



## Article

# Transcriptome Analysis of Sponge Gourd (*Luffa cylindrica*) Reveals Candidate Genes Associated with Fruit Size

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**Abstract:** Sponge gourd belongs to the Cucurbitaceae family and *Luffa* genus. It is an economically valuable vegetable crop with medicinal properties. The fruit size of sponge gourd presents distinct diversity; however, the molecular insights of fruit size regulation remain uncharacterized. Therefore, two sponge gourd materials with distinct fruit sizes were selected for a comparative transcriptome analysis. A total of 1390 genes were detected as differentially expressed between long sponge gourd (LSG) and short sponge gourd (SSG) samples, with 885 downregulated and 505 upregulated in SSG compared with LSG. KEGG pathway enrichment analysis revealed that the MAPK signaling pathway, biosynthesis of secondary metabolites, and plant hormone signal transduction were significantly enriched. The DEGs involved in the cell cycle and cell division, plant hormone metabolism, and MAPK signal transduction were crucial for sponge gourd fruit size regulation. Additionally, the transcription factor families of ERF, NAC, bHLH, MYB, WRKY, and MADS-box were associated with fruit size regulation. The qRT-PCR validation for selected DEGs were generally consistent with the RNA-Seq results. These results obtained the candidate genes and pathways associated with fruit size and lay the foundation for revealing the molecular mechanisms of fruit size regulation in sponge gourd.

**Keywords:** sponge gourd; fruit size; transcriptome; differentially expressed genes



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## 1. Introduction

Sponge gourd ( $2n = 2x = 26$ ), which belongs to the family Cucurbitaceae and genus *Luffa*, is an annual climbing herb originating from the subtropical and tropical regions of Asia [1]. The fruits of sponge gourd are edible and rich in nutrients, such as carbohydrates, protein, vitamin, crude fiber, and minerals [2]. Additionally, their extracts and isolated compounds have certain pharmacological effects (e.g., immunomodulatory, antioxidant, anticancer, and anti-inflammatory) that are beneficial for human health [3,4].

Fruit size, which is determined by fruit length, diameter, or the length:diameter ratio, is an important quality and yield trait for vegetables. Several genes controlling fruit size have been well studied in tomato, including *SUN*, *LC*, *FAS*, and *OVATE* [5,6]. The *LC* and *FAS* genes are associated with flat shape and fruit locule formation, whereas *SUN* and *OVATE* have crucial effects on fruit elongation [7,8]. Progress has been made regarding the characterization of the molecular mechanism underlying fruit size in Cucurbitaceae crops. The *CsACS2* gene, which encodes a truncated loss-of-function protein, is considered to be responsible for the development of cucumber elongated fruits [9]. The *CsSUN*, a homolog of tomato fruit shape gene *SUN*, was a candidate for *FS1.2* underlying cucumber fruit size variation [10]. The *SF1* (*Short Fruit 1*) of cucumber encodes a cucurbit-specific RING-type E3 ligase, which ubiquitinates and degrades both itself and *ACS2* (1-aminocyclopropane-1-carboxylate synthase 2) to control ethylene biosynthesis for a dose-dependent effect on fruit cell division and fruit elongation [11]. The *CsFUL1<sup>A</sup>* is a gain-of-function allele in long-fruited cucumber that acts as a transcriptional repressor of fruit elongation by repressing the expression of *SUPERMAN* and inhibiting the expression

of auxin transporters *PIN-FORMED1* (*PIN1*) and *PIN7* [12]. In melon, a genome-wide association analysis identified eight fruit size-related signals overlapping selective sweep regions, with *FL5.1* and *FL5.2* newly detected [13].

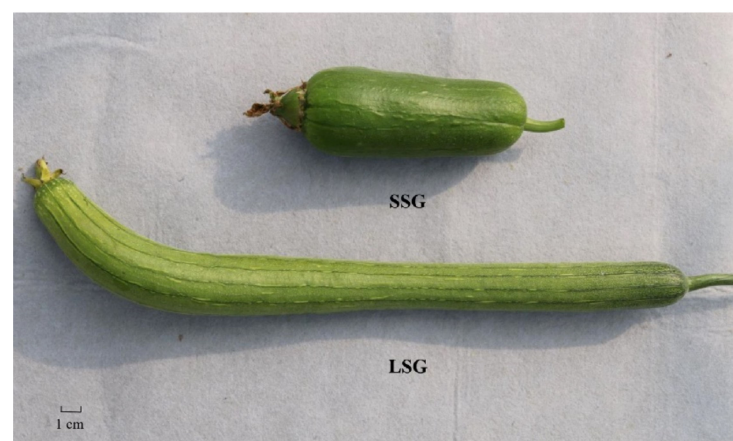
The development and utility of 'omics' have resulted in significant advances regarding fruit size in cucurbit crops. For example, a comparative transcriptome analysis of two pumpkin cultivars with extreme fruit size difference revealed candidate genes (e.g., *MYB*, *bHLH*, *AUX/IAA*, and *AP2*) associated with fruit morphology and size as well as genes involved in cell expansion and cell division [14]. Two bottle gourd accessions with distinct fruit size were analyzed using transcriptome sequencing technology, which identified the candidate genes related to cell wall metabolism, phytohormones, cell cycle, and cell division regulating the fruit size [15]. Additionally, the RNA-Seq technology has been applied to analyze the fruit development in cucumber [16], snake gourd [17], and melon [18,19].

Considerable diversity exists in the size and shape of sponge gourd fruits (e.g., long cudgel, short cudgel, long cylinder, short cylinder, oval, spindle, sickle, waist girdle, and snake); however, the molecular insights of fruit size regulation remain uncharacterized. In this study, two materials of sponge gourd with distinct fruit size were analyzed by RNA-Seq. The candidate genes and pathways associated with fruit size regulation were identified. The results of this study provide us with valuable information for future attempts at validating the genes and revealing their molecular mechanism of fruit size regulation in sponge gourd.

## 2. Materials and Methods

### 2.1. Plant Materials

The materials of sponge gourd used in this study were long sponge gourd (LSG) and short sponge gourd (SSG) (Figure 1). Plants were grown at the Yangdu Research and Innovation Base, Zhejiang Academy of Agricultural Sciences, under normal field management. Three fruits in each material were collected 10 days post pollination from three different plants, and the middle part of fruits were sliced and frozen in liquid nitrogen. The samples from each fruit were considered as a biological replicate and three biological replicates per material were used for subsequent RNA-Seq analysis.



**Figure 1.** The two materials of sponge gourd used in this study.

### 2.2. RNA Extraction

Total RNA was extracted using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), and genomic DNA was removed using DNase I (Takara, Shiga, Japan). The RNA quality was evaluated using the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), whereas the RNA quantity was determined using the ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). High-quality RNA samples ( $OD_{260}/OD_{280} = 1.8\text{--}2.2$ ,  $OD_{260}/OD_{230} \geq 2.0$ , RIN (RNA integrity number)  $\geq 6.5$ ,  $28S:18S \geq 1.0$ , and  $>10\text{ }\mu\text{g}$ ) were used to construct the sequencing libraries.

### 2.3. Library Preparation and Transcriptome Sequencing

The Illumina Truse RNA Sample Prep Kit (Illumina, San Diego, CA, USA) was used to prepare the transcriptome libraries from 1 µg total RNA. First, mRNA was isolated using oligo-(dT) beads and then fragmented in fragmentation buffer. The cDNA synthesis, end repair, A-base addition, and ligation of the Illumina-indexed adapters were performed as described by the manufacturer. Size-selected libraries comprising 200 to 300 bp cDNA target fragments were prepared by 2% agarose gel electrophoresis followed by a PCR amplification (15 cycles) using Phusion DNA polymerase (NEB, Beijing, China). The libraries were quantified using the TBS-380 fluorometer (Beijing Yuanpinghao Biotechnology Co., Ltd., Beijing, China) and then sequenced on the Illumina NovaSeq 6000 platform (2 × 150 bp paired-end reads) (BIOZERON Co., Ltd., Shanghai, China).

### 2.4. Read Quality Control Filtering and Mapping

The raw reads were trimmed to remove the reads containing adapter, reads containing ploy-N, and low-quality reads for quality control using Trimmomatic with parameters (SLIDINGWINDOW:4:15 MINLEN:75) [20] (version 0.36). The retained clean reads were separately aligned to the sponge gourd reference genome [21] using the default parameters of the HISAT2 software [22] (version 2.1.0). The quality of the data was assessed using Qualimap 2 software [23] (version 2.2.1). The read count of each gene was determined using HTSeq [24] (version 0.11.1).

### 2.5. Identification and Functional Analysis of Differentially Expressed Genes (DEGs)

To identify differentially expressed genes (DEGs) between samples, the expression level of each gene was calculated according to the fragments per kilobase of exon per million mapped reads (FPKM) method [25]. The DEGs between LSG and SSG were detected according to the following criteria:  $|\log_2(\text{fold-change})| > 1$  and  $p\text{-value} < 0.05$ . The heat map of DEGs were analyzed using  $\log_2(\text{FPKM} + 1e^{-2})$  at <https://www.omicstudio.cn/index> (accessed on 10 October 2021). Clustering analysis was performed for DEGs using the gplots package in R software (version 3.4.2; <http://www.r-project.org>) (accessed on 15 November 2020).

To functionally characterize the DEGs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted using Goatools [26] and KOBAS [27], respectively. The DEGs were assigned significantly enriched GO terms and KEGG pathways on the basis of a Bonferroni-corrected  $p\text{-value} < 0.05$ .

### 2.6. Validation of RNA-Seq Results by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

To verify the expression of DEGs, total RNA was extracted from sponge gourd fruit samples and then reverse transcribed into cDNA using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). The reaction mixture for the qRT-PCR analysis consisted of the following components: 10 µL 2 × TransStart Top Green qPCR SuperMix (TransGen Biotech), 2.0 µL diluted cDNA, 0.4 µL 50 × Passive Reference Dye I, 0.4 µL each primer (10 µM), and 6.8 µL ddH<sub>2</sub>O. The qRT-PCR analysis was performed in 96-well optical reaction plates and the StepOne Real-Time PCR System (ABI, Foster City, CA, USA). The PCR program was as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 55 °C for 15 s, and 72 °C for 10 s. Each sample was analyzed in triplicate. Relative gene expression levels were calculated using the  $2^{-\Delta\Delta C_t}$  method [28]. The UBQ was used as reference gene. Details regarding the qRT-PCR primers are listed in Table S1.

## 3. Results

### 3.1. Overview of the RNA-Seq Results

A total of 358,401,220 raw reads (53.76 Gb data) were obtained from all six samples using the Illumina NovaSeq platform (Table 1). After a quality control using Trimmomatic, 349,867,096 clean reads were retained, with the Q30 percentage exceeding 88.16% and the

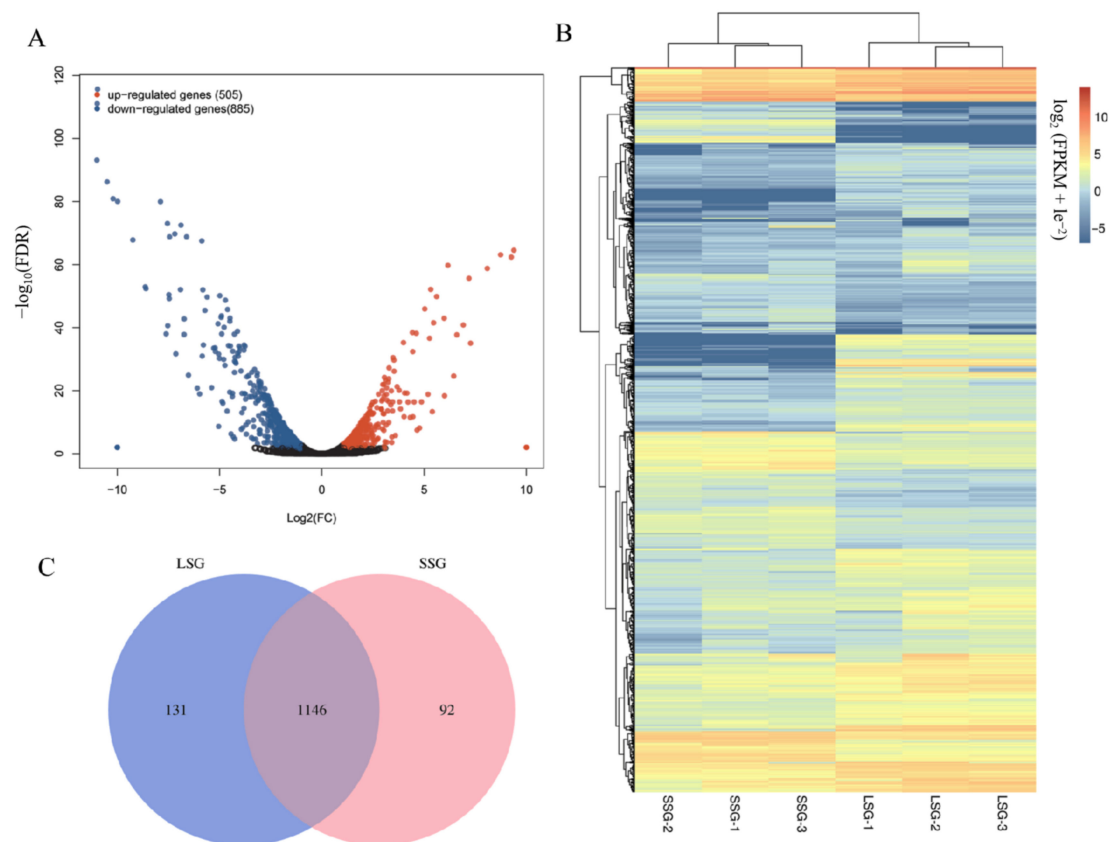
GC content ranging from 46.55% to 47.23% (Table 1). The clean reads in each sample were mapped to the sponge gourd reference genome using HISAT2, with the mapping rates ranged from 92.96% to 95.45% (Table 1).

**Table 1.** Overview of the sequencing data.

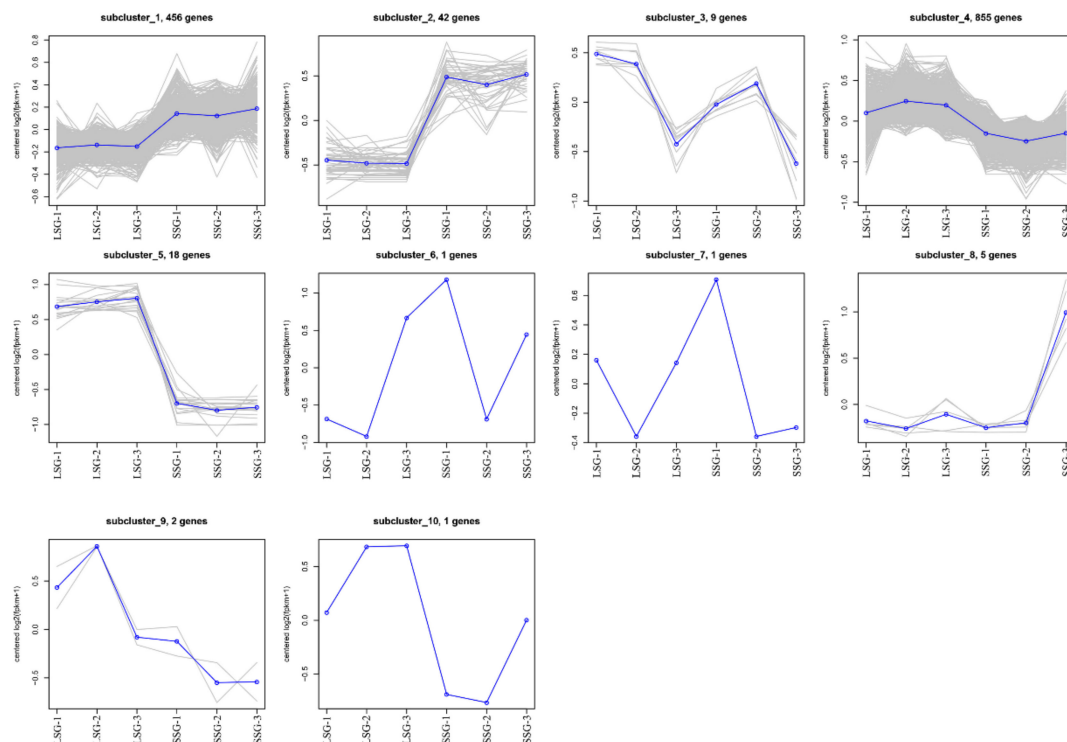
Samples	Raw Reads	Raw Bases (Gb)	Clean Reads	Mapped Reads	Mapped Rate (%)	Q30%	GC%
LSG-1	65,119,470	9.77	62,462,642	58,065,272	92.96	88.16	46.55
LSG-2	50,343,430	7.55	49,465,058	47,214,397	95.45	92.02	46.91
LSG-3	54,085,620	8.11	52,871,786	50,244,058	95.03	92.23	47.23
SSG-1	69,397,900	10.41	67,945,520	63,168,949	92.97	91.23	46.89
SSG-2	63,220,696	9.48	61,916,680	58,684,629	94.78	91.95	46.65
SSG-3	56,234,104	8.44	55,205,410	51,412,798	93.13	92.25	46.77

### 3.2. Identification of Differentially Expressed Genes (DEGs)

A total of 1390 genes were differentially expressed between LSG and SSG. Of these DEGs, 885 and 505 were respectively downregulated and upregulated in SSG compared with LSG (Table S2, Figure 2A,B). A Venn diagram analysis revealed 1146 DEGs commonly expressed in LSG and SSG. Moreover, 131 DEGs showed specific expression in LSG and 92 DEGs showed specific expression in SSG (Figure 2C). The DEGs were classified into 10 subclusters, with subcluster 4 (855 DEGs) and subcluster 1 (456 DEGs) the largest (Figure 3). The DEGs in subclusters 1 and 2 were upregulated in SSG for all libraries compared with LSG. However, the genes in subclusters 4 (855 DEGs), 5 (18 DEGs), and 9 (2 DEGs) had similar downregulated expression patterns in SSG compared with LSG for all libraries.



**Figure 2.** Analysis of DEGs between LSG and SSG. (A) Volcano map representing the number of DEGs between LSG and SSG. (B) Heat map of DEGs between LSG and SSG; the heat map of DEGs was analyzed using  $\log_2(\text{FPKM} + 1e^{-2})$  at <https://www.omicstudio.cn/index> (accessed on 10 October 2021). (C) Venn diagram of DEGs commonly or specifically expressed in LSG and SSG.



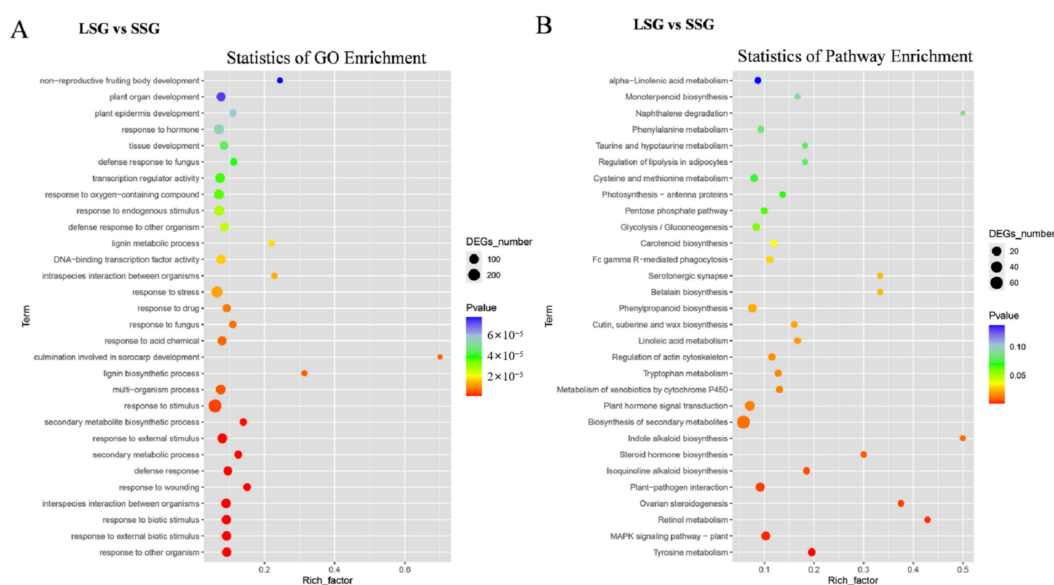
**Figure 3.** Cluster analysis of the DEGs between LSG and SSG.

### 3.3. GO and KEGG Enrichment Analyses of DEGs

To functionally characterize the DEGs, a GO enrichment analysis was performed, which identified 247 GO terms that were significantly enriched among the DEGs. More specifically, 210, 28, and 9 enriched GO terms were from the categories of biological process, molecular function, and cellular component, respectively. In the biological process category, the enriched GO terms included response to other organism (GO:0051707), response to external biotic stimulus (GO:0043207), response to biotic stimulus (GO:0009607), interspecies interaction between organisms (GO:0044419), and response to wounding (GO:0009611). In the molecular function category, the enriched GO terms included DNA-binding transcription factor activity (GO:0003700), transcription regulator activity (GO:0140110), and oxidoreductase activity, acting on paired donors (GO:0016709). In the cellular component category, the enriched GO terms included actin cortical patch (GO:0030479), endocytic patch (GO:0061645), caspian strip (GO:0048226), and Arp2/3 protein complex (GO:0005885) (Figure 4A and Table S3).

The KEGG pathway enrichment analysis of the DEGs identified 20 significantly enriched pathways. The top 10 enriched pathways were tyrosine metabolism (ko00350), MAPK signaling pathway-plant (ko04016), retinol metabolism (ko00830), ovarian steroidogenesis (ko04913), plant-pathogen interaction (ko04626), isoquinoline alkaloid biosynthesis (ko00950), steroid hormone biosynthesis (ko00140), indole alkaloid biosynthesis (ko00901), biosynthesis of secondary metabolites (ko01110), and plant hormone signal transduction (ko04075) (Figure 4B and Table S4).





**Figure 4.** GO and KEGG enrichment analysis of DEGs between LSG and SSG. (A) GO enrichment analysis of DEGs; (B) KEGG enrichment analysis of DEGs.

### 3.4. DEGs Related to Sponge Gourd Fruit Size Regulation

#### 3.4.1. Transcription Factors (TFs) Related to Sponge Gourd Fruit Size Regulation

In this study, 75 transcription factors (TFs) genes were differentially expressed between SSG and LSG, with 56 and 19 respectively downregulated and upregulated in SSG compared with LSG. The six most represented TF families were ERF, bHLH, MYB, NAC, WRKY, and MADS-box, which included 18 *ERF* genes (17 downregulated and 1 upregulated), 9 *bHLH* genes (8 downregulated and 1 upregulated), 9 *MYB* genes (7 downregulated and 2 upregulated), 7 *NAC* genes (3 downregulated and 4 upregulated), 7 *WRKY* genes (all downregulated), and 6 *MADS-box* genes (5 downregulated and 1 upregulated) (Table 2). The expression levels for most TF genes were downregulated in SSG compared with LSG.

#### 3.4.2. DEGs Involved in the Cell Cycle and Cell Expansion

A total of 15 DEGs related to the cell cycle and cell expansion were identified (Table 3), of which four DEGs (Maker00030282, Maker00038088, Maker00039350, and Maker00039481) encoding expansin (EXP) were downregulated in SSG compared with LSG. Five DEGs (Maker00006055, Maker00006010, Maker00006013, Maker00019798, and Maker00019916) encoding cyclin-dependent kinase were also downregulated in SSG compared with LSG, whereas Maker00013235, encoding cyclin, was upregulated in SSG compared with LSG. Additionally, five DEGs (Maker00007464, Maker00023770, Maker00029744, Maker00029948, and Maker00038114) encoding xyloglucan endotransglucosylase/hydrolase (XTH) were downregulated in SSG compared with LSG.

#### 3.4.3. DEGs Associated with Plant Hormone Metabolism

In this study, 22 DEGs related to plant hormone metabolism were identified (Table 4), among which six genes (Maker00000589, Maker00001150, Maker00014019, Maker00014874, Maker00015281, and Maker00023875) were encoding auxin-responsive protein. With the exception of Maker00023875, the remaining five auxin-responsive protein encoding genes were downregulated in SSG compared with LSG. Moreover, the expression levels of Maker00033389 (IAA-amino acid hydrolase), Maker00039293 (auxin transporter-like protein), and Maker00021979 (auxin-binding protein) were downregulated in SSG compared with LSG.

**Table 2.** DEGs encoding transcription factors.

Gene ID	Description	LSG-1	LSG-2	LSG-3	SSG-1	SSG-2	SSG-3	Log <sub>2</sub> FC	Regulation
Maker00022449	ethylene-responsive transcription factor 11-like	1.0	0.7	0.6	1.0	2.2	1.8	1.10	up
Maker00002693	ethylene-responsive transcription factor ERF109-like	4.0	34.3	27.8	3.1	2.9	9.4	−2.10	down
Maker00004871	ethylene-responsive transcription factor ERF113-like	2.3	59.2	10.6	8.9	6.4	19.6	−1.05	down
Maker00008702	ethylene-responsive transcription factor ERF110-like	5.8	72.3	24.1	16.6	8.4	24.3	−1.05	down
Maker00012352	ethylene-responsive transcription factor ABR1	0.2	3.8	0.5	0.4	0.3	0.9	−1.48	down
Maker00013906	ethylene-responsive transcription factor ERF084	2.9	1.8	0.7	0.8	0.6	0.0	−1.99	down
Maker00021217	ethylene-responsive transcription factor 5-like	12.6	29.3	27.9	9.1	7.8	10.1	−1.37	down
Maker00022236	ethylene-responsive transcription factor ERF017	2.8	8.8	11.3	2.1	2.7	5.4	−1.16	down
Maker00022840	ethylene-responsive transcription factor WIN1-like	0.9	3.0	4.8	3.3	0.1	0.6	−1.15	down
Maker00022955	ethylene-responsive transcription factor ERF105-like	20.5	48.3	57.3	9.9	10.0	14.9	−1.86	down
Maker00022958	ethylene-responsive transcription factor ERF106-like	12.1	10.7	22.0	8.3	3.9	7.0	−1.22	down
Maker00023340	ethylene-responsive transcription factor 1B-like	1.8	27.2	13.7	5.1	4.0	8.4	−1.28	down
Maker00024947	ethylene-responsive transcription factor TINY-like	1.5	2.0	1.7	1.6	0.5	0.2	−1.21	down
Maker00026990	ethylene-responsive transcription factor ABR1-like	0.1	1.6	0.1	0.0	0.1	0.2	−2.66	down
Maker00031851	ethylene-responsive transcription factor ERF098-like	1.0	2.1	1.0	0.3	0.0	0.2	−3.06	down
Maker00032087	ethylene-responsive transcription factor ERF071-like protein	3.4	3.4	3.7	1.5	1.3	1.5	−1.27	down
Maker00038032	ethylene-responsive transcription factor 1A-like	0.2	2.9	0.5	0.1	0.1	0.1	−3.91	down
Maker00039766	ethylene-responsive transcription factor ERF020-like	1.5	23.9	9.7	2.2	2.8	10.6	−1.17	down
Maker00012304	transcription factor bHLH133-like	2.3	0.6	0.8	1.0	4.8	5.2	1.54	up
Maker00002860	transcription factor bHLH118-like	0.0	1.0	0.6	0.0	0.1	0.0	−4.44	down
Maker00003086	transcription factor bHLH118-like	25.6	39.5	62.6	0.6	0.2	0.3	−6.89	down
Maker00008080	transcription factor bHLH117-like	1.9	1.8	0.1	0.4	0.4	0.1	−2.24	down
Maker00008913	transcription factor bHLH112-like isoform X1	16.4	17.9	21.5	0.0	0.0	0.0	−14.18	down
Maker00008926	transcription factor bHLH112-like isoform X3	9.7	12.4	9.9	0.0	0.0	0.0	−13.38	down
Maker00024818	transcription factor bHLH87-like	0.5	2.7	3.3	0.7	0.4	1.1	−1.60	down
Maker00031926	transcription factor bHLH67	4.5	3.7	8.1	3.2	1.3	2.5	−1.21	down
Maker00034132	transcription factor bHLH30-like	3.3	3.5	4.1	1.0	1.1	1.0	−1.81	down
Maker00038530	myb family transcription factor PHL5-like	0.3	0.2	0.1	8.1	8.5	11.1	5.47	up
Maker00039664	transcription factor MYB30-like	2.1	5.5	3.4	7.4	8.5	8.4	1.15	up
Maker00014658	transcription factor MYB83-like	0.8	1.4	1.0	0.8	0.2	0.5	−1.14	down
Maker00023477	MYB103-like protein	48.8	34.1	74.5	29.7	4.4	16.2	−1.65	down
Maker00025361	transcription factor MYB1-like	7.0	16.0	17.6	2.9	1.5	4.6	−2.17	down
Maker00029958	myb-related protein 308-like	1.3	0.2	0.2	0.1	0.1	0.2	−2.17	down
Maker00036073	transcription factor MYB86-like	1.5	5.5	3.6	2.3	0.2	1.4	−1.43	down
Maker00039070	transcription factor MYB20-like	14.0	14.1	19.3	7.5	4.8	4.2	−1.53	down
Maker00038509	transcription factor MYB46-like	1.1	1.5	1.6	0.7	0.5	0.5	−1.25	down
Maker00000545	NAC transcription factor 29-like	6.0	10.2	13.7	26.1	26.9	40.0	1.63	up
Maker00016510	NAC transcription factor 56-like	5.8	7.3	8.9	16.1	15.9	13.6	1.05	up
Maker00017754	NAC transcription factor 29-like	0.7	0.9	0.4	1.7	3.0	1.5	1.67	up
Maker00039552	NAC domain-containing protein 92-like	2.4	2.0	1.3	5.6	3.1	4.7	1.24	up
Maker00008337	NAC domain-containing protein 2-like	27.0	102.6	72.6	39.1	13.9	40.9	−1.11	down
Maker00029876	NAC domain-containing protein 55	3.2	2.2	7.7	1.0	0.3	1.9	−2.03	down
Maker00037829	NAC domain-containing protein 21/22-like	2.5	3.8	6.0	4.2	0.2	1.1	−1.17	down

Table 2. Cont.

Gene ID	Description	LSG-1	LSG-2	LSG-3	SSG-1	SSG-2	SSG-3	Log <sub>2</sub> FC	Regulation
Maker00005830	putative WRKY transcription factor 33	14.4	52.4	43.6	17.0	8.2	28.5	−1.04	down
Maker00013204	probable WRKY transcription factor 49	2.3	2.2	2.4	0.4	0.8	0.1	−2.51	down
Maker00028716	WRKY transcription factor 34	5.0	10.7	13.7	3.6	2.7	6.1	−1.25	down
Maker00031399	WRKY transcription factor 22-like	6.9	6.1	4.5	2.5	3.0	3.3	−1.00	down
Maker00033191	putative WRKY transcription factor 70	19.7	33.0	44.9	4.6	4.8	17.2	−1.88	down
Maker00037564	probable WRKY transcription factor 75	0.2	2.5	1.4	0.4	0.3	1.1	−1.14	down
Maker00038968	probable WRKY transcription factor 40 isoform X2	11.8	92.3	42.2	7.2	6.3	36.2	−1.56	down
Maker00002571	MADS-box transcription factor 6-like	5.5	2.1	2.9	37.1	6.9	47.1	3.12	up
Maker00004005	MADS-box protein JOINTLESS-like isoform X2	19.1	18.8	15.7	8.1	3.4	4.0	−1.79	down
Maker00013693	MADS-box protein EJ2-like	14.1	17.9	11.0	7.6	5.0	7.0	−1.13	down
Maker00014108	MADS-box protein EJ2-like	8.7	9.5	7.4	4.7	3.1	4.2	−1.10	down
Maker00034410	MADS-box protein AGL42 isoform X2	7.8	6.7	4.7	1.3	1.4	1.4	−2.22	down
Maker00034417	MADS-box protein AGL42 isoform X2	7.7	6.5	2.4	1.1	1.0	1.4	−2.24	down

Table 3. Differentially expressed genes associated with the cell cycle and cell expansion.

Gene ID	Description	LSG-1	LSG-2	LSG-3	SSG-1	SSG-2	SSG-3	Log <sub>2</sub> FC	Regulation
Maker00030282	expansin-A12	2.8	2.6	0.1	0.7	1.0	0.1	−1.56	down
Maker00038088	expansin-like B1	0.1	4.0	0.7	0.2	0.0	0.5	−2.95	down
Maker00039350	expansin A9	3.2	3.4	13.8	3.4	0.5	1.3	−1.96	down
Maker00039481	expansin-A10-like	67.0	98.9	110.9	47.7	7.6	22.2	−1.84	down
Maker00006010	cyclin-dependent kinase C-2-like	4.4	3.0	3.1	1.4	1.0	1.2	−1.54	down
Maker00006013	cyclin-dependent kinase C-2-like	9.5	8.5	6.3	4.0	3.1	3.0	−1.27	down
Maker00006055	cyclin-dependent kinase C-2-like	3.6	2.4	3.1	1.0	1.4	1.6	−1.18	down
Maker00019798	cyclin-dependent kinase C-2-like	24.5	18.6	19.0	6.6	5.5	7.2	−1.68	down
Maker00019916	cyclin-dependent kinase C-2-like	49.6	35.7	37.9	9.3	9.4	10.0	−2.10	down
Maker00013235	cyclin-D3-3-like isoform X1	10.9	8.7	9.1	17.3	22.2	19.2	1.03	up
Maker00007464	xyloglucan endotransglucosylase/hydrolase protein 22-like	40.3	112.6	106.1	63.5	14.9	47.6	−1.04	down
Maker00023770	xyloglucan endotransglucosylase/hydrolase protein 9-like	12.1	8.0	4.5	2.0	2.8	4.8	−1.35	down
Maker00029744	xyloglucan endotransglucosylase/hydrolase protein 6	2.4	6.0	6.2	0.6	0.1	0.7	−3.50	down
Maker00029948	xyloglucan endotransglucosylase/hydrolase protein 7	0.7	3.3	1.9	2.2	0.0	0.2	−1.28	down
Maker00038114	xyloglucan endotransglucosylase/hydrolase protein 30	5.1	4.3	2.2	1.4	1.0	1.6	−1.51	down

Four DEGs were involved in abscisic acid (ABA) metabolism, of which three DEGs (Maker00039228 and Maker00016867 encoding abscisic acid receptor; Maker00013728 encoding abscisic acid 8'-hydroxylase) were downregulated in SSG compared with LSG. In contrast, the expression level of Maker00017853 (abscisic acid 8'-hydroxylase) was upregulated in SSG compared with LSG. Three DEGs (Maker00012983, Maker00013515, and Maker00013263 encoding salicylic acid-binding protein) and two DEGs (Maker00007121 encoding gibberellin-regulated protein and Maker00004222 encoding gibberellin 2-beta-dioxygenase), involved in salicylic acid and gibberellin metabolism, respectively, were downregulated in SSG compared with LSG. Two DEGs were involved in ethylene metabolism, with Maker00029913 (ethylene synthase) and Maker00016907 (protein reversion-to-ethylene sensitivity 1) respectively downregulated and upregulated in SSG compared with LSG. Additionally, Maker00007027 (cytokinin riboside 5'-monophosphate phosphoribohydrolase) and Maker00031370 (jasmonic acid-amido synthetase), which were involved in cytokinin and jasmonic acid metabolism, respectively, were downregulated in SSG compared with LSG. These results showed that most of the DEGs involved in plant hormone metabolism were repressed in SSG compared with LSG.



**Table 4.** Differentially expressed genes associated with plant hormone metabolism.

Gene ID	Description	LSG-1	LSG-2	LSG-3	SSG-1	SSG-2	SSG-3	Log <sub>2</sub> FC	Regulation
Maker00012983	salicylic acid-binding protein 2-like	11.5	20.8	22.8	5.7	7.1	13.5	−1.07	down
Maker00013263	salicylic acid-binding protein 2-like	8.0	20.5	15.2	10.2	2.1	3.7	−1.45	down
Maker00013515	salicylic acid-binding protein 2	13.4	13.5	10.9	4.3	1.6	1.5	−2.35	down
Maker00029913	ethylene synthase	0.2	6.4	2.1	0.5	0.6	1.2	−1.94	down
Maker00016907	protein reversion-to-ethylene sensitivity1	26.6	38.0	37.0	71.7	100.8	87.4	1.36	up
Maker00004222	gibberellin 2-beta-dioxygenase 1-like	12.6	35.0	23.0	10.1	8.7	14.3	−1.09	down
Maker00007121	gibberellin-regulated protein 1-like	5.8	6.5	5.4	2.8	2.6	2.0	−1.26	down
Maker00013728	abscisic acid 8'-hydroxylase 4-like	4.3	2.0	8.1	2.1	1.9	2.3	−1.17	down
Maker00016867	abscisic acid receptor PYL2	14.1	27.5	39.6	15.5	8.5	8.5	−1.32	down
Maker00039228	abscisic acid receptor PYL4-like	2.9	1.4	0.6	0.4	0.6	0.4	−1.84	down
Maker00017853	abscisic acid 8'-hydroxylase 3-like	0.3	0.3	0.1	0.8	1.1	0.6	1.75	up
Maker00000589	auxin-responsive protein SAUR21-like	2.3	1.6	2.0	0.7	0.8	0.9	−1.26	down
Maker00001150	auxin-responsive protein SAUR23-like	12.2	7.3	5.0	3.9	5.6	2.4	−1.04	down
Maker00014019	auxin-responsive protein SAUR50-like	7.3	5.6	3.8	3.0	2.3	2.1	−1.18	down
Maker00014874	Auxin responsive SAUR protein	0.8	1.7	0.4	0.1	0.0	0.0	−4.27	down
Maker00015281	auxin-responsive protein SAUR50-like	1.8	2.3	1.8	1.2	0.6	0.9	−1.10	down
Maker00021979	auxin-binding protein ABP19a-like	108.2	125.9	305.9	112.0	9.6	58.6	−1.58	down
Maker00033389	IAA-amino acid hydrolase ILR1-like 3	36.1	49.4	48.1	9.9	9.9	9.9	−2.17	down
Maker00039293	auxin transporter-like protein 2	9.6	10.0	7.9	2.5	1.9	2.3	−2.03	down
Maker00023875	auxin-responsive protein IAA14	4.2	8.4	9.2	12.9	16.5	16.5	1.07	up
Maker00007027	cytokinin riboside phosphoribohydrolase LOG3-like	8.5	27.7	40.7	8.3	4.8	17.1	−1.35	down
Maker00031370	jasmonic acid-amido synthetase JAR1-like	12.1	58.4	45.1	24.7	7.2	20.1	−1.15	down

### 3.4.4. DEGs Involved in MAPK Signaling Pathway

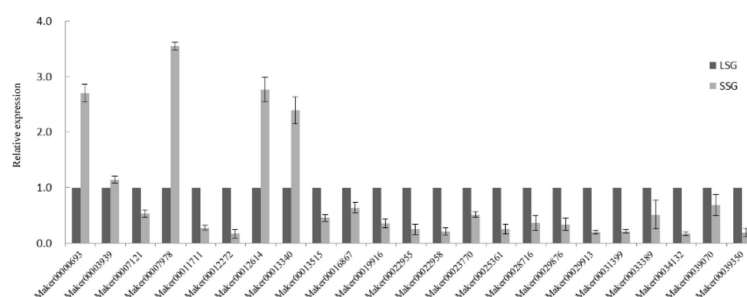
Fourteen DEGs were revealed to affect the MAPK signaling pathway (ko04016) (Table 5), of which the expression levels of the following 11 DEGs were downregulated in SSG compared with LSG: Maker00004003 (hypothetical protein), Maker00011711 and Maker00012272 (mitogen-activated protein kinase), Maker00016867 (abscisic acid receptor), Maker00009036 (STS14 protein-like), Maker00029408 (pathogenesis-related protein), Maker00029913 (ethylene synthase), Maker00023340 (ethylene-responsive transcription factor), Maker00005830 and Maker00031399 (WRKY transcription factor), and Maker00021373 (MYC2-like transcription factor). In contrast, Maker00020996 (MYC2-like transcription factor), Maker00016907 (protein reversion-to-ethylene sensitivity 1), and Maker00019397 (endochitinase 2-like) were more highly expressed in SSG than in LSG.

**Table 5.** DEGs involved in MAPK signaling pathway.

Gene ID	Description	LSG-1	LSG-2	LSG-3	SSG-1	SSG-2	SSG-3	Log <sub>2</sub> FC	Regulation
Maker00004003	hypothetical protein DKX38_014182	28.1	69.7	78.3	22.9	39.5	18.9	−1.12	down
Maker00005830	putative WRKY transcription factor 33	14.4	52.4	43.6	17.0	8.2	28.5	−1.04	down
Maker00009036	STS14 protein-like	219.9	140.9	84.9	83.1	62.2	47.1	−1.21	down
Maker00011711	mitogen-activated protein kinase kinase kinase 18-like	89.0	132.8	133.0	58.4	55.3	55.3	−1.07	down
Maker00012272	mitogen-activated protein kinase kinase kinase 17-like	0.3	15.2	3.1	0.3	0.5	1.5	−3.03	down
Maker00016867	abscisic acid receptor PYL2	14.1	27.5	39.6	15.5	8.5	8.5	−1.32	down
Maker00021373	transcription factor MYC2-like	3.5	14.8	17.3	5.6	2.7	7.3	−1.18	down
Maker00023340	ethylene-responsive transcription factor 1B-like	1.8	27.2	13.7	5.1	4.0	8.4	−1.28	down
Maker00029408	pathogenesis-related protein PRB1-2-like	0.9	1.9	4.7	0.2	0.0	0.7	−3.09	down
Maker00029913	ethylene synthase	0.2	6.4	2.1	0.5	0.6	1.2	−1.94	down
Maker00031399	WRKY transcription factor 22-like	6.9	6.1	4.5	2.5	3.0	3.3	−1.00	down
Maker00016907	protein reversion-to-ethylene sensitivity1	26.6	38.0	37.0	71.7	100.8	87.4	1.36	up
Maker00019397	endochitinase 2-like	0.1	0.2	0.7	1.6	1.1	1.0	1.78	up
Maker00020996	transcription factor MYC2-like	0.7	2.5	2.3	6.8	10.7	7.0	2.14	up

### 3.5. Validation of Fruit Size-Related DEGs by qRT-PCR Analysis

A total of 23 DEGs were selected for qRT-PCR validation (Figure 5). Five of the DEGs, namely, Maker00000693 (HVA22-like protein), Maker00003939 (beta-ureidopropionase), Maker00007978 (glutamate receptor), Maker00012614 (divaricata-like), and Maker00013340 (hypothetical protein Csa\_018195), were expressed at higher levels in LSG than in SSG. The following 18 DEGs were expressed at lower levels in LSG than in SSG: Maker00022955 (ERF105-like), Maker00022958 (ERF106-like), Maker00029876 (NAC protein 55), Maker00034132 (bHLH30-like), Maker00039070 (MYB20-like), Maker00025361 (MYB1-like), Maker00031399 (WRKY 22-like), Maker00028716 (WRKY 34), Maker00039350 (expansin A9), Maker00023770 (xyloglucan endotransglucosylase/hydrolase protein 9-like), Maker00019916 (cyclin-dependent kinase C-2-like), Maker00033389 (IAA-amino acid hydrolase ILR1-like 3), Maker00007121 (gibberellin-regulated protein 1-like), Maker00016867 (abscisic acid receptor PYL2), Maker00013515 (salicylic acid-binding protein 2), Maker00011711 (mitogen-activated protein kinase kinase kinase 18-like), Maker00029913 (ethylene synthase), and Maker00012272 (mitogen-activated protein kinase kinase kinase 17-like). The qRT-PCR data were generally consistent with the RNA-Seq results.



**Figure 5.** qRT-PCR analysis of DEGs related to sponge gourd fruit size.

## 4. Discussion

Fruit size is one of the important agronomic traits affecting the quality and commercial value of horticultural crops. In recent years, RNA-Seq analyses have been applied to identify the crucial genes associated with fruit size in several cucurbit vegetables [17–19]. However, there has been relatively little research on sponge gourd fruit size regulation. In this study, two sponge gourd materials with distinct fruit size underwent an RNA-Seq analysis, and the candidate genes and pathways related to sponge gourd fruit size regulation were identified.

### 4.1. Regulatory Roles of TFs Involved in Sponge Gourd Fruit Size

Transcription factors, which are proteins containing at least one specific DNA-binding domain, regulate the expression of target genes and play important roles in plant growth and development [29]. In this study, a total of 75 genes from multiple TF families (e.g., *ERF*, *NAC*, *bHLH*, *MYB*, *WRKY*, and *MADS-box*) were differentially expressed between SSG and LSG (Table 2).

The ERF TFs are associated with plant hormone signaling, mediating plant growth, and development as well as secondary metabolism [29]. ERF TFs are responsive to ethylene signals and contribute to the feedback regulation of ethylene synthesis in plant tissues [30]. In plum, seven *ERF* cDNAs were cloned and revealed to be differentially expressed in various flower and fruit developmental stages [31]. In tomato, 85 *ERF* genes have been identified, of which 57 genes influence fruit development [32]. Additionally, the tomato *ENO* gene, which encodes an ERF, regulates fruit size through the floral meristem development network; a mutation in this gene results in the production of large multi-compartment fruits [33]. In the present study, 18 ERF-encoding DEGs were identified, with all but one more highly expressed in LSG than in SSG, indicating that ERF may promote longer fruit formation in sponge gourd.

The NAC proteins form one of the largest TF families in plants. The C-terminal of these TFs contains a transcriptional regulatory region, whereas the N-terminal includes a conserved DNA-binding domain, both of which observe the activity of protein binding [34,35]. In tomato, NAC proteins regulate fruit ripening through the ethylene and ABA pathways [36]. In strawberry, six NAC TFs reportedly contribute to secondary cell wall and vascular development to modulate fruit development and ripening [37]. A recent genome-wide gene expression analysis and qRT-PCR analysis indicated that NAC expression is positively correlated with kiwifruit development [38]. In this study, four NAC genes (Maker00000545, Maker00016510, Maker00017754, and Maker00039552) were upregulated in SSG compared with LSG, whereas the opposite expression pattern was detected for three other NAC genes (Maker00008337, Maker00029876 and Maker00037829). Accordingly, NAC TFs may have diverse functions related to sponge gourd fruit development.

The basic helix–loop–helix (bHLH) contains a highly conserved bHLH domain with two distinct functional segments (i.e., the HLH region and the basic region) [39]. The bHLH family influences plant growth and development by regulating jasmonic acid responses, modulating light-regulated processes, and mediating cell cycle activities [40–42]. In Arabidopsis and rice, brassinosteroids regulate the conserved mechanism underlying plant development through HLH/bHLH [43]. In this study, eight *bHLH* genes (Maker00002860, Maker00003086, Maker00008080, Maker00008913, Maker00008926, Maker00024818, Maker00031926, and Maker00034132) were downregulated in SSG compared with LSG. Only one *bHLH* gene (Maker00012304) was upregulated in SSG compared with LSG. Therefore, bHLH might have positive effects on longer fruit formation in sponge gourd.

The MYB family, which forms one of the TF superfamilies, contains a MYB domain at its N-terminus [44]. The MYB family regulates the formation of the secondary cell wall by promoting/inhibiting the biosynthesis of xylan, cellulose, and lignin [45,46]. In Arabidopsis, the MYB-like DRMY1 can regulate cell expansion by directly affecting the cell wall structure and cytoplasmic expansion [47]. Moreover, MYB46 was reported serving as a critical regulator for the biosynthesis of all three major secondary cell wall components (hemicellulose, cellulose, and lignin) [48]. In this study, the following seven *MYB* genes were upregulated in LSG compared with SSG: Maker00025361 (MYB1-like), Maker00039070 (MYB20-like), Maker00038509 (MYB46-like), Maker00014658 (MYB83-like), Maker00036073 (MYB86-like), Maker00029958 (MYB-related protein 308-like), and Maker00023477 (MYB103-like protein). Hence, MYB may promote longer fruit formation by regulating secondary cell wall structural changes and cytoplasmic expansion in sponge gourd.

The WRKY family contains highly conserved domains and is involved in the regulation of various physiological processes in plants [49]. In Arabidopsis, WRKY46/54/70 coordinately function with the brassinosteroid-regulated BES1 to promote the brassinosteroid signaling and affect plant growth [50]. In watermelon, CIWRKY is involved in the growth and development of different tissues, and its diverse motifs and conserved or variable WRKY domains suggest it may play a variety of regulatory roles [51]. Our results indicated that seven genes encoding WRKY were upregulated in LSG compared with SSG. Thus, WRKY may positively regulate longer fruit formation in sponge gourd.

The MADS-box family participates in many plant developmental processes, especially those related to fruit development and maturation [52]. In Arabidopsis, *GORDITA* encodes an MADS-box that can affect fruit size by regulating fruit cell expansion [53]. The MADS-box encoded by *CsFUL1<sup>A</sup>* can bind to the *CsWUS* promoter, thereby modulating the cucumber fruit shape, size, and internal quality [54]. In apple, *MdPI* encodes an MADS-box that affects the apple floral organ, and ectopic expression of this gene can alter apple fruit tissue growth and shape [55]. In this study, five *MADS-box* genes (Maker00034417, Maker00034410, Maker00014108, Maker00013693, and Maker00004005) were more highly expressed in LSG than in SSG, whereas only one *MADS-box* gene (Maker00002571) was more highly expressed in SSG than in LSG. It was predicted that MADS-box genes might be positive regulators of longer fruit formation in sponge gourd.

#### 4.2. Cell Expansion and Cell Cycle Regulating Sponge Gourd Fruit Size

Fruit development is associated with cell expansion and the cell cycle. Cell expansion, which determines cell size, is regulated by EXPs, XTHs, and other important factors [56]. The EXPs induce cell wall extension and relaxation, leading to cell enlargement [57]. In watermelon, EXP-encoding genes (*ClEXP* genes) were differentially expressed in the fruit rind, fruit flesh, and seeds during various developmental stages, and their promoter regions contained many development-related elements [58]. Overexpression of cucumber *EXP* genes in maize grains revealed the synergistic effects of EXPs and cellulases during the deconstruction of complex cell wall substrates [59]. In addition to their cell expansion effects, XTHs also function as cell wall-loosening enzymes [60]. The *Brassica campestris* *BcXTH1* gene regulated cell expansion and was highly expressed in the epidermal cell layer [61]. Four DEGs encoding EXPs and five DEGs encoding XTHs were identified in this study. The expression levels of all these DEGs were upregulated in LSG compared with SSG, which implied that EXPs and XTHs positively regulated longer fruit formation in sponge gourd.

The cell cycle, which regulates cell division, determines the number of cells and is controlled by cyclins and cyclin-dependent protein kinases (CDKs) [62]. There are seven classes of cyclins (A, B, C, D, H, P, and T) in plants [63]. In terms of their effects on the cell cycle, cyclins in classes A, B, and D have been the most thoroughly studied. During the G2/M transition, the A-type and specific B-type CDKs can drive cell division after they bind to A-, B-, and D-type cyclins. Additionally, the plant D-type cyclins act as sensors of the G0-to-G1 phase transition [63]. Furthermore, DNA replication and mitosis are associated with CDK activities that control phosphorylation of the serine/threonine residues in specific substrates in eukaryotes [63,64]. In the present study, four CDK-encoding DEGs were upregulated in LSG compared with SSG, whereas one DEG encoding cyclin was downregulated in LSG compared with SSG. These results imply that CDKs are positive regulators of longer fruit formation in sponge gourd. However, the functions and effects of CDK and cyclin would need to be more precisely characterized in future investigations.

#### 4.3. Plant Hormones Regulating Sponge Gourd Fruit Size

Different plant hormones can form a complex network regulating various aspects of fruit development [65]. In strawberry, auxin (IAA) and ABA controlled the initial development and final ripening of fruits and the expression of underlying genes, and the IAA:ABA ratio was the main regulatory signal for fruit development [66]. In tomato, *Sl-ERF.B3* (ethylene response factor) integrates ethylene and auxin signals by regulating the expression of *Sl-IAA27* [67]. The interactions among gibberellins, salicylic acid, and ABA affect plant germination. Additionally, gibberellins modulate the salicylic acid pathway and influence salicylic acid biosynthesis [68]. Moreover, cytokinins interacted with TFs and phosphorylation cascades to control the expression of cytokinin target genes [69]. As a mobile signal in plants, gibberellins are involved in many developmental and adaptive growth processes [70]. Jasmonic acid is a lipid-derived stress hormone that regulates plant developmental processes (e.g., maternal control of embryo development in tomato) [71]. In this study, 21 DEGs associated with the metabolism of salicylic acid, ethylene, gibberellin, ABA, auxin, cytokinin, and jasmonic acid were identified. Most of these DEGs were upregulated in LSG compared with SSG. Therefore, it was speculated that the DEGs involved in plant hormone metabolism promoted longer fruit size formation in sponge gourd.

#### 4.4. MAPK Signaling Pathway Regulating Sponge Gourd Fruit Size

Mitogen-activated protein kinase (MAPK) cascades are ubiquitous and highly conserved signaling modules in eukaryotes. They are involved in embryogenesis, morphogenesis, senescence, abscission, and other aspects of plant growth and development [72]. The MAPK pathway consists of three major kinases: MAPK kinase kinase, MAPK kinase, and MAPK, which activate and phosphorylate downstream targets in a stepwise manner [73]. In addition to being activated during specific stages to regulate the cell cycle,



MAPK cascades are also crucial for basic physiological activities, including responses to hormones and abiotic stress signals as well as defense mechanisms [74]. In Arabidopsis, MAP3K17 and MAP3K18 activated the MAP2K (i.e., MKK3), thereby activating the C-clade MAPKs (MPK1, MPK2, MPK7, and MPK14) in response to ABA [75]. The MAPK pathway is also associated with ethylene signal transduction, with the MKK9–MPK3/MPK6 module functioning downstream of ethylene receptor [76,77]. In Arabidopsis, a bHLH transcription factor (MYC2/ZBF1) negatively regulated gene expression in response to blue and far-red light, and the MKK3–MPK6–MYC2 module affected seedling development [78]. In this study, 14 DEGs involved in the MAPK pathway were revealed to encode various proteins (e.g., MAPK, abscisic acid receptor, ethylene synthase, WRKY, MYC2, and ERF). The expression levels of most DEGs were upregulated in LSG compared with SSG, suggesting that the MAPK signaling pathway is important for longer fruit size formation in sponge gourd.

## 5. Conclusions

In this study, the RNA-Seq analysis was performed using two sponge gourd materials with distinct fruit sizes to reveal the crucial genes and pathways associated with fruit size regulation. A total of 1390 DEGs were identified (885 downregulated and 505 upregulated in SSG, relative to the expression levels in LSG). A functional analysis of these DEGs indicated they are mainly involved in the cell cycle and cell division, plant hormone metabolism, and the MAPK signal transduction pathway. Additionally, TFs belonging to the ERF, NAC, bHLH, MYB, WRKY, and MADS-box families play important roles in fruit size regulation. The findings of this study obtained the genes and pathways regulating fruit size in sponge gourd, which lay the foundation for revealing the molecular mechanisms conferring fruit size in cucurbit crops.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12081810/s1>, Table S1: The primers of qRT-PCR analysis for selected DEGs. Table S2: DEGs between LSG and SSG. Table S3: GO enrichment analysis for DEGs between LSG and SSG. Table S4: KEGG pathway enrichment analysis for DEGs between LSG and SSG.

**Author Contributions:** S.Q. carried out the experiment, data analysis, interpretation of the results and drafted the manuscript. Y.S. conceived and designed the study, supervised the work and revised the manuscript. Y.X. carried out the experiment. Q.H., W.D., S.H. and X.Q. give suggestions to the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Sequencing data from this article were deposited at the sequence read archive (SRA) of the National Center for Biotechnology Information (NCBI) under the accession number of SRR13618022, SRR13618023 and SRR13618024 for SSG, and SRR13618025, SRR13618026 and SRR13618027 for LSG.

**Conflicts of Interest:** The authors declare no conflict of interest.

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