Phylogenetic Analysis of the Membrane Attack Complex/Perforin Domain-Containing Proteins in *Gossypium* and the Role of *GhMACPF26* in Cotton Under Cold Stress

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The membrane attack complex/perforin (MACPF) domain-containing proteins are involved in the various developmental processes and in responding to diverse abiotic stress. The function and regulatory network of the MACPF genes are rarely reported in *Gossypium* spp. We study the detailed identification and partial functional verification of the members of the MACPF family. Totally, 100 putative MACPF proteins containing complete MACPF domain were identified from the four cotton species. They were classified into three phylogenetic groups and underwent multifold pressure indicating that selection produced new functional differentiation. Cotton MACPF gene family members expanded mainly through the whole-genome duplication (WGD)/segmental followed by the dispersed. Expression and cis-acting elements analysis revealed that MACPFs play a role in resistance to abiotic stresses, and some selected *GhMACPFs* were able to respond to the PEG and cold stresses. Co-expression analysis showed that *GhMACPFs* might interact with valine-glutamine (VQ), WRKY, and Apetala 2 (AP2)/ethylene responsive factor (ERF) domain-containing genes under cold stress. In addition, silencing endogenous *GhMACPF26* in cotton by the virus-induced gene silencing (VIGS) method indicated that *GhMACPF26* negatively regulates cold tolerance. Our data provided a comprehensive phylogenetic evolutionary view of *Gossypium* MACPFs. The MACPFs may work together with multiple transcriptional factors and play roles in acclimation to abiotic stress, especially cold stress in cotton.

**Keywords**: *Gossypium*, membrane attack complex/perforin (MACPF), cold stress, VIGS, WGCNA
INTRODUCTION

The membrane attack complex/perforin (MACPF) proteins are pore-forming proteins across the cellular membrane in plants and other organisms. They have a signature motif, Y/S-G-G/S-H-X7-G-G, and play important roles in mammalian immunity and bacterial pathogenesis (Hadders et al., 2007; Wade and Tweten, 2015; Johnson et al., 2017; Moreno-Hagelsieb et al., 2017; Ni and Gilbert, 2017; Yu et al., 2020). In Arabidopsis, the necrotic spotted lesion 1 (NSL1) protein, containing a putative MACPF domain, was involved in the negative regulation of programmed cell death (PCD) and defense response (Noutoshi et al., 2006). Moreover, loss function of the MACPF domain could trigger cell death through activating salicylic acid (SA) signaling in Arabidopsis (Morita-Yamamuro et al., 2005; Fukunaga et al., 2017).

Environmental stress is a major challenge in agricultural production, affecting plant photosynthesis efficiency, cell membrane homeostasis, and plant growth and development (Hasegawa et al., 2000; Odukoya et al., 2019). Plants have developed sophisticated physiological and biochemical adaptation that enables them to survive through the regulation of multiple hormones including salicylic acid (SA), ethylene, and abscisic acid (ABA) (Zhu, 2002; Vlot et al., 2009; Hasanuzzaman et al., 2017; Emanverdian et al., 2020). SA exerts key effects in disease resistance by modulating host cell death and defense gene expression (Vlot et al., 2009). An SA-responsive protein is known as constitutively activated cell death 1 (CAD1) has been found to have a conservative MACPF domain. The CAD1 mutant altered endophytic phyllosphere microbiota and displays leaf tissue damage in Arabidopsis. CAD1 is important for plant defense and likely negatively regulates plant immunity (Morita-Yamamuro et al., 2005; Tsutsui et al., 2006; Chen T. et al., 2020).

In recent years, different transcriptomic data showed that the MACPF genes were involved in growth, development, and response to abiotic stresses in sorghum, rice, and maize (Yu et al., 2020). However, the evolution and function of the MACPF genes in Gossypium spp. were still unknown. The underlying interaction among the MACPFs and other genes was also unclear. The weighted gene co-expression network analysis (WGCNA) was a useful method to identify the potential network through the transcriptomic analysis, which had been used to study development and abiotic stress in many core traits (Chen X. et al., 2020; Cheng et al., 2021).

As a pioneer crop in the barren land, cotton has a natural stress resistance through the complex genome structure (Li et al., 2015). Understanding the potential function of the MACPFs in cotton will be helpful to the cotton breeding and functional analysis. In this study, we performed a genome-wide identification of the MACPF family members in tetraploid species Gossypium hirsutum (AD1) and Gossypium barbadense (AD2) and their ancestor diploid Gossypium arboresum (A2) and Gossypium raimondii (D5) (Paterson et al., 2012; Du et al., 2018; Hu et al., 2019). The evolutionary relationships of the MACPFs were investigated and the putative interactions of the MACPFs with other genes were analyzed by the WGCNA. In total, 38 GhMACPFs, 33 GbMACPFs, 14 GaMACPFs, and 15 GrMACPFs were identified from the four Gossypium spp. genomes. Phylogenetic, conserved structural motif, the whole-genome duplication (WGD), non-synonymous substitution (Ka), and synonymous substitution (Ks) showed that the MACPFs were subjected to functional gene selection. Co-expression indicated that GhMACPFs played an important role in responding to abiotic stress. And Apetala 2 (AP2)/ethylene responsive factor (ERF), valine-glutamine (VQ), and WRKY transcription factors (TFs) were the co-expression genes of GhMACPFs in the regulation of cold stress response. In addition, silencing of GhMACPF26 in cotton enhanced the tolerance to cold stress. This study will lay a solid foundation for further exploration of the functions of GhMACPFs in cotton.

MATERIALS AND METHODS

Identification and Molecular Characterization of the MACPF Domain Family in Multiple Plants

A total of 15 genome databases, which contained Arabidopsis thaliana (L.) Heynh., Oryza sativa Linn., Solanum lycopersicum Linn., Vitis vinifera Linn., Ananas comosus (L.) Merr., Amborella trichopoda Baill., Carica papaya Linn., Theobroma cacao Linn., G. hirsutum, G. barbadense, Gossypium arboreum, G. raimondii, Brassica napus Linn., Brassica oleracea Linnaeus, and Brassica rapa Linn., were obtained from the CottonGen1 (Yu et al., 2014), the Joint Genome Institute (JGI) database2 (Goodstein et al., 2012), and the National Center for Biotechnology Information (NCBI) database3 (Sayers et al., 2010), respectively. Four AtMACPF genes, including AtMACPF01 (AT1G14780), AtMACPF02 (AT1G28380), AtMACPF03 (AT1G29690), and AtMACPF04 (AT4G24290), were used as query sequences to blast the selected proteins database via the protein-protein BLAST (BLASTP) method (E-value = 1 × 10−3). The hidden Markov model (HMM) profile of the MACPF domain (Pfam01823) was downloaded from the Pfam database4 (El-Gebali et al., 2019) and the NCBI conserved domain database5 (Marchlerbauer et al., 2015) were used to confirm the conservative MACPF domain. Finally, we identified the molecular characterization through the Softberry website6 and the ExPaSy7 website.

Phylogenetic and Collinearity Analysis of the MACPF Genes

The Multiple Alignment using Fast Fourier Transform (MAFFT) (version 7.4007) (Katoh and Standley, 2014), the Gblocks (version 1)

1https://www.cottongen.org/
2https://www.phytozone.net
3https://www.ncbi.nlm.nih.gov/
4https://pfam.xfam.org/
5https://smart.embl-heidelberg.de/
6https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
7https://linux1.softberry.com/berry.phtml
8https://web.expasy.org/translate/
cotton plants grown at the three-leaf stage were subjected to stress treatment. For the drought treatment, the seedlings of TM-1 were irrigated with 400 mM polyethylene glycol 600 (PEG 600) for 0, 1, 6, 12, and 24 h. For the cold stress, the seedlings were irrigated with continuous cold stress (4°C) in a plant incubator/illumination incubator for 0, 2, 4, 6, 8, 12, and 24 h. Leaf samples were collected from five uniform plants, then quickly frozen in liquid nitrogen and stored at −80°C. All the experiments were repeated three times.

Total RNA was isolated by using the RNAprep Pure Plant Kit (Polysaccharides and Polyphenolics-rich, DP441) (TIANGEN, Beijing, China). The Mir-X™ miRNA First-Strand Synthesis Kit (Takara Biotechnology Corporation Ltd., Dalian, China), the SYBR Green PCR Supermix Kit (Bio-Rad Laboratories), and the 7500 Real-Time PCR System (Applied Biosystems) were used to synthesize complementary DNA (cDNA) and analyze gene expression levels, respectively. GhUBQ7 (NCBI accession: DQ116441) (Tu et al., 2007) was used as an endogenous control. All the primers designed by Primer 6 were listed in Supplementary Table 1.

**RESULTS**

**Membrane Attack Complex/Perforin Domain Proteins in the Allotetraploid and Diploid Cotton**

All the protein sequences annotated as the MACPF in four Gossypium and other 11 species were performed. A total of 184 MACPF domain-containing sequences were retained for this study (Supplementary Table 2). The number of the MACPFs is approximately similar to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similarly, the number of the MACPF genes in each allotetraploid is nearly equal to the genome size of the evolution biodiversity (Qiao et al., 2019). Similar
group III (35 MACPFs) was specific and belonging to the Malvaceae, indicating that those genes likely experienced a sequence divergence event during polyploidization.

Basic information of the MACPF genes in *Gossypium* was listed in Supplementary Table 3 including genomic length, transcript length, GC count (%), exon number, molecular weight (MW), charge, and isoelectric point (PI). The genomic length of the MACPF genes was ranged from 790 (GhMACPF22) to 9,893 bp (GrMACPF14), the coding sequence (CDS) length of those range from 348 (GhMACPF22 and GbMACPF21) to 1,872 bp (GbMACPF19), and the average GC content of the transcript was 43.13. Moreover, the exon numbers varied from 2 (GhMACPF15, GbMACPF21, and GhMACPF22) to 9 (GbMACPF33), and more than half of the MACPFs containing seven exons. The MW value ranged from 12.884 (GbMACPF21) to 69.677 kDa (GbMACPF19) and the PI values varied from 6.228 (GhMACPF28) to 9.889 (GbMACPF15) (Supplementary Table 3).

### Conserved Characteristics Analysis in the Diploid and Allotetraploid Cotton

The 2,000-bp upstream from the initiation codon in the diploid and allotetraploid cotton was used for the cis-element analysis of the MACPF genes. Through the PlantCARE web analysis, 15 cis elements were identified from the allotetraploid cotton, 14 cis elements in *G. arboreum*, and 13 cis elements in *G. raimondii*, respectively. Among those, 13 cis elements were common. Five kinds of hormone-response cis-elements, including ABA-responsive element (ABRE), auxin-responsive element (TGA-element), gibberellin (GARE-motif, P-box, and TATC-box), MeJA-responsive element (CGTCA-motif, and TGACG-motif), and SA-responsive element (SARE and TCA-element) were identified in each gene. Totally, four regulatory elements, which contained the low temperature responsive element (LTR), MYB-binding site (MBS), MYB-binding site I (MBSI), and TC-rich...
repeats, were found to have a certain function in the cold, drought, and defense response in plants (Supplementary Figure 1). Furthermore, the RY elements (seed-specific regulation) and circadian elements (cis-acting regulatory element involved in circadian control) were also found in the promoters, indicated that the MACPF gene might be played a role in plant development. The diversity analysis of elements determined the response of the MACPFs in *Gossypium* spp. to endogenous hormones, abiotic stresses, and development.

Combining the phylogenetic, motif composition, and gene structure analysis in Figure 2, the MACPF domain-contained proteins were divided into three groups. In this study, with the 30 distinct conserved motifs set through the MEME program, motif 1 was shared in *GhMACPFs* and *GbMACPFs* with the conserved “(F/Y)GTH(F/Y)-X6-GG” structure (Supplementary Figure 2). Moreover, motif 20 and motif 28 were specific in group I and group II and motif dispersion is variable in group III (Figure 2). Multiple motif distribution suggested that those genes were conserved and might have similar functions in the allotetraploid cotton. Moreover, the gene structure and intronic phase were exhibited similar to the MEME dispersion in the diploid, especially in group I and group II (Supplementary Figure 3). As described, the MACPF members were more likely to have gene selection and expansion between the diploid and allotetraploid cotton.

### Orthologous Genes Analyses Between the Diploid and Allotetraploid Cotton

The chromosomal distribution of the MACPF genes in diploid cotton showed that 15 *GrMACPFs* were mapped to nine chromosomes of the *G. raimondii*. 14 *GaMACPFs* were also mapped to nine chromosomes of the *G. arboreum* (Supplementary Figure 4). A total of 38 *GhMACPFs* and 33 *GbMACPFs* were mapped to 19 chromosomes in the allotetraploid cotton, respectively. There was no MACPF gene located on chromosomes *GhA04*, *GhA07*, *GhA08*, *GhA11*, *GhD03*, *GhD08*, *GhD11*, *GbA04*, *GbA07*, *GbA08*, *GbA11*, *GbD03*, *GbD08*, and *GbD11* (Figure 3). Numerous genes were varied from 1 to 4 on the mapped chromosomes (Figure 3, Supplementary Figure 4, and Supplementary Table 3). Duplication event analysis in the MACPF family indicated that there were 12 WGD/segmental events in *Gh_At* (75%), *Gh_Dt* (54.5%), *Gb_At* (80%), and *Gb_Dt* (66.7%); 9 WGD/segmental events (64.3%) in *G. arboreum*; and 11 events (73.3%) in *G. raimondii*, respectively. However, the singleton and proximal event were few in the MACPF family. It was suggested that the WGD/segmental was the main reason for...
the expansion of the MACPF gene family in *Gossypium* spp. (Supplementary Table 4).

Orthologous analysis showed 31 MACPF gene pairs in the two allotetraploid pieces of cotton including 14 gene pairs in Gh_At and Gb_At subgenomes and 17 pairs in Gh Dt and Gb Dt subgenomes. Meanwhile, 12 paralogous gene pairs were found in *G. hirsutum* and 14 paralogous gene pairs were found in *G. barbadense*, respectively (Figure 3 and Supplementary Table 5). Subsequently, orthologous genes were also identified between the two allotetraploid pieces of cotton and two diploids. A total of 26 GhMACPFs and 26 GbMACPFs were orthologous genes in the two diploid cottons of which 13 gene pairs showed in *G. arboreum*, while 13 gene pairs showed in *G. raimondii* (Supplementary Figure 4 and Supplementary Table 5). Furthermore, the Ka/Ks ratios for the multi-MACPF pairs in *Gossypium* spp. were determined in Gb Dt-Gr, Gh Dt-Gr, Gb At-Ga, Gh At-Ga, Ga-Gr, Gb At-Gb Dt, Gb At-Gh At, Gh At-Gh Dt, and Gb Dt-Gh Dt. Among the 121 gene pairs, the Ka/Ks ratio of GbMACPF31-GhMACPF35, GbMACPF12-GaMACPF11, and GhMACPF12-GaMACPF11 were >1, the other 109 gene pairs were range from 0 to 0.94, indicating that these genes have undergone purifying selection (Supplementary Table 6).

**Expression Patterns and Co-expression of the Homologous MACPF Genes in the Allotetraploid Cotton**

To understand the potential function of GhMACPFs and GbMACPFs, we analyzed the expression patterns of the MACPFs in various tissues via the published RNA-seq data (Supplementary Figure 5 and Supplementary Table 7). Further, 27 GhMACPFs and 23 GbMACPFs were exhibited distinctive expression patterns in the different tissues. Most of the MACPFs were highly expressed in the anther, bract, filament, petal, pistil, and sepal (Supplementary Figures 5A,B). GhMACPFs and GbMACPFs were also highly expressed in the 5 DPA (day post anthesis), 10 DPA, and 15 DPA of the ovule, and low expression in fiber development stages (Supplementary Figures 5C,D). Different expression profiles of the MACPFs suggested that they widely participated in the various development stages of cotton. Furthermore, the expression pattern of the MACPFs in
the allotetraploid cotton was regulated by cold, heat, salt, and drought stresses (Figure 4). Most of the selected GhMACPFs were predominantly expressed at 24 h under cold treatment, especially GhMACPF04, GhMACPF13, GhMACPF26, and GhMACPF35. We further carried out qRT-PCR to confirm the RNA-seq data after the PEG and cold treatments (Figure 5). The results suggested that GhMACPFs were involved in response to the cold and PEG stresses in cotton.

Co-expression analysis was constructed to uncover the potential interaction of functional genes with the MACPFs in cotton under cold treatment. Figure 6A displayed nine GhMACPFs, including GhMACPF06, GhMACPF07, GhMACPF10, GhMACPF19, GhMACPF26, GhMACPF27, GhMACPF31, GhMACPF34, and GhMACPF39, which might be co-expressed with the AP2, GRAS, VQ, WRKY, and C2H2 TFs, which are widely involved in cold stress response (Supplementary Table 8). These results were confirmed with qRT-PCR analysis (Supplementary Figure 6). Moreover, Figure 6B (GhMACPF01, GhMACPF03, GhMACPF04, GhMACPF23, and GhMACPF35), Figure 6C (GhMACPF02, GhMACPF08, and GhMACPF11), and Figure 6D (GhMACPF18 and GhMACPF33) showed the other 10 GhMACPFs that were also participated in the cold responding interaction networks of TFs including AP2, p450, C2H2, basic helix-loop-helix protein (bHLH), and VQ domain-containing genes (Supplementary Table 8). GhMACPFs might interact with AP2, VQ, and WRKY TFs to enhance the cold resistance regulation of cotton.

Silencing GhMACPF26 Increased Tolerance to Cold Stress
To investigate the role of GhMACPF26 in response to cold stress, we performed a VIGS assay to decrease the GhMACPF26 expression in TM-1 plants. The albino phenotype ensured the success of the tobacco rattle virus (TRV)::CLA1 in cotton (Figure 7A) and the comparison of the expression level of TRV::00 and TRV::GhMACPF26 in cotton indicated that the gene expression had been successfully silenced (Figures 7B–D). TRV::GhMACPF26 and TRV::00 plants were subjected to cold stress for 24 h. The empty control plants (Figure 7B) and TRV::GhMACPF26 (Figure 7C) were similar before the cold treatment, but they contained MDA content that was significantly different based on the t-test analysis (Figure 7E). The phenotypic difference in the degree of leaf damage was clear between the TRV::00 (Figure 7F) and TRV::GhMACPF26 (Figure 7G) after cold treatments for 24 h. The MDA content in TRV::00 plants...
FIGURE 5 | Expression levels of 12 GhMACPF genes in response to the (A) cold and (B) PEG treatment. Gene expression was analyzed by quantitative real-time-PCR (qRT-PCR). Error bars represent the SD of the three biological replicates.
FIGURE 6 | The correlation network of 19 GhMACPF members in cold stresses. All the gene networks are constructed by the weighted gene co-expression network analysis (WGCNA) in which each node represents a gene. (A) (nine GhMACPFs), (B) (five GhMACPFs), (C) (three GhMACPFs), and (D) (two GhMACPFs) indicate GhMACPFs in the different modules.

was about 1.6 times more than in TRV::GhMACPF26 content (Figure 7H). Additionally, the MDA content was examined to the degree of cell damage (Jouve et al., 1993). Our results preliminarily proved that silencing of the GhMACPF26 gene improves cold tolerance in cotton.

DISCUSSION

Many MACPF domain-containing proteins were involved in the innate and adaptive immunity against pathogens through multiple pathways (Esser, 1994; McCormack et al., 2013; Spicer et al., 2017; Chen T. et al., 2020) and more and more pieces of evidence showed that the MACPF genes might participate in plant immune system, development, and abiotic stresses (Yu et al., 2020). In this study, 184 MACPFs were identified through a comprehensive analysis among the 15 genomes. Transcriptomic and co-expression analysis revealed that the MACPF genes were involved in the cold stresses, while silenced GhMACPF26 enhanced cotton plant tolerance to cold stress. These results indicated that the MACPF genes might play an important role in cotton adaptation to abiotic stress.
FIGURE 7 | Silencing GhMACPF26 via virus-induced gene silencing (VIGS) enhances the cold resistance regulation of cotton. (A) Plant albino phenotypes of TRV::CLA. (B,C) Phenotypes of TRV::00 and TRV::GhMACPF26 before cold stress. (D) GhMACPF26 expression levels in leaves of TRV::00 and TRV::GhMACPF26 plants. (E) The malondialdehyde (MDA) content of TRV::00 and TRV::GhMACPF26 before cold stress. (F,G) Phenotypes of TRV::00 and TRV::GhMACPF26 after cold treatment for 24 h. (H) The MDA content of TRV::00 and TRV::GhMACPF26 after cold stress. *p < 0.05, **p < 0.01, and ***p < 0.001 (Student’s one-tailed t test).

Phylogenetic, Duplication, and Structural Characteristics of the MACPFs in Gossypium spp.

The WGD or polyploidization is important for genome evolution due to the neofunctionalization and subfunctionalization of redundant genes (Blanc and Wolfe, 2004; Jiao et al., 2011; Qiao et al., 2019). Polyploidy on selection and domestication among the Gossypium spp. drives parallel gene expression in the development and abiotic stresses (Hu et al., 2019; Chen Z.J. et al., 2020). In this study, we found that the MACPF genes were shared with conserved gene numbers and similar duplication events by comparing the allotetraploid and diploid cotton. Totally, 38 GhMACPFs, 33 GbMACPFs, 14 GaMACPFs, and 15 GrMACPFs were identified from the four Gossypium spp. through a comprehensive gene family analysis, respectively. The number of the MACPFs in the allotetraploid cotton was almost two times the number in diploid cotton. The duplication event analysis showed that the WGD event was likely leading to the expansion of the MACPF genes in the allotetraploid cotton. Meanwhile, 184 MACPF genes were divided into three groups, of which 4 AtMACPFs and 7 OsMACPFs were evenly distributed in the Group I and Group II. The results showed that group I was conserved and belonged to the mallow (Figure 1). The conserved motif analysis in Gossypium spp. indicated that the “(F/Y)GTH(F/Y)-X6-GG” motif was widely distributed in GhMACPFs, GbMACPFs, GrMACPFs, and GaMACPFs (Figure 2 and Supplementary Figure 3). Orthologous analysis suggested that the evolution of the MACPF genes was not balanced between the allotetraploid and diploid genomes and there were more genes in the Dt subgenomes (Figure 3 and Supplementary Figure 4). The imbalance transcript indicated that the donors of the Dt subgenomes and G. raimondii were important to the abiotic stresses in the allotetraploid cotton and in the Brassica napus allotetraploids (Wang et al., 2019; Meng et al., 2020; Zhang et al., 2020). In addition, Ka/Ks values showed that most gene pairs have undergone purification selection during evolution (Supplementary Table 6) and the purification selection in WRKY and plant homeodomain (PHD) gene family (Hurst, 2002; Gu et al., 2018; Wu et al., 2021). These results suggested that the MACPF genes have been replicated in the ancient genome duplication events.

Membrane Attack Complex/Perforins Play Important Roles in Abiotic Stress

Cis-regulatory elements within the promoter analysis are important to understand transcriptional regulation, which contained the development regulation and environmental responses (Chen et al., 2018; Yan et al., 2019). Cis-acting elements on the promoter were recruited and bound by TFs and the expression of the gene was regulated (Liu et al., 2013).
As the previous results, the ABREs (ABA responsive elements) induced the expression of genes involved in the ABA signaling pathway (Hossain et al., 2010), while the GARE motif functions as a gibberellin-response element, TGA element acts as an auxin-response element, TGACG motif is the MeJA-response element (Chen and Qiu, 2020, and LTR is the low-temperature responsiveness element (Yamaguchi-Shinozaki and Shinozaki, 1994). ABRE, TGA, GARE- motif, CGTCA motif, TGACG motif, LTR, MBS, MBSI, and TC-rich repeats were also found in the MACPF promoters. These cis elements indicated that they participated in the different regulatory mechanisms of environmental adaptation including abiotic stress response (Supplementary Figure 1). Interestingly, the expression of the GhMACPFs and GbMACPFs widely responded to various abiotic stresses including the salt, PEG, cold, and heat treatments (Figure 4). In addition, most GhMACPFs and GbMACPFs were highly expressed in various tissues, and the ovule or fiber development, suggesting that the MACPFs also play an important role in the differentiation and fiber development of cotton tissue (Supplementary Figure 5). As reported in rice, maize, and Arabidopsis (Yu et al., 2020), MACPF genes were widely involved in abiotic stress response via the complex potential mechanism in Gossypium spp.

The Co-expression network was constructed to explore the potential connectivity of the genes involved in the plant development and abiotic stress response (Zhang and Horvath, 2005; van Dam et al., 2018; Chen P. et al., 2020; Cheng et al., 2020; Hu et al., 2020; Wang et al., 2020). TFs, including AP2/ERF, GRAS, VQ, and WRKY families, have recently been subjected to an intensive investigation because of increasing evidence of their response to abiotic stress (Mizoi et al., 2012; Grimplet et al., 2016; Gu et al., 2018; Chen P. et al., 2020). Constructing expression regulatory networks related to TFs is important for mining the candidate functional genes. Several GhMACPFs, including GhMACPF6, GhMACPF7, GhMACPF10, GhMACPF19, and GhMACPF26, were predicted to interact with AP2/ERF, WRKY, and VQ TFs (Figure 6 and Supplementary Table 8). The results indicated that the MACPF genes might act together with these TFs under cold stress in cotton.

Previous studies have shown that cold stress not only led to a decrease in growth and development but also affected plant metabolism and phytohormones (Thakur et al., 2010). Silencing GhMACPF26 indicated that the VIGS plant enhances the cold resistance via the measure of the MDA content (Figure 7). The MDA content in TRV://0 was significantly higher than the TRV://GhMACPF26 after 24 h cold treatment, indicating that the plasma membrane damage is more serious in the TRV://0 plant (Figure 7). The MDA content is usually used to measure the degree of damage to plant cells and it also acts as the product of lipid oxidation (Leshem, 1987; Jouve et al., 1993). Transcriptomic results suggested that GhMACPF was also participating in the regulation of cotton tissue, ovule, fiber growth, and development. Moreover, we hypothesized that the MACPF members related to cotton resistance, especially resistance to cold stress. However, the underlying molecular mechanism requires further elucidation.

CONCLUSION

In this study, a comprehensive analysis of the MACPF family was performed in the diploid and allotetraploid cotton via the phylogenetic, structural, orthologous, and transcriptomic analysis. Our data revealed that the WGD events might be the MACPF genes expansion in the allotetraploid cotton and genes on the Dt subgenome were related to stress resistance. Cis element, expression, and VIGS results indicated that the MACPF genes were activated in cotton response to cold stress. Co-expression analysis predicted that the GhMACPF genes might interact with AP2/ERF, WRKY, and VQ TFs to enhance the cold resistance of cotton. Silenced GhMACPF26 increased the cotton tolerance to cold treatment. This study could provide new insights into the MACPF gene function in cotton and their potential interactions with other TFs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

HW and SY designed the experiments. PC, HJ, and FW collected the sequences and analyzed the transcriptome analysis. AW, TH, XL, XG, JL, and LM participated in cotton culture processing and RNA extraction. LG, FW, and HW revised the language. PC performed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.684227/full#supplementary-material
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