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MSAP Marker Based Epigenetic Regulation in Symptomatic and Asymptomatic Floral Malady of Soybean (*Glycine max*)

Snehal Krishna Kad[1]
https://orcid.org/0000-0001-7875-9146

Mangesh Pradeep Moharil[1]
https://orcid.org/0000-0001-6811-271X

Pravin VishwanathraoJadhav[1]
https://orcid.org/0000-0003-2583-5373

Sanjay Bapuji Sakhare[1]
https://orcid.org/0000-0002-4004-1654

Pranita Prabhakar Thakur[1]*
https://orcid.org/0000-0002-6090-1682

Shyamsundar Shioram Mane[2]
https://orcid.org/0000-0003-3081-3777

Prashant Bhaskar Kale[1]
https://orcid.org/0000-0002-2043-2500

Rameshwar Baliram Ghorade[3]
https://orcid.org/0000-0002-3521-7862

Sayali Ganesh Magar[1]
https://orcid.org/0000-0002-7444-5841

Raviprakash Govindrao Dani[4]
https://orcid.org/0000-0001-9375-9232

[1]Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Post Graduate Institute, Biotechnology Centre, Akola, Maharashtra State, India; [2]Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Post Graduate Institute, Departmentof Plant Pathology, Akola, Maharashtra State, India; [3]Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Post Graduate Institute, Department of Agricultural Botany, Akola, Maharashtra State, India; [4]Namangan Engineering & Technological Institute, Agriculture Technology, Namangan, Uzbekistan.

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*Correspondence: pranitat03@gmail.com; Tel.: +91-7022247606(P.P.T.).

HIGHLIGHTS

- Cytological study for floral malady in soybean
- MSAP profiling in floral malady symptomatic and asymptomatic plant.
- Insights of pattern and extent of methylation.
- Epigentic gene regulation by MSAP approach.

Abstract: It has learned that soybean is being affected by a floral disorder known as floral malady where plants fail to develop pod and do notattendfull maturity. For this floral disorder, we present a new methylation-sensitive amplified polymorphism (MSAP) approach for the evaluation of relative quantitative characteristics of non-methylation, hyper-methylation, hemi-methylation, and full methylation status of CCGG sequences, which are recognized by the isoschizomers *Hpa*II and *Msp*I. We applied a technique to analyze alterations in the cytosine methylation a popular Indian soybean (*Glycine max* L.) genotype, JS-335.The result revealed that in the symptomatic plant, out of 392 MSAP sites, 281 (71.68%), 33 (8.41%),38 (9.69%), and 40(10.20%)

found to beun-methylated, hemi-methylated, fully methylated and hyper-methylated, respectively. Whereas, the MSAP profile of asymptomatic plants revealedout of 402MSAP sites, 330 (81.28%) was un-methylated, 22(5.41%) hemi-methylated,29(7.14%) fully methylatedand 25 (6.15%) hypermethylated. In comparison with asymptomatic(18.71%) plant, approximately 10% increased methylation was noted in symptomatic(28.31%) plantprofiles. The increased levels of methylation was recorded in the symptomatic plants about 28.31%and18.71% in asymptomatic. The study showed a higher epigenetic influence on JS-335 genotype of floral malady symptomatic than same genotype of asymptomatic plant. No pod formation in symptomatic plant induce genome wide changes either in promoter or coding region of gene(s) and DNA fragments showing polymorphism related to differences in pattern and extent of methylation associated with floral malady.

Keywords: Floral malady; Methylation; MSAP; Soybean; Epigenetic.

INTRODUCTION

Soybean is the second most important oilseed crop in India. Although it has a great potential to generate more revenue, it is challenged by various abiotic and biotic stresses. More than 100 biotic agents inclusive of five phytoplasma like organism cause disease in soybean, causing about 10-80 percent yield losses [1]. Floral malady is a peculiar budding malady prevailing in soybean with an average incidence ranging from 8.0 to 14.6% in different parts of central India [2]. Many different factors have been associated with floral malady, the phytoplasma despoil metabolic machinery of soybean outburst floral malady and causes morphological and metabolic changes which attracts insect for spread of disease. Phytoplasma triggers molecular signaling leading to mobilization of carbohydrate and protein, phyllody, abnormal pollen development, improved colonization of insect in host plants to spread the disease [3]. The symptoms in question are what triggers for floral malady and green staying plants without pods: Similar symptoms were characterized worldwide as pod set failure syndrome, bud blight, no-podding syndrome and bud proliferation syndrome [4, 5, 6, 7] and this causes leads to acute loss in productivity. Still now, the symptoms produced do not have resemblance with any of the documented disorders of soybean and are inconsistent in their distribution across the locations. Due to this ambiguity, till date, no effective control measures or management practices are reported to overcome the yield losses incurred due to this disorder.

Our previous transcriptomic study reveals abnormal flower development of floral bud distortion (FBD) or floral malady or witchs'-bloom disease at molecular level in terms of abnormal pollen development [3]. We report transcriptomic signature of 17,454 DEGs involving 139 pathways in manifestation of the FBD disease. The study reveals different molecular markers (SSR, SNP and InDel) for future MAS study as well as different chromatin structure based transcriptional factors and it showed differentially expressed gene and transcriptional factors leads to floral bud distortion (FBD). The gene expression and TFs are greatly influenced by plant growth and modification, which is influenced by DNA methylation that signifies the mechanism of controlling gene regulation without modifying DNA sequence through altering histone and chromatin structure and it plays important role in plant domestication and evolution [8, 9, 10]. Methylation sensitive amplified polymorphism is regularly preferred for the detection of DNA methylation in the eukaryotic organism [11]. In plants, DNA methylation occurs most commonly within three sequences viz., CG, CHG, and CHH (where H is A, C, or T), however, it varies depending on the level and pattern found within different genomic and intergenic regions. The frequency of cytosine methylation of CpG and CpNpG nucleotides vary with chromosome and regulate gene expression either at the gene level or else regionally to influence entire regions of chromosomes.

However, MSAP is a modified AFLP technique that efficiently identifies polymorphism in DNA methylation patterns [12]. This system is developed for determining the extent and pattern of cytosine methylation and being used in genomes of numerous crops like in rice [12], banana [13], cycads [14], fig [15], cotton [16] and almond [17]. As stated earlier inadequate attempts have been made to understand the floral malady of soybean at a molecular level and the single widely grown soybean genotype (JS-335) having symptoms of floral malady in our previous study [18] and MSAP marker variation in DNA methylation for floral malady provides needful information for future MAS study. This epigenetic modification in single genotype could result gene expression in responsive to symptomatic and asymptomatic floral malady of soybean. Keeping in view of unknown molecular insights associated with the floral malady, the investigation was aimed to determine pattern and extent of methylation associated with the floral malady in soybean.

MATERIAL AND METHODS

Plant material

Five each of floral bud distorted symptomatic and asymptomatic plants of widely grown soybean genotype JS-335 was collected at R5-R6 stage (pod containing a green seed that fills the pod capacity at one of the four uppermost nodes on the main stem) from the experimental field of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra State, India (20.42°N).

Assessment of pollen morphology, Pollen viability and pollen germination

Flowers, at R2-R3 stage were randomly collected from the symptomatic and asymptomatic plants of soybean genotype JS-335 grown in experimental fields. Fresh flowers collected before anthesis at 9.00 to 10.00 am and examined under scanning electron microscope. The dehydration of flowers was done for 30 min each in series of alcohol concentrations as described by methods [18]. After critical point drying, pollen were placed on aluminum stub having double adhesion tape and conducted with gold with JFC/100 sputter and pollen were examined under JSM 1100 JEOL scanning electron microscope. Pollen viability and pollen germination was studied as per the procedure described by method [19, 20].

MSAP profiling

Genomic DNA was isolated from the node and stem tissues of symptomatic and asymptomatic plants at the R6 stage using CTAB method. The methylation profile was developed using MSAP primers with pre-selective amplification and further used to carry out selective amplification using as a template. The genomic DNA samples of symptomatic and asymptomatic plants were digested sequentially with the isochizomers*Hpa*II and *Msp*I in replicates. The digestion reaction was performed using genomic DNA (~ 500 ng DNA) added with 45 µl buffer containing 10 units *Eco*RI, 10 units *Hpa*II, 5 units T4 DNA ligase, 5 pmol*Eco*RI adapter, 50 pmol *Hpa*II/*Msp*I adapter and 10 mM ATP. The mixture was formerly incubated at 37º C for 4 h. The reaction was stopped by incubating at 65º C for 10 min. and diluted 10 times in nuclease-free water for PCR amplification. The second digestion/ligation was carried out in the same way replacing *Msp*Iin place of *Hpa*II. The pre selective amplification was performed using 5 µl of the above-diluted mixture added to 15 µl reaction mixture with the final concentration of 10 X PCR buffer, 10 mM dNTP, 50 ng of *Eco*RI and *Hpa*II/*Msp*I adapter-directed primer, and 5U *Taq* polymerase. The PCR reaction was performed using the profiles at 65°C, and 1 min at 72ºC, followed by a touch-down profile (13 cycles with -0.7º C decrease in temperature for the annealing step in each successive cycle), followed by 18 cycles with annealing at 55.9ºC, and finally by an extension cycle of 10 min at 72º C. Selective amplification of the pre-selective amplified product were carried out using 22 primer combinations (Table 1). Each reaction consisting 5 µl of 1:10 diluted pre-selective amplified products were added to the selective amplification mix containing 50 ng of *Eco*RI primer, 50 ng of *Hpa*II/*Msp*I, 10 X PCR buffer,10 mM of dNTPs, and 0.5 unit of *Taq* DNA polymerase in a final volume of 20 µl. The temperature profile for selective amplification PCR reaction was the same as used for the pre-selection step. The selective amplified PCR products were separated on 8% polyacrylamide gel electrophoresis.

Table 1. List of primers and adapters

MSAP primers	***Eco*RI (E) (5'-3')**	***Hpa*II/*Msp*I (H/M) (5'-3')**
Adaptor-1	CTCGTAGACTGCGTACC	GATCATGAGTCCTGCT
Adaptor-2	AATTGGTACGCAGTC	CGAGCAGGACTCATGAC
Pre-amplification primers	GTAGACTGCGTACCAATTCA	ATCATGAGTCCTGCTCGG
Selective amplificationprimers	GACTGCGTACCAATTCAGC	CATGAGTCCGTGCTCGGTCAA
	GACTGCGTACCAATTCACA	CATGAGTCCGTGCTCGGCTAA
	GACTGCGTACCAATTCACT	ATCATGAGTCCTGCTCGGTCA
	GACTGCGTACCAATTCACG	ATCATGAGTCCTGCTCGGTTA
	GACTGCGTACCAATTCACC	ATCATGAGTCCTGCTCGGTGA

Profile scoring and data analysis

The amplicons were scored for data analysis and transformed into a binary character matrix, using “0” and “1” to indicate the absence and presence of a particular fragment, respectively. In MSAP profiles, the amplicons present in both EcoRI/HpaII and EcoRI/MspI lane were considered as Type I (non-methylated); in EcoRI/HpaII lanes but not in EcoRI/MspI were considered as Type II (hemi-methylated), in EcoRI/MspI but not in EcoRI/HpaII lane as Type III (fully-methylated) and absent in both the lanes as type IV (hyper-

methylated). A site was considered “methylation polymorphic” (MP) if it finds at least one sample where the site was methylated and at least one sample for which the site was not methylated

RESULTS

Morphology and cytological study for floral malady

To identify floral malady disorder an unusual floral malady, the morphological symptoms were recorded on randomly distributed plants exhibiting extended vegetative phase (stay green) where the symptomatic plant fails to produce pods at R_6 stage in the field (Figure 1). As well as, examination at cytological level depicted the significant difference between asymptomatic and symptomatic plant for pollen structure, pollen viability and pollen germination (Figure 2). Asymptomatic plant pollens were observed with dense cytoplasm, prominently stained nucleolus at the centre. The SEM observations revealed changes in symptomatic plant anther and pollen size and aberrated pollen structure where, anther width increased by 1.4-fold with low pollen count (Figure 2). The different dyes revealed pollen viability of affected plants (33.45%) with reduced size and spherical shape as compare to normal plants (89.28%). Likewise, pollen germination recorded in asymptomatic and symptomatic plant was 41.25% and 17.54% respectively after 24hr. Stigma found receptive in symptomatic and asymptomatic plants as in experimentation. The peroxidase activity showed reddish brown color on the both plant stigmas. Hence, normal stigma was found in both plants.

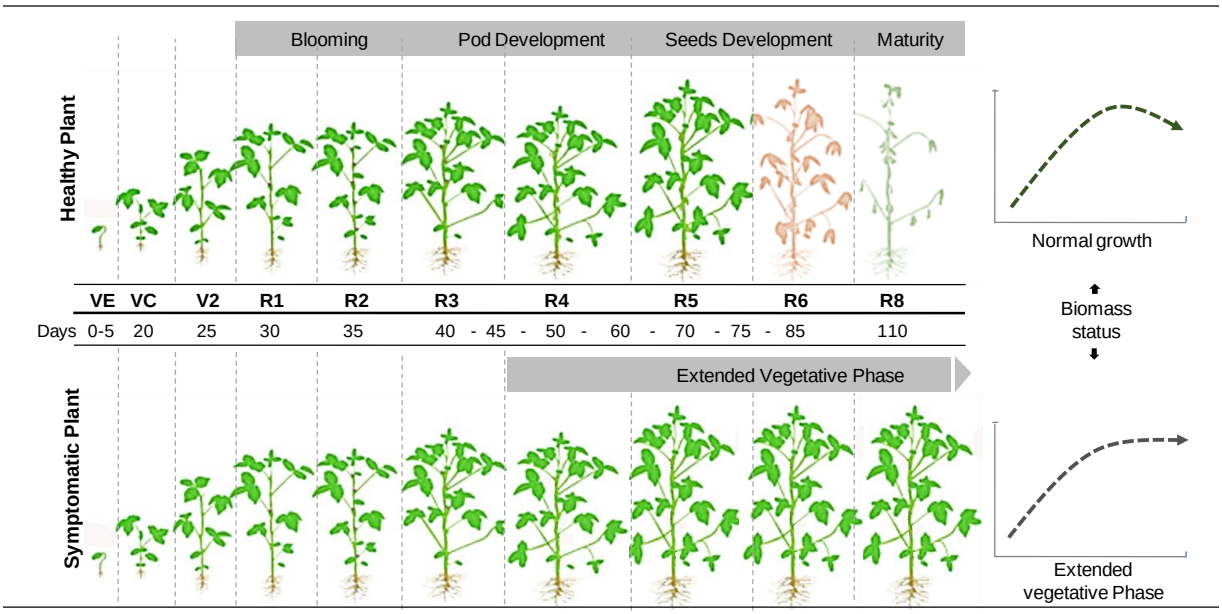

Figure 1. Comparative developmental stages of soybean: asymptomatic (healthy) *vs* symptomatic plant (disorted).

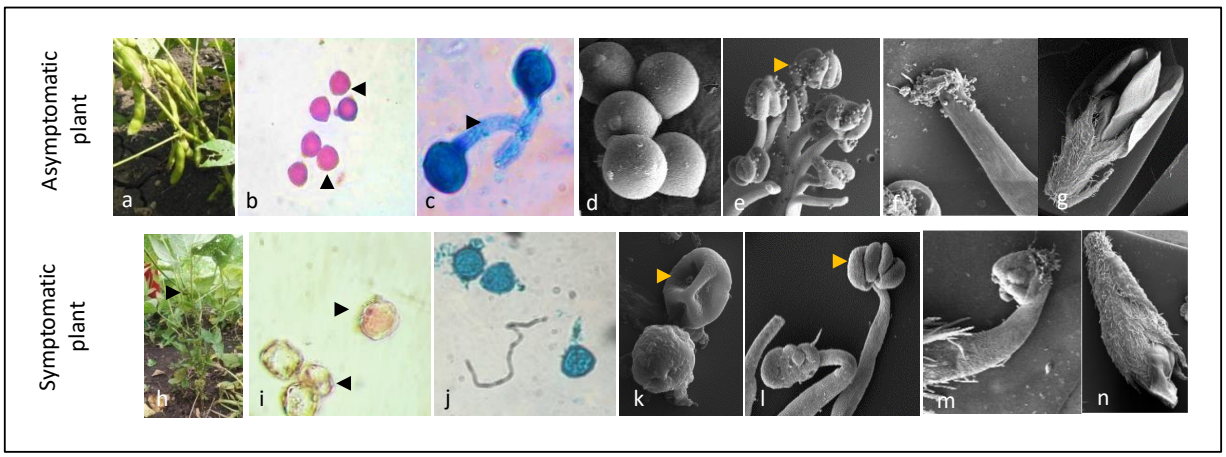

Figure 2. Morphological and cytological differences of floral organs between symptomatic and asymptomatic plant of genotype JS-335 at R5-R6 stage (50-55 days after sowing).

The Figures 2a and 2h show the morphological differences in asymptomatic and symptomatic plants at R5-R6 stage; 2b and2I: differences in pollen viability; 2c and 2j: pollen germination in asymptomatic and symptomatic plants after 24 hrs; 2d and 2k: structural differences in pollen of asymptomatic and symptomatic plants; 2e and 2l: anthers with pollengrain (asymptomatic) and without pollen (symptomatic); 2f and 2m: stigma in asymptomatic and symptomatic plant; 2g and 2n: flowers of asymptomatic and symptomatic plant.

MSAP profiling in floral malady symptomatic and asymptomatic plant

Twenty-two MSAP selective primer pair combinations produced in total 13 to 25sites in floral bud distorted plant and 12 to 26 sites in asymptomatic plants. The numbers of total,nonmethylated, hemi-methylated, and fully-methylated at CCGG sites were calculated based on theMSAP profiles. According to the expression patterns of methylation, sensitive fragments ofsymptomatic and asymptomatic plants were divided into the 4 types (Figure 3) *viz*., un-methylated, hemimethylated, fully methylated and hyper-methylated. In the symptomatic plant, out of total 392 MSAP amplified sites, 281 (71.68%) were un-methylated, 33(8.41%) hemimethylated, 38(9.69%) fully methylated and 40 (10.20%) hyper-methylated. The total methylation level in the symptomatic plant was 18.11% (varied 5.88% to 56.52%), which was comprised of 8.41% hemi-methylation at the external cytosine (varied from 0.0 to 39.13%), which consisted 9.69% full methylation at internal cytosine (varied between 0.0 to 27.77%). In particular, to the symptomatic plants, the highest total methylation sites (hemi-+ fully-methylated) was 13 (P9) followed by 7 and 5 (P21 and P22, respectively) and the lowest 1 (P18 and P11; Table 2). Similarly, in the asymptomatic plants, out of total 406 MSAP sites amplified, 330 (81.28%) were un-methylated, 22(5.41%) hemimethylated, 29 (7.41%) fully methylated and 25 (6.15) hyper-methylated. The total methylation level in the asymptomatic plant was 12.56 (varied between 0 to 29.41%), which was comprised of 5.41% hemi methylation at the external cytosines (ranged from 0.0 to 23.80), which comprised of 7.14% full methylation at the internal cytosine (varied from 0 to 29.41). Among the asymptomatic plant, the highest level of methylation sites were 5 (P11), followed by 5 and 3 (P10 and P14) and the lowest 0 (P18 and P20; Table 2). These results indicated substantially varied methylation status in symptomatic and asymptomatic plants of floral development stage (Table 3). The result was used to draw histograms which helped to interpret the details of the sites of methylation in both the tissue samples (Figure 4).

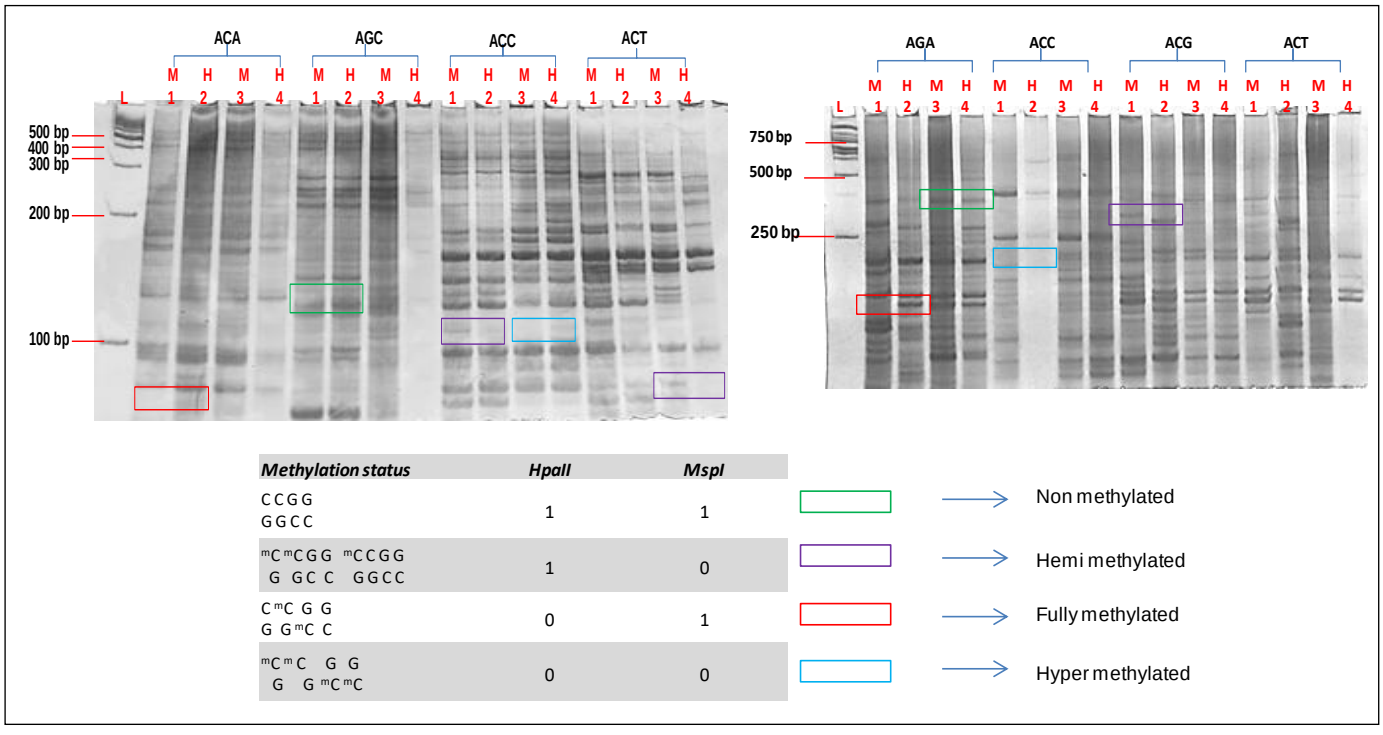

Figure 3. Examples of methylation pattern from comparing symptomatic and asymptomatic using eight primer combinations: MH-TTA and MH-TGA.

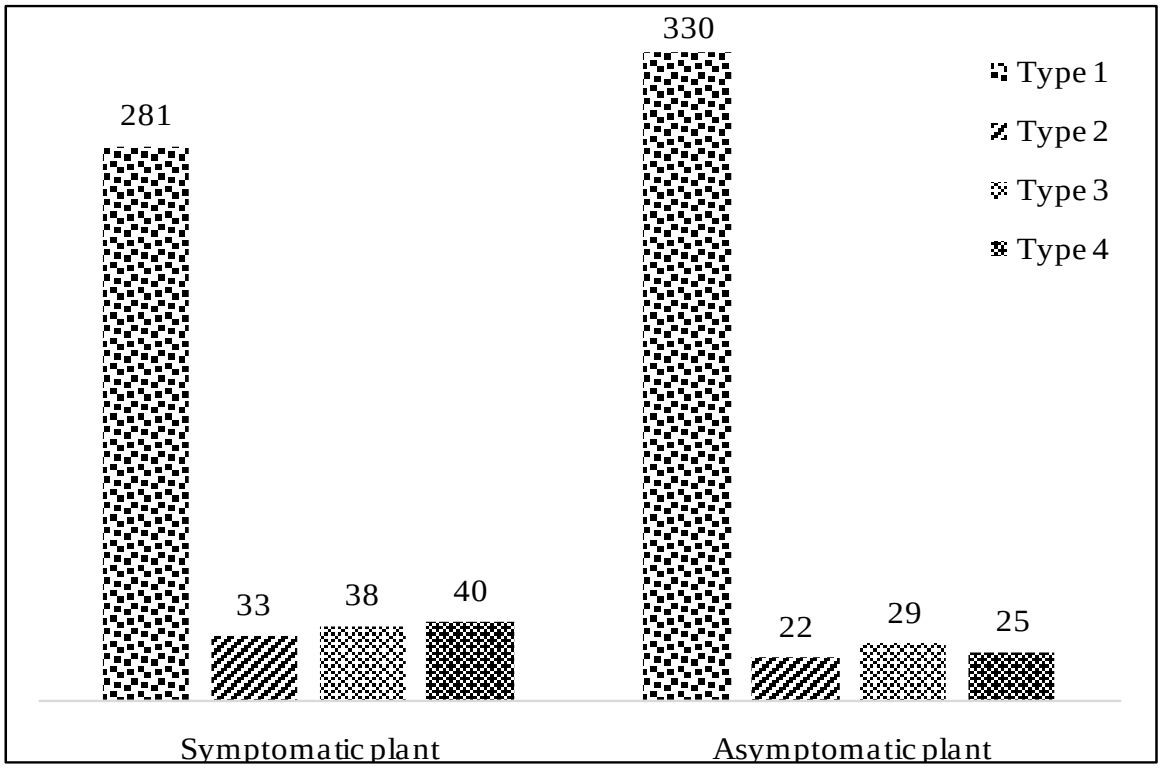

Figure 4. Histogram showing methylation sites of bands in symptomatic and asymptomatic plant.

Table 2. Alteration in cytosine DNA methylation level based on MSAP profile.

Symptomatic alteration in cytosine DNA Methylation						
Primer	**Total sites**	**Un-methylated sites (%)**	**Hemi-methylated sites (%)**	**Methylated CCGG Sites**		
				Fully-methylated sites (%)	**Hyper-methylated sites (%)**	**Total methylation in symptomatic plant (%)**
P1	21	17(80.95)	1(4.76)	2(9.52)	1(4.76)	3(14.28)
P2	14	11(78.57)	0	1(7.14)	2(14.28)	1(7.14)
P3	23	19(82.60)	3(13.04)	0	1(4.34)	3(13.04)
P4	21	11(52.38)	1(4.760	3(14.28)	6(28.57)	4(19.04)
P5	25	17(68)	2(8)	3(12)	3(12)	5(20)
P6	14	12(85.71)	2(14.28)	0	0	2(14.28)
P7	15	12(80)	0	1(6.67)	2(13.33)	1(6.67)
P8	13	9(69.23)	1(7.69)	1(7.69)	2(15.38)	2(15.38)
P9	23	9(39.13)	9(39.13)	4(17.39)	1(4.34)	13(56.52)
P10	20	15(75)	2(10)	1(5)	2(10)	3(15)
P11	17	13(76.47)	0	1(5.88)	3(17.64)	1(5.88)
P12	24	20(83.33)	1(12.5)	1(4.16)	2(8.33)	2(16.67)
P13	24	20(83.33)	3(23.07)	0	1(4.16)	3(23.07)
P14	15	9(60)	0	3(20)	3(20)	3(20)
P15	13	8(61.53)	0	2(15.38)	3(23.07)	2(15.38)
P16	13	9(69.23)	0	1(7.69)	3(23.07)	1(7.69)
P17	13	10(76.92)	0	2(15.38)	1(7.69)	2(15.38)
P18	17	14(82.35)	0	1(5.88)	2(11.76)	1(5.88)
P19	18	13(72.22)	3(16.67)	2(11.11)	0	5(27.77)
P20	17	14(82.35)	1(5.88)	1(5.88)	1(5.88)	2(11.76)
P21	18	11(61.11)	2(11.11)	5(27.77)	0	7(38.88)
P22	14	8(57.14)	2(14.28)	3(21.42)	1(7.14)	5(35.71)
Total	392	281(71.68)	33(8.41)	38(9.69)	40(10.20)	71(18.11)
Asymptomatic alteration in cytosine DNA Methylation						
P1	22	18(81.81)	1(4.54)	1(4.54)	2(9.09)	2(9.09)
P2	17	13(76.47)	1(5.88)	0	3(17.64)	1(5.88)
P3	24	22(91.66)	0	1(4.167)	1(4.16)	1(4.16)
P4	18	12(66.66)	0	2(11.11)	4(22.22)	2(11.11)
P5	23	17(73.91)	0	4(17.39)	2(8.69)	4(17.39)
P6	12	11(91.67)	0	1(8.33)	0	1(8.33)
P7	16	11(68.75)	1(6.25)	0	4(25)	1(6.25)
P8	14	11(78.5)	0	3(21.42)	0	3(21.42)
P9	19	15(78.94)	2(10.52)	1(5.26)	1(5.26)	3(15.78)
P10	21	15(71.4)	5(23.80)	0	1(4.76)	5(23.80)
P11	17	12(70.58)	0	5(29.4)	0	5(29.41)
P12	26	20(76.92)	2(7.69)	3(11.53)	1(3.84)	5(19.23)
P13	25	20(80)	3(12)	1(4)	1(4)	4(16)
P14	17	13(76.47)	2(11.76)	2(11.76)	0	4(23.52)
P15	15	13(86.67)	1(6.67)	1(6.67)	0	2(13.33)
P16	15	13(86.67)	0	2(13.33)	0	2(13.33)
P17	17	15(88.23)	0	1(5.88)	1(5.88)	1(5.88)
P18	18	16(88.89)	0	0	2(11.11)	0
P19	19	16(84.21)	2(10.52)	0	1(5.26)	2(10.52)
P20	17	16(94.11)	0	0	1(5.88)	0
P21	20	18(90)	1(5.88)	1(5)	0	2(10.88)
P22	14	13(92.85)	1(7.14)	0	0	1(7.14)
Total	406	330(81.28)	22(5.41)	29(7.14)	25(6.15)	51(12.56)

Table 3. Methylation patterns of HpaII- MspI digested genomic DNA from symptomatic and asymptomatic plant

Type of methylation status		Sensitivity of enzymes		Amplicon pattern obtained		Methylation type	Total number of amplified bands	
		*Hpa*II	*Msp*I	*Eco*RI/ *Hpa*II	*Eco*RI/ *Msp*I		Symptomatic	Asymptomatic
I.	C C G G G G C C	Active	Active	1	1	Non methylated	281	330
II.	^{m}C^{m}C G G^{m}C C GG G G CC G G CC	Active	Inactive	1	0	Hemi methylated	33	22
III.	C ^{m}C GG G G^{m}C C	Inactive	Active	0	1	Fully methylated	38	29
IV.	^{m}C^{m}C G G G G^{m}C^{m}C	Inactive	Inactive	0	0	Hyper methylated	40	25
Total amplified amplicon							392	406
Total methylated amplicon (II+III+IV)							111	76
Fully methylated amplicon (III+IV)							78	54
MSAP (%) [(II+III+IV)/(I+II+III+IV)]x100							28.31	18.71
Fully methylated ratio (%) [(III+IV)/(I+II+III+IV)] x100							19.89	13.30
Hemi methylated ratio (%) [(II)/(I+II+III+IV)]x100							8.41	5.41

Note: Type I indicated absence of methylation due to the presence of amplicon in both EcoRI/HpaII and EcoRI/MspI digest; type II amplicon appeared only in EcoRI/HpaII digestion but not in the EcoRI/MspI digest; type III generated amplicon obtained in EcoRI/MspI digest but not in the EcoRI/HpaII digest; and type IV represents the absence of band in both enzyme combinations.

Pattern of DNA methylation in the symptomatic and asymptomatic plant

Typically, MSAP amplicons (140) generating 14 different distribution patterns of cytosine methylation were grouped into two major types (Figure 4, Table 4). The MSAP amplicons were classified into two groups, Type I accounting for 2.85% (4/140) of the total number of amplicons. These sites were designated as monomorphic (either present or absent in tissues). Type I consisted of two classes, I-1 and I-2, whereas I-1 correspond to full methylation sites causing the cleavage by *Msp*I, but not by *Hpa*II, (Banding pattern as 0,1,0,1). Whereas, I-2 belong to hemi-methylation sites displaying a different banding pattern. Differences in Type II banding patterns were detected between symptomatic and asymptomatic plant and 97.14% (136/140) of the total number of MSAP amplicons were polymorphic sites. These polymorphic sites were clustered into five major classes, II-A, B, C, D, and E (Figure 4, Table 4). From 140 polymorphic amplicons class, II-A (52.14%, 73/140) was most frequently observed and consisted of the following three patterns-II-A1 (0, 0, 1, 1), II-A2 (0, 1, 1, 1), and II-A3 (1, 0, 1, 1). A common feature of patterns in class II-A was the detection of amplicon in both H and M lanes of asymptomatic plants, but those were either absent in H and M lanes of symptomatic plants or were only displayed in a single lane. This indicated that de-methylation might have occurred at these sites that to more in the symptomatic plants than the asymptomatic plants. For class II-B, 19 amplified fragments were recorded comprising following three patterns, II-B1 (1, 1, 0, 0), II-B2 (1, 0, 0, 0), and II-B3 (0, 1, 0, 0). These fragments were detected in one or both lanes of symptomatic plant, but not found in asymptomatic plants thus delineating that both external cytosine and internal cytosine methylation might have occurred in the asymptomatic plant only. Classes C and D accounted for 7.14% (10/140) and 22.14% (31/140), respectively. Class II-C corresponded to hyper-methylation; while, class II-D corresponded to a reduced level of methylation. Furthermore, class II-E indicated reduced level of methylation accounting to 2.14% (3/140), while most of these fragments were belonging to classes II-E1 (1, 0, 0, 1) and II-E2 (0, 1, 1, 0, Figure 5).

Table 4. Different patterns of methylation and their frequencies in symptomatic and asymptomatic plant through numbers of sites of methylation.

Methyl. type	Symptomatic		Asymptomatic		Site	Frequency (%)	Methy. type	Symptomatic		Asymptomatic		Site	Frequency (%)
	H	M	H	M				H	M	H	M		
Type I					4	2.85	II-C					10	7.14
I-1	0	1	0	1			II-C1	0	0	0	1	6	
I-2	1	0	1	0	2		II-C2	0	0	1	0	4	
Type-II					136	97.14	II-D					31	22.14
II-A					73	52.14	II-D1	1	1	0	1	13	
II-A1	0	0	1	1	22		II-D2	1	1	1	0	18	
II-A2	0	1	1	1	32		II-E					3	2.14
II-A3	1	0	1	1	19		II-E1	1	0	0	1	3	
II-B					19	13.57	II-E2	0	1	1	0	-	
II-B1	1	1	0	0	13								
II-B2	1	0	0	0	5								
II-B3	0	1	0	0	1								

Note: H-*HpaII*; M-*MspI*

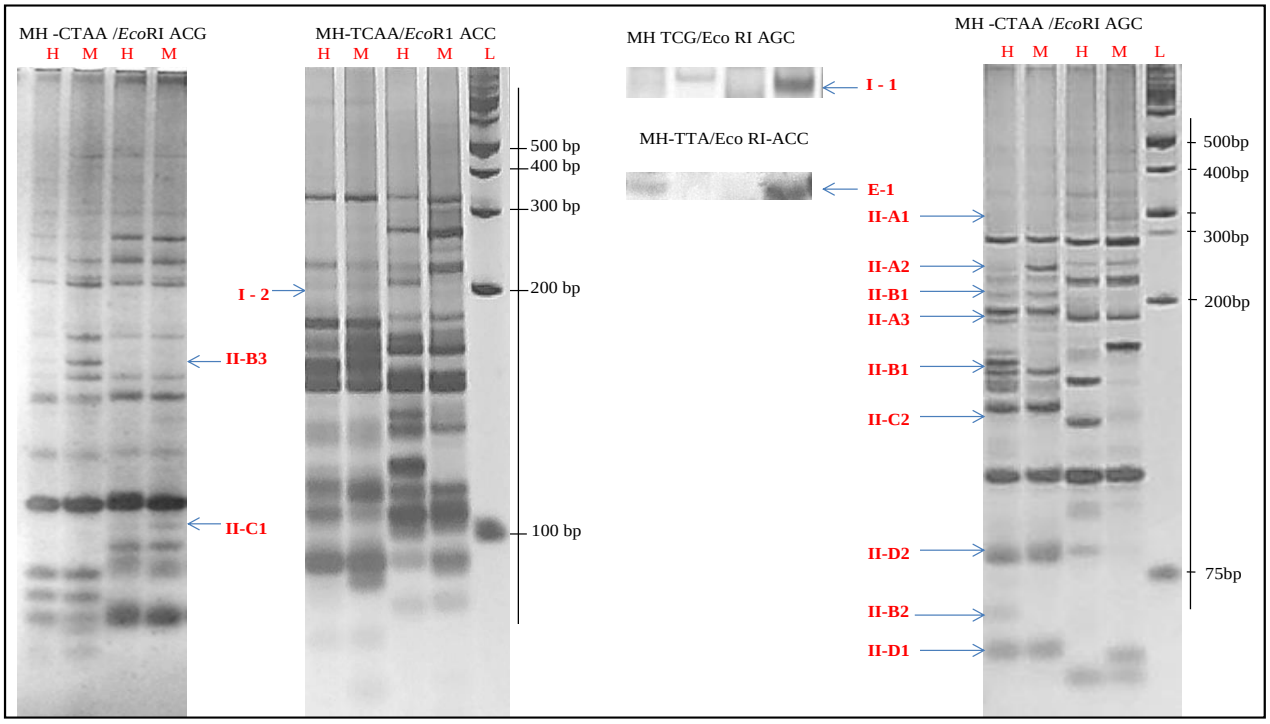

Figure 5. Electrophoretic profiles obtained from MSAP profiling and the arrows show different methylation patterns among fragments of both from symptomatic and asymptomatic plant.

DISCUSSION

Floral malady is an idiosyncratic disorder an unusual floral malady, limiting soybean yield in central India. The study was focused on epigenetic changes, especially reference to the complex interactions between the genome and environment. Today, this term is employed to refer alterations that are not due to changes in DNA sequence. Rather, epigenetic modifications, like DNA methylation, thereby regulating patterns of gene expression phenomenon. The floral malady per se molecular approaches have rarely been compared with cytological analysis, to know relation between the results of the cytological analysis and methylation patterns. The present investigation has undertaken to compare cytological screening with the pattern and extent of DNA methylation in symptomatic and asymptomatic plants. It revealed that cytological behavior in symptomatic plant may be due to difference at methylation in symptomatic and in asymptomatic plants. The results showed noteworthy difference in structural and functional behavior of pollens in symptomatic and asymptomatic plants. The pollens were shrunken and distorted than the normal pollens; delicate pollens were

diaphanous with less cytoplasm and thicker exine wall whereas, asymptomatic plants exhibited absolutely normal morphology of pollen. Neither one can predict its occurrence of the malady in the field nor recognize the symptomatic plants at an early age. The comprehensive field survey of central India revealed a 5–50% yield loss during 2010 and 2011 and cv. Samrat found as a most vulnerable genotype to the malady with an estimated incidence of 30%, followed by JS 93-05 (21%). The genotype JS-335 had moderate vulnerability with 13.2% incidence and recorded 13% for Sonia Gold [2]. In the past few years, 90% incidence was recorded in genotype JS-335 followed by JS-93-05 (58.8%) as reported by [2]. Hence, the genotype JS-335 was preferred for further MSAP profiling. Similarly, pollen cytology revealed a significant reduction in the number of pollen grains and the presence of sterile pollens in the symptomatic plants. Moreover, pollens were found non-viable, distorted in shape, and reduced germination ability. However, pollens in asymptomatic plants do not have flaws in their number, size, shape, and function [18].

During plant growth and development process, changes in DNA methylation play a key role in blooming, regulation of genes expression for vital functions, genomic defense, cell differentiation, and development [21] as well as to find epigenetic changes of conserved material [22]. The flowering process is shown to be accompanied by variations in the pattern of methylation in plants such as purple perilla (Perilla frutescens) and Silene armeria [23]. Similarly, the rate of DNA methylation in individual flowering plants is considerably lower than that of non-flowering plants and it has indicated that changes in the DNA methylation are closely related to the expression of flowering genes [24, 25]. The approach of MSAP profiling is widely being used to study the role of cytosine methylation in the regulation of gene expression during plant regeneration and development. MSAP facilitates the rapid identification of the methylated sequences in the genome. It is being successfully implemented in diverse plant species towards the detection of cytosine methylation [14, 26-29].

In the present investigated study, alteration of cytosine methylation in floral bud distorted and asymptomatic plant was found. Therefore, comparison of MSAP profile revealed that two main classes of differentially methylated genomic fragments: A) amplicons appeared after digestion with HpaII but not with MspI in all detected samples in the symptomatic plant, which were generated due to the DNA methylation at the CCGG sites; and B) amplicons appeared after digestion with MspI and not with HpaII in the asymptomatic plant, also the amplicons present in both the symptomatic and asymptomatic plant samples when digested with HpaII and MspI. Total alteration in cytosine methylation level in floral bud distorted plant was higher (5.88 to 56.52%) than that of asymptomatic plants (0.0 to 29.41%). Hence, methylation is a direct function of both environment and genotypes. In consensus to present findings, higher epigenetic variations in regenerants of potato (3.2% to 8.5%) was found [8] to that of their corresponding mother plants (0.0 to 3.4%) and higher epigenetic (12.56% to 26.13%) variations among the plantlets of potato derived from long term nodal tissue culture [30]. The findings revealed that there were greater differences in the level of genomic DNA methylation between the symptomatic and asymptomatic plants. During plant growth and developmental processes, changes in DNA methylation plays a crucial role in budding and regulation of gene expression for vital functions such as genomic defense, cellular differentiation, and development. This study demonstrates that the effect of environmental factors resulting in the epigenetic changes of soybean plants showed that the regulation of DNA methylation poses a great impact on the plant growth resulting in no pod formation in the plant.

The higher rates of methylation in symptomatic plants indicate hypermethylation in the promoter and/or coding region of a gene can inhibit the binding of transcription factor complexes in the symptomatic plants. This in turn inhibits gene expression, resulting in gene silencing; furthermore, demethylation in asymptomatic plants promotes gene expression. Similarly, the findings [31] revealed methylation of the FT promoter causes gene silencing and late flowering in Arabidopsis. The present study showed increased methylation in the symptomatic plants during the R6 stage. This is in accordance with earlier findings implying that methylation levels may change during cell differentiation and may be organized by regulatory mechanisms of gene expression during development and differentiation [32]. The MSAP technique assisted to investigate the percentage and the pattern of DNA methylation in floral malady symptomatic and asymptomatic plants. Pairwise comparisons of the symptomatic and asymptomatic samples exhibited that most of them had significantly different levels of methylation. The levels of methylation in symptomatic plant increased than the asymptomatic plant, delineating higher epigenetic influence in floral bud distorted plant. The MSAP is a reliable, low-priced, and fairly simple genome-wide method for the detection of genome regions with the putative alterations in DNA cytosine methylation in response to environmental and developmental aberrations. During plant growth and developmental processes, changes in DNA methylation perform an important role in plant development. This study demonstrated that the effect of biotic factor such as floral malady indicated the epigenetic changes via regulation of DNA methylation, has a great impact to such an

extent on the plant growth consequential to no pod formation. Either an increase or a decrease in DNA methylation level is the permanent imparting reduction in pod formation. These changes may also be accompanied by changes in the efficiency of gene transcription possibly controlled by regulation of methylation status in promoter regions or coding regions of genes and further needs to be studied.

CONCLUSION

In conclusion, it has been revealed that many endogenous genes are methylated either within their promoters or within their transcribed regions and that gene methylation is highly correlated with transcription levels. The methylated state is usually associated with inactivation of gene expression and vice versa. It has been demonstrated that several genes related to abiotic and biotic stress responses are differentially methylated. Indeed, we found that the levels of methylation in the symptomatic plants increased to about 28.31 % and in asymptomatic 18.71%. Thus, showing a higher epigenetic influence in floral bud distorted plant. The effect of environmental factors resulting in the epigenetic changes in soybean plants showed that the regulation of DNA methylation has a great impact on the plant growth leading to no pod formation to the plant. Either an increase or a decrease in DNA methylation level is permanent thus, showing the reduction in pod formation. These changes are also accompanied by changes in the efficiency in gene transcription possibly controlled by regulation of methylation status in promoter regions or coding regions of genes.

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