

RESEARCH ARTICLE

Identification and selection of resistance to *Bemisia tabaci* among 550 cotton genotypes in the field and greenhouse experiments

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Abstract Plants have developed sophisticated systems to cope with herbivore challenge, including morphological barriers and secondary metabolites to reduce damage. In this study, 550 *Gossypium* genotypes were evaluated for whitefly (*Bemisia tabaci*) resistance in five experiments including two in the field and three in the greenhouse, with 23 resistant and 19 susceptible genotypes selected. Whitefly-resistance index determination showed that a leaf having a high density of hairs had resistance to whitefly egg/nymph production. Longer leaf hairs were also important for resistance. This study revealed that okra shaped leaves reduced adult whitefly oviposition preference, while glabrous leaves and high hair density helped not only in the reduction of the adults but also decreased oviposition preference. Gossypol was also observed to be involved in the reduction of adult whitefly development and/or survival.

Keywords *Bemisia tabaci*, *Gossypium* genotypes, gossypol, leaf hair density, leaf hair length

1 Introduction

Cotton (*Gossypium*) is not only the source of the most important natural textile fiber, but also a significant oil-yielding crop grown all over the world. Generally, four cultivated species are grown^[1,2]. However, cotton production is severely affected by number of biotic and abiotic stresses^[3,4] and it has been reported that in total 1326 species of insects attack cotton plants^[5]. Some studies also reported that Bt-cotton plants provide substantial economic benefits and reduce the use of harmful insecticides^[6,7]. The secondary pests such as aphids, mirid bugs and whiteflies

are not susceptible to Bt-cotton plants and they directly benefit from reduced application of chemical insecticides^[8–12]. Specifically, the mirid bugs (Heteroptera: Miridae) have shown tendency to produce outbreaks and were suggested to be considered as a primary target for development forecasting and management strategies in Bt-cotton fields in China^[13–15]. Whiteflies are typical phloem-feeding insects and, as with other non-Bt target insects, tend to increase in Bt-cotton fields and have become a most devastating agricultural pest worldwide^[16–19]. Moreover, whiteflies and aphids carry geminiviruses, which result in the spread of viral diseases^[20–22]. The understanding of plant response mechanisms induced by herbivores can provide important information to assist in integrated pest management.

Plants and insects have coexisted for almost 350 million years and have evolved a variety of different interactions^[23,24]. In nature, herbivores usually deal with multiple predators and plants as well as more complicated trophic influences^[25,26]. Plants also have evolved distinct strategies to combat herbivores, physical barriers such as cell wall and cuticle as direct defenses and producing secondary metabolites in response to insect attack^[19,27,28]. These two defense mechanisms against pests might operate synergistically^[29]. The physical barriers, including hairs, trichomes, thorns, spines and thicker leaves on the surface of the plants, restrict or limit insect attack^[30]. While, the production of primary and secondary metabolites, such as allelochemicals, non-protein amino acids, terpenoids, alkaloids, anthocyanins, phenols and quinones, may help in the reduction of growth, fecundity and survival of the insects^[31–34]. It has been shown that phloem-feeding insects numbers are closely related to primary metabolite, such as amino acid and carbohydrate, concentrations in their host plants^[33,35,36]. However, an overall understanding of how *Bemisia tabaci* adapts to the cotton host plants is still to be developed.

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Among the development of plant defense mechanisms, the distinctive essential role of capitate and peltate trichomes has been reported^[37]. Capitate trichomes are the most important as they produce nonvolatile metabolic substances responsible for direct defense of plants^[38,39]. Glandular trichomes produce, store and secrete metabolites of various classes which are associated with plant resistance to herbivores^[17,37]. A negative correlation between trichome density and herbivore damage was found for this direct defense mechanism^[34]. Moreover, increased trichome density can also be induced by herbivores and plant hormones^[40]. Trichomes not only interfere with herbivore movement but also prevent egg attachment to plant tissues after ovipositioning^[28].

Leaf hair density has been linked to host plant resistance with lower populations of adult whiteflies being observed on glabrous leaves as compared to moderately hairy leaves giving a positive correlation between hair density and adult whitefly population^[41–43]. Moreover, hair density also has impact on whitefly eggs and nymphs^[42]. Whitefly oviposition preference has been compared across hairy, semi-glabrous and glabrous leaf isolines with a greater oviposition preference being found on hairy leaves by comparison with glabrous leaves^[44]. In addition, another study has demonstrated that leaf hair length seemed to have an important role in insect resistance, with higher density of hair providing resistance to jassids^[45]. Also, fewer whiteflies were observed on cotton leaves in the genotypes having okra shaped leaves compared to the normal leaf shape^[42,46,47]. Miyazaki et al.^[42] and Chu et al.^[47] compared normal-leaf upland cotton cultivars with okra-leaf genotypes, and lower numbers of adults, eggs and nymphs were found on okra-leaf genotypes because they provided a less desirable microenvironment with a more open canopy.

The glands of *Gossypium* plants produce a group of terpenoids, important secondary metabolites, which can protect plants from herbivorous insects^[48–52]. Terpenoids defend many plants, animals and microorganisms against predators, pathogens and competitors, and are involved in conveying messages to conspecifics and mutualists regarding the presence of food, mates and enemies^[53,54].

Gossypol is one of the main components of glands, as well as other terpenoid aldehydes produced by subepidermal glands. They protect plants from some pathogenic bacteria and insect attack^[55–57]. Cotton pigment glands and higher levels of gossypol resulted in a significant decrease in survival rates, larval weights and moth eclosion rates, and delayed not only the development of larvae and pupae, but also reduced the pupal weight of *Helicoverpa virescens* larvae^[58,59].

However, there have been few studies on the effect of pigment glands and gossypol on cotton resistance to whitefly. Our study assessed morphological traits (leaf shape, leaf hairiness, leaf hair length and gland density) and a secondary metabolite (gossypol) variation in a

large number of cotton genotypes in relation to whitefly attack.

2 Materials and methods

2.1 Identification of *Bemisia tabaci* biotype

Whitefly adults were sampled from mature plants in a greenhouse and field experiments in the year of planting. Nymphs were sampled from one greenhouse screening (Exp. 3, see below). As the specimens were preserved in alcohol, they were briefly washed in sterile double distilled water before homogenization. The method of Barro and Driver^[60] was used with modifications as follows: from each sample, 3–5 whiteflies were transferred to a 1.5-mL microcentrifuge tube and 100 μ L lysis buffer (1% SDS, 10 mmol·L⁻¹ Tris-HCl, 25 mmol·L⁻¹ NaCl pH 8.0, 25 mmol·L⁻¹ EDTA) added. Whitefly samples were ground using pestle and 100 μ L lysis buffer was used to clean the pestle, then 10 μ L of 20 mg·mL⁻¹ proteinase K was added to the tube and gently mixed. The homogenate was incubated at 60°C for 1 h (mixed every 20 min), then at 100°C for 5 min. After incubation, an equivalent volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the microcentrifuge tube to extract whitefly DNA. The tubes were shaken gently by hand for 5 min, then placed on ice for 10 min, then the homogenate was centrifuged for 10 min at 12000 r·min⁻¹. The supernatant was transferred to a new microcentrifuge tube, and pre-cooled isopropyl alcohol (400 μ L) added and gently mixed before freezing at -20°C for 20 min. After thawing it was centrifuged for 10 min at 12000 r·min⁻¹, the supernatant was discarded, and 75% ethanol was used three times to wash the white sediment and then blown dry. Finally, the DNA samples were stored in TE buffer (pH 7.8) at -20°C.

For purification, DNA extraction was replicated three times, then 30 μ L of TE buffer (PH 7.8) was added and these DNA samples were used for PCR. Following the method of Rao et al.^[61], primers for CI-J-2195 (5'-TTG ATT TTT TGG TCA TCC AGA AGT-3') and L2-N-3014 (5'-TCC AAT GCACTA ATC TGC CAT ATT A-3') synthesized by the Jinsirui Company (Nanjin, JiangSu, China) were used. The method of Rao et al.^[61] was modified using a reaction volume of 20 μ L with 16.1 μ L of double distilled water, 10 \times PCR buffer 2 μ L, dNTPs 0.3 μ L, PCR primer 0.2 μ L, Taq DNA polymerase 0.2 μ L and DNA template 1 μ L were added. The method of Simon et al.^[62] was used for the reaction procedure and PCR amplification. Agarose gels (1.5%) were used to run PCR product on electrophoresis.

2.2 Experimental design and plant genotype

Greenhouse screening experiments were conducted in August to December 2013 (Exp. 1), March to July 2014

(Exp. 2) and October 2014 to January 2015 (Exp. 3). Two field screening experiments were conducted, 2014–2015 growing season (Exp. 4) and 2015–2016 growing season (Exp. 5), at Wuhan, Hubei, China. The genotypes used in each experiment were 498 *Gossypium hirsutum*, 35 *G. barbadense*, 12 *G. arboreum*, and 5 *G. herbaceum* (Fig. 1). Among these genotypes were plants with differences in morphology of leaf shape, leaf hairiness, leaf hair length and possibly biochemistry.

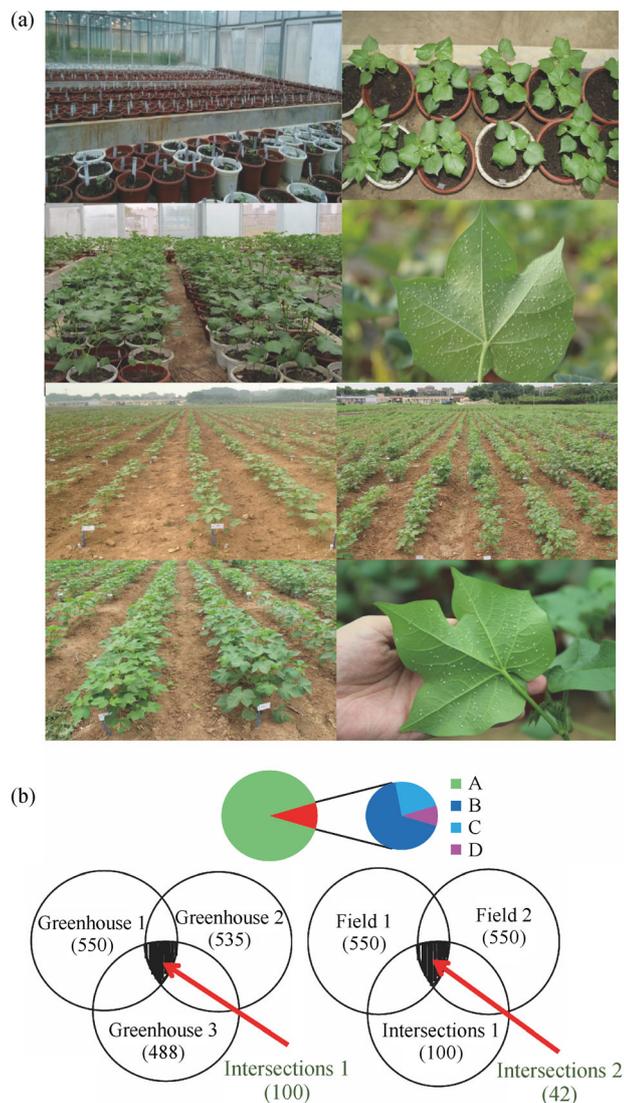


Fig. 1 Bioassay of 550 cotton genotypes under greenhouse and field conditions. (a) The greenhouse experiments were conducted from August to December 2013 (Exp. 1), March to July 2014 (Exp. 2) and October 2014 to January 2015 (Exp. 3). Each greenhouse had the same experimental treatment. The field experiments were conducted in the 2014–2015 growing season (Exp. 4) and 2015–2016 growing season (Exp. 5), each field plot was given the same treatment; (b) A, *G. hirsutum* ($n = 498$); B, *G. barbadense* ($n = 35$); C, *G. arboreum* ($n = 12$); D, *G. herbaceum* ($n = 5$). Red arrows indicate the number of genotypes in common for greenhouse (100) and field (42) experiments.

2.3 Design of greenhouse experiments 1–3

Five hundred and fifty cotton genotypes were grown in greenhouses that were completely cleaned and sprayed with insecticides and then closed for one week. Heat-treated soil was moved to greenhouse. The greenhouse experiment was a randomized complete block design with three blocks and 550 genotypes (550 in greenhouse 1, 535 in greenhouse 2, and 488 in greenhouse 3) (Fig. 1). Each genotype had three replicates and each replicate had at least two consistent growth opportunities. The experiments were conducted without any further pesticide treatments throughout the growing period. When the main cotton stem had five leaves, tobacco plants infested with whiteflies were uniformly distributed across the whole greenhouse to spread whiteflies.

Three weeks after infestation, the number of whitefly adults on the underside of five leaves from the top to bottom of the cotton plants were counted by turning the leaves over carefully. The counting time 06:30–08:30 and 17:30–19:30 because at those times the whiteflies were inactive. Also, a sample from the leaves from Exps. 2–3 were taken, mixed together and stored at -70°C for the determination of gossypol concentration.

2.4 Design of field experiments 4–5

In mid-April 2014 and 2015, fertile soil and a small propagating shed were used to ensure higher germination rate and uniformity. Cotton seedlings were transplanted into the field in the mid-May and no pesticides were applied during the whole growth period. The experiments were randomized complete block design with a buffer around the periphery and each genotype had 10 replications. On 20 June 2014, whitefly assessment commenced and was then conducted monthly throughout the growth period of cotton. During mid-August 2014, the whitefly population reached its peak. The population of adult whiteflies was counted on the underside of five leaves from the top to the bottom by turning the leaves over carefully. In both years, assessments were made between 06:00–08:00 and 18:00–20:00.

2.5 Design of greenhouse experiments 6–7

Two further greenhouse screening experiments were conducted in March to July 2014 (Exp. 6) and October 2014 to January 2015 (Exp. 7) that paralleled Exps. 2–3. Forty cotton genotypes identified and selected from Exp. 1 were used for the assessment of leaf hair length and density, leaf glands and gossypol concentration.

2.6 Assessment of egg and nymph density

Assessment of egg and nymph density was made in Exps. 2–3. Nymphs were counted after counting the adults

3 weeks after infestation by turning the leaves over and brushing all the adult whiteflies from the leaf. Eggs and nymphs were counted on the third main stem leaf from the top of cotton plants from photographs taken with a Leica stereo microscope. The mean number was calculated for each replicate at each assessment date. Leaf area was calibrated using photographs of Vernier calipers taken at the same scale. The final totals of eggs and nymphs were determined for each of the three biological replicates.

2.7 Assessment of leaf hair length and density and glands

Assessment of leaf hair length and density and the number of glands was conducted in Exps. 6–7. The main stem with five leaves is the preferred sampling position for commercial cotton crops^[63], so the third main stem node leaf from the top of cotton plant was selected from three stems to make a triangle. Scanning electron microscopy was used to study the leaf morphology. Photographs of the underside of the leaves were taken at the same magnification and were used to count the number of leaf hairs and glands, and to measure leaf hair length (Fig. 2). The means from the photographs were calculated for each replicate at each assessment. All genotypes were assessed three times. Finally, the photograph magnification was used to calculate leaf hair density.

2.8 Assessment of gossypol concentrations

Assessment of leaf gossypol was made in Exps. 6–7. High performance liquid chromatography (HPLC) was used to optimize the quantitative determination of free gossypol in the cotton leaves.

When the main stem node of cotton plants had five leaves, three samples were selected from the remaining four plants. Second and third main stem node leaves from the top cotton plant were selected. A vacuum freeze-drier was used to dry the cotton leaves at -50°C . The freeze-dried leaves were crushed with a mixer mill (MM 400, Retsch, Haan, Germany) with zirconia beads for 1.5 min at 30 Hz, 100 mg powder was weighed and extracted with acetonitrile and water (65:35, V/V) by ultrasound, vibration, centrifugation and suction filter processing before HPLC analysis.

The optimal conditions of HPLC were as follows: the column was Agilent TC-C (18) (150 mm \times 4.6 mm, 5 μm), and the mobile phase was a mixture of acetonitrile and 0.2% phosphoric acid (85:15, V/V) with a flow rate of 1.0 mL \cdot min⁻¹. The wavelength for UV detection was 238 nm, the injected sample volume 20 μL and the column temperature 25 $^{\circ}\text{C}$.

2.9 Statistical analysis

The data for adult whiteflies were obtained by determining the total number on the top five leaves of each cotton

genotype. The results were statistically analyzed by ANOVAR using SPSS 17.0. The number of nymphs, leaf hairs, glands and leaf hair length on each photo were calculated per cm². These densities and relationships were analyzed as previously published^[64]. Square-root transformed data for gland density was used for statistical analysis.

Relative weight analysis was used to estimate which factors were stronger correlation predictors^[65]. Relative weight analysis of the relationship between cotton morphological traits (leaf shape, and leaf hair density and length), the concentration of gossypol and number of glands on adult whitefly populations and nymph density was performed using R software v. 3.2.2 (R Core Team (2016). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

3 Results

3.1 Biotype identification of the *Bemisia tabaci*

PCR bands for the whitefly extract were found at about 680 bp (Fig. S1a). The whitefly mtDNA COI sequences (EU376994, AJ867555, AY686088, AY686089, EU376987, GQ139500, DQ365874, EF080823, EU760719 and EU099430) representative of different geographic populations was download from NCBI. Homology analysis showed that whiteflies from greenhouse and field experiments were both Q-biotypes and these sequences had significantly higher homology with EU376987 than other sequences: 99.1%, 98.8%, 99.0%, 99.7%, and 99.8% for Exps. 1–5, respectively. Molecular phylogenetic trees for *B. tabaci* constructed by the UPGMA method with bootstrap test in MEGA 6.0 (Fig. S1b) also indicated that *B. tabaci* from the experiments were closely related to GQ139500, EF080823 and EU376987.

3.2 Screening of resistant/susceptible cotton genotypes

A total of 550 cotton genotypes, selected from three main cotton cultivation regions in China, Yangzi River valley, Yellow River and Xinjiang, were infested with whiteflies in a greenhouse were sampled with three biological replicates and two bioassays in the field (campus of Huazhong Agricultural University) in three consecutive growing seasons (August 2013 to August 2015). In the greenhouse experiment, when the main-stem of cotton plants had five true leaves, tobacco plants with whiteflies were used to infest the cotton plants until the whiteflies were distributed uniformly. Three weeks later, the whitefly adults on each replicate of the 550 genotypes were counted.

Stability and comparative analysis of adult whitefly

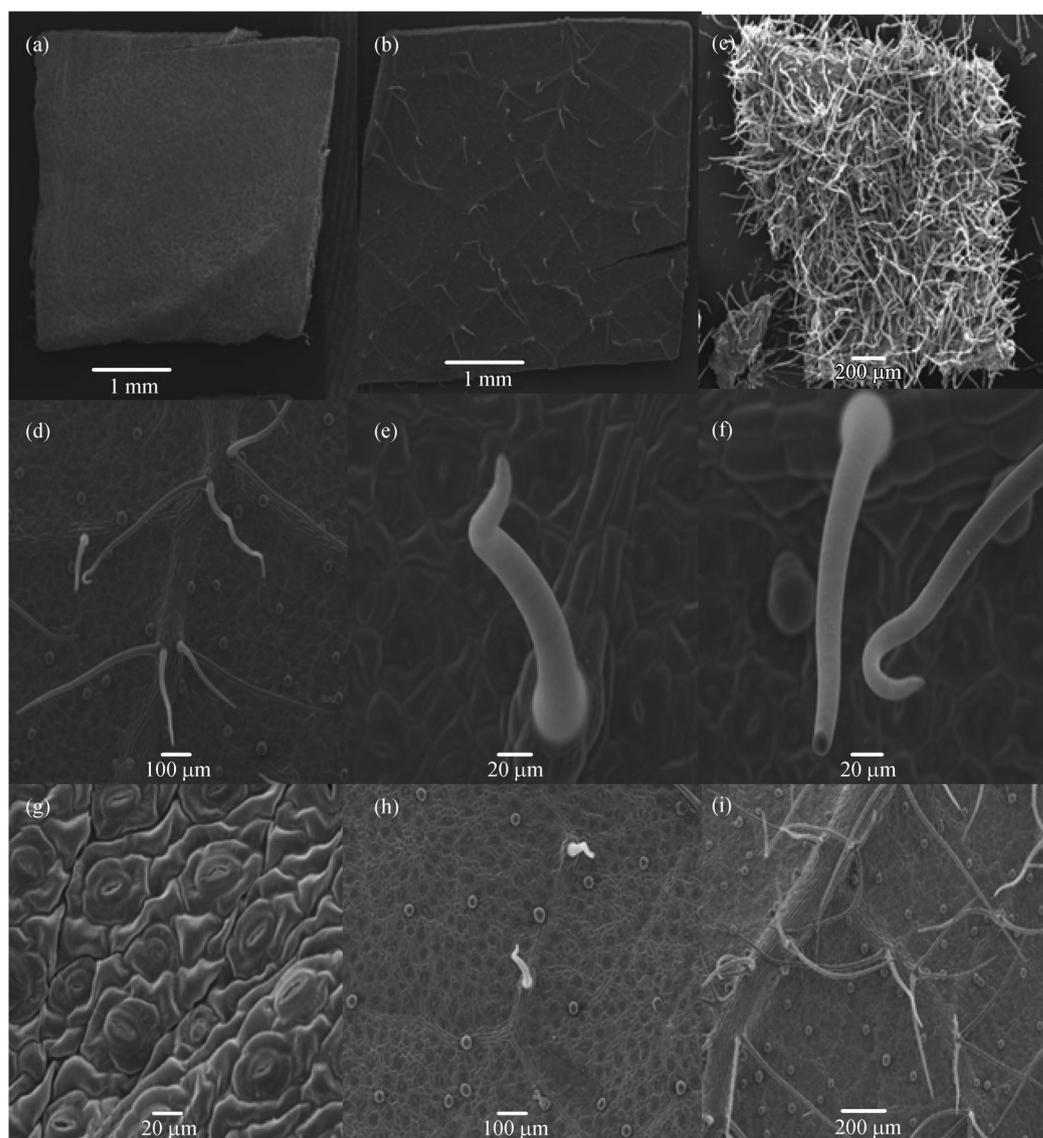


Fig. 2 Cotton leaf morphology related to resistance to whitefly in greenhouse Exps. 6–7. (a–c) Genotypes with different hair densities; (d–f) genotypes with different hair length; (g–i) gland on the leaf surface. All the photographs were taken by scanning electron microscopy.

populations with three biological replicates showed that 42 selected cotton genotypes (40 *G. hirsutum*s, one *G. arboreum* and one *G. herbaceum*), which included 23 resistant and 19 susceptible genotypes, exhibited a consistent performance in resistance and susceptibility in three greenhouses and two field experiments (Fig. 1a). The morphological traits (leaf shape, leaf hairiness, leaf hair length and gland density) (Table 1) and secondary metabolism (gossypol) were observed for each of these genotypes.

To further categorize resistance/susceptibility profiles of

these cotton genotypes, cluster analysis was performed on the Euclidean distance metric of each species and the cluster results were visualized in R with ‘cluster’ package^[66]. This analysis showed that the resistant genotypes were all clustered in one group while the susceptible species were in another (Fig. 3a). In addition, the reproducibility of these experiments was tested through the comparative analysis of adult whitefly populations differentiation from Exps. 1–3 in the greenhouse, and Exps. 4–5 in the field. The result showed a significance level of $P < 0.05$ (Fig. 3b).

Table 1 Details of genotypes identified and selected in three greenhouse experiments (Exps. 1–3 and two field (Exps. 4–5) experiments

Genotype	Experiment	Species	Leaf shape	Leaf hairs
SM8	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
DZMSR1	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
JM20	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
X16	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
ZLZ	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
GL1	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
Z1-59	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
37-30	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
38-36	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
39-38	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
PZYH	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
ACS50/2	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
3196	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
LBM	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Hairy
481GZ	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Hairy
74s-237	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Hairy
L1779	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Hairy
LM1	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Hairy
ZYZ4	1, 2, 3, 4, 5	<i>G. arboreum</i>	Okra	Hairy
DH77-116	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Hairy
MYm20	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Glabrous
HM4	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Glabrous
L901-902	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Glabrous
YJ2	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Glabrous
Z161	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Glabrous
LJM5	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Glabrous
L96-103	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Glabrous
13P022	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Glabrous
JN804	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
HMJw	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
GL3	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
RO	1, 2, 3, 4, 5	<i>G. herbaceum</i>	Okra	Normal
39-24	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
38-34	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
DGZ8-9	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
13p027	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
ZMS22	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
6919	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
QM465	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
ZM3	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
13p023	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal
HM11046	1, 2, 3, 4, 5	<i>G. hirsutum</i>	Normal	Normal

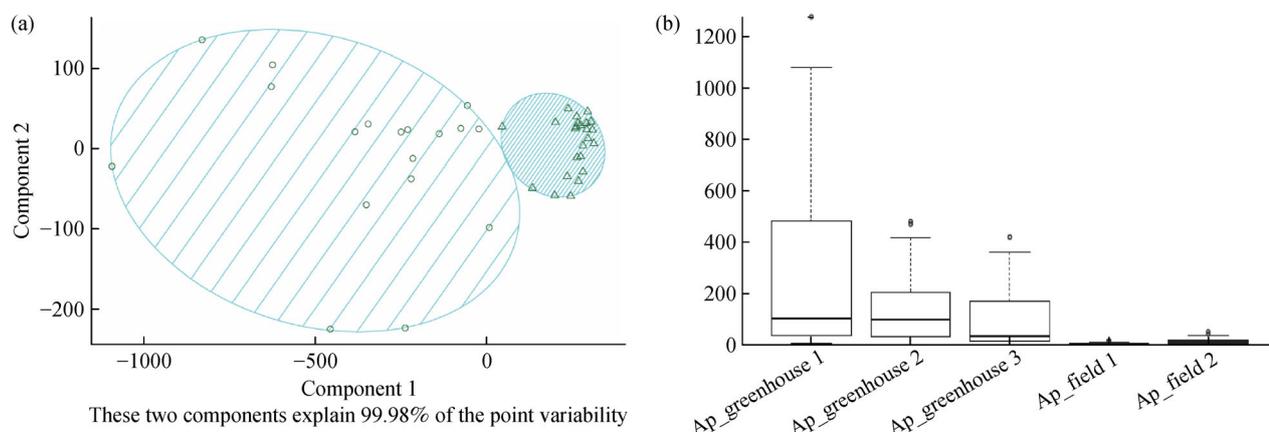


Fig. 3 Comparative analysis and Clusplot of adult whitefly populations in Exps. 1–3 in greenhouse and Exps. 4–5 in the field. (a) Clusplot of the first two components of 42 genotypes including susceptible and resistant genotypes with the population assessment of whitefly adults. To further categorize the resistance/susceptibility profiles of these different species of upland cotton, a cluster analysis was performed on the Euclidean distance metric of each species and the cluster results visualized in R with cluster package. The resistant species were clustered in one group and susceptible species in another group; (b) differentiation was supported by the *t*-test at a significance level of $P < 0.05$.

3.3 Screening of adult whitefly populations in greenhouse and field

The SPSS software was used to analyze the individual results for susceptible and resistant genotypes. The total number of adult whiteflies per five leaves in the greenhouse and field experiment plots was the highest in DH77-116 (mean of 447.133 adults per five leaves), which was five times higher than the lowest genotype, DGZ8-9 (mean of 89.8 adults per five leaves), from the susceptible genotypes (Table 2).

Adult whitefly populations combined with morphological traits of each genotype were used to ensure the stability and accuracy of resistant/susceptible genotypes. Susceptible genotypes of *G. hirsutum* with normal leaf shape had significantly higher numbers of adult whiteflies than other genotypes.

3.4 Nymph densitis in greenhouse experiments 2–3

Forty resistant/susceptible genotypes (some different from the 42 genotypes assessed for adult whiteflies) were assessed for nymph density. Of these, 25 genotypes common to the two groups were selected. When comparing the adult and nymphs density between (a) and (b) and between (c) and (d) in Fig. S2, no significant differences were detected among the selected genotypes. The nymph density across two experiments was the highest in DH77-116 (1450.571 and 3359.227 per cm^2 in Exps. 2–3, respectively), which had more than seven times the density of HM4 (2.00 and 4.30 per cm^2 in Exps. 2–3, respectively) (Table S1; Fig. 4), and had significantly higher densities than all other genotypes. However, the number of eggs and

nymphs on *G. hirsutum* genotype L1779, which had the highest leaf hair density and length, were significantly higher than all other *G. hirsutum* genotypes (Fig. 4).

3.5 Leaf hair density and length on the undersides of leaves

The 25 genotypes (10 susceptible and 15 resistant), which consisted of eight glabrous (HM4, MY4 and L901-902 being susceptible, and YJ2, 13P022, L96-103, LJM5 and Z161 being resistant), seven hairy (DH77-116, 481GZ, 74s-237 and LBM being susceptible, and L1779, ZLZ and ZYZ4 being resistant) and 10 normal genotypes, were assessed for leaf hair density. In greenhouse Exps. 2–3, the density of leaf hairs on three genotypes (DH77-116, 481GZ and 74s-237) was significantly higher than all other genotypes (susceptible listing in Table 3), and more than eight times the density of the normal genotype 6919 (Table 3). Furthermore, when comparing the leaf hair density (Table 3) with the number of whitefly eggs and nymphs (Table S1) in resistant/susceptible genotypes, it was found that the number of whitefly eggs and nymphs showed an increasing trend when the density of hairs was ultra-high (ZLZ had higher leaf hair density, and number of whitefly eggs and nymphs than JM20) or ultra-low (SM8 had lower leaf hair density but higher number of whitefly eggs and nymphs than L96-103) in resistant genotypes. In contrast, ultra high density of hairs resulted in a relatively low number of whitefly eggs and nymphs (481GZ had a higher density of hair but lower number of whitefly eggs and nymphs than DH77-116) in susceptible genotypes. Notably, all glabrous genotypes had fewer whitefly eggs and nymphs. So, it was concluded that low leaf hair density contributed to resistance to egg/nymph production.

Table 2 Mean number of adult whiteflies per five leaves on susceptible and resistant genotypes in greenhouse (Exps. 1–3) and field (Exps. 4–5) experiments

Phenotype	Genotype	Adults per five leaves											
		Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5		Mean	
Susceptible	MYm20	443.667	f	415.667	ab	288.333	b	5.333	cd	18	d	181.833	d
	JN804	641.667	d	480	a	213.333	cd	10.667	bc	34.333	bc	276	cd
	3196	256.333	h	224	bc	81.333	e	7.667	cd	22.667	cd	118.5	ef
	DH77-116	1277	a	470	ab	420	a	17.333	a	51.333	a	447.133	a
	X16	355.333	g	97.333	d	118.333	de	3.333	d	13.7	d	117.606	ef
	HMJw	364.667	g	130	bc	120.667	de	7.333	cd	21	cd	128.733	ef
	LBM	504	ef	177	bc	171.667	de	6.333	cd	19.7	cd	175.74	de
	GL3	885.667	c	213	bc	293.667	b	7	cd	27	c	285.267	c
	38-34	1081	b	243	bc	360	ab	11.333	bc	35	bc	346.067	b
	39-24	479	ef	232	bc	159.667	de	6.333	cd	20	cd	179.4	de
	DGZ8-9	255.667	h	93.3	d	85	e	4.333	d	10.667	d	89.793	f
	13p027	316.667	gh	116	c	105.333	e	4	d	12.667	d	110.933	ef
	ZMS22	880.667	c	240	bc	293.3	b	13	b	42.7	b	293.933	bc
	481GZ	481.667	ef	207	bc	160	de	5.667	cd	20.333	cd	174.933	de
	74s-237	589.333	de	301	b	195.333	cd	8.667	c	26.333	cd	224.133	d
	6919	420.333	fg	155	bc	140.333	de	5.667	cd	16	d	147.467	e
	HM4	521.667	e	185	bc	174	d	5.667	cd	19.333	cd	181.133	de
	L901-902	644	d	224	bc	214.333	c	6.667	cd	24.333	cd	222.667	d
	QM465	611.667	d	203	bc	204	cd	6.333	cd	20.333	cd	209.067	d
	LSD 5%												
Resistant	ZM3	92.667	c	131	ab	30.667	b	5.667	a	17.333	a	55.467	b
	ZLZ	61.667	de	33.667	cd	20.333	bc	3.667	bc	10.667	bc	26	de
	13p023	35.333	ef	47.667	c	11.667	c	2.333	c	7	bc	20.8	ef
	HM11046	26.333	fg	78.667	bc	8.667	c	0.3	d	1	d	22.993	de
	L1779	154.333	a	141	a	51.333	a	2	cd	14.333	ab	72.6	a
	YJ2	34.667	ef	3	d	11.667	c	1.333	cd	3.333	cd	10.8	f
	SM8	31.333	f	25.333	cd	10.333	c	1	cd	2.667	cd	14.133	f
	DZMSR1	62.333	de	18	cd	19.333	bc	1.667	cd	5.333	cd	21.333	ef
	Z161	47.667	e	25.333	cd	18.667	bc	2	cd	6	cd	19.933	ef
	JM20	37.667	ef	62.667	bc	12.667	c	1.667	cd	5	cd	23.934	de
	RO	7.667	g	36	cd	2.667	c	0	d	0.333	d	9.333	f
	LJM5	21.667	fg	12	cd	7	c	0.667	d	2.333	cd	8.733	f
	GL1	35.333	ef	93.333	b	10.333	c	4.333	ab	11.667	b	31	d
	ZYZ4	24.667	fg	35	cd	8.333	c	0	d	0	d	13.6	f
	L96-103	46.333	ef	66.667	bc	15.667	bc	3	bc	8.333	bc	28	de
	Z1-59	86.667	c	15.667	cd	29.667	b	4	b	11.333	bc	29.467	de
	13P022	33.333	ef	17.667	cd	12.667	c	2.333	c	6.333	c	14.467	f
	LM1	66.333	d	97.333	b	22.333	bc	2.667	bc	9.667	bc	39.667	c
	37-30	116.333	b	42.667	c	37.667	a	5.667	a	17.333	a	43.933	c
	38-36	15	g	20.667	cd	5.667	c	0.667	d	2	cd	8.8	f
39-38	56.333	de	25.333	cd	19	bc	3.333	bc	10.333	bc	22.866	e	
PZYH	59.667	de	30.667	cd	19.667	bc	4	b	12.333	ab	25.267	de	
ACS50/2	51.667	de	118	ab	13	c	1.889	cd	7.667	bc	38.445	cd	
LSD 5%													

Note: Each column followed by the same letter are not significantly different using LSD ($P < 0.05$) on ANOVA of square root transformed data.

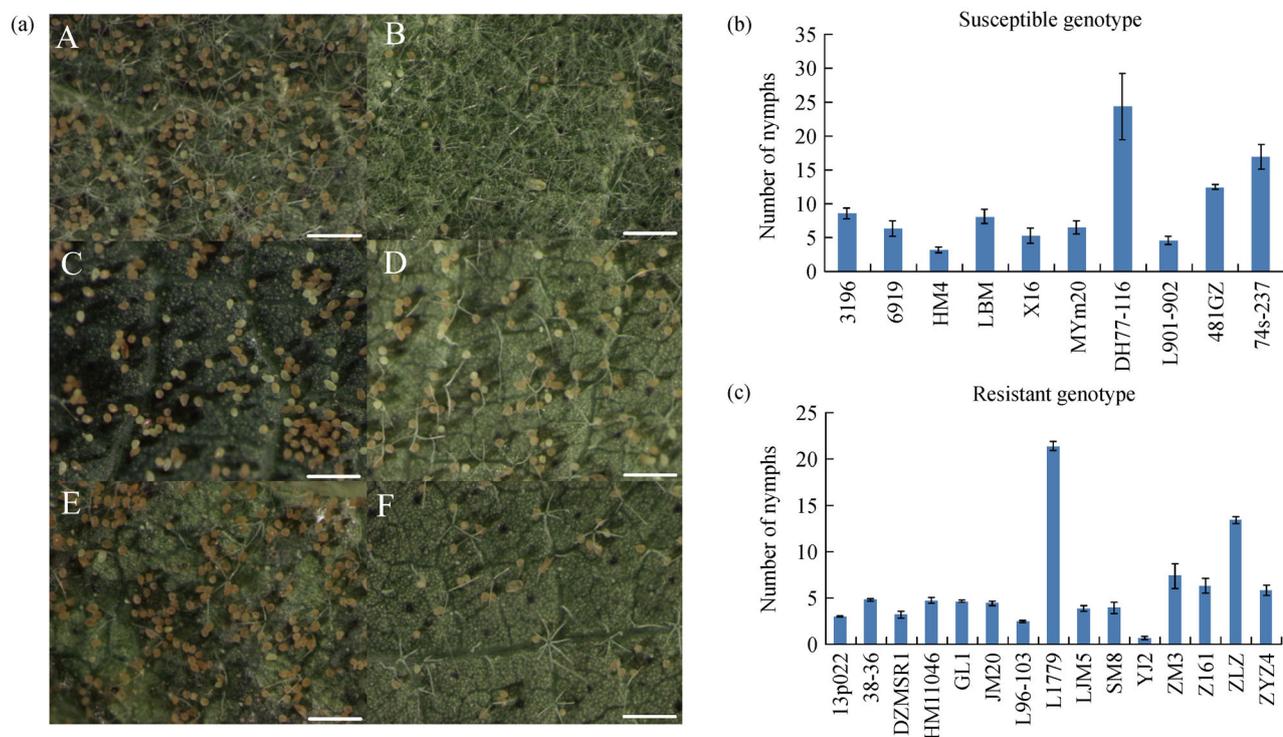


Fig. 4 Nymph densities on the leaves of 25 genotypes. (a) Nymphs on each leaf were counted using a Leica stereo microscope, samples were photographed from three positions to form a triangle. Vernier calipers were used to measure area of the leaves in the photographs with nymphs. Bars = 400 μm in A–F; (b, c) Mean number of whitefly eggs and nymphs per cm² on susceptible genotypes (10 genotypes) (b) and resistant (15 genotypes) (c) on the fifth leaf from the terminal in greenhouse Exps. 2–3. Back transformed means are plotted.

In the greenhouse experiments, differences in hair length on the underside of leaves were observed among different genotypes and experiments (Table 4). The resistant genotypes, HM11046, ZLZ, 38-36 and DZMSR1, had significantly longer leaf hairs than the other *G. hirsutum* and *G. arboreum* genotypes in these experiments. The results for Exps. 2–3 were mostly similar. Moreover, the same pattern was found among susceptible genotypes, especially, DH77-116 and 74s-237 that had longer leaf hairs than other genotypes.

3.6 Gossypol concentration and number of glands in cotton leaves

The concentration of gossypol, calculated using a standard curve that had a strong linear relationship ($y = 0.0136x + 0.072$, $R^2 = 0.999$), ranged from 2.8 to 14.0 μg·g⁻¹ (Fig. 5a). Gossypol concentration was approximately two times higher in the resistant genotypes than the susceptible ones in Exps. 2–3 (Table S2, Fig. 5b and 5c).

The mean number of glands on the underside of leaves in the resistant genotypes was about 81.2 per cm², which was significantly higher (about 1.7 times more) than susceptible genotypes (49.1 per cm²) (Table 5). In addition,

genotypes with fewer glands, e.g., X16 and MYm20, were susceptible genotypes, whereas those with more glands, e.g., 13p022 and Z161, were resistant genotypes.

The glands are important for the storage of gossypol. The analysis also showed that gossypol concentration had a strong positive relationship with glands density (Fig. S3).

3.7 The relationship between leaf shape and whitefly resistance

It was also found that *G. barbadense* genotypes, with larger leaves, had higher numbers of adult whiteflies than *G. hirsutum* genotypes, with normal leaf size. Genotypes with okra-shaped leaves had lower adult whitefly populations and nymph density (Tables 1–3). These relationships were observed in both greenhouse and field experiments.

3.8 Correlation and weight analysis of whitefly resistance in different cotton genotypes

The glabrous genotypes, without the effects of leaf hair length and density, were selected to study the correlation between gossypol concentration and glands density on whitefly resistance. The gossypol concentration and glands

Table 3 Mean leaf hair density of susceptible and resistant genotypes in the greenhouse Exps. 2–3

Phenotype	Genotype	Leaf hairs per cm ²					
		Exp. 2		Exp. 3		Mean	
Susceptible	3196	322.66	e	66.904	d	194.782	e
	6919	799.022	de	207.286	cd	503.154	de
	HM4	0		0		0	
	LBM	1451.129	d	357.899	c	904.514	d
	X16	213.788	e	12.437	d	113.113	e
	MYm20	0		0		0	
	DH77-116	4202.675	b	1088.381	b	2645.528	b
	L901-902	0		0		0	
	481GZ	6681.782	a	1595.488	a	4138.635	a
	74s-237	3211.371	c	854.999	b	2033.185	c
	LSD 5%						
Resistant	13p022	0		0		0	
	38-36	397.428	ef	97.199	cd	247.314	e
	DZMSR1	305.419	f	64.654	cd	185.037	ef
	HM11046	529.32	e	116.943	cd	323.131	de
	GL1	412.304	ef	91.487	cd	251.895	e
	JM20	977.039	d	104.227	cd	540.633	d
	L96-103	0		101.587	cd	50.793	ef
	L1779	10000	a	4744.234	a	7372.117	a
	LJM5	0		0		0	
	SM8	31.665	g	8.555	d	20.11	f
	YJ2	0		0		0	
	ZM3	314.944	f	115.256	cd	215.1	ef
	Z161	0		0		0	
	ZLZ	1485.31	c	471.308	c	978.309	c
	ZYZ4	5018.715	b	1396.312	b	3207.514	b
LSD 5%							

Note: Back transformed means in each column followed by the same letter are not significantly different using LSD ($P < 0.05$) on ANOVA of square root transformed data.

density were strongly negatively correlated with whitefly population in Exps. 2–3 (Table S3 ; Fig. 6).

Correlations between leaf hair length and density and whitefly nymphs in *G. hirsutum* was found for genotypes having a low concentration of gossypol (Table S4). Significant positive correlations between leaf hair density and length, and nymph numbers in Exps. 2–3 (Fig. 7) were also found.

Weight analysis was used to determine the proportion of multiple factors contributing to cotton resistance to whitefly. It was found that leaf hair density had a relative importance in resistance of 45.8% against adult whitefly populations, while glands density, gossypol concentration

and leaf hair length had relative importance of 31.3%, 5.6%, and 17.3%, respectively (Fig. 8a). For whitefly eggs and nymphs, leaf hair density had a relative importances of 52.5%, and leaf hair length, gossypol concentration and gland density had relative importances of 35.2%, 5.4%, and 6.9%, respectively (Fig. 8b).

4 Discussion

There is no doubt that insect resistance is controlled by multiple factors. Induced resistance describes the process by which changes occur in the host plant, in response to

Table 4 Mean leaf hair length of susceptible and resistant genotypes in greenhouse Exps. 2–3

Phenotype	Genotype	Mean leaf hair length/ μm					
		Exp. 2		Exp. 3		Mean	
Susceptible	3196	34.444	ab	59.305	ab	46.874	ab
	6919	34.154	b	55.516	ab	44.835	b
	HM4	0		0		0	
	LBM	34.097	b	55.332	ab	44.715	b
	X16	37.954	ab	41.882	b	39.918	b
	MYm20	0		0		0	
	DH77-116	44.435	a	68.372	a	56.403	a
	L901-902	0		0		0	
	481GZ	36.953	ab	56.823	ab	46.888	ab
	74s-237	42.568	ab	65.49	a	54.029	ab
	LSD 5%						
	Resistant	13p022	0		0		0
38-36		39.201	ab	55.815	ab	47.508	a
DZMSR1		34.023	b	60.196	ab	47.11	a
HM11046		0		63.796	a	31.898	b
GL1		32.533	b	54.542	ab	43.538	ab
JM20		23.745	c	51.039	ab	37.392	ab
L96-103		0		48.715	ab	24.357	b
L1779		45.555	a	42.95	ab	44.253	a
LJM5		0		0		0	
SM8		36.917	b	44.177	ab	40.547	ab
YJ2		0		0		0	
ZM3		36.979	b	52.584	ab	44.782	a
Z161		0		0		0	
ZLZ		39.11	ab	57.174	ab	48.142	a
ZYZ4		21.047	c	41.852	b	31.45	b
LSD 5%							

Note: Back transformed means in each column followed by the same letter are not significantly different using LSD ($P < 0.05$) on ANOVA of square root transformed data.

pest damage, it can increase the resistance of the plant to further herbivore attack^[68] and generally involves biochemical components. Gossypol is a kind of sesquiterpene, particularly involved in mediating interactions and protecting plants from herbivores and pathogens^[55,56,69]. Gossypol concentration and glands density were found to have strong negative correlations with the whitefly population which demonstrated their importance for whitefly resistance.

Leaf hair density has been implicated in resistance to spider mites^[70] and genotypes with high hair density or glabrous leaves had fewer mites, whereas those with intermediate densities had more mites^[71]. Also, the study

of population development together with B biotype adult whiteflies and oviposition preference revealed the glabrous leaf traits reduced oviposition preference^[42]. These experimental results are largely consistent with our results.

In weight analysis of multiple factors contributing to adult whitefly populations, we found that gland density was more important than gossypol concentration. It is also known that gossypol is one of the most abundant types of terpenoids in the glandular trichomes, which are involved in secondary metabolism synthesis, storage and release. This indicates that other metabolites synthesized and stored in the glands might also contribute to the resistance of cotton plants to adult whiteflies. However, for whitefly

Table 5 Mean number of glands on susceptible and resistant genotypes in greenhouse Exps. 2–3

Phenotype	Genotype	Gland density per cm ²					
		Exp. 2		Exp. 3		Mean	
Susceptible	3196	57	c	56	c	56.5	c
	6919	72.3	bc	84	b	78.2	bc
	HM4	75.3	b	84	b	79.7	bc
	LBM	32.3	d	24	d	28.2	d
	X16	0		0		0	
	MYm20	0		0		0	
	DH77-116	98	a	102	a	100	a
	L901-902	0		0		0	
	481GZ	90	ab	79	b	79.5	b
	74s-237	66	bc	52	c	59	c
	LSD 5%						
	Resistant	13p022	139.3	a	166	a	152.7
38-36		52.3	d	48	d	50.2	cd
DZMSR1		100.7	bc	104	b	102.4	bc
HM11046		77	c	86	bc	81.5	bc
GL1		102.7	bc	108	b	105.4	b
JM20		81	c	87	bc	84	bc
L96-103		29	d	28	d	28.5	d
L1779		81.3	c	64	cd	72.7	c
LJM5		109	b	99	b	104	b
SM8		81	c	99	bc	90	bc
YJ2		32	d	70.3	c	51.2	cd
ZM3		86	bc	70	c	78	c
Z161		129.3	ab	159	a	144.2	a
ZLZ		31	d	30	d	30.5	d
ZYZ4		86.3	bc	90	bc	88.2	bc
LSD 5%							

Note: Back transformed means in each column followed by the same letter are not significantly different using LSD ($P < 0.05$) on ANOVA of square root transformed data.

eggs and nymphs, the weight contribution of metabolites synthesized and stored in the glands was opposite. Some reports have stated that *G. hirsutum*, with a high gossypol concentration, is considered to be resistant to bollworm, *Helicoverpa zea*, and the tobacco budworm, *H. virescens*^[49]. Moreover, high gossypol concentration can significantly reduce the survival and reproduction of *Aphis gossypii* and *Propylaea japonica* by delaying the hatching of pupae, which greatly reduces the number of generations^[72]. Notably, the leaf hair densities were found to be highly important for adult whitefly populations as well as eggs and nymphs. Moreover, it has been shown that high leaf hair density can provide a favorable surface

environment for attachment^[73,74].

In this study over three years, we have conducted a detailed investigation and analysis of whitefly infestation throughout cotton growth and development in two field and five greenhouse experiments, using cotton genotypes from different parts of China. It was found that glabrous leaves were important for resistance to adult whiteflies, while low gossypol concentration and glands density contributed to susceptibility. It appears that within a certain range of leaf hair length and density, the larger and longer hairs allow whitefly adults and eggs to attach more easily to the leaf surface, and likewise for larger and smoother leaf types, but not for okra-shaped leaves.

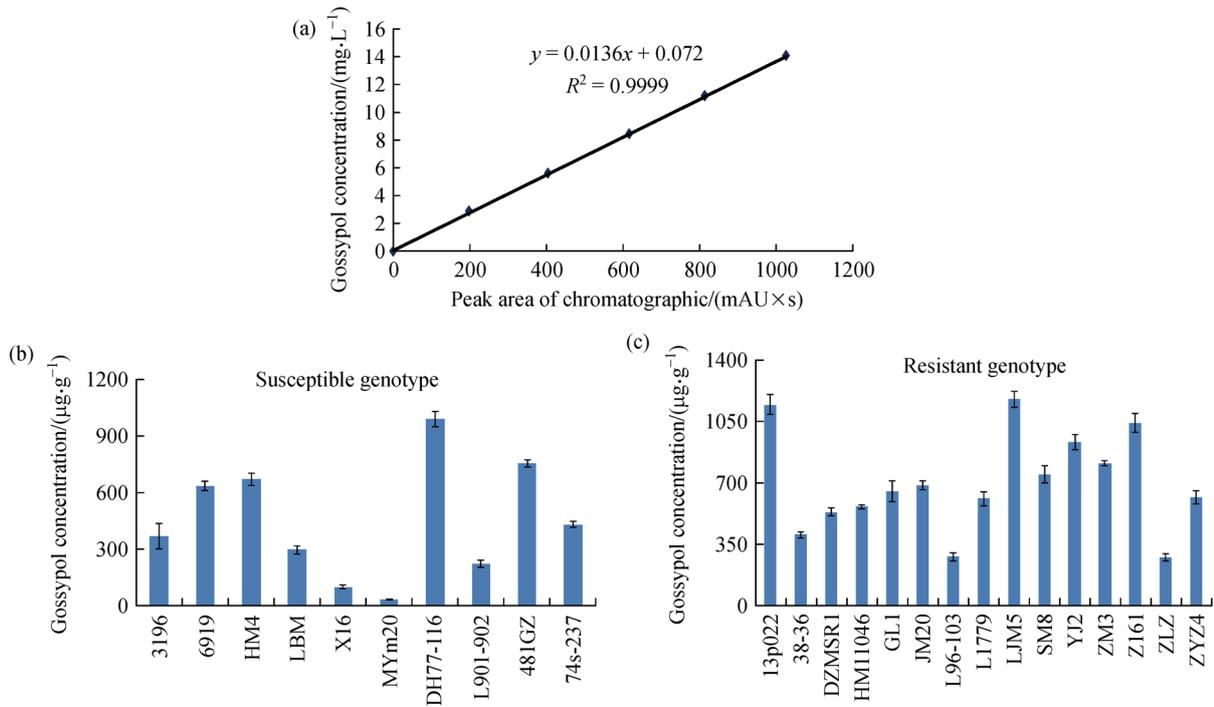


Fig. 5 Gossypol concentrations in the leaves of 25 genotypes quantified HPLC. (a) Six gossypol concentrations from 2.8 to 14.0 $\mu\text{g}\cdot\text{g}^{-1}$ were used to establish the standard curve; (b, c) gossypol concentration of 25 genotypes susceptible (b) and resistant genotypes (c) in greenhouse Exps. 2–3.

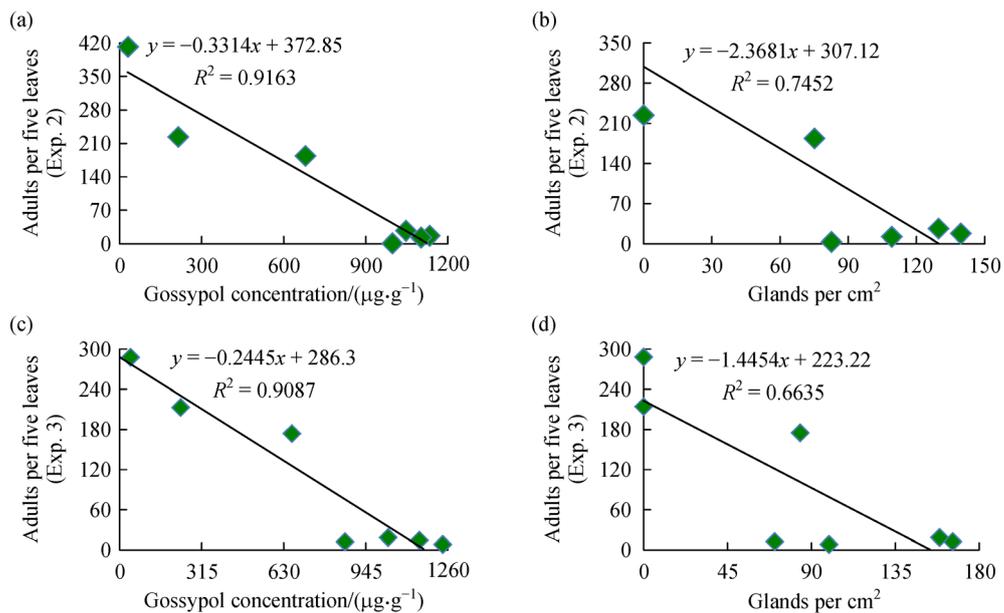


Fig. 6 Gossypol concentration and trichome density were negatively correlated with the population of whitefly per five leaves for glabrous genotypes. (a, b) Gossypol concentration ($\mu\text{g}\cdot\text{g}^{-1}$) (a) and glands per cm^2 (b) in greenhouse Exp. 2; (c, d) gossypol concentration ($\mu\text{g}\cdot\text{g}^{-1}$) (c) and glands per cm^2 (d) in greenhouse Exp. 3.

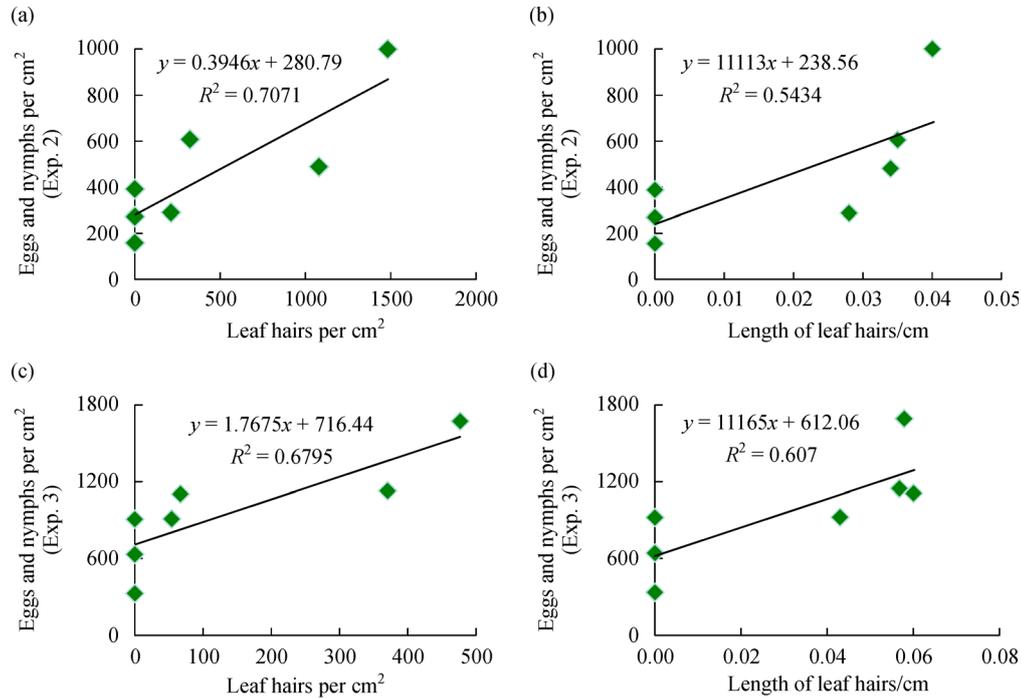


Fig. 7 Leaf hair density and hair length were positively correlated with the density of whitefly nymphs in genotypes with low concentrations of gossypol. (a, b) Leaf hairs per cm^2 (a) and leaf hair length (cm) (b) in greenhouse Exp. 2; (c, d) leaf hairs per cm^2 (c) and leaf hair length (cm) (d) in greenhouse Exp. 3.

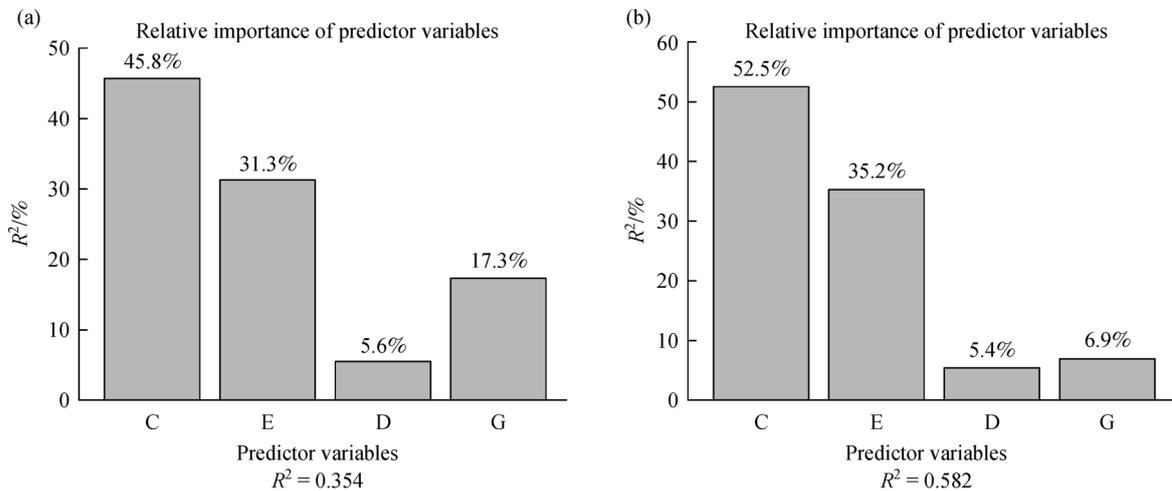


Fig. 8 Relative weight of predictor variables with number of adult whiteflies per five leaves (a) and whitefly nymphs per cm^2 (b). C, Leaf hair number per cm^2 ; E, Gland number per cm^2 ; D, Leaf hair length (cm); G, Gossypol concentration ($\mu\text{g}\cdot\text{g}^{-1}$). A multiple linear regression model was fitted and the all-subsets regression analysis in R with the regsubsets function from the leaps package^[67] was used to choose an optimal combination of the influence factors. Finally, the relative weight of each influence factor was calculated to define their contribution to adult whiteflies per five leaves (a) and whitefly nymphs per cm^2 (b).

5 Conclusions

This is the first report of a large-scale screening of *B. tabaci* resistance/susceptibility in cotton and the results revealed six different mechanisms of resistance: (1) Okra-shaped leaves reduce adult whitefly oviposition preference;

(2) Greater leaf hair length and densities reduce the density of whitefly eggs and nymphs for genotypes with low concentrations of gossypol through obstructing the feeding process; (3) Glabrous leaves help reduce adult numbers and oviposition preference; (4) Greater gossypol concentration and glands density reduce whitefly numbers on

glabrous genotypes; (5) Glands on the underside of cotton leaves have an important role in plant-insect interactions; (6) Gossypol in cotton leaves reduces the development and/or survival of whitefly adults. Although yield responses were not determined, these whitefly-resistant genotypes and traits should be considered as candidates for the development of host plant resistant genotypes.

Supplementary materials The online version of this article at <https://doi.org/10.15302/J-FASE-2018223> contains supplementary materials (Figs. S1–S3; Tables S1–S4).

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Compliance with ethics guidelines Lizhen Zhu, Jianying Li, Zhongping Xu, Hakim Manghwar, Sijia Liang, Suli Li, Muna Alariqi, Shuangxia Jin, and Xianlong Zhang declare that they have no conflicts of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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