Plant Science 335 (2023) 111813

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Using association mapping and local interval haplotype association analysis to improve the cotton drought stress response

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ARTICLE INFO

Keywords:
Cotton
Drought stress response
Kompetitive allele-specific PCR (KASP)
Local interval haplotype association analysis

ABSTRACT

Drought stress has a serious impact on the growth and development of cotton. To explore the relevant molecular mechanism of the drought stress response in cotton, gene mapping based on the QTL interval mapped by simplified genome BSA-seq of the drought-resistance-related RIL population was performed. A QTL region spanning 2.02 Mb on chromosome D07 was selected, and 201 resource materials were genotyped using 9 KASP markers in the interval. After local interval haplotype association analysis, the overlap of the 110 kb peak region confirmed the reliability of this region, and at the same time, the role of *GhGF14–30*, the only gene in the overlapping region, was modeled in the response of cotton to drought stress. qRT—PCR analysis of the materials and population parents proved that this gene plays a role in the drought stress response in cotton. Virus-induced gene silencing proved the importance of this gene in drought-sensitive materials, and drought-resistance-related marker genes also proved that the *GhGF14–30* gene may play an important role in the ABA and SOS signaling pathways. This study provides a basis for mining drought stress response functional genes in cotton and lays the foundation for the molecular mechanism of the *GhGF14–30* gene in response to drought stress in cotton.

1. Introduction

Cotton is an economically important crop worldwide. In recent years, with the change in the global climate, its cultivation and production have been affected by various environmental pressures. Drought stress is one of the most harmful abiotic stresses to cotton growth and production, and it is of great significance to explore the molecular mechanism of cotton's response to drought stress (Mahmood et al., 2019). Due to the complexity of drought resistance-related traits, the drought stress response of cotton is regulated by multiple genes. How to quickly and effectively identify key genes is an important basis for the genetic improvement of cotton (Ullah et al., 2017).

In plants, the fine mapping of QTL intervals has always been one of the best ways to effectively mine primary functional information, but the construction of secondary populations and the screening process of recombination and exchange of single plants often become difficult in experiments (Ijaz et al., 2019). In recent years, an increasing number of high-throughput sequencing techniques have been applied in the field of plant functional gene mining, and the ingenious combination of molecular marker-assisted selection (MAS) and genetics has become an important direction for breeders to identify key genes.

In some model plants or commercial crops, the means of key gene mining are constantly breaking through. For example, in tomato (Liu et al., 2019), Liu et al. performed fine mapping based on the leaf mold-related Cf-10 localization interval mapped by BSA-seq simplified genome sequencing. Utilizing the large F_2 population used in the positioning candidate interval, combined with the typing results of five effective Kompetitive Allele Specific PCR (KASP) markers, the positioning interval was further narrowed, and a main gene containing the characteristic motif of tomato leaf mold was identified. In wheat (Zhan et al., 2021), based on the phenotype of the $F_{2:3}$ family, Zhang et al. used BSR-seq technology to locate the candidate interval PmCH7087 related to wheat powdery mildew. Combined with 20 KASP markers, 183 $F_{2:3}$ families with extreme phenotypes were genotyped, and a T test was used

Abbreviations: **QTL**, Quantitative trait locus; **BSA**, Bulked Segregant Analysis; **RIL**, Recombinant Inbred Lines; **KASP**, Kompetitive Allele Specific PCR; **qRT-PCR**, Quantitative real-time polymerase chain reaction; **ABA**, Abscisic acid; **SOS**, Salt overly sensitive.

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https://doi.org/10.1016/j.plantsci.2023.111813
Received 19 April 2023; Received in revised form 26 July 2023; Accepted 31 July 2023
Available online 4 August 2023

to further locate the genes related to wheat powdery mildew. In cucumber (Xu et al., 2018), Xu et al. used high-throughput specific length amplified fragment sequencing (SLAF-Seq) technology to locate the candidate interval CsARN6.1 related to the waterlogging tolerance of cucumber. Through the continuous design of KASP marker genotyping in the interval and the process of screening recombination exchange individuals in 2274 F_2 individual plants, the interval was narrowed. Then, based on the resequencing results of the natural population, an association analysis was performed on the narrowed interval, and the gene at the SNP site with the highest effect value was further studied.

In cotton, based on the complex genome background of allotetraploids, an increasing number of high-throughput techniques have also been applied in the field of fine mapping of cotton, and the level of key gene mapping in cotton is also constantly improving (Fang et al., 2017; Mao et al., 2019; Zhang et al., 2022). In this study, KASP markers were selected for genotyping based on the QTL intervals mapped by the research group using drought resistance-related RIL populations. The method of local interval haplotype association analysis was used to mine the key genes in order to explore the possible important role of this QTL interval in the response to drought stress in cotton.

2. Material and methods

2.1. Plant material

The test materials in this experiment were 150 RIL populations with the upland cotton drought-resistant material "Shiyuan 321" and the upland cotton drought-sensitive material "Kui 85–174" as parents. In 2018 and 2019, drought resistance identification at the flowering and boll stage was carried out. In a total of 201 natural populations of upland cotton in 2021 and 2022, drought resistance identification at the flowering and boll stages was carried out (Fig. S1). Two common Xinjiang upland cotton resource materials, KK1543 (drought resistant) and Xinluzao 26 (XLZ26, drought sensitive) (Sun et al., 2021), were preserved by the Key Laboratory of Agricultural Biotechnology of Xinjiang Agricultural University.

2.2. Preliminary mapping of QTL intervals related to drought resistance

Based on the RIL population related to drought resistance of cotton constructed based on the drought-resistant material SY321 of upland cotton and the drought-sensitive material K85–174 as parents, BSA-seq (bulked segregant analysis) technology was used to construct extreme mixed pools and simplified genome sequencing (GBS, genotyping by sequencing), and the initial positioning of the QTL interval was carried out with reference genome TM-1_V2.1_ZJU version (Hu et al., 2019). Using the intersection of the results obtained by the two association analysis algorithms of Euclidean distance (ED) and SNP index, multiple candidate QTL intervals related to cotton drought resistance were located.

2.3. KASP genotyping

According to the candidate intervals initially located by QTL, the annotation results of polymorphic SNP sites were analyzed. The modified CTAB method was used to extract DNA from 201 leaves of natural population materials of upland cotton. Xinjiang Aidesen Biotechnology Co., Ltd. performed KASP genotyping detection on polymorphic SNP sites uniformly and randomly selected in the interval through the LGC high-throughput genotyping detection platform.

2.4. Local interval haplotype association analysis

Based on the KASP molecular markers, for which intervals can be genotyped, and the genotype and phenotype data for 201 natural populations of upland cotton, the general linear model GLM and the mixed linear model MLM in TASSEL 5.0 software were used to conduct local interval haplotype association analysis on the initially located QTL interval. According to the -$\log_{10}$(p) value in the association analysis results, the effect values of different SNP sites were compared, and the linkage disequilibrium (LD) map was drawn based on the SNP genotype frequency at the KASP marker using R language, and the initial positioning QTL interval was relocated to identify key genes.

2.5. Key genes virus-induced gene silencing (VIGS)

The parental materials Shiyuan 321 and Kui 85–174 were selected as the acceptor materials, and VIGS technology was used to construct key gene silencing vectors (Pang et al., 2013). Relatively important genes in the plant drought stress response pathway were selected as drought marker genes, and the possible roles of key genes in the cotton drought stress response pathway were explored. Based on the plants obtained from the VIGS experiment, simulated field drought stress treatment was performed, and normal plants, *p*TRV2::00 plants and *p*TRV2::key gene plants were used as controls. Soil samples were weighed daily. When the soil moisture content was 50 %, 40 %, 30 %, 20 %, and 10 %, samples were taken and stored in liquid nitrogen. The samples were used for qRT-PCR analysis of key genes and drought marker genes as well as for the measurement of MDA and Pro content (Kashyap et al., 2020) in silenced and pTRV2::00 plants, and the related phenotypes were photographed and recorded.

2.6. RNA extraction and qRT—PCR analysis

KK1543 (drought resistant), Xinluzao 26 (drought sensitive) (Sun et al., 2021) and *p*TRV2::00 plants of the parent material were selected as extreme materials for qRT-PCR experiments for key genes. For KK1543 and Xinluzao 26, 1/2 Hoagland nutrient solution was used for hydroponics under 12 h light/12 h dark conditions in greenhouse culture at 25 °C. When cotton seedlings were cultivated to the three-leaf stage, they were treated with 15 % PEG_{6000} (Fan et al., 2021) solution to simulate drought stress. Three cotton seedlings with the same growth were selected, and the roots, stems, leaves and other tissue parts were sampled at 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h and placed in liquid nitrogen for freezing.

The homologs of key genes and drought marker genes were compared using the BLASTn (Altschul et al., 1990) function in the CottonGen website (Yu et al., 2014), and specific primers were designed using DNAMAN (Lynnon Corporation, Canada) software. According to the manufacturer's instructions, the RNAprep Pure Polysaccharide and the Polyphenol Plant Total RNA Extraction Kit (Tiangen, Beijing, China) were used to extract total RNA from samples selected during each stress period, and cDNA was synthesized with a reverse transcription kit (abm). Cotton *Ubiquitin7* (UBQ7) was selected as an internal reference gene. The relevant genes were amplified by qRT—PCR using Applied Biosystems™ 7500 Fast Real-Time PCR Systems (three repetitions), and the relative expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Finally, GraphPad Prism version 8.0.1 for Windows was used to visualize the data.

3. Results

3.1. Interval positioning and selection

The use of high-throughput sequencing technology to assist in the positioning of key genes is a common method in molecular research at this stage (Jiang et al., 2019; Tomkowiak et al., 2021; Luo et al., 2022). In this study, based on the upland cotton drought-resistant material SY321 and the drought-sensitive material K85–174 as parents, a cotton RIL population related to drought resistance was constructed, and multiple candidate QTL intervals related to cotton drought resistance were located by using simplified genome sequencing and BSA-seq

technology (not yet published). Located at the physical position 43758089–45777408 of chromosome D07, a 2.02 Mb QTL interval was located and contained 28 candidate genes. This interval is the key research object of this study. Fig. 1A shows the distribution of ΔSNP index values. The ΔSNP-index peak area is located between the two red dotted lines, which is approximately 500 kb. The following three genes have been annotated here: *GH_D07G1923*, *GH_D07G1924*, and *GH_D07G1925*.

3.2. Local interval haplotype association analysis

As one of the main high-throughput genotyping technologies, KASP markers have extremely important prospects for their development and application (Li et al., 2022a; Jiang et al., 2021). KASP genotyping was used to find 9 polymorphic SNP sites that were typed in 201 natural populations of upland cotton and transformed into KASP markers. The 9 KASP marker pairs were used for local interval haplotype association analysis, and the results obtained are shown in Fig. 1B. The result analysis is mainly based on the effect value obtained from the association analysis. In particular, a 22021 marker on the far right was found to have the highest $-\log_{10}(p)$ value. However, combined with the ΔSNP-index value of this site in the BSA-seq analysis results and the degree of linkage with other markers in the LD in Fig. 1D, the marker is not sufficient. Moreover, this site is located at the boundary of the QTL interval initially identified by BSA-seq, and there may be some false positives, so this site was temporarily discarded.

Then, we continued to analyze other markers according to the $-\log_{10}(p)$ value and found that the area between the 22019 marker and the 22089 marker of the maximum value was of interest. First, LD showed that among the nine KASP markers that could be genotyped, the regional linkage strength between these two markers was extremely high. Second, the SNP annotation results of BSA-seq showed that the SNP sites at the 22018 markers were extremely important. It is located on the left side of marker 22019, and it is the only SNP site in the initially mapped QTL interval in which the genotypes in the extreme mixed pool and parents constructed by BSA-seq are homozygous genotypes consistent with drought resistance (K85–174: SY321: extreme drought-sensitive pool: extreme drought-resistant pool = 0/0:1/1:0/0:1/1). Finally, there is overlapping between this region and the ΔSNP-index peak interval when BSA-seq initially located the QTL interval, which is approximately 110 kb, and only one gene is annotated, namely, *GH_D07G1925*. The 22019 and 22089 markers are both located in the upstream and downstream intergenic regions of the gene, and there is a 22172 marker in the middle located in the exon region of the gene; this is the only nonsynonymous mutation site of the gene (Fig. S2). Fig. 1C is a gene structure diagram of the gene *GH_D07G1925*. Thus, the gene *GH_D07G1925* was selected as a key gene for follow-up research.

3.3. Nomenclature and qRT—PCR analysis of the GhGF14-30 gene

14–3–3 protein is the main soluble protein discovered by Moore and Perez (Moore, 1967) in bovine brain tissue in 1967. In plants, it was first found in Arabidopsis and other plants (Lu et al., 1992). Since this protein is often found as part of a complex bound to G-BOX, it is also called "G box factor 14–3–3", or "GF14" for short (Lu et al., 1994). Using the unique structural domain of the GF14 protein, 33 members of the GF14 protein family were compared in the upland cotton genome. The key gene is marked in red in the chromosome map in Fig. 2A, and the key gene was named *GhGF14–30* according to its sequence on the chromosome.

qRT—PCR analysis can be used to preliminarily explore the expression pattern of the *GhGF14–30* gene under drought stress. According to the qRT—PCR analysis results of the *GhGF14–30* gene in extreme materials (Fig. 2B), in the drought-resistant material KK1543 and the drought-sensitive material XLZ26, with the increase in drought stress, the relative expression of the *GhGF14–30* gene remained the same. The expression first increased and then decreased, then increased and decreased again. However, in the drought-sensitive material XLZ26, the relative expression level fluctuated, and the relative expression level was the highest at 6 h, which was significantly different from the relative expression level in the normal treatment stage. The 6 h stress period with the most significant difference was selected to analyze the tissue specificity of the *GhGF14–30* gene. The results of qRT—PCR (Fig. 2CD) showed that in the drought-sensitive material XLZ26, the relative expression of the *GhGF14–30* gene significantly decreased in the roots and stems but significantly increased in the leaves. In the drought-resistant material KK1543, the expression of the *GhGF14–30* gene significantly increased only in the roots and declined in the leaves and stems. These results indicated that the expression of the *GhGF14–30* gene can be affected by drought stress, and the specific expression sites in different resistant plants are different, which may also be the key to explain how the *GhGF14–30* gene changes the drought resistance of plants.

3.4. GhGF14-30 gene silencing analysis

VIGS is an extremely important method for gene function analysis (Rössner et al., 2022). In this study, a *GhGF14–30* gene silencing experiment was carried out based on two parent materials, K85–174 and SY321. After the albino phenotype appeared in the *p*TRV2::*CLA* plants (Fig. 3A), multiple *p*TRV2::00 plants and multiple *p*TRV2::*GhGF14–30* plants were randomly selected to determine the efficiency of *GhGF14–30* gene silencing. Taking the *p*TRV2::00 plant as a control, the results after calculating the relative expression level is shown in Fig. 3C. In the *p*TRV2::*GhGF14–30* plant based on the SY321 variety, the relative expression level of *GhGF14–30* was approximately 0.4; in the *p*TRV2::*GhGF14–30* plant based on the K85–174 variety, the relative expression level of *p*TRV2 was approximately 0.1. This result shows that there are certain differences in the silencing efficiency based on the VIGS experiments performed in the two parents, but the gene silencing was successful, which ensured the accuracy of subsequent experiments.

The absence of *GF14* gene expression may cause chlorosis of plant leaves under drought stress (Yan et al., 2004). Fig. 3B shows that the silencing of the *GhGF14–30* gene had significantly different effects on the two parental materials when the soil moisture content reached below 10 %. Under severe stress, all the materials showed wilting, yellowing and other characteristic phenotypes of water shortage. In the drought-resistant parent SY321, *p*TRV2::*GhGF14–30* plants were wilting and yellowing compared with *p*TRV2::00 plants, but the difference was not significant. In the drought-sensitive parent K85–174, the *p*TRV2::*GhGF14–30* plants wilted, turned yellow, and dried severely compared with the *p*TRV2::00 plants; furthermore, silencing of the *GhGF14–30* gene magnified this impact.

Functional verification based on the parental materials of the QTL mapping population can be used to better represent the characteristics of the population. Using the *p*TRV2::00 plants of the drought-sensitive parent material K85–174 and the drought-resistant parent material SY321, the expression pattern of the *GhGF14–30* gene was analyzed again, and the results were consistent with the results of the previous qRT—PCR analysis using extreme resource materials. In the process of increasing drought stress, expression of the *GhGF14–30* gene fluctuates, and the relative expression change trend in drought-sensitive materials was also significantly higher than that in drought-resistant materials.

The contents of malondialdehyde and proline in plants can also reflect the drought resistance of plants to a certain extent. The contents of malondialdehyde and proline in *p*TRV2::00 plants and *p*TRV2::*GhGF14–30* plants were measured. In the donor materials K85–174 and SY321, compared with the *p*TRV2::00 plants, the malondialdehyde content in the *p*TRV2::*GhGF14–30* plants was significantly increased, and it was significant in each stress stage. The proline content in the *p*TRV2::*GhGF14–30* plants with SY321 as the donor material was significantly decreased, but the proline content in the *p*TRV2::

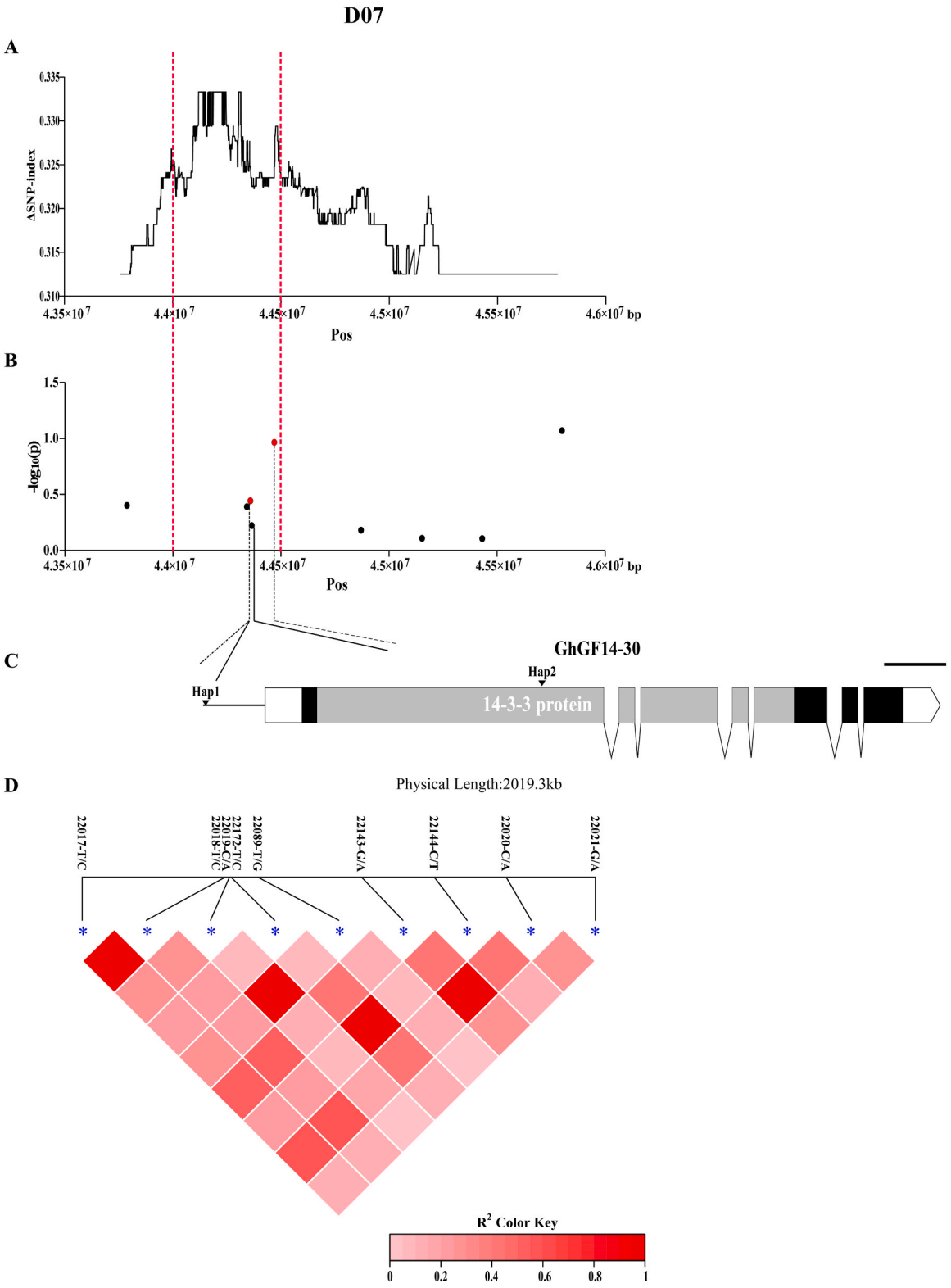

Fig. 1. Haplotype association analysis diagram of the initial mapping interval of chromosome D07. (**A**) Distribution of ΔSNP-index values. (**B**) Haplotype association analysis of 9 polymorphic SNP sites in the initial mapping interval of chromosome D07. The red point represents the point with the highest effect value and overlaps with the highest peak value of ΔSNP-index in the initial mapping interval of BSA-seq. (**C**) Gene structure diagram of the *GhGF14–30* gene. The black modules represent exons, the white modules represent the 5'UTR and 3'UTR, the middle interval region represents introns, and the gray region represents structural domains. (**D**) Linkage disequilibrium (LD) map constructed by 9 polymorphic SNP sites in the initial mapping interval of chromosome D07.

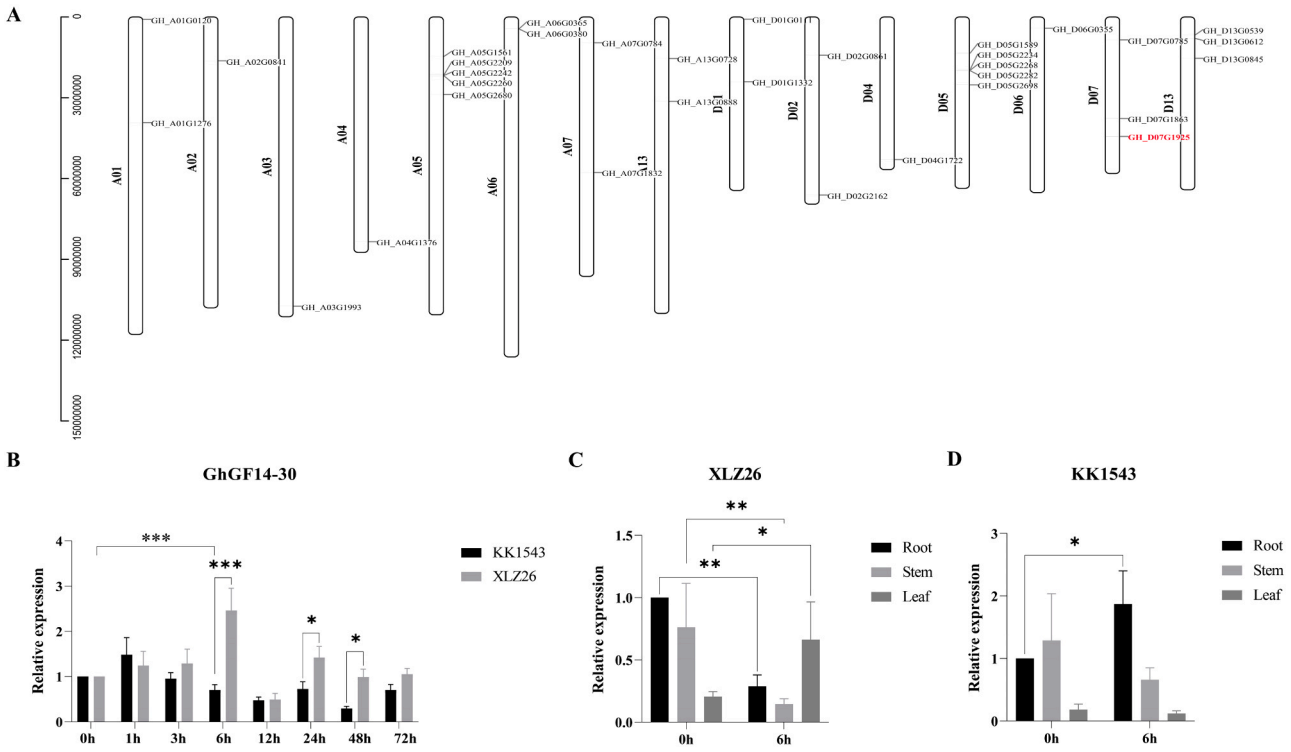

Fig. 2. Nomenclature and expression pattern analysis of the *GhGF14–30* gene. (**A**) Chromosomal location map of the *GhGF14–30* gene in the upland cotton genome. (**B**) Changes in relative expression of the *GhGF14–30* gene in the two extreme materials XLZ26 and KK1543 with deepening drought stress. (**C**) (**D**) Tissue specificity of *GhGF14–30* in XLZ26 and KK1543. One-way analysis of variance was used for statistics, * represents $P < 0.05$, significant difference; * * represents $P < 0.01$, very significant difference; * ** represents $P < 0.001$, extremely significant difference.

GhGF14–30 plants with K85–174 as the donor material was significantly increased. This result indicated that the effect of silencing the *GhGF14–30* gene on the change in malondialdehyde content was consistent with the change in plants under drought stress, which proved that the gene did play a role in the cotton drought stress response. However, the change in proline content in the drought-sensitive parental materials was relatively abnormal, and this situation needs further study.

3.5. Functional analysis of the drought resistance of GhGF14-30

The GF14 protein can interact with a series of proteins, enzymes or hormones related to the drought stress response, such as ion channel proteins (Booij et al., 1999), plasma membrane H^+-ATPase (Borch et al., 2002), ABA (Schoonheim et al., 2007), and SOS (Yang et al., 2019). In this study, *GhGF14–30* gene-silenced plants were subjected to drought stress, and by observing the relative expression changes in the marker genes in related pathways, we tried to explore the impact of *GhGF14–30* gene silencing on related pathways.

GhABF2 (Liang et al., 2016) participates in the ABA-regulated drought and salt stress response signaling pathway, is induced by ABA, and its overexpression can improve the drought resistance of cotton (Fig. 4AE). The experimental results showed that the relative expression of *GhABF2* showed a gradual increase and finally a decrease in expression in each material with the increase in the degree of stress. However, in the drought-sensitive parent K85–174, the relative expression level in *p*TRV2::*GhGF14–30* plants was significantly different compared with the relative expression level in *p*TRV2::00 plants.

ERF1 (Li et al., 2022b) is located upstream of the ABA signaling pathway, and its overexpression can inhibit the downstream gene *ABI5* and regulate the expression of genes containing the abscisic acid response element (ABRE) motif in the promoter region. With the increase in drought stress, the expression of *ERF1* (Fig. 4BF) in each material gradually decreased, while the expression of *ABI5* (Fig. 4DH) in each material gradually increased. Compared with *p*TRV2::00 plants, the relative expression level of *ERF1* decreased more significantly in *p*TRV2::*GhGF14–30* plants, and the relative expression level of *ABI5* increased more significantly. This indicates that the *GhGF14–30* gene may perform the same function as *ABI5* in the drought stress response.

PP2CA (Baek et al., 2018; Belda-Palazon et al., 2019) is the core component of ABA signal transduction, which negatively regulates ABA signal transduction and the stress response. ABA can promote the degradation of *PP2CA* through E3 ligase to enhance the tolerance of plants to salt and drought stress (Fig. 4CG). Experiments have shown that the relative expression of *PP2CA* increased gradually after being subjected to drought stress, and the relative expression of *PP2CA* in *p*TRV2::*GhGF14–30* plants significantly increased. In conclusion, silencing of the *GhGF14–30* gene may lead to unrepressed expression of *PP2CA*.

SOS2 plays an important role in the Salt-Overly Sensitive (SOS) signaling pathway (Yang et al., 2019). In rice, overexpression of *SOS2* can improve the drought tolerance of rice (Kumar et al., 2022) (Fig. 4IL). The aggravation of drought stress caused the expression of *SOS2* in each material to show the same trend. After the *GhGF14–30* gene was silenced, the expression of *SOS2* increased in both extreme parents. This is somewhat similar to the results of other studies showing that the GF14 protein can inhibit the activity of *SOS2* (Yang et al., 2019).

GhDPBF2 (Chen et al., 2021) and *GhNAC4* (Trishla and Kirti, 2021) are ABA signaling genes that are induced by ABA, and their overexpression improved plant tolerance to salt and drought stress (Fig. 4JM). The expression of *GhDPBF2* in SY321 and K85–174 showed the opposite fluctuating expression trend with the severity of drought stress, and the relative expression of *GhDPBF2* decreased significantly in *p*TRV2::*GhGF14–30* plants (Fig. 4KN). The expression of *GhNAC4* first increased and then decreased in each material. There was a significant increase in the *p*TRV2::*GhGF14–30* plants of SY321 and a significant

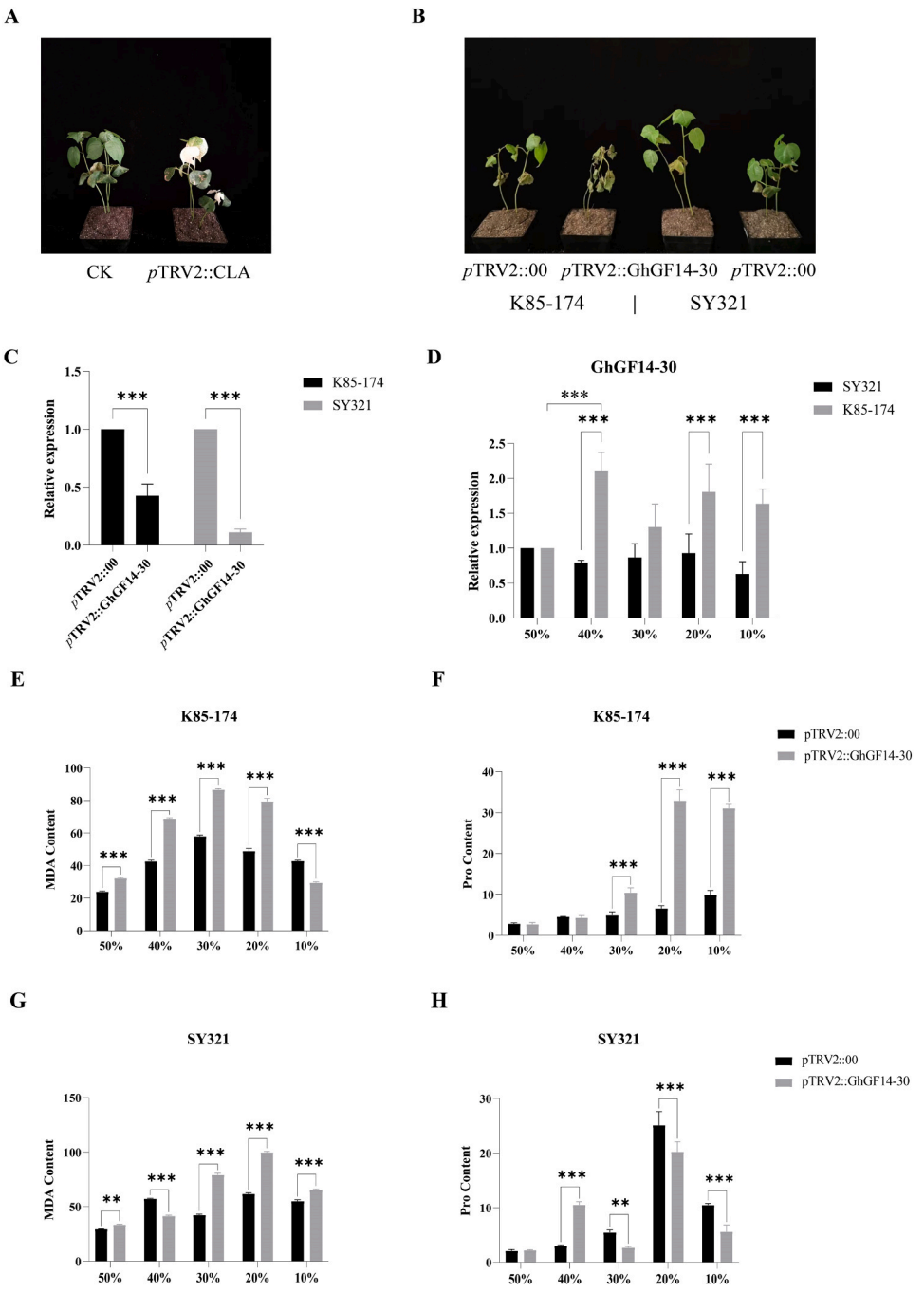

Fig. 3. GhGF14–30 gene VIGS test. (**A**) Normal plant and *p*TRV2::*CLA* albino phenotype. (**B**) Phenotype comparison of *p*TRV$_2$::00 and *p*TRV2::*GhGF14–30* materials at 10 % soil water content for 3 days. (**C**) Silencing efficiency assay. (**D**) Expression level of *GhGF14–30* in *p*TRV2::00 plants subjected to drought stress. (**E**) (**G**) MDA content in *p*TRV2::00 and *p*TRV2::*GhGF14–30* materials in K85–174 and SY321 under drought stress. (**F**) (**H**) Pro content in *p*TRV2::00 and *p*TRV2::*GhGF14–30* materials in K85–174 and SY321 under drought stress. One-way analysis of variance was used for statistics; * represents $P < 0.05$, significant difference; * * represents $P < 0.01$, very significant difference; * ** represents $P < 0.001$, extremely significant difference.

decrease in the *p*TRV2::*GhGF14–30* plants of K85–174. This indicates that silencing the *GhGF14–30* gene may indirectly or directly inhibit the expression of *GhDPBF2*, but the effect on *GhNAC4* has the opposite expression trend in different materials.

4. Discussion

Genetic populations can be used as important mapping tools for genetic analysis of population structure (Zhou et al., 2016; Rockman and Kruglyak, 2008). The population diversity and high recombination of natural populations are the key to improving the accuracy of positioning genes (Zeng et al., 2022; Mokrzycka et al., 2022). In this study, based on the initial QTL mapping intervals of the RIL populations, natural populations with a wide range of sources were selected, and further mining of key intervals was carried out by using the association between polymorphic KSAP markers and phenotypes. The overlap of some peak regions represents the authenticity of this QTL interval, in which there is only one gene, which is annotated as the GF14 protein.

As a functional regulatory protein, the GF14 protein mainly functions as a key regulator of primary metabolism and signal transduction in plants and participates in the regulation of plant growth and development and the response to stress. In Arabidopsis (Visconti et al., 2019), overexpression of the GF14 protein can improve the cold resistance and stress response of Arabidopsis plants. In maize (Jahn et al., 1997), Jahn T et al. found that the GF14 protein is a positive regulator of plasma membrane H^+-ATPase activity and is involved in regulating osmotic pressure and water transport in plants. In apple and wheat (Ren et al., 2019; Zhang et al., 2018), the GF14 protein can reduce the oxidative damage in cells by regulating the reactive oxygen species scavenging signaling pathway and the expression of stress-related genes and can act

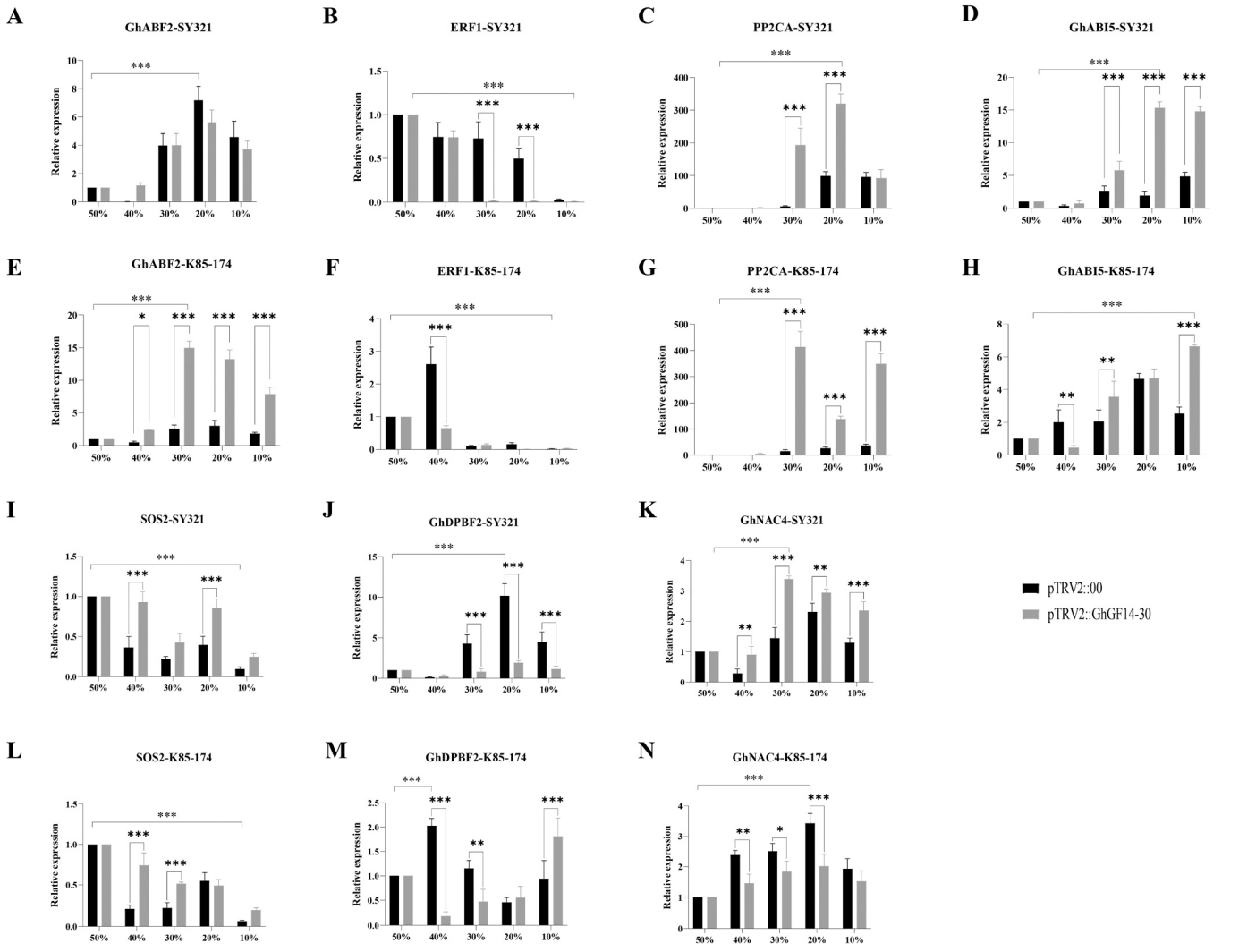

Fig. 4. qRT-PCR analysis of marker genes related to drought resistance. (**A-N**) Changes in relative expression of *GhABF2, ERF1, PP2CA, GhABI5, SOS2, GhDPBF2,* and *GhNAC4* in *p*TRV2::00 plants and *p*TRV2::*GhGF14–30* plants of SY321 and K85–174 under drought stress, respectively. One-way analysis of variance was used for statistics, * represents $P < 0.05$, significant difference; * * represents $P < 0.01$, very significant difference; * ** represents $P < 0.001$, extremely significant difference.

as a positive regulator of salt and drought stress responses. These results all indicate that the GF14 protein plays an important role in the process of plant growth and development and in coping with adversity, which is also the background basis for the in-depth study of the *GhGF14–30* gene after the location mining in this study.

In cotton, most of the recent research on the GF14 protein focus on fiber development (Shi et al., 2007; Zhang et al., 2010; Zhou et al., 2015). In this study, only one GF14 protein was found in the peak overlapping region based on QTL mapping, and we explored the role of the GF14 protein in cotton drought stress. Based on the qRT—PCR analysis of the QTL mapping population and the extreme resource materials, it was found that the drought stress response of the *GhGF14–30* gene was relatively strong, especially in the drought-sensitive materials, and the impact of VIGS before and after silencing was substantial. This proved that the GF14 protein is indeed involved in the response to drought stress in cotton and may play an important role in how cotton copes with drought stress. At the same time, the obvious difference in the phenotypes of the parental VIGS materials after drought stress also indicates that the *GhGF14–30* gene may not be the only gene that can enhance the drought resistance of plants in drought-resistant materials with good growth, but in the drought-sensitive materials with lower expression of strong drought-resistance genes, the *GhGF14–30* gene is extremely critical. This possibility may bring new life to some high-yield and high-quality cotton materials that are not resistant to stress.

The ABA pathway plays an important role in the response of plants to drought stress (Gong et al., 2020). In barley (Schoonheim et al., 2007), the GF14 protein is controlled by ABA and it also controls the action of ABA; furthermore, it interacts with genes such as the ABF protein family and the ABI5 transcription factor. In this study, ABA pathway-related marker genes were selected, and the relative expression changes in *p*TRV2::00 and *p*TRV2::*GhGF14–30* materials under drought stress were observed. It was discovered that the *GhGF14–30* gene may be involved in the transduction of ABA signals, and it regulates related stress response genes, which has an important impact upstream and downstream of the ABA signaling pathway.

The SOS pathway not only plays a role in the response to salt stress but also respond to drought stress (Kumar et al., 2022). In Arabidopsis (Zhou et al., 2014), under normal growth conditions, SOS2 phosphorylates and interacts with the GF14 protein, and the GF14 protein can inhibit the kinase activity of SOS2 to open the SOS pathway. At the same time, the GF14 protein also acts as a molecular switch for the calcium signal-induced SOS pathway, which is why SOS2 was selected as the marker gene in this study (Yang et al., 2019). In the *p*TRV2::*GhGF14–30* material, SOS2 expression appeared uninhibited, with a significant increase in expression compared to that in *p*TRV2::00 plants; this result is similar to the findings showing that GF14 and SOS2 can interact. These

results proved the importance of the *GhGF14–30* gene in the cotton drought stress response and laid the foundation for investigating the molecular mechanism of *GhGF14–30* gene regulation.

5. Conclusions

The local haplotype association analysis of natural populations combined with RIL population BSA-seq to locate QTL intervals and peak overlapping intervals, this study provides an important strategy for the rapid and accurate mining of key genes in cotton. qRT—PCR analysis and VIGS experiments helped characterize the role of the *GhGF14–30* gene in the drought stress response-related pathways of cotton and determine that the *GhGF14–30* gene can respond to drought stress. This study provides an important scientific basis for further research on the regulatory mechanism of the *GhGF14–30* gene's involvement in the drought stress response in cotton, and provides a potential strategy for further improving cotton stress adaptability and yield.

CRediT authorship contribution statement

Q.J.C. contributed to the conception of the study; W.G. and Q.C. have equal contributions to this study; W.G., Q.C., Y.Q., and Q.J.C. drafted and revised the manuscript. W.G., Q.C., J.F., J.H., F.S., and S.G. contributed to the data analysis. Y.W., J.Z., Y.X., and M.Z. contributed to the experiments. All authors reviewed and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

Acknowledgements

This work was supported by the Xinjiang Uygur Autonomous Region Natural Science Foundation Project (2021D01A92), Natural Science Youth Project of the Autonomous Region University Research Program (XJEDU2021Y020), The 69th batch of China Postdoctoral Science Foundation General Funding Project (2021M693901), Xinjiang Agricultural University Crop Science Key Discipline Development Fund Project (XNCDKY2021008), Autonomous Region Postgraduate Innovation Project (XJ2023G131), Development of Molecular Markers Related to Drought Resistance of Cotton GhGF14-30 Gene (dxscx2023014) and Xinjiang Uygur Autonomous Region International Science and Technology Program "Shanghai Cooperation Organization Science and Technology Partnership Program" "China Uzbekistan Seed Industry Science and Technology Innovation Center" (2020E01049).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111813.

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