RESEARCH ARTICLE

Transcriptomic analysis of differentially expressed genes in flower-buds of genetic male sterile and wild type cucumber by RNA sequencing

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Abstract Cucumber (*Cucumis sativus* L.) pollen development involves a diverse range of gene interactions between sporophytic and gametophytic tissues. Previous studies in our laboratory showed that male sterility was controlled by a single recessive nuclear gene, and occurred in pollen mother cell meiophase. To fully explore the global gene expression and identify genes related to male sterility, a RNA-seq analysis was adopted in this study. Young male flower-buds (1–2 mm in length) from genetic male sterility (GMS) mutant and homozygous fertile cucumber (WT) were collected for two sequencing libraries. Total 545 differentially expressed genes (DEGs), including 142 up-regulated DEGs and 403 down-regulated DEGs, were detected in two libraries (Fold Change ≥ 2 , FDR < 0.01). These genes were involved in a variety of metabolic pathways, like ethylene-activated signaling pathway, sporopollenin biosynthetic pathway, cell cycle and DNA damage repair pathway. qRT-PCR analysis was performed and showed that the correlation between RNA-Seq and

qRT-PCR was 0.876. These findings contribute to a better understanding of the mechanism that leads to GMS in cucumber.

Keywords Transcriptomics · RNA-seq · Genetic male sterility · Cucumber

Introduction

Hybridization is widely used to generate new fine properties in agriculture and horticulture. Conventionally, for cross pollination of plants especially main crops like rice and wheat, hand pollination is costly and difficult to ensure seed purity, which greatly limits the production of commercial hybrid seeds. So, the use of male sterile lines of a hybrid system that is unable to produce or release functional pollen can be free from artificial emasculation, lower labor costs and improve seed purity. Since the first male sterility gene *ms-1* was found, more and more cytoplasmic male sterility (CMS) and genetic male sterility (GMS) genes were reported, including *Orf*, *Cox2* and *Tms2* genes (Ducos et al. 2001; Pitnjam et al. 2008; Song et al. 1994). GMS-sterile lines are mainly controlled by allele usually recessive nuclear genes. In accordance with the anther development period and the form of pollen abortion, GMS genes can be summarized into the following categories: related to meiotic irregularities like *MSP1*, *PAIR1*, *PAIR2*, *AM1*, *Bub1*, *PPS1* (Nonomura et al. 2003, 2004; Pawlowski et al. 2009; Wang et al. 2012; Zhou et al. 2011); abnormal changes of callose distribution such as *CalS1*, *CalS5*, *AtGsl5*, *Osg1* (Che et al. 2011; Hong et al. 2001; Ostergaard et al. 2002; Wan et al. 2011); aberrant development of tapetum for instance *UDT1*, *OsTDL1A*, *TDR*, *PTC* (Jung et al. 2005; Li et al. 2006, 2011; Zhao et al.

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2008); anomalous development of pollen wall, including *CYP704B2*, *DPW*, *TDR*, *Wda1* (Jung et al. 2006; Shi et al. 2011; Zhang et al. 2008; Lia et al. 2010); abnormal anther dehiscence such as *OPR3*, *DAD1*, *MYB26*, *AID1* (Steiner-Lange et al. 2003; Stintzi et al. 2000; Xie et al. 1998; Zhu et al. 2004); and others like *OsGAMYB*, *BAM1&BAM2*, *CSA*, *P/TMS12-1* (Kaneko et al. 2004; Sun et al. 2007; Zhang et al. 2010; Zhou et al. 2012). However, the underlying anther development mechanism associated with gene regulation in male sterility is poorly investigated (Wei et al. 2013).

Cucumber (*Cucumis sativus* L.) is an economically and nutritionally important crop of the *Cucurbitaceae* family and has long been served as a primary model system for sex determination studies (Guo et al. 2010). Male sterility mutants have frequently been described in many other plants like maize, rice and cotton, but seldom reported in cucumber (Pawlowski et al. 2009; Wan et al. 2011; Wei et al. 2013). In spring of 2012, 36 male sterile plants were occasionally found in a selfing population of inbred line ‘YL-5’ obtained from 8 consecutive self-crossing and selection in plastic tunnel with day temperature 20–35 °C and night temperature 15–23 °C conditions. Further research work indicated that it was a spontaneous mutant of the wild type YL-5. Histological studies suggested that the main cause of the male sterility was defective microsporogenesis, resulting in no tetrad and microspores being formed, and the corresponding gene *ms-3* was further fine mapped through sequencing and KASP analysis (Han et al. 2017). However, the global differential gene expression profile remains unclear. Genome-wide expression studies provide breeders with an understanding of molecular basis of complex traits (Pawełkiewicz et al. 2016). This paper mainly describes differentially expressed genes in meiosis phase of male buds in GMS and WT cucumber by transcriptome Sequencing for comprehensive understanding of the mechanism of pollen abortion in GMS cucumber.

Materials and methods

Plant materials and RNA isolation

High-generation inbred line of cucumber ‘YL-5’ and its GMS mutant were grown in greenhouse of experimental farm of Tianjin kernel Cucumber Research Institute in Wuqing, Tianjin, China under normal conditions in October of 2014. The genotype is a monoecious in sex type. Young flower-buds (1–2 mm in length) from 56 homozygous fertile cucumber (WT), 56 GMS cucumber in the same background were collected respectively. The excised buds from 56 plants were mixed, frozen in liquid nitrogen

and then stored at – 80 °C. Total RNAs were isolated from the mixed flower samples using TaKaRa MiniBEST Plant RNA Extraction Kit referencing to the instructions.

cDNA library construction and sequencing

Poly (A) mRNA was isolated by Oligo-(dT) magnetic beads from total RNA, then fragmented by fragmentation buffer (Ambion, Austin, TX). The cDNA was synthesized by cDNA Synthesis Kit (Stratagene, Cedar Creek, USA) according to the manufacturer’s instruction, the QiaQuick PCR extraction kit (Qiagen, Valencia, CA) was used to purify the short fragments. Then PCR amplification was carried out to enrich the purified cDNA. The cDNA library was constructed and sequenced on the platform of Illumina HiSeq2500, concentration and insert size were evaluated with Qubit2.0 and Agilent 2100 Bioanalyzer.

Gene functional annotation and differential expression analysis

Raw reads were presented in fq data (fastq), and filtered to remove reads containing adaptors and low-quality. The process dealing with raw reads were as follows: (1) removal of adapter sequences. (2) removal of low-quality reads (reads with more than 50% bases Q < 10). (3) removal of low-quality data, ensure overall quality value Q30 > 85%. Then they were aligned to the genome of the cucumber (cucumber Chinese long genome v2) with *TopHat2* (Kim et al. 2013), parameters used in Tophat were mismatch = 2, other parameters as the default value. Mapped reads were assembled to transcript using Cufflinks software (<http://cole-trapnell-lab.github.io/cufflinks/>). These transcripts were then compared with known genes (defined genes in cucumber reference genome file). If a new transcript was in the intergenic region of known genes, it would be candidate new gene. Filter out new candidate genes with only single exons. Potential coding sequences were analyzed using get orf program for new genes identification. New genes were then aligned with the database of NR, SwissProt, GO, KEGG to annotate their functions. Differentially Expressed Genes (DEGs) were filtered following the criteria of Fold Change ≥ 2 and False Discovery Rate < 0.01 with DESeq (version1.10.1) (<http://www.bioconductor.org/packages/release/bioc/html/DESeq.html>), parameters set as follows: FC = 2, FDR < 0.01, other parameters as the default value.

Quantitative RT-PCR analysis

qRT-PCR analysis was used to verify the RNA-seq results and some genes of interest. Mixed buds from 56 WT plants and 56 GMS plants were repacked into small tubes, some

used for RNA-Seq and the rest for qRT-PCR analysis. Twelve DEGs annotated with male flower development or with unknown function but showing great differential expression were selected to subject qRT-PCR using Applied Biosystems® 7500 Real-Time PCR Systems (ThermoFisher, USA) with specific primers designed online (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and listed in the Table 1. The UBI-ep gene was used as the internal control for normalization of gene expression and SYBR Green (Takara, Japan) to detect PCR products (Wan et al. 2010). There were used three biological replicates and three technical replicates to ensure its reliability. The amplifications were performed according to the following protocol: 95 °C for 3 min, 40 cycles of 95 °C for 10 s and 58 °C for 30 s, followed by a thermal denaturing step to generate the melting curves of the amplified products. The relative expression levels were calculated with the $2^{-\Delta\Delta C_T}$ method and showed in histograms. The correlation between the RNA-seq and qRT-PCR results was evaluated by linear regression analysis based on the following equation: $\text{Log}_2(\text{qRT-PCR Fold Change}) = a \text{ Log}_2(\text{RNA-seq Fold Change}) + b$.

Results

Analysis of the transcriptome sequencing libraries

Previous researchers found that cotton male sterility of ‘Dong A’GMS mutant occurred in uninucleate microspore stage (Huang et al. 1981). Our original sequencing data

(SRA accession: SRP127536; Temporary submission ID: SUB3395363) had been uploaded to SRA database. However, our previous studies showed that male sterility in cucumber occurred earlier, tetrad cannot be formed in meiosis phase of pollen development. The GMS pollen aborted in this stage, when male flower bud length is approximately 1–2 mm. Thus, we mixed 1–2 mm long male buds to analyze the differentially expressed genes between the WT and the GMS mutants. The illumina HiSeq2500 platform was used to perform transcriptome sequencing of two cucumber cDNA libraries (WT and the GMS mutants).

The two libraries were sequenced and 9.98 Gb clean data were generated in total. Each library generated more than 4.33 Gb clean reads, both Q30 reached 86.46% (Table S1). All clean reads were aligned with reference genome of cucumber Chinese long genome v2.0 and mapping rate varied from 83.62 to 83.92% (Table S2). All mapped reads were assembled using cufflinks and aligned with databases of NR, Swiss-Prot, GO, COG, KEGG using BLAST. 479 new genes that were not annotated before were discovered and 381 of which were annotated in this test for functional annotation (Table S3).

After calculating gene expression levels of the unique mapped reads using RPKM (Reads Per Kilobases per Millionreads) values, DEGs were screened out between the MS and WT libraries at the criteria of at least two-fold change and Chi square test ($p < 0.05$, FDR < 0.01). A total of 545 DEGs were detected in our sequencing data (Table S4). Compared with WT plant, 142 genes were up-

Table 1 Genes selected and primers designed

Gene ID	Forward primer (5′–3′)	Reverse primer (5′–3′)
Cucumber_newGene_50	ATCGCCAGTAGTAGGAGCCA	TTTCAGACCCTGCCAAGTCC
Csa6G518270	AGCAAAAACAACTCCGCCA	GTCAGAGGATGAAGAGGAAGAACA
Csa1G537610	AGCTTCCCACAAGCACTGAA	GTTTCCAGTCTCCTCCAGCAA
Csa2G379350	GGAAGGTCTCCAATCAGGAT	CCGCTCAATCCCCACAATCT
Csa5G430000	AGGAGGAAGAGTCCAAGGATGA	CCGTGAGGCGTTGAGAGAAA
Csa6G518150	CTCCCTCTGCCATTTCCTAA	CGAAGATGACGCAATCAGCAA
Csa4G551130	AGGGCAACTACTGGCTTTT	TGTTTCATGCTTGGCCTTGGA
Csa6G448080	GTCCAGTGCTTGAATTGTCTGATG	CGTCACTCTGTTTCATCCTCGAAA
Csa7G043580	GGCAATAAATGGGCTCGTATGG	ACGTTTCATTCGGGTATTCCAA
Cucumber_newGene_606	AAAGTCCCTCTGCCATTCTGT	ACTTCCCATCCCCACAAGGA
Csa6G501860	CGCCAAGCTCTCCCATCTTA	GCATCATCCACTTTACCACACT
Csa5G291670	GGGGAGATTCCGAGAGAATGTAG	CGAAACGACCAACACCGAAAC
Csa6G502760	CCGCAGAGCAAGAATCCTCAA	TTGTCTGTCCACGATGGTT
Cucumber_newGene_1057	TGGAAAGGTGTATGCAGTCAAA	ACGTCTGAATGTCTTGGAGTAACA
Csa6G002350	ATCACCAACAACCCCTCAATCA	TTTCCCCTACATCTTCTCTCTTG
Csa3G164550	TGTGGGTAGCCTCGGAAAGT	AGCACTTGGGGGTGAATCTG
UBI-ep	CACCAAGCCCAAGAAGATC	TAAACCTAATCACCACCAGC

regulated in MS ones, but 403 genes were down-regulated in MS plant (Table S4).

GO and clustering analysis

To characterize the functions of genes associated with male sterility, a GO annotation was performed (<http://www.Arabidopsis.org/tools/bulk/go/index.jsp>). The proportions and comparisons between DEGs and all genes were summarized in three main functional categories: cellular component, molecular function and biological process (Fig. 1). 264, 280 and 298 DEGs were described respectively in above categories (Table S5). In each category, the height of yellow and blue columns represents all genes and DEGs enrichment degree. Categories that DEGs have more enrichment than all genes were picked out, including genes that associated with male sterility. Categories were ranked based on significance, and the top-ranking functions were given priority (Table S5). 41 DEGs were annotated in Cellular Component: integral component of membrane (GO: 0016021). Csa4G358690 and Csa1G006310 were two significant different genes between male sterile and fertile, then the genes were annotated in Molecular Function: tetrapeptides alpha-pyrone synthase activity (GO:

0090439). 23 DEGs participated in Biological Process: abscisic acid biosynthetic process (GO: 0009688). DEGs involved in sporopollenin and ethylene biosynthetic process were focused because these processes are very important for microsporogenesis.

KEGG pathway analysis of DEGs

The appearance of infertility gene brings out momentous changes in another genes expression. To determine genes involved in the specific metabolic pathways, total 60 DEGs were annotated with KEGG and assigned to 49 KEGG pathways (Fig. 2, Table S6). The most significant categories in our analysis are “protein processing in endoplasmic reticulum” (ko04141: a functional classification in the KEGG database) (Table S6). Heat shock protein family (Hsp40, Hsp70, Hsp90) involving in ER-related degradation and ubiquitin ligase complex in ko04141. Csa1G642540 was annotated in Heat Shock Protein 40 (HSP40) information database; Csa5G512930 and Csa5G514500 in Hsp70; Csa1G569270 and Csa3G183950 in Hsp90. Csa4G296190 was annotated in negative factor (*NEF*), Csa1G527870, Csa3G113300, Csa5G190560, Csa5G197120, Csa5G198120, Csa7G072770 in split-

Fig. 1 Histogram of several GO classifications of all genes and DEGs. Results are summarized in three main GO categories: biological process, cellular component and molecular function. All genes with gray bar graph and DEGs with black bar graph. The abscissa indicates various gene functional classification in the GO database, and the left ordinate indicates enrichment of the

differentially expressed genes in a class (that is, the ratio of DEGs in a certain functional category to the total DEGs); And enrichment of a gene in a responding class (that is, the ratio of genes in a functional category to the total genes screened). The right ordinate indicates the number of DEGs and genes. *DEG* differentially expressed genes, *GO* gene ontology

Fig. 2 DEGs in KEGG pathway. The numerals on the right side showed the numbers of DEGs in each category. *DEG* differentially expressed genes, *KEGG* kyoto encyclopedia of genes and genomes

hand/foot malformation (*sHSF*). Both *NEF* and *sHSF* participate in process of ER-related degradation. Csa4G361910 was annotated in Phosphodiesterase inhibitors (*PDI*s), which is involved in protein targeting pathway, Csa2G074200 and Csa6G521010 were annotated in ATP binding cassette subfamily B member 1 (*ABCB1*) in the pathway of ABC transporters (ko02010).

RNA-seq expression validation by qPCR

To confirm the reliability of the RNA-seq data, 12 DEGs were selected for qRT-PCR evaluation based on their functions and highest fold changes between 2 libraries. These candidate genes are involved in flower development biological processes of pollen germination (GO: 0009846), regulation of flower development (GO: 0009909), microsporogenesis (GO: 0009556), auxin biosynthetic process (GO: 0009851), pollen tube growth (GO:

0009860), auxin polar transport (GO: 0009926) and anther wall tapetum morphogenesis (GO: 0048655). Some candidate genes showed a considerable difference in gene expression level between WT and GMS mutants participating in biological processes of DNA-templated (GO: 0006355), hormone-mediated signaling pathway (GO: 0009755), oxidation–reduction process (GO: 0055114) and intracellular protein transport (GO: 0006886). And some were uncharacterized, but showing a significant difference in expression (Fig. 3, Table 2). All genes indicated by qRT-PCR agreed well with the RNA-seq patterns, and the correlation between RNA-seq and qRT-PCR was measured by scatter plotting log₂-fold changes (Fig. 4), which displayed a positive correlation coefficient (Pearson coefficient $R^2 = 0.8765$). Our results indicated a good reproducibility and thereby validated our expression profile analysis.

Fig. 3 Expression profiles of the selected genes measured by RNA-seq and qRT-PCR. Bars on each histograms or plots represent error bars. *RPKM* reads per kilobases per million reads

Discussion

DEGs related to sporopollenin biosynthetic pathway

Sporopollenin is the primary component of pollen exine, consisting of fatty acids and phenolic compounds, responsible for exine's adamant physical strength, protecting anther against various stresses (Yule et al. 2000). If there are abnormal changes in development of sporopollenin, the structure of cell wall may transform to abort the pollen (Piffanelli et al. 1998). Male Sterility 2 (MS2) protein is a fatty acyl-CoA reductase 2, first described in *Arabidopsis thaliana* (Aarts et al. 1997). MS2 plays a key role for the synthesis of sporopollenin. In ms2 mutant lines, pollen exine become thinner and the microspores are ultimately degraded. In our test, we found a homologous gene of ms2, fatty acyl-CoA reductase 2-like gene, Csa4G551130, 9.086 down-regulated in DEG set. It encodes male sterility-related protein in *Linum usitatissimum*, responses to cadmium ion and owns oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor (Table S5).

Other than MS2, cytochromes P450 CYP703A2 and CYP704B1, acyl-coenzyme A synthetase 5 (*ACOS5*), less adhesive pollen/polyketide synthase (*LAP5/PKSB* and *LAP6/PKSA*) are reported to be involved in the modification and accumulation of fatty acids, regulating exine formation of pollen (De Azevedo et al. 2009; Doan et al. 2009; Dobritsa et al. 2009; Grienberger et al. 2010; Kim

Fig. 4 Correlation of gene expression results obtained from qRT-PCR analysis and RNA-seq analysis

et al. 2010; Morant et al. 2007). We also found the cytochrome P450 family genes, including 704C1-like gene (Csa2G108690), CYP707A12v1 (Csa7G073680) and CYP707A13 (Csa4G639800), in DEGs set. In angiosperm, flavonoids play a key role in pollen germination and sterility. Chalcone synthase and flavonoid 3', 5'-hydroxylase are critical genes in flavonoids biosynthesis, vital for cell wall of pollen grains (Martens et al. 2010). Similar functional genes including acyl-coenzyme A thioesterase 8-like (Csa3G122430), Chalcone synthase A-like (Csa1G006310) and chalcone synthase J-like (Csa4G358690) were discovered upregulated in our DEGs. They all have tetraketide alpha-pyrone synthase activity, participate in polyketide biosynthetic process and sporopollenin biosynthetic process, ultimately contribute to pollen exine biosynthesis.

Table 2 DEGs selected for qRT-PCR

GeneID	Functions	log2FC
Cucumber_newGene_50	Flower morphogenesis (GO:0048439); regulation of flower development (GO:0009909)	4.613
Csa1G537610	Microsporogenesis (GO:0009556);	− 2.2
Csa2G379350	Inflorescence development (GO:0010229)	− 2.4
Csa5G430000	Pollen tube growth (GO:0009860);	− 3.014
Csa6G518150	Biological process: anther development (GO:0048653)	− 6.386
Csa4G551130	Pollen exine formation (GO:0010584)	− 9.086
Csa6G448080	Putative uncharacterized protein	− 7.009
Csa7G043580	Uncharacterized protein	− 6.004
Cucumber_newGene_606	Uncharacterized protein	5.608
Csa6G501860	Putative uncharacterized protein	5.129
Csa5G291670	Microgametogenesis (GO:0055046)	− 7.643
Csa6G502760	Uncharacterized protein	− 6.127

DEGs regulation in meiosis phase

Meiosis is important in eukaryotes replication and evolution. Meiotic disorder may lead to the male sterility of plants. In rice, *PAIR1*, *PAIR2* and *PAIR3* are essential for restoration of homologous chromosomes in meiosis I (Nonomura et al. 2004; Zhou et al. 2011). We also found abnormal expression level of genes like DNA cross-link repair protein *Cucumber_newGene_606* (up-regulated), *Csa5G266850* (down-regulated) and *Cucumber_newGene_78* (down-regulated) acting on DNA reproduction, recombination and repairing.

Pollen semi-sterility1 (*PPS1*) is reported to quantitatively encode a kinesin-1-like protein in rice panicle. When Arg (Arg-289) is substituted by His on-promoter region, the microtubule-stimulated ATPase activity is abolished, chromosome separation disordered and male sterility occurred (Zhou et al. 2011). In our data, we discovered two homologous genes of *PPS1*, *Cucumber_newGene_50* (up-regulated) and *Csa1G675040* (down-regulated), involved in anastral spindle assembly, chromosome recombination and segregation during the process of male meiosis in cucumber. *Cucumber_newGene_1098* (down-regulated) encodes a kind of separase that positively regulating sister chromatid cohesion, participating in the process of chromosome separation and cell division.

Prospect

The male sterility is not a signal biological trait. Understanding regulatory mechanism of morphogenesis and function of stamen is necessary for plant breeding. Any mutant gene in this developmental process may break the normal process. Therefore, it is reasonable and meaningful to further identify the differential expression gene between GMS plants and WT plants. In this study, we found the DEGs and new genes involved in sporopollenin biosynthetic pathway, meiosis phase and ethylene. However, we do not know which gene or genes break the normal stamen development. Further studies such as gene knock-in, knock-out should be conducted to identify the function of these genes in GMS strains.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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