Genome-Wide Identification and Comparative Analysis of the 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase (HMGR) Gene Family in Gossypium

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Abstract: Terpenes are the largest and most diverse class of secondary metabolites in plants and play a very important role in plant adaptation to environment. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is a rate-limiting enzyme in the process of terpene biosynthesis in the cytosol. Previous study found the HMGR genes underwent gene expansion in Gossypium raimondii, but the characteristics and evolution of the HMGR gene family in Gossypium genus are unclear. In this study, genome-wide identification and comparative study of HMGR gene family were carried out in three Gossypium species with genome sequences, i.e., G. raimondii, Gossypium arboreum, and Gossypium hirsutum. In total, nine, nine and 18 HMGR genes were identified in G. raimondii, G. arboreum, and G. hirsutum, respectively. The results indicated that the HMGR genes underwent gene expansion and a unique gene cluster containing four HMGR genes was found in all the three Gossypium species. The phylogenetic analysis suggested that the expansion of HMGR genes had occurred in their common ancestor. There was a pseudogene that had a 10-bp deletion resulting in a frameshift mutation and could not be translated into functional proteins in G. arboreum and the A-subgenome of G. hirsutum. The expression profiles of the two pseudogenes showed that they had tissue-specific expression. Additionally, the expression pattern of the pseudogene in the A-subgenome of G. hirsutum was similar to its paralogous gene in the D-subgenome of G. hirsutum. Our results provide useful information for understanding cytosolic terpene biosynthesis in Gossypium species.

Keywords: Gossypium; HMGR; terpene biosynthesis; gene expansion; pseudogene

1. Introduction

Terpenes are a type of natural compound, which are widely distributed in nature and have diverse structures and functions [1,2]. Thousands of terpenes and derivatives are a good example of metabolic plasticity that is essential to survive in changing environments [3,4]. Additionally, many terpenes are specialized compounds that are rich sources of commercial products widely used as flavors, fragrances and pharmaceuticals by humans [5,6].
In plant cells, terpenes are synthesized by two independent pathways: the mevalonate pathway (MVA pathway) in the cytosol and the 2-C-methyl-D-erythritol 4-phosphate pathway (MEP pathway) in the plastid [7,8]. The 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate (MVA), which is considered to be a rate-limiting enzyme of the MVA pathway and plays a key role in the biosynthesis of plant cytosolic terpenes [9,10]. Currently, HMGR genes have been isolated and cloned from many species of plants, such as Arabidopsis thaliana [11,12], rice [13], wheat [14], cotton [15], melon [16], medicinal plants Cymbopogon winterianus, [17] and Alisma orientale [18], and so on. Many experiments have shown that HMGR holds an important control point in the MVA pathway and genetic manipulation of HMGR indeed increased terpenes content in plants. The HMGR gene of Hevea brasiliensis was introduced into tobacco by Agrobacterium transformation, and then the activity of HMGR in transgenic plants increased by 4–8 times and the total amount of sterols increased by six times [19]. Ginsenosides are glycosylated triterpenoids, and overexpression of the HMGR gene in ginseng could significantly increase the amount of ginsenosides [20]. Transgenic spike lavender plants expressing the Arabidopsis HMGR gene accumulated more essential oil constituents, which were composed of monoterpenes and sesquiterpenes [21]. Additionally, more and more evidence shows that HMGR is not only critical for normal plant development but also very important in adapting to changing environments. HMGR was negatively regulated by protein phosphatase 2A (PP2A) in Arabidopsis plants during development and in response to stress conditions [22]. In Malus domestica, the various putative cis-acting elements were present in the promoter of MdHMGR1, MdHMGR2 and MdHMGR4 to response to different hormones, and the expression patterns of MdHMGR2 and MdHMGR4 were significantly induced by ethephon (ETH), methyl jasmonate (MeJA), and salicylic acid (SA) [23,24]. In Origanum vulgare subsp. gracile, the expression of the HMGR gene was directly affected by the changing environmental condition and was enhanced under water stress conditions [25].

The Gossypium (cotton) genus contains 50 species, of which 45 diploid (2n = 2x = 26) and five tetraploid (2n = 4x = 52) species, and all diploid cotton species are divided into eight genomes, A, B, C, D, E, F, G and K [26]. At present, two diploid cottons, Gossypium raimondii (D$_5$) [27,28] and Gossypium arboreum (A$_2$) [29], and the tetraploid cotton, Gossypium hirsutum ((AD)$_1$) [30,31] had completed whole genome sequencing. G. raimondii is a wild species belonging to the D-genome, and G. arboreum is a cultivar belonging to the A-genome [28,29]. They diverged from the same progenitor approximately 5–10 million years ago and G. arboreum underwent artificial domestication and selection [26,29]. Tetraploid cotton species are considered to be produced by interspecific hybridization between the African ancestor of an A-genome resembling G. arboreum and the American ancestor of a D-genome resembling G. raimondii approximately 1–2 million years ago [26,30]. G. hirsutum, as one of the tetraploid species, is domesticated to provide the world’s most natural textile fiber and become a major oilseed crop [30,31]. Additionally, Gossypium species serve as an ideal plant for studies of genome evolution and polyploidization [32,33]. Gossypium plants are also known to produce a specialized group of terpenes in the cytosol, including gossypol and related sesquiterpenoids, which could be used as phytoalexins in plant defense against pests and pathogens, as well as anticancer agents and male contraceptives in humans [34,35].

In previous studies, only a small portion of HMGR genes from cotton have been characterized, and our evolutionary analysis indicated that the HMGR genes underwent gene expansion and a unique gene cluster containing four HMGR genes was found in the diploid cotton, G. raimondii [36]. In order to explore the characteristics of the HMGR gene family in Gossypium genus, the HMGR gene family was identified in G. raimondii, G. arboreum, and G. hirsutum at a genome-wide level, and the phylogenetic relationship, chromosomal localization, gene structure, and protein motifs of HMGR genes in the three genomes were comprehensively analyzed in this study.
2. Results

2.1. Genome-Wide Identification of HMGR Genes in Gossypium

The candidate HMGR genes were identified from the Gossypium genome using the local blast program with the query sequences of Arabidopsis HMGR genes. The obtained sequences were further submitted to the Interpro database and validated to be the HMGR gene family membership (IPR004554). Finally, nine, nine and 18 putative HMGR genes were identified in G. raimondii, G. arboreum, and G. hirsutum, respectively (Table 1 and Supplementary Materials Table S3). The HMGR genes in G. raimondii were preferentially named GrHMGR1 to GrHMGR9, according to the published article [36]. Based on the orthologs in G. raimondii, the HMGR genes in G. arboreum were named GaHMGRs, with the same number as in G. raimondii. The HMGR genes in G. hirsutum were named GhHMGRs corresponding to the orthologs in G. raimondii and G. arboreum, and the D and A subgenomes were represented by suffixes D and A after each gene names, respectively. The genomic sequences with the upstream and downstream sequences of these gene loci were extracted and the coding sequences of these genes were re-annotated by the gene annotation tool FGENESH [37]. Then all the coding sequences were further manually verified by RT-PCR using gene-specific primers. As a result, 34 gene loci were confirmed to have the complete open reading frame (Supplementary Materials Table S4), while the G. arboreum GaHMGR1 and G. hirsutum GhHMGR1A were pseudogenes with a premature stop codon in their coding sequences. Compared with the initial annotation in genome database, the coding sequences of six HMGR genes (GhHMGR3A, GhHMGR4A, GhHMGR8A, GhHMGR8D, GaHMGR2, and GaHMGR9) were modified. Interestingly, the two gene loci (GhHMGR3A and GhHMGR4A) were originally found in one gene locus in the genome database of G. hirsutum, and their coding sequences were re-annotated subsequently. The coding sequences of GhHMGR8A and GhHMGR8D had a deletion according to the sequencing results. Additionally, the coding sequences of GaHMGR2 and GaHMGR9 had an insertion compared with their initial annotations. Overall, the results showed that G. raimondii and G. arboreum had the same number of HMGR loci (nine loci) and that G. hirsutum had twice as many HMGR loci as the other two species (18 loci).

One salient feature of the plant HMGR proteins is that they usually have two transmembrane movements across the endoplasmic reticulum membrane, and their catalytic domains are exposed to the cytosol during the process of performing function [38,39]. The transmembrane domains of Gossypium HMGR proteins were predicted by the online prediction tool TMHMM Server v. 2.0 (Supplementary Materials Figures S1–S3). The results showed that all the 34 HMGR proteins had two transmembrane domains at the N-terminus, and it was deduced that, as with other plant HMGR genes, it was necessary for Gossypium HMGR proteins to anchor on the membrane by two transmembrane movements in the catalysis reaction.

2.2. Chromosomal Distribution and Phylogenetic Analysis of Gossypium HMGR Genes

In G. raimondii, the nine HMGR genes were found in five chromosomes: four on chromosome 5, two on chromosome 2, and one on chromosome 8, 12 and 13 each (Supplementary Materials Figure S4). Although the chromosome distribution was found to be little different from the published article [36], there was also an HMGR gene cluster containing four genes on G. raimondii chromosome 5 (GrHMGR2, GrHMGR3, GrHMGR4 and GrHMGR5).
Table 1. The information of HMGR genes in *G. hirsutum*.

| Gene Name   | Gene Locus       | Chromosome | Location          | Strand | Protein Length | Mw(kDa) \(^a\) | pI \(^a\) |
|-------------|------------------|------------|-------------------|--------|----------------|----------------|----------|
| *GhHMGR1A*  | *HMGR* pseudogene| A01        | 39188679-39190582 | +      | 582            | 62.33          | 6.24     |
| *GhHMGR2A*  | Gh_A03G1497      | A03        | 95297747-95299778 | −      | 582            | 62.68          | 6.14     |
| *GhHMGR3A*  | Gh_A03G1496_1    | A03        | 95253091-95255136 | −      | 585            | 62.67          | 6.00     |
| *GhHMGR4A*  | Gh_A03G1496_2    | A03        | 95195182-95197211 | −      | 585            | 62.71          | 6.25     |
| *GhHMGR5A*  | Gh_A03G1495      | A03        | 95169197-95171221 | −      | 585            | 62.82          | 6.20     |
| *GhHMGR6A*  | Gh_A01G2017      | scaffold31 | 38993-42152       | +      | 585            | 62.62          | 6.24     |
| *GhHMGR7A*  | Gh_A12G0103      | A12        | 1425978-1428020    | −      | 585            | 62.62          | 6.24     |
| *GhHMGR8A*  | Gh_A04G1424      | scaffold1012 | 184892-187043     | −      | 581            | 62.26          | 6.69     |
| *GhHMGR9A*  | Gh_A13G0557      | A13        | 13047920-13050876  | −      | 628            | 67.10          | 6.26     |
| *GhHMGR1D*  | Gh_D01G1158      | D01        | 25640487-25642401  | +      | 560            | 60.40          | 6.53     |
| *GhHMGR2D*  | Gh_D02G1965      | D02        | 63580366-63582399  | −      | 582            | 62.33          | 6.43     |
| *GhHMGR3D*  | Gh_D02G1964      | D02        | 63566380-63569291  | −      | 585            | 62.54          | 6.00     |
| *GhHMGR4D*  | Gh_D02G1963      | D02        | 63558389-63560423  | −      | 583            | 62.40          | 6.17     |
| *GhHMGR5D*  | Gh_D02G1962      | D02        | 63549091-63551149  | −      | 585            | 62.78          | 6.25     |
| *GhHMGR6D*  | Gh_D01G0134      | D01        | 984873-987923      | +      | 585            | 62.79          | 5.83     |
| *GhHMGR7D*  | Gh_D12G0115      | D12        | 1451036-1453088    | −      | 585            | 62.44          | 6.43     |
| *GhHMGR8D*  | Gh_D04G2012      | scaffold3981 | 28507-30658       | +      | 581            | 62.26          | 6.49     |
| *GhHMGR9D*  | Gh_D13G0573      | D13        | 7846186-7848822    | +      | 628            | 67.46          | 6.50     |

\(^a\) The theoretical Mw (molecular weight) and pI (isoelectric point) of the full-length protein are predicted by ProtParam tool (http://web.expasy.org/protparam/). \(^b\) The coding sequences of genes are re-annotated. \(^c\) *GhHMGR1A* is a pseudogene identified in this study.
Likewise, in *G. arboreum*, the nine HMGR genes were found in five chromosomes (Supplementary Materials Figure S5). There was a single HMGR gene locus on chromosome 4, 6 and 13 each, and two HMGR gene loci on chromosome 7, but one of them was a pseudogene (*GaHMGR1*). Similarly, there was also an HMGR gene cluster on *G. arboreum* chromosome 5 (*GaHMGR2, GaHMGR3, GaHMGR4 and GaHMGR5*). Simultaneously, the 18 HMGR genes were found in *G. hirsutum*; nine for each of the D-subgenome and the A-subgenome (Supplementary Materials Figure S6). There was a pseudogene named as *GhHMGR1A* in the A-subgenome. Additionally, both of the two subgenomes had an HMGR gene cluster that contained four closely adjacent genes (*GhHMGR2D, GhHMGR3D, GhHMGR4D and GhHMGR5D* in the *G. hirsutum* D-subgenome, and *GhHMGR2A, GhHMGR3A, GhHMGR4A and GhHMGR5A* in the *G. hirsutum* A-subgenome), which was consistent with the two diploid cottons, *G. raimondii* and *G. arboreum*.

The genes encoding other related enzymes in upstream or downstream of HMGR in the MVA pathway were also identified in *Gossypium*. As a result, there were three genes encoding 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGS), two genes encoding mevalonate kinase (MK), one gene encoding phosphomevalonate kinase (PMK) and one gene encoding mevalonate diphosphate decarboxylase (MVD) in each of *G. raimondii* and *G. arboreum*, and six HMGS genes, two MK genes, two PMK genes and two MVD genes in *G. hirsutum* (Supplementary Materials Table S5). Phylogenetic trees based on the protein sequences of these genes in the MVA pathway of *Gossypium* were constructed to investigate the evolutionary relationships (Figure 1).

![Figure 1](image_url)  
**Figure 1.** Phylogenetic relationship of HMGS, MK, PMK, MVD and HMGR proteins from *G. raimondii*, *G. arboreum* and *G. hirsutum*. (a) HMGS; (b) MK; (c) PMK; (d) MVD; (e) HMGR. Numbers at the nodes represent bootstrap support values (1000 replicates). The bars in (a–e) indicate 0.5%, 5%, 0.2%, 0.1% and 5% sequence divergence, respectively.
The position of each gene and the homologous gene pairs were displayed intuitively with Circos diagrams (Figure 2). We identified 16 pairs of orthologous genes between G. raimondii and G. arboreum and 15 pairs of paralogous genes between the D-subgenome and the A-subgenome of G. hirsutum. It showed that there were one-to-one relationships between homologous genes of the MVA pathway in the two diploid cottons or in the two subgenomes of the tetraploid cotton. For the three HMGS genes and two MK genes in G. raimondii, they had orthologous genes in G. arboreum. In G. hirsutum, the three HMGS genes and one MK gene in the D-subgenome had corresponding paralogs in the A-subgenome. However, the orthologous genes of GaMK2 and GrMK2 were not found in G. hirsutum, indicating that they might be lost during the formation of G. hirsutum. For the PMK genes, including GaPMK, GrPMK, GhPMKA and GhPMKD, they exhibited the corresponding homologous relationships in G. raimondii and G. arboreum or the two subgenomes of G. hirsutum. Similarly, two MVD genes as a paralogous gene pair in G. hirsutum also had a corresponding ortholog in G. raimondii and G. arboreum. Furthermore, we elaborately detected the homologous relationships of HMGR genes, two HMGRs on chromosome 2, four HMGRs in a gene cluster on chromosome 5, three HMGRs on chromosome 8, 12 and 13 of G. raimondii had one to one orthologous relationships with two HMGRs on chromosome 7, four HMGRs in a gene cluster on chromosome 5, three HMGRs on chromosome 4, 6 and 13 of G. arboreum. Additionally, five HMGRs on chromosome 1, scaffold31_A01, scaffold1012_A04, 12 and 13, and four HMGRs in a gene cluster on chromosome 3 of the A-subgenome had one to one corresponding paralogous relationships with five HMGRs on chromosome 1, scaffold3981_D04, 12 and 13, and four HMGRs in a gene cluster on chromosome 2 of the D-subgenome of G. hirsutum. In general, it indicated that only the HMGR gene formed a gene cluster containing four genes and the gene cluster was present in all three cotton genomes (Figure 3).

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Locations and homologous relationships of the MVA pathway genes in G. raimondii and G. arboreum, and in the A-subgenome and D-subgenome of G. hirsutum. (a) Locations and orthologous relationships of the MVA pathway genes in G. raimondii and G. arboreum; (b) Locations and paralogous relationships of the MVA pathway genes in the D-subgenome and the A-subgenome of G. hirsutum. The chromosomes of G. raimondii, G. arboreum, G. hirsutum D-subgenome, and G. hirsutum A-subgenome are shown with different colors and labeled as Gr, Ga, Gh_Dt and Gh_At, respectively. The putative homologous gene pairs belonging to the HMGS, HMGR, MK, PMK and MVD gene families are connected by orange, purple, blue, yellow and grey lines, respectively. Several genes are located on the scaffolds that do not determine the exact locations and are placed next to the corresponding chromosomes.
2.3. Gene Structure and Conserved Protein Motifs of Gossypium HMGR Genes

The gene structure of Gossypium HMGR genes was determined (Figure 4). Except for GrHMGR1 and GaHMGR1D, the majority of protein-coding HMGR genes in the three Gossypium species had the typical gene structure with three introns and four exons, which was the same as the gene structure of most HMGR genes in plants [36]. GrHMGR1 and GaHMGR1D lacked the last intron, becoming the structure with two introns and three exons. Most of the HMGR genes had almost the same length of exons, only intron length varied greatly. The second and third introns of GaHMGR6, GhHMGR6A, GrHMGR6 and GhHMGR6D were relatively long, GaHMGR9, GhHMGR9A, GrHMGR9 and GhHMGR9D had also changed the gene structure. Although they had almost the same exon length as each other, their first exon was longer than other HMGR genes. Additionally, GaHMGR9 and GhHMGR9A had a short insertion in the second intron compared with GrHMGR9 and GhHMGR9D. The HMGR genes in the gene clusters had almost the same gene structure.

Figure 4. Phylogenetic relationship, gene structure, and conserved motifs of HMGR genes from G. raimondii, G. arboreum and G. hirsutum. Exons are represented by green boxes and introns by black lines. The two HMG-CoA binding motifs (EMPVGYVQIP and TTEGCLVA) and two NADP(H) binding motifs (DAMGMNM and GTVGGGT) are represented by red, light blue, yellow and dark blue boxes, respectively.
In the catalytic domain of the HMGR proteins, there were four highly conserved motifs: two HMG-CoA binding motifs (EMPVGYVQIP and TTEGCLVA) and two NADP(H) binding motifs (DAMGMNM and GTVGGGT) [36,40,41]. All 34 HMGR proteins of the three Gossypium species had the four conserved motifs, in which the relative position of these motifs was also conserved and remained constant (Figure 4 and Supplementary Materials Figure S7). Specifically, the first HMG-CoA binding motif (EMPVGYVQIP) was separated from the second HMG-CoA binding motif (TTEGCLVA) by 19 amino acid residues, there was the first NADP(H) binding motif (DAMGMNM) after 88 amino acid residues and the second NADP(H) binding motif (GTVGGGT) was at the C-terminus, separated from the first NADP(H) binding motif by 142 amino acid residues. The sequences of GaHMGR9A, GhHMGR9A, GrHMGR9 and GhHMGR9D at the N-terminus before the first HMG-CoA binding motif were longer than that of other HMGR genes, whereas GrHMGR1 and GhHMGR1D were shorter than other genes in this region.

2.4. Identification of HMGR Pseudogenes

We found a special HMGR gene locus GaHMGR1 in G. arboreum. The sequence alignment of GaHMGR1 and its orthologous gene in G. raimondii GrHMGR1 showed that their sequences were very similar, and there were only a few nucleotide insertion, deletion and substitution mutations. However, GaHMGR1 lacked a 10-bp fragment in the first exon region compared with GrHMGR1, leading to a premature stop codon (TGA) mutation (Figure 5).

![Figure 5](image-url)
Through cloning and sequencing, it was found that GaHMGR1 in G. arboreum could transcribe the full length RNA sequence similar to the GrHMGR1. However, the predicted protein sequence based on the RNA sequence showed that the translation was terminated prematurely due to the advance of the stop codon and the functional protein could not be translated.

Based on the genomic sequence of GaHMGR1, an HMGR pseudogene in the A-subgenome of G. hirsutum was identified by the Blast method and named GhHMGR1A. The sequence alignment revealed that its sequence was very similar to GaHMGR1. And both of them had the frameshift mutation and premature stop codon (TGA) due to the 10-bp deletion at the same position. These results indicated that GaHMGR1 and GhHMGR1A were really orthologous genes. In particular, we cloned GhHMGR1A from G. hirsutum (TM-1) using the seedling cDNA as the template, and sequencing results showed that its transcripts contained the whole sequence belonging to exons and introns in its protein-coding counterpart. In order to eliminate the effects of DNA contamination and alternative splicing and further confirm the result, we cloned the pseudogene using the cDNAs of roots, stems, cotyledons, leaves, and petals of TM-1, and at least four clones for the pseudogene from each of the materials were picked randomly and sequenced. Finally, the results indicated that the transcripts were consistent in all materials. Then in order to study the distribution of the HMGR pseudogene in Gossypium, the pseudogene was identified in several Gossypium species, including wild species of the D-genome, wild species and cultivars of A-genome, and wild species, semi-domesticated species and cultivars of the AD-genome. We collected leaves of these species and used their cDNAs to clone the pseudogene with gene-specific primers, and then four clones of each material were picked randomly for sequencing. It was found that all the A-genome species and tetraploid species (AD-genome) we used had the HMGR pseudogene (Table 2). Therefore, it could be deduced that the pseudogene was derived from an ancient A-genome species and transferred to the A-subgenome of the tetraploid species during the Gossypium evolution.

| Species                        | Type               | Genomic Group | HMGR Pseudogene |
|--------------------------------|--------------------|---------------|-----------------|
| G. raimondii                   | wild species       | D3            | Non-existence   |
| G. herbaceum race. africanum   | wild species cultivar | A1          | Existence       |
| G. arboreum cv. Shixiya1       | cultivar           | A2            | Existence       |
| G. darwinii                    | wild species       | (AD)3         | Existence       |
| G. mustelinum                  | wild species       | (AD)4         | Existence       |
| G. hirsutum race. latifolium   | semi-domesticated species | (AD)1       | Existence       |
| G. hirsutum cv. TM-1           | cultivar           | (AD)1         | Existence       |
| G. hirsutum cv. CCRH1          | cultivar           | (AD)1         | Existence       |
| G. barbadense cv. Xinhai21     | cultivar           | (AD)2         | Existence       |

Expression patterns of the two HMGR pseudogenes (GaHMGR1 and GhHMGR1A) and the homologous gene of GhHMGR1A in the D-subgenome of G. hirsutum (GhHMGR1D) were analyzed by qRT-PCR in roots, stems, cotyledons, leaves, petals, and ovules collected at 0 DPA, 10 DPA, 20 DPA, 30 DPA and 40 DPA of G. arboreum and G. hirsutum (Figure 6). The results showed that all the three genes displayed tissue-specific expression patterns. Both of the pseudogenes had the highest expression level in petals. In addition, GaHMGR1 had relatively high expression in roots, cotyledons, ovules at 40 DPA, and had the lowest expression in ovules at 20 DPA. GhHMGR1A was highly expressed in cotyledons and ovules at 30 DPA, and lowly expressed in roots, stems and ovules at 40 DPA. Moreover, GhHMGR1D as a protein-coding HMGR gene, which also was the paralog of the pseudogene GhHMGR1A in G. hirsutum, was expressed at a high level in roots, stems, cotyledons, petals and ovules at 30 DPA. Although GhHMGR1D had the highest expression level in cotyledons, its expression pattern was roughly similar to that of the pseudogene GhHMGR1A.
Figure 6. Expression patterns of GaHMGR1, GhHMGR1A and GhHMGR1D in different tissues. (a) GaHMGR1; (b) GhHMGR1A; (c) GhHMGR1D.
3. Discussion

It has been suggested that HMGR is a multigene family in cotton and there are seven to nine members in the tetraploid cotton according to the Southern blot technique [15,42]. Our previous studies have shown that there were nine HMGR genes in G. raimondii and the number of HMGR genes was significantly expanded compared with other plants [36]. In this study, another version of G. raimondii genome data [27] was used to further confirm that nine HMGR gene loci were included in G. raimondii. Unexpectedly, the chromosome distribution was little different. In the published study, GrHMGR6 was located on chromosome 7, but in this study it was on chromosome 2. In addition, the order of four genes in the HMGR gene cluster was exactly the opposite. The G. raimondii genome used in the previous study [36] was sequenced on the Illumina HiSeq 2000 platform at the BGI-Shenzhen and was assembled using the SOAPdenovo with a K-mer of 41 and SSPACE software [28], while the G. raimondii genome used in this study was sequenced on the Applied Biosystems 3730xl, Roche 454 XLR and Illumina Genome Analyzer (GA)IIx machines at the U.S. Department of Energy Joint Genome Institute and was assembled using the modified version of Arachne v.20071016 with specific parameters [27]. Considering that the two versions of G. raimondii genome data were independent sequenced and assembled, there might be numerous different assembling which might result in the difference of chromosomal distribution of HMGR genes. Furthermore, nine and 18 HMGR genes were identified in G. arboreum and G. hirsutum, respectively, using the recently published genome database [29,31]. Phylogenetic analysis showed that the nine HMGR genes in G. arboreum had one-to-one orthologous relationships with the nine HMGR genes in G. raimondii, which indicated that these HMGR genes were distributed to G. raimondii and G. arboreum with speciation from the common ancestor of them, and the number of HMGR genes has expanded in the ancestral species. The number of HMGR genes in G. hirsutum was just twice than that of G. raimondii and G. arboreum, indicating that all the HMGR genes were retained in the process of polyploidization.

Gene duplication, including segmental duplication and tandem duplication, has been recognized as the main mechanisms which contributed to expansion of gene families [43,44]. In Glycine max and Populus trichocarpa, the segmental duplication was the main reason for the expansion of MYB and WRKY gene families, but there were also some clusters resulting from the tandem duplication [45–48]. HMGR genes were generally distributed on chromosomes dispersedly in other plants [36]. However, there was a gene cluster containing four closely adjacent HMGR genes on the chromosome 5 of G. raimondii and G. arboreum. There was also a gene cluster on the chromosome 3 of the A-subgenome and chromosome 2 of the D-subgenome in G. hirsutum. The sequences of four HMGR genes in each gene cluster were very similar. It was speculated that the HMGR genes had underwent tandem duplication in the common ancestor of G. raimondii and G. arboreum, leading to the emergence of a HMGR gene cluster. Previous studies have shown that segmental duplication and tandem duplication play similar roles in the expansion of the HMGR gene family in G. raimondii [36]. In this study, it was found that there were nine corresponding HMGR gene loci in G. arboreum, which further indicated that this segmental duplication and tandem duplication had occurred in the common ancestor of G. raimondii and G. arboreum, resulting in the expansion of HMGR genes. Genomic evolution analysis showed that a whole genome duplication event was uniquely occurred for Gossypium after speciation from its closely related species, Theobroma cacao [29], which supported the inference that segmental duplication was one of the causes of HMGR gene expansion.

In this study, several related genes of the MVA pathway were identified in G. raimondii, G. arboreum and G. hirsutum. There were three HMGS genes, nine HMGR genes, two MK genes, one PMK gene, and one MVD gene in each of G. raimondii and G. arboreum, and six HMGS genes, 18 HMGR genes, two MK genes, two PMK genes, and two MVD genes in G. hirsutum. In the model plant Arabidopsis, there were one HMGS gene, two HMGR genes, one MK gene, one PMK gene and two MVD genes [49]. Compared with Arabidopsis, the number of HMGS, HMGR and MK gene loci was more in G. raimondii and G. arboreum, the number of PMK loci was the same as that in Arabidopsis, and the number of MVD loci was one less. In general, only the number of HMGR genes in Gossypium species was most
significantly expanded in the MVA pathway, and there was a unique gene cluster that might have resulted from tandem duplication. *Gossypium* species synthesize gossypol and related sesquiterpenoids uniquely by the MVA pathway in the cytosol and accumulate in roots and pigment glands of aerial tissues, to resist the invasion of pests and pathogens [35,50,51]. The proteins encoded by *HMGR* genes are a rate-limiting enzyme of the MVA pathway and are important regulatory sites for the biosynthesis of terpenes in the cytosol [9,10]. Therefore, it could be speculated that this increase in the number of *HMGR* genes in *Gossypium* species might be likely related to the biosynthesis of more terpenes including gossypol in the cytosol during their growth and development. In addition, this study found that *HMGR* gene expansion and a unique *HMGR* gene cluster were present in the three *Gossypium* species, and the four genes within the *HMGR* gene cluster had almost the same gene and protein structure, which indicated that the gene cluster was quite conserved in the evolutionary process.

After the number of genes was expanded, functional differentiation has three fates: pseudogenization, loss of gene function; neo-functionalization, access to new gene function; sub-functionalization, both of the two copies retain the function of ancestral gene [52]. *GrHMGR1* of *G. raimondii* and its orthologous gene in the D-subgenome of *G. hirsutum*, *GhHMGRID*, were lacking the third intron compared with other *HMGR* genes. Moreover, its orthologous gene in *G. arboreum*, *GaHMGR1*, and the orthologous gene of *GaHMGR1* in the A-subgenome of *G. hirsutum*, *GhHMGR1A*, had a 10-bp deletion at the same position, resulting in a frameshift mutation, and could not be translated into functional proteins. These results suggested that one functional member after expansion of *HMGR* genes in the common ancestor of *G. raimondii* and *G. arboreum*, might be differentiated by losing the third intron, then it became a pseudogene by losing the 10-bp fragment in the first exon in the ancestor of *G. arboreum* after the speciation of *G. raimondii* and *G. arboreum*. The pseudogene was identified in all the A-genome and AD-genome species collected in this study. Thus, it suggested that the pseudogene might be transferred from wild species to cultivars of the A-genome during process of domestication. Then, during the tetraploid formation by interspecific hybridization between the A-genome and D-genome, the pseudogene was transferred from the A-genome to the A-subgenome.

Previous study has found an *HMGR* pseudogene named *ψhmg5* in *G. hirsutum* and its transcript was detected in cotton embryos [53]. However, because of the lack of genome data of other *Gossypium* species, they were not sure whether the pseudogene arose before or after the polyploidization. Through sequence alignment, we found that the pseudogene identified in the study was the same as the one in the previous study [53]. However, more deeply, we provided a possible mechanism for the formation of the pseudogene by comparative genome analysis. After the expansion of *HMGR* gene in the progenitor of *Gossypium* species, one functional gene might become a new gene with significantly different gene structure attributed to selective excision of the third intron, and led to a pseudogene through a 10-bp deletion in the first exon after a series of evolutionary processes in the A-genome, then transferred to the A-subgenome with polyploidization. In addition, transcripts of the identified pseudogene in *G. hirsutum* contained the whole sequence belonging to exons and introns in its protein-coding counterpart and could be detected in all materials we had collected using the qRT-PCR method.

Pseudogenes do not code for protein, so they have long been labeled as “junk” DNA [54]. However, recent results demonstrated that some pseudogenes could influence their parent genes through their transcripts, including negative regulation and positive regulation [55,56]. For example, the transcripts of pseudogene can produce endogenous siRNAs and then silence the expression of parent gene by RNA interference. In soybean, the inhibition of seed coat pigmentation induced by the *I* gene results from posttranscriptional gene silencing (PTGS) of chalcone synthase (*CHS*) genes and leads to a uniform yellow color of mature harvested seeds [57]. *GmIRCHS* (*Glycine max* inverted-repeat *CHS* pseudogene) was identified as a candidate for the *I* gene [58]. The siRNAs derived from *GmIRCHS* cleaved the mRNA of all *CHS* genes to inhibit their function, and occurred in the seed coat, specifically [59]. Additionally, the pseudogene transcripts can positively regulate its homologous gene by competitively binding miRNAs. For example, *PTEN* is a tumor suppressor...
gene and maintaining PTEN protein levels can inhibit tumorigenesis. Its pseudogene PTENP1 is highly similar to the homologous coding gene PTEN at the 3′ untranslated region (UTR), which can bind miRNAs and reduce the cell concentration of miRNAs, leading to PTEN escape from miRNAs repression regulation [60]. In our study, the HMGR pseudogenes could still be detected after a long-term evolution, and the pseudogene GhHMGR1A showed tissue-specific expression and had the similar expression pattern with its paralogous gene GhHMGR1D in the other subgenome. Therefore, it suggested that the pseudogenes might have a potential role in regulation of other HMGR genes. Additionally, both of the pseudogenes had the highest expression level in petals. Many plants, such as snapdragon [61], Hedychium coronarium [62], and kiwifruit (Actinidia deliciosa) [63], can synthesize and emit lots of terpenes in their petals. It could be speculated that the high expression level of the two pseudogenes in petals might be related to the large demand for the precursors for terpene biosynthesis in petals of G. arboreum and G. hirsutum. Of course, the hypothesis requires further experimental evidence.

4. Materials and Methods

4.1. Identification of Genes in the MVA Pathway in Gossypium

The genome data of G. raimondii [27], G. arboreum [29] and G. hirsutum [31] were downloaded from the CottonGen database (https://www.cottongen.org/). Then, the local blast database was established for these genome data. The protein sequences of Arabidopsis thaliana genes in the MVA pathway were collected from the TAIR database (http://www.arabidopsis.org) [49,64]. BlastP and tBlastN programs were performed against the Gossypium genome local databases using the Arabidopsis protein sequences as queries with default parameters. All candidates were verified using the Pfam database [65] and InterPro database [66] to identify the members of gene family in the MVA pathway.

4.2. Sequence Alignment and Phylogenetic Tree Construction

Multiple sequence alignments were generated using ClustalX software (Version 2.1, Conway Institute UCD, Dublin, Ireland) [67] for the protein sequences with default parameters. Based on the result of multiple sequence alignment, phylogenetic trees were generated using the maximum likelihood method in MEGA software (Version 5.2, Biodesign Institute, Tempe, AZ, USA) [68], using the bootstrap method to assess the reliability with 1000 replicates.

4.3. Chromosomal Mapping, Protein Motif, and Gene Structure Analysis

The physical location data of identified genes were retrieved from the Gossypium genomes. MapInspect software (http://mapinspect.software.informer.com/) [69] and Circos software (Version 0.67, www.circos.ca) [70] were used to generate chromosomal distribution images for these genes in G. raimondii, G. arboreum and G. hirsutum according to their starting positions on chromosomes. The gene exon/intron structure was drawn using the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) online tool [71] by comparing the coding sequence (CDS) of each gene with its genomic sequence.

4.4. Cotton Plant Growth and Sample Collection

The seeds of G. arboreum cv. Shixiya 1, G. hirsutum cv. TM-1 and other species used in this study were supplied by Institute of Cotton Research, Chinese Academy of Agricultural Sciences (CAAS, Anyang, China). Whole seedlings, roots, stems, cotyledons and leaves were collected from two-week-old seedlings grown in a greenhouse. Petals were collected from plants on the day of flowering, and ovules were collected at 0, 10, 20, 30 and 40 days post anthesis (DPA). All samples were quick-frozen in liquid nitrogen and stored at −80 °C.
4.5. RNA Isolation and cDNA Synthesis

Total RNA was isolated from each sample using the RNA Extraction Kit (TIANGEN, Beijing, China). The RNA concentration was measured using a NanoDrop2000 microvolume spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the integrity of RNA was analyzed on 1% agarose gels. One microgram of total RNA was used for first strand cDNA synthesis using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China).

4.6. Reverse Transcription PCR (RT-PCR) and Quantitative Real-Time RT-PCR (qRT-PCR)

The gene-specific primers were designed based on the nucleotide sequences by Oligo software (Version 7.60, Molecular Biology Insights, Cascade, CO, USA) and synthesized by Suzhou GENEWIZ (Supplementary Materials Tables S1 and S2). The RT-PCR was carried out as follows: 94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min 30 s; then 72 °C for 10 min. The amplified fragments were purified with the MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, Dalian, China), cloned into the pMD18-Tvector (TaKaRa, Dalian, China) and verified by sequencing. The qRT-PCR was performed using a LightCycler480 system (Roche, Basel, Switzerland) with SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China) and the cotton UBQ7 gene was used as an internal control. The amplification parameters were as follows: stage 1: 95 °C, 5 min; stage 2: 40 cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s; stage 3: extension at 72 °C for 10 min. Three biological replicates were used for each sample and the results were analyzed using the $2^{-\Delta\Delta CT}$ method [72].

5. Conclusions

We performed a genome-wide identification of the HMGR gene family in Gossypium and analyzed their structure, conserved motif, and evolution. The results revealed that the HMGR genes were obviously expanded in the common ancestor of Gossypium mainly by segmental duplication and tandem duplication, and a gene cluster containing four closely adjacent genes was highly conserved during evolution. There was a pseudogene in G. arboreum and the A-subgenome of G. hirsutum, and they displayed tissue-specific expression patterns. This study is the first to characterize the HMGR gene family in Gossypium species and lays an important foundation for further study of cytosolic terpene biosynthesis in cotton.

Supplementary Materials: The following are available online. Table S1. Primers for reverse transcription PCR. Table S2. Primers for quantitative real-time PCR. Table S3. The information of HMGR genes in G. raimondii and G. arboreum. Table S4. The coding sequences of HMGR genes in G. raimondii, G. arboreum and G. hirsutum. Table S5. The information of HMGS, MK, PMK and MVD genes in G. raimondii, G. arboreum and G. hirsutum. Figure S1. Predicted transmembrane domain for G. raimondii HMGR proteins. Figure S2. Predicted transmembrane domain for G. arboreum HMGR proteins. Figure S3. Predicted transmembrane domain for G. hirsutum HMGR proteins. Figure S4. Chromosomal distributions of HMGR genes in G. raimondii. Figure S5. Chromosomal distributions of HMGR genes in G. arboreum. Figure S6. Chromosomal distributions of HMGR genes in G. hirsutum. Figure S7. Phylogenetic relationship and distribution of conserved motifs in HMGR proteins from G. raimondii, G. arboreum and G. hirsutum.

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Author Contributions: S.Z. and Z.M. conceived and designed the research. W. Liu, W.Z. and L.J. performed the experiments. W. Liu, Z.Z., W. Li and L.L. analyzed the data. W. Li, Z.R. and Z.W. prepared the figures. W. Liu and Z.Z. wrote the manuscript. S.Z. and Z.M. revised the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare that they have no competing interests.

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**Sample Availability:** Samples of the *Gossypium* species are available from the authors.