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COTTON SNP 63K ARRAY FOR THE IDENTIFICATION AND VALIDATION OF MARKERS FOR JASSID RESISTANCE: GENETIC MAPPING

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ABSTRACT

Cotton crops are highly vulnerable to the pest Jassid (*Amrasca biguttula*) during their early growth stages. Developing resistant cotton varieties requires understanding disease-resistant genes and integrating them into elite cultivars. However, the cotton genome's limited diversity and complexity have made this challenging. Recent advancements have identified numerous single nucleotide polymorphisms (SNPs), enabling the use of high-throughput genotyping tools like the Cotton SNP 63K array for more consistent analysis. In our study, we used the SNP array to examine genetic variations between resistant and susceptible cotton cultivars, aiming to link SNP markers with Jassid resistance traits. We initiated crossbreeding with wild cotton lines (7076 and 7082) to create resistant cotton lines (BR-B). After a backcross, resistance traits were successfully transferred into the elite line (BS-1). Using the SNP array, we analysed 2462 polymorphic markers between resistant and susceptible lines. The contribution of each donor parent was assessed, revealing that alleles from 7076 and 7082 contributed 2.68% of the total alleles. After evaluating gene activity, we selected 148 SNP markers for further analysis, which were confirmed as authentic through Sanger sequencing. Using single-marker analysis, we identified a Quantitative Trait Locus (QTL) linked to pest resistance, which could accelerate the selection of other beneficial traits in cotton breeding.

Keywords: Jassid, Cotton, QTL, SNP, Phenotypes, Markers

INTRODUCTION

Over 95% of the cotton grown worldwide is Upland cotton (*Gossypium hirsutum L.*), a variety that has been subjected to numerous insect pests, which pose serious economic challenges for farmers. Cotton has been affected by arachnid pests for centuries, since it was domesticated at least 3,000 years ago (**Jabran *et al.*, 2019**). Among the many arthropod species that infest cotton, fewer than 40 are considered significant pests (**Wagan *et al.*, 2023; Jabran *et al.*, 2019**). Invertebrate pests alone can cause substantial losses to cotton yields, sometimes as high as 40% (**Alves *et al.*, 2020**). Even with the implementation of management techniques, pest damage is estimated to result in an ongoing loss of approximately 12% of the crop yield (**Naeem-Ullah *et al.*, 2020**).

The threat posed by sap-sucking insects remains particularly severe for genetically modified *Bacillus thuringiensis* (Bt) cotton, which is engineered for pest resistance. Farmers often resort to chemical pesticides to manage these insect infestations (**Ahmad *et al.*, 2021**). However, the widespread application of chemical insecticides comes with significant risks, including potential harm to human health, the emergence of secondary pests, environmental contamination, and the destruction of natural predators (**Zhang *et al.*, 2018**). Commonly cultivated cotton varieties are increasingly overwhelmed by sucking pests like whiteflies, thrips, and jassid. To keep these pests below the economic damage threshold, farmers must rely on chemical control methods. Thus, there is a pressing need for alternative cotton varieties that are less susceptible to insect infestations while maintaining high seed yields.

One promising area of research is plant-based pest control mechanisms, such as basal defense systems, which are found in almost all plants. These systems activate when the plant encounters pests or pathogens and can include morphological, physiological, or molecular responses (**Yassin *et al.*, 2021**). In some cases, cotton plants may naturally exhibit pest resistance (**Wani *et al.*, 2022**). Additionally, plants produce secondary metabolites that act as natural repellents, and these metabolites can be harnessed as environmentally friendly bio-pesticides (**Nikolaou *et al.*, 2021**). These metabolic processes serve to deter pest activity and reduce the need for harmful chemicals.

In terms of genetic research, Simple Sequence Repeats (SSRs) have been a preferred method for identifying genotypic variations in cotton. SSR markers are advantageous because they can detect multiple alleles per locus, leading to higher polymorphism and requiring fewer markers for analysis. However, SSR-based genotyping can be labor-intensive and costly (**Kumar *et al.*, 2022**). Single Nucleotide Polymorphisms (SNPs) are now more commonly used in cotton research due to their bi-allelic nature, which allows for high-throughput and cost-effective genotyping of numerous SNPs simultaneously. As the number of SNPs increases, the ability to differentiate between similar cultivars improves, thus enhancing the precision of genetic diversity studies (**Anu *et al.*, 2022**).

SNPs, which are scattered across the genome, have rapidly replaced SSR markers in many genetic studies due to their ability to provide a higher marker density and more efficient analysis methods. High-throughput DNA sequencing technologies have made it easier and more affordable to discover and identify SNPs, which are vital for marker-assisted breeding, map-based cloning, quantitative trait loci (QTL) mapping, and genetic and physical map construction. However, in polyploid crops like cotton, the presence of homoeologous or paralogous loci can complicate the accurate identification and validation of SNPs (**Dar *et al.*, 2019**).

Cotton has only recently begun to fully exploit the availability of SNP markers, lagging behind other field crops in this area (**Anu *et al.*, 2022**). This is especially true for allotetraploid crops like cotton, where identifying SNPs can be particularly challenging. However, recent advancements have led to the development of high-throughput genotyping methods, such as the CottonSNP63K array, which allows researchers to analyze a large number of SNP markers simultaneously and obtain consistent results (**Hou *et al.*, 2018**). This array has proven useful for identifying SNP markers associated with resistance to pests like jassid. The current research aims to identify, authenticate, and genotype SNP markers in a bi-parental population of cotton to pinpoint loci linked to resistance traits.

This growing body of research on SNP markers and pest resistance in cotton offers exciting possibilities for improving cotton cultivars that are more resilient to insect pests, ultimately reducing the reliance on chemical pesticides and improving sustainability in cotton farming. Thus, the present work aims to identify, authenticate, and genotype SNP markers in a bi-parental population.

MATERIALS AND METHODS

Plant materials and genomic DNA isolation

An F2 population of 1,478 individuals was created by crossing BS-1 and R-1, followed by selfing the F1 generation. Genomic DNA was extracted from the young leaves of resistant and susceptible plants, as well as from the donor plants 7076 and 7082. DNA extraction was performed using Nucleo Spin Plant II kits (MACHEREY-NAGEL, USA) on the young leaves. For the screening of the F2

population, DNA was isolated from 1,478 plants using the cetyltrimethylammonium bromide (CTAB) method (**Paterson *et al.*, 1993**).

Phenotyping

Jassid tolerance was assessed in the mapping population, with 267 F2 plants selected for phenotyping from a total of 1,478. These plants were cultivated in the same field, located at 17.59' N and 78.49' E, approximately 577 meters above sea level, with an average annual temperature of 33°C. The typical signs of damage caused by the Jassid pest were evaluated during phenotyping. The plants were rated according to the criteria outlined in Table 1, with each rating reflecting the impact of the pest on the plant, including symptoms such as leaf cracking, curling, and yellowing, ranging from minimal to severe damage.

Table 1 Phenotypingevaluation of the usual sucking pest (Jassid) damage signs.

Grade I	Completely green foliage that isn't browning or breaking.
Grade II	A few leaves in the lowest part of the plant are curling and crinkling, and they are also somewhat yellowing.
Grade III	Nearly all of the plant's leaves are wrinkling and curling, and plant development is impeded.
Grade IV	Extreme leaf drying, cracking, yellowing, and curling.

Jassid resistance screening under controlled conditions:

Resistant and susceptible materials, along with donor parents and known checks (as shown in Table 2), were assessed under controlled conditions. Three replications were conducted for each line, and data was recorded 51 days after planting to evaluate Jassid damage.

Table 2 Jassid screening of different cotton entries under poly house conditions

S. No.	Entry No	No of Replicates	R1	R2	R3	Grading Total	Ranking	Reaction
1	BS1	3	4	3	4	11	3.67	Grade IV
2	BR_A	3	1	2	1	3	1.33	Grade II
3	BR_B	3	3	1	1	1	1	Grade I
4	7076 Donor	3	1	2	1	4	1.33	Grade II
5	7082 Donor	3	1	1	1	3	1	Grade I
6	RCH-2 (Check)	3	3	3	2	8	2.67	Grade IV
7	RP-278(Check)	3	1	1	1	3	1	Grade I

Genotyping

A total of 45,103 SNPs from G. hirsutum, 2,226 from G. mustelinum, 9,579 from G. barbadense, 1,862 from G. longicalyx, 2,397 from G. tomentosum, and 1,890 from G. armorianum were genotyped using the Illumina iScan system and the Cotton 63K SNP bead chip (**Hinze *et al.*, 2017**).

Tetra-primer ARMS-PCR amplification

The tetra primer ARMS-PCR method was developed to support SNP markers in single samples from a mapping population due to its simplicity, speed, cost-effectiveness, and ability to differentiate between homozygous and heterozygous SNPs (**Raja *et al.*, 2018**). Primers for the SNPs were designed using the website http://cedar.genetics.soton.ac.uk. For each selected SNP locus, two outer primers and two inner primers (forward and reverse) were synthesized. PCR amplification was carried out using the Eppendorf VP thermal cycler (Germany).

The PCR reaction mixture contained 50 ng of template DNA, 0.4 µL of each outer and inner primer, 20 µL of reaction mixture (10x), 2.5 mM MgCl2, 200 mM dNTPs, 2 µL of 10x buffer, and 0.5 U of Taq DNA polymerase. The PCR conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 34 cycles of denaturation at 95°C for 30 seconds, annealing at 58–68°C for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The PCR products were then analyzed for SNP allele presence by electrophoresing the samples on a 3% agarose gel with ethidium bromide. The band patterns were evaluated to determine whether the SNP was homozygous or heterozygous.

QTL analysis and mapping

QTL analysis was performed using the ICIM-ADD method (**Meng *et al.*, 2015**). For linkage mapping, the parameters were set as follows: LOD threshold of 3, ordering algorithm - nnTwoOpt, rippling criteria - SARF with a window size of 5. During QTL mapping with the ICIM-ADD method, the step size was set to 1 cM, and a permutation of 500 was used to determine the LOD threshold, which was set to greater than 3.

RESULTS

Genotyping using Cotton 63k Chip array

The Cotton SNP 63K array (Illumina, USA), which includes 63,058 SNP Infinium II assays (Table 3), was used to generate SNP genotypes for each individual sample. Five samples were sent to Scigenom labs for genotyping (**Hinze *et al.*, 2017**).

To identify markers related to Jassid resistance, an Illumina high-throughput genotyping platform and the Illumina 63K Array chip were utilized for both resistant and susceptible cultivated cotton, as well as its wild relatives. Approximately 2,475 polymorphic SNPs were found between the resistant and susceptible cotton lines.

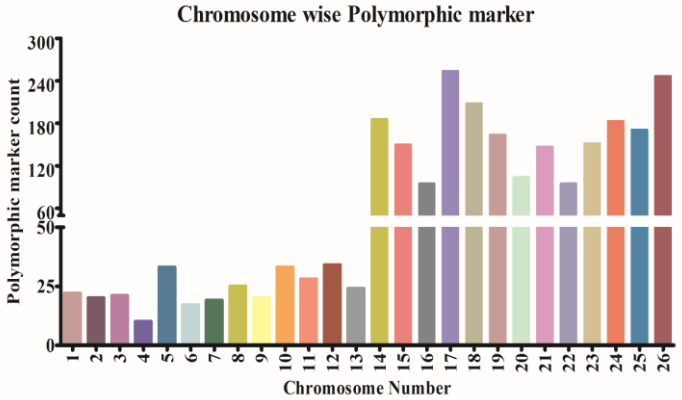

Figure 1 Chromosome wise polymorphic SNP marker distribution for resistant and susceptible lines.

Allele contribution

Two donor parents were used for introgressing the resistant trait, so the focus was on identifying the combined and individual allele contributions from both parents. Donor 7076 contributed 390 alleles, while donor 7082 contributed 385 alleles. The total number of common alleles shared by both parents was approximately 1,687.

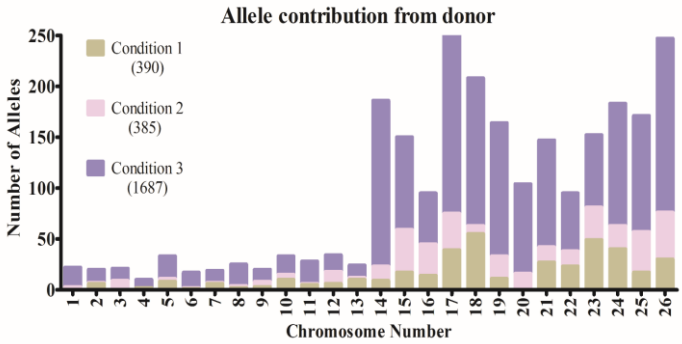

Figure 2 Allele contributions from a donor for Number of Alleles and chromosomes.

Gene annotation

The cotton genome was queried against filtered homozygous allele sequences on Uniport.org to identify the biological and molecular functions of the genes. In condition 1, 214 gene annotations were obtained from a total of 390 alleles in the 7076 donors. In condition 2, 198 out of 385 alleles were informative. For condition 3, 826 gene annotations were retrieved from the 1,687 alleles.

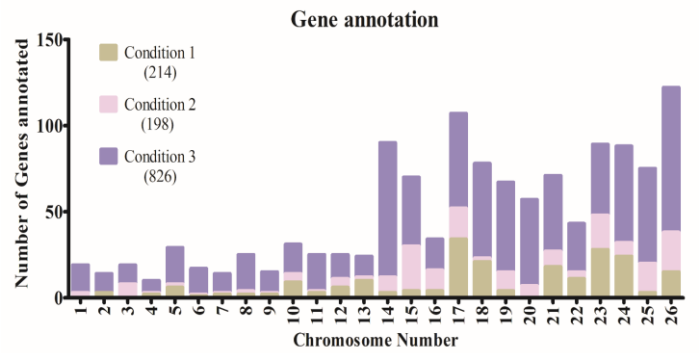

Figure 3 Chromosome wise Gene annotations for resistant and susceptible lines.

Table 3 Origin of the 63K array's single nucleotide polymorphism (SNP) and the SNP distinguishing resistant from susceptible lines.

SNP origin	No. of SNPs in 63K array	No. of polymorphic markers	Polymorphism (%)
G. hirsutum (Gh)	45104	2000	4.43
G. barbadense (Gb)	9579	294	3.07
G. tomentosum (Gt)	2397	74	3.09
G. mustelinum (Gm)	2226	43	1.93
G. armorianum (Ga)	1890	40	2.12
G. longicalyx (Gl)	1862	11	0.59
Total	63058	2462	

Polymorphic markers reconfirmation

The markers were refined to 145 SNPs based on gene ontology. To verify the results and eliminate false positives, Sanger sequencing was employed. All 145 markers were re-amplified and sent for Sanger sequencing, with 63 of the SNPs yielding positive results.

SNP validation

SNP validation for trait-related markers

The tetra-primer ARMS-PCR method was modified for the associated SNP markers to genotype individual samples from the segregating population, aiming to validate the F2 population method. The figure illustrates the sizes of the DNA fragments amplified using these primers.

Single marker analysis results

Marker Trait Association (MTA)

All five screened markers were analyzed for marker-trait association, and the results revealed that three markers exhibited significant variation. These markers, i01143Gh, i02359Gh, and i03879Gh, showed high LOD values of 3.4216, 4.4174, and 10.1173, respectively. Additionally, these markers had higher PVE values of 6.5784 and 10.308, compared to the others. The results of the Marker Trait Association (MTA) analysis are summarized in Table 4. The MTA findings suggest that these three markers can be utilized for screening and selection in breeding programs.

Chromosome no 4

Table 4 For QTL mapping with ICIM-ADD method Step- 1 cM, permutation -500 to decide LOD threshold.

Trait Name	Chrom	Pos	Marker Name	LOD	PVE (%)	Add	Dom
Jassid	4	0	i01143Gh	3.0282	3.4216	-0.3515	0.0586
Jassid	4	27.32	i03879Gh	9.4566	10.1173	-0.4038	0.1143
Jassid	2	0	i02359Gh	3.9402	4.4174	-0.424	-0.0081

DISCUSSION

The emergence of next-generation sequencing (NGS) technologies has significantly transformed genetic analysis by enabling the detection of numerous markers, particularly single nucleotide polymorphisms (SNPs). This advancement has greatly enhanced our ability to investigate genetic diversity and traits associated with specific characteristics, providing a powerful tool for the advancement of plant breeding efforts. In this study, we utilized the CottonSNP63K array to assess genetic variation in various species of Gossypium. Our research focused on a range of wild germplasm lines alongside refined Gossypium cultivars (**Hinze *et al.*, 2017**).

Cotton (*Gossypium hirsutum* L.) is severely impacted by Jassid (*Amrasca biguttula*) during its early growth stages, making the development of resilient cotton varieties essential. This requires a thorough understanding of disease-resistant genes and their incorporation into elite cultivars. However, the cotton genome's complexity and the limited genetic diversity have posed challenges in identifying these resistance genes. Recent advancements in identifying numerous SNP markers and the development of high-throughput genotyping technologies like the CottonSNP63K array present a promising solution (**You *et al.*, 2018**).

In this study, we leveraged the SNP array to explore genetic variations, particularly between resistant and susceptible cotton cultivars. Through single marker analysis, we aimed to identify correlations between SNP markers and phenotypes, as well as to pinpoint loci associated with Jassid resistance traits.

To develop resistant cotton lines (BR-B), a crossbreeding process was initiated using wild cotton lines (7076 and 7082), resulting in the successful incorporation of resistance traits into the elite line (BS-1) after a single backcross. Subsequently, both the resistant and susceptible cotton lines, along with the donor parents, were subjected to the Cotton63K SNP array (**Ma *et al.*, 2018**). This analysis revealed 2,462 polymorphic markers when comparing R-1 and BS-1 lines. The contribution of each donor parent was assessed based on the total homozygous alleles. Alleles from the 7076 and 7082 parents contributed 0.62% and 0.61%, respectively, making a total contribution of 2.68% from both parent lines. After evaluating biological activity and gene functionality, 148 SNP markers were selected as the final set of polymorphic markers. These markers were further validated using Sanger sequencing, resulting in 63 confirmed SNP markers.

Using a biparental population derived from the cross of resistant and wild lines, along with donor parents, genotyping was performed using the Cotton SNP 63K array. Among the resistant and susceptible lines, 2,462 polymorphic markers were identified. The contribution of each donor parent was evaluated, and 148 SNP markers were chosen based on biological activity and gene function. Further confirmation through Sanger sequencing led to the identification of 63 validated polymorphic markers. A single marker analysis was conducted to identify markers associated with the Jassid resistance trait, which resulted in the identification of a significant quantitative trait locus (QTL). This discovery provides a pathway for the rapid identification of QTLs of interest using marker-assisted selection in cotton breeding programs. The successful application of single-marker analysis has led to the identification of markers associated with resistance to sucking pests, culminating in the discovery of a single QTL. This holds significant promise for accelerating the identification of QTLs linked to other desirable traits using marker-assisted selection in cotton breeding.

The study also offers valuable insights into the challenges faced by cotton cultivation due to pest infestations. Over 95% of the world's cotton is grown in plateau regions (**Cao *et al.*, 2020**), which are vulnerable to various insect pests, including Jassid. These pests have a considerable economic impact, potentially causing losses of up to 40% from invertebrate pests alone. Pest control efforts often rely on pesticides, which have harmful effects on human health and the environment. Therefore, developing pest-resistant cotton varieties, as explored in this study, is crucial for the sustainability of cotton farming.

The use of high-throughput genotyping technologies, such as the Cotton SNP 63K array, represents a major advancement in cotton breeding. This technology enables efficient analysis of large numbers of SNP markers, allowing researchers to identify and select desirable traits more effectively.

CONCLUSION

In conclusion, this study provides an in-depth analysis of genetic variations associated with Jassid resistance in cotton cultivars. The identification of SNP markers and the discovery of a QTL linked to resistance traits represent significant progress toward the development of pest-resistant cotton varieties. These findings extend beyond cotton breeding and contribute to the broader goals of sustainable agriculture and effective pest management. Continued research in this area has the

potential to enhance cotton yields and reduce the dependence on chemical pesticides, ultimately benefiting both farmers and the environment.

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