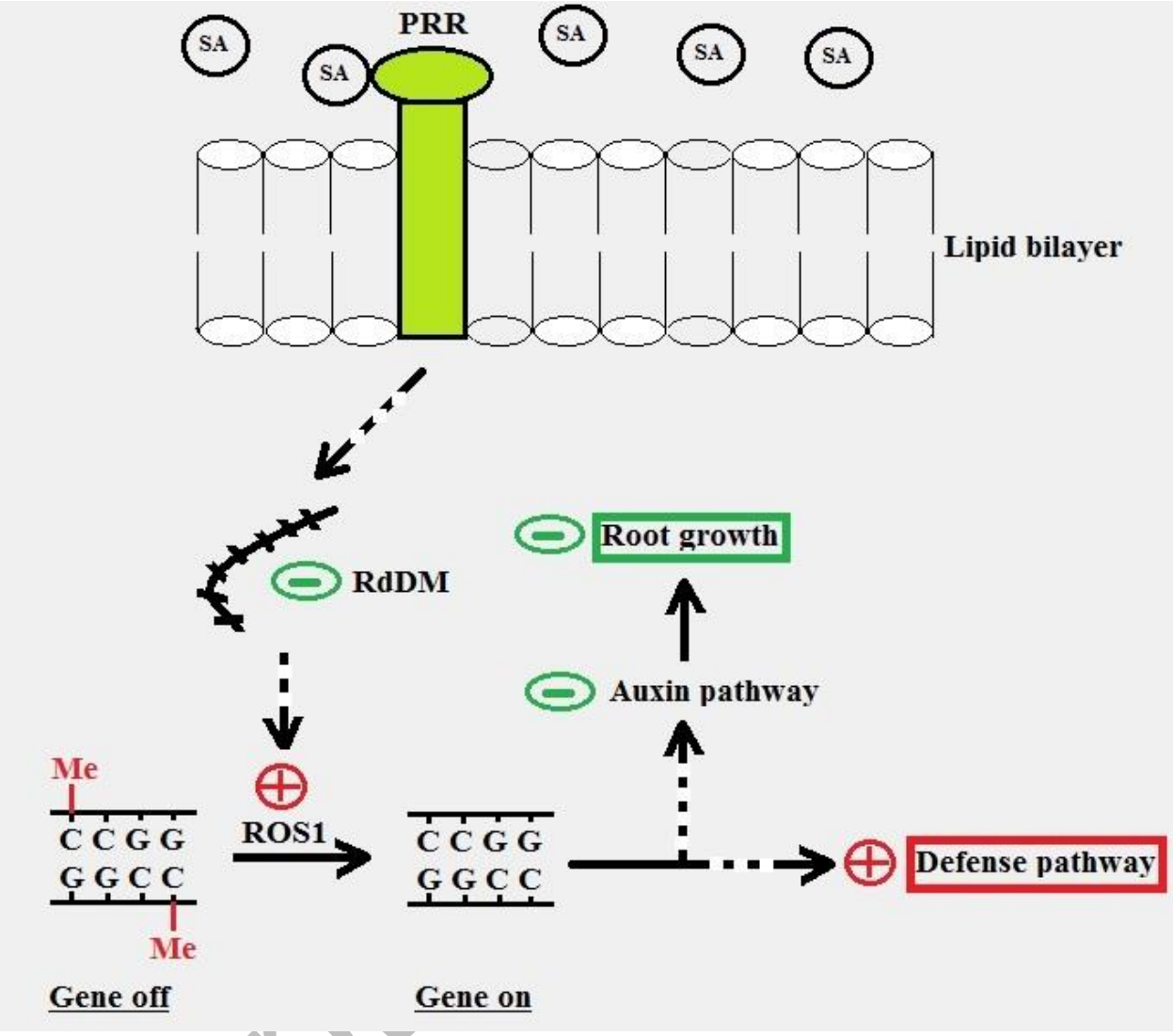

Figure 4: Theoretical actions of exogenous SA on Pearl millet. SA application is detected by Pattern Recognition Receptors (PRRs). This initiates a downregulation of the RNA-directed DNA Methylation (RdDM) and an induction of Repressor Of Silencing 1 (ROS1) demethylase activity that activates R genes. Some R genes could control some genes implicated in early root growth (Auxin), minimizing its expression. Me: methyl group; SA: salicylic acid; CCGG: target region.