Multi-responses of O-methyltransferase genes to salt stress and fiber development of Gossypium species

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Abstract

**Background:** O-methyltransferases (OMTs) are an important group of enzymes that catalyze the transfer of a methyl group from S-adenosyl-L-methionine to their acceptor substrates. OMTs are divided into several groups according to their structural features. In *Gossypium* species, they are involved in phenolics and flavonoid pathways. Phenolics defend the cellulose fiber from dreadful external conditions of biotic and abiotic stresses, promoting strength and growth of plant cell wall.

**Results:** An OMT gene family, containing a total of 192 members, has been identified and characterized in three main *Gossypium* species, *G. hirsutum*, *G. arboreum* and *G. raimondii*. Cis-regulatory elements analysis suggested important roles of OMT genes in growth, development, and defense against stresses. Transcriptome data of different fiber developmental stages in Chromosome Substitution Segment Lines (CSSSLs), Recombination Inbred Lines (RILs) with excellent fiber quality, and standard genetic cotton cultivar TM-1 demonstrate that up-regulation of OMT genes at different fiber developmental stages, and abiotic stress treatments have some significant correlations with fiber quality formation, and with salt stress response. Quantitative RT-PCR results revealed that GhOMT10 Dt and GhOMT70 At genes had a specific expression in response to salt stress while GhOMT49 At, GhOMT49 Dt, and GhOMT48 At in fiber elongation and secondary cell wall stages.

**Conclusions:** Our results indicate that O-methyltransferase genes have multi-responses to salt stress and fiber development in *Gossypium* species and that they may contribute to salt tolerance or fiber quality formation in *Gossypium*.

**Background**

Cotton (*Gossypium* Species) has the importance for natural fiber all over the globe. The primary goals of upland cotton (*G. hirsutum*) perspectives have been always to achieve better quality with higher yield [1]. Mostly *G. hirsutum* bears staple fibers 25-40 mm in length and 15 μm in thickness at their full maturity. Fiber cells must undergo four distinct but partially overlapped developmental stages, including initiation, elongation, secondary cell wall deposition, and maturation. The secondary cell wall of fiber, which is mainly composed of cellulose, is important especially for fiber quality perspective. However, some studies have shown that secondary cell wall of fibers of flax (*Linum usitatissimum* L.), ramie (*Boehmeria nivea* L.), and Spanish broom (*Spartium junceum* L.) also contain phenolics along with cellulose. Their fibers are known for their physical properties such as length and strength and have been used for textile purposes. A thicker secondary cell wall was estimated to contain no less than 70% cellulose content while the cotton fiber contains almost 90% cellulose [2, 3]. Lignin is another important component in cell wall [4]. It provides strength to plant cell wall and response to biotic and abiotic stresses in vascular plants [5]. The presence of lignin, which is reported at lower level in secondary cell wall of cotton fibers [6], negatively regulates fiber elongation and secondary cell wall synthesis in cotton. Studies demonstrated that the cotton plants that accumulate less lignin and lignin-like phenolics in mature fibers tend to have longer and stronger fibers [7]. From an active perspective, lignin and phenolics defend the cellulose fiber against dreadful conditions and increase the ability of response to biotic and abiotic stresses, and thus influence the growth and strength of plant cell walls [8]. Previous studies in herbaceous plants demonstrated the involvement of O-methyltransferases (OMTs) in lignin biosynthesis [9]. The involvement of OMTs mediate normal plant growth in the presence of lignin [10]. The initial OMT cDNA was described in 1991 [11], then a series of OMT cDNAs have been cloned from diverse plants species, including *Zea mays*, *Arabidopsis thaliana*, *Iris hollandica*, and *Nicotiana tabacum* [12].

According to substrate classification, plant methyltransferases have three major categories, I. O-methyltransferases (OMTs), II. N-methyltransferases (NMTs), and III. C-methyltransferases (COMTs). Category I OMTs are further classified into five sub-categories. Sub-category I-a comprises caffeoyl coenzyme A 3-O-methyltransferase (CCoAOMT) and caffeic acid 3-O-methyltransferases (COMTs), which are involved in methylation in phenylpropanoids. Sub-categories I-b, I-c, and I-d act in methylation of hydroxyl in flavonoid, alkaloids, and myoinositol, respectively. The fifth sub-category I-e takes part in methylation of carboxyl of diverse acids. The results of a study discovered the crystal structure of OMTs from *Medicago sativa* [13]. In the light of the explanations, the OMT gene that was cloned and characterized from a medicinal plant *Ligusticum chuanxiong* and contained higher ferulic acid was named as *LcCOMT*. The differential expression of *LcCOMT* gene under chilling stress was more than 6-fold higher than that under controlled conditions, suggesting that ferulic acid may increase plant tolerance to cold stress. BLAST analysis showed that *LcCOMP* was 23.9–40.2% similar to OMTs of alkaloid, flavonoid, isoavonoid, and phenylpropanoids [14].
In the whole life cycle of cotton plant, it undergoes various environmental conditions from the cold spring in April when it is sowed to hot mid-summer when it grows rapidly in vegetation and reproduction and to late freezing autumn when it gets mature and is harvested. During the whole growth procedure, the cotton plant maintains an exquisite molecular controls and regulations. But little is known what roles the OMT family genes have played in cotton plant especially in early or late growth stage when season transition occurs, or in various stress conditions. Therefore, in this study, we identified the OMT family genes in the genome-wide scale and made detailed bioinformatics analysis of gene structure, chromosomal distribution, selection pressure during their evolution, sub-cellular localization, cis-regulatory elements etc, together with their expression profiling in different developmental stages and in responses to various stresses. Their expression profiling in developing fiber cells was verified using RNA sequencing data from RILs, CSSLs, and TM-1 at different fiber development stages. This study could open the way to comprehend the functions of OMTs in fiber quality advancement and in cotton plant responses to abiotic stresses, and thus could assume a noteworthy part for further investigation in the molecular mechanism of fiber improvement and stress tolerance.

Results

Genomewide identification and characterization of OMT genes

A genome wide analysis was conducted to characterize OMT family genes in three Gossypium species. A total of 192 OMT members were identified, including 82 in G. hirsutum, 55 in G. arboreum, and 55 in G. raimondii (Table S3. Sheet A). For phylogenetic analysis [15], 33 OMT members in A. thaliana, and 26 members in T. cacao species were also retrieved (Table S3. Sheet B). Retrieving information of OMT genes in G. hirsutum revealed that GhOMT75_Scaf, which was detected in scaffold, coded the smallest protein of 62 amino acids (aa) with a molecular weight of 6.642 kDa. While GhOMT33 Dt, which was identified on chromosome D02, coded the largest protein of 969 aa with a molecular weight of 108.296 kDa among all OMT members in three Gossypium species.

In domain analysis of OMT family genes in Gossypium species, the results revealed that 64, 45 and 47 OMTs in G. hirsutum, G. arboreum and G. raimondii contained Pfam domain Pf00891, and that only 20, 10 and 9 OMTs in G. hirsutum, G. arboreum and G. raimondii contained Pfam domain Pf01596. In A. thaliana and T. cacao, 25, 24 OMTs contained Pf00891 domain, and 8, 2 OMTs contained Pf01596 domain respectively.

Chromosomal distribution, collinearity, duplication, and loss of OMT genes

The analysis of chromosomal positioning was performed by using TBtool software [16]. A total of 161 OMT genes were positioned on their respective chromosomes, while seven of G. raimondii, one of G. arboreum, and 23 of G. hirsutum were positioned in scaffolds (Figure S1). In G. raimondii (D genome), chr11 was mapped with 13 genes followed by chr08 with nine genes. The minimum number of genes in a chromosome was one in chr2, chr6, and chr10 respectively. There was no OMT family members identified in chr01 and chr07 (Figure S1.a). In G. arboreum (A-genome) (Figure S1.b), 54 OMT genes were mapped in all chromosomes except chr1. Chr10 harbored 13 OMT genes which were the highest per chromosome, followed by chr12 and chr04 with 10 and 9 genes respectively. The minimum number of genes located in a chromosome was one in chr02 and chr11 respectively. In G. hirsutum (A4D1 genome) (Figure S1.c), unexpectedly, there were no OMT genes in A02, A05, A07, D03, D09, and D11 chromosomes. The distribution of genes in D4 sub-genome (33 genes) was higher than in A4 sub-genome (26 genes). The maximum number of genes in a chromosome was seven in D04 and A12, followed by four in D10 and A10 chromosomes, respectively. D01, D05, A01, A06, and A11 only had one OMT gene, and D06, D07, A03, A08, A09, A13 two OMT genes and D02, D08, and D13 three OMT genes respectively (Figure S4.c). A collinearity analysis of the OMT family genes in Gossypium species chromosomes was shown in Figure 1. The results demonstrated a pair wise collinearity of OMT genes between the chromosomes on which OMT family genes were mapped. Noticeably, a number of available genes in A4 and D4 scaffolds were collinear with their homologues in A and D genomes suggesting the collinearity of the DNA fragments between the scaffolds and chromosome where these OMT genes locate (Figure 1). Taken the OMT gene numbers identified in each A/D genome or A4/D1 sub-genome, collinearity analysis also revealed that there were totally 21 and 19 OMT genes exclusively detected in A and D genomes respectively. Their homologous counterparts in A4D1 genomes of G. hirsutum are lost. There are also a few OMT genes that are exclusively detected in A4D1 genome of G. hirsutum without homologous counterparts in A and D genomes (Figure S1).
According to previous studies there are five types of duplications including singleton, dispersed, proximal, tandem, and segmental or whole-genome duplication [17]. In the present study, the analysis of gene pairs duplication events predicted a total of 31, 28, and 54 gene pairs of D_D, A_A, and D_A genomes from their common ancestor, 33 gene pairs of A_D subgenomes in segmental duplication, and 5 gene pairs of A_D subgenomes in tandem duplication events (Table S4 Sheet A).

**Analysis of selection pressure**

In genetics, the Ka/Ks ratio used to estimate the balance between neutral mutations, purifying selections, and positive mutations based on a set of homologous genes [18]. The ratio of the number of non-synonymous substitutions per non-synonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks) represents selection pressure of the gene [19]. Ka/Ks<1 demonstrates purifying selection pressure, while Ka/Ks=1 and Ka/Ks>1 show neutral and positive selection pressures respectively. Analysis of Ka/Ks ratio of homologous OMTs in three Gossypium species revealed that they are under purifying selection pressure. The Ka/Ks ratio of homologous OMTs in G. raimondii and G. arboreum ranged from 0.09 to 0.8, in G. raimondii and G. hirsutum ranged 0 to 0.7, and in A and D of G. hirsutum ranged 0.4 to 0.7 (Table S4 Sheet B).

**Sequences alignment, phylogenetic Analyses, conserved motifs and gene structure**

The sequence alignment of 251 OMT genes, including 192 genes from three Gossypium species, 33 from A. thaliana, and 26 from T. cacao species was performed to understand the phylogenetic relationship of these genes. The evolutionary relationship of OMT genes in three Gossypium species was monophyletic (Figure 2.a), and the member of A. thaliana and T. cacao were distributed in paraphyletic manner (Figure 2.b). According to the topology of constructed tree, the OMT gene family is divided into five clades (I, II, III, IV, and V) in Gossypium, A. thaliana, and T. cacao species. The results showed that each clade of OMT genes were symmetrically distributed within Gossypium species (Figure 2.a), while in A. thaliana and T. cacao, OMT genes were identified in cluster forms (Figure 2.b). The results demonstrated that these Gossypium OMT members might be evolutionary close within respective species and their identified clades.

To examine the conserved motifs of each clade, the analysis of representative motif logo and conserved motifs prediction were conducted (Figure S2). The results revealed that motif 1, enriched with leucine, valine, and glycine, motif 2, enriched with leucine and valine, and motif 3, motif 4, motif 5, and motif 6 were common in clades I, II, III, IV and clade V. While motif 7 was found missing in some members of clade V, which was then replaced with motif 8 at same positions (Figure S2). The enriched amino acid residues of conserved motif1 (L/VDVGGG/TG) was previously identified in S-adenosyl-l-methionine (SAM)-dependant OMTs that shared 95% similarity with G. hirsutum OMT [20].

Investigation of gene structure has uncovered the different number of exons and introns of OMT genes. Exon and intron number of OMT genes varied from the least one exon and no intron to the most 7 to 9 exons and 6 to 8 introns (Table S5). Two members in G. hirsutum including GhOMT82_At and GhOMT82_Dt contain nine exons as the highest (Table S5). Same as, two members including GaOMT82_A in G. arboreum, and GrOMT82_D in G. raimondii contain 9 exons (Table S5). Gene structure analysis revealed that the OMT genes with higher number of exons had a shorter exons and introns, and vise versa. These results demonstrated that OMT members possess different structural patterns in accordance with their features.

**Identification of cis-regulatory elements in OMT family**

The promoter regions of the OMT family contain precisely a large number of cis-regulatory elements. The analysis of cis-regulatory elements revealed the enrichment of MYB cis-regulatory elements, which was detected more than 350 times in OMT genes (Figure S3). The MYC was another important element that was found 183 times in enlisted OMT genes. Box 4 (part of a conserved DNA module involved in light responsiveness) was found 152 times in 43/82 genes in G. hirsutum. ABRE elements was detected 119 times in 29/82 OMT genes in G. hirsutum. The ERE element was detected 113 times in 37/82 and G-Box 97 times in 37/82 G. hirsutum OMT genes. An auxin RR-core and cis-acting regulatory element involved in the MeJA-responsiveness (TGACG-motif) were also observed in Gossypium OMT genes where this element was identified 48 times in 25/82 genes. Some other important cis-regulatory elements including wun-motif 44 times in 26/82, W-box 39 times in 31/82, GATA-motif 32 times in 27/82, O2-site 30 times in 22/82 OMT genes respectively, in G. hirsutum (Figure S3). These cis-regulatory elements might function collectively in accordance with their specific roles and with specific conditions as well as growth and development stages (Figure S3).
Sub-cellular localization prediction of OMT genes

Understanding and determining the sub-cellular localization of proteins is an important strategy to identify the function of protein at cellular level [21]. This approach includes proteomic-based experiments and microscopic high throughputs [22, 23]. Several sequence-based approaches have been developed to predict the sub-cellular localization by providing amino acid sequences including PSORT [24], Yloc [25], BaCelLO [26], LOCtree [27]. According to CELLO prediction, most of OMT genes were located in the cytoplasm (Table 1), while seven genes were predicted in periplasm, including, GhOMT45_At, GhOMT45 Dt, GhOMT46 Dt, GhOMT48 At, GhOMT48 Dt, GhOMT49 At, and GhOMT49 Dt. Five OMTs were predicted to be localized in both periplasm and cytoplasm, including GhOMT47 At, GhOMT47 Dt, GhOMT53 At, GhOMT54 Dt and GhOMT68 At. Two genes GhOMT82 At and GhOMT82 Dt were predicted in the outer membrane. Only GhOMT55 At was predicted in inner membrane and cytoplasm (Table 1). The results of Wolf Psort were highly in agreement with those of CELLO analysis regarding the presence of most of the OMT genes in cytoplasm, however, with exceptions of GhOMT48 At, GhOMT82 At, GhOMT82 Dt, which were predicted in chloroplast and one gene GhOMT76 Dt in mitochondria (Table 1). The function of the OMT genes might be related to their predicted localizations, though the experimental approach is still needed for further confirmation.

GO enrichment and KEGG pathway analyses

To understand the functional annotations of OMT family genes of G. hirsutum, 82 genes in G. hirsutum were undergone through gene ontology (GO) enrichment, kyoto encyclopedia of genes and genomes (KEGG Pathway), and InterPro analyses. GO term analysis verified their O-methyltransferase activity of all 82 OMT genes, while 62 of the 82 genes were also enriched in methyltransferase activity and 53 of the 82 genes in protein dimerization activity (Figure 3a). KEGG Pathway analysis revealed that these OMTs were involved in different metabolic pathways. Twenty-nine OMTs were involved in monolignol biosynthesis, phenylpropanoid, secondary metabolism, and metabolic pathways respectively. Eleven genes were involved in phenylalanine and flavonoid biosynthesis pathways respectively (Figure 3b). InterPro analysis (http://www.ebi.ac.uk/interpro/) categorized these 82 OMT genes as functional genes of S-adenosyl-L-methionine-dependent methyltransferase (Figure 3c). Sixty-two genes were also predicted in categories of methyltransferase_2 and O-methyltransferase COMT-type respectively (Figure 3c), while fifty-seven in winged helix-turn-helix DNA-binding domain, fifty-three in plant methyltransferase dimerization (Figure 3c).

Expression profiling of OMT genes and their homologues in fiber development and salt stress

In order to verify the biological functions of OMT family genes, several transcriptome data sets including TM-1 [28], G. arboreum, G. raimondii, CSSLs [29], and RILs [30], were applied to analyze their expression profiles in different developmental stages, organs, or tissues, and responses to various abiotic stress treatments. The transcriptome clusters showed that the OMT genes can be assorted into three basic groups (Figure 4A): Those that have a broad responses to different developmental stages from germination to fiber maturation, typical examples of which included GhOMT48 At, GhOMT48 Dt, GhOMT49 At and GhOMT49 Dt; those that have specific responses to root development, including GhOMT1 At, GhOMT2 At and GhOMT40 At; and those that have responses to early germination in seed, cotyledon, root and stem, including GhOMT47 At, GhOMT9 Dt, and GhOMT58 Dt. When fiber specific transcriptome data sets of G. arboreum, G. raimondii were applied to observe the expression profiling diploid OMT family genes, the result also supported specific expression profiling of some OMT genes in diploid species of G. arboreum (Figure 4B) and G. raimondii (Figure 4C).

The gene expression profiling was further verified with transcriptome datasets of RILs (Figure 5A) two CSSLs (Figure 5B and 5C). The results showed that the genes that had specific expressions during fiber development (Figure 4A) also had specific expressions in fiber development of RILs and CSSLs materials. These genes had a highly consistent expression profiling among the different cotton cultivars and lines during fiber development. Some selected GhOMT examples genes, GhOMT49 At (Figure 5D), GhOMT70 At (Figure 5E), GhOMT48 At (Figure 5F), GhOMT10 Dt (Figure 5G), and GhOMT49 Dt (Figure 5H), were verified through qRT-PCR using sGK9708 and 0-153, the two parental lines of the RIL population with different fiber quality traits. The results showed that GhOMT48 At, GhOMT49 At, and GhOMT49 Dt were significantly up-regulated during fiber development in sGK9708 than in 0-153 (Figure 5D, 5F and 5H) and that GhOMT70 At and GhOMT10 Dt did not show differences between the two cultivars (Figure 5E and 5G). Noticeably, GhOMT49 At and GhOMT49 Dt reached the highest expression levels at 20 DPA and their high expression lasted in a short time as compared with that of GhOMT48 At. GhOMT48 At had a rapid expression increase from 10 DPA to 15DPA and then its expression steadily increased until 25 DPA when it reached its highest expression level.
Based on the expression profiling of the OMT gene family in responses to cold, hot, osmotic, and salt stress treatments (Figure 6.A), two genes specific in salt stress responses, GhOMT7_0_At and GhOMT10_0Dt, and three genes specific in fiber development, GhOMT48_0At, GhOMT49_0At, and GhOMT49_0Dt, were verified by qRT-PCR with RNA samples extracted from salt treatment. The results indicated that both GhOMT7_0At and GhOMT10_0Dt showed an elevated expression in salt treatments in salt-tolerant cultivar as compared to the control treatments (Figure 6.B and 6.C). These two genes had different expression profiles from 2h to 6h after salt treatment. GhOMT7_0At had the highest expression at 2h and then its expression went down at 6h; whereas GhOMT10_0Dt had an increasing expression pattern from 2h to 6h. Both genes had much higher expression in roots than in stem or leaf.

**Discussion**

**A genome-wide survey of OMTs**

A genome wide search of *G. hirsutum* [28], *G. arboreum* [31], and *G. raimondii* [32] resulted in the identification of 192 genes (82 in *G. hirsutum*, 55 in *G. arboreum*, and 55 in *G. raimondii*). Recent study testified that modern allotetraploid *Gossypium* species were developed from a natural hybridization between the ancestors of two diploid species of *G. raimondii* (D-genome) [33] and *G. arboreum* (A-genome) [31] 1.7 to 1.9 million years ago [28]. The results of current study revealed a loss of quite a large number of OMT genes in *G. hirsutum* A_D1 genome as compared to the total number of OMT genes in A and D genomes. Possibly 19 OMT genes in A1 sub-genome and 17 in D1 sub-genome in *G. hirsutum* (Figure S1) were lost during the evolution procedure after it arose from above mentioned hybridization [28]. Gene losses can be the result of premature stop codon, disruption of genes as compared to their orthologous [34], and rapid genome re-organization during polyploidization and diploidization process [35-37]. Previous studies have evidenced that polyploidization processes may result in losing of homologous members or altered expression profiles of the homologous genes or both [38-41]. Similar phenomenon was noticed in the expression profiling of homologous OMT genes between A1 and D1 in *G. hirsutum*, which clued that these genes might have experienced abovementioned events during evolution processes. Collectively, a higher number of genes were also identified in whole genome duplication event. The whole genome duplication may have resulted from an organism that inherited two genomes from each parent. Whole genome duplication events results duplicate genes that may lost through fractionation [42]. Besides the whole genome duplication, segmental duplication events were also identified with a large number in OMT gene pairs. Segmental duplication is widespread in flowering plants, which might lead to the evolution of novel genes and their functions [43].

Phylogenetic analysis showed high similarity and monophyletic distribution of OMTs within *Gossypium* species that might support the conservative evolution mode of OMT genes within five phylogenetic clades. Previous study also reported five clades of OMT genes in *Catalpa bungei* [44]. Analyses of selection pressures revealed that most of OMT genes in *Gossypium* species were under a purifying selection pressure. The purifying selection pressure might suggest the importance of OMT genes in *Gossypium* species. But noticeable exceptions were also observed in some interspecific homologous pairs, in which their Ka/Ks values were >1, indicating these homologous pairs were under a positive selection pressure. These homologous pair exceptions included GrOMT52_D-GhOMT52_Scaf and GrOMT29_D-GhOMT29_At in *G. raimondii* and *G. hirsutum*, GaOMT30_A-GhOMT30_At in *G. arboreum* and *G. hirsutum*, GrOMT63_D-GaOMT64_A and GrOMT29_D-GaOMT29_A in *G. raimondii* and *G. arboreum*. These results suggested that the OMT genes might have experienced positive selection pressures during the evolution from diploids to tetraploids. Previous studies have evidenced that the positive selection pressure might be associated with the onsets of new functions in genes [45, 46]. Considering the fact that quite a proportion of OMT genes were lost during the formation and evolution of allotetraploid cotton (see afore discussion and Figure S1). In the current study, six OMT family members in *G. hirsutum*, one in *G. arboreum* and nine in *G. raimondii* were characterized as (R,S)-reticuline 7-O-methyltransferase. 7OMTs convert reticuline to laudanine in tetrahydrobenzylisoquinoline biosynthesis in the opium poppy *Papaver somniferum*, however, this enzymatic activity is unknown in most higher plants [47]. Therefore, how these genes function is still open to discussion. Taken all findings together, the results might suggest that the OMTs that experienced positive selective pressure be lost or take on some novel functions in *G. hirsutum* during the processes of its evolution and ancestor formation.

Previous findings have reported that the *G. raimondii* (D-genome) and *G. arboreum* (A-genome) are the closest relatives to the D1 and A1 sub-genomes of allotetraploids, respectively [28]. Each gene in A or D genome will always have a homolog in the correspondent A1 or D1 sub-genomes of *G. hirsutum* [48]. However, in both A and D genomes we detected quite a large number of OMT genes that do not have homologs in their relative A1 and D1 sub-genomes (Figure S1). Previous studies evidenced that such
homolog loss could result from two possible reasons: one is that the homologs were lost during the procedure of polyploidization from diploids to tetraploid; the other is that after the tetraploid formation, the OMT members in each genome started their separate evolution procedure. This separate evolution procedure makes the newly evolved members have no homologs in its relative genomes [28]. Previous studies revealed that in A, D, A_D, genomes do not maintain same speed of evolution. A faster evolution rate was observed in allotetraploid cottons than in diploid cottons [28]. Taken the fact that OMT genes undergo purifying selection procedures (Table S4. Sheet B), the first reason is possibly endorsed as the main cause for the current evolution status of OMT gene family and the second reason may also played a role.

**Function prediction of OMT candidates**

**OMTs are involved in diverse cis-regulatory elements**

Plants encounter various biotic and abiotic stresses during their entire life cycles that negatively affect growth, development, and productivity [49]. Under exposure of these stresses, plants require some potential mechanism, which can be activated in critical circumstances, to support whole plant life cycle [50]. Excessive salinity is also a major factor that affects the cotton production all around the world [51]. Identification of cis-regulatory elements revealed that the OMT genes are enriched with important cis-regulatory elements that are essential against negative environmental stresses. Some important regulatory elements, including W-box, MYB, MYC, DRE, ABRE, G-Box, MBS [52], were identified in OMT genes. W-box is important to regulate the expression of genes and to bind WRKY TFs. WRKY TFs are important to mediate plants to defense against chilling, wounding, drought, salinity and heat stresses [53-61]. MYB and MYC have been identified as involved in dehydration-response [62]. DRE [63], which up-regulate gene expression under cold stress and increase the tolerance of plants was also identified in these specific genes. ABRE is an important regulatory element that enhances salt stress tolerance in plants. It plays a key role in dehydration and in response to salinity stress in *Arabidopsis thaliana*, soybean and rice, and in response to chilling or cold in *Paeonia suffruticosa* [64]. G-box is identified in several gene promoters in previous studies and it contributes to development, hormone response, and tolerance against fungal infections in plants. Besides, a gibberellins response element (GARE) was also identified to be important to promote flowering in plants. The auxin hormones play a major role in growth and development of diverse plant species [65]. These results were in accordance with our findings. Especially the repetitively identified cis-regulatory elements might have biological functions in plants under specific conditions and development stages.

**OMTs are possibly involved in secondary metabolic pathways**

The KEGG pathways enrichment analysis revealed the involvement of OMT genes in secondary metabolism and metabolic pathways including monolignol, phenylpropanoid, flavonoid, and phenylalanine metabolisms. Secondary metabolic pathways are demonstrated to have exceptional impacts on biotic and abiotic stresses. Secondary metabolites are phytochemicals, which are synthesized through secondary metabolism. In plants, phenylpropanoids are categorized in several groups such as phenolic acids, flavonoids, and lignins, which are involved in diverse physiological processes and tolerance under unfavorable conditions [66-70]. The activity of secondary metabolites increases during the response of abiotic stresses. These phenolics provide plants with higher tolerance against heavy metals [71, 72], salinity [73], drought [74], and temperature stresses [69]. These pathways also play an important role in plant cell elongations [75, 76]. Same as, plant OMT genes have been identified in secondary metabolism [77]. Higher expression of secondary metabolic pathways related genes in developing cotton fiber is reported in previous studies [78, 79]. Importantly, OMT genes were reported to be involved in lignin synthesis and to be induced by inoculation of *Verticillium dahliae* in cotton [80-82]. During the inoculation of pathogens, changes in the expression patterns of phenylpropanoid related OMT genes were identified. These identified OMT genes included *GhOMT53_At*, *GhOMT58_Dt*, *GhOMT61_Dt*, and *GhOMT78_Dt* that were found significantly expressed in 12 and 48 hours postinoculation *V. dahliae* [83]. In the current study, these genes were down-regulated under abiotic stresses and in fiber development stages (Figure 4). Previous reports have evidenced that desoxyhemigossypol-6-O-methyltransferase (dHG-6-OMT) catalyzed the biosynthesis of terpenoid and provided an effective defense mechanism to cotton plant against biotic stresses including insects and pathogens [84]. In response to *V. dahliae* (V991) in CSSLs lines CCR36 and MBI8255, diverse genes were found differentially expressed in lignin biosynthesis including *CCoAOMT*, which can adequately utilize lignin and has been characterized in several previous studies [85, 86]. Another study also reported that *CCoAOMT* was up-regulated in response to *Verticillium* pathogen in cotton and rendered cotton plants a comparable phenotypic resistance as compared to control plants [87]. A RNA-seq analysis based research identified differential expression patterns of *CCoAOMT* in response to *V.*
from cotton functional genomic database (Genome data of three)

**Methods**

Identification of *OMT* protein family members, sequences alignment, and phylogenetic tree construction

Genome data of three *Gossypium* species including *G. arboreum* (CRI), *G. raimondii* (JGI), and *G. hirsutum* (NAU) were downloaded from cotton functional genomic database (https://cottonfgd.org/) [99]. Genome data of *A. thaliana* (Athaliana/TAIR10,
https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Athaliana# [100] and T. cacao (Tcacao/v2.1, https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=Tcacao# [101]) were also downloaded for comparative analysis of OMT genes. The hidden Markov model profiles (PF00891 and PF01596) were downloaded from Pfam database (https://pfam.xfam.org/). The hmmsearch program of HMMER 3.0 software [102] was used to search for protein sequences of three Gossypium species with the E-value of 1e−5. OMT protein sequences of A. thaliana and T. cacao were also retrieved from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html) for phylogenetic analysis. The proteins with absence of required domains were manually removed. Other features of OMT genes including protein length (aa), and molecular weight (kDa) were characterized by using cotton functional genomic database (http://www.cottonfgd.org/) [99]. The full length amino acid sequences of G. hirsutum, G. arboreum, G. raimondii, A. thaliana, and T. cacao encoded by OMT genes were aligned with clustalx2 software (http://www.clustal.org/) [103] with default parameters for the neighbor-joining phylogenetic tree as 1000 bootstraps. Subsequently, two neighbor-joining phylogenetic trees were generated by using Mega7 [104]. The topology of both phylogenetic trees was confirmed to understand the phylogenic relationship within the five plant species.

Nomenclature of these members was based on their chromosomal locations and numbers in each Gossypium species.

**Chromosomal mapping and collinearity analysis**

TBtool was used to perform the chromosomal mapping of the given OMT genes, to search the homologous pairs of OMT genes between genomes of the three Gossypium species through protein-protein blast (E-value 1e-5). Circle gene viewer model of TBtool software was used to visualize the results of collinearity between homologous gene pairs [16].

**Gene structure and conserved motifs**

The structure of the OMT genes was analyzed using the online server of Gene Structure Display (GSDS 2.0, http://gsds.cbi.pku.edu.cn) [105]. The conserved motifs were predicted online in MEME web based motif prediction tool version 5.0.5 (http://meme-suite.org/) by providing protein sequences of OMT genes [106].

**Selection pressure, cis-regulatory elements, sub-cellular localization and gene enrichment analysis**

The CDS of homologous gene pairs of G. hirsutum (NAU), G. arboreum (CRI), and G. raimondii (JGI) were assigned to TBtools software to estimate the Ka/Ks ratio to predict selection pressure between the genes of each pair in genomes and sub-genomes [16]. The upstream sequences (2000bp) of OMT genes were retrieved through cotton functional genomic database (http://www.cottonfgd.org) and were submitted to PlantCARE database [107] to obtain the cis-regulatory elements. Sub-cellular localization of genes was predicted using online bioinformatics tools CELLO v.2.5 and Wolf Psort with their protein sequences [24, 108]. KEGG IDs of OMT family genes were downloaded from cotton functional genomic database (http://www.cottonfgd.org), then annotation was performed by providing KEGG IDs in kyoto encyclopedia of genes and genomes database (https://www.genome.jp/kegg/) [109]. Gene ontology (GO) annotation IDs of OMT family genes were downloaded from cotton functional genomic database (http://www.cottonfgd.org) and were submitted to Gene ontology database (http://geneontology.org/) to perform GO analysis [110].

**Expression profiling of OMT genes**

The different sets of RNA sequencing data including TM-1, a genetic standard line of G. hirsutum (Nanjing Agricultural University, Nanjing, Jiangsu, China) (PRJNA248163) [28, 41], 69307 and 69362 (selected lines from a RIL population sGK79708×0-153, Institute of Cotton Research, Anyang, Henan, China) (PRJNA542946) [30], MBI7747, MBI7561, and MBI7285 (selected lines from CSSL population CRI45×Hai1, SRP084203) [111, 112], and MBI9915 and MBI9749 (selected lines from CSSL population CRI36×Hai1, SRX2843778) (Institute of Cotton Research, Anyang, Henan, China) [29, 112] were included in this study to observe the expression pattern of OMT family genes at different growth stages, under abiotic stress treatment stages, ovule development, and in different fiber development stages of cotton. Briefly, 69307, 0-153, MBI7747, MBI7561, MBI9915, MBI9749, and Hai1 have high fiber quality traits, while 69362, MBI7285, sGK79708, CRI36, and CRI45 have low fiber quality traits. Detailed information of these referenced materials is presented in Table S1.
Transcriptome data of *G. arboreum* (PRJNA179447) [31], and *G. raimondii* (PRJNA79005) [33] were also included to compare the comparative expression of these OMT genes.

**Plant material, RNA isolation, cDNA synthesis, and qRT-PCR**

Upland cotton cultivar 0-153 had elite fiber quality while sGK9708 had high yield potential and wide adaptability. They are successfully used to tag fiber quality and yield QTLs in our previous reports [113-115]. In the current study, sGK9708 and 0-153 (Table S1) were planted in April 2018 in the experimental fields of the Institute of Cotton Research, Chinese Academy of Agricultural Sciences, Anyang, Henan. Flowers were tagged on the day of anthesis for fiber sampling in July 2018. Bolls of tagged flowers were sampled in the morning between 9:00 and 10:00 AM at 10, 15, 20, and 25 days’ post anthesis (DPA). The fibers were dissected from the developing seeds right after boll picking and immediately stored at -80 °C for RNA extraction.

To examine the expression profiling of OMT genes under salt stress, seeds of sGK9708 cultivar were germinated in wet filter papers for 72 hours and then were transferred to hydroponic conditions. The seedlings were treated with 200mM NaCl at three leaves stage. The true leaves, stems, and roots were sampled at 0 hr, 2 hrs, and 6 hrs of the treatment. The 0h of treatment was considered as control sample to compare the expression profiling with treated samples.

Total RNA isolation was performed with the RNAprep Pure Plant Kit by (Tiangen, Beijing, China). To eliminate the genomic DNA contamination, the RNA samples were treated with DNase1. RNA concentration and integrity was observed on Nano Drop 2000 spectrophotometer (Thermo scientific, USA) and 1% agarose gel electrophoresis. cDNAs of the RNA samples that the A260/280 ratio reached 2.00 were synthesized using PrimeScript® RT Reagent Kit (Perfect Real Time, Takara Biotechnology Co., Ltd., Dalian, China). qRT-PCR was performed with ABI 7500 fast Real-Time PCR system (Applied Biosystems, USA), with *Gh-Histone3* gene was used as reference to normalize the relative expression level. Primers pairs of five OMT genes were designed by using Oligo 7 [116] (Table S2). \(2^{-\Delta\Delta Ct}\) method was used to calculate the gene expressions [117].

**Abbreviations**

aa: amino acid; DPA: days post anthesis; CCoAOMT: caffeoyl coenzyme A 3-O-methyltransferase; CMTs: C-methyltransferases; COMTs: caffeic acid 3-O-methyltransferases; NMTs: N-methyltransferases; OMT: O-methyltransferase; kDa: kilodalton; GO: gene ontology; KEGG: kyoto encyclopedia of genes and genomes; Ka: the number of non-synonymous substitutions per non-synonymous site; Ks: the number of synonymous substitutions per synonymous site; CSSLs: chromosome segment substitution lines; RILs: recombinant inbred lines; SAM: S-adenosyl-l-methionine; Ga: *Gossypium arboreum*; Gh: *Gossypium hirsutum*; Gr: *Gossypium raimondii*; qRT-PCR: quantitative real-time polymerase chain reaction

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Competing interests**

Authors declare that they have no competing interests for the publication of the manuscript.

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Author contributions

AH and WG designed the project, QG, QZ, MSI and XD performed the formal analysis, JL, JG, YS, HS, RL, AL and AR provide the methodology, QZ and QG performed the software, YY and WG supervised the study, AH and WG drafted the manuscript, MA, YY and WG reviewed & edited the manuscript. All authors have read and approved the manuscript.

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**Table**

**Table 1. Predicted Subcellular localization of OMT genes of G. hirsutum**
| Gene ID   | CELLO  | C_Reliability | Wolf | P_Reliability | Gene ID   | CELLO  | C_Reliability | Wolf | P_Reliability |
|-----------|--------|---------------|------|---------------|-----------|--------|---------------|------|---------------|
| hOMT31_At | Cp     | 3.712         |     | 1             | GhOMT10Dt| Cp     | 3.384         |     | 6             |
| hOMT33_At | Cp     | 3.597         |     | 9.5           | GhOMT7_Dt| Cp     | 4.717         |     | 11            |
| hOMT74_At | Cp     | 2.985         |     | 7             | GhOMT8_Dt| Cp     | 4.717         |     | 7             |
| hOMT40_At | Cp     | 3.897         |     | 12            | GhOMT9_Dt| Cp     | 4.735         |     | 9             |
| hOMT68_At | Pp/Cp  | 2.094/2.624   |     |               | GhOMT19 Dt| Cp     | 3.973         |     | 10            |
| hOMT71_At | Cp     | 4.545         |     | 5             | GhOMT81_Dt| Cp     | 3.924         |     | 6.5           |
| hOMT78_At | Cp     | 4.122         |     | 13.5          | GhOMT78_Dt| Cp     | 2.531         |     | 2             |
| hOMT81_At | Cp     | 2.707         |     | 5             | GhOMT54_Dt| Pp/Cp  | 1.967/2.566   |     | 8             |
| hOMT33_At | Cp     | 4.033         |     | 2             | GhOMT76_Dt| Cp     | 3.273         |     | 8             |
| hOMT10_At | Cp     | 4.822         |     | 6             | GhOMT32_Dt| Cp     | 4.607         |     | 12            |
| hOMT12_At | Cp     | 4.154         |     | 4             | GhOMT30_Dt| Cp     | 3.946         |     | 1             |
| hOMT6_At  | Cp     | 4.234         |     | 8             | GhOMT31_Dt| Cp     | 4.695         |     | 6             |
| hOMT67_At | Cp     | 4.275         |     | 11.5          | GhOMT49_Dt| Pp     | 3.275         |     | 8             |
| hOMT32_At | Cp     | 4.675         |     | 8             | GhOMT57_Dt| Cp     | 4.391         |     | 7             |
| hOMT30_At | Cp     | 4.743         |     | 6             | GhOMT77_Dt| Cp     | 2.352         |     | 3             |
| hOMT49_At | Pp     | 3.107         |     | 11            | GhOMT58_Dt| Cp     | 4.675         |     | 5             |
| hOMT77_At | Cp     | 3.838         |     | 7             | GhOMT63_Dt| Cp     | 3.572         |     | 10            |
| hOMT57_At | Cp     | 2.229         |     | 9             | GhOMT62_Dt| Cp     | 2.968         |     | 4             |
| hOMT52_At | Cp     | 1.577         |     | 10            | GhOMT61_Dt| Cp     | 3.224         |     | 4             |
| hOMT33_At | Pp/Cp  | 1.995/1.743   |     | 6.5           | GhOMT17_Dt| Cp     | 4.112         |     | 2             |
| hOMT62_At | Cp     | 3.577         |     | 2             | GhOMT13_Dt| Cp     | 4.124         |     | 7             |
| hOMT65_At | Cp     | 3.508         |     | 8             | GhOMT70_Dt| Cp     | 4.385         |     | 13            |
| hOMT24_At | Cp     | 4.529         |     | 8             | GhOMT1_Dt | Cp     | 4.927         |     | 10            |
| hOMT14_At | Cp     | 4.404         |     | 12            | GhOMT55_Dt| Cp     | 2.781         |     | 1             |
| hOMT29_At | Cp     | 4.069         |     | 1             | GhOMT82_Dt| OM     | 2.226         | Chp | 9             |
| hOMT41_At | Cp     | 4.655         |     | 6             | GhOMT45_Dt| Pp     | 3.910         |     | 6             |
| hOMT70_At | Cp     | 3.408         |     | 8             | GhOMT46_Dt| Pp     | 2.490         |     | 10            |
| hOMT1_At  | Cp     | 4.921         |     | 7             | GhOMT47_Dt| Pp/Cp  | 1.961/2.316   |     | 10            |
| hOMT55_At | IM/Cp  | 1.807/2.478   |     | 1             | GhOMT48_Dt| Pp     | 4.276         |     | 8             |
| hOMT82_At | OM     | 2.374         |     | 9.5           | GhOMT72_Dt| Cp     | 4.474         |     | 11.5          |
| hOMT45_At | Pp     | 4.340         |     | 7             | GhOMT60_Dt| Cp     | 4.312         |     | 7             |
| hOMT47_At | Pp/Cp  | 2.121/2.358   |     | 12            | GhOMT59_Dt| Cp     | 4.534         |     | 6             |
| hOMT48_At | Pp     | 4.154         |     | 6             | GhOMT5_Scaf | Cp     | 4.641         |     | 11            |
| hOMT2_At  | Cp     | 4.870         |     | 5             | GhOMT11_Scaf | Cp     | 4.885         |     | 4             |
| hOMT72_At | Cp     | 4.493         |     | 13.5          | GhOMT68_Scaf | Cp     | 3.973         |     | 8             |
| hOMT60_At | Cp     | 4.637         |     | 5             | GhOMT52_Scaf | Cp     | 2.277         |     | 4             |
| hOMT51_Dt | Cp     | 3.636         |     | 2             | GhOMT46_Scaf | Cp     | 2.154         |     | 11            |
| hOMT37_Dt | Cp     | 4.092         |     | 6             | GhOMT15_Scaf | Cp     | 3.576         |     | 9             |
| hOMT35_Dt | Cp     | 4.228         |     | 4             | GhOMT75_Scaf | Cp     | 2.090         |     | 5             |
| hOMT33_Dt | Cp     | 3.130         |     | 8             | GhOMT73_Scaf | Cp     | 3.123         |     | 6             |
| hOMT71_Dt | Cp     | 4.475         |     | 11.5          |                            |        |             |     |
| hOMT6_Dt  | Cp     | 4.619         |     | 8             |                            |        |             |     |

Cp: Cytoplasmic, Pp: Periplasmic, OM: outer membrane, IM: inner membrane, Chp: Chloroplast, Mc: Mitochondria
C_Reliability: Lower reliability values show the stronger possibility of predicted localization
P_Reliability: Higher reliability values show the stronger possibility of predicted localization

**Figures**
Figure 3

Enrichment analysis of OMT family genes in G. hirsutum. a. The functional annotations of Gene Ontology. b. The functional annotations of KEGG pathways. c. The functional annotations of Interpro. The scales indicate the enriched gene number in respective categories.