ECO-TILLING revealed SNPs in GhSus genes that are associated with fiber- and seed-related traits in upland cotton

Yan-Da Zeng1, Jun-Ling Sun2, Su-Hong Bu1, Kang-Sheng Deng1, Tao Tao1, Yuan-Ming Zhang3, Tian-Zhen Zhang1, Xiong-Ming Du2 & Bao-Liang Zhou1

Cotton is the most important textile crop in the world due to its cellulose-enriched fibers. Sucrose synthase genes (Sus) play pivotal roles in cotton fiber and seed development. To mine and pyramid more favorable alleles for cotton molecular breeding, single nucleotide polymorphisms (SNPs) of GhSus family genes were investigated across 277 upland cotton accessions by ECO-TILLING. As a result, a total of 24 SNPs in the amplified regions of eight GhSus genes were identified. These SNPs were significantly associated with at least one fiber- or seed-related trait measured in Nanjing, Anyang and Kuche in 2007–2009. Four main-effect quantitative trait nucleotides (QTNs) and five epistatic QTNs, with 0.76–3.56% of phenotypic variances explained by each QTN (PVE), were found to be associated with yield-related traits; six epistatic QTNs, with the 0.43–3.48% PVE, were found to be associated with fiber quality-related traits; and one main-effect QTN and one epistatic QTN, with the PVE of 1.96% and 2.53%, were found to be associated with seed oil content and protein content, respectively. Therefore, this study provides new information for molecular breeding in cotton.

Cotton fiber is not only the most important textile material worldwide, but also the ideal experimental system for studying the mechanism of cell development due to its single-celled profile1. Cotton fibers originate from the epidermal cells of ovules, and its growth and development is a highly regulated process with four distinct, but overlapping, stages: initiation, elongation, secondary wall synthesis, and maturation2. During the development of cotton fiber, the importation and metabolism of sucrose is considered to be a major factor in determining the sink strength of tissues3. In higher plants, sucrose is the major product of photosynthesis, the main form of translocated carbon, and the main substrate of sink metabolism4. Sucrose metabolism and accumulation in plant cells involves two key enzymes: sucrose synthase (Sus, EC 2.4.1.13) and invertase (INV, EC 3.2.1.26). Sucrose synthase catalyzes a reversible reaction, but preferentially cleaves sucrose to bond uridine diphosphate (UDP) into UDP-glucose and fructose5. Sucrose synthase plays important roles in metabolic processes, including starch storage6–10, cellulose synthesis, sugar import11,12, environmental stress responses13,14, and nitrogen fixation, as well as arbuscule maturation and maintenance in mycorrhizal roots of legumes15,16.

Sucrose synthase (Sus) genes have been proposed to be involved in controlling cotton fiber development17. Sus activity is responsible for cellulose synthesis by supplying the UDP-glucose substrate, which is essential for cell wall thickening and cotton fiber cell development18–20. Suppressing Sus activity in the ovule epidermis led to a fiberless phenotype and to fewer fiber initials or shrunken or collapsed fibers in those ovules in cotton21. Over-expressing a Sus gene from potato in cotton gave rise to enhanced leaf development, improved early seed development, and promoted fiber elongation22. Thus, a new strategy was proposed to increase cotton fiber yield by improving seed development. As demonstrated in many plants, Sus isoforms are encoded by a multiple gene family. For example, maize and pea each contain three distinct Sus family members6,23. Six different Sus genes were identified in Arabidopsis thaliana, Oryza sativa, Hevea brasiliensis, and Citrus unshiu13,24–26. Populus has seven Sus

1State Key Laboratory of Crop Genetics & Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China. 2Institute of Cotton Research, Chinese Academy of Agricultural Sciences, Anyang 455000, China. 3College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China. Correspondence and requests for materials should be addressed to X.-M.D. (email: dxm630723@163.com) or B.-L.Z. (email: baoliangzhou@njau.edu.cn)
In two diploid cotton species (\textit{Gossypium arboreum} L. and \textit{G. raimondii} Ulbr.), eight \textit{Sus} genes for each species have been found\cite{29}. The tetraploid cotton, \textit{G. hirsutum} L., has the largest \textit{Sus} family to date, containing fifteen \textit{Sus} genes\cite{29}. Examination of their expression patterns indicated that \textit{Sus} gene family members have different expression in various tissues or organs in cotton species. Additionally, their functional analyses and isolations were confined to a limited number of cotton accessions, their diverse functions and sequence variations have not been clarified in natural populations, implying that some favorable alleles are yet to be identified for cotton.

EcoTILLING is a fast and cost-effective method of detecting rare SNPs, small insertions, and deletions (indels) in target genes in natural populations, and was adapted from Targeting Induced Local Lesions in Genomes (TILLING)\cite{30}. Compared to direct sequencing, EcoTILLING has the following advantages: high-throughput, accuracy, and cost-effectiveness. To date, EcoTILLING has been used for many purposes, including mapping\cite{31,32}, candidate gene discovery\cite{33,34}, evaluation of nucleotide diversity\cite{35}, and population genetics\cite{36,37}.

In this study, EcoTILLING technology was used to reveal sequence diversities and identify SNPs in \textit{GhSus} family genes in a natural population of upland cotton accessions. The SNPs were then used for association analysis of phenotype variations in plants from nine environments over three years and three locations, and for the identification of favorable \textit{GhSus} alleles that confer high yield and high fiber and seed quality traits.

### Results

**Nucleotide polymorphisms.** Fifteen genes in the \textit{GhSus} family were analyzed by EcoTILLING. As a result, eight out of 15 genes showed a total of 24 putative natural variation sites in the amplified regions across 277 upland cotton accessions, and no SNPs were detected in the other seven genes (Table 1). The minor allele frequency of polymorphic sites in the 277 accessions ranged from 0.018 to 0.755, with an average 0.214. Samples containing each of the polymorphic sites were randomly sequenced to determine the position and identity of these polymorphic sites in the targeted region of genes. Twenty four putative SNPs were confirmed by sequencing in the targeted region of the eight \textit{GhSus} family members. Of the 24 SNPs, 12 were in the coding regions, including seven synonymous variations and five non-synonymous variations. Of the five non-synonymous variations, two SNPs were predicted by the SIFT program to severely affect protein function (Table 1).

| Gene       | Nucleotide polymorphism\(^a\) | Ratio\(^b\) | Variation of amino acid\(^c\) | SIFT\(^d\) |
|------------|-------------------------------|-------------|-------------------------------|----------|
| \textit{GhSus1At} | A918G                          | 0.612       | No                            |          |
|            | G178SC                         | 0.342       | No                            |          |
|            | A2940T                          | 0.338       | T678=                         |          |
| \textit{GhSus1Dt} | T650C                          | 0.089       | No                            |          |
|            | G751A                          | 0.089       | Y153I                         | 0        |
| \textit{GhSus3At} | T881A                          | 0.072       | No                            |          |
|            | T1320C                         | 0.072       | No                            |          |
|            | G1327A                         | 0.072       | No                            |          |
|            | T2294C                         | 0.018       | L504S                         | 0        |
| \textit{GhSus4Dt} | A2472C                         | 0.018       | No                            |          |
|            | A1886T                         | 0.025       | No                            |          |
|            | T2167C                         | 0.025       | E388=                         |          |
| \textit{GhSus5Dt} | G491T                          | 0.029       | L108F                         | 1        |
|            | C2648G                          | 0.025       | P660A                         | 1        |
| \textit{GhSus6At} | G3143T                         | 0.159       | No                            |          |
|            | C4655T                         | 0.209       | No                            |          |
|            | G4697A                         | 0.209       | W755=                         |          |
|            | G5005A                         | 0.209       | No                            |          |
| \textit{GhSus7Dt} | T192C                          | 0.755       | T64=                          |          |
|            | C404T                          | 0.755       | D109=                         |          |
|            | T407C                          | 0.755       | E110=                         |          |
| \textit{GhSus8Dt} | C577T                          | 0.09        | No                            |          |
|            | T1334C                         | 0.069       | G298=                         |          |

\(^a\)The first letter indicates bp at this site in TM-1 sequence, followed by the position of the SNP in the TM-1 sequence, and then the nucleotide that is the rare variant at this site.  
\(^b\)Ratio was calculated by dividing the number of similar nucleotide changes identified on the EcoTILLING gel by 277 upland cotton accessions.  
\(^c\)The first letter indicates the common amino acid at this site, followed by the position of the SNP within the predicted protein sequence and the amino acid change induced by the variant nucleotide polymorphism; “=” means no change in amino acid encoded by that codon (synonymous variation); “No” means that SNP was in intron.  
\(^d\)A non-synonymous SNP is predicted to be damaging to the encoded protein if the SIFT score is <0.05 (bold).
Haplotype diversity analysis. The haplotype distribution for each of the amplicons is shown in Table 2. The GhSus1At amplicon had the largest number of haplotypes in the GhSus gene family, with eight haplotypes detected, while the amplicons GhSus4Dt and GhSus7Dt each had only two haplotypes. The levels of haplotype diversity (HD) varied markedly in different amplicons of the GhSus family. The highest level of HD was in amplicon GhSus1At, with 0.574, while the lowest was in amplicon GhSus4Dt, with only 0.049. One major haplotype was detected in each GhSus gene sequence except for amplicon GhSus1At, for which no haplotype exhibited a frequency higher than 0.5 (Supplementary Table S1).

Population structure. Analysis of the population structure composed of 277 upland cotton varieties showed that the log probability of data [ln P(D)] value corresponding to each hypothetical number of subpopulations (k) continued to increase with k value and did not reach a peak. An ad hoc statistic Δk value was then calculated according to the method described by Evanno et al.39. The values showed a much higher likelihood at k = 2 than at k = 3–10, suggesting that the whole panel could be divided into two major subpopulations, SP1 and SP2, respectively. The SP1 group contained 89 accessions; 16 from America, three from Europe, seven from North regions of China, 12 from Northwest regions of China, 32 from the Yellow River region of China, 15 from the Yangtze River regions of China, and four from other Asian regions. The SP2 group consisted of 188 accessions; four from Australia, five from Africa, 34 from America, two from Europe, six from North regions of China, 3 from Northwest regions of China (Supplementary Table S2), 101 from the Yellow River region of China, 33 from the Yangtze River regions of China. The corresponding Q matrix at k = 2 was then used for the subsequent association analysis.

Complete linkage disequilibrium of SNPs was detected as follows within five single GhSus genes: GhSus1Dt-T650C and GhSus1Dt-G751A in GhSus1Dt-G751A; GhSus4Dt-A1886T and GhSus4Dt-T2167C in GhSus4Dt; GhSus3At-T818A, GhSus3At-T1320C, and GhSus3At-G1327A in GhSus3At; GhSus3At-T2294C and GhSus3At-A2472C in GhSus3At; GhSus6At-C4655T, GhSus6At-G4697A, and GhSus6At-G5005A in GhSus6At; GhSus7Dt-T192C, GhSus7Dt-C404T, and GhSus7Dt-T407C in GhSus7Dt. All SNPs in complete linkage disequilibrium were combined into a single haplotype and those were used for further analysis (here, for easy description of the results, only one SNP name from each haplotype was used instead of haplotype). Although ten of those SNPs (for example, GhSus1At-A918G and GhSus1At-G1783C) result in synonymous variations or are located in intron regions, they may be in complete linkage disequilibrium with other non-synonymous mutations, which could have functional relevance to the organism. Therefore, these SNPs were further analyzed. Thus, a total of 15 SNPs were included in the association analysis, namely, three SNPs in GhSus1At (GhSus1At-A918G, GhSus1At-G1783C, and GhSus1At-A2940T), each two SNPs in GhSus1Dt (GhSus1Dt-G751A and GhSus1Dt-G7290C), in GhSus3At (GhSus3At-T818A and GhSus3At-T2294C), in GhSus4Dt (GhSus4Dt-G4697A and GhSus4Dt-G5005A), in GhSus5Dt (GhSus5Dt-G4191T and GhSus5Dt-C404T), in GhSus6At (GhSus6At-G3143T and GhSus6At-C4655T), and in GhSus8Dt (GhSus8Dt-C577T and GhSus8Dt-T1334C), and each one in GhSus4Dt (GhSus4Dt-A1886T) and in GhSus7Dt (GhSus7Dt-T192C).

Association between SNPs and fiber- or seed-related traits. In this study, eleven SNPs were found to be significantly associated with traits (four SNPs were main-effect QTNs and eleven pairs of SNPs were involved in epistatic QTNs) (Table 3). Of them, six QTNs were associated with fiber quality traits. Three epistatic QTNs (GhSus1At-A918G × GhSus5Dt-C2648G, GhSus1At-A918G × GhSus6At-C4655T, and GhSus1Dt-G751A × GhSus3At-T881A) were associated with fiber length (FL), which explained 3.48%, 1.09%, and 1.50% of the phenotypic variation (PVE), respectively. Two epistatic QTNs (GhSus1Dt-G751A × GhSus8Dt-T1334C and GhSus1Dt-G7290C × GhSus8Dt-C577T) were associated with fiber micronaire (FM), which explained 1.27% and 0.89% of PVE, respectively. One epistatic QTN (GhSus3At-T881A × GhSus7Dt-T192C) was associated with fiber strength (FS), which explained 0.43% of PVE. Nine QTNs were associated with yield traits. One main-effect QTN (GhSus1At-A2940T) was associated with lint percentage (LP), which explained 3.43% of PVE. Two main-effect QTNs (GhSus1At-G1783C and GhSus3At-T881A) and two epistatic QTNs (GhSus1At-A2940T × GhSus1Dt-G2709C and GhSus4Dt-C4655T × GhSus7Dt-T192C) were associated with boll weight (BW), which explained 3.56% and 1.76%, and 2.03% and 0.76% of PVE, respectively. One main-effect QTN (GhSus3At-T881A) and three epistatic QTNs (GhSus1At-A918G × GhSus1Dt-G2709C, GhSus1At-G1783C × GhSus1Dt-G2709C, and GhSus1Dt-G751A × GhSus1Dt-G2709C) was associated with seed index (SI), which explained 1.32% and 2.10%.

Table 2. Gene length, number of SNPs, number of haplotypes and haplotype diversity (HD) for the GhSus family genes.

| Gene   | Length (bp) | Number of SNP | Number of Haplotype | HD     |
|--------|-------------|---------------|---------------------|--------|
| GhSus1At | 3650        | 3             | 8                   | 0.574  |
| GhSus1Dt | 3648        | 3             | 4                   | 0.290  |
| GhSus3At | 3491        | 5             | 4                   | 0.143  |
| GhSus4Dt | 4790        | 2             | 2                   | 0.049  |
| GhSus5Dt | 3167        | 2             | 4                   | 0.064  |
| GhSus6At | 5176        | 4             | 4                   | 0.386  |
| GhSus7Dt | 3428        | 3             | 2                   | 0.372  |
| GhSus8Dt | 3762        | 2             | 4                   | 0.201  |
Table 3. Association analysis with fiber- or seed-related traits of SNPs in the GhSus family genes. * and ** at the 0.05 and 0.01 levels of significance on the genome, respectively. LP, lint percentage (LP); BW, boll weight; SI, seed index; FL, fiber length; FM, fiber micronaire; FU, fiber uniformity; FS, fiber strength; PC, protein content; OC, oil content. \( r^2 \) is the phenotypic variance explained by each QTN.

| Trait | QTN | LOD | \( r^2(\%) \) | F | \( P \) | Favorable allele |
|-------|-----|-----|----------------|---|-------|-----------------|
|       |     |     |                |   |       | Alleles         | Effect         |
| LP    | GhSus1At-A2940T | 16.59 | 3.43 | 15.16 | 1.01E-04** | GhSus1At-A2940T | 0.95%          |
|       | GhSus1At-G1783C | 14.60 | 3.56 | 12.71 | 3.71E-04** | GhSus1At-1783C | 0.18 g         |
| BW    | GhSus3At-T881A | 15.30 | 1.76 | 77.09 | 3.02E-18** | GhSus3At-881A | 0.23 g         |
|       | GhSus1At-A2940T × GhSus1Dt-G2709C | 10.64 | 2.03 | 21.42 | 6.03E-10** | GhSus1At-A2940T × GhSus1Dt-2709C | 0.14 g         |
|       | GhSus6At-C4655T × GhSus7Dt-T192C | 8.44 | 0.76 | 33.00 | 1.04E-08** | GhSus6At-4655T × GhSus7Dt-192C | 0.08 g         |
| SI    | GhSus3At-T881A | 10.82 | 1.32 | 65.96 | 7.25E-16** | GhSus3At-881A | 0.40 g         |
|       | GhSus1At-A918G × GhSus1Dt-G2709C | 10.98 | 2.10 | 10.83 | 1.01E-03** | GhSus1At-918G × GhSus1Dt-2709C | 0.26 g         |
|       | GhSus1At-G1783C × GhSus1Dt-G2709C | 17.73 | 2.27 | 28.49 | 5.91E-13** | GhSus1At-1783C × GhSus1Dt-2709C | 0.29 g         |
|       | GhSus1Dt-G751A × GhSus1Dt-G2709C | 6.58 | 0.89 | 40.02 | 2.98E-10** | GhSus1Dt-751A × GhSus1Dt-2709C | 0.25 g         |
| FL    | GhSus1At-A918G × GhSus5Dt-C2648G | 16.53 | 3.48 | 14.62 | 4.90E-07*** | GhSus1At-918G × GhSus5Dt-2648G | 0.42 cm         |
|       | GhSus1At-A918G × GhSus6At-C4655T | 7.98 | 1.09 | 31.51 | 2.21E-08** | GhSus1At-918A × GhSus6At-4655C | 0.23 cm         |
|       | GhSus1Dt-G751A × GhSus3At-T881A | 11.97 | 1.50 | 22.29 | 2.57E-10** | GhSus1Dt-751A × GhSus3At-881A | 0.39 cm         |
| FS    | GhSus3At-T881A × GhSus7Dt-T192C | 4.81 | 0.43 | 14.22 | 7.24E-07** | GhSus3At-881A × GhSus7Dt-192C | 0.19 cm/tex     |
| FM    | GhSus1Dt-G751A × GhSus8Dt-T1334C | 5.15 | 0.89 | 14.56 | 5.17E-07** | GhSus1Dt-751A × GhSus8Dt-1334C | 0.07           |
|       | GhSus1Dt-G2709C × GhSus8Dt-C577T | 5.11 | 1.27 | 6.72 | 1.23E-03** | GhSus1Dt-2709G × GhSus8Dt-577C | 0.08           |
| PC    | GhSus1At-A918G × GhSus1Dt-G2709C | 9.57 | 2.53 | 11.11 | 1.62E-05** | GhSus1At-918A × GhSus1Dt-2709G | 0.49%          |
| OC    | GhSus6At-C4655T | 3.87 | 1.96 | 15.21 | 4.04E-07** | GhSus6At-4655C | 0.54%          |

Favorable QTN alleles mined in upland cotton accessions. The phenotypic effects of each QTN allele were estimated by the method of Liu et al., and a total of 17 desirable alleles were identified. Phenotypic effects for each favorable allele are shown in Table 3. Among the favorable alleles, GhSus3At-881A had the most positive phenotypic effects for BW and SI, and increased BW and SI by 0.23 g and 0.40 g, respectively. One epistatic QTN, between GhSus1At-918G and GhSus5Dt-2648G, increased FL by 0.42 mm; another epistatic QTN, between GhSus1Dt-2709G and GhSus8Dt-577C, decreased FM by 0.08; GhSus1At-2940T increased LP by 0.95%; the epistatic QTN between GhSus3At-881A and GhSus7Dt-192C increased FS by 0.19 cm/tex; the epistatic QTN between GhSus1At-918A and GhSus1Dt-2709G increased PC by 0.49%; and GhSus6At-4655C increased OC by 0.54%.

Expression analysis of GhSus gene at different developmental stages. To better understand the potential functions of GhSus isoforms, their expression patterns were analyzed in fiber and ovule at different developmental stages using upland cotton TM-1 published RNA-seq data. Most of GhSus genes were expressed (fragments per kilobase of transcript per million reads sequence (FPKM) > 1) in both fiber and ovule except for GhSus4A and GhSus4D which were not expressed in either tissue. The expression levels of GhSus1A and GhSus1D were both high in fiber at 5 DPA (days post-anthesis), then declined notably at 20 DPA (Fig. S1). The transcripts of GhSus5D and GhSus8D showed relatively higher in fiber from 5 to 25 DPA, and were very weak in ovule (Fig. S1).

Discussion. To understand genetic variation and mine more favorable alleles, 277 upland cotton accessions from China and another 5 geographic areas were analyzed for allele diversity in fifteen GhSus family genes by EcoTILLING. The results revealed 24 SNPs among the family genes after analysis of 14,957,446 bp sequences. Of the 24 SNPs, half (12/24) were in coding regions, including seven synonymous variations and five nonsynonymous variations. Of the five nonsynonymous variations, two SNPs were predicted by the SIFT program to severely affect protein function. Although SIFT has a 20% false-positive error and some mutations predicted to be deleterious may be functionally neutral, these scores may be useful in prioritizing mutations for further study and for the analysis of possible contributions of the GhSus family gene members to stress tolerance.
Sus is a key enzyme in plant sucrose metabolism. In many plants, Sus isoforms are encoded by a small multi-gene family \(^{3,12}\). Fifteen Sus genes have been detected in \(G. \) hirsutum so far; previous reports showed that some of these genes were related to the initiation, elongation, and secondary cell wall deposition of the single-celled fibers \(^{31-34}\). However, little is known about the underlying genetics and molecular biology of the Sus genes and their physiological functions during fiber growth and development.

In the present study, eleven SNPs in the \(GhSus\) family gene members were significantly associated with at least one agronomic trait. Five main-effect QTNs and twelve epistatic QTNs containing these eleven SNPs were detected for eight traits in upland cotton. Although most of the QTNs were associated with SNPs that are expected to result in synonymous mutations or be located in intron sequences, these SNPs may be in complete linkage disequilibrium with other non-synonymous mutations, which could have functional relevance to the organism, or these SNPs could become the targets of other genes, leading to effects on agronomic traits. All of these are waiting for further experimental confirmation.

Using 2,878 QTLs mapped in a previous study \(^{40}\), we found that only five out of eleven SNPs in our study were located in the same regions as previous reports. For example, the locus \(GhSus1Dt-G751A\) associated with FL on chromosome (Chr.) 19 was located in the region of qFL-D5-1 identified by Hu et al. \(^{45}\). The loci \(GhSus1Dt-G751A\) and \(GhSus1Dt-G2709C\), both associated with FM on Chr. 19, were located in the region of qFM-C19-1 identified by Sun et al. \(^{46}\). The locus \(GhSus1Dt-G2709C\) associated with BC on Chr. 19 was located in the region of qBC-19-1 identified by Yu et al. \(^{47}\). The locus \(GhSus5Dt-C2648G\) associated with SC on chromosome 25 was located in the regions of qSC-C25-1 and qSC-C25-2 identified by Sun et al. \(^{46}\). The locus \(GhSus7Dt-T192C\) associated with BW on chromosome 16 was located in the region of qBW-C16-1 identified by Wu et al. \(^{47}\). The locus \(GhSus7Dt-T192C\) was also associated with FS, which was located in the regions of F2.3-qFS-c16-1 and F2.3-qFS-c16-2 identified by Yu et al. \(^{46}\). This comparison demonstrates that our association analysis further confirmed the previous results of QTL mapping suggesting that these QTLs are stable, repeatable, and reliable.

The quality and productivity of cotton fiber is mainly affected by two biological processes: fiber initiation and fiber elongation \(^{49}\). The fiber elongation process (~3–23 days post anthesis, DPA) determines the fiber final length and strength. Fiber strength also depends on formation of the secondary cell wall (~16–40 DPA), which contains >95% of the dry weight of cellulose in the mature cotton fiber. As \(GhSus\) genes are vital for fiber cell development, suppression experiments result in a disruption of fiber and seed development \(^{43}\). Due to the severity of the phenotypic effects, it was difficult to analyze the impact of different \(GhSus\) family genes on fiber quality from pleiotropic developmental effects on fiber growth. As a reverse genetic research method, EcoTILLING technology provided a new approach for \(GhSus\) family gene function analysis.

The expression patterns of \(GhSus\) family genes during fiber cell development were analyzed using RNA-seq data from \(G. \) hirsutum TM-1 \(^{45}\). All seven genes associated with the fiber-related traits (Table 3) were highly expressed in fiber cells during the fiber elongation stage or secondary cell wall formation stage. Five \(GhSus\) genes (\(GhSus1At\), \(GhSus1Dt\), \(GhSus5Dt\), \(GhSus6At\), and \(GhSus6At\)) associated with fiber length were found to be highly expressed during the fiber elongation process (Supplementary Fig. S1). Two genes (\(GhSus3At\) and \(GhSus7At\)) associated with fiber strength had relatively high expression in the secondary cell wall formation stage (~16–40 DPA) (Supplementary Fig. S1). Sucrose synthase (Susy) is the major enzyme of Sucrose (Suc) hydrolysis to UDP-glucose that could be used as a substrate for cellulose synthesis in cotton \(^{43}\). The combination of \(GhSus\) gene expression patterns and QTL analysis in our study further confirmed that \(GhSus\) family genes play important roles in fiber development.

Six SNPs (\(GhSus1At-A918G\), \(GhSus1At-G1783C\), \(GhSus1Dt-G751A\), \(GhSus1Dt-G2709C\), \(GhSus3At-T881A\), and \(GhSus6At-C4655T\)) were found to be associated with seed-related traits (SI, PC, and OC) in addition to the SNPs associated with fiber-related traits. In a previous study, \(Sus\) was found to be abundant in transfer cells located at the innermost layer of the seed coat and in developing filial tissues, where \(Sus\) is involved in transfer cell wall ingrowth \(^{41}\) and endosperm cellularization \(^{41}\). Suppressing the expression of \(Sus\) in cotton seed coats leads to a fiberless phenotype \(^{41}\), and silencing its expression in the filial tissue results in stunted and unserviceable seed and loss of transfer cells \(^{45,35}\). Xu et al. \(^{22}\) overexpressed the potato \(Sus\) gene in cotton, and revealed that increased \(Sus\) activity in cotton could improve seed development, leading to enhanced seed weight and number. Overexpression of \(Sus\) could also enhance sink strength and sucrose supply by expanding leaves \(^{42}\). Jiang et al. \(^{42}\) overexpressed \(GhSusA1\) in transgenic cotton and found this gene increased fiber length and strength. These findings provide an indication that it is valuable to increase fiber yield by enhancing fiber length and seed number. The new \(GhSus\) alleles found in our study may be used to increase fiber yield.

Materials and Methods

Plant materials. A set of 277 Upland cotton varieties possessing diverse fiber qualities, kindly provided by the Cotton Research Institute, Chinese Academy of Agricultural Sciences (CRI-CAAS), were subjected to EcoTILLING analysis. Of the 277 upland cotton accessions, four were from Australia, five from Africa, five from Europe, 50 from America, and 213 from Asia. Of the 213 accessions from Asia, two were from Pakistan, two from Vietnam, and 209 from China. The 209 accessions from China were from four geographic regions (the North region, Yellow River region, Yangtze River region, and northwest inland region). Detailed information on all accessions is shown in Supplementary Table S2.

Phenotypic data. All 277 accessions were sown in three consecutive growing seasons (2007–2009) simultaneously at three main ecological cotton-growing areas of China: Nanjing in Jiangsu Province \((31^{\circ}14'\text{~}32^{\circ}37'N; 118^{\circ}22'\text{~}119^{\circ}14'E)\); the average annual frost free period lasts 227–278 days, the average active accumulated temperature was 3500–5500 °C, annual rainfall was about 1000–1600 mm; Yangtze River valley cotton growing region), Anyang in Henan Province \((35^{\circ}12'\text{~}36^{\circ}22'N; 113^{\circ}37'\text{~}114^{\circ}58'E)\); the average annual frost free period
were acquired, semi-nested PCR primers were designed to further improve the PCR specificity and increase the

Designation and verification of subgenome-specific primers for duplicated gene amplification.

To amplify target fragments of \( \text{GhSus} \) gene family sequences, gene-specific primers were designed according to the published DNA sequences of \( \text{GhSus} \) gene family members\(^{29,52} \) (Supplementary Tables S4 and S5). The expression patterns of \( \text{GhSus} \) family genes were analyzed from the public RNA-seq data\(^{44} \).

Since cultivated Upland cotton is allotetraploid, where many single nucleotide diversities exist between subgenomes, subgenome-specific primers were designed for \( \text{GhSus} \) gene family members to avoid errors caused by SNPs derived from duplicated genes in the A- and D-subgenomes. SNAPER software (http://ausubellab.mgh.harvard.edu/) was employed to design the subgenome- specific primers. All primers were evaluated for their subgenome specificity using PCR. Firstly, DNA templates from two diploid species, \( \text{G. raimondii} \) and \( \text{G. herbaceum var. africanum} \) (A genome) and \( \text{G. hirsutum acc. TM-1} \) (AD genome), were amplified. The PCR products were then subjected to polyacrylamide gel electrophoresis (PAGE) to verify the primer specificity. When the PCR products only existed in two species, \( \text{G. hirsutum acc. TM-1} \) and \( \text{G. hirsutum acc. TM-1} \), but not in \( \text{G. raimondii} \), the primers were considered to be At subgenome-specific primers. When the PCR products only existed in \( \text{G. raimondii} \) and \( \text{G. hirsutum acc. TM-1} \), but not in \( \text{G. hirsutum acc. africanum} \), the primers were considered to be Dt-subgenome-specific primers. After subgenome-specific primers were acquired, semi-nested PCR primers were designed to further improve the PCR specificity and increase the product concentration.

PCR amplification and EcoTILLING assays.

Celery juice extract (CEL I enzyme) was extracted from celery following the method described by Till et al.\(^{54} \). CEL I enzymatic activity was evaluated by the digestion of heteroduplexes formed by control substances C and G, which were both supplied in the Surveyor Mutation Discovery kit. The enzyme digestion conditions followed those described in the Surveyor Mutation Discovery kit protocol (Transgenomics).

To identify nucleotide diversities by EcoTILLING, gene- and subgenome- specific primers were designed based on the \( \text{GhSus} \) family gene sequences and used in a semi-nested PCR. To avoid base mismatch during PCR, an ExTaq (TAKARA, China) enzyme with high fidelity was used in three replicates. The first PCR was performed with gene- and subgenome- specific primer pairs. The PCR amplified products were then used as templates for the second semi-nested PCR reaction. Both steps of the PCR reaction used the same forward primer but distinct reverse primers (Supplementary Table S6).

After PCR, an EcoTILLING protocol was employed for SNP screening, as described by Kadaru et al.\(^{55} \) with some modifications. First, the semi-nested PCRs were performed, and then the PCR products of each cotton variety were mixed with that of TM-1 and incubated at 99 °C for denaturing 10 min, followed by annealing with 28 cycles of 20 sec at 72 °C with a decrement of 0.3 °C per cycle and then cooling to 42 °C with a decrement of 1 °C per cycle, before finally being cooled to 24 °C for 1 min. Finally, if SNPs existed between the cotton variety and TM-1, base-mismatched DNA heteroduplexes would be formed in the mixture of the two PCR products and could be digested specifically at mismatched base position by endonuclease, CEL I. The digestion of heteroduplexes generated several small-sized DNA bands that were visualized using PAGE, implying that SNPs existed between the cotton variety and TM-1 gene sequences. Otherwise, if no SNPs existed between the cotton variety and TM-1, heteroduplexes would not be formed and could not be digested by CEL I. No small-sized DNA fragments generated. Once a polymorphic band was identified, the corresponding DNA samples were amplified using gene- and subgenome- specific primers. The resulting PCR fragments were directly sequenced for each polymorphic site from at least three accesses to confirm that only two alleles segregated at any specific site.

Nomenclature of SNP.

Here, SNP was named as “gene + subgenome + dash + base in TM-1 + base position + base in the accession analyzed. For example, the SNP, \( \text{GhSus1Dt-G751A} \), demonstrates that G base on the position of Dt subgenome in TM-1 is substituted by A base in the accession analyzed (Supplementary Table S8).
DNA sequencing and statistical analysis. Once polymorphisms were found for a targeted region of the \( \text{GhSus} \) family genes by EcoTILLING, accessions that showed polymorphisms on the gel were randomly selected for sequencing by a commercial company (Genscript Biotech Co. China) to confirm the polymorphisms. ClustalX software was used to find the SNP sites in the obtained sequences. The SIFT (Sorting Intolerant from Tolerant) method was used to predict deleterious effects of SNPs to protein function. Haplotype diversity (HD) was analyzed using DnaSP v5.157.

Calculation of gene expression levels. Genomic (as reference) and RNA-seq data of upland cotton TM-1 was obtained from the NCBI SRA database (accession codes: PRJNA248163). RNA-seq reads were mapped to the TM-1 genome (Version 1.1) using Tophat (Version 2.0.8). To measure gene expression level in fiber and ovule, we calculated the expression of each gene using FPKM ( Fragments per Kilobase of exon model per Million mapped reads) with Cufflinks (Version 2.1.1).  

Association analysis between SNPs and agronomic traits. A possible population structure was depicted using 258 simple sequence repeat (SSR) markers by STRUCTURE software version 2.3. The length of the burn-in period and the number of Markov chain Monte Carlo replications after burn-in were all assigned at 100,000 with an admixture and allele frequencies correlated model. The hypothetical number of subpopulations (k) ranged from 1 to 10 was performed at five independent run iterations. The log probability of data from the STRUCTURE output was used to estimate the k according to the method described by Evanno et al. Based on the k, the population structure matrix (Q) was generated for further marker-trait association analysis. 

The tentative association between nucleotide variations in the \( \text{Sus} \) gene family with agronomic traits was performed by an epistatic association mapping (EAM) approach proposed by Lu et al. The genetic model extended was as follows:

\[
y = \mu + X_p \beta_p + X_E \beta_E + Z_G \gamma_G + Z_{GE} \gamma_{GE} + Z_{GG} \gamma_{GG} + \varepsilon
\]

where \( y \) is the phenotypic value of agronomic traits, \( X_p \) is the Q matrix for the population structure, \( X_E \), \( Z_G \), \( Z_{GE} \), and \( Z_{GG} \) are the design matrices of the environment effect, QTL effect, QTL-by-environment interaction effect, and QTL-by-QTL interaction effect, respectively, and \( \beta_p \), \( \beta_E \), \( \gamma_G \), \( \gamma_{GE} \), and \( \gamma_{GG} \) are the corresponding effects. Note that \( \gamma_G \) includes additive and dominant effects, \( \gamma_E \) includes additive-by-environment and dominant-by-environment effects, and \( \gamma_{GE} \) includes additive-by-dominant, additive-by-dominant, and dominant-by-dominant effects. All the effects in the above model were estimated by empirical Bayes. Results from Monte Carlo simulation studies showed that these estimates were unbiased. The significance threshold of the logarithm of odds (LOD) score was set at 3, which is equivalent to the significance level of 0.0002.

References

1. Ruan Y. L. Rapid cell expansion and cellulose synthesis regulated by plasmodesmata and sugar: insights from the single-celled cotton fibre. *Funct. Plant Biol.* **34**, 1–10 (2007).
2. Basra A. S. & Malik C. P. Development of the cotton fibre. *Int. Rev. Cytol.* **89**, 65–113 (1984).
3. Pugh D. A., Offler C. E., Talbot M. J. & Ruan Y. L. Evidence for the role of transfer cells in the evolutionary increase in seed and fiber biomass yield in cotton. *Mol. Plant* **3**, 1075–1086 (2010).
4. Farrar J., Pollock C. & Gallagher J. Sucrose and the integration of metabolism in vascular plants. *Plant Sci.* **154**, 1–11 (2000).
5. Geigenberger P. & Stitt M. Sucrose synthesis catalyses a readily reversible reaction in vivo in developing potato tubers and other plant tissues. *Planta* **189**, 329–339 (1993).
6. Barratt, D. H. P. et al. Multiple, distinct isoforms of sucrose synthase in pea. *Plant Physiol.* **127**, 655–664 (2001).
7. Chourey, P. S., Taliercio, E. W., Carlson, S. J. & Ruan, Y. L. Genetic evidence that the two isozymes of sucrose synthase present in developing maize endosperm are critical, one for cell wall integrity and the other for starch biosynthesis. *Mol. Genet. Genom.* **259**, 88–96 (1998).
8. Fu, H. & Park, W. D. Sink- and vascular-associated sucrose synthase functions are encoded by different gene classes in potato. *Plant Cell* **7**, 1369–1385 (1995).
9. Tang, G. Q. & Sturm, A. Antisense repression of sucrose synthase in carrot (*Daucus carota L.*) affects growth rather than sucrose partitioning. *Plant Mol. Biol.* **41**, 465–479 (1999).
10. Zrenner, R., Salanoubat, M., Willmitzer, L. & Sonnewald, U. Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). *Plant J.* **7**, 97–107 (1995).
11. Klotz, K. L., Finger, F. L. & Shavel, W. L. Characterization of two sucrose synthase isoforms in sugar beet root. *Plant Physiol. Biochem.* **41**, 107–115 (2003).
12. Sun, J., Loboda, T., Sung, S. J. & Black, C. C. Sucrose Synthase in Wild Tomato, Lycopersicon chmielewskii, and Tomato Fruit Sink Strength. *Plant Physiol.* **98**, 1163–1169 (1992).
13. Bieniawska, Z. et al. Analysis of the sucrose synthase gene family in Arabidopsis. *Plant J.* **49**, 810–828 (2007).
14. Harada, T., Satoh, S., Yoshioka, T. & Iizukura, K. Expression of sucrose synthase genes involved in enhanced elongation of pondweed (*Potamogeton distinctus*) turions under anoxia. *Ann. Bot.* **96**, 683–692 (2005).
15. Baier, M. C. et al. Knockdown of the symbiotic sucrose synthase *MtSucS1* affects arbuscule maturation and maintenance in mycorrhizal roots of Medicago truncatula. *Plant Physiol.* **152**, 1000–1014 (2010).
16. Hohnjec, N., Perlick, A. M., Puhler, A. & Kuster, H. The Medicago truncatula sucrose synthase gene *MtSucS1* is activated both in the infected region of root nodules and in the cortex of roots colonized by arbucular mycorrhizal fungi. *Mol. Plant Microbe In*. **16**, 903–915 (2003).
17. Lee, J. J., Woodward, A. W. & Chen, Z. J. Gene expression changes and early events in cotton fibre development. *Ann. Bot.* **90**, 107–115 (2002).
18. Albrecht, G. & Mustroph, A. Localization of sucrose expression in wheat roots: increased *in situ* activity of sucrose synthase correlates with cell wall thickening by cellulose deposition under hypoxia. *Planta* **217**, 252–260 (2003).
19. Fujii, S., Hayashi, T. & Mizuno, K. Sucrose synthase is an integral component of the cellulose synthesis machinery. *Plant Cell Physiol.* **51**, 294–301 (2010).
20. Nolte, K. D., Hendrix, D. L., Radin, J. W. & Koch, K. E. Sucrose synthase localization during initiation of seed development and trichome differentiation in cotton ovules. *Plant Physiol.* **109**, 1285–1293 (1995).
21. Ruan, Y. L., Llewellyn, D. J., Furbank, R. T. & Chourey, P. S. The delayed initiation and slow elongation of fuzz-like short fibre cells in relation to altered patterns of sucrose synthase expression and plasmodesmata gating in a lintless mutant of cotton. J. Exp. Bot. 56, 977–984 (2005).
22. Xu, S. M., Brill, E., Llewellyn, D. J., Furbank, R. T. & Ruan, Y. L. Overexpression of a potato sucrose synthase gene in cotton accelerates leaf expansion, reduces seed abortion, and enhances fiber production. Mol. Plant 5, 430–441 (2012).
23. Duncan, K. A., Hardin, S. C. & Huber, S. C. The three maize sucrose synthase isozymes differ in distribution, localization, and phosphorylation. Plant Cell Physiol. 47, 959–971 (2006).
24. Hirose, T., Scaffold, G. N. & Terao, T. An expression analysis profile for the entire sucrose synthase gene family in rice. Plant Sci. 174, 534–543 (2008).
25. Islam, M. Z., Hu, X. M., Jin, L. F., Liu, Y. Z. & Peng, S. A. Genome-wide identification and expression profile analysis of citrus sucrose synthase genes: investigation of possible roles in the regulation of sugar accumulation. PLoS One 9, e113623 (2014).
26. Xiao, X. et al. Overexpression of a potato sucrose synthase gene in cotton accelerates leaf expansion, reduces seed abortion, and enhances fiber production. Mol. Plant 5, 430–441 (2012).
27. Xu, S. M., Brill, E., Llewellyn, D. J., Furbank, R. T. & Ruan, Y. L. Overexpression of a potato sucrose synthase gene in cotton accelerates leaf expansion, reduces seed abortion, and enhances fiber production. Mol. Plant 5, 430–441 (2012).
28. Zhang, X. et al. Overexpression of a potato sucrose synthase gene in cotton accelerates leaf expansion, reduces seed abortion, and enhances fiber production. Mol. Plant 5, 430–441 (2012).
29. Zou, C. et al. Genome-wide analysis of the Sus gene family in cotton. J. Integr. Plant Biol. 55, 643–653 (2013).
30. Comai, L. et al. Efficient discovery of DNA polymorphisms in natural populations by Ecotilling. Plant J. 37, 778–786 (2004).
31. Raghavan, C. et al. Rapid method for detecting SNPs on agarose gels and its application in candidate gene mapping. Mol. Breed. 19, 87–101 (2006).
32. Rungis, D. et al. Efficient genetic mapping of single nucleotide polymorphisms based upon DNA mismatch digestion. Mol. Breed. 16, 261–270 (2005).
33. Xia, Y. et al. Allelic variations of a light harvesting chlorophyll a/b-binding protein gene (Lhcb1) associated with agronomic traits in barley. PLoS One 7, e37573 (2012).
34. Xu, S. et al. Identification of rice transcription factors associated with drought tolerance using the Ecotilling method. PLoS One 7, e30765 (2012).
35. Cordeiro, G., Eliott, F. G. & Henry, R. J. An optimized ecotilling protocol for polyploids or pooled samples using a capillary electrophoresis system. Anal. Biochem. 355, 145–147 (2006).
36. Gilchrist, E. J. et al. Use of Ecotilling as an efficient SNP discovery tool to survey genetic variation in wild populations of Populus trichocarpa. Mol. Ecol. 15, 1367–1378 (2006).
37. Raksolut, S. et al. Large-scale DNA polymorphism study of Oryza sativa and O. rufipogon reveals the origin and divergence of Asian rice. Theor. Appl. Genet. 114, 731–743 (2007).
38. Evanno, G., Regnaut, S. & Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14, 2611–2620 (2005).
39. Lü, H., Liu, X., Wei, S. & Zhang, Y. Epistatic association mapping in homozygous crop cultivars. PLoS One 6, e17773 (2011).
40. Zhang, T. et al. Sequencing of allotetraploid cotton (Gossypium hirsutum L. acc. TM-1) provides a resource for fiber improvement. Nat. Biotechnol. 33, 531–537 (2015).
41. Bai, W. Q. et al. Gibberellin overproduction promotes sucrose synthase expression and secondary cell wall deposition in cotton fibers. PLoS One 9, e96537 (2014).
42. Brill, E. et al. A novel isoform of sucrose synthase is targeted to the cell wall during secondary cell wall synthesis in cotton fiber. Plant Physiol. 157, 40–54 (2011).
43. Jiang, Y., Guo, W., Zhu, H., Ruan, Y. L. & Zhang, T. Overexpression of GhSau1 increases plant biomass and improves cotton fiber yield and quality. Plant Biotech. J. 10, 301–312 (2012).
44. Ruan, Y. L., Llewellyn, D. J. & Furbank, R. T. Suppression of sucrose synthase gene expression represses cotton fiber cell initiation, elongation, and seed development. Plant Cell 15, 952–964 (2003).
45. Hu, W., Zhang, X., Zhang, T. & Guo, W. Molecular tagging and source analysis of QTL for elite fiber quality in upland cotton. Acta Agron. Sin. 34, 578–586 (2008).
46. Sun, F. et al. QTL mapping for fiber quality traits across multiple generations and environments in upland cotton. Mol. Breed. 30, 569–582 (2011).
47. Wu, J., Gutierrez, O. A., Jenkins, J. N., McCarty, J. C. & Zhu, J. Quantitative analysis and QTL mapping for agronomic and fiber traits in an RI population of upland cotton. Euphytica 165, 231–245 (2008).
48. Xu, S. et al. Identification of quantitative trait loci across interspecific F1, F2, and testcross populations for agronomic and fiber traits in tetraploid cotton. Euphytica 191, 375–389 (2013).
49. Shi, Y. H. et al. Transcriptome profiling, molecular biological, and physiological studies reveal a major role for ethylene in cotton fiber cell elongation. Plant Cell 18, 651–664 (2006).
50. Pang, L. et al. Transcriptomic analysis of fiber strength in upland cotton chromosome introgression lines carrying different Gossypium barbadense chromosomal segments. PLoS One 9, e94642 (2014).
51. Ruan, Y. L. et al. Expression of sucrose synthase in the developing endosperm is essential for early seed development in cotton. Funct. Plant Biol. 35, 382 (2008).
52. Paterson, A. H., Brubaker, C. L. & Wendel, J. F. A rapid method for extraction of cotton (Gossypium spp.) genomic DNA suitable for RFLP or PCR analysis. Plant Mol. Biol. Rep. 11, 122–127 (1993).
53. Zhu, H. et al. Structure, expression differentiation and evolution of duplicated fiber developmental genes in Gossypium barbadense and G. hirsutum. BMC Plant Biol. 11, 40 (2011).
54. Till, B. J., Zerr, T., Comai, L. & Henikoff, S. A protocol for TILLING and Ecotilling in plants and animals. Nat. Protoc. 1, 2465–2477 (2006).
55. Kadaru, S. B., Yadav, A. S., Fjellstrom, R. G. & Oard, J. H. Alternative ecotilling protocol for rapid, cost-effective single-nucleotide polymorphism discovery and genotyping in rice (Oryza sativa L.). Plant Mol. Biol. Rep. 24, 3–22 (2006).
56. Ng, P. C. & Henikoff, S. SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res. 31, 3812–3814 (2003).
57. Librado, P. & Rozas, J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452 (2009).
58. Trappell, C., Pachter, L. & Salberg, S. L. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105–1111 (2009).
59. Trappell, C. et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28, 511–515 (2010).
60. Pritchard, J. K., Stephens, M. & Donnelly, P. Inference of population structure using multilocus genotype data. Genetics 155, 945–959 (2000).
61. Xu, S. An expectation-maximization algorithm for the Lasso estimation of quantitative trait locus effects. Heredity 105, 483–494 (2010).
62. Bu, S. H. et al. Interacted QTL mapping in partial NCII design provides evidences for breeding by design. PLoS One 10, e0121034 (2015).
63. Wang, S. B. et al. Improving power and accuracy of genome-wide association studies via a multi-locus mixed linear model methodology. Sci. Rep. 6, 19444 (2016).
Acknowledgements
This work was financially supported in part by the National Natural Science Foundation of China (30971824), the National Key Technology Support Program of China during the Twelfth Five-year Plan Period [grant number 2013BAD01B03-04], Jiangsu Collaborative Innovation Center for Modern Crop Production. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions
B.-L.Z., X.-M.D. and T.-Z.Z. conceived and designed the experiments, Y.-D.Z. and J.-L.S. performed the experiments, Y.-D.Z., S.-H.B. and Y.-M.Z. analyzed the data, Y.-D.Z., K.-S.D. and T.T. contributed reagents/materials/analysis tool, J.-L.S. performed the phenotype investigation, B.-L.Z. and Y.-D.Z. wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zeng, Y.-D. et al. EcoTILLING revealed SNPs in GhSus genes that are associated with fiber- and seed-related traits in upland cotton. Sci. Rep. 6, 29250; doi: 10.1038/srep29250 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/