Methylation-sensitive amplified polymorphism analysis of Verticillium wilt-stressed cotton (*Gossypium*)

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ABSTRACT. In this study, a methylation-sensitive amplification polymorphism analysis system was used to analyze DNA methylation level in three cotton accessions. Two disease-sensitive near-isogenic lines, PD94042 and IL41, and one disease-resistant *Gossypium mustelinum* accession were exposed to Verticillium wilt, to investigate molecular disease resistance mechanisms in cotton. We observed multiple different DNA methylation types across the three accessions following Verticillium wilt exposure. These included hypomethylation,

hypermethylation, and other patterns. In general, the global DNA methylation level was significantly increased in the disease-resistant accession *G. mustelinum* following disease exposure. In contrast, there was no significant difference in the disease-sensitive accession PD94042, and a significant decrease was observed in IL41. Our results suggest that disease-resistant cotton might employ a mechanism to increase methylation level in response to disease stress. The differing methylation patterns, together with the increase in global DNA methylation level, might play important roles in tolerance to Verticillium wilt in cotton. Through cloning and analysis of differently methylated DNA sequences, we were also able to identify several genes that may contribute to disease resistance in cotton. Our results revealed the effect of DNA methylation on cotton disease resistance, and also identified genes that played important roles, which may shed light on the future cotton disease-resistant molecular breeding.

Key words: Cotton; *Verticillium* wilt;
Methylation-sensitive amplification polymorphism

INTRODUCTION

With the development of the cotton textile industry, it is more and more important to breed cotton varieties with elite fiber quality and disease resistance. Upland cotton (*Gossypium hirsutum* L.) provides the majority of the cotton production in the world. However, a narrow genetic diversity restricts cotton breeding by limiting the range of valuable traits available for use and also leads to genetic vulnerability to biotic and abiotic stresses. Diseases such as Fusarium wilt and Verticillium wilt are the main threats to cotton production. Therefore, disease-resistant breeding is one of the most important purposes of cotton breeding programs. Cotton Verticillium wilt is a soil-borne vascular disease caused by a soil-inhabiting fungus, *Verticillium dahliae* Kleb., which mainly resides in the catheter of cotton plants in the form of mycelia and conidia, causing abscission of cotton leaves and bolls. Verticillium wilt can survive in the soil in the form of microsclerotia, making the fungus difficult to prevent or cure. Therefore, the most economical and effective solution is to grow disease-resistant varieties. Most commercial cultivars of upland cotton are susceptible or show only slight resistance to cotton wilt diseases. It is therefore necessary for cotton breeders to improve Verticillium wilt resistance in *G. hirsutum* by conducting introgression of resistance genes from diverse resistance sources (Zhao et al., 2014). Exploring the molecular mechanisms of Verticillium wilt resistance in cotton is of great importance to cotton disease breeding. So far, most studies have focused on screening quantitative trait loci or new resistance genes, whereas little attention has been paid to the epigenetic profiling of the resistance responses.

In this study, we used a capillary electrophoresis (CE)-based methylation-sensitive amplified polymorphism (MSAP, Xiong et al., 1999; Xu et al., 2000) method. This has been shown to be an effective and reliable method for detecting whole-genome DNA methylation (Wang et al., 2015, 2016). The DNA methylation levels after disease exposure and in a no-disease control were detected in three cotton accessions; two *G. hirsutum* near-isogenic lines and one *Gossypium mustelinum* accession. Our aim was to elucidate the molecular mechanism of disease resistance in cotton from the viewpoint of epigenetics.

MATERIAL AND METHODS

Cotton materials

The cotton materials used in this study were *G. mustelinum*, *G. hirsutum* accession PD94042, and its near-isogenic line IL41 with introgression of *G. mustelinum* segments in a PD94042 background.

Verticillium wilt resistance assay under greenhouse conditions

A defoliating *V. dahliae* isolate, V991, which is prevailing in the Yellow and Yangtze River cotton growing regions (Ning et al., 2013), was used for the disease infection. *V. dahliae* isolates were grown on potato dextrose agar plates at 25°C for 7-10 days. For the inoculum preparation, a conidial suspension was spread on plates and incubated at 25°C for 6-7 days. Conidia were then collected and diluted to a concentration of 1-5 x 10^7 cells/mL. The conidial suspension was prepared immediately before inoculation used.

The Verticillium wilt resistance evaluation was conducted in a greenhouse. Acid-delinted seeds were planted in 10-cm-diameter pots with one seed per pot and 30 pots per cotton accession. The pots were filled with vermiculite. At the four-true leaf stage, 20 healthy and uniformly growing plants were selected from each cotton accession that were then divided into four groups consisting of five plants per group. The groups formed two replicates for the disease treatment and two control replicates, respectively. The bottom of the pot was gently torn off with scissors. The two disease treatment groups were placed on plates containing 100 mL *V. dahliae* conidial suspension, whereas the other two groups were treated with 100 mL fresh water (no-disease control). When all the conidial suspension had been absorbed, the seedlings were planted in a seedling bed in a greenhouse. Five days after inoculation, the 4th leaf from each plant was collected and stored at -80°C for later use. The plants were irrigated once a week. The average temperature was 24°-27°C in the daytime and 18°-20°C at nighttime. Three weeks after inoculation, disease reactions were scored as described by Ning et al. (2013). Disease grades of 0, 1, 2, 3, and 4 for leaf disease symptoms corresponded to the following: 0: no disease symptoms observed; 1: <25.0% of the leaf surface exhibited disease symptoms; 2: 25.1 to 50.0% of the leaf surface exhibited disease symptoms; 3: 50.1 to 75.0% of the leaf surface exhibited disease symptoms; and 4: >75.0% of the leaf surface exhibited disease symptoms, with plants completely defoliated or dead. A Verticillium wilt disease index was calculated using the following formula:

$$R = (\sum_{i=1}^{n} X_i Y_i) / 4\alpha \qquad \text{(Equation 1)}$$

in which R is the Verticillium wilt disease index of each cotton accession, X is the disease grade between 0 and 4, Y is the number of plants with that disease grade, and Y is the total number of investigated plants of each cotton accession. The differences of the disease resistance levels between the three cotton accessions were analyzed by using SPSS ver. 19.0.

DNA isolation and MSAP assay

DNA of the three cotton accessions was extracted following the protocols described

by Paterson et al. (1993). First, any RNA was digested using 20 mg/mL RNase for 30 min at room temperature. MSAP detection with the introduced CE was performed as described by Wang et al. (2016). The robustness of the three biological replicates for each sample was identified using multiple primers and identical MSAP bands among all three replicates were detected (data not shown). The subsequent MSAP analysis was then conducted pooling the samples from both replicates. A total of 32 amplified fragment length polymorphism primer combinations, including four *Eco*RI and eight *Hpa*II-*Msp*I primers, were used in this study. The detailed sequence information about the adapters and primers used in MSAP is provided in **Table S1**.

The scored MSAP bands were then transformed into a binary character matrix, where the absence and presence of particular sites were indicated with "0" and "1", respectively. Although both *Hpa*II and *Msp*I recognize the tetranucleotide sequence 5'-CCGG, *Hpa*II cleaves the hemimethylated sequence (only one methylated DNA strand) but is inactive if one or both cytosines are fully methylated (both strands methylated), whereas *Msp*I cleaves $C^{5m}CGG$ but not $^{5m}CCGG$ (McClelland et al., 1994). The resulting MSAP patterns from digestions with the isoschizomers for each single DNA sample were divided into the following four band types: Type I: present only for *Eco*RI/*Msp*I (0,1), representing full methylation of internal cytosine of 5'-CCGG on both strands; Type II: present only for *Eco*RI/*Hpa*II (1,0), suggesting a hemimethylated state of 5'-CCGG sites owing to methylation in one DNA strand (external cytosine or both external and internal cytosines of 5'-CCGG) but not in its complementary strand; Type III: absent from both enzyme combinations (0,0), indicating full methylation of both strands where at least one external cytosine of 5'-CCGG was methylated; and Type IV: present for both enzyme combinations (1,1), representing no methylation, or a hemimethylated state at 5'-CCGG sites caused by methylation of internal cytosine of 5'-CCGG on one DNA strand but not on its complementary strand. Type IV was usually treated as indicating no methylation in the subsequent analyses even though it may underestimate the actual methylation level. The following formula was used to calculate the percentage of polymorphic MSAP bands:

$$MSAP\ (\%) = [(I + II + III) / (I + II + III + IV)] \times 100 \quad \text{(Equation 2)}$$

Cloning and BLAST search of differentially methylated fragments

The surplus samples used for the CE-based MSAP were separated on 10% conventional polyacrylamide gels and the excised bands of interest were then cloned. The detailed procedure of DNA recovery, vector ligation, and transformation of DH5α *Escherichia coli* competent cells were performed as described by Guo et al. (2003). For each amplified fragment, at least three positive clones were simultaneously sequenced by Shanghai Generay Biotech Co., Ltd., China. For each cloned sequence, both the nucleotide and deduced amino acid sequences were used in a homology search on the National Center for Biotechnology Information BLAST server (USA).

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

The EASY spin RNA plant kit (Aidlab, Beijing, China) was used to extract total RNA following the manufacturer instructions. Leaves were sampled at the same time-points as the DNA methylation analyses. RNA was extracted from 100 mg leaf tissue and stored at -80°C.

After quantification by UV spectrophotometry, the RNA was converted to cDNA using the M-MLV reverse transcriptase kit (Invitrogen Biotechnology Co., Ltd., Shanghai, China). The RT-qPCR procedure was performed using the iCycler iQTM real-time PCR detection system (Bio-Rad) according to the manufacturer instructions. The Primer3 software (http://www.simgene.com/Primer3) was used to design primers, which were subsequently synthesized and purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The detailed sequences of the primers are listed in **Table S2**. The cotton *GhGAPDH* gene (GenBank accession No. FJ415206) was used as an endogenous control, for normalization. All PCRs were performed in triplicate, and the specificity of the reactions was detected by melting-curve analysis at the dissociation stage. Each target gene was comparatively quantified based on cycle threshold (CT) normalized to *GhGAPDH*, using the ΔΔCT method (Cui et al., 2015).

RESULTS

Wilt resistance assay

The resistance of the three cotton materials to Verticillium wilt strain V991 are shown in Table 1. We found that PD94042 and IL41 were both disease-sensitive, with disease indices of 53.8 and 52.1, respectively. In contrast, *G. mustelinum* was disease-resistant with a disease index of 18.8. There was no significant difference between the PD94042 and IL41 disease indices, whereas *G. mustelinum* had a significantly lower disease index compared to the other accessions.

Table 1. Verticillium wilt (VW) disease resistance level of three different cotton accessions (*Gossypium mustelinum*, *Gossypium hirsutum* accession PD94042, and *G. hirsutum* accession IL41).

Cotton material	VW disease index	Disease resistance level
PD94042	53.8[a]	Sensitive
IL41	52.1[a]	Sensitive
G. mustelinum	18.8[b]	Resistant

Superscripted lower case letters indicate significant differences at $P \leq 0.05$.

DNA methylation level

The DNA methylation levels were identified with a CE-based MSAP. In total 3440 CCGG sites were detected in the three accessions (Table 2). In PD94042, 2989 (86.9%) and 3038 (88.3%) sites were methylated in the control and disease treatments, respectively. The average number of sites detected per primer combination was 93.4 and 94.9, respectively, with no significant difference between them. In *G. mustelinum*, the average number of detected sites per primer combination were 98.4 and 100.1 in the control and disease treatments, respectively. The methylation level in the disease treatment was significantly higher than that of the control ($P < 0.05$). For the type III methylation, 72.6 and 77.1 methylated sites per primer combination were detected in the control and disease treatments, respectively. The methylation level in the disease treatment was significantly higher than in the control ($P < 0.01$). In accession IL41, the total methylation level was significantly lower in the disease treatment, compared to in the control ($P < 0.05$). Likewise, the Type I methylation level was also significantly decreased in the disease treatment ($P < 0.01$), whereas the Type III methylation level was significantly increased ($P < 0.01$) (Table 2).

Table 2. DNA methylation patterns of different cotton accessions (*Gossypium mustelinum*, *Gossypium hirsutum* accession PD94042, and *G. hirsutum* accession IL41) detected by MSAP.

Type	Enzyme digestion[a]		Methylation pattern[b]	Number or ratio (%) of loci[c]					
	H	M		PD94042		IL41		*G. mustelinum*	
				Control	Disease treatment	Control	Disease treatment	Control	Disease treatment
I	0	1	C<u>C</u>GG	679	677	950	684	550	478
			GG<u>C</u>C						
II	1	0	<u>C</u>CGG or C<u>C</u>GG	306	341	493	437	275	259
			GGCC GGCC						
III	0	0	<u>C</u>CGG or C<u>C</u>GG or C<u>C</u>GG or C<u>C</u>GG or C<u>C</u>GG	2004	2020	1435	1673	2324	2467
			GG<u>C</u>C GG<u>C</u>C GG<u>C</u>C GG<u>C</u>C GG<u>C</u>C						
IV	1	1	<u>C</u>CGG or C<u>C</u>GG	451	402	562	646	291	236
			GGCC GGCC						
Type I locus No./Primer combination				21.2	21.2	29.7**	21.4	17.2	14.9
Type II locus No./Primer combination				9.6	10.7	15.4	13.7	8.6	8.1
Type III locus No./Primer combination				62.6	63.1	44.8**	52.3	72.6**	77.1
Methylation loci No./Primer combination				93.4	94.9	89.9*	87.3	98.4*	100.1
Total methylation loci (I+II+III)/Ratio to total loci				2989	3038	2878	2794	3149	3204
				86.9%	88.3%	83.7%	81.2%	91.5%	93.1%
Total amplified loci (I+II+III+IV)				3440	3440	3440	3440	3440	3440

[a]H and M represent fragments produced by digestion with *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI, respectively; 1: band present; 0: band absent; [b]<u>C</u> represents methylated cytosine; the ten methylation patterns were divided into four types (I-IV) based on the enzyme digestion results; [c]Significance levels indicated as *P ≤ 0.05 and **P ≤ 0.01, respectively.

Variation in DNA methylation status among the control and disease treatments

The differential methylation levels between the disease and control treatments for each cotton accession could be categorized into four patterns (A-D, Figure 1). Pattern A suggested that the same methylation status was found in both the disease and control treatments. Pattern B indicated a decreased DNA methylation (hypomethylation) in the disease treatment compared to the control. Pattern C indicated an increased DNA methylation level (hypermethylation) identified in the disease treatment. Pattern D indicated an altered DNA methylation status between the disease and control treatments in which the difference in methylation level could not be identified. Our results suggested that banding pattern A was the most commonly found in all three cotton accessions, whereas banding pattern D was the least common (Figure 1). Significant differences among the different cotton accessions were found for each specific banding pattern (Figure 1).

Differentially methylated DNA sequences and gene homology

Cloning and BLAST search analysis of the methylated DNA sequences identified six sequences with a high similarity to known genes/ESTs (Table 3), which were related to disease resistance in plants.

Gene expression analysis of selected MSAP fragments

The six gene homologues to the cloned MSAP fragments were analyzed for their transcript levels, using RT-qPCR. Transcript abundance was assessed in all treated samples and normalized against controls. XM_007047137.1, the *Theobroma cacao* Transducin/WD40

repeat-like superfamily protein isoform 4 (TCM_000575) mRNA (Table 3) was mildly up-regulated in the disease treatment in PD94042, and was significantly up-regulated in IL41 and *G. mustelinum* (Figure 2).

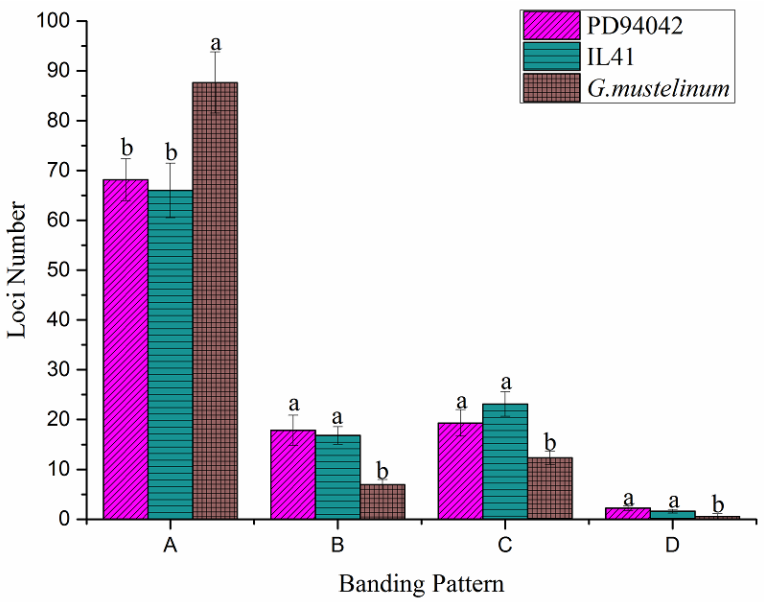

Figure 1. Variation of methylation banding pattern between three cotton accessions (*Gossypium mustelinum*, *Gossypium hirsutum* accession PD94042, and *G. hirsutum* accession IL41). A: Same methylation level between control and disease treatment; B: methylation level decreased under disease treatment (hypomethylation); C: methylation level increased under disease treatment (hypermethylation); D: methylation pattern changed. Lower case letters indicate significant differences at $P \leq 0.05$ among the different accessions. The columns indicate means ± SE.

Table 3. BLASTn results for part of the methylated sequences.

Sequence No.	Description	E-value	Accession No.
W123	*Theobroma cacao* Transducin/WD40 repeat-like superfamily protein isoform 4 (TCM_000575) mRNA	2.00E-74	XM_007047137.1
W124	*Gossypium hirsutum* gypsy-type retroelement	2.00E-72	AY395702.1
W127	*Gossypium hirsutum* actin depolymerizing factor 2 (ADF2) mRNA	1.00E-42	DQ402077.1
W135	*Gossypium hirsutum* gibberellin 20-oxidase (GA20ox3) gene	4.00E-08	FJ623274.1
W138	*Gossypium herbaceum* isolate GH31_2 retrotransposon Gorge3 reverse transcriptase (pol) gene	8.00E-48	EU098633.1
W161	*Theobroma cacao* kinases, ubiquitin-protein ligases isoform 2 (TCM_045033) mRNA	1.00E-37	XM_007010857.1

The DQ402077.1, *G. hirsutum* actin depolymerizing factor 2 (ADF2), mRNA was significantly up-regulated in all the three cotton accessions. Another three genes (AY395702.1, EU098633.1, and XM_007010857.1) were significantly up-regulated in PD94042 and IL41, whereas they were significantly down-regulated in *G. mustelinum* (Figure 2). Finally, FJ623274.1 was significantly up-regulated in PD94042 and IL41, whereas no significant difference was found in *G. mustelinum* (Figure 2).

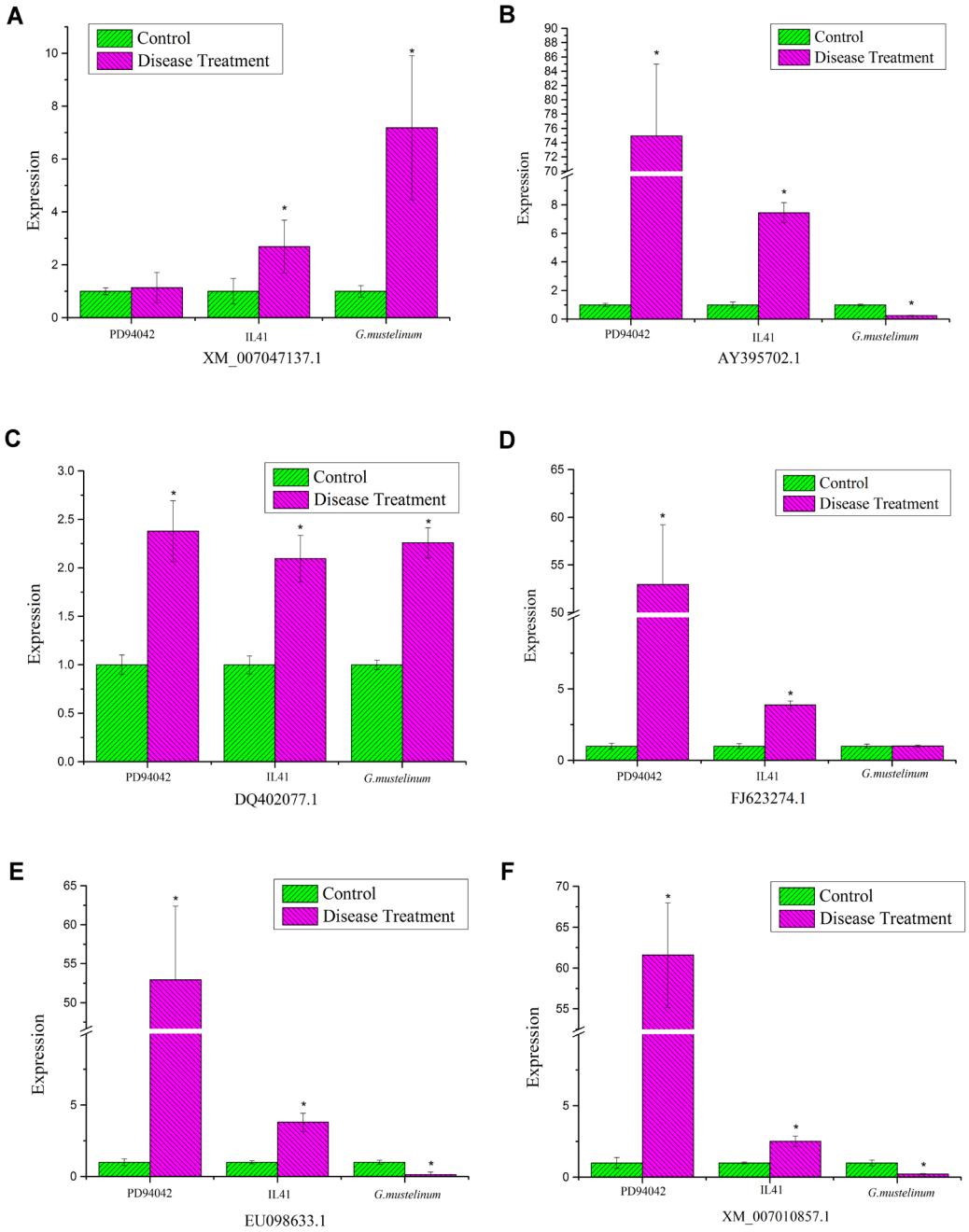

Figure 2. Expression of differentially methylated genes (**A.** XM_007047137.1, **B.** AY395702.1, **C.** DQ402077.1, **D.** FJ623274.1, **E.** EU098633.1, and **F.** XM_007010857.1) detected by RT-qPCR in the three cotton accessions *Gossypium mustelinum*, *Gossypium hirsutum* accession PD94042, and *G. hirsutum* accession IL41. The transcript levels were normalized to that of *GhGAPDH*, and the level of each gene in the control samples was set at 1.0. Error bars represent standard errors of the mean for three independent experiments.

DISCUSSION

DNA methylation and disease stress

DNA methylation is a modification that occurs at the fifth carbon position of a cytosine ring. Cytosine is primarily methylated in the CG dinucleotide context in both plants and animals. It has been observed that methylation in the 5' or 3' portions of a gene may inhibit gene expression (Grativol et al., 2012), and that the least expressed genes are more likely to be methylated than more highly expressed genes (Zemach et al., 2010).

Numerous stress-induced differentially methylated regions associated with differentially expressed genes have been found in plants exposed to abiotic (Zhang et al., 2013; Al-Lawati et al., 2016; Wang et al., 2015, 2016) and biotic stresses (Dowen et al., 2012; Shao et al., 2015). Previous studies have identified different trends of DNA methylation under disease stress conditions. For example, Dyachenko et al. (2006) found that the level of CHG methylation in the halophyte *Mesembryanthemum crystallinum* increased in satellite DNA when switching-over from C3-photosynthesis to the crassulacean acid metabolism pathway of carbon dioxide assimilation under disease stress. Other studies also found that gene demethylation is an active epigenetic response to disease stress in rice and cotton (Wang et al., 2011; Zhao et al., 2014). Notably, Peng and Zhang (2009) pointed out that biotic stresses, such as pathogenic infection, can lead to two contrasting effects at the methylation level in plants: hypomethylation of resistance-related genes and hypermethylation on the genome-wide level. Both phenomena may contribute to the adaptation of plants to stress. In addition, the progeny of stressed plants often show genomic hypermethylation, although specific loci that are beneficial in times of stress may be hypomethylated (Migicovsky and Kovalchuk, 2013).

In our study, we found that the methylation level significantly increased in the disease treatment, compared to the no-disease control in the disease-resistant *G. mustelinum* (Table 2). This contrasts with the effects observed in the two disease-sensitive cotton accessions, in which no significant differences were found in PD94042, whereas the methylation level was significantly lower in the disease-exposed IL41 than in the control. The significant increase in global DNA methylation in the disease exposed *G. mustelinum* but not in its disease-sensitive parents suggests that disease-resistant cotton might have a mechanism of increasing the methylation level in response to disease exposure. We also noted that different methylation types were observed among the disease and control treatments (Table 2). The different methylation types, as well as the increase in overall level of DNA methylation in the cotton genome, may play important roles in cotton disease resistance.

Comparing the variation in DNA methylation patterns revealed that the same methylation level between control and disease treatment (Pattern A) occurred most often in all three cotton accessions. At the same time, the locus number of Pattern A in the disease-resistant cotton accession *G. mustelinum* was significantly higher than that of the two disease-sensitive accessions (Figure 1). For Patterns B, C, and D, in which the DNA methylation status changed, there was no significant difference between the two disease-sensitive cotton accessions, but their locus numbers were significantly higher than that of *G. mustelinum*. These results suggest that the methylation status of disease-resistant accessions is more stable than that of disease-sensitive accessions, which might contribute to disease resistance in cotton.

Variation in gene expression related to disease stress in cotton

Cloning and BLAST search analysis of methylated DNA sequences identified 68 sequences with high similarity to known genes/ESTs (Table 3), including important genes related to disease resistance. Transducin/WD40 repeat proteins are prominent features within proteins that mediate diverse protein-protein interactions (Gibson, 2009). Repeated WD40 domains play central roles in plant developmental processes by regulating ribosomal structural features, activities, and biogenesis in plant cells (Gachomo et al., 2014). Zhang et al. (2009) proposed that genes encoding transducin expressed specifically in the resistance lines may be involved in barley yellow dwarf virus resistance. Here we found that XM_007047137.1, the *T. cacao* Transducin/WD40 repeat-like superfamily protein isoform 4 (TCM_000575) mRNA (Table 3) was mildly up-regulated in the disease treatment in PD94042, and significantly up-regulated in IL41 and *G. mustelinum* (Figure 2). This indicates that its up-regulation might be part of the cotton disease resistance.

The primary role of ADFs is to sever filamentous actin, generating pointed ends, which in turn are incorporated into newly formed filaments, thus supporting stochastic actin dynamics. Porter et al. (2012) suggest a novel role for ADF4 in controlling gene-for-gene resistance activation. Henty-Ridilla et al. (2014) further provided genetic and cytological evidence for the inhibition of ADF activity regulating actin remodeling during innate immune signaling. Fu et al. (2014) suggested that a wheat ADF gene, *TaADF7*, might contribute to wheat resistance against avirulent *Puccinia striiformis* f. sp *tritici* (*Pst*) infection. In the present study, the DQ402077.1 gene of *G. hirsutum* ADF2 mRNA was significantly up-regulated in all the three cotton accessions. This also indicates that the ADF2 gene might play a positive role in cotton Verticillium wilt resistance.

Plant retrotransposons are transcribed at low levels under normal conditions and have been found to be responsible for only a few mutations. However, several stress conditions, including pathogen infections, have been reported to activate them through transcription activation, which is known to induce the plant defense response (Asíns et al., 1999). We identified that two genes related to retroelements and retrotransposons, AY395702.1 and EU098633.1, were significantly up-regulated in the two disease-sensitive accessions PD94042 and IL41. These same genes were significantly down-regulated in the disease-resistant *G. mustelinum*, suggesting that retroelements and retrotransposons are involved in cotton disease resistance and that their expressions correlate to disease resistance levels.

MYB30-Interacting E3 Ligase1 (MIEL1) is an *Arabidopsis* RING-type E3 ubiquitin ligase that interacts with ubiquitinate MYB30. Marino et al. (2013) found that following bacterial inoculation, repression of MIEL1 expression resulted in sufficient MYB30 accumulation in the inoculated zone to trigger the hypersensitive response and restrict pathogen growth. In our study, the XM_007010857.1 gene, *T. cacao* kinase, ubiquitin-protein ligases isoform 2 (TCM_045033) mRNA was significantly up-regulated in PD94042 and IL41, whereas it was significantly down-regulated in *G. mustelinum*. This provides further support that ubiquitination plays an important role in controlling the hypersensitive response and highlights the sophisticated fine-tuning of plant responses to pathogen attacks.

Plant hormones, including abscisic acid and gibberellins (GAs), have emerged as crucial regulators of plant-microbe interactions, and it has been shown that these hormones influence disease outcomes. Some studies have shown that GAs are linked to suppression of defense-related gene expression and phytoalexin biosynthesis (De Vleesschauwer et al., 2013). Qin

et al. (2013) found that GA20ox gene (*OsGA20ox3*) RNAi lines showed enhanced resistance against rice pathogens causing rice BLAST and bacterial blight and increased expression of defense-related genes. Conversely, *OsGA20ox3*-overexpressing plants were more susceptible to these pathogens compared with wild-type plants. We found that the FJ623274.1 gene was significantly up-regulated in the two disease-sensitive cotton accessions PD94042 and IL41, whereas there was no significant difference in the disease-resistant *G. mustelinum* (Figure 2, Table 3). This suggests that *G. hirsutum OsGA20ox3* is negatively related to cotton disease resistance, which is consistent with previous studies.

In general, our study explored the molecular genetic basis of Verticillium wilt resistance in cotton. The results indicated that besides some key genes that function in disease resistance, the different methylation patterns, together with the increase in global DNA methylation level, might play important roles in resistance to Verticillium wilt in cotton.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. Adaptors and primers used for the MSAP analysis.

Table S2. Primers used in Real-time qPCR.