RESEARCH ARTICLE

Genome-wide comparative analysis of RNA-binding Glycine-rich protein family genes between *Gossypium arboreum* and *Gossypium raimondii*

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Abstract

*RB-GRP* (RNA-binding Glycine-rich protein gene) family belongs to the fourth subfamily of the GRP (Glycine-rich protein gene) superfamily, which plays a great role in plant growth and development, as well as in abiotic stresses response, while it has not been identified in cotton. Here, we identified 37 and 32 *RB-GRPs* from two cotton species (*Gossypium arboreum* and *Gossypium raimondii*, respectively), which were divided into four distinct subfamilies based on the presence of additional motifs and the arrangement of the glycine repeats. The distribution of *RB-GRPs* was nonrandom and uneven among the chromosomes both in two cotton species. The expansion of *RB-GRP* gene family between two cultivars was mainly attributed to segmental and tandem duplication events indicated by synteny analysis, and the tandem duplicated genes were mapped into homologous collinear blocks, indicated that they shared a common ancestral gene in both species. Furthermore, most *RB-GRPs* in two cotton species undergone stronger negative selective pressure by evolutionary analysis of *RB-GRP* orthologous genes. Meanwhile, *RB-GRPs* participated in different abiotic stresses (Abscisic acid, salt and Polyethylene glycol) responses and tissues at different developmental stages between two cotton species were showed by gene expression analysis. This research would provide insight into the evolution and function of the *RB-GRPs* in *Gossypium* species.

Introduction

Glycine-rich proteins (GRPs) are a group of proteins mainly constituted with glycine, which was first discovered in *Petunia* and *Cucurbita* [1]. GRPs could govern gene expression at transcriptional or post-transcriptional levels of RNA during plant development. Moreover, they also
participate in the post-transcriptional regulation triggered by different environmental stresses, such as water, temperature, light or low-oxygen stress [2]. In plants, GRPs were grouped into four classes (class I, II, III and IV) by the presence of additional motifs and the arrangement of the glycine repeats [3]. Here, Class IV GRPs will be elaborated in this study which are also known as RNA-binding GRPs (RB-GRPs), which contain either an RNA-recognition motif (RRM) or a cold-shock domain (CSD), in addition to the CCHC (cysteine-cysteine-histidine-cysteine) zinc-fingers and the glycine-rich domain [4]. RB-GRPs could also be subdivided into four subgroups, IVa (which contains an RRM), IVb (one RRM and a CCHC zinc-finger), IVc (a CSD and two or more zinc-fingers), and IVd (two RRMs) based on the diversity of domain arrangements [4].

In the past decade, numerous RB-GRP encoding genes have been isolated and identified among different plants sequentially. For instance, eight and six RB-GRPs have been discovered in Arabidopsis and rice by genome analysis [5, 6], 23 RB-GRPs in maize [7]. RB-GRPs play great important roles in plant growth, development, and stress resistance, as the presence of RRM, CSD, or CCHC domains [8]. AtGRP2 and AtGRP7 could enhance the tolerance of cold and freezing in Arabidopsis [9]. Eight ZmRB-GRPs significantly responded to cold, salt and ABA stresses, and they also be involved in other physiological processes of maize under multiple stresses according to expression profile analysis [7]. RB-GRP2 could promote the germination of Arabidopsis seed and growth of seedlings [10]. The transcription of MhGR-RBP1 were remarkably inhibited after exogenous JA (jasmonic acid) and ABA treatment [11], while expressions of three RB-GRPs (GRP2, GRP4 and GRP7) increased significantly during plant acclimation to cold [12]. CSDPs (cold shock domain proteins), contain one cold shock domain at N-terminus and glycine-rich regions interspersed with CCHC-type zinc finger at the C-terminal, play a significant role in plant growth and development as well as resistant to cold stress [13, 14]. CSDP1 from Arabidopsis thaliana could complement the cold sensitivity of BX04 mutant Escherichia coli, and resulted in better survival rate than control at low temperature, which implied that CSDP1 could exhibit RNA chaperone activity during the cold adaptation process [15].

Cotton (Gossypium spp.) is an important economic crop that produces the most important natural resources for the textile industry [16], while it also conducts as a model plant for study of polyploidy cell elongation and cell wall synthesis in scientific research [17]. Researches on genome of diploid cotton were increasing in recent years [17, 18, 19, 20], provides help for the extensive identification of gene family. As is known to all, all tetraploid cotton species were derived from interspecific hybridization between A (G.arboreum) and D-genome species (G.raimondii) [21]. Thus, G.arboreum (A₂) and G.raimondii (D₂) were assumed to be the donor material tetraploid cotton. However, the function of RB-GRPs in cotton remains unknown. Here, a systematic study of RB-GRP gene family in G.arboreum and G.raimondii to identify the characterization and phylogenetic relationships between the two species was conducted and predicted. The development of cotton fiber consists of four stages, including fiber initiation, cell elongation, secondary wall deposition and maturation [22], while the RB-GRPs whether participate in development of cotton is still unknown, thus, expressions of RB-GRPs in fiber and seed during different developmental stages were discussed in this study. Moreover, the expression patterns of RB-GRPs under different stress conditions were also surveyed. It would offer a diploid reference for the analysis of cotton agronomic traits, such as the quality of fiber and resistance to stress.

Materials and methods

Data resource

The G.arboreum gene information was downloaded from CGP (http://cgp.genomics.org.cn), and the G.raimondii genome database was obtained from phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Graimondii). Arabidopsis thaliana genomic data was
downloaded from TAIR10 (http://www.arabidopsis.org). *Theobroma cacao* data was downloaded from Ensemblplants (http://plants.ensembl.org). Maize (*Zea mays* L.) genomic data was downloaded from Gramene (http://maizesequence.org). RRM, RRM-related and CSD hmm files (PF00076, PF04059, PF08777, PF10378, PF10598, PF13893, PF14259, PF00313) were retrieved from pfam (http://pfam.sanger.ac.uk/).

**Identification and classification of RB-GRPs in *G. aboreum* and *G. raimondii***

In the draft genome of *G. aboreum*, RB-GRPs were identified using Hidden Markov Model (HMM) profile corresponding to the Pfam RRM family (PF00076, PF04059, PF08777, PF10378, PF10598, PF13893, PF14259) and CSD family PF00313 through the HMMER 3.0 package with the E-value $< 1 \times 10^{-4}$ as threshold. From the selected protein sequence screened through RRM domain and CSD domain, the RBPs containing high content of glycine residues (more than 50% residues within any 20-amino acid peptide are glycine) were obtained using perl script. Then, the RB-GRPs were confirmed within the database SMART (http://smart.embl-heidelberg.de/) and INTERPRO (http://www.ebi.ac.uk/interpro/) according to their conserved domain architecture. The RB-GRPs were divided into different subgroups, Sector allotment was based on their conserved motif composition as described previously. Molecular weight (MW), theoretical isoelectric point (pI), and size of the RB-GRPs were investigated with the online tool ExPASy (http://expasy.org/tools/). Subcellular locations were predicted by software WoLF PSORT (http://wolfpsort.org/).

**Chromosome distribution of RB-GRPs**

The positions of the RB-GRP genes were physically mapped to the 13 chromosomes in each genome with GFF file downloaded from http://cgp.genomics.org.cn, respectively. After that, Mapchart was used to draw the physical maps of RB-GRPs on chromosomes with SVG module.

**Multiple sequence alignment and phylogenetic analyses of RB-GRP genes**

To explore the evolution relationship of RB-GRP genes, conserved domains among five species have been extracted using perl script and aligned according to the "hmalign" module by HMMER V3.0 programmer, and the files containing conserved domain sequences should be converted into a format that MEGA 5.0 can recognize. Then the resulting sequences were used to construct a phylogenetic tree using the N-J method in MEGA 5.0 with the random seed of phylogeny test, poisson correction and pairwise deletion option parameters enable. A bootstrap test with 1,000 replicates was tested to obtain the reliability of the trees, and only a test value higher than 50% in the clades was selected for the conserve tree.

**Exon/intro structure analysis and motif prediction of RB-GRPs between *G. aboreum* and *G. raimondii***

The existing gff files of *G. aboreum* and *G. raimondii* were downloaded from http://cgp.genomics.org.cn, and then extracted the files contained candidate RB-GRP genes to analyze the exon/intro structures by GSDS (http://gsds.cbi.pku.edu.cn). Conserved motifs and zinc finger structures of the selected protein sequences were confirmed by SMART database (http://smart.embl-heidelberg.de/) and INTEPRO (http://www.ebi.ac.uk/interpro/scan.html), respectively.

**Detection of collinear tandem arrays**

A tandem array at an ancestral locus which termed collinear tandem array, may imply positional gene family expansion. In this study, BLASTP was provided for detection of collinear
tandem arrays of RB-GRP genes between *G. arboreum* and *G. raimondii*. In any BLASTP hit, the two genes are relabeled as ‘tandem duplicates’ if they have a difference of gene rank = 1.

### Identification and non-synonymous/synonymous substitution (Ka/Ks) ratios of orthologous gene pairs of BP-GRP genes between *G. arboreum* and *G. raimondii*

A two-step method was used to obtain the orthologous gene pairs of BP-GRP genes to detect the evolutionary relationship between different cotton species. Firstly, MCscanX was employed to identify the orthologous regions between *G. arboreum* and *G. raimondii*. Secondly, orthologous gene pairs of RB-GRP genes were then extracted according to orthologous regions containing RB-GRP genes with small Ks value. PAML package was used to calculate the orthologous rate of Ka and Ks to characterize the collinear genes between *G. arboreum* and *G. raimondii*.

### Plant materials and growth condition

*G. arboreum* and *G. raimondii* were grown in soil mixture in a climate-controlled greenhouse (16 h light/8 h dark at 30˚C). 250 mM NaCl, 100 mg/L ABA and 10% PEG (Polyethylene glycol) were treated after the expansion of the first true leaf to induce salt stress, hormone stress and drought stress, respectively. For each induction treatment, we collected leaf samples at five time points (0 as control, 6, 12, 24 and 48 h). To analyze the expressions of RB-GRPs in different tissues, plants were tagged on the day of flowering (0 DPA), fiber was separated from 50 plants at 0 DPA (ovule), 3 DPA, 6 DPA, 10 DPA and 15 DPA, and seed at 10 DPA, 20 DPA, 30 DPA and 40 DPA. And then immediately frozen in liquid nitrogen and stored at -80˚C freezer for RNA extraction. Three biological replicates were conducted for each sample.

### RNA isolation and qPCR analysis

Total RNA of all the collected samples were extracted using the RNAprep Pure Plant kit (Aidlab, Beijing, China). A total of 2 μg of RNA was used as the template, and the first-strand cDNAs were synthesized using the Takara Reverse Transcription System (TaKaRa, Shuzo, Otsu, Japan). The gene expressions of all RB-GRP genes were detected by the 170-8792iCycler iQ Calibration Kit qPCR (Quantitative Real-time polymerase chain reaction) system (Bio-Rad, USA). SYBR Green Real-time PCR Master Mix (Toyobo) was used to perform the reaction. The details of the protocol were as follows: (Step 1) initial denaturation step of 30 s at 95˚C, (Step 2) 40 cycles of 5 s at 95˚C, 34 s at 60˚C and (Step 3) melting curve analysis, and the comparative Ct (2^(-ΔΔCt)) method was used to calculate gene expression levels. The β-actin gene was chosen as the reference gene. The primer sequences are shown in S1 Table. Specificity of primers used in this study was verified by subcloning the generated amplicons using the TOPO TA Cloning Kit (Thermo Fischer Scientific, Reinach, Switzerland), and then using them for sequencing (data not shown). Gradient dilution of validated plasmids was then used to construct a standard curve. Amplification efficiency of primer pairs of all genes we detected were no less than 98%. Each experiment was repeated three times.

### RNA-sequencing analysis

Total RNA was extracted using the RNAprep Pure Plant kit (Aidlab, Beijing, China).CA, USA) from different cotton tissues during different development stages. The RNA samples were sent to the Beijing Berrygenomics for sequencing on an Illumina HiSeq2000 sequencing platform. The DEGseq package was used for identifying genes differentially expressed between paired
samples pairings, and P-values were adjusted according to the Benjamini and Hochberg method [23].

Results
Identification and classification of BP-GRP genes in G. aboreum and G. raimondii

Until now, 23 and 8 glycine-rich RNA-binding protein genes were identified in the genomes of Zea mays and Arabidopsis thaliana, respectively [5, 7]. 434 and 405 non-redundant RNA-binding protein (RBP)-coding genes were identified by the HMM profile from the Pfam database in the genome assemblies of G. aboreum and G. raimondii, respectively. 50 and 47 RNA-binding glycine-rich protein genes were then selected according to presence of (Gly)n-X repeats in the 434 and 405 RB-GRPs. The protein sequences of above candidate genes were then confirmed within the SMART database (http://smart.embl-heidelberg.de/) and BLASTP according to the conserved domains of their own. Finally, 37 and 32 RB-GRP genes were selected from G. aboreum and G. raimondii (S1 and S2 Figs). Meanwhile, we have identified 13 and 15 glycine-rich RNA-binding protein genes in the genomes of Arabidopsis thaliana and Theobroma cacao using the same method (Table 1). Then we categorized these RB-GRP encoding genes into four subtribes (IVa, IVb, IVc and IVd) according to domain motif consistent with previous principles of classification (Table 1) [4]. According to Table 1, numbers of Class IVa in genomes of five different plant species were all bigger than any other subtribes, followed by the Class IVd. In addition, the numbers of RB-GRP genes in two cotton species were bigger than other plant species. GaRB-GRP1 to GaRB-GRP37 and GrRB-GRP1 to GrRB-GRP33 were ordered according to Tables 2 and 3.

Chromosome location of RB-GRPs between G. aboreum and G. raimondii

The 37 GaRB-GRP genes were located on the 13 G. aboreum chromosomes (Fig 1). Normally, the number of GaRB-GRP genes on each chromosome varied widely. Chromosome 5 and chromosome 13 have a maximum of five GaRB-GRP genes, respectively. Four GaRB-GRP genes on chromosome 1, 6, 7 and 8, followed by on chromosome 10 which three members were found. Chromosome 2, and 4 contained two genes each, whereas each only single GaRB-GRP gene was localized on chromosome 3, 11 and 12 (Fig 1). Obviously, they were distributed unevenly among 13 chromosomes, except for no GaRB-GRP gene was found on chromosome 9 (Fig 1). Four pairs of GaRB-GRPs were linked on the same chromosome. The rest genes were found as singletons on chromosomes (Fig 1).

Like the case in G. aboreum, the 32 GrRB-GRP genes distributed unevenly across the 13 chromosomes in G. raimondii (Fig 2). Chromosomes 2 had a maximum of six GrRB-GRP genes, five GrRB-GRP genes each on chromosome 5 and 10, respectively, three on chromosome 1 and 7, two on chromosome 9 and 8, one each distributed on the other four

| Class | Domain originat | Ga | Gr | Tc | Zm | At |
|-------|----------------|----|----|----|----|----|
| a     | RRM            | 14 | 11 | 6  | 6  | 8  |
| b     | RRM-C2HC       | 4  | 6  | 0  | 6  | 3  |
| c     | CSD-C2HC-C2HC  | 7  | 6  | 0  | 2  | 2  |
| d     | RRM-RRM        | 12 | 9  | 9  | 9  | 0  |
| Total |                | 37 | 32 | 15 | 23 | 13 |

Note: Ga, G. aboreum; Gr, G. raimondii; Tc, Theobroma cacao; Zm, Zea mays; At, Arabidopsis thaliana.
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chromosomes, respectively (Fig 2). Five pairs of GrRB-GRPs in G. raimondii were linked on the same chromosome (Fig 2).

**Phylogenetic analysis of RB-GRP gene family**

To investigate the molecular evolution of RB-GRP gene family comprehensively and systematically, all the putative RB-GRPs (with protein conserved domains) from two cotton species, as well as the RB-GRPs from Arabidopsis thaliana, Theobroma cacao and Zea mays, were aligned.

Table 2. The information of RB-GRP genes from G. arboreum.

| Gene name | Gene identifier | Genomics position | Domain | Class | Size(aa) | Mw(kDa) | pI  | SL |
|-----------|----------------|--------------------|--------|-------|----------|---------|-----|----|
| GaRB-GRP1 | Cotton_A_18641  | Chr12: 121743510–121745456 | RRM | IVa   | 1947     | 154.40  | 4.99| Nucl|
| GaRB-GRP2 | Cotton_A_35587  | Chr10: 63405763–63408849 | RRM | IVa   | 1741     | 150.76  | 5.43| Nucl|
| GaRB-GRP3 | Cotton_A_13382  | Chr06: 99467833–99469938 | RRM | IVa   | 620      | 67.81   | 5.61| Nucl|
| GaRB-GRP4 | Cotton_A_19121  | Chr01: 83119516–83121726  | RRM | IVa   | 204      | 17.97   | 3.33| Chlo|
| GaRB-GRP5 | Cotton_A_25290  | Chr05: 63500417–63500418 | RRM | IVa   | 160      | 16.00   | 7.84| Nucl|
| GaRB-GRP6 | Cotton_A_30104  | Chr02: 39345847–39347785 | RRM | IVa   | 277      | 27.60   | 1.43| Chlo|
| GaRB-GRP7 | Cotton_A_00739  | Chr05: 77395952–77402949 | RRM | IVa   | 150      | 17.49   | 5.58| Nucl|
| GaRB-GRP8 | Cotton_A_10822  | Chr06: 66607573–66608404 | RRM | IVa   | 180      | 20.37   | 1.43| Nucl|
| GaRB-GRP9 | Cotton_A_18468  | Chr08: 44375570–44376091 | CSD | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP10 Cotton_A_09110 | Chr13: 49095157–49096462 | RRM | C2HC  | 176      | 19.71   | 10.85| Nucl|
| GaRB-GRP11 Cotton_A_38392 | Chr13: 48986706–48987861 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP12 Cotton_A_29989 | Chr01: 14518612–14518725 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP13 Cotton_A_22958 | Chr08: 124343839–124346641 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP14 Cotton_A_23360 | Chr08: 67682667–67685020 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP15 Cotton_A_35063 | Chr08: 87050125–87051448 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP16 Cotton_A_09110 | Chr13: 49095157–49096462 | RRM | C2HC  | 176      | 19.71   | 10.85| Nucl|
| GaRB-GRP17 Cotton_A_00105 | Chr07: 51641998–51643980 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP18 Cotton_A_38392 | Chr13: 48986706–48987861 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP19 Cotton_A_29989 | Chr01: 14518612–14518725 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP20 Cotton_A_22958 | Chr08: 124343839–124346641 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP21 Cotton_A_23360 | Chr08: 67682667–67685020 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP22 Cotton_A_23360 | Chr08: 67682667–67685020 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP23 Cotton_A_00105 | Chr07: 51641998–51643980 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP24 Cotton_A_29989 | Chr01: 14518612–14518725 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP25 Cotton_A_22958 | Chr08: 124343839–124346641 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP26 Cotton_A_22958 | Chr08: 124343839–124346641 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP27 Cotton_A_22958 | Chr08: 124343839–124346641 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP28 Cotton_A_22958 | Chr08: 124343839–124346641 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP29 Cotton_A_22958 | Chr08: 124343839–124346641 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP30 Cotton_A_15644 | Chr12: 1209807–1210081 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP31 Cotton_A_35535 | Chr12: 23068372–23072184 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP32 Cotton_A_15644 | Chr12: 1209807–1210081 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP33 Cotton_A_35535 | Chr12: 23068372–23072184 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP34 Cotton_A_15644 | Chr12: 1209807–1210081 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP35 Cotton_A_35535 | Chr12: 23068372–23072184 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP36 Cotton_A_35535 | Chr12: 23068372–23072184 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|
| GaRB-GRP37 Cotton_A_35535 | Chr12: 23068372–23072184 | RRM | HCIVb | 173      | 18.08   | 8.56| Nucl|

Note: Mw, Molecular weight; SL, Subcellular localization. The same is below.

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Table 3. The information of RB-GRP genes from *G. raimondii*.

| Gene name | Gene identifier | Genomics position | Domain | Class | Size(aa) | Mw(kDa) | pI | SL |
|-----------|-----------------|-------------------|--------|-------|----------|---------|----|----|
| GrRB-GRP1 | Gorai.012G173800 | Chr12: 34341214–34347064 | RRM | IVa | 648 | 69.15 | 5.75 | Nucl |
| GrRB-GRP2 | Gorai.007G038400 | Chr07: 2655660–26596 | RRM | IVa | 718 | 76.46 | 5.49 | Nucl |
| GrRB-GRP3 | Gorai.008G195400 | Chr08: 48022470-48026788 | RRM | IVa | 710 | 75.35 | 5.64 | Nucl |
| GrRB-GRP4 | Gorai.001G077000 | Chr01:7977562-7980895 | RRM | IVa | 294 | 30.59 | 4.99 | Chlo |
| GrRB-GRP5 | Gorai.005G028100 | Chr05: 2458681-2460986 | RRM | IVa | 143 | 15.22 | 7.90 | Nucl |
| GrRB-GRP6 | Gorai.003G101300 | Chr03: 31097257-31100241 | RRM | IVa | 257 | 26.78 | 4.63 | Nucl |
| GrRB-GRP7 | Gorai.005G243900 | Chr05: 6243216-62433175 | RRM | IVa | 150 | 15.28 | 6.31 | Nucl |
| GrRB-GRP8 | Gorai.007G039400 | Chr07: 2741633-2744829 | RRM | IVa | 278 | 28.06 | 4.76 | Chlo |
| GrRB-GRP9 | Gorai.013G036800 | Chr13: 2909216-2910436 | RRM | IVa | 169 | 17.09 | 7.84 | Nucl |

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to generate an unrooted phylogenetic tree separately with Neiboring-Joining method. According to the position among the protein sequence, the domain sequence naming scheme was added a suffix N, M and C behind the original sequence name, such as GaRB-GRP37 N, GaRB-GRP37 M and GaRB-GRP37 C. There are two domains (RRM and CSD) existed in RB-GRPs, while the RRM-type RB-GRPs accounted for the majority. Thus, the RRM-type phylogenetic tree of RB-GRP proteins from *G. raimondii* or *G. arboreum*, *Arabidopsis thaliana*, *Theobroma cacao* and *Zea mays* was established (Fig 3). It suggested that most of RRM-type RB-GRPs of five species were divided into two subgroups according to the position of RRM domain (C-terminal or N-terminal), expect for two GaRB-GRPs (GaRB-GRP28 and GaRB-GRP29), two GrRB-GRPs (GrRB-GRP5 and GrRB-GRP26) and TcRB-GRP 10.

### Gene structure of GrRB-GRPs and GaRB-GRPs

To investigate the possible structural evolution of RB-GRP gene family in the two diploid cotton species, the gene structures of GaRB-GRPs and GrRB-GRPs were compared separately. In
In general, the exon/intron organizations of RB-GRPs were consistent with the phylogenetic subfamilies showed in Fig 4. In general, the exon/intron organizations of RB-GRPs were consistent with the phylogenetic subfamilies showed in Fig 4, and the gene structures were conserved within the same group. Most members of RB-GRPs possessed two or more exons, GaRB-GRP19 had 22 exons, which is the maximum in all the RB-GRP genes, followed by GaRB-GRP18 and GrRB-GRP18. The gene structures of RB-GRP orthologous pairs were almost identical with only minor differences, with the exception of GrRB-GRP23/GaRB-GRP24, GrRB-GRP13/GaRB-GRP16, GrRB-GRP10/GaRB-GRP13, GrRB-GRP19/GaRB-GRP20 and GrRB-GRP2/GaRB-GRP2.

**Whole genome collinearity analysis of RB-GRPs between G.arboreum and G.raimondii**

The RB-GRP gene family in G.arboreum and G.raimondii have established a close relationship of collinear and synteny for each other (Fig 5), exploited by Circos software. The analysis of...
collinear blocks of two cotton species indicated that the large-scale syntenies contained 27 GaRB-GRPs, 26 GrRB-GRPs and one gene (Gorai.009G401300) in G.raimondii genome that was identified as not RB-GRP gene found to share synteny with G.arboreum. 10 RB-GRPs were
single *G. arboreum*-to-*G. raimondii* orthologs, which indicated these genes should have been in the genome of the last common ancestor of *G. arboreum* and *G. raimondii*. While the rest genes
showed one-to-more or more-to-one correspondence, for example, twelve cases that two *G. arboreum* genes corresponded to one *G. raimondii* gene, thirteen cases that one *G. arboreum* gene corresponded to multiple *G. raimondii* genes.

Fig 5. Collinear gene pairs of orthologous RB-GRP genes between *G. arboreum* and *G. raimondii*. The duplicated gene pairs and orthologous relationships between the genomes are represented by Circos figure.

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Expansion pattern of RB-GRP genes in *G. arboreum* and *G. raimondii*

Detection of collinear orthologs is important for understanding gene evolution. Segment and tandem duplications are the main mechanism resulting in gene family expansion. Distribution of paralogs could be used to analyse the potential duplications and the evolution of RB-GRPs between *G. arboreum* and *G. raimondii*. Sixteen paralogs GaRB-GRPs and twelve GrRB-GRPs were detected based on the bootstrap value in the phylogenetic analysis, and all of them randomly distributed on chromosomes of *G. arboreum* and *G. raimondii*, which indicated that segmental duplication is predominant during RB-GRP gene family expansion. Meanwhile, *Cotton_A_22861/Cotton_A_22864, Gorai.005G053000/Gorai.005G052700 and Gorai.002G019510/Gorai.002G019500* were six tandem duplication genes, and *Cotton_A_22861/Gorai.005G052700* belonged to the orthologous pair of single *G. arboreum*-to-*G. raimondii* gene correspondence. It illustrated expansion of RB-GRP gene family was also associated with tandem duplication event, and the expansion pattern of RB-GRPs in *G. arboreum* is consistent with *G. raimondii*.

Selective pressure analysis of orthologous gene pairs for RB-GRP genes

To investigate the selective constrains on RB-GRP genes, the non-synonymous (Ka) to synonymous (Ks) substitution ratio for orthologous gene pairs for RB-GRPs were calculated. As is known to all that the ratio (Ka/Ks) indicated neutral mutation when Ka equals to Ks, negative (purifying) selection when ka is less than Ks, and positive (diversifying) selection when Ka exceeds Ks [24]. In this study, 20 orthologous gene pairs in the RB-GRP gene family of *G. arboreum* and *G. raimondii* were investigated (S3 Fig). For RRM-type, the mean Ka/Ks ratio of all of orthologous genes was 0.154 between *G. arboreum* and *G. raimondii*, with most of them being even < 0.3, which suggested that they had experienced strong purifying selection pressure (S3 Fig). 3 out of 4 orthologous gene pairs had undergone purifying selection pressure, and only one pair (*Cotton_A_18468-Gorai.010G048300*) with a ratio > 1 were found in CSD-type (S3 Fig). Those observations reflected that the functions of RB-GRPs in different cottons did not diverge much during subsequent evolution. And the purifying selection might contribute mainly to the maintenance of function in RB-GRP gene family.

Expression analysis of RB-GRPs during fiber and seed development between *G. arboreum* and *G. raimondii*

We used RNA-seq analysis to compare the gene expression profiles in fiber or seed during different development, the result showed that 10 GaRB-GRPs and 11 GrRB-GRPs were participated in fiber cell development (Fig 6), 7 GaRB-GRPs and 11 GrRB-GRPs were then participated in seed development of *G. arboreum* and *G. raimondii*, respectively (Fig 7). Expressions of above genes during different development were further analyzed by q-PCR. Expression of *Cotton_A_00105, Cotton_A_09110, Cotton_A_11378, Cotton_A_25290, Cotton_A_34922* and *Cotton_A_35063* were higher in the earlier development stage of fiber, especially *Cotton_A_34922* and *Cotton_A_35063* had the highest expression level at 0–3 DPA, and decreased with fiber development, while *Cotton_A_10822* presented a gradually increasing trend during 0–15 DPA (Fig 8). Unlike GaRB-GRPs, most GrRB-GRPs presented stable high expression during 0–3 DPA (Fig 9), which means that GrRB-GRPs played an important role in earlier fiber development of *G. raimondii*. In addition, while two (*Gorai.002G19510 and Gorai.013G03680*) kept high expressions during 0–15 DPA (Fig 9), indicated that they may participated in the initiation and elongation of cotton fiber. There are many RB-GRPs differentially expressed during the seed development of cotton (Figs 10 and 11). In *G. arboreum*, expressions
of most RB-GRPs showed a decline trend with seed development of cotton, while Cotton_A_19718 kept high and stable expression during whole seed developmental stage, and Cotton_A_35063 showed a dramatic increase at 40 DPA (Fig 10). But in G. raimondii, only Gorai.013G03680 showed a high stable expression in seed during different developmental stages. Expressions of Gorai.002G01580, Gorai.002G19500, Gorai.002G19510, Gorai.005G02810, Gorai.005G24390, Gorai.005G25320, Gorai.006G03410, Gorai.008G19320, Gorai.010G12110 and Gorai.013G03680 presented transient increase in seed at 40 DPA (Fig 11).

Expression analysis of RB-GRPs between G. arboreum and G. raimondii under different stress conditions

To understand stress responsiveness of GaRB-GRPs and GrRB-GRPs, eight GaRB-GRPs and GrRB-GRPs were chosen for expression profile analysis under different stress conditions, ABA, NaCl and PEG. Six genes (Cotton_A_19718, Cotton_A_19121, Cotton_A_00739, Cotton_A_35063, Cotton_A_16121, Cotton_A_22297 and Cotton_A_18468) in G. arboreum were upregulated at first then downregulated, while Cotton_A_39551 and Cotton_A_10822 had the least changes under the stress of ABA (Fig 12). There were also six genes (Gorai.010G13910, Gorai.002G00350,
Gorai.013G03680, Gorai.002G11140, Gorai.002G23920 and Gorai.002G16700) in G.raimondii participated in ABA response, and presented the same trend as stress prolonging, while Gorai.009G24350 and Gorai.001G07470 showed insensitive to ABA stress (Fig 12). As shown in Fig 13, all the eight RB-GRPs both in two cotton species responded to salt stress treatment. Expressions of Cotton_A_19121, Cotton_A_000739 and Cotton_A_18468 were increased, while that of the others upregulated at first then downregulated (expect for Cotton_A_10822) (Fig 13). In comparison, GrRB-GRPs in G.raimondii are relative insensitivity to salt stress (Fig 13). Four GrRB-GRPs (Gorai.010G13910, Gorai.002G11140, Gorai.002G23920 and Gorai.002G16700) rose at first then decreased, two GrRB-GRPs (Gorai.002G00350, Gorai.013G03680) increased under salt stress, and expressions of other GrRB-GRPs (Gorai.009G24350, Gorai.001G07470) showed lower than control (Fig 13). Expressions of Cotton_A_19121, Cotton_A_00739 and Cotton_A_18468 were up-regulated, while three GaGB-GRPs (Cotton_A_19718, Cotton_A_16121 and Cotton_A_22297) and five GrGB-GRPs (Gorai.010G13910, Gorai.002G00350, Gorai.013G03680, Gorai.002G23920 and Gorai.002G16700) increased at first then decreased after PEG treatment (Fig 14).

Discussion

RB-GRP genes in different cotton species

RB-GRP family is characterized by the presence of a glycine-rich domain arranged in (Gly)n-X repeats and a RRM [7]. A number of RB-GRPs have been reported in different plants, such as eight in Arabidopsis [25] and six in rice [26], which play important roles in plant growth, development, and stress resistance [8, 9]. In our study, 37 GaRB-GRPs and 32 GrRB-GRPs
Fig 8. Expression patterns of the selected RB-GRP genes in fibers of *G. arboreum* during different developmental stages.
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Fig 9. Expression patterns of the selected RB-GRP genes in fibers of *G. raimondii* during different developmental stages.
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were identified in genomes of *G. arboreum* and *G. raimondii*. Conserved protein sequence analysis indicated that the GaRB-GRPs and GrRB-GRPs could be divided into four subgroups (Class IVa, IVb, IVc and IVd), which was similar to that in other plant species [3]. However, two GaRB-GRPs (*Cotton_A_35374* and *Cotton_A_38093*) and one GrRB-GRP (*Gorai.002G167100*) belong to Class IVb contained one RRM domain and two ZnF_RanBP2, which is not found in other plant species.

The species phylogenetic tree displayed the value of synonymous substitution/site (Ks) among *G. arboreum*, *G. raimondii* and *T. cacao*, and the divergence time of cotton species can be calculated by Ks values. The result of divergence time was consistent with that the common ancestor of two diploid cotton species have diverged from *T. cacao* 18–58 million years ago [19]. To sum up, we could speculated that RB-GRP gene in cotton was in an expansion trend after speciation from the *T. cacao* lineage.

**Tandem duplication and segmental duplication contributed to the expansion of the RB-GRP gene family in *G. arboreum* and *G. raimondii***

Gene family expansion mainly caused by tandem duplication, segmental duplication and transposition events [7]. Through the analyses of 50 gene families in *Arabidopsis thaliana*, tandem duplication is the most prominent, whereas segmental and transposition events occurred
Fig 11. Expression patterns of the selected **RB-GRP** genes in seeds of *Graimondii* during different developmental stages.

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Fig 12. Expression patterns of the selected **RB-GRP** genes in *G.arboreum* (A) and *G.raimondii* (B) under ABA treatment.

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more frequently in other plants [27]. Due to a recent and an ancient whole-genome duplication event occurring in G.arboreum and G.raimondii genomes, and the whole-genome duplication event cannot be ruled out as the cause of gene family expansion in G.arboreum and G.raimondii. In our study, no orthologous genes in G.raimondii could be found corresponding to 11 GaRB-GRPs, and 8 GrRB-GRPs have no corresponding orthologous genes in G.arboreum, which may result from losing or deleting of orthologous after whole genome duplication event, and the missing genes may not survive the evolutionary selection pressure. Moreover, the two cotton species experienced a retrotransposon insertion event, and the insertion was the fundamental reason for the different size between A and D genome [28], including the losing or deleting of orthologous genes. The number of copies of the orthologous genes between G.arboreum and G.raimondii was not increased by the whole genome duplication. Thus, whole genome duplication of cotton did not contribute to expansion of RB-GRPs in G.arboreum and G.raimondii.

Only two (5.4%) and four (12.5%) tandem duplication genes were found in RB-GRPs among G.arboreum and G.raimondii, while seven pairs of paralogous RB-GRP genes in G.
arboreum and five pairs of paralogous RB-GRP genes in G. raimondii randomly distributing on chromosomes indicated that segmental duplication is predominant in the three duplication events, which was consistent with the expansion of the ZmRB-GRPs in maize [7]. Overall, both tandem duplication and segmental duplication contributed to expansion of RB-GRP gene family between G. arboreum and G. raimondii.

Role of the GaRB-GRPs and GrRB-GRPs during fiber and seed development of cotton

Cotton plays a crucial role in human life, the world economy, and scientific research, and the fiber of cotton is an essential raw material for the textile industry [29, 30], and the seed of cotton, a by-product of fiber, is increasingly recognized to have excellent potential as a source of food and biofuel [31]. A large number of genes are believed to be involved in fiber and seed developmental process, such as WLIM1a, Sus (Sucrose synthase), GhRDL1 [32, 33, 34]. The tissue-specific expression patterns of the selected RB-GRP genes under normal condition reflected that they might play versatile functions in the growth and development of cotton (Dong et al. 2016). Comparing to G. arboreum, there were more RBGRPs involved in the cotton fiber development of G. raimondii, while more RBGRPs differently expressed in seed of G. arboreum. Although most of the expression patterns between GaRB-GRP encoding genes and GrRB-GRP encoding genes were different in same tissue between G. arboreum and G. raimondii, few orthologous genes presented similar expressions between two cotton species, which implied their functional conservation.

Role of the GaRB-GRPs and GrRB-GRPs under different stresses tolerance

Most of Arabidopsis, rice and maize RB-GRPs were involved in response to stresses [5, 6, 7]. In this study, expressions of 8 GaRB-GRPs and 8 GrRB-GRPs in two cottons under different stresses were analyzed by qPCR. Cotton_A_19718, Cotton_A_19121, Cotton_A_00739, Cotton_A_16121, Cotton_A_22297, Cotton_A_18468 in G. arboreum and Gorai.010G13910, Gorai.002G00350, Gorai.013G03680, Gorai.002G11140, Gorai.002G23920, Gorai.002G16700 in G. raimondii were significantly responded to ABA, NaCl and PEG response, while Cotton_A_39551, Cotton_A_10822 in G. arboreum and Gorai.009G24350, Gorai.001G07470 in G. raimondii were involved in the early stages of different stress, while showed no obvious significant difference with the prolongation of treatment, which implied that they were involved in rapid response to environmental stress, and other RB-GRPs play an important role in stress resistance. The gene expression patterns can provide important clues for gene function [35]. However, their detailed roles in stress responses need to be further studied in future.

Conclusion

The RB-GRP gene family has been extensively studied in model plant species such as Arabidopsis, rice and maize [5, 6, 7], while there has been a lack of systematic analysis of RB-GRP family genes in cotton. Here, we identified and compared the RB-GRP gene family members between the two cotton species (G. raimondii and G. arboreum). The RB-GRP genes in cotton likely experienced tandem and segmental duplication events, similar to other species. Most RB-GRPs in two cotton species undergone stronger negative selective pressure by evolutionary analysis of RB-GRP orthologous genes. qRT-PCR analyses revealed that RB-GRPs have crucial functions during fiber and seed development of cotton, and may be involved in ABA, NaCl and PEG response. The results have provided a basis for further assessment of physiological roles of different RB-GRP genes in response to stresses in cotton species.
Supporting information

S1 Fig. Phylogenetic tree of RB-GRP genes in *G. arboreum*. (TIF)

S2 Fig. Phylogenetic tree of RB-GRP genes in *G. raimondii*. (TIF)

S3 Fig. Comparative analysis of Ka/ks ratio value for RRM-type and CSD-type RB-GRP genes between *G. arboreum* and *G. raimondii*. (TIF)

S1 Table. RB-GRP gene primer pairs used in the q-PCR experiments. (DOCX)

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