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EFFECTS OF *CERCOSPORA SOJINA* HARA STRESS ON DNA METHYLATION IN SOYBEAN

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Abstract

Epigenetic mechanisms significantly influence plant stress responses, yet their role in soybean (*Glycine max*) responses to stress triggered by *Cercospora sojina* Hara infection remains unexplored. In this study, soybean strains Longpin 09-85 and Yulong 09-521, with respective high susceptibility and high resistance to *C. sojina* Hara infection, were analysed for differences in infection-induced DNA methylation and changes in gene expression using the methylation-sensitive amplified polymorphism (MSAP) technique. Post-infection, both strains exhibited increased genomic DNA demethylation that was significantly more pronounced in the Longpin 09-85 strain, thereby reducing the Longpin 09-85:Yulong 09-521 DNA methylation ratio. Furthermore, the difference between stress-induced methylation and demethylation changes in DNA of the susceptible strain was significantly greater than that of the resistant strain, suggesting an important role for genomic DNA methylation in soybean resistance to stress induced by *C. sojina* Hara infection. Analysis of the total of 22 MSAP products that were differentially methylated between strains revealed four products that encoded proteins with homologies to proteins with known functions, as well as diverse types of methylation changes occurring in response to *C. sojina* Hara infection-induced stress. These findings establish a foundation on which to build future investigations of molecular mechanisms governing soybean responses to *C. sojina* Hara-induced stress.

Key words: *Cercospora sojina* Hara stress; DNA methylation; MASP; Homology analysis.

Introduction

Various environmental stressors, including drought, disease, salinity, extreme temperatures and biological stressors, such as *Cercospora sojina* Hara infection, significantly diminish crop yields. *C. sojina* Hara, a fungal crop pathogen initially reported in Japan, is now also found in the United States, Brazil and other agricultural countries (Ma & Han, 2014; Liu *et al.*, 2021*),* where it causes outbreaks of soybean grey leaf spot disease. This pathogen can infect soybean leaves, stems, pods and grains, with infections of leaves and grains most detrimental to crop growth and yield (Gu *et al.,* 2021*)*. Notably, this disease is highly prevalent in Heilongjiang Province, China within an area covering nearly one million hectares (Wu & Morris, 2001; Ding *et al.*, 2003).

In recent years, DNA methylation has garnered considerable attention for its pivotal roles in regulating gene expression, maintaining genome stability and facilitating normal eukaryotic cell growth and development (Sun *et al.,* 2021*)*. In both animals and plants, conversion of cytosine to 5-methylcytosine is an important epigenetic modification associated with silent chromatin. Silent chromatin participates in diverse cellular activities, including silencing of expression of transposable elements (TEs), regulating gene expression, X-chromosome inactivation, somatic clonal variation, gene imprinting, and growth and development (Soppe *et al*., 2000; Sanjida *et al*., 2004; Pham *et al*., 2015; Luo *et al*., 2018; Francisco *et al*., 2018; Vishwakarma *et al*., 2020). Importantly, once a cytosine residue undergoes DNA methylation, this modifications can persist as a genomic epigenetic change across many generations, contributing to stress-induced gene evolution (Ganesan, 2018; Soodeh & Jacqueline, 2019). Understanding these alterations may reveal pivotal genes and molecular pathways involved in soybean adaptation to *C. sojina* infection-induced stress to guide the development of new soybean varieties with enhanced resistance to this pathogen.

The methylation-sensitive amplified polymorphism (MSAP) analysis technique, a variant of the widely used amplified fragment length polymorphism (AFLP) analysis method, is a simple method frequently employed to detect cytosine methylation changes at CCGG sites across entire genomes of plants, including maize, rapeseed and peony (Portis *et al*., 2004; Tan, 2009; Fu *et al*., 2009; Zhang *et al*., 2021; Luo *et al*., 2022). MSAP technology has been instrumental in detecting genome methylation level increases, up-regulated methyltransferase gene expression and nuclear chromatin structural changes occurring in plants under stress (Greco *et al*., 2014). These findings have shed light on the role of chromatin structural changes in the maize adaptive response to stress induced by grey leaf spot disease. Specifically, these structural changes have been shown to govern the genome-wide balance between up-regulation and down-regulation of gene expression by altering key genomic DNA methylation patterns within distinct chromatin regions (Portis *et al*., 2004).

In this study, we employed MSAP analysis to explore changes in genome methylation profiles of soybean plants under stress induced by *C. sojina* Hara infection with the aim of elucidating epigenetic molecular mechanisms involved in the soybean stress response triggered by exposure to this pathogen. For this analysis, disease-susceptible and disease-resistant soybean strains Longpin 09-85 and Yulong 09-521, respectively, served as

experimental materials. In addition to methylation patterns, we investigated functions of proteins encoded by genes found within differentially methylated DNA fragments obtained from leaves of stressed plants of both strains to uncover the relationship between disease resistance and DNA methylation differences between strains, as well as methylation-related mechanisms governing gene expression changes associated with the soybean response to stress induced by *C. sojina* Hara infection. These findings pave the way for future studies of soybean stress response mechanisms and may guide the development of soybean varieties with resistance to grey leaf spot disease.

Material and Methods

Plant materials, enzymes and reagents: The soybean Sensitive strains (Long pin 09-85) and Resistant strains (Yu nong 09-521) were provided by Jiamusi Branch of Heilongjiang Academy of Agricultural Sciences.Sensitive strains (Long pin 09-85) for 110 days from emergence to maturity, the active accumulated temperature should be Aricultural Sciences 2150°C.It is an early maturing and high protein soybean variety. It has pointed leaves, white flowers, gray hairs and light brown pods. The plant is about 89 cm high, with branches. The seeds are round, yellow and glossy, and the hilum is yellow. The 100 seeds weight is about 19.6 g. Early May, the yield per hectare is about 2480.5 kilograms. Resistant strains (Yu nong 09-521) for 125 days from emergence to maturity, the active accumulated temperature should be Aricultural Sciences 2550°C.It is an early maturing and high protein soybean variety. It has pointed leaves, purple flowers, gray hairs and light brown pods. The plant is about 89 cm high, with branches. The seeds are round, yellow and glossy, and the hilum is yellow. The 100 seeds weight is about 24.9 g. Early May, the yield per hectare is about 3075.8 kilograms. Main experimental reagents are shown in Table 1.

Plant Materials Treatments

Activation and culture of soybean *Cercospora sojina* Hara: First of all, soybean *Cercospora sojina* Hara physiological speciesnumber 1, number 3, number 7, number 11 and number 15 were inoculated into PDA medium and cultured at 28℃ for 7-10 days. Then, mycelia were selected from PDA medium and inoculated into sorghum grain medium. Add sterile water to the sorghum grains covered with mycelia, disinfect them with 70% alcohol, then gently rub the sorghum grains until the mycelia is washed off the surface. Finally, put the sorghum grains on gauze to dry thoroughly, and store in a cool and dry place at room temperature.

Preparation of spore suspension: Before inoculation, sorghum grains were soaked in sterile water for 12 h, then taken out and placed on the yarn net to drain the water. The sorghum grains were covered with several layers of wet gauze (the gauze should be kept wet all the time) to induce new spore production. After 5 days, the sorghum grains were put into a basin and bowl, and sterile water was added to the sorghum grains, and then the sorghum grains were rubbed down to wash the black spores on the surface. Then the sorghum grains were taken out, and the water was the suspension of spores. It is appropriate to adjust the concentration of the spore suspension to 5-10 spores in each field of view under a 10×10 times microscope. The spore suspension of different physiological races of *Cercospora sojina Hara* was mixed, and 3% sucrose was added to the suspension to increase the concentration of the spore suspension.

Soybean inoculation and sampling: The soybean lines were planted alternately in the soil with good moisture retention effect, and inoculated when the second compound leaf was three out. The spore suspension was evenly sprayed on the soybean leaves twice with a sprayer, with an interval of 7 days. The control group was sprayed with 3% sucrose solution in the same way. One month after inoculation, leaves of susceptible and highly resistant soybean plants with the same growth status and leaves of control plants were used for genomic DNA extraction.

Extraction of soybean genomic DNA: The leaves of high-susceptible soybean strains (Longpin 09-85) and high-resistant soybean strains (Yunong 09-521) inoculated with *Cercospora sojina Hara* and uninoculated control were respectively 0.1 g and placed in a 2.0mL centrifuge tube. The leaves were frozen and ground with liquid nitrogen, and DNA was extracted by SDS. The concentration and purity of DNA were determined by UV spectrophotometry and detected by 1% agarose gel electrophoresis.

Methylation-sensitive amplified polymorphism (MSAP) analysis: The methylation-sensitive amplified polymorphism (MSAP) analysis method used in this study was based on the method reported by Pan *et al*, 2009 and modified. Methylation-sensitive restriction enzymes Hpa II and Msp I were used in combination with restriction enzymes EcoR I (E+H, E+M), and then treated and control genomic DNA was double digested with each pair of enzymes. The sequences of adaptor and pre-amplified primers and selective amplified primers are shown in Table 2. Each reaction system and reaction conditions are shown in Table 3. PCR products were separated by 5% denaturing polyacrylamide gel electrophoresis and stained with 0.1% silver nitrate. After digital scanning and imaging of the gel, the number of electrophoretic bands in each lane of each primer combination was counted, with bands marked as 1 and without bands marked as "0". The results of three experiments were analyzed to determine the statistical significance.

Data collation: Cut the differential bands from the glue plate with a clean blade, put them into a 1.5ml centrifuge tube, add 20μL sterilized deionized water, and take a 70℃ water bath for 90-120 min. After cooling, take 2μL supernatant as template, and use pre-amplification primers for PCR amplification (amplification conditions are the same as pre-amplification). PCR products are recovered with DNA glue recovery kit. It was ligated with pMD18-T vector and transformed into *E. coli* DH5α. After screening for ampicin resistance, positive clones were selected and sent to Shanghai Sangong for sequencing. The sequences obtained were searched by BLAST program on NCBI.

Table 1. Main reagents used in this study.

Reagent name	Manufacturer (Product Model)
Tris base	CAISSON LABS, Beijing, China (CAS77-86-1)
EDTA Na_2	BIOSHARP, Shanghai, China (CAS139-33-3)
Acrylamide	Beijing Hifi Technology Co., Ltd, Beijing, China (CAS79-06-1)
N'.N'-Methylene-bis-acrylamide	Aoran Fine Chemical Research Institute, Tianjin, China (CAS110-26-9)
Ammonium persulfate	Longxi Chemical Co., Ltd, Longxi, China (CAS7727-54-0)
Acetic acid glacial	Tianjin Fuyu Fine Chemical Co., Ltd, Tianjin, China (CAS64-19-7)
Silver nitrate	Shanghai No. 1 Reagent Factory, Shanghai, China (CAS7761-88-8)
Hpa II DNA Restriction Enzymes	TAKARA, Dalian, China (1053A)
Msp I DNA Restriction Enzymes	TAKARA, Dalian, China (1053A)
*Eco*R I DNA Restriction Enzymes	TAKARA, Dalian, China (1040A)
dNTP	TAKARA, Dalian, China (639132)
T_4 DNA ligase	TAKARA, Dalian, China (2011A)
Taq DNA polymerase	TAKARA, Dalian, China (RR001A)
MiniBEST Plant DNA Extraction Kit	TAKARA, Dalian, China (9763)
DNA glue recovery kit	TAKARA, Dalian, China (639138)

Table 2. Adapter and primer sequences.

Adaptor and primer		Sequence (5'→3')
Adaptors	*Eco*R I adaptors (F)	CTCGTAGACTGCGTACC
	*Eco*R I adaptors (R)	AATTGGTACGCAGTCTAC
	H/M adaptors (F)	GATCATGAGTCCTGCT
	H/M adaptors (R)	CGAGCAGGACTCATGA
Preamplification primers	E0 (E+A)	GACTGCGTACCAATTCA
	HM0 (HM+0)	ATCATGAGTCCTGCTCGG
Selecting amplification primers	E1 (E+AAC)	GACTGCGTACCAATTCAAC
	E2 (E+AAG)	GACTGCGTACCAATTCAAG
	E3 (E+ACA)	GACTGCGTACCAATTCACA
	E4 (E+ACT)	GACTGCGTACCAATTCACT
	E5 (E+ACG)	GACTGCGTACCAATTCACG
	E6 (E+AGC)	GACTGCGTACCAATTCAGC
	E7 (E+AGT)	GACTGCGTACCAATTCAGT
	E8 (E+AGA)	GACTGCGTACCAATTCAGA
	E9 (E+ATC)	GACTGCGTACCAATTCATC
	HM1 (HM+TAC)	ATCATGAGTCCTGCTCGGTAC
	HM2 (HM+TCT)	ATCATGAGTCCTGCTCGGTCT
	HM3 (HM+TCG)	ATCATGAGTCCTGCTCGGTCG
	HM4 (HM+TCC)	ATCATGAGTCCTGCTCGGTCC
	HM5 (HM+TGA)	ATCATGAGTCCTGCTCGGTGA
	HM6 (HM+TGT)	ATCATGAGTCCTGCTCGGTGT
	HM7 (HM+TGC)	ATCATGAGTCCTGCTCGGTGC
	HM8 (HM+TTA)	ATCATGAGTCCTGCTCGGTT
	HM9 (HM+TTC)	ATCATGAGTCCTGCTCGGTTC
	HM10 (HM+TTG)	ATCATGAGTCCTGCTCGGTTG

Notes: H/M: *Hpa* II and *Msp* I

Analysis of DNA methylation level and pattern change: Isomerases *Hpa* II and *Msp* I have different sensitivities to the methylation status of DNA cleavage sites. MSAP analysis of the two cleaved fragments can reveal the methylation status of individual CCGG sites, as well as the overall degree of cleaved site methylation across the genome. DNA samples were cleaved by *Eco*R I/*Hpa* II(H) and *Eco*R I/*Msp* I(M) enzymes to obtain four methylation band types. Type I bands are H and M gel bands, indicating no methylation at CCGG sites. Type II bands had bands in lane H but no bands in lane M, indicating single-stranded side methylation (hemimethylation /CHG methylation) at the CCGG site. The type III bands were in lane H and lane M respectively, indicating double-stranded medial methylation (full methylation /CG methylation) at the CCGG site. Type IV bands showed no bands in lane H and lane M, suggesting double-stranded side methylation at the CCGG site. Only three band types I, II and III could be detected in polyacrylamide gel.

Table 3. Reaction system and conditions in methylation sensitive amplified polymorphism (MSAP) analysis.

System	Reaction system	Reaction conditions
Restriction enzyme digestion	*Eco*R I 5 U, *Hpa* II/*Msp* I 5 U, 10×Buffer 3μL, DNA 400 ng. Add appropriate amount of ddH_2O to a total capacity of 30μL	Digestion at 37°C for 6 h, inactivation at 65°C for 20 min and storage at 4°C until use
Ligation	double-cut DNA product 20 μL, *Eco*R I adaptors 5 pmol, H/M adaptors 50 pmol, T4 DNA ligase 1 U, 10×Buffer 4 μL. Add appropriate amount of ddH_2O to a total capacity of 40μL.	Ligation reactions proceeded at 16°C for 13 h followed by inactivation at 65 °C for 10 min and storage at -20°C for later use.
Pre-amplification	ligated product 2 μL, E0 primer 5 pmol, H/M0 primer 5 pmol, dNTP (2.5 mM) 1.6 μL, 10×Buffer 2 μL, Taq polymerase 0.5 U. Add appropriate amount of ddH_2O to a total capacity of 20μL.	Pre-denaturation at 94°C for 5 min followed by 30 cycles of (denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min) and a final extension step of 72°C for 5 min. Completed reactions were stored at -20°C for later use.
Selective amplification	Pre-amplification product diluted (10×) 3 μL, E primer 5 pmol, H/M primer 5 pmol, dNTP (2.5 mM) 1.6 μL, 10 × Buffer 2 μL, Taq polymerase 0.5 U. Add appropriate amount of ddH_2O to a total capacity of 20μL.	Pre-denaturation 94°C 5 min, denaturation 94°C 30 s, annealing 65°C 30 s, extension 72°C 1 min, then a temperature reduce of 0.7°C per cycle for 13 cycles; followed by 23 cycles of (94°C 30 s, 56°C 30 s, 72°C 1 min) and a final extension step at 72°C for 10 min. Amplification reactions were stored at -20°C.

Table 4. Effects of *Cercospora sojina* Hara stress on soybean leaf genomic DNA methylation level.

MSAP amplified bands type	Sensitive strains (Long pin 09-85)		Resistant strains (Yu nong 09-521)	
	Control	Stress	Control	Stress
I type	1746	1841	1805	1812
II type	103	65	79	65
III type	396	410	373	373
Total bands	2245	2316	2257	2250
Total bands with methylation	499	475	452	438
Ratio of hemi-methylation (%)	4.59	2.80	3.50	2.89
Ratio of global methylation (%)	17.64	17.70	16.53	16.58
Ratio of total methylation (%)	22.23	20.51	20.03	19.47

Note: Total amplified bands=I+II+III; Total methylated bands=II+III; Semi-methylation rate =II/total amplified bands; Full methylation rate = III/total amplified bands; Methylation polymorphism ratio = Total methylated bands / total amplified bands

Results and Discussion

Effects of *Cercospora sojina* Hara stress on DNA methylation levels: In our MSAP analysis, we utilised 36 primer combinations to examine DNA methylation patterns in genomic DNA of *C. sojina*-inoculated and uninoculated soybean plants of susceptible and highly resistant strains. Statistical analysis of relative frequencies of methylated and unmethylated amplified DNA products obtained from leaves of pathogen-inoculated and uninoculated plants of both strains revealed that of the total 2245-2316 amplified DNA products obtained from all experimental groups, 438-499 (19.47%-22.23%) were methylated. Notably, the DNA products of the susceptible strain exhibited a higher global methylation rate (22.23%) as compared to that of the highly resistant strain (20.03%), with similar trends noted for hemimethylation and total methylation rates. Following inoculation with the fungal pathogen, both strains displayed decreased global methylation and hemimethylation rates as compared to their uninoculated counterparts, although total methylation rates of both strains remained largely unchanged after inoculation (Table 4).

DNA methylation serves as a common epigenetic mechanism utilised by organisms to cope with stress. As such, plants adapt to drastic environmental changes by altering their genomic DNA methylation levels and patterns to regulate and enhance their stress resilience (Chen *et al*., 2021b; Wang *et al*., 2021) For example, Pleurotus tuber-regium has been shown to adapt to bacterial stress through methylation-mediated regulation of gene or protein expression levels (Wu *et al*., 2022). Similarly, maize sheath blight has been shown to trigger an overall decrease in the genomic DNA methylation level of the highly susceptible inbred maize line 478, while that of the highly resistant maize inbred line R15 was observed to increase after infection (Ma & Han, 2014).

In this study, total DNA methylation rates of *C. sojina* Hara-infected leaves of soybean strains with high susceptibility and high resistance to leaf spot disease were both decreased after infection, with a more pronounced decrease observed in the susceptible strain. These results prompted us to speculate that decreased total DNA methylation rates may influence expression patterns of stress-related genes that may differ between stress-susceptible and stress-resistant strains. For example, in our study the highly susceptible strain exhibited less DNA

methylation than the resistant strain, as consistent with results of a previous study demonstrating that insufficient DNA methylation in environmentally stressed plants led to abnormal phenotypes (Xu *et al*., 2014). Consequently, our findings suggest that the significant decrease in DNA methylation rate in the highly susceptible soybean strain following *C. sojina* Hara infection potentially contributed to heightened susceptibility to infection.

Effects of *Cercospora sojina* Hara stress on DNA methylation patterns: Comparison of MSAP results between soybean strains with high and low susceptibility to grey leaf spot disease MSAP analysis of *C. sojina*-inoculated or uninoculated susceptible and resistant soybean strains revealed 13 different band patterns between the two strains (Table 5, Fig. 1). Differences in stress-induced DNA methylation pattern changes between strains mainly included monomorphic and polymorphic changes (Table 5). A monomorphic change refers to an unchanging band type between control and inoculated leaves (type D), indicating that the methylation status of CCGG site did not change after inoculation, with type D1 referring to an unmethylated site and types D2 and D3 referring to hemimethylated sites.

In contrast, a polymorphic change refers to different methylation patterns between control and inoculated leaves indicating altered CCGG site methylation status after inoculation. Polymorphic changes are of three types: methylation (type A), demethylation (type B) and indefinite (type C). Within the CCGG site, the medial cytosine methylation site is referred to as the CG site, the site undergoing lateral cytosine methylation is referred to as the CHG site and the site undergoing both medial and lateral methylation modifications is both a CG and CHG (CG/CHG) site. Regarding type A changes, A1 and A2 indicate elevated CG site methylation levels at CG sites, A3 and A4 indicate elevated methylation at CHG sites and A5 indicates elevated methylation at CG/CHG sites. Our results revealed increased type A soybean leaf genomic methylation levels in response to stress induced by *C. sojina* Hara infection.

Table 5. Changes of methylation pattern of soybean genome under *Cercospora sojina* Hara stress.

Status type	Type				Methylation status		Sites	
	Control		Stress		Control	Stress	Sensitive strains (Long pin 09-85)	Resistant strains (Yu nong 09-521)
	H	M	H	M				
A1	1	1	0	1	CCGG GGCC	C<u>C</u>GG GG<u>C</u>C	8	12
A2	1	0	0	0	<u>CC</u>GG<u>CC</u>GG GGC<u>C</u> GCC	<u>CC</u>GG GG<u>CC</u>	8	23
A3	1	1	1	0	CCGG GGCC	<u>CC</u>GG<u>CC</u>GG GGC<u>C</u>GGCC	9	13
A4	0	1	0	0	C<u>C</u>GG GG<u>C</u>C	<u>CC</u>GG GG<u>CC</u>	50	26
A5	1	1	0	0	CCGG GGCC	<u>CC</u>GG GG<u>CC</u>	6	84
B1	0	1	1	1	C<u>C</u>GG GG<u>C</u>C	CCGG GGCC	10	21
B2	0	0	1	0	<u>CC</u>GG GG<u>CC</u>	<u>CC</u>GG<u>CC</u>GG GGC<u>C</u>GGCC	26	19
B3	1	0	1	1	<u>CC</u>GG<u>CC</u>GG GGC<u>C</u>GGCC	CCGG GGCC	60	24
B4	0	0	0	1	<u>CC</u>GG GG<u>CC</u>	C<u>C</u>GG GG<u>C</u>C	70	34
B5	0	0	1	1	<u>CC</u>GG GG<u>CC</u>	CCGG GGCC	34	76
C1	0	1	1	0	C<u>C</u>GG GG<u>C</u>C	<u>CC</u>GG<u>CC</u>GG GGC<u>C</u>GGCC	1	2
C2	1	0	0	1	<u>CC</u>GG<u>CC</u>GG GGC<u>C</u>GGCC	C<u>C</u>GG GG<u>C</u>C	1	2
D1	1	1	1	1	CCGG GGCC	CCGG GGCC	1707	1668
D2	1	0	1	0	<u>CC</u>GG<u>CC</u>GG GGC<u>C</u>GGCC	<u>CC</u>GG<u>CC</u>GG GGC<u>C</u>GGCC	31	31
D3	0	1	0	1	C<u>C</u>GG GG<u>C</u>C	C<u>C</u>GG GG<u>C</u>C	329	322
Total amplified bands (A +B+ C +D)							2350	2357
Total polymorphic bands (A +B+ C)							283	336
Polymorphic bands ratio of polymorphic bands (%)							12.04	14.26
Type A band ratio of polymorphic bands (%)							3.45	6.7
Type B band ratio of polymorphic bands (%)							8.51	7.38

Note: H: the lane of *Eco*R I + *Hpa* II enzyme combinations; M: the lane of *Eco*RI + *Msp* I

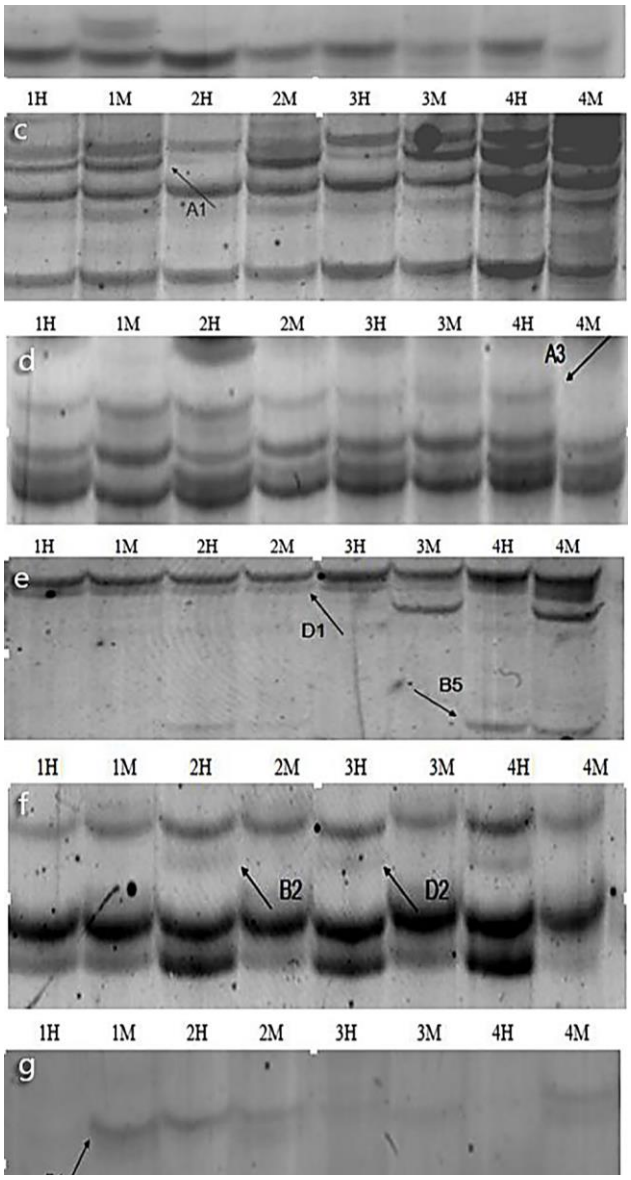

Fig. 1. Soybean leaf DNA methylation banding patterns
Note:H: *Eco* R I+*Hpa* II combination; M: *Eco*R I+*Msp* I combination;1: Long pin 09-85(control);
2: Long pin 09-85 (vaccination); 3: Yu nong 09-521 (control); 4: Yu nong 09-521 (vaccination)

With regard to type B changes, B1 and B2 indicate decreased CG site methylation levels, B3 and B4 indicate decreased CHG site methylation levels and B5 indicates decreased methylation levels at CG/CHG sites at the same time. Our results revealed decreased type B methylation levels in genomic DNA of soybean leaves under *C. sojina* Hara infection-induced stress. Regarding type C changes, type C is assigned when the difference in DNA methylation level between control and inoculated leaves cannot be determined.

Here, CHG site methylation was the main stress-induced change observed in the highly susceptible strain, while CG/CHG site methylation was the main stress-induced change observed in the highly resistant strain. Regarding methylation changes of inoculated susceptible and resistant strains as compared to uninoculated controls, methylated sites (type A) accounted for 3.45% and 6.70% of total MSAP site changes, while demethylated sites (type B) accounted for 8.51% and 7.38% of total MSAP site changes and total DNA methylation polymorphism rates were 12.04% and 14.26%, respectively.

Importantly, soybean leaf genomic DNA methylation patterns of stress-susceptible and stress-resistant strains underwent varying degrees of change in response to *C. sojina* Hara-induced stress, with demethylation emerging as the prevailing trend in both strains. Notably, a significant difference in methylation and demethylation change rates was observed in the highly susceptible strain, but not in the highly resistant strain. However, it is notable that the methylation polymorphism rate in the highly resistant strain surpassed that of the highly susceptible strain.

The regulation of gene expression in plants is chiefly governed by two methylation modes, heavy methylation and demethylation, with *in vivo* changes in these methylation modes closely linked to plant resilience to stress. In this vein, a previous study highlighted a significant correlation between DNA methylation rate and susceptibility to various diseases affecting plant growth, development and flowering period, as well as stress resistance, gene regulation efficiency and genome stability (Niu *et al*., 2020). Generally, DNA methylation levels are negatively correlated with gene expression, since methylation tends to silence gene expression and demethylation tends to promote gene expression.

Infection by pathogenic bacteria leads to demethylation of specific sites in plants. When *Arabidopsis thaliana* was infected with tomato bacterial leaf spot, several differentially expressed regions were identified near plant defense genes. The demethylation of these regions can promote the expression of adjacent genes, thereby enhancing the immunity of *Arabidopsis* and enabling the plant to effectively combat bacterial infection (Dowen *et al*., 2012).

DNA methylation also plays a role in animal resistance to viral infection. For example, in a study of hepatitis B virus (HBV), CIC, CIT and CIF directly inhibited hepatitis B replication in infected cells, thereby reducing viral load, while also exerting anti-inflammatory, immunoregulatory, and lipid metabolism-modulating anti-HBV effects by regulating DNA methylation activity (Zhang *et al*., 2022).

Here we observed alterations in methylation patterns of soybean strains with high-level susceptibility or high-level resistance to grey leaf spot disease-induced stress induced by *C. sojina* Hara infection, with a greater methylation polymorphism observed for the resistant strain. These findings indicate a potential link between the heightened disease resistance observed in the highly resistant strain and its amplified methylation reaction to stress induced by *C. sojina* Hara infection. The more rapid stress response of the resistant strain may be intricately connected to inter-strain differences in DNA methylation patterns that, in turn, lead to differences in expression of stress-response genes between strains.

Here we conducted MSAP analysis using 36 primer pairs to amplify 36 soybean genomic DNA fragments from a soybean strain that was highly resistant to grey leaf spot disease and a strain that was highly susceptible to the disease. Our MSAP findings revealed greater numbers of demethylation events in leaf genomic DNA of both strains after exposure to *C. sojina* Hara infection-induced stress, signifying stress-induced global changes in DNA

methylation patterns. These global changes predominantly involved DNA demethylation, which tends to turn on expression of genes functionally related to dynamic stress-response mechanisms that may ultimately minimise infection-induced toxicity.

Intriguingly, a significant difference between methylation and demethylation rates of the highly susceptible strain was observed post-infection (3.45% and 8.51% respectively), with no significant rate difference observed for the highly resistant strain (6.70% and 7.38% respectively). Therefore, we speculated that the greater resistance of the highly resistant strain to *C. sojina* Hara infection may be attributed to more pronounced demethylation-induced activation of genes associated with disease resistance and to enhanced methylation-induced genomic stability of this strain versus corresponding features of the susceptible strain.

Sequencing and homology analysis of methylated DNA fragments: Functional analysis of proteins encoded by infection stress response-related, differently methylated genomic DNA fragments obtained from highly resistant and susceptible strains.

To elucidate the functional roles of genes associated with differentially methylated soybean genomic sequences between strains with high and low resistance to leaf spot disease, we analysed predicted sequences of proteins encoded by 22 differentially methylated genomic fragments. Using the NCBI Blast search tool (https://blast.ncbi. nlm.nih.gov/Blast.cgi) we compared these sequences to those of known proteins, yielding insightful results (Table 6). Predicted proteins from 8 of the 22 fragments were homologous to proteins with known, diverse functions, such as cation/H+ antiporter, kinesin, long interspersed nuclear element-1 (LINE-1) reverse transcriptase, pentatricopeptide repeat (PPR), Frigida-Essential 1, tubulin, 2-aminoethiol dioxygenase and tryptophan-aspartic acid (WD) repeat proteins. Conversely, the remaining 14 fragments encoded three proteins without known functions and sequences of 11 fragments lacked homologues in the database.

Plant DNA methylation, particularly that occurring in response to stress, plays a pivotal role in regulating gene expression by altering gene interactions with transcription factors or by modulating chromatin structure. For instance, a study on rice cold tolerance (Han *et al*., 2017) highlighted differences in genomic DNA methylation patterns and transcriptomes of rice grown under diverse climatic conditions that were correlated with variations in cold tolerance. The regulation of cold tolerance-associated gene expression in rice is mediated by C-repeat binding (CBF) transcription factors, which orchestrate transcriptional pathways influenced by the methylation level of the *ICE1* gene. Intriguingly, expression levels of *ICE1* and its downstream regulated gene targets *CBF1* and *CBF3* are positively correlated with tolerance to cold temperatures, while a negative correlation exists between *ICE1* promoter region methylation and cold tolerance. Collectively, these results suggest a regulatory interplay between methylation levels and gene expression that ultimately dictates plant cold tolerance capacity.

Methylation also serves a critical function in plant adaptation to drought stress, as evidenced by a study conducted by (Kim *et al*., 2008), which demonstrated heightened methylation of histones, particularly through H3K4me3 modification of histone H3 protein. This modification facilitates interactions between histones and DNA sequences of stress-responsive genes *RD20* and *RD29A* during drought stress. Furthermore, under drought conditions, the Arabidopsis histone methylase Arabidopsis trithorax-like protein 1 (ATX1) has been shown to induce up-regulated expression of the gene encoding ABA (abscisic acid) biosynthetase 9-cis-epoxycarotenoid dioxygenase 3 (NCED3). This up-regulated expression is induced through H3K4me3 modifications of histones that interact with target sequences within these genes, leading to their increased transcription (Ding *et al*., 2011). These findings underscore the capacity of stress to modulate expression levels of DNA methylation-related genes, thereby regulating plant adaptation to stress.

Table 6. Blast result of DNA differential methylation bands.

Fragment number	Fragment length	Variation type	Strains	GenBank Login ID	Blast X	E-Value
S2	261	A2	Yu	ref|XP_006586547.1|	PREDICTED: uncharacterized protein LOC102669688 [Glycine max]	3 e-14
S3	166	A4	Long	ref|XP_003547155.2|	PREDICTED: protein FRIGIDA-ESSENTIAL 1-like [Glycine max]	2 e-22
S4	235	A4	Yu	gb|KHN25997.1|	Cation/H(+) antiporter 4 [Glycine soja]	2e-13
S6	359	B1	Yu	gb|KHN20030.1|	Kinesin-related protein 4 [Glycine soja]	2 e-23
S7	259	B3	Long	gb|KHN25562.1|	LINE-1 reverse transcriptase like [*Glycine soja*]	8e-31
S9	210	B1	Long	ref|XP_006589853.1|	PREDICTED: uncharacterized protein LOC100792312 [Glycine max]	3 e-16
S13	114	B4	Yu	ref|XP_006606279.1|	PREDICTED: uncharacterized protein LOC100791652 isoform X1 *[Glycine max]*	1 e-05
S19	117	B3	Long	ref|XP_008664343.1|	PREDICTED: tubulin beta-5 chain-like *[Zea mays]*	7 e-13
S20	122	A1	Long	gb|KHN28518.1|	Pentatricopeptide repeat-containing protein [*Glycine soja*]	8 e-12
S21	271	A1	Yu	ref|XP_003543744.1|	PREDICTED: 2-aminoethanethiol dioxygenase-like [*Glycine max*]	4 e-13
S22	204	B5	Long	ref|XP_003521420.1|	PREDICTED: WD repeat-containing protein 3-like[Glycine max]	8 e-12

Note: Long: Long pin 09-85; Yu: Yu nong 09-521

Our results uncovered three gene sequences that were demethylated in the susceptible soybean strain after *C. sojina* Hara inoculation. These sequences were homologous to LINE-1 reverse transcriptase (S7), β-tubulin (S19) and WD-repeat protein (S22). The main role of β-tubulin is to modulate cellular metabolism and function (Liu *et al*., 2018), while WD-repeat protein plays important roles in signal transduction, regulation of cell cycle, RNA splicing and transcription (Chen *et al*., 2021a), thereby affecting plant growth and development.

One demethylated gene sequence identified in resistant plants, S6, encodes a protein with homology to kinesin, a class of molecular motor proteins that primarily controls cellular activities related to mitosis and transport of intracellular substances (Mishra *et al*., 2021). This finding suggests that *C. sojina* Hara infection-induced methylation-related modifications of several genes may turn on expression of stress response genes. However, few studies have explored the functions of proteins encoded by these genes, their regulatory roles in soybean structural adaptations to infection, and whether soybean regulatory mechanisms conferring resistance to infection overlap with mechanisms conferring resistance to other environmental stresses. Understanding these regulatory mechanisms could offer valuable insights into the intricate interplay between plant responses to pathogenic stressors and broader environmental adaptability.

Conclusion

Under the stress of *Cercospora sojina* Hara,the overall level of genomic DNA methylation (total methylation rate + hemimethylation rate) decreased in both resistant and susceptible strains, and the decrease trend was obvious in highly susceptible strains.The methylation pattern variation was mainly demethylation, but there was a big difference between the methylation variation and demethylation variation in the highly susceptible strain, but not in the highly resistant strain, and the methylation polymorphism in the highly resistant strain was higher than that in the highly susceptible strain.DNA methylation variants occurred in a wide range, and the differences showed high homology with soybean genome, and most of them were related to soybean response to stress. The functional study of these genes will be helpful for the development of soybean varieties resistant to *Cercospora sojina* Hara stress.

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