Comparative Transcriptome Analysis between a Novel Allohexaploid Cotton Progeny CMS Line LD6A and Its Maintainer Line LD6B

Jie Zheng 1, Xiangjun Kong 1, Bin Li 1, Aziz Khan 1©, Zhiling Li 1, Yiding Liu 1, Haodong Kang 1, Farman Ullah Dawar 2 and Ruiyang Zhou 1,*

1 Key Laboratory of Plant Genetics and Breeding, College of Agriculture, Guangxi University, Nanning 530006, China; zhengjieself@163.com (J.Z.); kongxiangjun201010@163.com (X.K.); 18697998850@163.com (B.L.); aziz.hzau@gmail.com (A.K.); 18404968530@163.com (Z.L.); liuyiding1988@163.com (Y.L.); khd2414630992@163.com (H.K.)
2 Department of Zoology, Kohat University of Science and Technology, Kohat 26000, Pakistan; farmandawar@kust.edu.pk
* Correspondence: ruiyangzhou@aliyun.com

Received: 10 October 2019; Accepted: 1 December 2019; Published: 5 December 2019

Abstract: Cytoplasmic male sterility (CMS) is an important agronomic feature and provides an effective tool for heterosis utilization of crops. This study reports the comparative transcriptomic sketches between a novel allohexaploid cotton progeny CMS line LD6A and its maintainer line LD6B using de novo transcriptome sequencing technology at the pollen abortion stage. A total of 128,901 Unigenes were identified, in which 2007 were upregulated and 11,864 were downregulated. The significantly differentially expressed genes (DEGs) in LD6A show a distant and diverse genetic nature due to their distant hybrid hexaploidy progeny. Further analysis revealed that most of the DEGs participated in the tricarboxylic acid (TCA) cycle, oxidative phosphorylation, histone acetyltransferase activity, sepal development, stigma development, cotyledon development and microsporogenesis. A highly differentially expressed toxic protein, Abrin, was identified in the CMS line LD6A, which can catalyze the inactivation of ribosomes and consequently lead to cell death through the mitochondrial pathway in human cells. Twelve DEGs were selected randomly to validate transcriptome data using quantitative reverse-transcribed PCR (qRT-PCR). This study will contribute to new ideas and foundations related to the molecular mechanism of CMS and the innovation of cotton germplasm resources.

Keywords: cotton; allohexaploid; cytoplasmic male sterility; transcriptome; differentially expressed genes

1. Introduction

Cotton is widely cultivated for fiber and oil seed production globally [1]. In China, cotton production has been improved by artificial emasculation and pollination, however this required a long time, intensive labor and cost to achieve [2]. Cytoplasmic male sterility (CMS) is a classical and convenient way to improve crop yields in cotton [3–5], kenaf [6], sweet orange (Citrus sinensis) [7], rape (Brassica napus) [8] and chili pepper (Capsicum annuum L.) [9]. Producing superior varieties using the hybridization method improves crop yields, however, interspecific hybrid incompatibility occurs in distant hybridization. Ancient natural allopolyploidization events and the superior properties of modern crop plants provides a driver of speciation and environmental adaptation. These methods of distant hybridization and allopolyploidization are the classical way to develop new germplasms [10]. In this study, a new CMS line LD6A was produced through the comprehensive utilization of distant
hybridization, polyploidization and backcross, which is the first CMS germplasm that has been produced in this way in cotton. However, the molecular mechanism of CMS is still unclear.

In 1976, the carrier of the CMS factor was found to be mitochondrial DNA [11]; more than 50 mitochondrial genes were identified as CMS-relevant in various plants [12–15]. Several metabolic pathways which influence biological processes that cause CMS via the action of mitochondrial genes coupled with nuclear genes were identified [16]. Studies have been done regarding global transcriptional networks from big data analysis to explore CMS genes, which have been shown to be associated with the tricarboxylic acid (TCA) cycle, respiratory electron transport chain, oxidative phosphorylation and toxic proteins [6,17–20].

In the backcrossing process, progeny of distant hybridization with chromosomes that come from wild cotton could not be completely replaced [21]. Moreover, the genome sequences of the allohexaploid progeny of Gossypium raimondii (DD) [22], Gossypium arboreum (AA) [23], and Gossypium hirsutum (AADD) [24] revealed unclear gene annotation. The single reference genome of diploid and tetraploid cotton could not completely restore the basic features of the transcriptome; rather, the de novo assembly method could annotate the transcriptomic data.

However, limited sources of the CMS line and its negative effects on the cytoplasm inhibited cotton yield. In this context, identification of the molecular mechanism and development of new CMS germplasm resources are essential.

To understand the molecular mechanism of LD6A pollen abortion, we analyzed the gene expression at the tetrad stage (abortion stage) in a novel CMS line and its maintainer line on a global scale using a de novo assembly transcriptome. Our study reveals that Abrin, BTB/POZ and TAZ family genes, MDH, WRKY40, and atp8 were closely related to CMS. Our findings are interesting and facilitate understanding of the CMS molecular mechanism, which will provide further knowledge for germplasm innovation and heterosis utilization.

### 2. Results

#### 2.1. De Novo Transcriptome Analysis

In this study, the transcriptome sequencing of the CMS lines LD6A and LD6B at the tetrad stage was conducted using an Illumina Hi-Seq 4000. A total of 39.57 gigabytes of raw data (73.88 million raw reads) was obtained from six libraries. After filtering, 65.96 million clean reads were identified. The de novo profiles were performed with all clean reads using Trinity software (v2.4.0) (Table 1). After assembling, a total of 709,971 transcripts were identified, which has 680,840,189 bases (Table 1). The N50 statistics showed that more than 50% of transcripts were longer than 1541 bp. The N50 of all genes was 2036 bp, while the average length of all genes was 1434 bp (Table 1). The length distribution of all assembled cotton genes is shown in Figure 1, which shows that 24.9% of the total Unigenes and 10.4% of the total CDS were longer than 2000 bp.

| Type                          | LD6B         | LD6A         |
|------------------------------|--------------|--------------|
| Total raw reads              | 73.88 Mb     | 73.88 Mb     |
| Total trinity transcripts    | 709,971      | 1541         |
| N50 (transcripts)            | 1541         | 1541         |
| Total assembled bases (transcripts) | 680,840,189 | 680,840,189 |
| Total trinity genes          | 128,901      | 2036         |
| N50 (genes)                  | 128,901      | 2036         |
| Average length (genes)       | 1434         | 1434         |
| Total assembled bases (genes) | 184,861,957 | 184,861,957 |
| GC (%)                       | 40.66        | 40.66        |

Table 1. Overview of the assembled cotton transcriptome. Mb = megabytes.
2.2. Annotation of the Assembled Cotton Genes

All sequences were aligned against public databases: 109,903 (NR: 85.26%), 119,364 (NT: 92.60%), 77,480 (SwissProt: 60.11%), 83,201 (KOG: 64.55%), 81,970 (KEGG: 63.59%), 75,860 (GO: 58.85%) and 71,567 (Pfam: 55.52%) for functional annotation (Figure 2). GO annotation shows that 17,037, 4416 and 33,804 genes were involved in cellular process, metabolic process and catalytic activity, respectively (Figure 3). Furthermore, genes responsible for antioxidant activity (398 genes), toxin activity (8 genes) and response to stimuli (2035 genes) were identified in this study (Figure 3). The top five KEGG pathways were “global and overview maps” (18,738 genes), “carbohydrate metabolism” (7371 genes), “translation” (6271 genes), “folding, sorting and degradation” (6155 genes), and “signal transduction” (5554 genes). The Unigene sequences were annotated into the KOG database to obtain classification information of gene homologies. “General function prediction only” (18,372 genes), “signal transduction mechanisms” (9774 genes), “function unknown” (7574 genes), “post-translational modification, protein turnover, chaperones” (6504 genes) and “transcription” (5680 genes) were the top five classes. Multiple annotation perspectives of the assembled cotton transcriptome were performed to deeply understand the differences in microsporogenesis and the physiological and biochemical characteristics between the CMS line and its maintainer line. In these annotations, 85,312 CDS were detected by TransDecoder (https://transdecoder.github.io). At the same time, 29,807 SSRs were detected in 23,774 Unigenes, among which 5141 Unigenes encoding transcription factors (TF) were predicted. Meanwhile, 9537 out of 128,901 Unigenes were predicted to encode proteins that have not been annotated.
2.3. Annotation Difference Analysis of the Allohexaploid Progeny of Cotton

The distribution of species aligned by the assembled allohexaploid progeny of cotton shows that 46,034 genes were from *Gossypium hirsutum*, 32,736 from *Gossypium raimondii*, 24,485 from *Gossypium arboretum*, 1794 from *Theobroma cacao*, 1097 from *Herrania umbratica* and 3751 were found from other species (Figure 4). The CMS line showed more annotated transcripts and genes compared to the maintainer line (Figure 5).
Figure 4. Distribution of species aligned by the assembled cotton.

Figure 5. Number of transcripts and Unigenes annotated in different samples.

2.4. Analysis of Differentially Expressed Genes (DEGs)

Three biological replicates of both the CMS and maintainer line were pooled using the DEseq2 method (fold change $\geq 2.00$ and adjusted $p$ value $\leq 0.05$), in which 13,871 DEGs were obtained from LD6A vs. LD6B (Figure 6, Supplementary Materials 1). Among the DEGs, the top 10 upregulated CMS-related genes and top 10 downregulated fertile-specific genes were detected. These highly upregulated genes encoded nitrate or di/tri-peptide transporters NRT1/PTR FAMILY 3.1 (CL14392.Contig3_All), non-specific lipid-transfer protein A (CL3338.Contig1_All), repetitive proline-rich cell wall protein 1
(Unigene17011_All), repetitive proline-rich cell wall protein 2-like (Unigene17016_All), ATP synthase protein YMF19 (CL1583.Contig3_All), zinc finger protein constants-like 16 (CL5056.Contig4_All, CL5056.Contig2_All), cup-sharp cotyledon3 (CL4436.Contig2_All) and an uncharacterized protein (CL14868.Contig5_All, CL14585.Contig1_All) which are associated to CMS (Figure 7a). The top 10 genes (Figure 7b) that were identified in the maintainer line and downregulated in the CMS line that may be related to fertility encode polygalacturonase (CL2518.Contig9_All, CL2518.Contig6_All), pectinesterase (CL1505.Contig10_All), Parus major synaptotagmin 11 (CL9169.Contig1_All), transcript variant X1 arabinogalactan peptide 23 (CL10526.Contig6_All), non-specific lipid-transfer protein 2 (CL642.Contig3_All), uncharacterized ncRNA (CL9396.Contig5_All), L-ascorbate oxidase homolog (CL11928.Contig2_All), putative pectinesterase 63 (CL3983.Contig4_All), and pollen allergen Che a 1 (CL929.Contig9_All). In addition, differentially expressed genes were also identified that encode various protein products, including the toxic protein Abrin, transcription factors BTB/POZ and TAZ, cytoplasmic malate dehydrogenase, stress-induced transcription factors (such as WRKY40 which influences toxic activity), histone acetyltransferase, components of the tricarboxylic acid (TCA) cycle and the development of sepals, stigmas and cotyledons, respectively.

**Figure 6.** Comparison of gene expression levels between LD6A and LD6B according to the DEseq2 method. DEGs = differentially expressed genes.
Figure 7. (a) Indicates highly expressed genes exclusively identified in LD6A. (b) Top 10 highly expressed genes identified exclusively in LD6B samples. FPKM = fragments per kilobase of transcript per million fragments mapped.

2.5. GO Annotation and Pathway Analysis of DEGs

To better understand the relationship between DEGs and CMS, GO classification and functional enrichment were carried out to perform the functional analysis of DEGs (Supplementary Materials 2–4). “Catalytic activity”, “binding”, “membrane”, “membrane part” and “cellular process” were the top five terms among the 48 GO terms classified from 7648 DEGs. Most of the DEGs were allied with catalytic activity, binding, transporter activity and molecular function regulator.

Moreover, among the significant GO terms (Table 2), 2742, 198, and 285 DEGs were involved in “integral component of membrane” (GO:0016021), “carbohydrate metabolic process” (GO:00056975) and “extracellular region” (GO:005576), respectively. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification and functional enrichment of DEGs was used to identify metabolic networks and biological pathways. Furthermore, 4500 DEGs were annotated and classified into 133 groups and 27 KEGG pathways were significantly enriched with \( p \leq 0.05 \) and \( Q \leq 0.05 \) (Table 3). The 285 DEGs were involved in endocytosis (ko04144), starch and sucrose metabolism (262, ko00500), amino sugar and nucleotide sugar metabolism (230, ko00520), pentose and glucuronate interconversions (224, ko00040) and phenylpropanoid biosynthesis (209, ko00940).

| Type                  | ID          | Term                       | Number | Rich Ratio   | \( p \) Value   |
|----------------------|-------------|----------------------------|--------|--------------|-----------------|
| **Biological process** | GO:0045490  | pectin catabolic process    | 89     | 0.028507367  | \( 1.52 \times 10^{-26} \) |
|                      | GO:0042545  | cell wall modification     | 61     | 0.019538757  | \( 4.79 \times 10^{-26} \) |
|                      | GO:005975   | carbohydrate metabolic process | 198   | 0.063420884  | \( 3.23 \times 10^{-16} \) |
|                      | GO:0071555  | cell wall organization     | 108    | 0.034539209  | \( 3.49 \times 10^{-10} \) |
|                      | GO:0016042  | lipid catabolic process     | 57     | 0.018257527  | \( 3.77 \times 10^{-9} \)  |
|                      | GO:0050101  | actin filament bundle assembly | 15    | 0.004163997  | \( 1.60 \times 10^{-8} \)  |
| **Cellular component** | GO:0016021  | integral component of membrane | 2742  | 0.637377964  | \( 2.30 \times 10^{-25} \) |
|                      | GO:005576   | extracellular region        | 144    | 0.034372803  | \( 3.17 \times 10^{-23} \) |
|                      | GO:005618   | cell wall                  | 92     | 0.021385402  | \( 5.67 \times 10^{-10} \) |
| **Molecular function** | GO:004857   | enzyme inhibitor activity   | 69     | 0.011591984  | \( 1.53 \times 10^{-28} \) |
|                      | GO:0030599  | pectinesterase activity    | 61     | 0.010212623  | \( 5.75 \times 10^{-26} \) |
|                      | GO:004330   | aspartyl esterase activity | 61     | 0.010212623  | \( 5.75 \times 10^{-26} \) |
|                      | GO:0004650  | polygalacturonase activity | 54     | 0.009040683  | \( 1.65 \times 10^{-20} \) |
|                      | GO:0030570  | pectate lyase activity     | 32     | 0.005357442  | \( 6.03 \times 10^{-17} \) |
|                      | GO:005096   | GTPase activator activity  | 59     | 0.009877783  | \( 2.61 \times 10^{-14} \) |
|                      | GO:003779   | actin binding              | 63     | 0.010547464  | \( 5.97 \times 10^{-11} \) |
|                      | GO:0045735  | nutrient reservoir activity | 21     | 0.003515821  | \( 7.83 \times 10^{-11} \) |
|                      | GO:004575   | sucrose alpha-glucosidase activity | 14    | 0.002343881  | \( 1.11 \times 10^{-9} \) |
|                      | GO:0015299  | solute: proton antipporter activity | 34    | 0.005692282  | \( 2.99 \times 10^{-9} \) |
|                      | GO:0020337  | heme binding               | 132    | 0.022099448  | \( 7.05 \times 10^{-8} \) |
Table 3. Statistical enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways ($p \leq 0.05$, $Q \leq 0.05$).

| Pathway                                      | Pathway ID | Genes with Pathway Annotation | p Value        | Q Value          |
|----------------------------------------------|------------|-------------------------------|----------------|-----------------|
|                                              |            | DEGs (2934)                  |                |                 |
|                                              |            | All Genes (19,296)           |                |                 |
| Biosynthesis of other secondary metabolites  |            |                               |                |                 |
| Flavone and flavonol biosynthesis           | ko00944    | 8                             | 5.749022 x 10^{-3} | 2.874511 x 10^{-2} |
|                                              |            | 28                            |                |                 |
| Stilbenoid, diarylheptanoid and gingerol biosynthesis | ko00945  | 27                            | 2.693941 x 10^{-3} | 1.454728 x 10^{-2} |
|                                              |            | 150                           |                |                 |
| Phenylpropanoid biosynthesis                | ko00940    | 209                           | 2.371287 x 10^{-4} | 1.684862 x 10^{-3} |
|                                              |            | 1613                          |                |                 |
| Carbohydrate metabolism                     |            |                               |                |                 |
| Glycolysis/gluconeogenesis                  | ko00010    | 119                           | 2.607198 x 10^{-3} | 1.454728 x 10^{-2} |
|                                              |            | 902                           |                |                 |
| Inositol phosphate metabolism               | ko00562    | 98                            | 1.457824 x 10^{-3} | 8.945738 x 10^{-4} |
|                                              |            | 709                           |                |                 |
| Starch and sucrose metabolism               | ko00500    | 262                           | 4.067709 x 10^{-5} | 3.922434 x 10^{-4} |
|                                              |            | 2022                          |                |                 |
| Galactose metabolism                        | ko00052    | 134                           | 5.676684 x 10^{-6} | 8.515026 x 10^{-5} |
|                                              |            | 896                           |                |                 |
| Amino sugar and nucleotide sugar metabolism | ko00520    | 230                           | 2.096337 x 10^{-9} | 7.075137 x 10^{-8} |
|                                              |            | 1532                          |                |                 |
| Pentose and glucuronate interconversions    | ko00040    | 224                           | 2.589022 x 10^{-30} | 3.495180 x 10^{-28} |
|                                              |            | 993                           |                |                 |
| Digestive system                            |            |                               |                |                 |
| Cholesterol metabolism                      | ko04979    | 26                            | 2.363962 x 10^{-4} | 1.684862 x 10^{-3} |
|                                              |            | 122                           |                |                 |
| Energy metabolism                           |            |                               |                |                 |
| Carbon fixation in photosynthetic organisms | ko00710    | 71                            | 1.431960 x 10^{-3} | 8.945738 x 10^{-3} |
|                                              |            | 485                           |                |                 |
| Oxidative phosphorylation                   | ko00190    | 131                           | 1.187092 x 10^{-4} | 1.001609 x 10^{-3} |
|                                              |            | 930                           |                |                 |
| Photosynthesis                              | ko00195    | 39                            | 1.710027 x 10^{-5} | 2.098670 x 10^{-4} |
|                                              |            | 189                           |                |                 |
| Lipid metabolism                            |            |                               |                |                 |
| Glycerophospholipid metabolism              | ko00756    | 134                           | 5.903167 x 10^{-4} | 3.984638 x 10^{-3} |
|                                              |            | 992                           |                |                 |
| Linoleic acid metabolism                    | ko00591    | 30                            | 1.108358 x 10^{-4} | 9.975222 x 10^{-4} |
|                                              |            | 143                           |                |                 |
| Ether lipid metabolism                       | ko00565    | 61                            | 3.005129 x 10^{-5} | 3.120711 x 10^{-4} |
|                                              |            | 351                           |                |                 |
| Arachidonic acid metabolism                 | ko00590    | 37                            | 2.341729 x 10^{-5} | 2.634445 x 10^{-4} |
|                                              |            | 178                           |                |                 |
| Glycerolipid metabolism                     | ko00561    | 122                           | 1.313738 x 10^{-5} | 1.773546 x 10^{-4} |
|                                              |            | 814                           |                |                 |
| Steroid biosynthesis                        | ko00100    | 51                            | 1.062244 x 10^{-8} | 2.390049 x 10^{-7} |
|                                              |            | 216                           |                |                 |
| Cutin, suberine and wax biosynthesis        | ko00073    | 59                            | 3.884301 x 10^{-9} | 1.048761 x 10^{-7} |
|                                              |            | 260                           |                |                 |
| Metabolism of other amino acids             |            |                               |                |                 |
| Cyan amino acid metabolism                  | ko00460    | 136                           | 2.254576 x 10^{-4} | 1.684862 x 10^{-3} |
|                                              |            | 986                           |                |                 |
| Diterpenoid biosynthesis                     | ko00904    | 36                            | 1.022848 x 10^{-2} | 4.931589 x 10^{-2} |
|                                              |            | 236                           |                |                 |
| Monoterpenoid biosynthesis                  | ko00902    | 24                            | 5.264254 x 10^{-3} | 2.733363 x 10^{-2} |
|                                              |            | 135                           |                |                 |
Table 3. Cont.

| Pathway                              | Pathway ID | Genes with Pathway Annotation | p Value   | Q Value     |
|--------------------------------------|------------|-------------------------------|-----------|-------------|
|                                      |            | DEGs (2934) | All Genes (19,296) |            |             |
| Signal transduction                  |            |                |            |             |
| Phosphatidylinositol signaling system| ko04070    | 104            | 770        | 2.261160 × 10^{-3} | 1.327203 × 10^{-2} |
| Transcription                        |            |                |            |             |
| RNA polymerase                       | ko03020    | 166            | 795        | 3.529257 × 10^{-19} | 1.588166 × 10^{-17} |
| Transport and catabolism             |            |                |            |             |
| Endocytosis                          | ko04144    | 285            | 2152       | 3.517584 × 10^{-6} | 5.935923 × 10^{-5} |
| Phagosome                            | ko04145    | 111            | 697        | 2.018673 × 10^{-6} | 3.893155 × 10^{-5} |
2.6. Validation of DEGs by qRT-PCR

qRT-PCR was used to verify the reliability of RNA sequencing (Illumina sequencing). Twelve DEGs (six upregulated and six downregulated) were selected randomly. The results indicated that the Illumina sequencing data were reliable (Table 4). The fold change of some DEGs confirmed by qRT-PCR was different to the fold change detected by RNA-Seq, which may be due to the different computing methods of the two technologies: RNA-Seq calculates absolute quantification, whereas qRT-PCR detect relative expression.

| Gene ID          | Protein Identity                          | Fold Change | RNA-Seq | qRT-PCR |
|------------------|-------------------------------------------|-------------|---------|---------|
| LOC107915747     | cytochrome P450 83B1-like                 | 5.16        | 4.40    |
| LOC107892026     | zinc finger protein CONSTANS-LIKE 16-like | 6.06        | 5.83    |
| LOC107903815     | methyltransferase-like protein            | 5.21        | 1.5     |
| LOC107893683     | mitochondrial uncoupling protein 3-like   | 5.56        | 3.13    |
| LOC107911279     | transcription factor MYB114-like          | 5.04        | 5.03    |
| LOC107926337     | probable calcium-binding protein CML49    | 6.69        | 0.19    |
| LOC107905948     | probable calcium-binding protein CML13    | -10.76      | -2.74   |
| LOC107941823     | pollen allergen Che a 1-like              | -12.94      | -26.59  |
| LOC107942901     | pollen-specific protein-like A4g18596     | -11.48      | -8.12   |
| LOC107915309     | plasma membrane ATPase 4-like             | -10.61      | -1.30   |
| LOC107908343     | V-type proton ATPase subunit G1-like (ATP6V1G1) | -10.45      | -4.71   |
| LOC107903454     | cytochrome P450 76A2-like                 | -10.59      | -4.71   |

2.7. Candidate Genes Associated with CMS

We further analyzed the DEGs in different categories that might be related to CMS, toxic proteins, HATs (histone acetyltransferase) related proteins, TCA cycle related proteins, transcription factors, and some other proteins [6,17–20].

2.7.1. Abrin

In this study, eight genes were identified that encoded proteins with toxic activity. Interestingly, one gene (Unigene9082) was differentially expressed (Figure 8) and was significantly upregulated in the CMS line compared with the maintainer line. After the sequence was aligned against NCBI Blast (https://blast.ncbi.nlm.nih.gov/BlasT.cgi), it was predicted to be a toxic protein called Abrin that can be found in cotton.

![Figure 8. The heat map of key candidate genes associated with CMS.](https://example.com/figure8.png)
2.7.2. Histone Acetyltransferase (HATs)

Differential gene expression analysis revealed that two genes (Unigene33219 and CL2338.Contig1) were associated with histone acetyltransferase. These genes were significantly downregulated in the CMS line (Figure 8).

2.7.3. TCA Cycle

Gene expression analysis showed a significantly differentially expressed gene called Unigene27290 (Figure 8). After alignment to NCBI, it was predicted to be a cytoplasmic malate dehydrogenase (MDH) gene. Molecular identification and characterization also showed it to be malate dehydrogenase (MDH, EC 1.1.1.37), which belongs to the class A dehydrogenase family. This can form a highly conserved NAD (P) \( \sim \) dependent gene family and can catalyze the reversible conversion of oxaloacetate and malate.

2.7.4. WRKY40

GO analysis of genes retrieved from sepal, stigma and cotyledon development revealed that three genes (Unigene29896, unigene17074 and unigene17081) were highly differentially expressed (Figure 8). All encoded a transcription factor family called WRKY40, a stress-inducible transcription factor gene that plays an important role in stress.

2.7.5. Oxidative Phosphorylation

There were 45 DEGs associated with oxidative phosphorylation, including six upregulated and 39 downregulated. Oxidative phosphorylation is the most important process of energy metabolism and closely related to organ development and microspore development. Inadequate energy supply during microspore development causing microspore abortion is the main cause of CMS. Interestingly, an upregulated gene encodes a subunit of the ATP synthase (Figure 8), a mitochondrial gene called atp8, which plays an important role in the respiratory chain.

2.7.6. Transcription Factors

We found 5141 cotton genes with potential of encoding TFs, falling into 59 categories. We detected AP2–EREFP, NAC, bHLH, MYB, WRKY, MADS, BBR/BPC to analyze gene expression, respectively (Figure 9). These selected transcription factors are associated with biological development, energy metabolism, toxic activity protein and other functions.

Figure 9. The heatmap of key transcription factor family genes related to CMS.
3. Discussion

In this study, RNA sequencing technology was used to explore gene expression profiles of cotton associated with CMS compared to a maintainer line. The CMS line was obtained from the progeny of an allohexaploid cotton produced by distant hybridization of hexaploidy wild cotton and tetraploid upland cotton. The isogenic lines were produced by backcrossing with maintainer lines for many generations. Therefore, the CMS line LD6A is more suitable for studying the molecular mechanism of CMS and chromosome evolution.

Traditionally, near-isogenic lines are similar at the nuclear gene level, however the current transcriptomic study showed that their gene expression levels are quite different (13,871 DEGs out of 128,901 assembled genes), which indicated that some genetic material from the wild cotton (*Gossypium stocksii*) could not be totally replaced in the backcross process [21]. It plays an important role in gene expression and may be the key factor for microspore abortion in the CMS line (LD6A). Previously, sources of the CMS and maintainer line used for comparative analysis were quite different, even compared with the other near-isogenic lines [6,17]. Since the first cotton reference genome was published [22], more assembly reference genomes of diploid and tetraploid cotton have been developed [23–26] and the evolution of the cotton genome has been more focused [27,28]. However, little is known about the chromosome background of allohexaploid cotton. In this context, the current de novo assembly method was necessary, which annotated the full transcriptome data of LD6A.

The CMS line and its restoration system are the main pollination control systems for hybrid production by crop heterosis [29]. However, limited CMS and restorer germplasm resources, lack of effective pollination media and photo-temperature sensitivity of the restorer system prevent the increase of cotton yield. Identification of the molecular mechanism and development of new CMS germplasm resources are vital.

Histone acetyltransferases (HATs) play an important role in the structural modification of chromosomes and regulation of gene expression [30]. HATs and HDAs (histone deacetylases) are emerging as important components of protein complexes that affect the dynamics of chromatin folding during gene transcription [31,32]. HATs and HDAs are co-regulated to keep a dynamic balance between histone acetylation and histone deacetylation. A BTB/POZ and TAZ domain-containing protein with HAT activity was identified, which can affect the function and stability of many proteins. In the process of polyploidization and backcrossing, this balance may be destroyed by the differential expression of HATs, which may cause CMS.

During microspore development, an inadequate energy supply and reactive oxygen species (ROS) play a key role leading to pollen abortion. This energy is supplied by the mitochondria, which are the core sites for energy metabolism [33]. Therefore, we focused on the DEGs related to energy metabolism and biological development. A cytoplasmic malate dehydrogenase (MDH) gene related to the TCA cycle was found to be significantly downregulated in the CMS line, located in the cytoplasm. This participated in many metabolic pathways such as carbohydrate metabolism and lipid metabolism [34]. It has many biological functions in the glyoxylic acid cycle, tricarboxylic acid cycle, glucose synthesis, amino acid synthesis and redox stability [35]. In addition, malate dehydrogenase is a plant stress resistance and a candidate gene to breed cotton cultivars for increasing insoluble P absorption [36].

Meanwhile, a mitochondrial gene called *atp8*, related to oxidative phosphorylation, was upregulated in the present study but downregulated in UG93 [6,37]. This indicates that excessive ATP production and inadequate ATP consumption may lead to reactive oxygen species (ROS) explosion, which may cause cell toxicity and microspore abortion.

In this study, a toxic protein called Abrin was identified, which can cause mitochondrial apoptosis via the ribosomal pathway in human cells [38]. Toxic protein production is considered to be the cause of CMS [39]. The Database of Interacting Proteins (DIP) showed two proteins that interact with Abrin. One is glycosylation related, UDP-N-acetyl-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase (Gly5, DIP: 26207N), which is located on the Golgi apparatus. Another is acetylglutamate kinase (NAGK, DIP: 5348N), which is a catalytic enzyme for the second
step of arginine biosynthesis located in plastids and regulates gametophytic function and embryonic development [40]. This provides a new direction for studying the molecular mechanism of CMS and mitochondrial programmed cell death. Further molecular based research is recommended to explore the function of Abrin in cotton.

Transcription factors (TFs) play several roles in plant physiology throughout life. AP2-EREBP is a key regulator of several developmental processes, such as floral organ identity determination, to form part of the mechanisms used by plants to respond to various types of biotic and environmental stress [41]. NAC transcription factors interact directly or indirectly with other proteins by binding to DNA, participating in the plant biological and abiotic stress response, hormone signaling pathway transduction, apoptosis and other processes [42]. bHLH transcription factors are essential for the normal growth and development of plants, and act against various abiotic stresses in plants [43]. The MYB transcription factor is widely involved in plant metabolic regulation [44]. The WRKY gene family plays essential roles in diverse stress responses, and developmental and physiological processes [45]. A transcription factor family protein called WRKY40 is related to sepal, stigma and cotyledon development. It acts as a stress-inducible transcription factor gene and plays an important role in stress. We speculated that WRKY40 may be related to the accumulated ROS stress response. MADS transcription factors play an important role in flower development [46]. BBR/BPC transcription factors respond to ethylene with DNA-binding transcription factor activity and sequence-specific DNA binding [47]. The relationship between CMS and TFs with abnormal transcription level needs further study.

4. Materials and Methods

4.1. Plant Materials

The CMS line is a progeny, which was developed by distant hybridization and the allopolyploidization method. Its parents, Zhongmian16 (Gossypium hirsutum, tetraploid, AADD, 2n = 52) and a wild cotton (Gossypium stocksii, diploid, EE, 2n = 26) provided distant hybridization that yielded a triploid branch (provided by the National Wild Cotton Nursery). To obtain a double genome, we treated the branch with 0.1% colchicine solution. After self-pollination, hexaploidy seeds were obtained and backcrossed with Zhongmian16, which yielded a CMS mutant progeny. Thereafter, the CMS mutant was backcrossed with Zhongmian16 (9 generations and, named LD6A. Zhongmian16 was named LD6B as its maintainer line (Figure 10).

Cotton were sown (April–October) at the experimental farm of Guangxi University (Nanning, summer) and the National Wild Cotton Nursery (Sanya, winter). After microscopic study, floral buds were collected from LD6A and LD6B. The pollen abortion stage (tetrad stage, 4–5 mm in diameter) were frozen in liquid nitrogen and stored at −80 °C for RNA isolation.

4.2. RNA Extraction, cDNA Library Construction and Sequencing

Total RNA of both LD6A and LD6B lines were extracted from the floral bud (tetrad stage) using an RNA Isolation Kit (TransGen Biotech, Beijing, China). From each sample, 3 µg of the total RNA (RIN ≥8) was used for transcriptome cDNA library construction with a TruSeq™ RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA). RNA purification beads with oligo (dT) were used to separate mRNA from the total RNA. After breaking the mRNA into short fragments in fragmentation buffer, double-stranded cDNA was synthesized (Super ScriptII reverse transcriptase, Invitrogen, Carlsbad, CA, USA) and purified (Agencourt AMPure XP-Medium, Agencourt, Carlsbad, CA, USA). The short cDNA fragments were end-repaired with an A-tail addition and connected with adapters. After agarose gel electrophoresis, suitable fragments were used as templates for PCR amplification. The Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and an ABI StepOnePlus Real-Time PCR System were respectively used for quantification and qualification of the sample cDNA library. Solexa sequencing was performed by BGI (Shenzhen, China) using an Illumina HiSeq 4000 platform. The raw sequencing
files of these six samples (FASTQ files) are accessible from the NCBI Sequence Read Archive (SRA) database under Accession Number PRJNA577562.

4.3. De Novo Transcriptome Assembly and Gene Expression Profile

Raw sequence data were processed to obtain clean reads by SOAPnuke (self-developed by BGI, version: v1.4.0) and trimmomatic (version: v0.36) for filtering low-quality reads (quality score <20). Trinity (version v2.0.6) was used for de novo assembly, and then Tgicl (https://github.com/trinityrnaseq/trinityrnaseq/wiki) was used to cluster the assembled transcripts in Unigene. All coding sequences were predicted from Unigene using TransDecoder (version v3.0.1, https://transdecoder.github.io). Unigene was detected for SSR sequences by MISA (version v1.0, http://pgrc.ipk-gatersleben.de/misa), and primers were designed by Primer3 (version v2.2.2, http://bioinfo.ut.ee/primer3). High-quality clean reads were mapped with reference gene sequences by Bowtie2 (v2.2.5, http://bowtie-bio.sourceforge.net/Bowtie2/index.shtml). The expression levels of genes and transcripts were calculated by RSEM (version: v1.2.8 http://deweylab.biostat.wisc.edu/rsem/rsem-calculate-expression.html).

4.4. Gene Functional Annotation Analysis

For gene functional annotation, data from Unigene were aligned against various databases (KEGG, http://www.genome.jp/kegg; GO, http://geneontology.org; NR, ftp://ftp.ncbi.nlm.nih.gov/blast/db; NT, ftp://ftp.ncbi.nlm.nih.gov/blast/db; Pfam, http://pfam.xfam.org; SwissProt, https://www.uniprot.org; KOG, ftp://ftp.ncbi.nih.gov/pub/COG/KOG/kvva) using a combination of BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), HMMER (http://hmmer.janelia.org/), Blast2GO (http://www.blast2go.com/b2ghome), and KEGG Automatic Annotation Server (KAAS; http://www.genome.jp/kegg/kaas/). According to the results of NR annotation, the proportion of different species on the annotation was counted and the species distribution figure was drawn.

4.5. Transcription Factor Analysis

In order to determine plant transcription factors, the ORF in Unigene was detected using getorf (version EMBOSS:6.5.7.0, http://emboss.sourceforge.net/apps/cvs/emboss/apps/getorf.html). Further, hmmsearch (version v3.0, http://hmmmer.org) was used to compare ORFs with the domain of transcription factor proteins (data from TF). The previous two steps enabled us to identify the ability of Unigene to identify the characteristics of the transcription factor family described by the plant TFDB (http://plntfdb.bio.uni-potsdam.de/v3.0/).
4.6. Individualized Bioinformatics Analysis

All clean data were analyzed by Dr. Tom, an online software developed by BGI (http://report.bgi.com). Cluster heatmap, GO enrichment and KEGG enrichment were carried out, followed by standard process.

4.7. Verification of Gene Expression by qRT-PCR

The relative expression of the DEGs was verified by qRT-PCR and analyzed using the $2^{\Delta\Delta Ct}$ method [48], where the 18s gene was considered as the endogenous control. All the primers were designed by Primier 5.0 (Supplementary Materials 5) and synthesized by BGI (Shenzhen, China). RT-PCR and qRT-PCR were performed according to the method previously used [17].

5. Conclusions

In this study, the transcriptomes of the allohexaploid progeny cotton CMS line LD6A and its maintainer line LD6B were investigated using detailed RNA sequencing methodology. Thousands of DEGs were assessed between LD6A and LD6B focusing on their key biological processes and energy metabolism. The toxic protein, Abrin, and several other key DEGs such as BTB/POZ and TAZ family genes, MDH, WRKY40 and atp8 were found to be closely related to CMS. These genes are mainly involved in the TCA cycle, respiratory electron transfer chain, and oxidative phosphorylation, which were considered to be candidate CMS genes. Our findings will improve understanding of the gene regulation mechanism in CMS and the evolution of chromosomes.

Supplementary Materials: The following are available online at http://www.mdpi.com/1422-0067/20/24/6127/s1.

Author Contributions: J.Z. performed the experiments and drafted the manuscript. X.K., B.L. and Z.L. participated in the experiments. A.K., Y.L., H.K., F.U.D. participated in the revision of the manuscript. R.Z. conceived, designed and supervised the study. All authors read and approved the final manuscript.

Funding: This work was supported by a grant from the National Natural Science Foundation of China (Grant No. 31360348). The authors are thankful for the financial support from Weng Hong-Wu and Weng Hongwu Original Research Fund of Peking University of China (WHW201809).

Acknowledgments: We acknowledge Ming Tinghui (graduated from our team as a master) for her earlier basic morphology research on LD6A.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Campbell, B.T.; Saha, S.; Percy, R.; Frelichowski, J.; Jenkins, J.N.; Park, W.; Mayee, C.D.; Gotmare, V.; Dessauw, D.; Giband, M.; et al. Status of the Global Cotton Germplasm Resources. *Crop Sci.* 2010, 50, 1161. [CrossRef]
2. Fan, S. Progresses in Research on Cotton High Yield Breeding in China. *Sci. Agric. Sin.* 2016, 49, 3465–3476.
3. Yang, P.; Han, J.; Huang, J. Transcriptome sequencing and de novo analysis of cytoplasmic male sterility and maintenance in JA-CMS cotton. *PLoS ONE* 2014, 9, e112320. [CrossRef] [PubMed]
4. Zhou, B.; Chen, P.; Khan, A.; Zhao, Y.; Chen, L.; Liu, D.; Liao, X.; Kong, X.; Zhou, R. Candidate Reference Genes Selection and Application for RT-qPCR Analysis in Kenaf with Cytoplasmic Male Sterility Background. *Front. Plant Sci.* 2017, 8, 1520. [CrossRef] [PubMed]
5. Yang, L.; Wu, Y.; Zhang, M.; Zhang, J.; Stewart, J.M.; Xing, C.; Wu, J.; Jin, S. Transcriptome, cytological and biochemical analysis of cytoplasmic male sterility and maintainer line in CMS-D8 cotton. *Plant Mol. Biol.* 2018, 97, 537–551. [CrossRef] [PubMed]
6. Liao, X.; Zhao, Y.; Chen, P.; Zhou, B.; Diao, Y.; Yu, M.; Huang, Z.; Zhou, R. A Comparative Analysis of the atp8 Gene between a Cytoplasmic Male Sterile Line and Its Maintainer and Further Development of a Molecular Marker Specific to Male Sterile Cytoplasm in Kenaf (*Hibiscus cannabinus* L.). *Plant Mol. Biol. Rep.* 2016, 34, 29–36. [CrossRef]
7. Zheng, B.B.; Wu, X.M.; Ge, X.X.; Deng, X.X.; Grosser, J.W.; Guo, W.W. Comparative Transcript Profiling of a Male Sterile Hybrid Pumello and Its Fertile Type Revealed Altered Gene Expression Related to Flower Development. *PLoS ONE* 2012, 7, 43758. [CrossRef]

8. Yan, X.; Dong, C.; Yu, J.; Liu, W.; Jiang, C.; Jia, L.; Hu, Q.; Fang, X.; Wei, W. Transcriptome profile analysis of young floral buds of fertile and sterile plants from the self-pollinated offspring of the hybrid between novel restorer line NR1 and Nsa CMS line in *Brassica napus*. *BMC Genom.* 2013, 14, 26. [CrossRef]

9. Liu, C.; Ma, N.; Wang, P.Y.; Fu, N.; Shen, H.L. Transcriptome Sequencing and De Novo Analysis of a Cytoplasmic Male Sterile Line and Its Near-Isogenic Restorer Line in Chili Pepper (*Capsicum annuum* L.). *PLoS ONE* 2013, 8, e65209. [CrossRef]

10. Yu, Z.; Haage, K.; Streit, V.E.; Gierl, A.; Ruiz, R.A. A large number of tetraploid *Arabidopsis thaliana* lines, generated by a rapid strategy, reveal high stability of neo-tetraploids during consecutive generations. *Theor. Appl. Genet.* 2009, 118, 1107–1119. [CrossRef]

11. Levings, C.S., III; Pring, D.R. Restriction Endonuclease Analysis of Mitochondrial DNA from Normal and Texas Cytoplasmic-Male-Sterile Maize. *Science* 1976, 193, 158–160. [CrossRef]

12. Dewey, R.E.; Timothy, D.H.; Levings, C.S. A mitochondrial protein associated with cytoplasmic male sterility in the T cytoplasm of maize. *Proc. Natl. Acad. Sci. USA* 1987, 84, 5374–5378. [CrossRef]

13. Handa, H.; Gualberto, J.M.; Grienenberger, J.-M. Characterization of the mitochondrial orf B gene and its derivative, orf 224, a chimeric open reading frame specific to one mitochondrial genome of the “Polima” male-sterile cytoplasm in rape seed (*Brassica napus* L.). *Curr. Genet.* 1995, 6, 546–552. [CrossRef]

14. Song, J.; Hedgoth, C. A chimeric gene (orf256) is expressed as protein only in cytoplasmic male-sterile lines of wheat. *Plant Mol. Biol.* 1994, 26, 535–539. [CrossRef]

15. Moneger, F.; Smart, C.J.; Leaver, C.J. Nuclear restoration of cytoplasmic male sterility in sunflower is associated with the tissue-specific regulation of a novel mitochondrial gene. *EMBO J.* 1994, 13, 8–17. [CrossRef]

16. Chase, C.D. Cytoplasmic male sterility: A window to the world of plant mitochondrial—nuclear interactions. *Trends. Genet.* 2007, 23, 81–90. [CrossRef]

17. Kong, X.; Liu, D.; Liao, X.; Zheng, J.; Diao, Y.; Liu, Y.; Zhou, R. Comparative Analysis of the Cytology and Transcriptomes of the Cytoplasmic Male Sterility Line H276A and Its Maintainer Line H276B of Cotton (*Gossypium barbadense* L.). *Int. J. Mol. Sci.* 2017, 18, 2240. [CrossRef]

18. Chen, G.; Ye, X.; Zhang, S.; Zhu, S.; Yuan, L.; Hou, J.; Wang, C. Comparative Transcriptome Analysis between Fertile and CMS Flower Buds in Wucai (*Brassica campestris* L.). *BMC Genom.* 2018, 19, 908. [CrossRef]

19. Mei, S.; Liu, T.; Wang, Z. Comparative Transcriptome Profile of the Cytoplasmic Male Sterile and Fertile Floral Buds of Radish (*Raphanus sativus* L.). *Int. J. Mol. Sci.* 2016, 17, 42. [CrossRef]

20. Liu, X.; Zhang, R.; Ou, H.; Gui, Y.; Wei, J.; Zhou, H.; Tan, H.; Li, Y. Comprehensive transcriptome analysis reveals genes in response to water deficit in the leaves of Saccharum narenga (Nees ex Steud.) hack. *BMC Plant Biol.* 2018, 18, 250. [CrossRef]

21. Han, J.; Zhou, B.; Shan, W.; Yu, L.; Wu, W.; Wang, K. A and D genomes spatial separation at somatic metaphase in tetraploid cotton: Evidence for genomic disposition in a polyploid plant. *Plant J.* 2015, 84, 1167–1177. [CrossRef]

22. Wang, K.; Wang, Z.; Li, F.; Ye, W.; Wang, J.; Song, G.; Yue, Z.; Cong, L.; Shang, H.; Zhu, S.; et al. The draft genome of a diploid cotton *Gossypium raimondii*. *Nat. Genet.* 2012, 44, 1098–1103. [CrossRef]

23. Li, F.; Fan, G.; Wang, K.; Sun, F.; Yuan, Y.; Song, G.; Li, Q.; Ma, Z.; Lu, C.; Zou, C.; et al. Genome sequence of the cultivated cotton *Gossypium arboreum*. *Nat. Genet.* 2014, 46, 567–572. [CrossRef]

24. Wang, M.; Tu, L.; Yuan, D.; Zhu, D.; Shen, C.; Li, J.; Liu, F.; Pei, L.; Wang, P.; Zhao, G.; et al. Reference genome sequences of two cultivated allotetraploid cottons, *Gossypium hirsutum* and *Gossypium barbadense*. *Nat. Genet.* 2019, 51, 224–229. [CrossRef]

25. Wang, S.; Chen, J.; Zhang, W.; Hu, Y.; Chang, L.; Fang, L.; Wang, Q.; Lv, F.; Wu, H.; Si, Z.; et al. Sequence-based ultra-dense genetic and physical maps reveal structural variations of allopolyploid cotton genomes. *Genome Biol.* 2015, 16, 108. [CrossRef]

26. Li, F.; Fan, G.; Lu, C.; Xiao, G.; Zou, C.; Kohel, R.J.; Ma, Z.; Shang, H.; Ma, X.; Wu, J.; et al. Genome sequence of cultivated Upland cotton (*Gossypium hirsutum* TM-1) provides insights into genome evolution. *Nat. Biotechnol.* 2015, 33, 524–530. [CrossRef]

27. Wang, M.; Wang, P.; Lin, M.; Ye, Z.; Li, G.; Tu, L.; Shen, C.; Li, J.; Yang, Q.; Zhang, X. Evolutionary dynamics of 3D genome architecture following polyploidization in cotton. *Nat. Plants* 2018, 4, 90–97. [CrossRef]
28. Hu, Y.; Chen, J.; Fang, L.; Zhang, Z.; Ma, W.; Niu, Y.; Ju, L.; Deng, J.; Zhao, T.; Lian, J.; et al. Gossypium barbadense and Gossypium hirsutum genomes provide insights into the origin and evolution of allotetraploid cotton. *Nat. Genet.* 2019, 51, 739–748. [CrossRef]
29. Fu, D.; Xiao, M.; Hayward, A.; Fu, Y.; Liu, G.; Jiang, G.; Zhang, H. Utilization of crop heterosis: A review. *Euphytica* 2014, 197, 161–173. [CrossRef]
30. Wang, Z.; Qin, G.; Zhao, T.C. Histone Deacetylase 4 (HDAC4): Mechanism of Regulations and Biological Functions. *Epigenomics* 2014, 6, 139–150. [CrossRef]
31. Servet, C.; Conde E Silva, N.; Zhou, D. Histone Acetyltransferase AtGCN5/HAG1 Is a Versatile Regulator of Developmental and Inducible Gene Expression in Arabidopsis. *Mol. Plant* 2010, 3, 670–677. [CrossRef]
32. Zheng, H.; Zhang, F.; Wang, S.; Su, Y.; Ji, X.; Jiang, P.; Chen, R.; Hou, S.; Ding, Y. MLK1 and MLK2 Coordinate RGA and CCA1 Activity to Regulate Hypocotyl Elongation in Arabidopsis thaliana. *Plant Cell* 2018, 30, 67–82. [CrossRef]
33. Brookes, P.S.; Yoon, Y.; Robotham, J.L.; Anders, M.W.; Sheu, S. Calcium, ATP, and ROS: A mitochondrial love-hate triangle. *Am. J. Physiol. Cell Physiol.* 2004, 287, C817–C833. [CrossRef]
34. Muhammad, I.; Zhang, B.; Tang, K.; Liu, J. Molecular Identification and Characterization of Cotton Malate Dehydrogenase Gene Family; Tsinghua University: Beijing, China, 2017.
35. Mohan, A.; Muthukrishnan, S.; Hunt, M.C.; Barstow, T.J.; Houser, T.A. Kinetics of Myoglobin Redox Form Stabilization by Malate Dehydrogenase. *J. Agric. Food Chem.* 2010, 58, 6994–7000. [CrossRef]
36. Wang, Z.A.; Li, Q.; Tang, K.; Liu, J.; Su, Y.; Ji, X.; Jiang, P.; Chen, R.; Hou, S.; Ding, Y. MLK1 and MLK2 Coordinate RGA and CCA1 Activity to Regulate Hypocotyl Elongation in Arabidopsis thaliana. *Plant Cell* 2018, 30, 67–82. [CrossRef]
37. Kong, X.; Liu, D.; Zheng, J.; Khan, A.; Li, B.; Diao, Y.; Zhou, R. RNA editing analysis of ATP synthase genes in the cotton cytoplasmic male sterile line H276A. *Biol. Res.* 2019, 52, 6. [CrossRef]
38. Servet, C.; Conde E Silva, N.; Zhou, D. Histone Acetyltransferase AtGCN5/HAG1 Is a Versatile Regulator of Developmental and Inducible Gene Expression in Arabidopsis. *Mol. Plant* 2010, 3, 670–677. [CrossRef]
39. Levings, C.R. Thoughts on Cytoplasmic Male Sterility in cms-T Maize. *Plant Cell* 1993, 5, 1285–1290. [CrossRef]
40. Huang, J.; Chen, D.; Yan, H.; Xie, F.; Yu, Y.; Zhang, L.; Sun, M.; Peng, X. Acetylglutamate kinase is required for both gametophyte function and embryo development in Arabidopsis thaliana. *J. Integr. Plant Biol.* 2017, 59, 642–656. [CrossRef]
41. Sharoni, A.M.; Nuruzzaman, M.; Satoh, K.; Shimizu, T.; Kondoh, H.; Sasaya, T.; Choi, I.; Omura, T.; Kikuchi, S. Gene Structures, Classification and Expression Models of the AP2/EREBP Transcription Factor Family in Rice. *Plant Cell Physiol.* 2011, 52, 344–360. [CrossRef]
42. Olsen, A.N.; Ernst, H.A.; Leggio, L.L.; Skriver, K. NAC transcription factors: Structurally distinct, functionally diverse. *Trends. Plant Sci.* 2005, 10, 79–87. [CrossRef]
43. Zhou, Q.; Anderson, D.J. The bHLH Transcription Factors OLG2 and OLG1 Couple Neuronal and Glial Subtype Specification. *Cell 2002, 109*, 61–73. [CrossRef]
44. Dubos, C.; Stracke, R.; Grotewold, E.; Weisshaar, B.; Martin, C.; Lepiniec, L. MYB transcription factors in Arabidopsis. *Trends. Plant Sci.* 2010, 15, 573–581. [CrossRef]
45. Chen, L.; Song, Y.; Li, S.; Zhang, L.; Zou, C.; Yu, D. The role of WRKY transcription factors in plant abiotic stresses. *Biomed. Biophy. Acta* 2012, 1819, 120–128. [CrossRef]
46. Shao, S.; Li, B.; Zhang, Z.; Zhou, Y.; Jiang, J.; Li, X. Expression of a cotton MADS-box gene is regulated in anther development and in response to phytohormone signaling. *J. Genet. Genom.* 2010, 37, 805–816. [CrossRef]
47. Prewitt, S.F.; Ayre, B.G.; McGarry, R.C. Cotton CENTRORADIALIS/Terminal Flower 1/Self-Pruning genes functionally diverged to differentially impact plant architecture. *J. Exp. Bot.* 2018, 69, 5403–5417. [CrossRef]
48. Livak, K.; Schmittgen, T. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2^(-ΔΔCT) Method. *Methods* 2000, 25, 402–408. [CrossRef]