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Assessment of corn transcriptome in two susceptible and tolerant genotypes to *Fusarium veticillioides* at grain filling under non-inoculated conditions

Sakineh Pesaraklu[1], Hassan Soltanloo[1*], Seyyede Sanaz Ramezanpour[1], Afshin Mosavat[2], and Elahe Tavakol[3]

[1]Department of Plant Breeding and Biotechnology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.
[2]Field and Horticultural Crops Research Department, Golestan Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Gorgan, Iran.
[3]Department of Crop Production and Plant Breeding, School of Agriculture, Shiraz University, Shiraz, Iran.
[*]Corresponding author; soltanlooh@gau.ac.ir

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Abstract

Objective: Fusarium is one of the most important causes of corn rot. Fusarium corn infection generally exists in corn seeds and causes a decrease in plant vigor, and leading to loss of seedlings. Given the significance of corn and the necessity to investigate the genes related to resistance against this pathogen, it is essential to acquire more comprehensive genomic and transcriptomic information. This study aimed to investigate the transcriptomic change of the two corn genotypes 15 days after flowering.

Methods: In this study, transcriptome analysis of two C7 and MO17 corn lines was conducted using RNA-Seq technology and the Illumina HiSeq 2500 sequencing system. The C7 and MO17 lines have shown the highest differences in resistance and susceptibility to Fusarium rot, respectively. After sequencing and deletion of the low-quality reads, 1078 significant differential expressions were observed.

Results: The analysis of gene ontology revealed that in the grouping of differential genes based on molecular function, the catalytic activity and binding groups accounted for the highest number of genes in both genotypes. Also, in classifying genes based on the biological process, the two groups of metabolic process and cellular process had the highest percentage of differential genes in both genotypes. In the KEGG pathway analysis, the most significant pathways belonged to the metabolic pathway, protein processing in the endoplasmic reticulum, and biosynthesis of secondary metabolites. The KEGG analysis of biological pathways in genotype C7 showed that a total of 144 differential expressions were assigned to 68 pathways. *HB*, *MYB*, and *Bzip* transcription factor families were among the important transcription factors that showed differential expression in this analysis.

Conclusion: The seed development stage is controlled by transcription factors. *ZmEREB167*, as an endosperm-specific transcription factor, affects starch accumulation and grain size.

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Introduction

Corn (*Zea mays* L., 2n = 2x = 20) is an important food crop, which has allocated the highest production among crops in the world. The growing world population and the need to meet the demand for food necessitate an increase in the production of crop plants. According to the predictions, by 2050 the demand for food will increase to twice the current level (FAO 2018). Increasing grain yield is one of the main goals of corn breeding programs. The number and weight of seeds are the most critical components of grain yield. Borrás and Otegui (2001) indicated that grain weight is influenced by seed filling rate, agrotechnical limitations, pests, and climatic conditions.

Fusarium verticillioides (Sacc), formerly known as *F. moniliforme*, is one of the most common pathogens affecting corn fields globally (Battilani *et al*. 2003). This fungus leads to grain and ear rot in corn and is exacerbated by dry and hot climates (Eller *et al*. 2008).

RNA-Seq is a rather new molecular technology that uses cDNA sequencing technology on a large scale. This technology can identify gene isoforms, translocation events, gene variants (including types of SNPs and SSRs), and post-transcriptional changes (Wang *et al*. 2007). The RNA-Seq method was developed in 2008 and was first used to analyze yeast, mouse, human, and Arabidopsis transcriptomes. It is expected to be an excellent alternative to the microarray method because of its high sensitivity and resolution, and ability to sequence longer ranges of genes (Marioni *et al*. 2008). In this technique, the researcher is informed of the content of total RNA expressed under specific test conditions, including mRNA, non-coding RNA, differentially spliced transcripts, post-transcriptional mutations, and other gene events (Maher *et al*. 2009). One of the most important goals of RNA-seq experiments is to detect gene expression changes in two or more different conditions. The expression level of each RNA is determined by measuring the number of fragments sequenced for a specific transcript (Tarazona *et al*. 2011). The RNASeq method was applied for the first time to explore the molecular events related to the development of resistance to *F. verticillioides* in corn (Lanubile *et al*. 2010). Also in this study, the expression profile of intra-genotypic and line differences was investigated, and the CO441 genotype showed the highest increase in differential expression.

Many studies have been conducted to discover the process of grain development and expression of related genes for grain growth in rice (Xu *et al*. 2012; Gao *et al*. 2013), *Arabidopsis thaliana* (Le *et al*. 2010; Belmonte *et al*. 2013), soybean (Jones and Vodkin 2013), and *Tropaeolum majus* (Jensen *et al*. 2012). The grain endosperm transcriptome was first sequenced by Lai *et al.* (2004), using the sequence tag method. The dynamic gene expression was identified in the seed development stage with the microarray-based technique of 3445 genes with differential expression (Liu *et al*. 2008), and Li *et al*. (2013) re-examined the transcriptome of corn embryo and endosperm with RNA-Seq. Several other studies have also used the RNA-Seq technology in corn (Bi *et al*. 2014; Lang *et al*. 2014; Lanubile *et al*. 2014; Niu *et al*. 2015).

The genetic control of the early stages of grain development after double fertilization was a complex and unknown process until recently. Yi *et al.* (2019) investigated the transcriptome by studying 31 corn samples collected in the first six days of grain development and in 4-6 hours. The results of the survey showed 22,790 differential genes in this stage, which included four groups: cellularization, coenocyte formation, differentiation, and double fertilization.

The present study aimed to investigate the transcriptional gene expression changes 15 days after pollination at the grain filling stage in the two corn genotypes MO17 and C7.

Materials and Methods

This research complied with relevant institutional, national, and international guidelines and legislation.

Plant materials

The two genotypes used in this study have shown the highest differences in resistance and susceptibility to Fusarium corn rot (Table 1). The tolerant C7 genotype was obtained from CIMMYT, and the susceptible MO17 line was provided by the Seed and Plant Improvement Institute, Karaj, Iran (Table 1). The seeds were planted in a seedling tray with field soil, Aeolian sand, and rotten manure in a ratio of 3:1:1, respectively. After 20 days, the seedlings were transferred to the field in the Gorgan Agricultural Research Station (25° 54' longitude and 54° 36' latitude), Gorgan University of Agricultural and Natural Resources, Gorgan, Iran. The between-row and within-row spacing were 75 cm and 25 cm, respectively. Seed sampling was performed 15 days after manual pollination. The samples were frozen in liquid nitrogen and stored at -80 °C for extraction.

Table 1. Specifications of the corn genotypes used in the study.

Line	Line origin	Response to the disease
MO17	USA	Susceptible (S)
C7	CIMMYT	Very Resistant (VR)

RNA isolation and transcriptome sequencing

The extraction of total RNA was carried out using a p-BIOZOL kit (Biofax, Japan) and then treated with the DNase I enzyme. The quantity and quality of the extracted RNA were evaluated by spectrophotometry at 260 nm and 1.5% agarose gel, respectively. This was done at the BGI company (Shenzhen, China) using the RNA-seq and NextFlex kit. The cDNA library was constructed on the Illumina HiSeqTM2500 (Illumina, USA) platform as paired-end at a read of 150 nt. Based on the bioanalyzer instrument, all samples had RIN values of greater than 7.5 and, thus, were suitable for constructing the cDNA library and sequencing.

Next-generation RNA sequencing and bioinformatics analysis

Raw data were controlled for quality and edited by the FastQC and Trimmomatic software. The reads having adapter sequences were omitted. Also, to acquire high-quality reads, the reads with unknown bases of greater than 5% and low quality were filtered. These reads were aligned against the B73 reference genome (ZmB73_RefGen_v2; http://www.maizesequence.org) by the Hisat2 software (version 2.2.1.0). Cufflinks v2.0.2 and HtSeq were used to assemble the mapped reads from each sample. The gene alignment process was performed by Cufflinks 2.02 software (Trapnell *et al.* 2010), which assembles isoform transcripts and quantitative expression values, for example, fragments per kilobase of known exons per million mapped reads (FPKM), and new genes were identified using a reference genome assembly (AGPv3)[1]. These Cufflinks assemblies were merged by Cuffmerge. Then, the outputs were used for differential expression analysis by Cufdif and EdgeR packages, and differentially expressed genes (DEGs) with a false discovery rate (FDR) threshold of <0.001 were considered. The expressed functional DEGs were classified through the gene ontology (GO). To classify the genes based on their molecular role, biological process, and cellular compartment, the gene ontology DEGs were identified by the NCBI (www.ncbi.nlm.nih.gov) BLASTx (Nr) database, and the list of GOs of DEGs was analyzed by the AgriGO and gprofiler (http://biit.cs.ut.ee/ gprofiler/) online software. The analysis of pathway enrichment was conducted by the following database:

[1] http://ftp.ensemblgenomes.org/pub/plants/release-25/fasta/zea_mays

https://string-db.org/cgi/input?sessionId=bOY6Uufuj0j2&input_page_active_form=multiple_identifers. Critical pathways were selected through Fisher's exact test at the FDR of <0.001.

Results

Analysis of RNA-Seq datasets

***Transcriptome sequencing and mapping*:** Among the total reads, 26,178,434 transcripts in the C7 line and 28,654,220 transcripts in the MO17 were explicitly mapped to the reference genome by Star Aligner (Table 2).

Table 2. The results of sequencing, quality control, and mapping at grain filling stage of two corn genotypes C7 and MO17.

Sample	C7	MO17
Raw reads	26178434	28654220
Clean reads	26021603	28465424
Raw base (G)	7.85	8.60
Clean base (G)	7.81	8.54
Effective rate (%)	99.40	99.34
Error rate (%)	0.01	0.01
Sequencing depth (%) (Q20)	96.5	96.68
GC content (%)	55.71	57.2

***Identification of DEGs*:** The DEGs of the C7 and MO17 lines were identified by R software. The use of the TMM and edgeR methods resulted in 1953 DEGs, considering FDR <0.001 and Log 2 FC |2|. Of these DEGs, 1113 and 840 genes had significant up- and down-regulation at the grain filling stage, respectively. This comparison also identified 303 new codogenetics. The function of the novel genes was annotated using the NCBI non-redundant (NR) database, and some of them remained unknown. In total, 178 unique differentially expressed genes were successfully mapped to the reference genome.

***DEGs and gene ontology*:** DEGs were identified using Agri GO (Figure 1) and were classified into three main categories. Grouping of differential genes based on the cell component showed that in both genotypes, the cell and cell parts groups had the highest number of genes. At the molecular level, the catalytic activity and binding groups accounted for the largest number of genes in both genotypes. In the classification of genes based on biological process, the two groups, metabolic process and cellular process, involved the highest percentage of differential genes in the two genotypes. Response to the stimulus gene ontology group, which includes genes related to stresses, showed a higher increase in the expression in the tolerant parent C7, compared to MO17. Among the genes of the

response to stimulus group, the genes related to the transcription factors (TFs) that are effective in the vital processes of the plant have been extracted and listed in Table 3. The results of the correlation network analysis between transcripts for the differential genes are shown in the Supplementary Figures 1, 2, and 3.

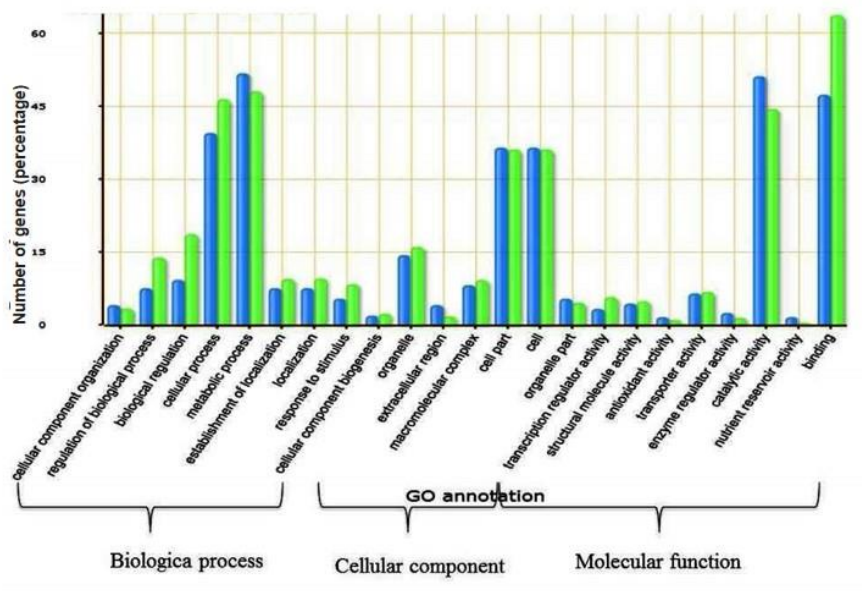

Figure 1. Gene ontology for up- and down-expressed genes in the C7 and MO17 corn genotypes.

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Table 3. Genes related to the response to stimulus group that are expressed more as transcription factors in response to stress in corn.

Transcription factor	Line	Position	Gene ID
E2F	MO17	Chromosome 1	Zm00001d033566
AP2/ERF-E	C7	Chromosome 9	Zm00001d047339
AP2/ERF-E	C7	Chromosome 10	Zm00001d026448
BZIP	C7	Chromosome 10	Zm00001d025589
MYB	C7	Chromosome 2	Zm00001d003412
NAC	C7	Chromosome 7	Zm00001d022517
WRKY	MO17	Chromosome 8	Zm00001d009619

The analysis of pathway enrichment using the KEGG database revealed the active biological pathways concerning the grain filling stage in corn. In our study, 53 and 46 pathways were identified in the C7 and MO17 lines, respectively. The most important pathways were protein processing in the endoplasmic reticulum, metabolic pathway, and biosynthesis of secondary metabolites. A total of 144 annotated DEGs were assigned to 68 pathways, using the KEGG database on biological pathways in the C7 genotype. Among the 53 pathways, the most significant pathways were metabolic (27 genes), protein processing in the endoplasmic reticulum (5 genes), biosynthesis of amino acids (4 genes), and

hormone signal transduction (4 genes). The KEGG analysis in the MO17 line revealed 122 DEGs involved in the biosynthesis of secondary metabolites (20 genes), energy metabolism pathways such as ribosome (6 genes), and phenylalanine, tyrosine, and tryptophan biosynthesis (6 genes) (Figure 2).

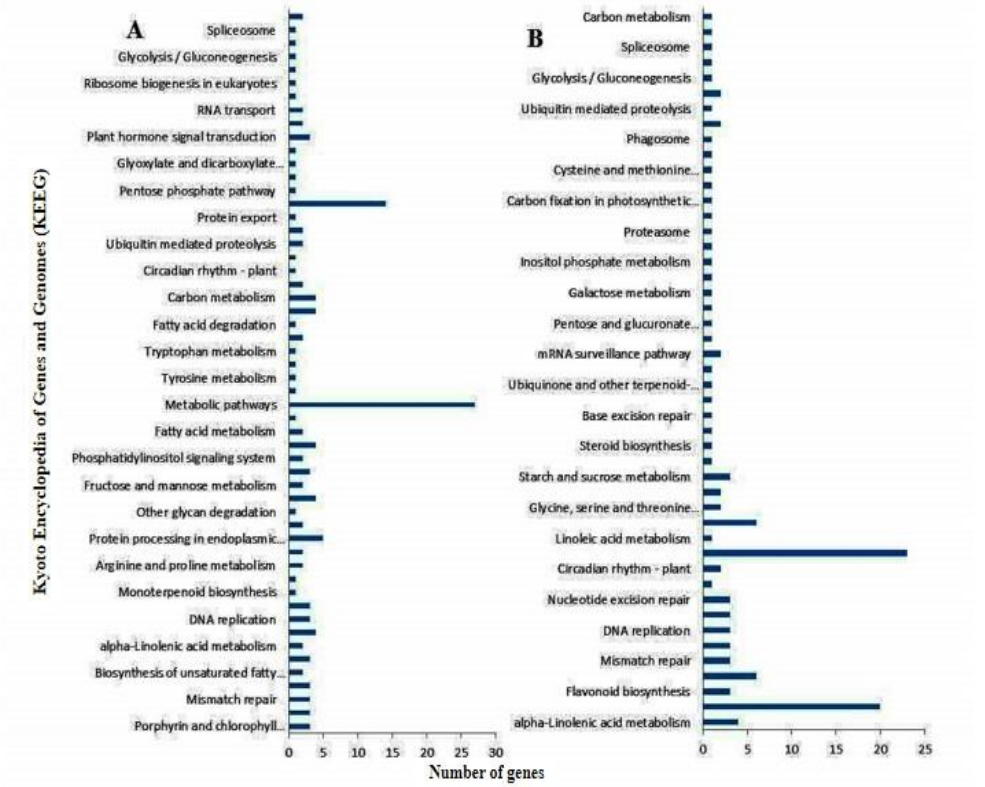

Figure 2. Analysis of KEGG metabolic pathways for differential expression of genes of C7 (A) and MO17 (B) corn genotypes at the grain filling stage.

Food metabolism pathways such as the metabolism of glutathione, fatty acids, alanine, beta-alanine, aspartate, glutarate, peroxisome, and autophagy regulators were also significantly enriched (Figure 3). These cases have been related to the energy metabolism pathways, such as ribosome, metabolic pathways such as photosynthesis, carbohydrates, and biosynthesis of secondary metabolites, and show the expression of specific genes in storing metabolites in different stages of seed development. The significance of these pathes shows their importance in the grain-filling stage.

***Transcription factors, protein kinases, and transcriptional regulators analysis*:** In two lines, 36 transcription factor-related genes were recognized. The top 21 transcription factor (TF) families were presented in the Supplementary Figure 4. The most significant members of the TFs belonged to the *AP2/ERF* family (8), followed by *Bhlh* (4), *MYB*-related (3), *NAC* (2), *MYB* (2), Bzip (2), and *C2C2-Dof*(2). In the comparison of the two lines, a total of 31 genes encoding protein kinases were identified among the differentially expressed genes, which belonged to four families (Supplementary Figure 5).

These genes were expressed in the C7 and MO17 lines, respectively; 17 and 14 genes were related to protein kinases with increased differential expression. The *TKL* kinase gene family was present only in the C7 genotype.

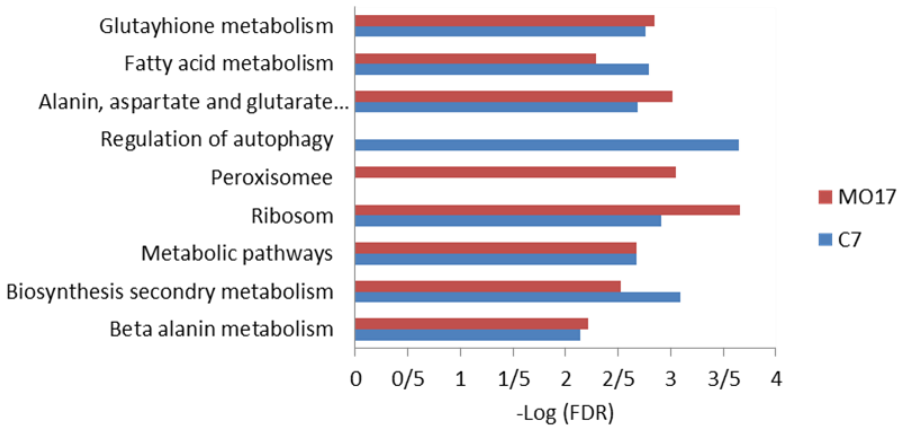

Figure 3. Differential expression enrichment of pathways associated with the grain filling stage of C7 and MO17 corn genotypes; FDR: false discovery rate.

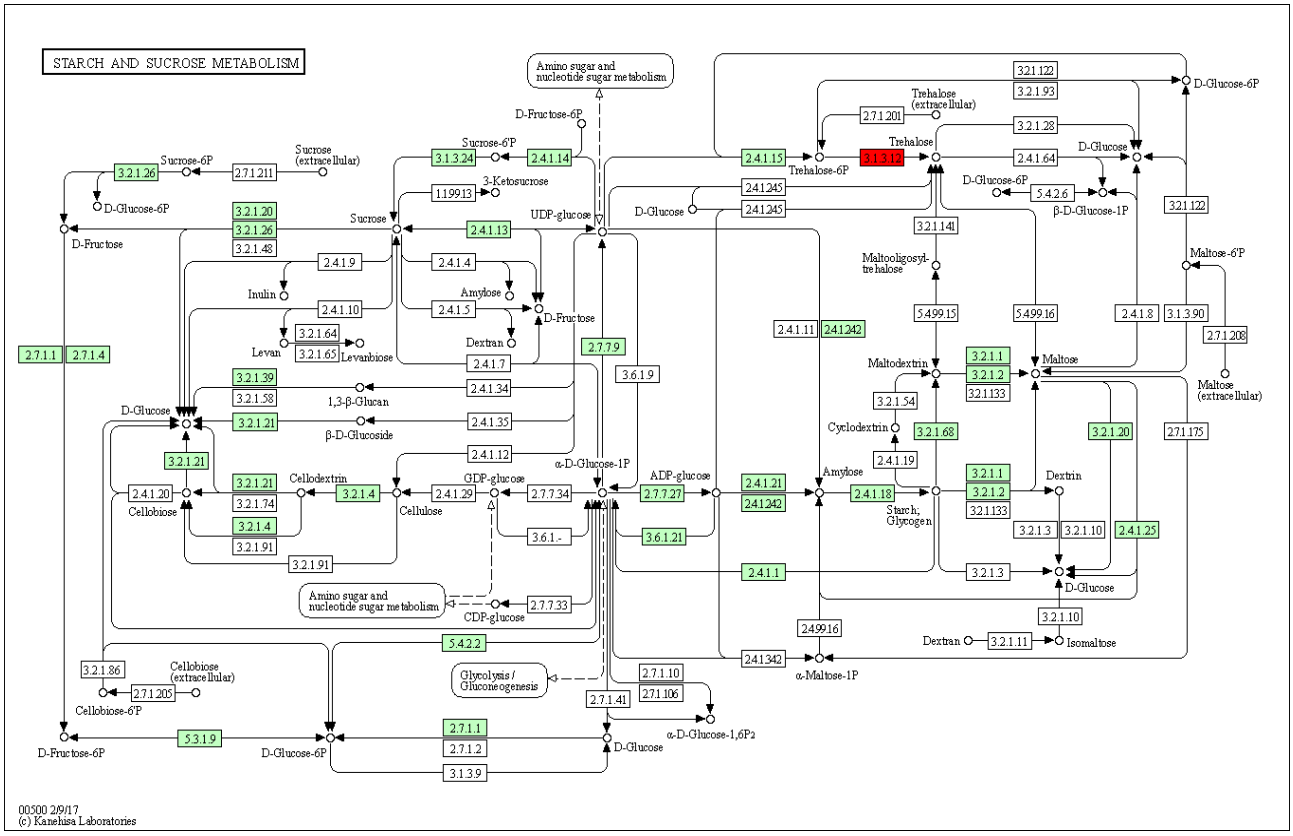

Figure 4. The KEGG enrichment pathway of starch and sucrose metabolism.

Discussion

About 50% of the identified grain-specific genes were highly expressed in the course of endosperm differentiation (144 h after pollination). These genes are further divided into two subgroups of cellularization and differentiation (Chen *et al*. 2014). Among these genes, specific *ESR*s and basal endosperm transfer cell layer (*BETLs*) might have an essential impact on the endosperm cell differentiation (Yi *et al.* 2019). For example, *Esr6* is a defensin gene, specifically expressed in the embryo surrounding region (ESR), which governs a protective role (Balandín *et al*. 2005). Several *BETL*[2]-specific genes, such as *Betl3*, hamper the entry of the pathogen into the developing seed using a specific defense response. By examining this stage, it may be possible to understand the deployment of defense mechanisms in differentiated tissues. BETL-specific genes are a set of genes expressed in the basal endosperm transfer layer (BETL) of maize seeds, a specialized cell layer crucial for nutrient transport between the maternal and filial tissues. These genes are often coordinately expressed during seed development and play a role in nutrient uptake and partitioning. (Magnard *et al*. 2003; Barrero *et al*. 2006). The study of Xie *et al.* (2018) showed that in two mutants, *ZmDA1* and *ZmDAR1*, the transgenic plants outyielded the wild-types by 15% because they had higher grain number, grain weight, and starch content. The over-expression of *Zmda1* and *Zmdar1* genes resulted in a more developed BETL than the wild type.

Transcription factors, as critical regulatory proteins, control the expression of several downstream genes. These genes play strategic roles in the plant responses to stress (Joshi *et al*. 2016). The control and regulation of the expression of several stress-related genes have made transcription factors suitable candidates for stress tolerance genes in genetic engineering and plant breeding. Isolation of CIPK25 and CarNAC4 transcription factors from chickpea and their transfer to tobacco and Arabidopsis plants, respectively, increased tolerance to water-deficit and salinity stresses (Meena *et al.* 2015; Yu *et al.* 2016). Acyl gene expression led to increased expression of free fatty acid synthesis under drought stress in wheat; fatty acids play a role in cell membrane repair and biosynthesis (Kazerani and Navabpour 2019). In a study, Yi *et al.* (2019) identified 22,790 expressed genes, including 1415 TFs, in the early stages of corn seed development. They were mainly involved in biological processes like grain filling (e.g., MYB81, BZIP46, and HB118). These transcription factors are activated to express BETL genes in the endosperm differentiation. According to Xiao *et al.* (2017), the MYB155 TF was highly expressed in the corn endosperm and involved in starch

[2]Basal Endosperm Transfer Layer

biosynthesis. López-González *et al.* (2022) also showed that the bZIP113 and ABI51 TFs could be involved in the regulation of sugar transport in the top internode of the corn stem.

The stage of seed development is controlled by transcription factors. For example, *ZmEREBs* also play important roles in the development process in corn. There are 240 *AP2/ERFs* in maize, named *ZmEREB1* to *ZmEREB240* (Qi *et al*. 2023). *OPAQUE11* directly regulates the expression of key TFs in nutrient accumulation and endosperm development, such as *O2*, *Naked endosperm 2* (*NKD2*), *Prolamin-box binding factor 1* (*PBF1*), and DNA-binding with one finger TF (*ZmDOF3*) (Feng *et al*. 2018). DOF36 directly binds and activates the expression of starch biosynthesis genes Du1 and Su2, which in turn promotes the accumulation of starch in the endosperm (Qi *et al.* 2017). Also, several family members are active in embryonic development, germination, and metabolic pathways (Kato *et al*. 2007). Zinc finger protein activity has been reported in plant and animal development, and mutations in this family result in abnormal growth of the embryo and other morphological changes (Guan *et al*. 2019).

The ubiquitin-proteasome pathway plays an essential role in the regulation of biological processes, protein metabolism, and recently, in the regulation of grain size (Li and Li 2014). The ubiquitin-proteosome system is one of the most essential mechanisms in the seed development process (Rangan *et al*. 2017) .

In the KEEG pathway analysis, one and three significant differential genes were identified for the proteasome pathway and ubiquitin-mediated proteolysis, respectively. Guan *et al.* (2019) identified 26 differential genes for these two pathways in wheat plants. Also, the enrichment of hormone signal transduction, sucrose and starch metabolism, and gluconeogenesis pathways corresponds to the expression pattern of grain development and increased grain size. These two pathways are activated through the response to auxin, gibberellin, ethylene, and jasmonic acid.

Starch is one of the most essential components of corn as a source of energy in the diet (Wang *et al*. 2014), and its synthesis is a complex process. In this study, some genes involved in this pathway were identified, including sucrose-phosphate synthase and sucrose synthase. Also, three differential expression genes of fructose-bisphosphate aldolase were identified; these genes play a crucial role in glycogenesis. Sucrose produces starch by sucrose synthase and invertase (Weschke *et al*. 2000), and the decrease in the biosynthesis of starch genes leads to a reduction in grain starch and finally the phenotypic dishevelment of the grain (Scofield *et al*. 2002). An increase in glucose leads to the expression of *ZmRP1* genes, which are responsible for regulating the transcription of *BTEL* genes (Sosso *et al*. 2015). Studies have shown that the development of transporter cells also increases the transfer of nutrients into the endosperm and increases the expression of genes involved in the sucrose

or glucose transport, which also improves the grain performance (Saalbach *et al*. 2014; Sosso *et al*. 2015).

Yin *et al.* (2019) used the bulked-segregant RNA-sequencing (BSR-seq) analysis and identified eight genes that showed differential gene expression patterns at several time points. Two genes, GRMZM2G391936 and GRMZM2G008263, are involved in the biosynthesis of secondary metabolites and the sucrose and starch metabolism. The results of the study by López-González *et al*. (2022) showed that in the early stages of seed development, the topmost female inflorescence, leaf blade, and leaf sheath had higher starch accumulation. The starch in the female inflorescence is synthesized in sink tissues to prepare for grain filling (Weise *et al.* 2011; Nagler *et al.* 2015; Scialdone and Howard 2015).

Photosynthesis and carbohydrate metabolism are essential metabolic processes that directly affect grain yield (Kriedemann 1966; Evans and Rawson 1970). Current hypotheses about the possible mechanisms of source-to-sink interactions are those involving sugars as signaling molecules. Current models show that many independent sugar-sensing pathways exist in plants. The seed weight depends on the photosynthetic capacity, and the remobilization of assimilates from the stems also determines the rate and length of grain filling, which ultimately determines the weight of the seeds (Ghassemi *et al*. 2020). In the study by Tarinejad *et al*. (2023) on rice, most of the gene ontologies were involved in the response to abiotic stresses and photosynthetic and metabolic processes. Also, sucrose synthesis and carbon partitioning throughout the plant considerably affect these processes (Hofius *and* Bornke 2007). Nitrogen availability is essential in the critical period of silking and during grain filling. Increasing the amount of dietary nitrogen leads to the activation of the trios-phosphate *ZmSps1* gene (Ning *et al*. 2018); this gene is located in the inner membrane of the chloroplast and plays a crucial role in regulating the carbon flux from the chloroplast to the cytosol (Zeeman *et al*. 2007; 2010). This action causes the synthesis of sucrose and ultimately the production of starch. In this study, porphyrin, chlorophyll metabolism, and carbon fixation by photosynthesis showed a significant differential expression increase in parent C7. Between the genes related to the response *to F. verticillioides*, a correlation has been observed with the genes involved in cell wall changes, lignin metabolic processes, and glycosyltransferase activity (Lanubile *et al*. 2014). In this study, 15 days after pollination, the abundance of glycosyltransferase genes (including Zm0000d039642) was expressed; this gene is effective in developing the plant's defense response to a stressful environment. Phosphohexokinase and hexokinase type IV glucokinase are the key regulatory genes involved in carbohydrate metabolism and show differential expression in the early and late grain-filling stages. Transaminase converts serine to hydroxy-pyruvate, leading to glycerate formation. Glycerate is

regarded as the key differentially expressed gene during early-grain filling, and it is associated with the photorespiration process. Phosphohexokinase and hexokinase type IV glucokinase are also involved in the respiratory metabolism (Rangan *et al*. 2017).

Conclusion

The transcriptome analysis of the lines C7 and MO17, resulted in 1078 significant differential expressions at the time of seed filling. Differentially expressed genes were implicated in pathways related to sugar and amino acid metabolism, carbon metabolism, metabolic pathways, and protein production process in the endoplasmic reticulum. Most of the genes involved in the grain filling, showed more differential expression in the C7 line. KEGG metabolic pathway analysis also showed that sucrose and starch metabolism pathway, plant hormone signal transduction, and glycogen synthesis were enriched with 14 differential genes. Next generation sequencing (NGS)-based transcriptome analysis (RNA-Seq) together with functional annotation are robust tools in identifying novel genes governing yield and metabolic pathways, which can improve our understanding of the complex metabolic networks. The information obtained from the complex metabolic networks, will facilitate the selection for genes governing grain filling. Also, the information obtained from the differentially expressed genes can be used to improve the yield and quality of the grain. A focus on silencing the genes that are involved in photorespiration might help to improve grain yield by reducing photorespiration, which in turn, will result in the increased efficiency of photosynthesis.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data availability

The raw data for this article can be found online at: https://www.ncbi.nlm.nih.gov/sra/PRJNA1062052

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