High-density 80 K SNP array is a powerful tool for genotyping G. hirsutum accessions and genome analysis

Caiping Cai†, Guozhong Zhu†, Tianzhen Zhang† and Wangzhen Guo1,2*

Abstract

Background: High-throughput genotyping platforms play important roles in plant genomic studies. Cotton (Gossypium spp) is the world’s important natural textile fiber and oil crop. Upland cotton accounts for more than 90% of the world’s cotton production, however, modern upland cotton cultivars have narrow genetic diversity. The amounts of genomic sequencing and re-sequencing data released make it possible to develop a high-quality single nucleotide polymorphism (SNP) array for intraspecific genotyping detection in cotton.

Results: Here we report a high-throughput CottonSNP80K array and its utilization in genotyping detection in different cotton accessions. 82,259 SNP markers were selected from the re-sequencing data of 100 cotton cultivars and used to produce the array on the Illumina Infinium platform. 77,774 SNP loci (94.55%) were successfully synthesized on the array. Of them, 77,252 (99.33%) had call rates of >95% in 352 cotton accessions and 59,502 (76.51%) were polymorphic loci. Application tests using 22 cotton accessions with parent/F1 combinations or with similar genetic backgrounds showed that CottonSNP80K array had high genotyping accuracy, good repeatability, and wide applicability. Phylogenetic analysis of 312 cotton cultivars and landraces with wide geographical distribution showed that they could be classified into ten groups, irrelevant of their origins. We found that the different landraces were clustered in different subgroups, indicating that these landraces were major contributors to the development of different breeding populations of modern G. hirsutum cultivars in China. We integrated a total of 54,588 SNPs (MAFs >0.05) associated with 10 salt stress traits into 288 G. hirsutum accessions for genome-wide association studies (GWAS), and eight significant SNPs associated with three salt stress traits were detected.

Conclusions: We developed CottonSNP80K array with high polymorphism to distinguish upland cotton accessions. Diverse application tests indicated that the CottonSNP80K play important roles in germplasm genotyping, variety verification, functional genomics studies, and molecular breeding in cotton.

Keywords: Single nucleotide polymorphism (SNP), Array, Upland cotton, Genotyping identification, Genome-wide association studies (GWAS), Molecular breeding

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Background
Cotton (Gossypium spp.) is the largest source of renewable fiber in the world and also a significant oil crop. Upland cotton accounts for more than 90% of the world crop. However, modern upland cotton cultivars have narrow genetic diversity, due to their development from the limited quantity of resources in the United States [1]. Molecular markers have been successfully used in genotyping identification of cotton accessions and genomics-based cotton improvement. Among these, simple sequence repeat (SSR) markers are the most widely used type of molecular marker in cotton. So far, 19,010 SSR markers have been registered in CottonDB (http://cottondb.org/), and 100,290 microsatellites have been mined in the genome of G. hirsutum, where 77,996 SSR markers been developed [2]. However, SSR markers have disadvantages; for example, they are low-throughput, labor-intensive and time-consuming as a genotyping platform. In addition, the low density of SSR markers could cause the loss of linkages between markers and target trait locigenes [3]. Hence, high-throughput molecular markers are imperative to saturate cotton genetic maps, and to significantly improve genequantitative trait loci (QTL) mapping for genomic studies and marker-assisted selection (MAS) breeding [4].

Single nucleotide polymorphisms (SNPs) are the most abundant DNA sequence variation present in plant genomes, with virtually unlimited, evenly distributed along the genome, bi-allelic and co-dominant characteristics. In cotton, several studies on genome-wide SNP marker development and utilization have been reported recently. Using BAC-end sequences of 12 G. hirsutum lines, one G. barbadense acc. 379 and one G. longicalyx line, genome-wide SNPs were mined. Of them, a total of 132,262 intraspecific SNPs have been developed in G. hirsutum, while 223,138 and 470,631 interspecific SNPs have been identified between G. hirsutum and G. barbadense or G. longicalyx, respectively [5]. By means of genotyping-by-sequencing (GBS) of 6071 SNPs and 223 SSRs, genome-wide association studies (GWAS) analysis was conducted using 547 recombination inbred lines (RILs) of a multi-parent advanced generation inter-cross (MAGIC) population in four environments to identify genesQTLs associated with fiber quality traits. As a result, a QTL cluster associated with four fiber quality traits, short fiber content (SFC), strength (STR), length (UHM) and uniformity (UI), was identified on A07, and a candidate gene, GhRBB1_A07, encoding a regeneration of bulb biogenesis 1 protein was further investigated [6]. Based on the specific-locus amplified fragment sequencing (SLAF-seq) of 355 upland cotton accessions, a total of 81,675 SNP markers was used for GWAS analysis of lint percentage [7] and early maturity [8]. Taken together, these findings suggest that SNPs play important roles in cotton genetics and breeding studies.

Compared to re-sequencing analysis, SNP arrays can produce large-scale genotyping data through one hybridization procedure at a relatively low cost. So far, there are nearly a hundred different genotyping techniques to meet different SNP array needs, with different sample sizes and numbers of SNP loci. In high-density SNP genotyping, Illumina (Infinium technology) and Affymetrix (Affymetrix GeneTitan technology) are two of the most widely used SNP genotyping platforms in a variety of species. In agricultural crops, a variety of SNP arrays using different SNP resources have been developed using appropriate platforms. For example, in rice, RiceSNP6K [9], RiceSNP50K [10] and HDRA700K [11] were developed based on the Illumina Infinium platform; 1536 SNPs [12] based on the GoldenGate platform; 384-plexes [13] based on the BeadXpress platform; and GeneChip Rice 44 K [14] and OsSNPnks 50 K chip [15] were developed using the Affymetrix platform. In cotton, only one array (CottonSNP63K) has been developed based on the Illumina Infinium platform [16]. The array loci were identified from 13 different discovery sets that represent a diverse range of G. hirsutum germplasms, as well as five other species. Using the array, 188 RIL populations from an intra-specific cross (HS46 and MARCABUCAG8US-1-88) were genotyped, and 71 QTLs for fiber quality and yield traits were detected [17]. Recently, GWAS analysis of 719 diverse accessions of upland cotton was conducted using multiple environment tests of fiber quality traits with the CottonSNP63K SNP array, and 10,511 polymorphic SNPs and forty-six significant SNPs associated with five fiber quality traits were detected [18]. In addition, the population structure and genetic basis of the agronomic traits of upland cotton has been investigated in 503 G. hirsutum accesses using 11,975 quantified polymorphic SNPs in the CottonSNP63K SNP array [19]. These results indicate that the SNP array is a robust and highly efficient tool for use in cotton genetic studies and breeding improvement.

The availability of data on the whole-genome of four different cotton species, two diploid cotton species, G. raimondii and G. arboreum [20, 21], the upland cotton genetic standard line, G. hirsutum acc. TM-1 [22, 23], and two sea-island cottons, G. barbadense cv. Xinhai21 and G. barbadense acc. 379 [24, 25], made it possible to develop SNP markers at a genome-wide level. In our previous studies, an ultra-dense inter-specific genetic map of G. hirsutum and G. barbadense containing 519 framework SSRs and 4,999,048 SNPs, was constructed [26]. Further, genomic re-sequencing data from 100 different upland cotton cultivars were produced and provided millions of polymorphic SNP information [27]. In this study, based on the high quality reference sequence of the TM-1 genome [22] and re-sequencing data from different upland cotton cultivars [27], we focused on the
development of a high-density upland cotton SNP array for intraspecific genotyping detection, with the advantages on addressable SNPs, genome-wide distribution, high-effective genotyping detection. We selected 82,259 SNPs for the array development using Illumina’s Infinium platform, and named this the CottonSNP80K. Our tests showed that this array had wide applicability, good repeatability and high efficiency of genotyping identification. Phylogenetic relationship and GWAS analysis of salt stress traits indicated the CottonSNP80K play important roles in germplasm genotyping, variety verification, genetic relationship identification, functional genomics studies, and molecular breeding in cotton.

Results

Development and characteristics of the CottonSNP80K array

Based on the high quality reference sequence of the TM-1 genome [22] and re-sequencing data from 100 different cultivars of *G. hirsutum* with an average coverage of 5× in the genome [27], we developed a high-density SNP array for high-throughput intraspecific upland cotton genotyping identification. Following the SNP selection process (Additional file 1: Figure S1), 82,259 SNPs were selected out of 1,372,195 putative intraspecific SNPs with minor allele frequencies (MAFs) >0.1, and 77,774 of these (94.55%) were successfully synthesized on the array. We named the array CottonSNP80K. In this array, the average distance between adjacent SNPs was 24.9 Kb (Fig. 1a). In detail, 45,183 (58.10%) and 32,591 loci (41.90%) were located in the At- and Dt-subgenomes, and one SNP existed every 25.7 Kb and 23.8Kb, respectively. The highest SNP density was on A08, which contained 7773 SNPs (~1 SNP every 13.3 Kb), followed by chromosomes D06, D07, D09 and A13 (the number of SNPs ranged from 2938 to 4340, with an average of 1 SNP every 15–20 Kb). Chromosomes A02, A03, A04, and D04 had the relatively low SNP density, with an average distance of more than 40 Kb between two adjacent SNPs (Table 1). The distances between the loci of 63.62% (49,477) of the SNPs were less than 10 Kb, and only 7891 SNPs (10.15%) had gaps that were >50 Kb. The largest gap between two adjacent SNPs was 3148 Kb, and this was on chromosome A03 (Fig. 1b). In CottonSNP80K, 16,642 SNPs (21.40%) were tagged in the genic region of 9902 genes, and 61,132 SNPs (78.60%) in intergenic regions. Of the genic region SNPs, 44.62% (7426), 31.42% (5228) and 23.96% (3988) were distributed within introns, exons and untranslated regions (UTRs), respectively (Fig. 1c).

Fig. 1 Description of SNPs in CottonSNP80K array. a SNPs distributions on the 26 chromosomes of upland cotton. A01-A13 and D01-D13 in vertical axis are the serial number of 26 chromosomes; the horizontal axis shows chromosome length (Mb); the red region depicts SNP density (the number of SNPs per window). b Distances between the SNPs. The vertical axis represents distances range (Kb) of SNPs. c Distribution of genic and intergenic regions of selected SNPs.
SNP genotype calling in allotetraploid cotton

The genome-wide CottonSNP80K array was used to genotype 352 cotton cultivars/accessions (Additional file 2: Table S1). GenomeStudio Genotyping software (V2011.1, Illumina, Inc.) was used to provide a default clustering file based on bi-allelic SNPs, and all 77,774 SNP markers corresponding to the three separated signal clusters, AA, AB and BB, were genotyped. From an evolutionary point of view, the polyploidy cotton originated from an interspecific hybridization event between A- and D-genome diploid species about 1–2 million years ago, and the two extant progenitor relatives diverged from a common ancestor 5–10 million years ago [28]. In addition, upland cotton is a type of cross-pollination allotetraploid crop with a 10–15% natural hybridization rate. Based on this, some SNP loci in upland cotton could contain five genotypes (AAAA, AAAB, AABB, ABBB and BBBB). When these genotyping signals gather >3 clusters, automatic SNP calling might make mistakes, therefore we confirmed these loci genotypes with the manual adjustment.

We grouped the SNP cluster graphs of 352 samples into three types. In the first type, the loci of most samples could be accurately recognized with default clustering by GenomeStudio software. Markers of this type primarily produced four distinct clustering patterns. Those with the first and second patterns (Fig. 2a, b) all fell in a single cluster, representing a monomorphic locus. Those with the third and fourth patterns (Fig. 2c, d) were markers that showed two (AA, BB) and three (AA, AB, AB) clearly definable clusters, respectively, and were homozygous genotype clusters located near 0 and 1. The second type of SNP cluster graph consisted of markers that had partial or whole samples with uncalled genotypes. The partial uncalled markers may be caused by structural variance (SV) or insertion-deletion (In-Del), and were noted as “NG” rather than “–” (Fig. 2e, f). In addition, data for 165 SNPs was missing from all samples, because these SNPs showed complex cluster graphs that could not be accurately clustered even with manual adjustment or the NormR >0.2 in these SNPs (Fig. 2g, h). In the third type, most loci needed to be adjusted for accurate genotyping (Fig. 2i-2l). Some monomorphic loci that were relatively close together by GenomeStudio genotyping were adjusted to produce two cluster groups (AA and AB), of which the only possible genotypes were AAAA and AAAB in allotetraploid cotton (Fig. 2i ). By adjusting cluster AB to AA, the heterozygous rate significantly reduced for some loci with high heterozygous rates, of which the possible genotypes were AABB, ABBB and BBBB (Fig. 2j). A small number of loci showed 4–5 distinct clusters (Fig. 2k, l): their genotypes might be AAAA, AAAB, AABB, or BBBB, in which two closer groups (such as AAAA and AAAB or AABB and BBBB) would be merged into one. Through the adjustment, a more accurate clustering file was produced to improve the genotyping efficiency of the 352 cotton samples.

The genotype data revealed that the 352 tested accessions possessed an average call rate of 99.23%. Of the 77,774 SNPs in the array, 77,252 (99.33%) had high call rates >95% in these tested accessions. Of them, 59,502 SNPs (76.51%) showed polymorphic loci, with MAFs > 0.05 for 57,071 loci (95.91%) and MAFs > 0.1 for 48,940 (82.25%), respectively. Compared to the Yellow River Valley and the introduced accessions, the Yangtze River Valley and Northwestern inland region accessions had more SNPs with MAFs > 0.1 (Table 2).
The number of polymorphic SNPs was very similar in the Yangtze River Valley (59,428) and the Yellow River Valley accessions (59,402), however, the number of SNPs with MAFs > 0.1 was higher in the Yangtze River Valley accessions (46,434) than in the Yellow River Valley accessions (40,540), indicating the higher genetic diversity in the accessions from the Yangtze River Valley than that in the Yellow River Valley. We also found that the number of rare alleles (those with a frequency of less than 5%) in the Yellow River Valley accessions was greater than that in the Yangtze River Valley accessions.

### Evaluation of the CottonSNP80K array

To evaluate the genotyping accuracy, reproducibility, and distinguishability of the CottonSNP80K array, 22 cotton materials were selected for genotyping analysis.

### Table 2 Summary of the SNP polymorphisms detected in the different cotton subpopulations

| Origin                         | Accession No. | Accessions with phenotype data | Polymorphic SNPs | SNPs MAF > 0.05 | SNPs MAF > 0.1 |
|--------------------------------|---------------|--------------------------------|------------------|-----------------|----------------|
| The Yellow River Valley        | 182           | 166                            | 59,402           | 49,975          | 40,540         |
| The Yangtze River Valley      | 79            | 75                             | 59,428           | 55,337          | 46,434         |
| Northwestern inland region     | 22            | 22                             | 57,294           | 51,875          | 46,656         |
| Northern specifically early maturation region | 16 | 16                             | 56,085           | 53,888          | 45,126         |
| The introduced landraces       | 13            | 9                              | 52,113           | 49,884          | 40,745         |
| Outgroup                       | 20            | 0                              | 54,310           | 51,404          | 43,336         |
| Total                          | 332           | 288                            | 59,502           | 57,071          | 48,940         |

*MAF, minor allele frequency; SNP, single nucleotide polymorphism

Accessions with phenotype data for GWAS analysis between SNP loci and salt-tolerance related traits

Outgroup was involved in 5 wild cotton, 13 semi-wild cotton and 2 sea-island cotton materials
(Additional file 2: Table S1). First of all, we analyzed the SNPs of TM-1 and Hai7124 by comparing their array detection with the re-sequencing results from genomic data of 62 × TM-1 and 39 × Hai7124 [26]. The SNPs that could be detected by both methods were used for comparison, and the consistency of the two SNP calling results was 95.40% for TM-1 and 97.19% for Hai7124.

To determine the reproducibility of this array, G. hirsutum acc. W0 DNA samples were loaded three times on different chips and exhibited 100% identical genotype calls. Two biological replications of TM-1 plants from different seed sources also exhibited 99.99% consistency. Further, the consistencies of two biological replications of Zhongmiansuo12, the immature fiber mutant and Xuzhou-142 were 94.96%, 90.06% and 93.00%, respectively. In addition, interspecific polymorphism between G. hirsutum and G. barbadense was also detected; with the polymorphic rate of 32.70% between TM-1 and Hai7124, and 32.85% between TM-1 and Junhai1 (Table 3). Taken together, these results show that the CottonSNP80K array has a very good genotyping efficiency and accuracy.

To detect the distinguishability of this array, the polymorphic SNP loci of several mutants and their corresponding donors with similar genetic backgrounds were mined (Table 3). The near-isogenic lines Xinxiangxiaoji linted-fuzzless and Xinxiangxiaoji lintless-fuzzless yielded a 16.11% polymorphic rate. The polymorphic rates were 19.04% between Xuzhou-142 and the Xuzhou-142 lintless-fuzzless mutant, and 25.01% between 7235 and the 7235 mutant. We also detected relatively high polymorphic rates between TM-1 and four fiber development mutants (SL1–7-1, MD-17, N1, and n2), which ranged from 22.73% to 30.62%. In addition, a 21.00% polymorphic rate was identified between TM-1 and the immature fiber (im) mutant, far higher than the 1.28% polymorphic rate from SSR markers reported previously [29]. In general, the CottonSNP80K array showed high distinguishability when genotyping upland cotton accessions with similar genetic backgrounds.

To confirm the accuracy of the heterozygous loci, we compared genotype data from three parent/F1 combinations: (V1 × TM-1), (V3 × TM-1), and (V8 × TM-1). The results showed that the heterozygous rate increased by 14.07–19.91% in F1, compared with the corresponding parents. In the parents, 99.02%, 98.55% and 99.11% of the polymorphic SNP loci showed perfect heterozygous loci in the corresponding F1 (Fig. 3), indicating the potential application of the array in the identification of different varieties and hybrids.

**Table 3** Polymorphism rate and the similarity analysis using CottonSNP80K array

| Materials | Polymorphic rate (%) | Materials | Similarity (%) |
|-----------|----------------------|-----------|----------------|
| TM-1 vs Hai7124 | 32.70 | Three W0 DNA samples | 100.00 |
| TM-1 vs Junhai1 | 32.85 | Two TM-1 biological replicates | 99.99 |
| 7235 vs 7235 mutant | 25.01 | Two Xuzhou 142 biological replicates | 93.00 |
| Xinxiangxiaoji linted-fuzzless vs Xinxiangxiaoji lintless-fuzzless | 16.11 | Two Zhongmiansuo12 biological replicates | 94.96 |
| Xuzhou 142 vs Xuzhou 142 lintless-fuzzless mutant | 19.04 | Two im mutant biological replicates | 90.06 |
| TM-1 vs SL1–7-1 | 26.82 | | |
| TM-1 vs MD-17 | 30.62 | | |
| TM-1 vs N1 | 22.73 | | |
| TM-1 vs n2 | 25.71 | | |
| TM-1 vs im mutant | 21.00 | | |

**Phylogenetic tree and linkage disequilibrium analysis of upland cotton core collections**

Phylogenetic analysis and principal component analysis (PCA) were used to compare the genetic diversity between wild cotton, semi-wild cotton and upland cotton accessions. A neighbor-joining tree was constructed using 57,071 polymorphic SNP markers with MAFs > 0.05 (Fig. 4). As a result, the 332 samples were clustered into two groups. One contained 20 accesses of wild, semi-wild and sea-island cotton clustered into one branch as outgroup. The other contained 312 G. hirsutum accessions, including 299 from modern improved Chinese cultivars/accessions and 13 from the introduced landraces. We further classified the group into ten clusters, clusterI to clusterX. We found that the classification of 299 upland cotton accessions from four different ecological areas (the Yellow River Valley, the Yangtze River Valley, the Northwestern inland region and the Northern specifically early maturation region), was irrelevant to their geographical distribution, but highly related to the introduced landraces. Several landraces, from the United States and other countries, were assigned to different groups, respectively. For example: Uganda3, Stoneville4, DPL16, King, Stoneville2B, DPL15, and Foster6 were grouped into clustersl-V1 andX, respectively, indicating that these landraces have high genetic diversity and contributed to the development of different breeding populations in China during the improvement of modern varieties. Further, PCA analysis also agreed well with the clustering results in the phylogenetic tree (Fig. 5a).

Linkage disequilibrium (LD) analysis was performed for cotton accessions of different origins using PLINK software. Pairwise LD was estimated using squared allele frequency correlations ($R^2$). The results showed that the distance of LD decay in the outgroup was shorter than
Fig. 3 Verification of heterozygous loci in three F₁ combinations. a (V₁ × TM₁) F₁; b (V₃ × TM₁) F₁; c (V₈ × TM₁) F₁. The red labels on the chromosomes displayed the expected heterozygous loci which showed different homozygous alleles in two parents. Otherwise labeled in blue.

Fig. 4 Phylogenetic analysis of 332 cotton accessions based on the CottonSNP80K genotyping array. A neighbor-joining tree was constructed using 57,071 polymorphic SNP markers.
in other groups, implying that cultivated upland cotton, especially that from the northern group, may have been under higher selective pressure during evolution. Further, the accessions from the Yangtze River Valley, the Yellow River Valley and the Northwestern inland groups showed the same LD patterns, indicating the narrow genetic diversity of modern improved cultivars. On average, the distance of LD decay in the 332 cotton accessions was ~700 K (Fig. 5b).

**Application in genome-wide association studies for salt stress traits**

Based on previous investigations of 10 salt stress traits that provided phenotype data for 288 *G. hirsutum* accessions [30] (Additional file 3: Table S2), GWAS analysis was performed using a total of 54,588 SNPs (MAFs > 0.05) and stress traits. Eight significant SNPs for three salt stress traits were detected at the $P < 1 \times 10^{-5}$ level (Fig. 6). Among those, two loci located on D5 (TM57102 and TM57104), were significantly associated with relative chlorophyll content (RCC); one locus located on A2 (TM5633) and four loci located on D9 (TM70162, TM70169, TM70170, TM70171) were significantly associated with relative MDA content (RMDA); and one locus located on A12 (TM43002) was significantly associated with relative germination rate (RGR) (Table 4). Considering the LD decay distance of ~700 K identified in this study, we further analyzed the sequence information in 500 Kb regions flanking each peak SNP, and tagged candidate genes in the four significant SNP regions associated with salt stress traits. One LD block was associated with RCC on chromosome D05 (8.41–9.41 Mb) and RGR on A12 (83.73–84.73 Mb), and two LD blocks were associated with RMDA on chromosomes A02 (79.77–80.77 Mb) and D09 (3.10–4.31 Mb) (Fig. 6). A total of 308 genes were annotated in the four different regions (Additional file 4: Table S3), and 36 and 21 genes were annotated as response to stimulus and stress, respectively (Additional file 5: Table S4).

**Discussion**

**Development of CottonSNP80K**

High-density SNP arrays have been developed for a number of economically important crops, such as rice...
maize [31, 32], soybean [33], wheat [34], and cotton [16]. These chips have been successfully used for functional genomics studies and molecular breeding. To date, only one array (CottonSNP63K) has been reported in cotton [16]. In this study, we developed a new upland cotton genotyping SNP array (CottonSNP80K), an improved version of CottonSNP63K for upland cotton intraspecific genotyping detection. Of the 82,259 SNPs selected, 77,774 SNPs (94.55%) were successfully synthesized on this CottonSNP80K array. Compared to the CottonSNP63K array [16], the CottonSNP80K array shows several significant highlights and improvements. Firstly, compared to SNP loci in CottonSNP63K array, which were collected from 13 different discovery sets of G. hirsutum germplasm and five other species, the SNP

\[ P < 1 \times 10^{-5} \] were marked in red. The pair-wise LD between the SNP markers is indicated as D' values, where dark red indicated a value of 1 and light yellow indicated 0. a SNPs associated with relative chlorophyll content (RCC) at the peak region (8.41–9.41 Mb) on chromosome D05. b SNPs associated with relative MDA content (RMDA) at the peak region (79.77–80.77 Mb) on chromosome A02. c SNPs associated with relative MDA content (RMDA) at the peak region (3.10–4.31 Mb) on chromosome D09. d SNPs associated with relative germination rate (RGR) at the peak region (83.73–84.73 Mb) on chromosome A12.

**Fig. 6** GWAS analysis for salt-tolerance related traits in cotton. Local Manhattan plot (top) and LD heatmap (bottom) surrounding the peak of candidate loci. The significant SNPs \( P < 1 \times 10^{-5} \) were marked in red. The pair-wise LD between the SNP markers is indicated as D' values, where dark red indicated a value of 1 and light yellow indicated 0. a SNPs associated with relative chlorophyll content (RCC) at the peak region (8.41–9.41 Mb) on chromosome D05. b SNPs associated with relative MDA content (RMDA) at the peak region (79.77–80.77 Mb) on chromosome A02. c SNPs associated with relative MDA content (RMDA) at the peak region (3.10–4.31 Mb) on chromosome D09. d SNPs associated with relative germination rate (RGR) at the peak region (83.73–84.73 Mb) on chromosome A12.

| Traits \(^{a}\) | SNPs | Chr. | Position (bp) | Allele | MAF | \(-\log_{10}(P)\) | Allele's effect |
|-----------------|------|------|---------------|--------|-----|----------------|----------------|
| RCC             | TM57102 | D5   | 8,908,622     | A/G    | 0.36(G) | 5.23          | 0.06           |
|                 | TM57104 | D5   | 8,918,010     | A/C    | 0.14(C) | 5.23          | 0.06           |
| RMDA            | TM5633  | A2   | 80,274,022    | A/G    | 0.18(A) | 5.00          | −0.28          |
|                 | TM70162 | D9   | 3,595,148     | A/G    | 0.47(A) | 6.53          | −0.13          |
|                 | TM70169 | D9   | 3,793,378     | A/C    | 0.48(A) | 6.53          | −0.13          |
|                 | TM70170 | D9   | 3,801,945     | A/G    | 0.47(A) | 6.53          | −0.13          |
|                 | TM70171 | D9   | 3,812,331     | A/T    | 0.47(T) | 6.53          | −0.13          |
| RGR             | TM43002 | A12  | 84,233,507    | A/G    | 0.17(G) | 5.34          | 0.13           |

| Traits \(^{a}\) | SNPs | Chr. | Position (bp) | Allele | MAF | \(-\log_{10}(P)\) | Allele's effect |
|-----------------|------|------|---------------|--------|-----|----------------|----------------|
| RCC             | TM57102 | D5   | 8,908,622     | A/G    | 0.36(G) | 5.23          | 0.06           |
|                 | TM57104 | D5   | 8,918,010     | A/C    | 0.14(C) | 5.23          | 0.06           |
| RMDA            | TM5633  | A2   | 80,274,022    | A/G    | 0.18(A) | 5.00          | −0.28          |
|                 | TM70162 | D9   | 3,595,148     | A/G    | 0.47(A) | 6.53          | −0.13          |
|                 | TM70169 | D9   | 3,793,378     | A/C    | 0.48(A) | 6.53          | −0.13          |
|                 | TM70170 | D9   | 3,801,945     | A/G    | 0.47(A) | 6.53          | −0.13          |
|                 | TM70171 | D9   | 3,812,331     | A/T    | 0.47(T) | 6.53          | −0.13          |
| RGR             | TM43002 | A12  | 84,233,507    | A/G    | 0.17(G) | 5.34          | 0.13           |

**Table 4** Information on eight SNPs associated with salt-tolerance related traits based on GWAS analysis

\(^{a}\) RCC relative chlorophyll content, RMDA relative MDA content, RGR relative germination rate
accessions, indicating that more rare alleles exist in the Yangtze River accessions than in the Yellow River Valley. The number of SNPs with MAF > 0.1 was higher in the Northwestern inland and Northern groups. In addition, the Yangtze River Valley showed a higher degree of polymorphism than areas. The Yangtze River Valley and the Yellow River Valley account for more than 90% of the world's cotton production, however, modern upland cotton cultivars have narrow genetic diversity. The CottonSNP80K array is of high quality with high levels of consistency (100%) and different levels of variability were detected with biological replication types. Considerable genetic variation existed in cotton lines of different origins, which might be related to the cross-pollination nature of upland cotton with a 10–15% natural hybridization rate. Similarly, this variation was also found in other array projects [16, 35]. Thus, the CottonSNP80K array provides an efficient tool to characterize the inconsistencies between upland cotton accessions despite their similar genetic backgrounds. In addition, the CottonSNP80K array is of high quality with high levels of convenience and cost-effectiveness, and could be widely used in diverse types of research.

Applications of CottonSNP80K
Cotton (Gossypium spp.) is the world's most important fiber crop plant. While most of the >50 Gossypium species are diploid (n = 13), five are allopolyploids (n = 26), originating from an interspecific hybridization event between A- and D-genome diploid species [36]. There are three wild tetraploid cotton species (G. tomentosum, G. mustelinum and G. darwinii) and two cultivated species, upland cotton (G. hirsutum) and sea-island cotton (G. barbadense). In addition, upland cotton consists of seven semi-wild races, G. hirsutum race punctatum, morrilli, yucatanense, richmondii, marie-galante, latifolium and palmeri, and the domesticated upland cotton cultivars, which constitute about 90% of the world cotton production. In this study, using the CottonSNP80K array, 5, 13 and 312 cotton accessions from wild species, G. hirsutum races and cultivated upland cotton accessions, respectively, as well as 2 sea-island cotton accessions, were selected for phylogenetic analysis. We found that 20 accessions from wild, semi-wild and sea-island cotton were clustered together, with closer genetic relationships between 2 sea-island cotton accessions and wild species, and more similarity between semi-wild species and cultivated upland cotton. This might be because these SNP loci originated from intraspecific upland cotton variation. We also found that all 312 G. hirsutum accessions were sub-clustered ten groups, but these groupings were not related to their geographical distribution. A similar
analysis was reported in a previous study that used 81,675 SNPs from the SLAF-seq of 355 cotton accessions, and population structure analysis showed that the tested accessions could be separated into nine subpopulations with no obvious geographic relationship. In the present study, we grouped 312 G. hirsutum accessions, including 299 from modern improved Chinese cultivars/accessions and 13 from the introduced landraces, into ten clusters, cluster1 to clusterX. We found that the different introduced landraces were clustered in different subgroups, such as King (in the 1920s), Foster6 (in 1933), Stoneville2B (in 1947), DPL15 (in 1950) and DPL16 (in 1970), which were successively introduced into China from the USA and were grouped into clusters IV, X, V, VI and III, respectively. In addition, Stoneville 4, which was introduced from the USA in 1934 was grouped into cluster II. Uganda3, which was introduced from Uganda in 1959, was grouped into cluster V. Junmian1 was developed from the filial generation of multi-parents involved in C1470, C3521, and 147ϕ, which was introduced from the former Soviet Union in 1960s, and was grouped to cluster IX. These results suggest that the introduced upland cotton landraces have a high genetic diversity, which provides rich genetic resources for breeding modern improved cotton varieties in China.

Soil salinization, one of the main factors leading to soil desertification and land degradation, has become a serious threat to agricultural production and ecological environments throughout the world. More than 800 million hectares (~6%) of world’s total land area are salt affected. Compared with several other crops such as rice, maize and soybean, cotton is a pioneer crop in saline-alkali land. Based on SSR markers, some of the QTLs related to salt tolerance traits have been reported by family-based QTL mapping and association mapping approaches. To date, no studies have reported the identification of genes/QTLs associated with salinity tolerance in cotton using high-density SNP arrays. In previous studies, we carried out the large-scale identification of ten salt tolerance related traits in 304 upland cotton cultivars/accessions. Here, we integrated 288 cotton accessions with genome-wide SNP genotyping data, and a total of 54,588 SNPs (MAF > 0.05) were used for GWAS analysis of salt stress related traits. We detected eight significant SNPs for three salt stress traits at the \( P < 1 \times 10^{-5} \) level (Table 4, Fig. 6). Further, in these SNPs peak region, 36 and 21 genes were annotated as response to stimulus and response to stress, respectively. Of these candidate stress response genes, mitogen-activated protein kinase kinase 5 (MKK5), which acts upstream of MPK3/MPK6, plays key roles in mediating many different stress signals and in plant development. Overexpression of MKK5 in wild-type plants enhanced the tolerance to salt treatments, while mkk5 mutants exhibited hypersensitivity to salt stress during germination on salt-containing media in Arabidopsis. Another candidate gene, lesion simulating disease 1 (LSD1), together with phytoalexin deficient 4 (PAD4) and enhanced disease susceptibility 1 (EDS1), comprise a molecular hub that integrates plant responses to several stresses such as hypoxia, drought, cold and oxidative stress. A reactive oxygen species (ROS) scavenging related gene, peroxidase (POD), was also found amongst the candidate genes. ROS, typically in the forms of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \), can be rapidly generated in plants when exposed to adverse environments, such as high salinity, drought, heat or cold. An excess of ROS leads to oxidative damage of cellular components, such as proteins, lipids, carbohydrates and nucleic acids. High ROS level also disturb protein synthesis and the cellular membrane, resulting in cellular and tissue damage. Therefore, ROS scavenging capability is closely related to plant stress tolerance. Transcription factors (TFs) are considered as upstream regulatory proteins that play a major role in cellular metabolism and abiotic stress responses. One TF, named homeobox 7 (HB7), which encodes a putative transcription factor that contains a homeodomain closely linked to a leucine zipper motif, was found in an associated region with relative germination rate (RGR) in this study. AthB7 has essential functions in the primary response to drought, as mediators of a negative feedback effect on ABA signaling in the plant response to water deficit. Independent expression of AthB7 resulted in improved stress tolerance in Arabidopsis. In summary, with the identification of increasing numbers of genes/QTL related to important traits, high-throughput genotyping platforms will provide an effective pathway for high-resolution dissection of complex traits and molecular breeding by design in cotton.

With reduced computational requirements for downstream data processing, high call frequency, low error rates and ease of use, high-density SNP arrays are an attractive genotyping tool, which are widely applied in diversity studies and high-resolution dissection of complex traits, variety verification, trait introgression and genome-wide association studies. Compared with genome-wide sequencing technology, the SNP loci in the array is known and addressable, the data generation and analysis are more convenient and cost-effective. In the study, CottonSNP80K showed to work efficiently for variety verification and genome-wide association studies for salt stress traits, indicating great application potential in future cotton molecular breeding.

**Conclusion**

Based on reference sequence of the TM-1 genome and re-sequencing data from 100 different cultivars of G. hirsutum, high-density SNP arrays of CottonSNP80K provide rich genetic resources for breeding modern improved cotton varieties in China.
was developed. By genotyping 352 cotton cultivars/accessions, the CottonSNP80K showed excellent efficiency with an average call rate of 99.23%. Application tests using cotton accessions with parent/F1 combinations or with similar genetic backgrounds showed that this array had high genotyping accuracy, good repeatability, and wide applicability. Phylogenetic relationship and GWAS analysis of salt stress traits showed that the CottonSNP80K played important roles in variety verification, genetic relationship identification, and molecular breeding in cotton. Compared to the previously reported CottonSNP63K array, the CottonSNP80K array showed higher SNP density with genome-wide distribution, more accurate addressable loci, and higher upland cotton intraspecific polymorphism, which will be a powerful tool for genetics and molecular breeding by design in cotton.

**Methods**

**Plant materials**

A total of 352 cotton materials were collected for the evaluation and application of the CottonSNP80K array in this study (Additional file 2: Table S1). To evaluate the CottonSNP80K array, 22 cotton materials (named as E1-E22 in Additional file 2: Table S1), comprising the sequenced reference lines (G. hirsutum acc. TM-1 and G. barbadense cv. Hai 7124), duplicated DNA samples, parent/F1 combinations, several mutants and their corresponding donors with similar genetic background, G. hirsutum cv. Zhongmiansuo12 and G. barbadense cv. Junhail 1, were genotyped. To investigate the genetic diversity and phylogenetic relationship, a core collection of 332 cotton accessions were chosen (named as C1-C332 in Additional file 2: Table S1), including 5 wild and 13 semi-wild G. hirsutum race materials, 312 cultivated upland cotton accessions and two sea-island cotton accessions. For the GWAS analysis, 288 G. hirsutum accessions (selected from C21-C332 in Additional file 2: Table S1) with salt-stress phenotyping identified in our previous study [27] (Additional file 3: Table S2) were chosen for marker-trait associations of salt stress traits.

**Selection of SNPs and development of the cotton SNP array**

To develop the genome-wide CottonSNP80K chip, an Illumina Infinium array, as well as intraspecific SNPs data from sequencing of the allotetraploid cotton G. hirsutum acc. TM-1 [22] and re-sequencing of 100 different cultivars in G. hirsutum with 5x coverage on average [27] were used. In total, 1,372,195 putative intraspecific SNPs with MAF > 0.1 were detected and chosen for inclusion on the array. When designing the array, subsequent filtering steps included the following: (1) genotype accuracy was required to be >99.12%; (2) SNPs in repeat regions were filtered; (3) no other SNPs or InDels were permitted in the 50 bp flanking the SNP site; (4) heterozygosity rates were required to be <15%; (5) SNP cluster analysis was carried out. After these filters were applied, 175,192 SNPs remained and were submitted through the Illumina Assay Design Tool to determine array design scores for each marker. SNPs in gene regions with Illumina design scores >0.7, and SNPs in intergenic regions with Illumina design scores >0.9 remained. Further, the intermarker distance flanking the SNPs was >2100 bp. The remaining 82,259 SNP markers were used for the manufacture of the CottonSNP80K array by Illumina (Additional file 6: Table S5). The scheme of CottonSNP80K development is shown in Additional file 1: Figure S1.

**Array hybridization and SNP genotyping**

Genomic DNA from young leaf tissue of the 352 cotton materials involved in this study was isolated as described by Paterson et al. (1993) [52]. According to the Illumina protocols, qualified DNA was hybridized to the CottonSNP80K array. The Illumina iScan array scanner was used to scan arrays, and GenomeStudio Genotyping software (V2011.1, Illumina, Inc.) was used to cluster SNP alleles and for genotyping. Among the 82,259 SNP markers, the CottonSNP80K array successfully synthesized 77,774 SNP markers (94.55%). That is, 4485 SNPs failed to meet bead representation thresholds and were removed. At first, the default clustering file was provided based on bi-allelic SNPs. Next, all 77,774 SNPs were tested and manually adjusted as described by Hulse-Kemp et al., (2015b) [16]. Ultimately, an adjusted clustering file was produced and was used to call SNP genotypes for the allotetraploid cottons in the study.

**Phylogenetic trees and linkage disequilibrium analysis**

PLINK V1.90 software [53] was employed to conduct similarity analysis of 332 cotton accessions for cluster analysis. Based on the distance matrix data (1-IBS, identity-by-state), phylogenetic trees were constructed using neighbor software, and visually edited by figtree software. Principal component analysis (PCA) was performed using an iBS matrix data. The correlation coefficient (r²) of alleles was calculated to measure LD in each upland cotton group level using PLINK V1.90, and LD blocks containing SNP loci associated with target traits were generated using the R software package “LDheatmap”.

**GWAS analysis and identification of candidate genes**

A total of 54,588 SNPs (MAF > 0.05) were used for the GWAS analysis of 288 upland cotton accessions. We carried out GWAS analysis with TASSEL 5.0, which uses a Mixed Linear Model (MLM) with Q + K model for association tests [54]. Population structure (Q) was performed using admixture 1.3 [55] with k = 3, and kinship matrix (K) was performed with Tassel 5.0. Significant levels of association were estimated considering an
adjusted $P$ value of $1/n (1.8 \times 10^{-5})$ after the Bonferroni correction, which $n$ represented the number of SNP markers. To get more reliable results, the SNPs with $P$ values lower than $1 \times 10^{-5}$ were selected as finally significant trait-associated SNPs. Candidate genes were identified within 500 Kb upstream or downstream of peak SNPs (the most significant SNPs). Manhattan plots were performed using the R software package “qgman”. GO analysis was implemented using AgriGO, and genes in “response to stimulate” or “response to stress” terms were selected as candidate genes.

Additional files

- **Additional file 1: Figure S1.** The flow chart of developing the CottonSNP80K array. (TIFF 229 kb)
- **Additional file 2: Table S1.** Basic information and their phylogenetic classification on cotton materials in this study. (XLSX 25 kb)
- **Additional file 3: Table S2.** Phenotype data of 10 salt-stress traits in the 288 tested cotton accessions. (XLSX 53 kb)
- **Additional file 4: Table S3.** Gene information of SNPs associated with salt-tolerance related traits within 1 Mb regions. (XLSX 31 kb)
- **Additional file 5: Table S4.** Information on candidate genes response to stimulus and stress. (XLSX 12 kb)
- **Additional file 6: Table S5.** Information on SNP loci in the CottonSNP80K array. (XLSX 9317 kb)

Abbreviations

EDS1: enhanced disease susceptibility 1; GBS: genotyping-by-sequencing; GWAS: genome-wide association studies; HB7: homebox 7; In-Del: insertion-deletion; LD: Linkage disequilibrium; LSD1: lesion simulating disease 1; MAFs: minor allele frequencies; MAGIC: multi-parent advanced generation intercross; MAS: marker-assisted selection; M KK: mitogen-activated protein kinase kinase; PAD4: phytoalexin deficient 4; PCA: principal component analysis; POD: peroxidase; QTL: quantitative trait loci; RCR: relative chlorophyll content; RGR: relative germination rate; RILs: recombinant inbred lines; RMDOA: relative malondialdehyde content; ROS: reactive oxygen species; SLAF-seq: specific locus amplified fragment sequencing; SNP: single nucleotide polymorphism; SSR: simple sequence repeat; SV: structural variation; TFs: transcription factors; UTRs: untranslated regions

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Availability of data and materials

All supporting data were included as additional files.

Authors’ contributions

Experiments were designed by WZG with suggestion by TZZ. Experiments were performed by CPC, GZZ and WZG. CPC, WZG and GZZ drafted the manuscript, WZG revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

A total of 352 cotton materials, including 332 G. hirsutum cotton accessions, 5 wild cotton and 13 semi-wild G. barbadense race materials, were collected in this study. All necessary permits for collecting 332 G. hirsutum and two G. barbadense cotton accessions were obtained from Nanjing Agricultural University, China. All necessary permits for 5 wild cotton and 13 semi-wild G. hirsutum race materials were obtained from the Institute of Cotton Research, Chinese Academy of Agricultural Science, China. These 352 materials were planted in the liangpu experimental station of Nanjing Agricultural University, Nanjing, Jiangsu Province, China, for reproduction and sampling. All necessary permits for the field evaluations of these accessions were obtained from Nanjing Agricultural University, China. All the field evaluations were not relevant to human subject or animal research. Therefore, they did not involve any endangered or protected species.

Consent for publication

Not applicable.

Competing interests

The authors declared that they had no competing interests.

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