

Article

# 22KD Zein Content Coordinates Transcriptional Activity during Starch Synthesis in Maize Endosperm

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**Abstract:** Starch, the main form of stored energy in plants, plays an important role in maize (*Zea mays* L.) kernel development. The *Shrunken-2* (*Sh2*) gene encodes the large subunit of the rate-limiting starch biosynthetic enzyme ADP-glucose pyrophosphorylase (AGPase). The *sh2* mutant exhibits impaired AGPase activity, resulting in the partial or complete loss of starch synthesis. Here, we investigated the transcriptional regulatory framework of *sh2* through transcriptome and co-expression network analysis using an F<sub>2</sub> population derived from the maize reference line B73 and sweet corn inbred line HZ508. We identified 5175 differentially expressed genes (DEGs), including 2878 upregulated and 2297 downregulated genes in *sh2* mutant lines. DEGs are associated with various biological processes including nutrient reservoir activity, transferase activity, catalytic activity, water deprivation and glycogen metabolism. At the genetic level, 2465 DEGs, including 357 transcription factors, were involved in transcription. In addition, the maize floury and opaque mutant genes *fl1*, *ndk2*, *o7* and *o2*, which regulate the biosynthesis of 22KD zein, were co-expressed with the differential expressed transcription factor genes, thus suggesting that zein content might be a key regulator coordinating the expression of genes determining starch accumulation in maize endosperm.

**Keywords:** endosperm development; maize; *sh2*; starch accumulation; zein

## 1. Introduction

Starch regulation draws the attention of the scientific community, as this polymer is essential to meet energy needs in early plant development, human diet, feedstock and bioenergy. It accounts for up to 70% of the seed storage components in cereal endosperm and is thus a major contributor to yield as well as the main source of energy for seed germination and seedling growth. Starch production is a multistep mechanism starting with the conversion of Glc-1-P and ATP into ADP-Glc by ADP-glucose pyrophosphorylase (AGPase). This step is particularly important, as the inhibition of AGPase activity results in the partial or complete termination of starch biosynthesis, which greatly reduces yields [1,2]. AGPase is made of two subunits known as the large subunit (LSU) and small subunit (SSU). Genes encoding for AGPase subunits depend on the organ and tissue where they are localized. The fate of LSU and SSU are determined by *Agpslzm* and *Agpllzm* in the leaf, *shrunken2* (*Sh2*) and *brittle2* (*Bt2*) in the endosperm while *Agplsmzm* and *Agplemzm* are specifically expressed in the embryo [3]. Knocking down of *Sh2* and *Bt2* genes yields the starch-deficient mutant with a pericarp shrivel upon maturation [4]. As the mutant *sh2* and *bt2* genes behave in a complementary

manner, the complete loss of function, which is lethal, requires a double homozygous mutant [4]. Heterologous expression of the gene encoding *Escherichia coli* glgC AGPase increased tuber yield by 35% in potato (*Solanum tuberosum* L.) [5], grain yield by 11% in rice (*Oryza sativa* L.) [6] and seed weight by 13% to 25% in maize [7]. Likewise, the inhibition of AGPase activity using site-specific mutagenesis system [8] and overexpression of *Sh2* [9] also lead to an increase of maize seed weight by 11%–18%. Heterologous expression of a modified form of maize *Sh2* (*Sh2r6hs*) increased yield by 38% in wheat (*Triticum aestivum* L.) [10], 23% in rice [11] and 64% in maize [12]. Although AGPase is crucial for starch biosynthesis and seed development in maize, the genetic and molecular mechanisms regulating this process need to be further clarified.

In this study, we aimed to get further insights into the genetic regulation of starch biosynthesis during maize kernel development.

We performed transcriptomic analysis using mutant *sh2* kernels and wild-type kernels collected at 15 days after pollination (DAP), as well as co-expression network analysis using RNA-seq data from some well-known maize kernel mutants. Finally, we confirmed the reliability of our RNA-seq data via qRT-PCR using various differentially expressed genes (DEGs) related to starch or zein biosynthesis. Our analyses uncovered huge changes in the transcriptome of *sh2* and point to four zein-related genes as major coordinators of transcriptional activity occurring during endosperm development in maize kernels.

## 2. Materials and Methods

### 2.1. Plant Materials and Sample Collection

Maize kernels harboring the starch deficiency (shrunken) phenotype in the HZ508 background (HZ508<sup>sh2</sup>) were received from the agricultural academy of Enshi Tujia and Miao autonomous prefecture. This material was crossed with the maize inbred B73 to succinctly produce the F<sub>1</sub> and F<sub>2</sub> population used to carry this experiment.

The F<sub>1</sub> plants were self-crossed; 15 days after pollination (DAP), samples were collected on developing F<sub>2</sub> ears. On each ear, 10 homozygous mutant kernels (*sh2sh2*) and homozygous wild-type kernels (*Sh2Sh2*) were snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The identity of each kernel was confirmed by genotype analysis, and each set of 10 kernels constitute a sample that was replicated three times.

### 2.2. RNA Extraction and Library Construction

Total RNA was extracted from pooled wild-type and *sh2* mutant kernels obtained from the same F<sub>2</sub> ears derived from the cross B73 × HZ508<sup>sh2</sup> at 15 DAP (10 kernels per sample) using TRIzol reagent, followed by cleanup and DNase I treatment using a Qiagen RNeasy Mini Kit, according to the manufacturer's protocol. We used the Illumina NEBNext Ultra RNA Library Prep Kit (cat.no E7530L New England Biolabs, MA, USA) to generate RNA-seq libraries following the manufacturer's recommendations. These libraries were sequenced on the Illumina NovaSeq platform by Novogene.

### 2.3. Identification of DEGs

The sequence reads were trimmed using Trimmomatic (version: 0.33) and mapped to the B73 reference genome (RefGen\_v4.34) using HISAT2 (version: 2.1.0). StringTie (version: 1.3.3b) was employed to reconstruct the transcripts and to estimate gene expression levels [13]. HTSeq (version: 0.6.1) was used to count the number of reads per gene. When using the DESeq2 package, we applied the *p*-value adjusted for multiple testing (padj) as a threshold. Significant DEGs are those with a padj of differential expression above the threshold (*p* < 0.05, absolute value of log<sub>2</sub> (fold change) > 0.5). Gene ontology (GO) enrichment was performed for the significant DEGs using the agriGO Singular Enrichment Analysis tool with default parameters (<http://bioinfo.cau.edu.cn/agriGO/>) and REVIGO [14].

#### 2.4. Confirmation of DEG

qRT-PCR was performed on 33 genes using SYBR Select Master Mix (Aidlab) on a CFX96 real-time PCR system (Bio-Rad, C1000 Touch). Relative expression levels were calculated using the comparative Ct method [15] and the maize actin gene (*Zm00001d010159*) as the internal control. All primers used are listed in Table S1.

#### 2.5. Co-Expression Network Analysis of Well-Known Maize Kernel Mutant Transcriptomes

To construct a co-expression network, RNA-seq data from several well-known maize kernel mutants were obtained from the sequence read archive SRA database. Raw data filtering, mapping to the reference genome and gene expression level estimation were performed as described for RNA-seq data analysis. The weighted correlation network analysis (WGCNA) package in R was used to construct the gene co-expression network using the TPM matrix from StringTie. The analysis was conducted using an unsigned type of topological overlap matrix (TOM). For any gene pair (*i* and *j*), we applied the exponential adjacency function in the WGCNA algorithm to measure their relation index, the exponential weighted  $\beta$  square of the correlation coefficient ( $a_{ij} = \text{power}(S_{ij}, \beta) = |S_{ij}|^\beta$ ). Exponential weighted  $\beta$  is the power of the correlation coefficient. We selected  $\beta = 12$  after the analysis (fit value  $R^2$  to approximately 0.9). After determining the adjacency function parameter  $\beta$ , the correlation matrix  $S = [S_{ij}]$  was switched into the adjacency matrix  $A = [a_{ij}]$  and converted into the topological overlap matrix  $\Omega = [\omega_{ij}]$ .  $k_i$  or  $k_j$  indicate the sum of one node's adjacency coefficients. Genes for which the sum in each sample was below 80 ( $<80$ ) and the standard deviation (SD) was below 100 ( $<100$ ) or above 6000 ( $>6000$ ) were filtered out, yielding 14,528 genes for WGCNA co-expression network analysis.

The node is a gene (*i* or *j*). The hierarchical clustering tree was built using the dissimilarity coefficient, and the different branches represent the gene modules. We used the function of *cutreeDynamic* in WGCNA package to identify gene modules. The soft thresholding power was 12, the relatively minimum module size was 30 and the absolute value of correlation was greater than 0.25.

### 3. Results

#### 3.1. Isolation of Shrunk Mutant and DEGs

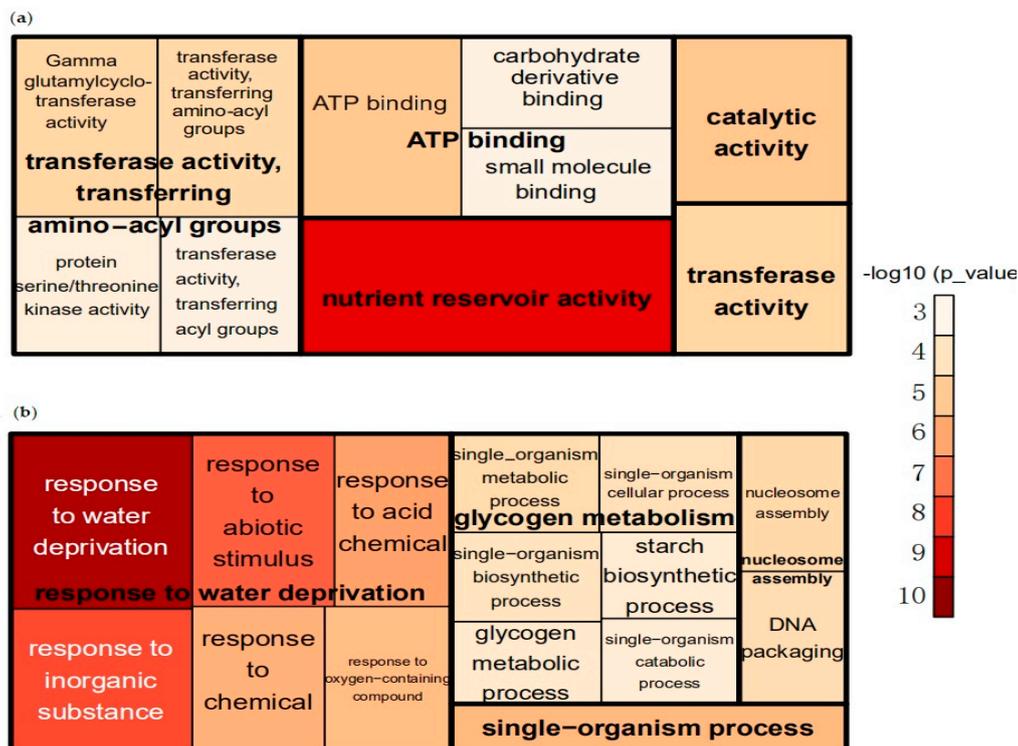
Using total RNA from the endosperm of *sh2* mutant kernels and wild-type kernels at 15 DAP, we generated three mRNA libraries. After high-throughput sequencing and filtering of reads, we obtained 24.8–27.3 million clean reads (Table S2). We mapped these clean reads to the B73 reference genome (RefGen\_v4.34). More than 91% of the clean reads were uniquely mapped to the reference genome for each sample (Table S3), and more than 94% of the mapped reads aligned to exons (Table S4). Comparing the structure of *Sh2* gene in pooled wild-type and  $F_2$  samples, we observe that the structure of *Sh2* gene was altered in this mutant (Figure S1). The wild-type *Sh2* line has 15 transcribed exons while *sh2* mutant line has only one exon. That difference also served as a basis for separating between wild-type and mutated shrunk kernels.

Identification of differentially expressed genes started by calculating the relative expression level of each gene using the unique mapped reads. Then, we normalized relative expression levels to the number of fragments per kilobase of the transcript sequence per million base pairs sequenced (FPKM) [16]. Principal component analysis based on these FPKM values showed that the three biological replicates of each sample were clustered into a single group (Figure S2).

Using DEGseq2 to identify DEGs, we detected 2878 upregulated genes and 2297 downregulated genes in *sh2* under the threshold of  $\text{padj} < 0.05$  and  $\log_2(\text{FoldChange}) > 0.5$  and  $\log_2(\text{FoldChange}) < -0.5$  (Figure S3).

### 3.2. GO Enrichment Analysis of DEGs

Out of the 2297 downregulated genes in *sh2*, 74.44% could be functionally annotated using AgriGO. Most of the significantly enriched gene ontology (GO) terms belong to the molecular function category, including genes involved in nutrient reservoir activity, transferase activity, ATP binding, and catalytic activity. The biological process-related DEGs are involved in glutathione catabolic processes, organonitrogen compound catabolism, and sulfur compound catabolic processes. There were no significantly enriched GO terms in the cellular component category (Figure 1a, Table S5).



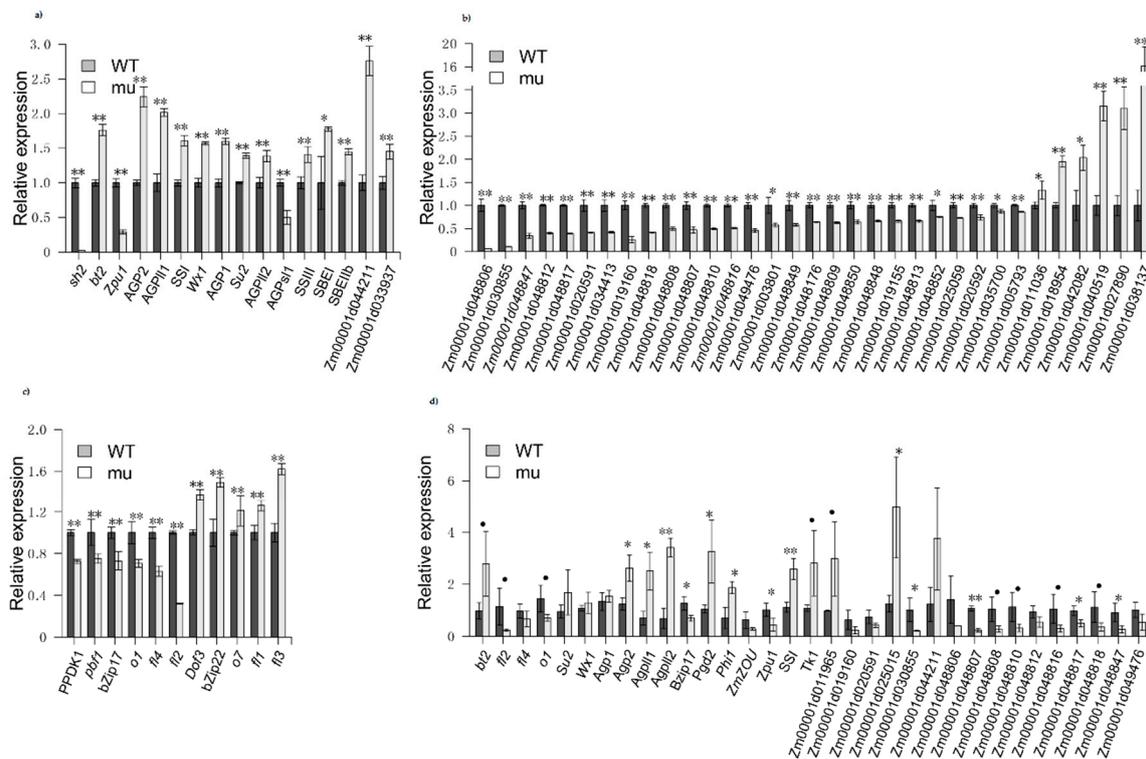
**Figure 1.** Gene ontology (GO) analysis of differentially expressed genes (DEGs) via AgriGO [17] and REVIGO [14]. The size and color of each square/rectangle indicate the level of enrichment ( $-\log_{10} p$ -value). (a) Molecular function of downregulated genes in *sh2*. (b) Biological processes of the upregulated genes in *sh2*.

Out of the 2878 upregulated genes in *sh2* mutant lines, 74.08% could be functionally annotated. DEGs in the biological process category include genes involved in the response to water deprivation, glycogen metabolism, nucleosome assembly and single-organism processes. Cellular-component-related genes are enriched in various GO categories, including nucleosome, cell junction, plasmodesmata and cell periphery. Molecular-function-related genes are involved in protein heterodimerization activity, oxidoreductase activity, cofactor binding, coenzyme binding, monooxygenase activity, acid phosphatase activity and DNA binding (Figure 1b, Table S6).

### 3.3. Starch Biosynthesis and Zein-Related Genes Are Differentially Regulated in *sh2* Kernels

We identified seven AGPase-encoding DEGs between mutant and wild-type kernels. Out of these DEGs, five were upregulated and two were downregulated genes along with *Sh2* in mutant lines. We identified nine additional DEGs involved in starch biosynthesis, including one phosphoglucosyltransferase (PGM) gene, three glycogen synthase genes, three glucan-branching enzyme (GBE) genes, and one starch debranching enzyme gene. Out of these, only the starch debranching enzyme gene was downregulated in *sh2* mutant lines (Figure 2a). In addition, 27 zein genes were downregulated and five were upregulated, which may lead to the reduced zein levels in the mutant kernels (Figure 2b). Many

genes influencing zein accumulation and maize kernel development were differentially expressed between mutant and wild-type kernels, such as the classic semi-dominant maize endosperm mutant genes *fl1*, *fl2*, *fl3* and *fl4* [18–21]; the classic recessive maize endosperm mutant genes *o1* and *o7* [21,22]; and the maize endosperm mutant genes *pbf1*, *bZIP17*, and *bZIP22* [23,24] and *PPDK1* (Figure 2c), all of which could affect storage protein gene expression [25].



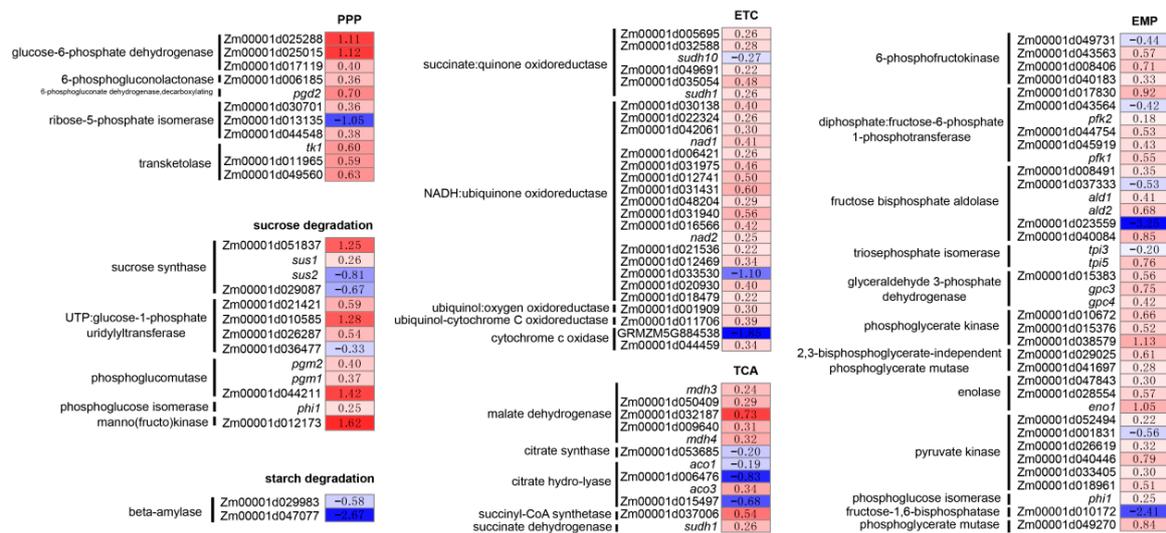
**Figure 2.** Expression levels of starch biosynthesis, zein and maize endosperm genes in *sh2* mutant kernels (mu) and wild-type kernels (WT). (a) Genes related to starch biosynthesis based on RNA-seq data (FPKM values); (b) Zein-encoding genes based on RNA-seq data (FPKM values); (c) Endosperm genes based on RNA-seq data (FPKM values); (d) Results of qRT-PCR. All expression levels were normalized to that of the wild type. The data are from three biological replicates per sample and are presented as mean values  $\pm$  SD. (Student's *t*-test, “•” represents *p*-value < 0.1, “\*\*” represents *p*-value < 0.05, “\*\*\*” represents *p*-value < 0.01).

To confirm the reliability of the RNA-seq data, we analyzed the expression levels of individual DEGs using qRT-PCR analysis in mutant and wild-type kernels and found a high association between the two datasets (Figure 2d). The expression levels of many genes related to starch and zein biosynthesis were altered in the mutant, suggesting that the levels of starch and zein were altered due to the dysfunction of AGPase activity.

### 3.4. Carbohydrate Catabolism and Electron Transport Chain Related Genes Are Differentially Regulated in *sh2* Kernels

As the weight of mature *sh2* kernels was substantially reduced, we focused on genes in the carbohydrate catabolism pathway. We identified 103 DEGs in various carbohydrate catabolism pathways. For every enzyme in the Embden–Meyerhof–Parnas (EMP) pathway, at least one gene was differentially expressed, and most of these DEGs were upregulated in *sh2* (Figure 3). Some of these enzymes are related to energy supply and maize kernel development, such as phosphofructokinase [26], and there is a competitive relationship between the EMP pathway and starch biosynthesis [27].

For the pentose phosphate pathway (PPP), 11 genes encoding five enzymes were differentially expressed, 10 of which were upregulated in *sh2* mutant lines (Figure 3). For every enzyme in the sucrose degradation pathway, at least one gene was differentially expressed, and most were upregulated in *sh2* (Figure 3). *Sus1* and *Sus2* play important roles in sucrose metabolism and plant growth, but the functions of these genes are different, perhaps explaining why they had opposite expression patterns in mutant and wild-type kernels [28]. No starch degradation genes were upregulated in mutant kernels (Figure 3).



**Figure 3.** Pathway analysis of the DEGs involved in carbohydrate catabolism and the electron transport chain. The data and the color of the rectangles indicates mean log<sub>2</sub> (fold change) of the DEGs; plant metabolic pathway databases [29] were used for pathway analysis described above from left to right: Pentose phosphate pathway (PPP), sucrose degradation, starch degradation, Electron Transport Chain (ETC), Tricarboxylic Acid Cycle (TCA) and the Embden–Meyerhof–Parnas pathway (EMP).

We identified 12 and 27 DEGs involved in the tricarboxylic acid cycle (TCA) and electron transport chain (ETC) (Figure 3), respectively, which help supply energy for plant growth and function in stress resistance, but only 17.9% (7 of 39) of these were downregulated.

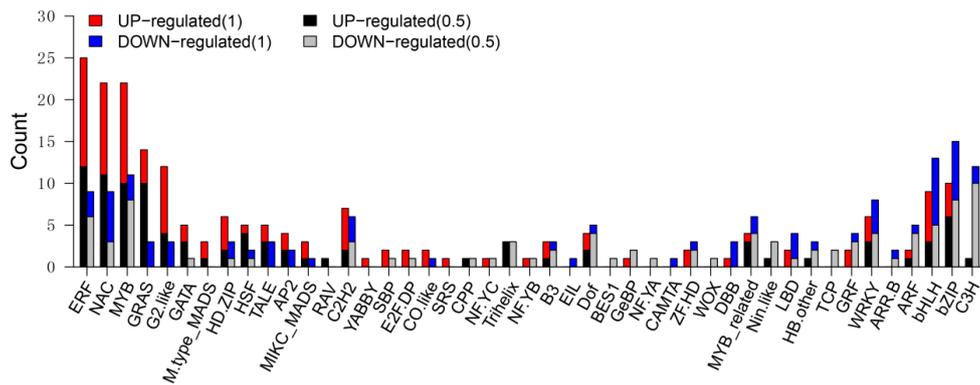
### 3.5. Hormone-Related DEGs

Hormones play important roles in regulating plant growth and kernel development. Ethylene negatively regulates those genes encoding enzymes related to starch biosynthesis [30]. We identified nine DEGs related to the ethylene metabolism pathway (Figure 4), and 34 ERFs (ethylene-responsive transcription factors) were differentially expressed between mutant and wild-type kernels, as revealed by RNA-seq (Table S7).

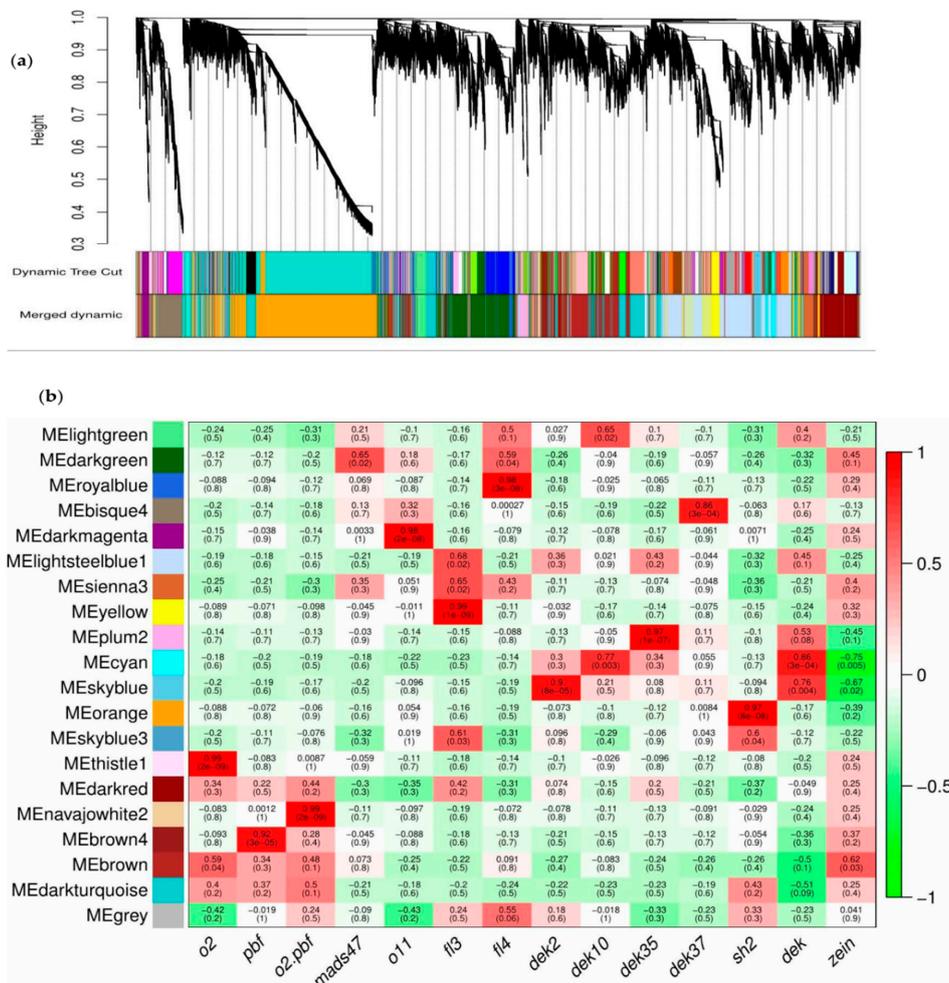
Abscisic acid (ABA) functions in abiotic stress resistance, and its levels of accumulation are highly correlated with starch accumulation [31]. Four genes related to ABA biosynthesis pathways and three genes related to ABA catabolic pathways were differentially expressed between mutant and wild-type kernels (Figure 4), including *nced2*, which codes for 9-cis-epoxycarotenoid dioxygenase (NCED) and catalyzes the conversion of 9-cis-epoxycarotenoids to xanthoxin, a key regulatory step in ABA biosynthesis [32]. Three genes (*abh1*, *abh2* and *abh5*) encoding for ABA 8'-hydroxylase, an enzyme involved in the ABA catabolic pathway, were also differentially expressed [33].

Auxin regulates rice grain size [34]. The main form of auxin in plants is indole-3-acetic acid (IAA). We identified 12 DEGs related to the IAA biosynthesis pathway (Figure 4), including six indole-3-pyruvate monooxygenase genes, three 2-oxoglutarate aminotransferase genes, two indole-3-acetamide amidase genes and one nitrilase gene.





**Figure 5.** Distribution of differentially expressed TF genes between wild-type and mutant kernels. The graph shows the number of upregulated genes ( $\log_2$  (fold change) > 1 or  $\log_2$  (fold change) < -1) in each family identified in the mutant (red) and wild-type (blue) and the number of upregulated genes ( $\log_2$  (fold change) > 0.5 or  $\log_2$  (fold change) < -0.5) in each family identified in the mutant (black) and wild-type (gray).



**Figure 6.** WGCNA using RNA-seq data for 12 endosperm mutants. (a) Hierarchical cluster analysis showing the co-expression modules identified by WGCNA; (b) Module-trait associations. Each row corresponds to a module. Each column shows one mutant (*dek* represents the four *dek* mutants used in this study, similarly *zein* stands for the seven *zein* mutants). The values in each cell represent the corresponding correlation coefficient and *p*-value.

**Table 1.** Modules identified by weighted correlation network analysis (WGCNA).

Module	Gene Number	Module	Gene Number	Module	Gene Number
Bisque4	527	Dark turquoise	438	Royal blue	86
Brown	429	Light green	3	Sienna3	26
Brown4	77	Light steel blue1	1230	Sky blue	71
Cyan	62	Navajo white2	21	Skyblue3	14
Dark green	622	Orange	2465	Thistle1	21
Dark magenta	104	Plum2	89		
Dark red	115	Yellow	327		

The “orange” module had a high module membership (MM) with *sh2*, with a correlation coefficient of 0.97 and a *p*-value of  $8 \times 10^{-8}$  (Figure 6b, Figure S4). We subjected the genes in that module to GO enrichment analysis and identified only one significant GO term: TF activity (sequence-specific DNA binding; GO:0003700, *p*-value =  $3.5 \times 10^{-9}$ ). This implies that many genes related to TF activity are included in this module. Out of the 2465 genes found in this module, 249 are TF genes, which include 70 upregulated and 15 downregulated DEGs in *sh2*, as determined by annotation using PlantTFDB (Figure S4). Most of these genes encode members of the ERF, bZIP, bHLH, GRAS, Trihelix, MYB, GATA, C2H2 and NAC TF families, and nine TF families include more than 10 members. The bZIP TF family, with 24 members, includes *o2*, a zein mutant known to network regulate maize endosperm development by directly regulating zein and other TF genes [24,42]. We identified 22 bHLH TF genes, including *o11*, encoding a key regulator of endosperm development and nutrient metabolism [43]. Twenty MYB and MYB-related TF genes are included, such as MYB14, which regulates the starch biosynthesis pathway in maize by regulating *ZmBT1* promoter activity [44]. Other genes in the “orange” module, such as *Zm00001d021537* and *Zm00001d038288*, are targets of *o11* in the MYB TF family [43]. Two targets of *o11*, *Zm00001d023294* and *Zm00001d049860*, encode NAC TFs [43]. Although transcriptional regulation is essential in starch synthesis, its global network is yet to be identified.

### 3.8. Co-Expression Network Analysis

To further understand the transcriptional mechanism regulating starch biosynthesis in early endosperm development (15 DAP) in maize, we selected four maize endosperm mutants belonging to the “orange” module (Figure 6). RNA-seq data from these mutants (*fl1*, *o7*, *nkd2* and *o2*) were downloaded from the SRA database. The imprinted floury1 mutant gene *fl1* presented greater co-expression with genes related to transcriptional activity (Figure 7). It is followed by *nkd2* and *o7*, while *o2* is only associated to five genes.

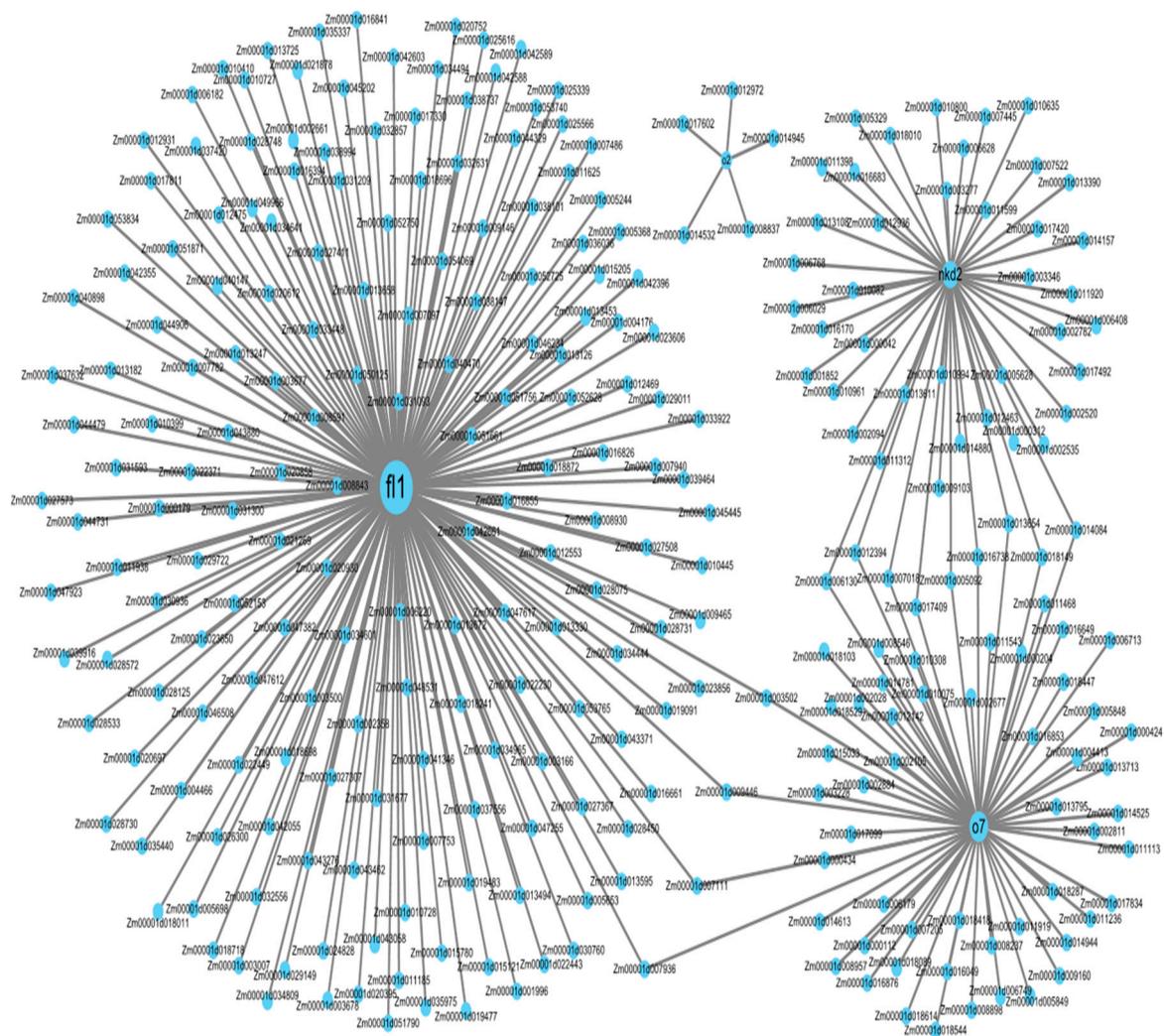


Figure 7. Visualization of the network connections among *fl1*, *o7*, *nkd2* and *o2*.

#### 4. Discussion

Malfunction of AGPase resulting from loss of *Sh2* function causes disruption of starch biosynthesis pathway, resulting in an increase of soluble sugar in kernels that are not fully formed and causing mature kernels to have reduced weight [45,46]. Insight into the transcriptional regulatory framework of *sh2* mutant in immature maize kernels can improve our understanding of genetic regulation of starch accumulation during endosperm development. To be enzymatically active, AGPase requires the complementary activity of its two subunits encoded by *Bt2* and *Sh2* genes in maize endosperm [4,47,48]. The effect of mutational changes in these genes leads to various phenotypes, which range from similarity to wild-type to lethality depending on the combination of the *Bt2* and *Sh2* alleles [49]. We identified a *sh2* allele in which only one exon was transcribed, while the wild type had 15 exons transcribed in the B73 background (Figure S1). RNA-seq analysis performed on this mutant at 15 DAP revealed 5175 DEGs identified from *sh2* mutant line kernels and wild-type kernels. DEGs related to endosperm include 11 genes for kernel development, 16 genes for starch biosynthesis and 32 zein-encoding genes. DEGs related to starch, including *bt2*, were upregulated (Figure 2). This alteration of the *shrunken2* gene also affects the expression of protein-related genes. Among the 32 zein-encoding genes identified in our experimental conditions, 25 genes were downregulated (Figure 2b). These data support that starch and zein synthesis are strongly associated biological processes which likely depend on transcript content of AGPase-related genes [48,50,51].

Starch synthesis occurs at 10–35 DAP, when the carbohydrate, storage protein and amino acid metabolism enzymes have accumulated [52]. Transcriptional regulation of expression of proteins was initially suggested to be the regulatory mechanism determining this coordinated expression [52]. Our co-expression network analysis shows that the module with the highest module membership had 2465 genes involved in transcriptional activity. A total of 357 TF genes, or 6.89% of the genome-wide DEGs, were identified, thus supporting the importance of transcriptional control in the regulation of starch biosynthesis [43,50,53,54]. In addition, four known mutants *fl1*, *o7*, *nkd2* and *o2* are co-expressed (Figure 7). The mutant of floury gene 1 (*fl1*) shows the greatest amount of co-expressed genes. Previous work reports that *fl1* encodes a zein protein body membrane accumulating 22-kD  $\alpha$ -zeins in the  $\gamma$ -zein-rich periphery and center of the core rather than their normal discrete location in a ring at outer edge of the core [18]. This mutation results in the formation of starchy endosperm, while the wild-type allele *Fl1* causes the vitreous endosperm. This is consistent with a putative role as a coordinator of transcriptional activity in starch synthesis at 15 DAP. The maize opaque 7 mutant gene (*o7*) appear to be the second TF gene highly co-expressed with maize endosperm genes at this developmental stage. In addition to decreasing starch  $\alpha$ -zein contents, *o7* influences amino acid biosynthesis by affecting  $\alpha$ -ketoglutaric acid and oxaloacetic acid both in maize and rice [22,55]. Although less important than *fl1*, the naked endosperm 2 (*nkd2*) and the opaque 7 (*o7*) mutant TF genes are also co-expressed with a wide number of genes in the “orange” module. *ndk2* TF is a member of the indeterminate domain (IDD) TF family, which is implicated in many biological functions, including the regulation of carbohydrate metabolism, gravitropism, seed germination, lateral organ morphogenesis, gravitropism, hormone signaling or DNA binding factor in Arabidopsis [56]. In maize, characterization of *ndk1 ndk2* double mutant into B73 background revealed that *ndk1* and *ndk2* influence 6.7% of transcriptome in starchy endosperm (at 15 DAP) [57], and NDK2 activates transcription from the 22KD zein promoter. The fourth connector is *o2*, a gene that was previously identified in the regulation of starch and protein synthesis in maize endosperm at 16 DAP [23]. However, the genetic network regulated by *o2* is reduced to five genes only in our experimental conditions. The difference is likely due to the pathway controlled by these two genes. While *sh2* controls starch synthesis by regulating the activity of AGPase, the transcription factor *o2* induces the downregulation of genes involved in the pentose phosphate pathway. Altogether, these data confirm that coordinated expression of starch is controlled by protein content. Our co-expression data highlight the specific role of 22KD zein content in *sh2*-deficient mutant.

## 5. Conclusions

Control of starch production and storage in the endosperm determines seed weight and kernel properties. In maize endosperm, this mechanism is regulated by several enzymes, among which AGPase plays a determinant role. Transcriptome profiling supports that the modification of *sh2* transcript content influences not only AGPase activity, but also the expression of genes controlling starch and protein synthesis during endosperm development. This regulation is carried out through a transcriptional network capped by four TF, namely the floury mutant gene 1 (*fl1*), the opaque 7 gene, the naked endosperm 2 (*nkd2*) gene and opaque 2 TF gene. Previous functional characterization of these genes demonstrated that they activate the transcription of genes involved in the storage of 22KD zein, thus supporting that accumulation of zein is a determinant for starch accumulation.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4395/10/5/624/s1>, Figure S1: Reads aligned to *sh2*, Figure S2: Principal component analysis of FPKM, Figure S3: MA-plot shows the log<sub>2</sub>-fold changes, Figure S4: Venn diagram of the number of TF genes in the DEG set and “orange” module, Table S1: List of Primers Used in this Study Table S2: Raw Data and Data Filtering, Table S3: RNA-Seq Data Mapping, Table S4: RNA-Seq Mapped Events, Table S5: Downregulated Genes Identified by GO Enrichment Analysis, Table S6: Upregulated Genes Identified by GO Enrichment Analysis, Table S7: List of annotated TFs, Table S8: Data Used for Co-expression Network Analysis.

**Author Contributions:** F.Q., A.M.N.S. and H.Z. conceived and designed the experiments; A.M.N.S., H.Z., Y.Q. and Q.S. performed the experiments; A.M.N.S., H.Z., Y.Q., D.G. and Z.P. analyzed the data; A.M.N.S., H.Z., Y.Q. and F.Q. wrote and revise the article. All authors have read and agreed to the published version of the manuscript.

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

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