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Association mapping of resistance to Verticillium wilt in *Gossypium hirsutum* L. germplasm

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Verticillium wilt is a major disease affecting the growth of cotton. For screening the resistant genes, 320 *Gossypium hirsutum* germplasms were evaluated in Verticillium nursery, and association mapping was used to detect the markers associated with the Verticillium wilt resistance. 106 microsatellite marker primer pairs were used to estimate the genetic diversity, population structure and linkage disequilibrium (LD) of the germplasm. Polymorphism (PIC) was found to be 0.53, and population structure were detected to be three subgroups (K=3). LD decay rates were estimated to be 13 to 15cM (r^2 ≥0.20). Significant associations between polymorphic markers and Verticillium wilt resistance traits were observed using the general linear model (GLM) and mixed linear model (MLM). Four loci showed positive effects on the phenotype which meant that these loci could promote the Verticillium wilt resistance of cotton, and thirteen loci showed negative effects in GLM. The results displayed that association mapping could complement and enhance quantitative loci (QTLs) information for marker-assisted selection in cotton breeding.

Key words: Cotton germplasm, verticillium wilt, simple sequence repeats (SSR) markers, linkage disequilibrium (LD), association analysis.

INTRODUCTION

Verticillium wilt is one of major diseases that has been found in most cotton cultivated areas and caused severe yield losses in the popular field. This disease is induced by the soil-borne fungus *Verticillium dahliae* Kleb that can survive in the soil for long periods of time (Wilhelm, 1955). In view of incurability for fungicides, breeding and using of disease-resistant cultivars are the mainly available method of protecting cotton from the infection of pathogen. Understanding the genetic events of this disease at the molecular level will help us to utilize existing resistance in cotton germplasm for breeding. The genes of resistance to Verticillium wilt were firstly considered to be recessive according with the model of Mendel in the populations of G. *hirsutum* (Brinkerhoff and

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Ashagari,1970; Roberts and Staten, 1972). Ma et al., (1996) and Zhang et al., (2000) concluded that the resistance to Verticillium wilt was controlled by a dominant gene. However, Devey and Roose (1987) and Wang et al., (2004) deduced a conclusion that the resistance to Verticillium wilt was quantitative trait and was controlled by minorgene. Quantitative loci (QTLs) of Verticillium wilt resistance were detected in G. barbadense and in G. hirsutum in the past years (Yang et al., 2007, 2008, 2009; Jiang et al., 2009), some important genes have been cloned (Simko et al., 2004).

Molecular markers or QTLs linked with important traits have been identified in many crops based on the linkage analysis of F2- , RIL-, or DH (double haploid)-derived mapping populations using molecular marker technology. However, some important QTLs might not be detected because of linkage disequilibrium (LD) in mapping and breeding populations. Association mapping could be valuable for validating and detecting more QTLs in complex-pedigree population relevant linkage analyses. This method has been successfully used in different crops to identify markers and genes associated with a variety of phenotypes based on the nature population. *tb1* was the first gene that was found to be associated with short branches of maize using the approach of association mapping in plant (Doebley et al., 1997; Wang et al., 1999; Jaenicke-Després et al., 2003). Then, a large number markers were discovered to be associated with kernel size in wheat (Breseghello and Sorrels, 2006), heading date and water-stress tolerance in barley (Kraakman et al., 2004; Ivandic et al., 2003), resistance to late blight in potato (Gebhardt et al., 2004; Karolina et al., 2009), and fiber quality in *G. hirsutum* (Abdurakhmonov et al., 2008, 2009).

Association mapping was usually affected by the population structure (Pritchard et al., 2000a). False markers would be detected because of populations which were composed of individuals deriving from a complex pedigree. The use of population structure could significantly correct the number of false positives in plant studies (Thornsberry et al., 2001). Currently, STRUCTURE was the main software to evaluate the population structure (Pritchard et al. 2000b), especially the population of allogamy plants. In this study, we analyzed the association of simple-sequence repeat (SSR) markers with resistance to Verticillium wilt in a collection of cotton germplasm from all over the world, and aimed to provide theory basis and feasible method for the molecular marker-assisted selection.

MATERIALS AND METHODS

Plant material

For the association analysis, 320 *G. hirsutum* germplasm were used. These cotton germplasm were collected from all over the world and conserved in the Gene Bank of Cotton Research Institute at Chinese Academy of Agricultural Sciences. All these cotton germplasm have been strictly self-pollinated during the past years.

Phenotype evaluation

V. dahliae VD race were isolated from the soil of local field in Anyang which was moderate pathogenic and defoliating (ND) pathotypes. Then the soil of nursery was inoculated with *V. dahliae* cotton seed cultivation.

These cotton germplasm were grown in the disease nursery of Cotton Research Institute at Chinese Academy of Agricultural Sciences. Each individual was sown in one row with three repeats and randomized completely in different blocks. One line named Jimian 11 that was susceptible to Verticillium wilt was used as the control cultivars, which was usually used in the evaluation of Verticillium wilt resistance (Wu et al.,1999; Du and Zhou, 2005). The plant spacing was maintained at 0.7×0.30 m. The damage of leaf and vascular tissue in seedlings and maturity stage were classified into five grades, which was the national standard of evaluation of Verticillium wilt resistance in China (Wu et al.,1999). The grades scored as 0, 1 and 2 were considered as resistance to Verticillium wilt, and grades 3 and 4 as susceptibility to Verticillium wilt. The index of damage was calculated as Wu et al. described (1999): $DI = \sum(d_i \times n_i) / (n_t \times 4) \times 100$, d_i was the grade of damage, n_i was the number of seedlings in the corresponding grade of damage, n_t was the total seedlings. When the damage index of sensitive cultivar was about 50, the damage index of other individuals must be evaluated as soon.

Genotyping with SSR markers

DNA was extracted from the young and fully expanded leaves of each species (Paterson et al., 2003). The sequences of SSR primers were downloaded from CMD (Cotton Marker Database, www.cottonmarker.org/cgi-bin/panel.cgi). Polymorphic SSRs were screened from a standard panel including upland cotton cultivars and other tetraploid species (Blenda et al., 2006). 106 SSRs were selected from the polymorphic primers. PCR reacted in 10 µL volumes included 1.0 µL10×Buffer (consisting of 20 mM $MgSO_4$, 100 mM KCl, 80 mM $(NH_4)_2SO_4$, 100 mM Tris-HCl, pH 9.0, 0.5% NP-40), 50 ng template DNA, 0.5 mM dNTP, 0.4 units of *Taq* DNA polymerase, 0.5µM forward and reverse primers. The PCR amplification program included 3 min pre-denature at 95□C, 30 cycles of 94°C 45 s, 57°C 45 s, 72°C 1 min, and 7 min extension at 72°C. The reactions were completed by PTC-100TM thermocycler. The PCR product was stored at 4°C before being run on the 8% non-denature PAGE gel (Sambrook et al., 1992). The gel was dyed by referring to Zhang et al.'s method (2000), and then was photographed using SYNGENE gel system.

Allele diversity and population structure

When markers produced a single band, each allele was scored with "1". Whereas markers produced more than two bands, alleles were scored with "1", "2", "3"and "4" representing the numbers of bands, respectively. The missing data was represented with "-9". Diversity and heterozygosity were calculated based on 106 polymorphic SSR data in 320 lines. Allele frequencies were calculated using SpaGeDi ver.1.3 software (Hardy and Vekemans, 2002). The polymorphic information content (PIC) was analyzed using the PowerMarker 3.25 software package (Liu and Muse, 2005). The genetic distance (GD) was estimated using Neighbor Joining (N-J) algorithms with the minimum evolution objective function.

Table 1. Summary statistics of verticillium resistance index.

Parameter	Mean	Standard deviation	Minimum	Maximum	P-value of normality test[a]	Q1[b]	Median	Q3[c]
Verticillium resistance index	37.46	10.45	10.41	76.91	0.97	29.51	36.08	44.61

[a] Shapiro–Wilk test, [b] Quantile 25%, [c] Quantile 75%

Table 2. Mean squares of the ANOVA of verticillium resistance index.

Parameter	Degree of freedom (d.f.)	Verticillium resistance
Cultivar	321	328.08*
Replicate	2	29.61
Error		53.74*
R^2 of model		0.75

* indicate significance at the probability levels of 0.001.

Population structure analysis

Bayesian was estimated using STRUCTURE software for the population structure analysis (Pritchard et al., 2000b). The number of populations tested was assumed as K where K varied from 1 to 10. The length of running time was 100 000, and replication after burning was 10 000 for the STRUCTURE with admixture model. However, we did not find distinct clusters and could not determine a significant number of K populations using STRUCTURE. So we built a graph of Pn to find a proper value of K following the method of Evanno et al. (2005).

Linkage disequilibrium

LD parameter r^2 was estimated using Tassel 2.1 software (http://www.maizegenetics.net). LD between all pairs of SSR alleles was analyzed with MAF filtered datasets, where SSRs alleles with a 0.05 frequency in genotyped accessions were removed before conducting LD analyses, because minor alleles are usually problematic and biased for LD estimates between pairs of loci (Mohlke et al., 2001; McRae et al., 2001). The MAF removal was performed using the TASSEL site filtration function. LD was estimated by a weighted average of squared allele-frequency correlations between SSR loci. The significance of pairwise LD (p-values≤0.005) among all possible SSR loci was evaluated using TASSEL with the rapid permutation test in 10 000 shuffles. The LD values between all pairs of SSR loci were plotted as LD plots using TASSEL to estimate the general view of genome-wide LD patterns and evaluate 'block-like' LD structures.

Association studies

The general linear model (GLM) association test was performed after incorporating index of damages, SSRs genotype, and Q matrix using the TASSEL 2.1 software (Bradbury et al., 2007). The Q of population was set as covariate, and 1 000-time permutations were set for the correction of multiple testing. The Q matrix was created with K = 3 as determined by STRUCTURE. The phenotypic allele effect was estimated using the method described by Breseghello and Sorrells (2006): $a_i = \sum x_{ij} / n_i - \sum N_k / n_k$ where a_i was the phenotype effect of specific i allele, x_{ij} was the phenotype value of j individual with i allele, n_i was the total individuals with i allele, N_k was the phenotype value of j individual with null i allele and n_k was the total individuals with null i allele.

RESULTS

Morphological traits

Phenotype values of Verticillium wilt resistance showed a wide range variation, and revealed that the data of traits was favorable for the association analysis (Table 1, Table 2). The lowest value was 10.40 meaning that the individual was the most resistant to the Verticillium wilt. The highest value was 76.9 presenting that the individual was the most sensitive.

Diversity and structure

In the population, 106 SSR markers detected 278 loci and 333 SSR alleles with an average of 3.1 alleles per marker (from 2 to 6 alleles) (Table 3); whereas the average effective alleles were 2.4 varying from 1.2 to 4.9. The polymorphic information content (PIC) was calculated, and the average PIC of the population was found to be 0.53 ranging from 0.17 to 0.79. The genetic distance (GD) was estimated using the NTSYSpc Version 2.1, and the average GD was got to be 0.26 ranging from 0.04 to 0.57, which demonstrated the significant ranges of genetic diversity of the population.

The structure of the population was estimated with the

Table 3. Summary of SSR polymorphisms.

Number of polymorphic SSRs				Polymorphic information content (PIC)		Genetic distance	
Locus	Average allele/ marker	Effective Allele	Rare Allele (%)	Range	Average	Range	Average
278	3.1	2.4	23	0.17-0.79	0.53	0.04–0.57	0.26

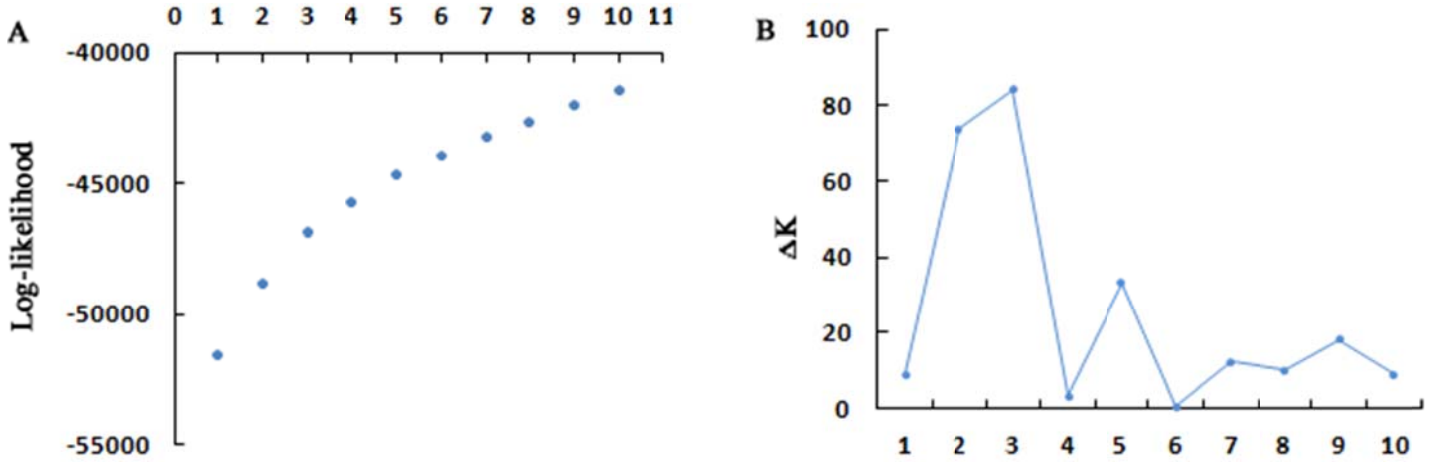

Figure 1. Analysis of the population structure. The numbers of subgroups were calculated using STRUCTURE (Pritchard et al. 2000b); A: Graph about the log-likelihood. The log-likelihood increased with the number of groups (K) increasing. B: Graph about **Δ**K. ΔK= m(|L(K + 1) − 2 L(K) + L(K − 1)|)/s[L(K)] was used to assess the number of groups (K) (Evanno et al. 2005). A clear peak was detected for K =3.

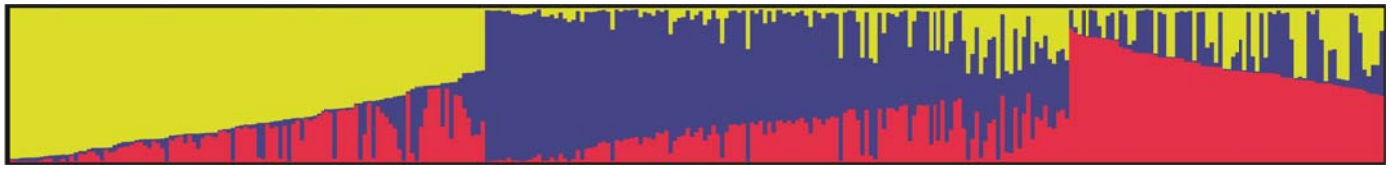

Figure 2. Population structure and cluster analysis: The bar plots of Q-matrix estimates for the variety accessions: Groups were represented in different colors (Blue for group 1, Green for group 2, Red for group 3).

STRUCTURE 2.3. The most appreciable value of K was found to be K = 3 after calculating the second-order change in log-likelihood described by Evanno et al. (Figure 1). Thus, 320 lines were assigned to three sub-populations, which were presented as three different bar plots (Figure 2). The bar plots showed that some individuals owned at least 50% of the single ancestral genetic background. So these individuals were assigned to three subgroups that were consisted of 101, 126 and 63 lines separately and were labeled as group1, group 2 and 3, respectively. The remaining 30 lines that showed a probability lower than 50% were assigned to a mix group.

Linkage disequilibrium analysis

The LD of the population was estimated using Tassele2.1 for the accurate analysis of association. 11.6 percent of SSR loci pairs were found to be linkage disequilibrium (p≤0.01, r2≥0.01), where 10.9 percent of loci pairs were found to be decayed over the long terms of cotton cultivar selection (38503 pairwise comparisons).

LD parameter r^2 was estimated for the calculation of the LD decated rate, and haplotypic LD was studied in the genome with 278 loci covering most of the chromosomes. It was found that most of r^2 ranged from 0.0 to 0.1, and

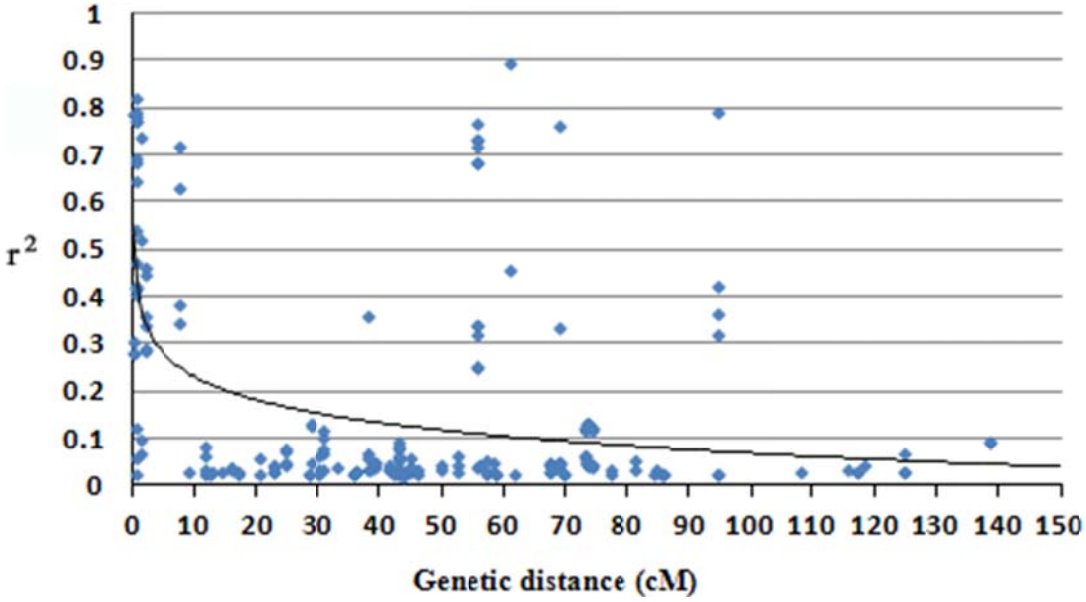

Figure 3. The decays of linkage disequilibrium by r^2 against genetic distance between all pairs of SSR loci. LD-decay is considered at the threshold of $r^2 \leq 0.2$ based on trend line.

most of LD ($r^2 \geq 0.2$) located in the distance of less than 15 cM. These results meant that the LD of the population decayed with the genetic distance (Figure 3).The LD decay rate was usually measured using the chromosomal distance when r^2 dropped to half its maximum value, and was used to evaluate the level of the LD decay. In this study, the LD decay rates of the population were found to be 7 to 8 cM and 13 to 15 cM, where the r^2 dropped to 0.25 and 0.20, respectively.

Association mapping of Verticillium resistance

The general linear model (GLM) and mixed linear model (MLM) were used to detect the SSR markers associated with Verticillium wilt resistance among the *G. hirsutum* population. Seventeen loci were found to be associated with Verticillium wilt resistance in GLM at the significant threshold $p \leq 0.01$ after screening the total diversity markers (Table 4). However, only six loci were detected to be associated with Verticillium wilt resistance in MLM when incorporated both population structure and kinship among individuals ($p \leq 0.01$). Some loci such as NAU2265_382, NAU2277_60, BNL1694_415, NAU2741_282, and NAU5099_280, were found to be significantly associated with the traits in both of GLM and MLM. Well, MUSS440_401 was only detected in the MLM, and not was found in the GLM.

Allele effects on the phenotype were calculated (Table 4). Most of the loci showed significant negative effects on the Verticillium wilt resistance. NAU5099_280 appeared the most obvious effect that could reduce 6.22 point of the Verticillium resistance. However, NAU2277_60, BNL1694_415, NAU2741_282 and MUSS440_401 performed the especially abilities of promoting the Verticillium wilt resistance of cotton, which could increase the Verticillium wilt resistance 7.79 and 10.28 respectively ($p \leq 0.01$). The allele effects estimated in this test would be useful for making an accurate selection in the proceeding of molecular marker assistant breeding.

DISCUSSION

In this study, association mapping was firstly used to identify the genetic markers associated with Verticillium wilt resistance in cotton. The whole genome was scanned with 106 SSR markers that located in the 26 chromosomes. High genetic diversity of population was certified after calculating the PIC and genetic distance of the group. STRUCTURE was used to determine the structure of population, Three subgroups were found with K=3, and then the whole population was assigned to three subgroups and one mixed group separately. The genetic diversity and structure of the sample showed highly effects on the level of LD and the accuracy of the associated loci (Breseghello and Sorrells, 2006). The closely related cultivars in the group would violate the assumptions of the algorithm of Structure (Pritchard, 2000a) and inflate LD among unlinked loci. Thus, high polymorphic cultivars were necessary for the association analysis.

The level of LD was estimated in the population. 11.6%

Table 4. SSR markers associated with verticillium resistance among the *G. hirsutum* population.

Association markers	GLM	MLM	Effect for phenotype
	p-value	p-value	
NAU3419_252	$6.5\times10^{-3*}$	0.0757	-0.89
NAU2265_382	$6.3\times10^{-4**}$	$9.5\times10^{-3*}$	-1.18
NAU2277_60	$4.4\times10^{-3*}$	$5.2\times10^{-3*}$	1.82
NAU2277_72	$3.8\times10^{-3*}$	0.0652	-0.96
NAU1190_228	$9.4\times10^{-3*}$	0.1846	-0.91
NAU2679_218	$5.1\times10^{-3*}$	0.0155	-1.53
BNL1694_415	$1.5\times10^{-3*}$	$6.5\times10^{-3*}$	1.15
TMB1963_218	$5.7\times10^{-3*}$	0.0734	-0.94
TMB1963_243	$2.7\times10^{-3*}$	0.0415	1.02
NAU2437_245	$2.9\times10^{-3*}$	0.0244	-1.17
BNL1694_235	$2.4\times10^{-3*}$	0.0265	-1.16
NAU1102_230	$3.0\times10^{-3*}$	0.016	-1.26
NAU3110_224	$3.5\times10^{-3*}$	0.0155	-1.22
NAU3110_292	$3.6\times10^{-3*}$	0.0174	-1.22
NAU3110_318	$3.6\times10^{-3*}$	0.0174	-1.22
NAU2741_282	$5.3\times10^{-3*}$	$3.1\times10^{-3*}$	7.79
NAU5099_280	$8.3\times10^{-3*}$	$8.4\times10^{-3*}$	-6.22
MUSS440_401	0.0101	$6.4\times10^{-3*}$	10.28

*, ** indicate significance at the probability levels of p<0.01 and 0.001 respectively.

of loci pairs were found to be linkage disequilibrium. Haplotype LD decayed with the genetic distance of alleles in the sample. The decay rate was about 13 to 15cM (r2<0.2), which was similar to the report of Abdurakhmonov in the landrace stocks germplasm (2009). The decayed distance of LD usually determined the density of markers for the association mapping. Longer decayed distance needed fewer markers to cover the whole genome. In cotton, the total recombination length of genome was 5 200 cM with an average 400 kb per cM (Paterson and Smith, 1999). Our result showed that the LD decay rate of the population was 13 to 15 cM (r2≥0.2), which meant that 300~400 polymorphic loci were required for the association mapping. Though 278 loci detected with 106 markers were fewer than those of theoretical prediction, the number could be reduced to 80~100 if the LD rate was set at the r2≥0.1 threshold with the decayed distance 55 to 65 cM.

The General Linear Model (GLM) and the Mixed Linear Model (MLM) were used to detect the markers of associated with Verticillium wilt. We found that seventeen loci were associated with Verticillium wilt resistance at the significant level of p≤0.01 in GLM. In those associated loci, thirteen loci showed negative allele effects, and four loci showed positive allele effects. When the factor of kinship was incorporated in MLM, most of those loci that were screened in the GLM were filtered, and only six loci were remained to be associated with Verticillium wilt resistance, including four positive effect loci and two negative effect loci. MUSS440_401 locus was only detected in MLM, and not was found in the GLM, which meant that population stratification affected marker–trait association significantly. MLM could filter most of the significant markers detected by GLM, and reduced both false-positive and false-negative rates by adding population structure and kinship as covariate.

The QTLs of Verticillium wilt resistance had been successfully detected through F2 and RIL group based on the method of combination interval mapping in the past years (Wang et al., 2004; Yuksel et al., 2005; Yang et al., 2007, 2008, 2009). Different results were obtained using different linkage group. Four QTLs were detected to be located on chromosome A5, A7 and A8 at the seedling stage, and three QTLs were detected to be located on chromosome A5, A7 and A9 at the mature stage in the combination group of *G. barbadense* × *G. hirsutum* (Yuksel et al., 2005; Yang et al., 2007, 2008). Three QTLs were found to be located on the LG01 linkage group and chromosomes D8 and D7 at the seedling stage, and four QTLs were detected on the LG01 chromosomes A11, D8 and D7 at the mature stage in the group of *G. hirsutum*×*G. hirsutum* (Yang et al., 2007, 2009). Some important markers associated with Verticillium wilt resistance were screened, which were also found to be located on the chromosomes 11, 16, 17, 19, and26 (Zhao et al., 2014). D9 and D7 were found to host most of the

QTLs that were resistant to the *Verticillium* VD8, BP2 and T9 races (Jiang et al., 2009). In present research, all of the loci associated with Verticillium wilt resistance were firstly reported. However, the Verticillium wilt resistance was affected by a complex genetic system including the different *Verticillium* race. We used mixed races to infect the cottons, and calculated the index of resistance after evaluating from the seedling to mature stage. Therefore, the loci associated with Verticillium wilt resistance that we screened were_horizontal resistance, which were useful for MAS in the breeding.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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