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Methylation analysis revealed salicylic acid affects pearl millet defense through external cytosine DNA demethylation

Baba Ngom [a], Edward Mamati[b], Mame Fatoumata Goudiaby[a], Josphert Kimatu[c], Ibrahima Sarr[a], Diaga Diouf [d] and Ndjido Ardo Kane[e]

[a]Institut Sénégalais de Recherches Agricoles, CNRA, Bambey, Sénégal; [b]Department of Horticulture, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya; [c]Department of Biology, South Eastern Kenya University, Kitui, Kenya; [d]Faculté des Sciences et Techniques, Laboratoire Campus de Biotechnologies Végétales, Université Cheikh Anta Diop, Dakar, Sénégal; [e]Institut Sénégalais de Recherches Agricoles, LNRPV, Dakar, Sénégal

ABSTRACT

The cytosine DNA methylation and demethylation have a role in regulating plant responses to the environment by affecting the promoter regions of most plant defense-related genes through the CpG islands or the CCGG motifs. Salicylic acid, a defense and signaling plant hormone, is seen playing crucial role in the variation of the methylome. In this study, the effects of salicylic acid and feeding of the millet headminer (*Heliocheilus albipunctella* de Joannis) on pearl millet DNA methylome changes were evaluated through MSAP epigenotyping during panicle development. The results showed that millet headminer feeding increased the level of genomic methylation while application of salicylic acid caused DNA demethylation occurring mostly at external cytosine and accompanied by a decrease of the number of larvae per panicle. This suggests that hemimethylation (external cytosine methylation) has key role in regulating defense responses and conferring tolerance to pearl millet through salicylic acid application.

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DNA demethylation; DNA methylation; salicylic acid; MSAP epigenotyping; external cytosine; millet headminer

Introduction

Plants responds differently during pest attacks. Feeding of insects on plants is a dynamic ecological interaction, with characteristics of the insect and the plant affecting feeding behavior. During attacks, larval pest release chemical cues from oral secretions which are detected by the plants through the pattern recognition receptors, and this enhances the efficacy of the responses (Stahl et al. 2017). For instance, in tomatoes, *Nesidiocoris tenuis* feeding triggers defense response induction (Naselli et al. 2016). The plant response affects feeding, growth, and survival of herbivores and include development of structural barriers, toxic chemicals, and attraction of natural enemies of the target pests (Howe and Jander 2008; War et al. 2012).

However, some insects develop survival strategies that neutralize plant defense by suppressing the plant immune responses (Musser et al. 2005). The plant responses are controlled by a series of coordinated epigenetic events, which involve DNA methylation (Baulcombe and Dean 2014; Gijzen et al. 2014; Espinas et al. 2016). The DNA methylome variation directs expression of plant defense-related genes. A decrease and increase of methylation level is associated with upregulation or downregulation of genes during pest stress. *Heliocheilus albipunctella*, the millet headminer feeding may act like methylating agents on the epigenome. Usually, the CpG islands which 95% of CG dinucleotides are dispersed throughout 99% of the genome and are typically methylated and found in half of all promoters (Vinson and Chatterjee 2012).

Epigenetic defense is also influenced by salicylic acid (SA), a multifaceted plant hormone that have eliciting effects in plant defense (Vicente and Plasencia 2011; Ngom et al. 2017; Razmi et al. 2017). Usually, accumulation of SA in the nucleus as a defense signal is induced by biotic stresses (Gao et al. 2015), but its exogenous application has been observed to enhance the plant defense mechanism (Zehra et al. 2017). However, SA is mostly linked with abiotic and pathogen stresses (Zehra et al. 2017), and not directly to pest stress.

This study focused on evaluating the effects of applied SA on pearl millet or *Pennisetum glaucum* (L.) R. Br. methylome using MSAP epigenotyping targeting the CCGG motifs during the millet headminer (*H. albipunctella* de Joannis) infestation. The millet headminer is an important pest in pearl millet causing crop losses especially in the semi-arid and arid areas in the Sahel (Pattanashetti et al. 2016; Amadou et al. 2017).

Materials and methods

Plant and animal materials

Four varieties of *P. glaucum* (L.) R. Br used in this study were Souna3 (PMS3), Gawane (PMG), IBV8004 (PMI8), and Thialack2 (PMT2). These varieties reach maturity stage between 85 and 95 days from the planting date. The seeds were provided by the Senegalese Institute of Agricultural Research, Bambey (Senegal). Under the traditional field growing conditions, millet headminer start flying in millet agroecosystem one month after a first

CONTACT Baba Ngom sadikh_ngom@yahoo.fr
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significant rain of 20 mm and the female starts egg laying at the panicle initiation period. The plants were grown during the rainy season under natural infestation. Foliar application of SA was performed at panicle initiation and repeated on the third and sixth day later about 56 days after planting. Millet headminers were collected from the panicles in each of the treatments.

Experimental design and sampling

Split plot design was used, and the field was stratified to improve the representativeness of the sample by reducing sampling error as the millet headminer behavior is aggregative. Each stratum corresponded to one variety. Then, the stratum was divided into three plots where the treatments were applied (0, 1.5, and 3 mM of salicylic acid). A selective random sampling was carried out in each plot where 15 panicles were harvested during the panicle stage to determine the number of larvae per panicle. At the same time, five leaves from treated SA and control strata were collected for epigenetic analysis. For the control stratum (no salicylic acid treatments), plants infested with headminer larvae (feeding) were considered as positive control and without larvae (no feeding) as negative control.

Msap epigenotyping

In each treatment, five leaves from each variety were harvested for DNA extraction using ZR plant/seed DNA miniprep (Zymo Research, Cat No. D6020) following the company protocol and, an alcohol isoamyl-chloroform purification was performed to increase DNA quality. The methylation analysis was performed using the methylation-sensitive amplified polymorphism (MSAP) targeting the CCGG regions on the genome (Al-Lawati et al. 2016). The experiment was replicated four times. Two isoschizomers, Msp*I* and Hpa*II* used have different sensitivity to cytosine DNA methylation. Two series of experiments were conducted *i.e.* one for each isoschizomers. One hundred (100 ng) DNA samples were digested using 10 U EcoR*I* at 37°C for two hours and deactivated by heating at 65°C for 20 min. The digested products were subjected to each isoschizomers (10 U) at 37°C overnight and deactivated by heating at 80°C for 15 min. Ligation was performed using T4 DNA ligase (10 U) with EcoR*I* adaptors (10 mM) plus Msp*I*/Hpa*II* adaptors (10 mM) and incubated at room temperature for two hours. Pre-selective amplification was performed in a 50 µL reaction volume using 10 mM of EcoR*I* and Msp*I*/Hpa*II* primers, diluted restriction-ligation DNA and One Taq standard buffer. The pre-selective amplification was carried out 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 two minutes. Finally, a second amplification was realized by selectively amplifying methylated DNA fragments using different MSAP primer combinations to generate an MSAP fingerprint. The PCR conditions were as follows: 94°C for 30 s, 12 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s, 23 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 60 s. A 2% gel electrophoresis was performed to identify the different band size (Al-Lawati et al. 2016).

Data scoring and analysis

The MSAP profile was captured into binary matrix for data scoring, 1 as presence of band and 0 as absence of a band (Schulz et al. 2013). The internal cytosine methylation and the external cytosine methylation (hemimethylation) were considered in this study. The raw data from the MSAP profile were used for epigenetic analysis. Analysis of variance was performed to compare the effects of larvae infestation and SA on methylome regulations and least significance difference (LSD) was used for mean separation, as well as the larval density (number of larvae per panicle) using R (version 3.2.5). The raw data from the MSAP profile were analyzed using RMSAP 1.1.8 (Perez-Figueroa 2013) to determine the level of methylation as well as the non-target and mutations. Methylation diversity was also evaluated to determine epigenetic markers for each variety through the principal coordinates analysis (PcoA) using R version 3.2.5.

Results and discussion

Effects of salicylic acid on headminer attacks

Foliar treatments of SA were carried out before the initiation of the first panicle expansion stage. The ANOVA showed a significant difference between the control (without SA application) and the treatments ($p < 0.05$). Varieties PMT2, PMI8 and PMS3 remained susceptible to *H. albipunctella*, whereas treatment with SA significantly reduced the larval density per panicle. Larval density of *H. albipunctella* in PMG variety increased after applications of 1.5 mM SA. Therefore, at the control level, the tolerance of the varieties to the millet headminer was different, with PMS3 and PMT2 being more sensitive in *H. albipunctella* attacks. The larvae density in SA concentrations (1.5 and 3 mM) did not vary significantly, except for PMG (Table 1).

Exogenous application of salicylic acid reduced the number of larvae of *H. albipunctella*, suggesting an eliciting effect on pearl millet defense. This has been seen in pathogen and abiotic stresses (Fragniere et al. 2011; Khan et al. 2015). It seems now that SA-eliciting pathway is more complex and has a cross-talk with the defense mechanisms involved during herbivore attacks. These mechanisms involve gene-related defense regulations controlled at epigenetic level, which interact with oral secretion from larvae feeding. The same SA role is seen in plants subjected to insect elicitor treatments (Engelberth et al. 2011).

Effects of salicylic acid and millet headminer on methylome variation

Analysis was performed to determine the CCGG methylation level following the millet headminer feeding (positive control),

Table 1. Effects of salicylic acid treatments on the millet headminer. SA (0, 1.5 and, 3 mm) was applied on four pearl millet varieties. Results showed SA decreased the larval density for the varieties tested. Means with the same letter in the same line are no significant difference at 0.05 probability level.

	Salicylic acid doses (mm)		
Varieties	0	1.5	3.0
PMS3	0.6 a	0.2 b	0.4 b
PMG	0.4 a	1.2 b	0.4 a
PMI8	1.8 a	0.8 b	0.6 b
PMT2	1.6 a	0.4 b	0.4 b

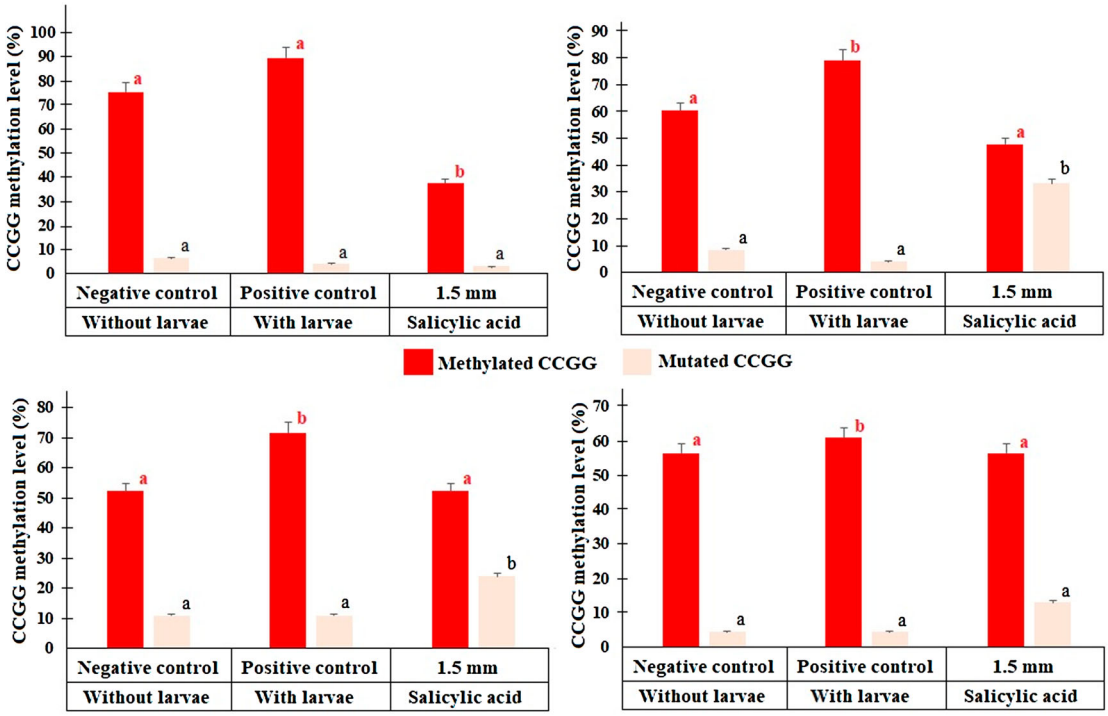

Figure 1. Methylation level of pearl millet following headminer feeding and salicylic acid treatments. Methylation level and mutated CCGG sites of each treatment was determined using MSAP analysis and RMSAP respectively. Millet headminer feeding increased the level of methylation, while SA treatments decreased. The CCGG mutation level was higher in PMG and PMI8 during salicylic acid application. Means with the same letter are no significant difference at 0.05 probability level. (a) PMS3; (b) PMG; (c) PMI8; (d) PMT2.

without feeding (negative control) and salicylic acid treatment at 1.5 mM. The methylation level varied significantly for all varieties ($p < 0.05$). The methylation level was higher during *H. albipunctella* feeding (positive control) and decreased after SA application in all varieties. The average methylation level (negative control) of varieties was 61%, while individually the highest (75.5%) was found in PMS3. Moreover, the level of mutated and non-targeted CCGG motifs were low in PMS3 and PMT2 varieties while it increased during salicylic acid treatment for PMG and PMI8 (Figure 1).

The significant difference ($p < 0.05$) between the negative (without larvae feeding) and positive (with feeding) controls revealed an increase of methylation level during the headminer feeding, suggesting downregulation of resistance gene expression. This inhibition of plant defense through increase of DNA methylation level could be from *H. albipunctella* infestation. In the class of Lepidoptera in which belong the headminer, some larvae have oral secretion contain suppressors acting as methylating agents or effectors that inhibit the host defense responses (van Kleeff et al. 2016; Acevedo et al. 2017). This strategy has been reported in *Helicoverpa zea* (Eichenseer et al. 1999; Musser et al. 2005), in *Leptinotarsa decemlineata* (Chung et al. 2013), and mite species (Villarroel et al. 2016). Moreover, this is accompanied by an inhibition of the salicylic acid pathway (Zarate et al. 2006; Sarmento et al. 2011).

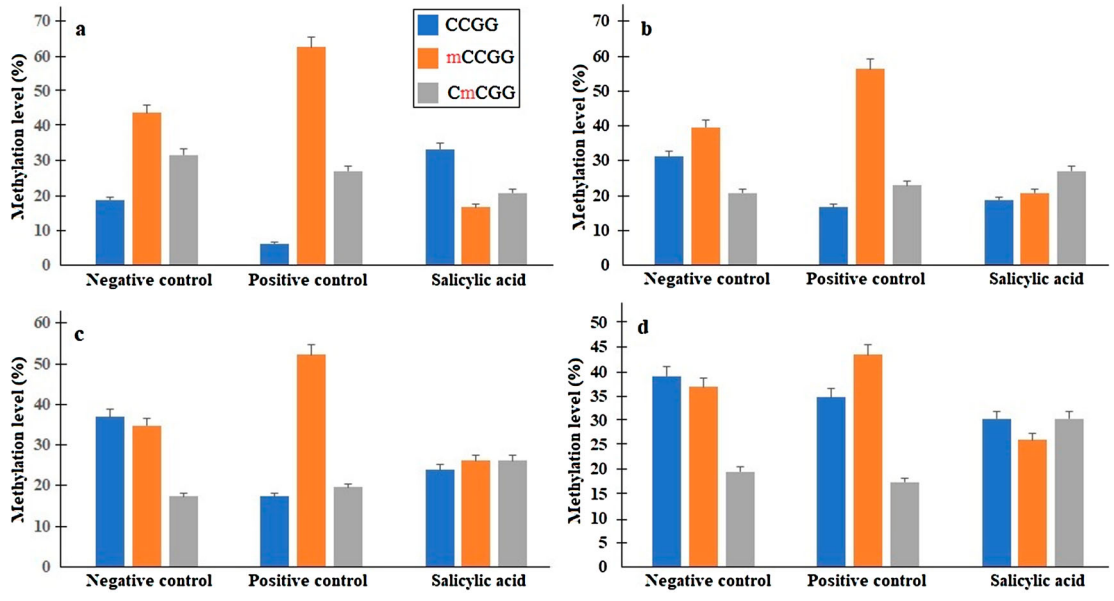

Figure 2. Occurrence of types of methylation. the results showed most of the methylation occurred at the external cytosine. Results shown as percentage ± s.d. (a) PMS3; (b) PMG; (c) PMI8 and (d) PMT2. CCGG: unmethylation; mCCGG: external cytosine methylation; CmCGG: Internal cytosine methylation.

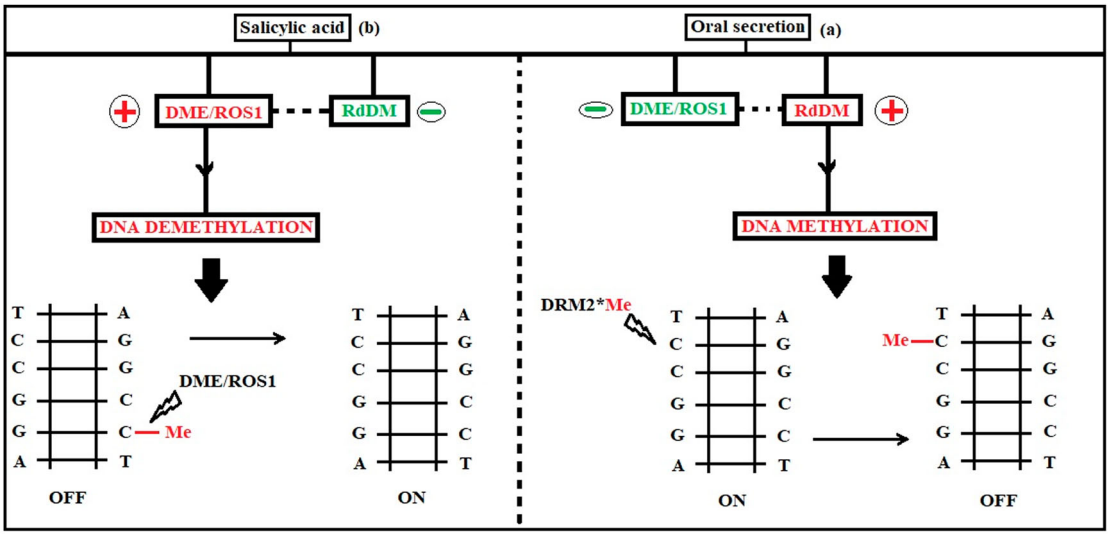

Figure 3. Hypothetical model of the effects of salicylic acid and oral secretion on the external cytosine. Oral secretion and salicylic acid causes external cytosine gene methylation and demethylation respectively through different pathways. DME: Demeter; ROS1; Repressor of Silencing 1; RdDM: RNA-directed methylation; DMR2: Domains rearranged methyltransferase 2; Me: Methyl group.

Occurrence of methylation types

The level of the different types of methylation occurring during headminer feeding and salicylic acid application was determined. The results showed that most of the methylation occurred at the external cytosine (hemimethylation, *mCCGG*) which is higher during insect feeding. Additionally, the hemimethylation level decreased after salicylic acid treatment. In each variety, the level of unmethylated CCGG sites decreased after larvae infestation while application of salicylic acid seemed to reestablish the CCGG pattern (Figure 2).

The high occurrence of the hemimethylation during pest feeding could reveal important role of the external cytosine in plant tolerance. Additionally, the demethylation process occurred after salicylic acid treatment which was mostly at the external cytosine. The external cytosine may be important for identification of de novo methylation events during pest stress. This hemimethylation is very unstable and mainly due to pest feeding and salicylic acid that could affect its pattern. However, the cellular external cytosine DNA methylation and demethylation is far from being explained. Both oral secretions and salicylic acid could direct the expression of enzymes involved in DNA methylation and demethylation, respectively (Figure 3).

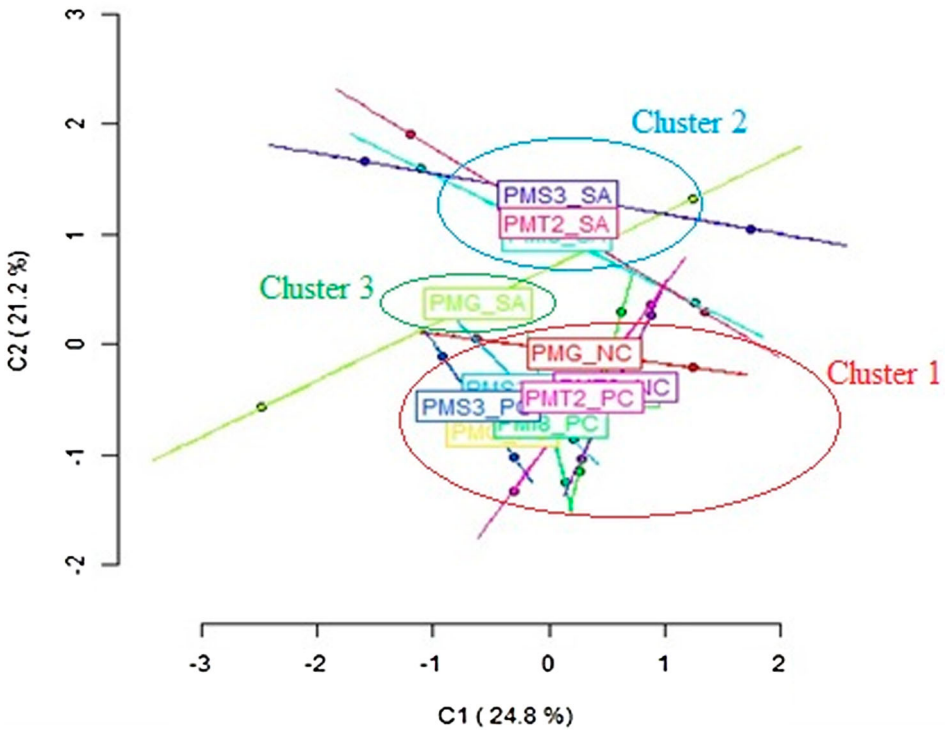

Figure 4. Principal coordinate analysis of methylome loci. Three groups appeared: the negative and positive controls, the SA group (PMS3_SA, PMT2_SA, and PMI8_SA) and the single PMG_SA group. SA = salicylic acid; NC = negative control; PC = positive control. C1 = 24.8% and C = 21.2%. Cluster 1 gathers the feeding and non-feeding groups; Cluster 2 gathers the SA treatments except for PMG; Cluster 3 with the isolated SA for PMG.

Epigenetic diversity and clustering

The principal coordinate analysis (PcoA) revealed three separated clusters. The larvae feeding (positive control) and the healthy plants (negative control) clustered separately from the others. The SA treatment formed a group with only PMS3, PMT2, and PMI8, with PMG being isolated as a single cluster (Figure 4).

The clustering analysis with three groups from which PMG was isolated indicates a perfect correlation with the results found during the application of salicylic acid in pearl millet panicle stage. Indeed, larval density increased after salicylic acid treatment (1.5 mM) only in variety PMG while it decreased it in the other varieties. This may due to the high level of mutated CCGG motifs after salicylic treatment.

Conclusion

This study offers comprehensive insights into the methylome variation during pest stress and salicylic acid signaling. The plant-insect interactions provide interesting perspectives in understanding its defense mechanisms, especially at epigenetic level. *H. albipunctella* larvae infestation increases the DNA methylation and this could negatively affect the pearl millet defense mechanisms. This process is counteracted by salicylic acid through demethylation pathway by reducing the pest infestation. The mechanism in which the larvae inhibit the plant defense and the salicylic acid act as an elicitor could be driven by the methylation-demethylation pathways on the external cytosine (hemimethylation, mCCGG), but further works are needed to investigate in depth these complex pathways.

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Disclosure statement

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ORCID

Baba Ngom http://orcid.org/0000-0003-3909-6436
Diaga Diouf http://orcid.org/0000-0002-3262-1763

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