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Enrichment Analysis of Differentially Expressed Genes during Endosperm Grouting Periods in Non-waxy and Waxy Foxtail Millets

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Abstract The content of amylose and amylopectin is an important trait affecting cooking and eating quality in millet. During process of kernel grouting, the synthesis of starch involves different pathways and components in non-waxy and waxy millets. Immature grouting grains of waxy millet 'Gonggu 68' and non-waxy millet 'Chigu 4' (grouting period 1 and 5 days) were used to analyze their transcriptome sequences by using Illumina Hiseq4000. The results showed that: (1) GBSSI enzymes activity of waxy and non-waxy millet was low-high-low. There were some differences between the two activities. 665 up-regulated differentially expressed genes were screened on day 5 and day 1 during grouting period in waxy cultivar 'Gonggu 68', there were 431 more up-regulated genes than down-regulated genes. There were 97 more up-regulated genes than down-regulated genes in non-waxy cultivar 'Chigu 4' on day 5 and day 1 in grouting period. (2) In the A_2-VS-A_1 waxy comparison group, the differential genes were mainly GO enriched in 7 functions such as the seed oil body biogenic function, 17-β-ketosteroid reductase activity function and so on. it was mainly enriched in biological processes and molecular functions. In the B_2-VS-B_1 non-waxy comparison group, the differential genes were mainly GO enriched in 8 functions such as the light harvesting in photosystem I function and the pigment binding function for non-waxy millet. (3) Differentially expressed genes were mainly KEGG enriched in caffeine metabolism pathway, linoleic acid metabolism pathway, anthocyanin biosynthesis pathway, aflatoxin biosynthesis pathway in waxy A_2-VS-A_1, but which were mainly KEGG enriched in the synergy-antenna protein pathway, the linoleic acid metabolic pathway, the caffeine metabolic pathway, the brassinosteroid biopathway in non-waxy B_2-VS-B_1. These two comparative groups were enriched Caffeine metabolism pathway and linoleic acid metabolism pathway appeared in the process. (4) Three (*SSII-3*, *PHO1*, *AS*) and four (*PHO1-1*, *AS*, *AGP16*, *WAXY*) genes with significant differences and related to waxy and non-waxy millet were screened. With Actin (Si001873) as the internal reference gene, the above seven differentially expressed genes were verified by qRT-PCR, which was consistent with the transcriptome results, indicating that the differentially expressed genes were related to waxy or non-waxy endosperm.
Keywords Non-waxy millet; Waxy millet; Enzyme activity; Grouting period; Transcriptome; qRT-PCR

Setaria italica (L.) Beauv. belongs to the Poaceae Barnhart, also known as Daliang or Xiaomi in China. *Setaria italica* (L.) Beauv. is the earliest cultivated grain in primitive agriculture in northern China. For thousands of years, *Setaria italica* (L.) Beauv. has been widely planted because of its favorable characteristics such as drought tolerance, fast growth and strong adaptability, which is distributed from Xinjiang in the west to the whole Yellow River Basin (You, 1993, Agricultural History of China, (3): 1-13). *Setaria italica* (L.) Beauv. is rich in protein, fat, carbohydrates, vitamins and minerals and provides six basic nutrients for the human body (Qiao and Wang, 2015, Agricultural Science and Technology and Equipment, (11): 41-42). Due to different planting areas and varieties, the content of each nutrient may be different (Xue et al., 2008). In recent years, people pay more and more attention to eating habits and dietary structure, and *Setaria italica* (L.) Beauv. is favored by more and more people. Therefore, it has gradually become an indispensable part of the diet and been one of the most popular food crops in China.

In higher plants, starch can be divided into amylose and amylopectin according to different functions and structures. According to amylose content, it can be divided into non-waxy millet and waxy millet. The amylose content of non-waxy millet is >17.0%, and that of waxy millet is 0~3.5% (Nakayama et al., 1998). *Waxy* gene encodes granule-bound starch synthase (GBSSI), which is a key enzyme for amylose synthesis (Yang et al., 2007) and widely exists in rice, corn and other food crops (Gao et al., 2006). Deletion or mutation of *waxy* gene will affect the activity of GBSSI, which in turn affects the synthesis of amylose (Zhang et al., 2009). The content of amylose and amylopectin is an important trait that affects the cooking and eating quality of millet (Jia et al., 2010). Waxy millet is deeply loved by consumers because of its rich nutrition and diverse tastes. Therefore, it is necessary to study the mechanism of waxy millet and explore genes related to waxy phenotype. At present, there are no reports about the use of transcription sequencing technology to discover the differentially expressed genes during grouting periods in millets in China.

In this study, 'Chigu 4' (non-waxy millet) and 'Gonggu 68' (waxy millet) were selected to determine the activity of granule-bound starch synthase (GBSSI) during grouting periods. The grain samples corresponding to the time points with the highest and lowest enzyme activity were selected for transcriptome sequencing, screening, verification and enrichment analysis of differentially expressed genes.

1 Results and Analysis

1.1 The determination of GBSSI enzyme activity

The enzyme activities of 'Chigu 4' and 'Gonggu 68' were lowest on day 1 during grouting periods, which were 200 and 300 IU respectively (Figure 1), and the GBSSI activities of the two varieties were highest on day 5, which were 660 and 560 IU respectively.

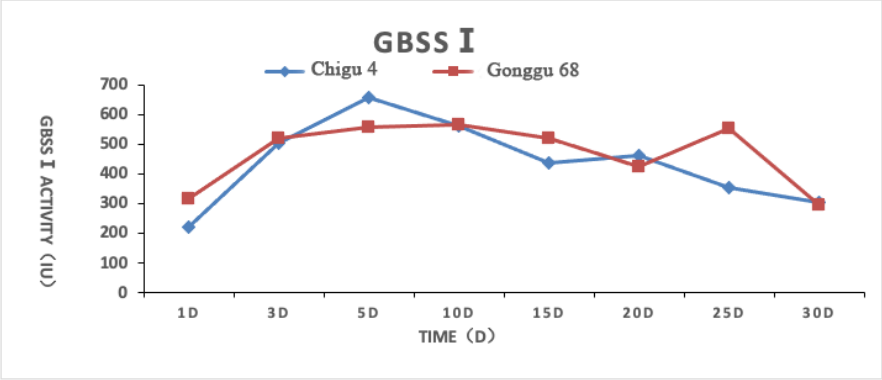


Figure 1 Dynamic changes of granule-bound starch synthase (GBSSI) activity

1.2 The analysis of differentially expressed genes (DEGs)

From the number distribution of differentially expressed genes in waxy and non-waxy millet cultivar (Figure 2), it can be seen that 665 up-regulated and 234 down-regulated differentially expressed genes were screened from the A_2-VS-A_1 comparison group on day 5 and day 1 during grouting period of waxy cultivar 'Gonggu 68', there were 431 more up-regulated genes than down-regulated genes. While 1 586 up-regulated and 1 489 down-regulated differentially expressed genes were screened from the B_2-VS-B_1 comparison group on day 5 and day 1 during grouting period of non-waxy cultivar 'Chigu 4', there were 97 more up-regulated genes than down-regulated genes.

1.3 GO enrichment analysis of differentially expressed genes

In the A_2-VS-A_1 waxy comparison group, differential expressed genes (DEGs) were enriched in many pathways (Figure 3). The enrichment factors that were larger than 0.1 included the following six pathways, namely seed oilbody biogenesis function, 17-beta-ketosteroid reductase activity function, 17-beta-hydroxysteroid dehydrogenase (NADP+) activity function, killing of cells of other organism function, central vacuole function, lipid storage function. Among them, seed oilbody biogenesis function, killing of cells of other organism function and lipid storage function were biological processes, while 17-beta-ketosteroid reductase activity function and

17-beta-hydroxysteroid dehydrogenase (NADP+) activity function were molecular functions. In the B_2-VS-B_1 non-waxy comparison group, the enrichment factors that were larger than 0.15 also included the following six pathways, namely light harvesting in photosystem I function, pigment binding function, chlorophyll binding function, pectinesterase activity function, pectinesterase inhibitor activity function, plastoglobule function, cellular transition metal ion homeostasis function, transition metal ion binding function and so on. Among them, light harvesting in photosystem I and cellular transition metal ion homeostasis function were biological processes, while pigment binding, chlorophyll binding, pectinesterase activity, pectinesterase inhibitor activity, plastoglobule function, and transition metal ion binding and extracellular regional function were molecular functions.

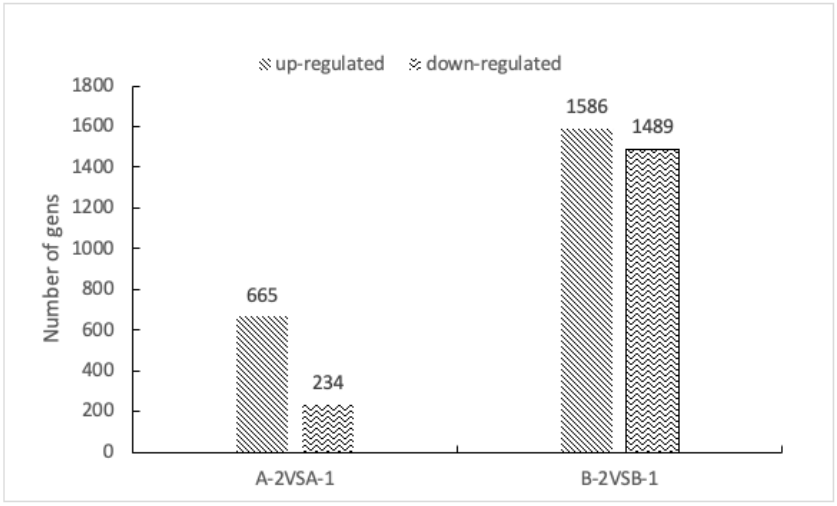
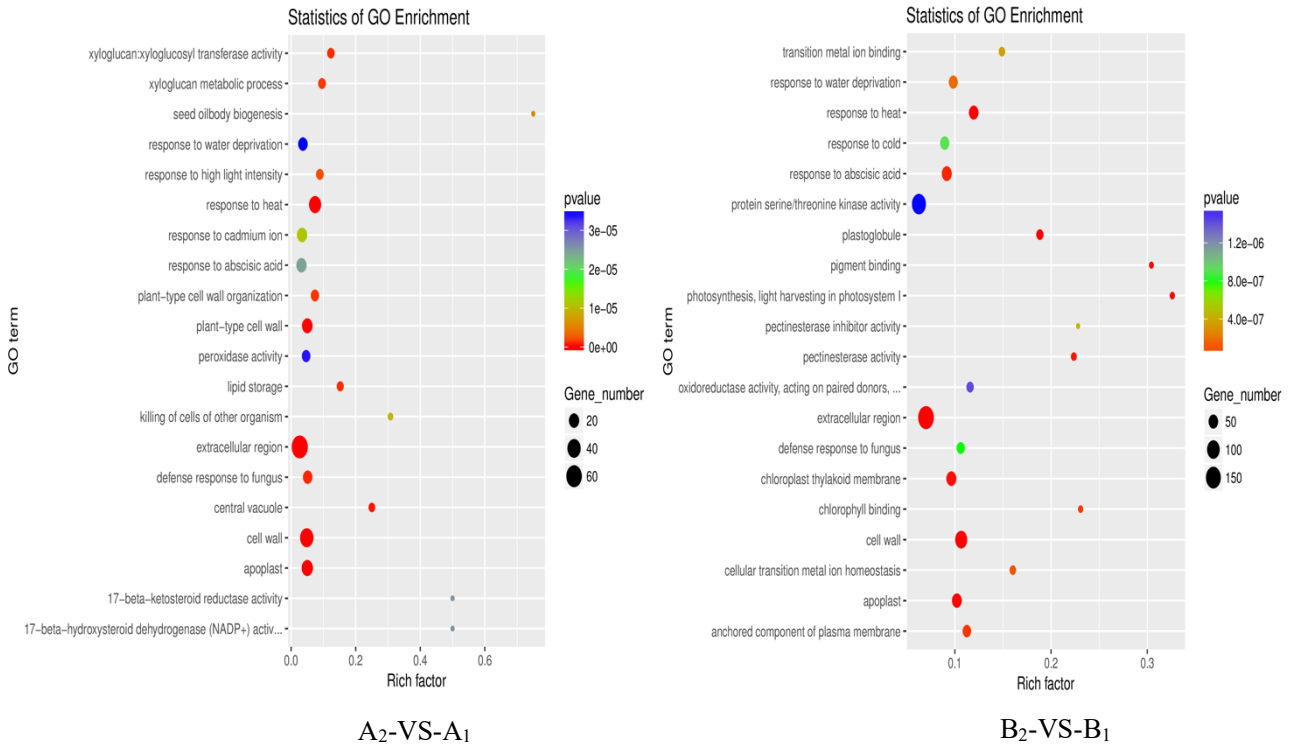


Figure 2 Number distribution of differentially expressed genes in waxy and non-waxy millet cultivar



A_2-VS-A_1 B_2-VS-B_1

Figure 3 GO enrichment analysis of differentially expressed genes

1.4 KEGG enrichment analysis of differentially expressed genes

In the A_2-VS-A_1 waxy comparison group (Figure 4), the enrichment factors of the differentially expressed genes that were larger than 0.06 included caffeine metabolism pathway, linoleic acid metabolism pathway, anthocyanin

biosynthesis pathway, aflatoxin biosynthesis pathway and so on. In the B_2-VS-B_1 non-waxy comparison group, the enrichment factors of the differentially expressed genes that were larger than 0.06 included photosynthesis-antenna protein pathway, linoleic acid metabolism pathway, caffeine metabolism pathway and brassinosteroid biosynthesis pathway.

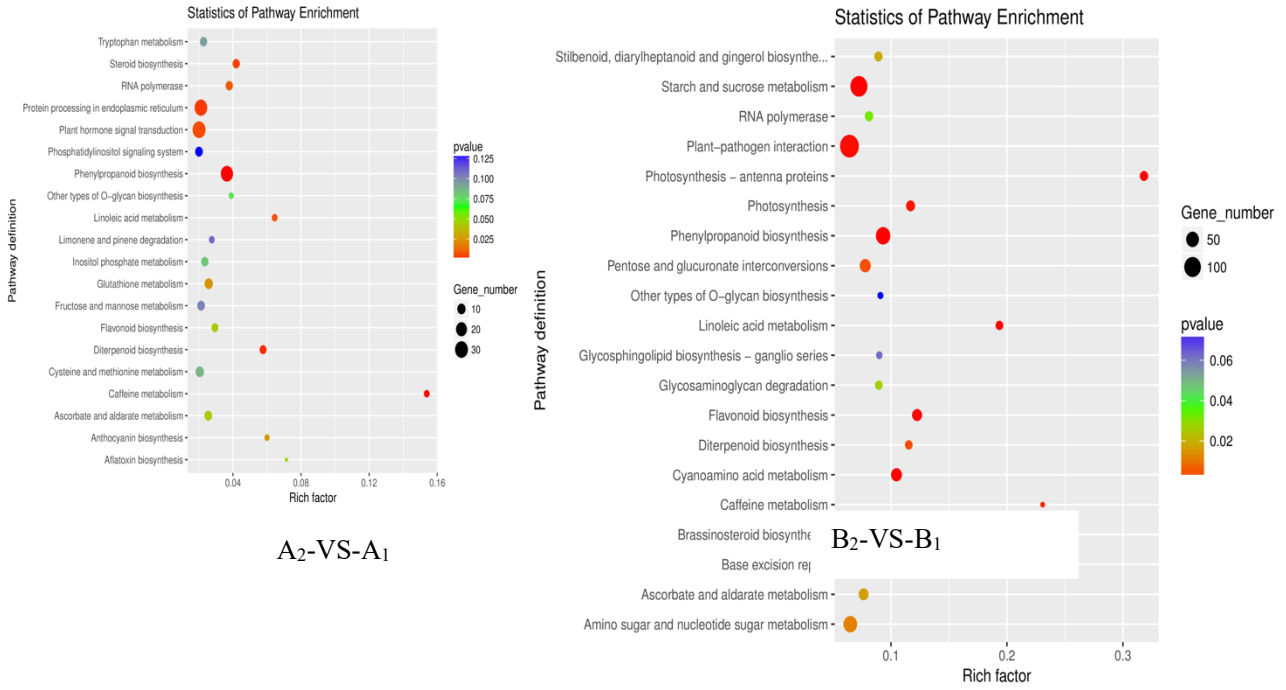


Figure 4 KEGG enrichment analysis of differentially expressed genes

1.5 Screening and verification of differentially expressed genes

Using |log2FC|>1, p<0.05 as the screening conditions, the differential expressed genes in the two comparison groups were screened. There were 899 and 6 150 differential expressed genes in A_2-VS-A_1 and B_2-VS-B_1, respectively. According to the gene function annotation, 36 genes related to the waxy and non-waxy millet were screened, among which, 12 was in A_2-VS-A_1 group and 24 was in B_2-VS-B_1 group. The results (Table 1) showed that 3 differential genes with FDR<0.01 and high expression (TPM), related to the waxy and non-waxy millet, were screened in A_2-VS-A_1 comparison group, including *SSII-3*, *PHO1* and *AS*, while in B_2-VS-B_1 group, 4 differential genes were screened, including *PHO1-1*, *AS*, *AGP16* and *WAXY*.

Table 1 Differential genes and their expression quantity

Name	TPM_1	TPM_2	log2FC	p value	FDR	Up-regulation/Down-regulation
SSII-3	12.37	43.66	-1.73	0.00	0.00	Down
PHO1	124.20	387.64	-1.70	0.00	0.00	Down
AS	5.66	0.13	5.57	0.00	0.00	Up
PHO1-1	3.12	20.79	-2.80	0.00	0.00	Down
AS	8.76	0.56	4.46	0.00	0.00	Up
AGP16	0.00	3.42	-8.32	0.00	0.00	Down
WAXY	99.77	402.75	-1.65	0.00	0.01	Down

According to the screening results of differential genes, the differential genes were verified by real-time fluorescence quantitative qRT-PCR with *Actin* (Si001873) as the internal reference gene. The total RNA of the sample was extracted, and the quality and purity of the sample were detected by 1.5% agarose gel electrophoresis (Figure 5). The differential genes were performed t test at the 0.05 level by SPSS 19.0 (Figure 6).

In A_2-VS-A_1 waxy comparison group (Figure 6), A_1 was the control group, and the expression of differential gene *SSII-3* and *PHO1* in A_2 was lower than that in A_1, showing down-regulation expression; The differential gene *AS* showed up-regulation expression. In B_2-VS-B_1 non-waxy comparison group, B_1 was the control group, and the

expression of differential genes *PHO1-1*, *AGP16* and *WAXY* in B_2 were lower than that in B_1, showing down-regulation expression, but the expression of differential gene *AS* in B_2 was higher than that in B_1, showing up-regulation expression.

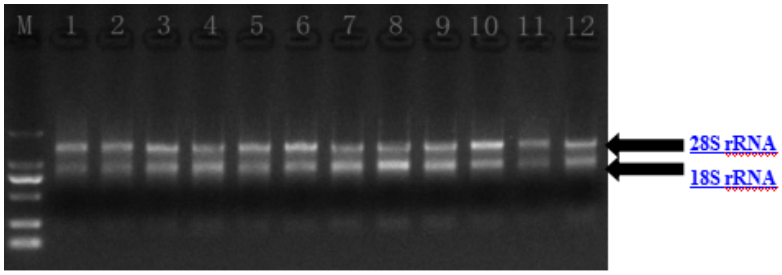


Figure 5 Agarose gel electrophoresis of total RNA extraction

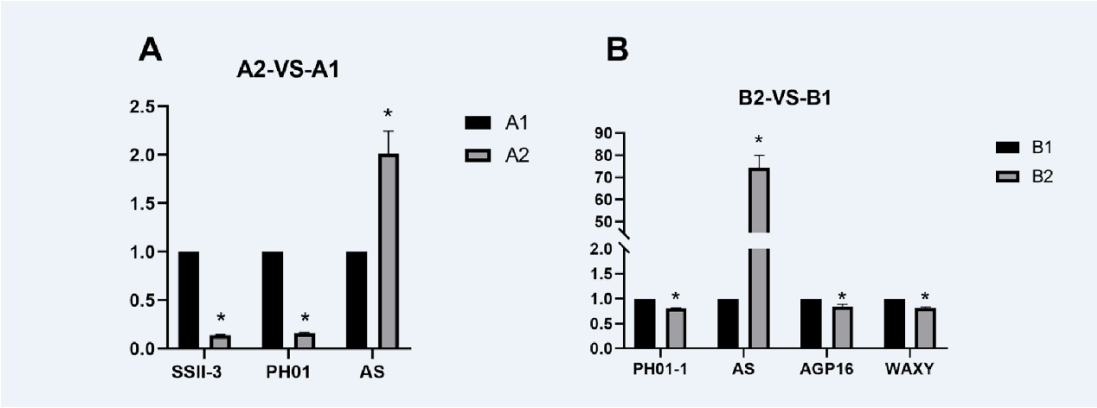


Figure 6 Candidate genes and their expression levels in non-waxy and waxy comparable group

Note: A: Candidate genes and their expression levels in waxy comparison group; B: Candidate genes and their expression levels in non-waxy comparison group

2 Discussion

The differentially expressed genes can be identified by comparing the high-throughput transcription and sequencing data of different tissues. New transcripts, variable splicing and gene structure variation can be identified and discovered with the help of a large number of gene annotation data (Qi et al., 2011). With the application of bioinformatics in the field of life science, transcriptome sequencing has become an important research method of transcriptome. Transcriptome sequencing technology is relatively mature, low-cost and widely used, which comprehensively and quickly reflects the transcriptome data of a certain tissue or cell at a specific time and specific location (Zhou et al., 2019). The researchers obtained the anthocyanin metabolic structure gene and regulatory gene sequence of *Hyacinthus orientalis* L. variety 'Black Baccara' through transcriptome sequencing. The expression patterns of 'Black Baccara' in white, red, blue and black were analyzed by qRT-PCR. It was found that *HoF3H*, *HoMYB4, HoMYB6* and *HoMYB7* were four key genes affecting anthocyanin accumulation of *Hyacinthus orientalis* L. (Li et al., 2020, http://kns.cnki.net/kcms/detail/46.1068.S.20200226.1514.004.html). Wang et al. (2018) used high-throughput RNA-seq technology to sequence the transcriptome of drought-stressed millet, and the samples treated 6 d were compared with that treated 6 hours. It was found that 206 genes were up-regulated and 611 genes were down-regulated. Zu et al. (2015) obtained a WRKY transcription factor SiWRKY36 by sequencing the transcriptome of drought-stressed millet.

In this study, the high-throughput sequencing technology Illumina Hiseq4000 was used to sequence the transcriptome of immature grains at a specific time during the grouting period of non-waxy millet ('Chigu 4') and waxy millet ('Gonggu 68'). There were 431 more up-regulated genes than down-regulated genes in the waxy comparison group A_2-VS-A_1, while there were 97 more up-regulated genes than down-regulated genes in the non-waxy comparison group B_2-VS-B_1. The results showed that the number of up-regulated genes was

significantly larger than that of down-regulated genes in the early grouting period of waxy millet, and there was less difference between up-regulated genes and down-regulated genes in the early grouting period of non-waxy millet.

In the A_2-VS-A_1 waxy comparison group, the differential genes were mainly GO enriched in 7 functions such as the seed oil body biogenic function, 17-β-ketosteroid reductase activity function and so on. In the B_2-VS-B_1 non-waxy comparison group, the differential genes were mainly GO enriched in 8 functions such as the light harvesting in photosystem I function and the pigment binding function for non-waxy millet. The two comparison groups were mainly enriched in biological processes and molecular functions.

In the A_2-VS-A_1 waxy comparison group, the differential genes were mainly KEGG enriched in caffeine metabolism pathway, linoleic acid metabolism pathway, anthocyanin biosynthesis pathway, aflatoxin biosynthesis pathway and so on. In the B_2-VS-B_1 non-waxy comparison group, the differential genes were mainly KEGG enriched in photosynthesis-antenna protein pathway, linoleic acid metabolism pathway, caffeine metabolism pathway and brassinosteroid biosynthesis pathway. Both the non-waxy millet and waxy millet included caffeine metabolism pathway and linoleic acid metabolism pathway.

Soluble starch synthase genes include *SSI*, *SSII*, and *SSIII*, which participate in amylopectin synthesis together with branching enzyme genes and debranching enzyme genes (Ohdan et al., 2005; Pandey et al., 2012). The *SSII-3* gene is the main gene that controls the gelatinization temperature of rice (Gao et al., 2003, Science in China (Serices C: Life Sciences), 33(6): 481-487). *SSII-3* has two genotypes, and different genotypes have different effects on eating quality. Type I is high gelatinization temperature and low amylose, and type II is low gelatinization temperature and high amylose (Tian et al., 2009). In this study, the expression of *SSII-3* gene was down-regulated in A_2-VS-A_1 group, that is, the expression of *SSII-3* gene in the middle grouting period of 'Gonggu 68' was lower than that in the early grouting period, indicating that the amylopectin synthesis in waxy millet was more active in the early grouting period, which may be an important period to determine the non-waxy and waxy millet and a period that has a great impact on the eating quality of millet.

AS is one of glycosyltransferase genes. *AS* can accept a variety of compounds with diverse structures as its substrate, which is a rare multifunctional glycosyltransferase with multi-substrate acceptance ability (Hefner et al., 2002). In this study, the expression of *AS* on day 5 was higher than that on day 1 in the two comparison groups, indicating that the expression of *AS* increased continuously in the early grouting period, which was more important in the process of grain formation.

Starch phosphorylase plays a dynamic regulatory role in the carbohydrate metabolism of higher plants, that is, the synthesis and degradation of starch (Schneider et al., 1981). In this study, the *PHO1* gene showed a down-regulation trend in the two comparison groups, and waxy millet had an obvious down-regulation trend, indicating that the expression of starch phosphorylase gene was higher in non-waxy millet and waxy millet in the early grouting period, which was more important for starch synthesis and degradation.

In the process of starch synthesis, adp-glucose pyrophosphorylase (AGP) catalyzes glucose-1-phosphate (G-1-P) and adenosine triphosphate (ATP) to synthesize adenosine diphosphate glucose (ADPG) and release pyrophosphate (PPi) (Gao and Huang, 1998). The effect of genotype on AGP activity and starch content is simultaneous, and AGP activity has a direct effect on starch content (Zhao et al., 2013).

In this study, the expression of differential gene *AGP16* was down-regulated in B_2-VS-B_1 group, indicating that the expression of differential gene *AGP16* in the middle grouting period was lower than that in the early grouting period, and the early grouting period was an important stage for starch formation. *AGP* has a variety of isozymes, and *AGP16* gene may have similar function to *AGP* gene, which may be its isozyme gene.

Waxy gene controls the amylose synthesis in endosperm and encodes a key enzyme - granule-bound starch synthase (GBSSI) for plant starch synthesis. The hindrance of amylose synthesis in endosperm and the decrease of content is caused by the deletion or mutation of *Waxy* gene or the decrease of GBSSI enzyme activity (Liu et al.,

2010). In this study, the expression of differential gene *WAXY* was down-regulated in B_2-VS-B_1 group, indicating that the expression of differential gene *WAXY* in the middle grouting period of non-waxy millet was lower than that in early grouting period, and the early grouting period of non-waxy millet was an important stage for amylose synthesis. The formation of waxy endosperm is caused by the deletion and mutation of *WAXY* gene.

In this study, 7 differentially expressed genes in different periods of non-waxy and waxy millet were obtained by transcriptome sequencing, and the differentially expressed genes were verified by qRT-PCR, which provided an experimental basis for millet quality genetic breeding, germplasm identification and molecular marker assisted breeding.

3 Materials and Methods

3.1 Plant materials

The materials of this study were two spring millet varieties introduced from China: 'Gonggu 68' (waxy variety, AC was 0, selected by Jilin Academy of Agricultural Sciences) and 'Chigu 4' (non-waxy variety, AC was 20.10%, selected by Chifeng Academy of Agricultural Sciences).

3.2 Sampling and enzyme activity determination

In this study, the two experimental materials were designed according to the random block. The plot area was 25 m^2 and repeated for 3 times, which was planted on the Inner Mongolia Agricultural Teaching Farm. The 12^{th} day after millet flowering was selected as the first day of grouting period. The sampling time points were day 1, day 3, day 5, day 10, day 15, day 20, day 25 and day 30. After liquid nitrogen quick freezing, transfer to ultra-low temperature refrigerator to save for testing. The enzyme activity was measured by using the GBSSI kit from Suzhou Comin Biotechnology Co., Ltd. According to the level of enzyme activity, comparison groups were set for the highest and lowest points of enzyme activity of waxy millet and non-waxy millet, named A_2-A_1, B_2-B_1, and transcriptome sequencing was performed on the corresponding grain materials to compare the expression of differential genes in the same variety at different grouting periods.

3.3 Analysis of differentially expressed genes (DEGs) and gene function and classification annotation

According to the expression of genes in the comparison groups (TPM) in the sequencing results, DEGs were screened, and the genes were annotated with GO and KEGG pathways.

3.4 Real time fluorescence quantitative PCR analysis

The total RNA was isolated from the corresponding grain materials and its quality and purity were determined by agarose gel electrophoresis. Taking *Actin* (Si001873) as the internal reference gene, the gene sequence primers were designed by PrimerQuest tool (Table 2). The expression of DEGs was calculated by $2^{-\Delta\Delta Ct}$ method and plotted by GraphPad Prism 8.0.1.

Table 2 Name and sequence of primers for quantitative PCR of candidate genes

Gene name	Primer	Sequence (5'-3')	Product size (bp)
Actin (Si001873)	Forward	GGCAAACAGGGAGAAGATGA	
	Reverse	GAGGTTGTCGGTAAGGTCACG	
SSII-3	Forward	ATTCCGCATTCATCGGTCTC	110
	Reverse	GCTCAACCCACTACCATTCTAC	
PHO1	Forward	GTGCAGAGGCACATGAAATTG	110
	Reverse	AAAGACACCACTGCGGATAAA	
AS	Forward	GGTAGGCGAGGAGTAGTAGTT	150
	Reverse	CATAGGCGAGAGTTTGGAGATG	
PHO1-1	Forward	GTGGACGAGGACTAACGTACTA	144
	Reverse	AGGAACTACTACGCGCATAAAC	
AGP16	Forward	GTGCAATCCTTGGTACCTCTAC	103
	Reverse	GAACCTTTGGTATGTGTTGCATTA	
WAXY	Forward	GAGTGCTGTGGCTATACATTCA	108
	Reverse	ATGCGCTCGAACTTCTTCTT	

Authors' contributions

GSH, LQ, and LT were the designers of the experiments and executors of the research; LQ and LT completed the data analysis and wrote the first draft of the manuscript; BYT and LXC participated in designing the experiments and analyzing the experimental results; GSH was the conceiver and person in charge of the project, directing experimental design, data analysis, manuscript writing and revision. All authors read and approved the final manuscript.

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