Transcriptomic and anatomic profiling reveal germination process of different wheat varieties in response to waterlogging stress

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Abstract

Background Waterlogging is one of the most serious abiotic stresses affecting wheat-growing regions in China. There are considerable differences in waterlogging tolerance among different wheat varieties, and the mechanisms governing the waterlogging tolerance of wheat seeds during germination have not been elucidated. Methods To reveal the adaptability of wheat to waterlogging stress during germination, we analysed the germination rate and anatomical structure of three wheat seeds, 'Zhoumai 22'(ZM22), 'Bainong 207'(BN207) and 'Bainong 607'(BN607). At the same time, Illumina sequencing technology was used to determine the transcriptome of these three wheat varieties during germination.

Results The results showed that there was no significant difference between the germination rate of BN607 after 3 days of waterlogging treatment and that of the control seeds. However, under waterlogging stress, the degree of emulsification and degradation of endosperm cells was higher than that of the control treatment, and amyloplasts in endosperm were significantly reduced. Transcriptomic data were obtained from seed samples (a total of 18 samples) of three wheat varieties under the waterlogging and control treatments. A total of 2775 differentially expressed genes (DEGs) were identified by comprehensive analysis. In addition, by analysing the correlation between the expression levels of DEGs and seed germination rates in three wheat varieties under waterlogging stress, it was found that the relative expression levels of 563 and 398 genes were positively and negatively correlated with the germination rate of wheat seeds, respectively. The GO and KEGG analyses found that the difference in waterlogging tolerance of the three wheat varieties was related to the abundance of key genes involved in the glycolysis pathway, the starch and sucrose metabolism pathway, and the lactose metabolism pathway. The alcohol dehydrogenase (ADH) gene in the endosperm of BN607 was immediately induced after short-term waterlogging, and the energy provided by the glycolysis pathway enabled the seeds of BN607 to germinate as early as possible, while the expression of the AP2/ERF transcription factor was upregulated to further enhance its waterlogging tolerance.

Conclusions Taken together, the results of this study help to elucidate the mechanisms by which different wheat varieties respond to waterlogging stress during germination.

Background

Hypoxia is one of the most important stresses on plants in the soil or the roots of plants. Hypoxia caused by waterlogging can inhibit the growth of roots and stems, dry matter accumulation and final yield [1]. Relevant studies show that waterlogging may affect the hormone content in plant roots and stems, possibly by impacting the production of ethylene [2], and then reduce the absorption of mineral nutrients by plant roots [3]. Wheat is an important food crop widely planted in the world, and it is ranked first in the harvest area among the three major food crops (rice, wheat and corn). China is the largest wheat-producing and -consuming country in the world, and wheat is of great significance to China's food security and farmers' increasing incomes. According to the World Food and Agriculture Organization (FAO), approximately 10% of the world's land area is affected by waterlogging to varying degrees [4]. In the Mediterranean region, the germination and growth stage of winter wheat is vulnerable to waterlogging because approximately 40% of the annual rainfall occurs during the sowing period of winter wheat [5]. The wheat-growing areas in northern China also face the same problems. For example, some areas often encounter continuous rainy days during the wheat-planting period. In addition, uneven terrain or poor drainage systems of farmland easily cause water logging or waterlogging in the soil, which often leads to a lack of oxygen supply to the soil and the inhibition of seed germination, thereby reducing the germination rate of wheat [6] and resulting in wheat reductions.

Seed germination is the first step of plant life. Flooding leads to loss of potassium and phosphorus, leakage of carbohydrates and amino acids, starvation of seedlings and proliferation of pathogenic microorganisms [7]. There are many topics on waterlogging of rice, such as the quiescent strategy by the SUB1 gene [8, 9], avoidance strategy with SNORKEL 1 and 2 genes [10], anaerobic respiration mechanism, hormone response [11] or redistribution of storage materials [12]. Researchers believe that plants have formed a set of new mechanisms in anatomy, morphology and physiology to adapt to waterlogging environments [13], including the production of aerenchyma, the formation of adventitious roots, the elongation of petiole speed, the growth of stem hypertrophy, the growth of hypocotyls, the increase of plant height, the closure of stomata, the reduction of transpiration and the inhibition of photosynthesis [2, 4, 14]. In research on wheat, more attention has been paid to the response of plants to waterlogging at the seedling and filling stages [3, 15]. There are few reports concerning the response of wheat to waterlogging stress at the germination stage.

Transcriptome sequencing technology can identify and clarify the pathway of metabolite synthesis and accelerate research on plant metabolism. In rice, RNA-Seq technology is used to analyse the differential expression profile of waterlogging-tolerant and waterlogging-sensitive varieties during germination to quickly screen several important candidate genes, such as OsTPP7, OsHXK7 and OsPGM, which may affect the early stage of rice growth [16]. In this study, the phenotype and anatomical structures of different wheat genotypes during seed germination were analysed, and then the transcriptional profile during germination was analysed. By comparing the structural and transcriptional levels of three kinds of wheat under the waterlogging and control treatments, several key DEGs involved in important

metabolic pathways under the waterlogging treatment were revealed. This study establishes a foundation for further study on the functional characteristics of key waterlogging resistance genes in wheat and is of great significance for the genetic improvement of waterlogging resistance varieties in wheat.

Results

Phenotypic analysis of different wheat varieties under the waterlogging treatment

The phenotypes of three wheat seeds (ZM22, BN207 and BN607) under the waterlogging treatment after three days of germination were shown in Fig. 1. Under the control treatment, there was no significant difference in phenotype among the three wheat varieties (Fig. 1A). The germination rate and coleoptile height of the three kinds of wheat were above 95% and approximately 3.5 cm, respectively (Fig. 1B and D). Although there were no significant differences among the roots of ZM22, BN207 and BN607, the germination rate and coleoptile height of the three wheat varieties changed significantly under the flooding treatment. The germination rate of BN607 was the highest (94.02%) followed by BN207 (78.85%), and the germination rate of ZM22 was the lowest (61.21%) (Fig. 1C and 1E). In addition, compared with the control treatment, the coleoptile height of ZM22 and BN207 under the waterlogging treatment was significantly lower with an average length of approximately 2 cm, while that of BN607 under the waterlogging treatment was 3.5 cm without a significant difference from that of the control treatment (Fig. 1E). Based on the above analysis, we known that the waterlogging tolerance of the three wheat varieties from high to low was BN607, BN207 and ZM22.

Anatomical analysis of different wheat seeds under the waterlogging treatment

Germination of seeds was mainly dependent on amyloplast in the endosperm [17], therefore, we focused our analysis on structural changes of the endosperm at the seed germination stage. Through structural anatomical analysis of the seeds, it was found that the endosperm was emulsified and degraded during seed germination. Endosperm began to emulsify from the endosperm adjacent to the embryo, and the place close to the embryo was emulsified into a cavity (Fig. 2A). After 3 days of waterlogging treatment, the emulsification degree of the endosperm of the three wheat varieties was different. The endosperm emulsification degree of BN607 was the highest followed by BN207 and ZM22 (Fig. 2A). By scanning the slices of three wheat varieties, we found that there was no significant difference in the starch grain size of ZM22 between the waterlogging and control treatments, but the number of amyloplasts under the waterlogging treatment was significantly lower than that of the control treatment (Fig. 2B). In BN607, the length and width of amyloplasts under the waterlogging treatment were lower than those of the control treatment, and the number of amyloplasts under the waterlogging treatment was significantly lower than that of the control treatment (Fig. 2C). In BN207, there was no significant difference in starch size between the waterlogging and control treatments. Surprisingly, the number of amyloplasts in ZM22 under the waterlogging treatment was significantly higher than that in the control treatment (Fig. 2C). We concluded that amyloplasts in ZM22, BN207 and BN607 might have varying degrees of responses to waterlogging stress.

Transcriptomic analysis of 3 wheat varieties under the waterlogging and control treatments

To study the response mechanism of wheat seeds to waterlogging stress during germination, samples were taken from BN607, BN207 and ZM22 under the waterlogging and control treatments. Three treatments were repeated for each variety, and a total of 18 libraries were constructed. After we performed transcriptome sequencing using Illumina NovaSeq™ 6000. In this study, the wheat genome was used as the reference genome with an average of 6.40 million reads per sample. By removing low mass, joints, and potentially contaminated parts, each sample obtained 7.41–10.38 GB of data, the GC value accounted for 53.00%-54.00%, and the Q30 range was 97.62%-98.44% (Table S1). The ratio of exons to reference genomic libraries averaged 92.35%, introns averaged 2.67%, and intergene spacers averaged 4.98% (Table S2).

Through principal component analysis (PCA) of all genes in 18 libraries (Fig. S1), we found that principal component 1 (PCA1) explained 79.09% and principal component 2 (PCA2) explained 11.85%. The three biological replications of each treatment were largely clustered together along with the waterlogging and normally cultured wheat varieties. These results showed that the biological replications of the samples were good and that the sequencing results were reliable.

Analysis of differentially expressed genes (DEGs) in response to waterlogging stress in three wheat varieties

A total of 107891 genes were obtained from transcriptome sequencing data, among which ZM22, BN207 and BN607 had 479, 637 and 1800 DEGs under waterlogging stress, respectively (Fig. 3A). Through the gene ontology-enrichment analysis of these DEGs (Fig. S2, Table S3), the results showed that they were divided into ‘biological processes’, ‘cellular components’ and ‘molecular functions’, among which oxidation-reduction process, extracellular regions and oxidoreductase activity were the most abundant. The DEGs of three wheat varieties were compared and analysed, and the results showed that the gene ontology enrichment of ZM22 was similar to that of BN607; however, the

"molecular function" of BN607 is mainly concentrated in "transcription factor activity." The "abscisic acid response" of "biological process", the "cellular solute" of "cellular component" and "molecular function" of "molecular function" were enriched. KEGG enrichment analysis showed that 13 DEGs in ZM22 were significantly enriched in the pathways of "amino sugar and nucleotide sugar metabolism" (Fig. S3, Table S4) followed by the "glyceride metabolism" and "base sugar and nucleotide sugar metabolism" pathways. In BN607, 40 genes were significantly enriched in the "phenylpropanoid biosynthesis" and "starch and sucrose metabolism" pathways under waterlogging stress followed by the "amino sugar and nucleotide sugar metabolism" pathway.

A total of 2775 genes were significantly expressed in three wheat varieties under waterlogging stress. In ZM22, 378 genes were significantly downregulated, and 103 genes were significantly upregulated (Fig. 3A). In BN207, 302 genes were significantly downregulated and 335 genes were significantly upregulated (Fig. 3A). In BN607, there were 1116 genes with significantly downregulated expression and 684 genes with significantly upregulated expression (Fig. 3A). The Venn diagram of 2775 DEGs showed that the expression levels of 4 genes (TraesCSU02G032900, MSTRG.18850, TraesCS6D02G038500 and TraesCS1A02G139900) were downregulated in three varieties under waterlogging stress, and the expression level of TraesCS5B02G445500 was upregulated under waterlogging stress (Fig. 3B, Table S5), suggesting that these genes might play an important role in three wheat varieties under waterlogging stress.

To validate the reliability of the expression profiles obtained from RNA-Seq, we randomly selected 10 DEGs with various degrees of expression for qRT-PCR. Pearson's correlation coefficients showed that the qRT-PCR data for these genes and the RNA sequencing results were highly correlated (Fig. 3C). The correlation coefficient was 0.8936, indicating a positive correlation between the RNA sequencing data and qRT-PCR data (Fig. 3C).

Selection of gene set response to waterlogging stress by hierarchical clustering based on DEGs

According to the expression differences of DEGs in three waterlogging-resistant varieties (BN607 > BN207 > ZM22), DEGs were stratified and clustered (Fig. 4A). The results showed that these DEGs were divided into eight clusters, Cluster 1 to Cluster 8, with different expression patterns (Fig. 4B, Table S7). Among these clusters, cluster 7 had 563 DEGs, whose expression in three varieties was positively correlated with germination rate (R^2 = 1.00), while cluster 2 contained 398 DEGs, whose expression in three varieties was negatively correlated with germination rate (R^2=-0.97) (Fig. 4C). The results of the above analysis suggestted that DEGs in cluster 2 and cluster 7 played important roles in the waterlogging stress response in the three wheat cultivars.

Main metabolic pathways involved in wheat germination under waterlogging stress

Based on the hierarchical clustering analysis of DEGs, we focused on analysing the DEGs involved in waterlogging stress in clusters 2 and 7. Through GO enrichment and KEGG enrichment, it was found that these genes were mainly involved in the glycolysis pathway, the starch and sucrose metabolism pathway and the lactose metabolism pathway.

Analysis of differentially expressed genes (DEGs) related to the glycolytic pathway

Through KEGG enrichment, it was found that there were many DEGs involved in the glycolysis pathway in three kinds of wheat under the waterlogging and control treatments (Fig. 5A). In clusters 2 and 7, we screened four DEGs (2 alcohol dehydrogenase genes, 1 L-lactate dehydrogenase gene, and 1 glycosyltransferase gene) that were closely related to the germination rate (Fig. 5B). The expression levels of two genes (TraesCS5D02G196300 and TraesCS4B02G106400) encoding alcohol dehydrogenase (EC:1.1.1.1) decreased successively in ZM22, BN207 and BN607 (Fig. 5B). In addition, the expression trend of the L-lactate dehydrogenase gene (TraesCS2B02G341200), which catalysed the conversion of pyruvate to L-lactate (EC:1.1.1.27), was opposite to that of the alcohol dehydrogenase gene in the three wheat varieties. Glycosyltransferase was used to catalyse the conversion of α-D-glucose into β-D-glucose (EC:5.1.3.3). The expression of the gene (TraesCS2B02G341200) encoding this function decreased successively in ZM22, BN207 and BN607.

Analysis of differentially expressed genes (DEGs) related to starch and sucrose metabolism pathways

KEGG enrichment revealed that under the waterlogging and control treatments, a large number of DEGs were involved in starch and sucrose metabolism pathways in three wheat varieties (Fig. 6A), among which 18 DEGs were screened for starch and sucrose metabolism pathways in cluster 2 and cluster 7 (Fig. 6B). A gene (TraesCS5B02G015800) from cluster 7 was annotated as alpha-amylase, and its expression abundance increased successively in ZM22, BN207 and BN607, indicating that this gene might play an important role in starch decomposition. We screened the gene (TraesCS7B02G093800) encoding maltitol glycosylase (EC:2.4.1.21), whose expression level was successively increased in ZM22, BN207 and BN607. At the same time, the expression abundance of TraesCS5D02G09100 encoding the conversion of starch/glycogen to dextrin was decreased successively in ZM22, BN207 and BN607. This result indicated that the ability of ADP-glucose to control amylose synthesis was gradually enhanced. Chitinase was used to catalyse the decomposition of β-D-glucose or fibre to D-glucose (EC:3.2.1.21). The expression of six genes encoding this function decreased gradually in ZM22, BN207 and BN607, but three

genes increased gradually (Fig. 6B). In this study, the expression levels of genes encoding glucan-1,3-glucosidase (EC: 3.2.1.39) and endocellulase (EC: 3.2.1.4) decreased gradually in ZM22, BN207 and BN607 (Fig. 6B), indicating that these genes may play an important role in the waterlogging tolerance of different varieties.

Analysis of differentially expressed genes (DEGs) related to the lactose metabolic pathway

Through the comprehensive analysis of the DEGs in the lactose metabolism pathway in cluster 2 and cluster 7 of three wheat varieties (Fig. 7A), it was found that there were two genes (TraesCS1A02G266600 and TraesCS1D02G266700) encoding glucuronosyltransferase (inositol galactoside synthase) (EC:2.4.1.123). This protein catalyse the synthesis of inositol galactoside from UDP- galactose and myoinositol, which is the first key step in the synthesis of raffinose family oligosaccharides (RFOs) and the most critical regulatory step in RFO synthesis. The expression levels of these two genes were decreased successively in ZM22, BN207 and BN607. There were three genes encoding inositol galactoside-sucrose galactosyltransferase (raffinose synthase) (EC:2.4.1.82). This protein mainly catalysed inositol galactoside and sucrose to synthesize raffinose. The expression level of the TraesCS7D02G236700 gene was gradually decreased in ZM22, BN207 and BN607, while that of the TraesCS7B02G035200 and TraesCS7D02G133500 genes was gradually increased in ZM22, BN207 and BN607. There was one gene (TraesCS5B02G557400) encoding β-D-fructofuran glycoside hydrolase (EC:3.2.1.26), which mainly catalysed the decomposition of stachyose and raffinose into melibiose and the decomposition of sucrose into glucose and fructose. The expression level of TraesCS5B02G557400 was gradually increased in ZM22, BN207 and BN607. β-galactosidase (EC:3.2.1.23) mainly catalysed the decomposition of lactose and raffinose into melibiose. We found that the expression levels of two genes (TraesCS7A02G0363600 and TraesCS1B02G278400) in ZM22, BN207 and BN607 gradually increased.

Analysis of differentially expressed genes (DEGs) related to transcription factor analysis

By analysing the DEGs in cluster 2 and cluster 7, it was found that 23 transcription factors were involved in waterlogging stress (Table 1). These transcription factors were distributed in 12 families, including WRKY, MYB, MADS, ZIP, and ERF (Table 1). Among the WRKY family, the expression levels of five genes decreased gradually in ZM22, BN207 and BN607, while the expression levels of one gene increased gradually in ZM22, BN207 and BN607. A total of four MYB family genes were found; the expression level of one gene gradually decreased in ZM22, BN207 and BN607, and the other three genes increased gradually. In addition, the expression levels of MSTRG.6000, TraesCS1B02G392300 and MSTRG.32294 were gradually enhanced in ZM22, BN207 and BN607, which were all ethylene-related transcription factors (ERF, AP2/ERF, ABR) belonging to cluster 7. This finding indicated that these transcription factors might play an important role in the response to waterlogging stress in different wheat varieties.

Table 1

Response of transcription factors to waterlogging stress

Category	Gene_id	Cluster	Log_2(Fold change)			Swissprot_name	Swissprot_description
			W72_ZM22 vs C72_ZM22	W72_BN207 vs C72_BN207	W72_BN607 vs C72_BN607		
WRKY	TraesCS1A02G197400	C2	-0.13	-0.38	-1.55	WRKY35	WRKY transcription factor 35
	MSTRG.12348	C2	-0.4	-0.57	-1.83	WRKY24	WRKY transcription factor WRKY24
	MSTRG.27655	C2	-0.99	-1.07	-2.16	WRKY71	WRKY transcription factor WRKY71
	MSTRG.29100	C2	-0.98	-1.4	-2.14	WRKY71	WRKY transcription factor WRKY71
	TraesCS2B02G410400	C2	-0.13	-0.61	-1.51	WRKY12	WRKY transcription factor 12
	TraesCS3D02G113300	C7	-2.2	-0.49	0.15	WRKY72	WRKY transcription factor 72
MYB	MSTRG.3642	C2	0.04	-0.65	-1.68	MYB86	Transcription factor MYB86
	MSTRG.36482	C7	-3.69	-1.34	-0.03	MYB44	Transcription factor MYB44
	MSTRG.5463	C7	-1.46	-0.46	0.19	MYB306	Myb-related protein 306
	MSTRG.5321	C7	0.7	0.78	1.03	EFM	Myb family transcription factor EFM
ERF	MSTRG.6000	C7	-0.06	0.32	1.12	ERF114	Ethylene-responsive transcription factor ERF114
	TraesCS1B02G392300	C7	-0.41	0.73	1.95	AP2/ERF	AP2/ERF and B3 domain-containing protein
	MSTRG.32294	C7	-0.2	0.39	1.08	ABR1	Ethylene-responsive transcription factor ABR1
MADS-box	MSTRG.23752	C2	0.17	0.06	-2.07	MADS13	MADS-box transcription factor 13
	MSTRG.21330	C7	0.07	0.96	1.16	MADS51	MADS-box transcription factor 51
ZIP	MSTRG.6529	C2	0.14	-0.34	-1.26	ZIP3	Zinc transporter 3
	MSTRG.2818	C2	-0.14	-0.34	-1.36	ZIP5	Zinc transporter 5
BHLH	MSTRG.10317	C7	-1.1	-0.56	1.62	BHLH113	Transcription factor bHLH113
	TraesCS5D02G411600	C7	-2.61	0.24	1.16	BHLH84	Transcription factor bHLH84

NOTE: C72_ZM22, ZM22 for 72 hours after germination under the control treatment; W72_ZM22: ZM22 for 72 hours after germination under the waterlogging treatment; C72_BN207, BN207 for 72 hours after germination under the control treatment; W72_BN207, BN207 for 72 hours after germination under the waterlogging treatment; C72_BN607, BN607 for 72 hours after germination under the control treatment; W72_BN607, BN607 for 72 hours after germination under the waterlogging treatment.

Supplementary information

Category	Gene_id	Cluster	Log_2(Fold change)			Swissprot_name	Swissprot_description
			W72_ZM22 vs C72_ZM22	W72_BN207 vs C72_BN207	W72_BN607 vs C72_BN607		
IAA	TraesCS7A02G322000	C2	-0.74	-0.99	-1.55	IAA25	Auxin-responsive protein IAA25
ARF	MSTRG.13431	C2	-0.1	-0.64	-1.34	ARF1	Auxin response factor 1
HSFA	MSTRG.9725	C7	-1.72	-0.81	0.53	HSFA3	Heat stress transcription factor A-3
NAC	MSTRG.5265	C7	0.24	0.69	1.24	NAM-B1	NAC transcription factor NAM-B1
NOTE: C72_ZM22, ZM22 for 72 hours after germination under the control treatment; W72_ZM22: ZM22 for 72 hours after germination under the waterlogging treatment; C72_BN207, BN207 for 72 hours after germination under the control treatment; W72_BN207, BN207 for 72 hours after germination under the waterlogging treatment; C72_BN607, BN607 for 72 hours after germination under the control treatment; W72_BN607, BN607 for 72 hours after germination under the waterlogging treatment.							
Supplementary information							

Discussion

Response of germination rate to waterlogging stress in different wheat varieties

In the environment of field irrigation, plants will suffer from waterlogging due to sudden heavy rain. Waterlogging often occurs in all stages of plant growth; therefore, the evaluation of plant waterlogging tolerance at each stage is highly important. Studies of physiological and molecular mechanisms on the occurrence of waterlogging in wheat at the seedling stage [15, 9], filling stage [18] and maturity stage [19] have been reported. Some studies found that wheat, barley and rape were much more likely to suffer from waterlogging between 2 and 6 weeks after germination than between 6 and 14 weeks after germination. In addition, Cannell et al. [20] found that after wheat germination, all seedlings died after 16 days of waterlogging at 12 °C, while the survival rate of wheat seedlings after 6 days of waterlogging was 12-38% compared to the control treatment. There were also considerable differences in waterlogging tolerance among different varieties in different regions. For example, Takeda and Fukuyama [21] found that some varieties from China, Japan, South Korea and Nepal, as well as some varieties from North Africa, Ethiopia and southwestern Asia, tended to show higher "waterlogging tolerance", while barley varieties from western India showed weaker "waterlogging tolerance" [22]. The plant height, SPAD, number of tillers, and biomass of stems and roots of non-waterlogging wheat varieties were lower than those of waterlogging-tolerant wheat varieties [23]. In this study, it was found that the germination rates of Zhoumai 22, Bainong 207 and Bainong 607 increased gradually after waterlogging treatment. The coleoptile height and root length of Zhoumai 22 were seriously inhibited after 3 days of waterlogging treatment; however, the growth of Bainong 607 was not affected by waterlogging treatment (Fig. 1 and Fig. 3), indicating that there were notable differences in "flood tolerance" among different varieties.

Anatomy of grain endosperm in wheat response to waterlogging stress

Endosperm is the main place of nutrients stored in cereal seeds and mainly contains starch. Starch degraded by amylase is transported to the germ and radicle in the form of simple compounds (such as sucrose), provides a matrix for respiration and provides a material basis and energy source for radicle, germ growth and organ construction [17, 24, 25]. At the initial stage of wheat seed germination, the cell wall of endosperm cells was dissolved, and the endosperm cells were separated. Then, the starch and cellulose gradually decomposed under a series of enzymes, which loosened the whole endosperm structure and led to endosperm liquefaction. Therefore, the activity of amylase in seed germination might be determined by the degree of endosperm emulsification and degradation. In this study, it was found that the endosperm cells of three kinds of wheat seeds changed significantly under waterlogging stress, in which the degree of emulsification between embryo and endosperm in BN207 and BN607 was higher than that of ZM22, and the degree of emulsification of BN607 was the highest (Fig. 2B, 2C). The difference in the number and size of amyloplasts among ZM22, BN2607 and BN607 may be due to the inconsistency of the degradation rate. For example, compared with the control treatment, the size of amyloplast in ZM22 remained unchanged, but the number of amyloplasts decreased under the waterlogging treatment; for BN207, the size of amyloplasts decreased with no significant difference between the control

and waterlogging treatments; the number of amyloplasts increased, and starch degradation was completed quickly, which provided nutrients for embryo growth.

Alcohol dehydrogenase (ADH) gene plays an important role in the response to waterlogging tolerance in wheat

Seed germination requires an energy supply, and energy metabolism is activated only when the seed is imbibed. It was found that some proteins related to energy metabolism were phosphorylated in wheat embryos. For example, the upregulation of the expression of the alcohol dehydrogenase gene (ADH) under waterlogging stress was a positive signal. Plant alcohol fermentation was activated under low oxygen stress conditions: pyruvate decarboxylase (PDC) first converted pyruvate to acetaldehyde, and then alcohol dehydrogenase (EC 1.1.1.1) converted acetaldehyde to ethanol. It had been reported that in the early stage of soybean growth and development, the expression of *ADH* and *PDC* in soybean roots was significantly upregulated under waterlogging stress [26-28]. It had also been found that hypoxia stress could lead to the production of fermentation alcohol products [PDC, lactate dehydrogenase (LDH), ADH] in *Arabidopsis thaliana* [29]. Komatsu et al. [30] reported that there were at least six *ADH* genes in soybean in response to waterlogging stress, of which one *GmADH2* gene was specifically expressed in soybean root tissue. Similarly, two *ADH* genes were cloned in cotton, only one of which was expressed under waterlogging stress [31]. Zhang et al. [32] found that the 2/3 *ADH* genes in kiwifruit were significantly upregulated in the roots after waterlogging treatment. Tougou et al. [28] found that the inhibitory effect of waterlogging stress on the growth of soybean seedlings was decreased in overexpressing *GmADH2* transgenic soybeans, while the expression and activity of ADH in transgenic soybeans were higher than those of the control, suggesting that *GmADH2* transgenic soybean waterlogging stress might induce changes in glycolysis and alcohol fermentation and improve the germination rate of transgenic soybeans. The transcriptional levels of metabolites in the embryo and endosperm of rice seeds were higher than those in other periods at 12 h after anaerobic treatment, while the change in a large number of metabolites was highest at 48 h [33]. This finding showed that the enzyme activity-catalysed energy metabolism was induced or activated at the beginning of germination, and the late changes of metabolites might be driven by transcription and translation because they occurred after the change of transcriptional abundance. In this study, we found that compared with the control treatment, the transcriptional levels of *ADH2* (*TraesCS5D02G196300*) and *ADH3* (*TraesCS4B02G106400*) (Fig. 7) decreased successively in ZM22, BN207 and BN607 after 3 days of the waterlogging treatment, while the transcriptional levels of these genes increased gradually after 1 day of the waterlogging treatment (not published). This finding suggested that the *ADH* genes in the endosperm of BN607 were induced quickly after short waterlogging, and the energy was provided by glycolysis to cause the seeds of BN607 to germinate early. However, the *ADH* genes of ZM22 were induced to be highly expressed after 3 days of the waterlogging treatment. It is possible that the accumulation of toxic substances, such as acetaldehyde, in the early stage inhibited the germination of seeds.

Changes in sugar metabolism in wheat endosperm and endosperm

Anaerobic respiration is the main form of ATP production in plants under hypoxia or waterlogging conditions to adjust the energy crisis caused by the external hypoxic environment. The pathway of energy metabolism is not only related to alcohol dehydrogenase of glycolysis but also related to starch and sucrose metabolism and the lactose metabolism pathway [34, 35]. We found that the expression levels of genes related to glycolysis, starch and sucrose metabolism and lactose metabolism in the embryo and endosperm of BN607 seeds were lower than those of ZM22 after 3 days of the waterlogging treatment. Among these genes, the expression levels of genes related to hydrolytic enzymes, such as chitinase, glucan-1,3-glucosidase and endocellulase, decreased successively in ZM22, BN 207 and BN607. The change trend of these genes was largely consistent with that of *ADH* gene expression. The anatomical structure of the three wheat species and the expression levels of genes indicated that the process of emulsification and dissolution of endosperm in BN607 began early after waterlogging treatment, while the process of emulsification and dissolution of embryo and endosperm of ZM22 seeds occurred relatively late. The transcriptional expression of mRNA of ZM22 seeds was inhibited by waterlogging stress, and it was relatively late to use starch and sugar hydrolysis to provide energy and complete the growth of coleoptile.

Coleoptile is the protective tissue of young cotyledons of crops. The length and elongation rate of coleoptile affect the early growth of crops. Coleoptile growth is the extension growth of coleoptile cells, and the power of extension growth is from continuous swelling and pressure. The extended growth of cells is limited by the cell wall; therefore, relaxation of the cell wall is necessary in the process of coleoptile growth [36]. The relaxation of the cell wall is related to the degradation of hemicellulose, in which a single hydrolase, such as β-glucanase, expansin, α-amylase and β-galactosidase, can induce cell wall relaxation. The pectin polysaccharides in the cell wall are hydrolysed by β-galactosidase, which is positively correlated with cell relaxation [37]. This study also found that the coleoptile height of ZM22, BN207 and BN607 increased successively after 3 days of waterlogging, while the expression levels of the two genes encoding β-galactosidase increased successively (Fig. 7B). At the same time, we found that the expression level of a gene encoding α-amylase in cluster 7 also increased successively in ZM22,

BN207 and BN607. This finding showed that the elongation of wheat coleoptile under waterlogging stress might be closely related to the changes in cell wall composition and the synthesis and activity of induced enzymes related to cell wall relaxation.

Role of transcription factors in the response to waterlogging stress

Previous studies reported that WRKY-type genes played an important role in the response to drought, high salinity, cold injury, and heat damage, such as external abiotic stress [38]. At the same time, there were also some reports about WRKY-type genes in response to waterlogging stress. Nanjo et al. [27] found that the expression levels of WRKY-type transcription factors in soybean roots and hypocotyls were significantly upregulated under the waterlogging treatment. The expression levels of 11 WRKY transcription factor genes in the roots of kiwi fruit seedlings were significantly upregulated under the waterlogging treatment [32]. In this study, it was found that the expression levels of six WRKY transcription factors decreased successively in ZM22, BN207 and BN607 under the waterlogging treatment (Table 1), indicating that the seed germination of ZM22, BN207 and BN607 under the waterlogging treatment was negatively regulated by WRKY transcription factors. AP2/ERF played an important role in the response to external biotic and abiotic stresses [39-41]. SUB1A, an ERF transcription factor, could inhibit ethylene synthesis and the response to gibberellin in rice under waterlogging stress, reduce carbohydrate consumption and improve waterlogging tolerance [42, 43]. Lee et al. [44] found that *CIPK15* expression was induced by hypoxia and a lack of glucose during rice germination under water flooding, which caused a series of potential chain reactions downstream by activating the energy and stress receptive factor SnRK1. This effect could enhance the expression of amylolytic enzyme genes and the synthesis of alcohol dehydrogenase, promoting anaerobic respiration to ensure the metabolic energy needed for cell growth. At the same time, the coleoptile could also be rapidly elongated. In this study, the expression of one AP2/ERF transcription factor (*TraesCS1B02G392300*) increased successively in ZM22, BN207 and BN607 (Table 1), indicating that the expression level of the AP2/ERF transcription factor could be used as an important reference index to evaluate the difference in waterlogging tolerance among three wheat varieties. Further research on the regulatory mechanism of AP2/ERF transcription factors is needed in the future.

Materials And Methods

Experimental materials and treatment methods

In this experiment, three wheat (*Triticum aestivum* L.) varieties, 'Zhoumai 22' (ZM22), 'Bainong 207' (BN207) and 'Bainong 607' (BN607), were selected as materials. They were provided by the School of Life Science and Technology, Henan Institute of Science and Technology. Intact seeds with uniform sizes and no pests and diseases were selected for the following treatment: the seeds were first disinfected with 5% hydrogen peroxide for 5 min, then washed with steam water 3-5 times, and finally placed into distilled water for 24 h to allow them to absorb water sufficiently.

Experimental treatment: Fifty plump seeds were placed in a beaker with a water depth of 10 cm for waterlogging treatment. After 72 h of waterlogging, the seeds were removed and germinated in a Petri dish (to ensure that the seeds were in contact with water but not submerged by water). Control treatment: Germination was conducted on the seeds that were suctioned for 24 h. The experimental and control treatments were given 5 repetitions. All seeds were grown in a growth chamber at 25 ℃ and 75% relative humidity (16 h light / 8 h dark cycle). Finally, the germination rate of seeds after 72 h of germination was calculated. In addition, we removed the root and coleoptile of the seed 72 h after germination and used part of the remaining samples for seed anatomy and the other part for transcriptomic sequencing.

Mensuration of germination rate and coleoptile length of wheat seeds

After 72 h of germination, the germination rates (GR) and the coleoptile height (CH) of seeds in the waterlogging and control treatments were measured.

Structural anatomy of wheat seeds

For the anatomy of wheat seeds, we first fixed the seeds in FAA (38% formaldehyde: glacial acetic acid: 70% alcohol = 1:1:18 volume ratio) solution. Then, the fixed seeds were sliced and embedded in paraffin, and the slicing was processed as follows: first, the sections were sequentially placed in xylene I for 20 min, xylene II for 20 min, absolute ethanol I for 5 min, absolute ethanol II for 5 min, 75% alcohol for 5

min, and tap water for 5 min to deparaffinize the paraffin sections until dehydration. Second, the sections were sequentially stained with periodic acid, Schiff's reagent, and naphthol yellow S (Wuhan Servicebio Technology Co. Ltd.). After that step, the sections were dehydrated and mounted with anhydrous ethanol, xylene and neutral gum. Finally, the sections processed above were examined by image acquisition and analysis.

Total RNA isolation, RNA library construction and transcriptome sequencing

To explore the response of wheat seeds to waterlogging stress, transcriptome analysis was performed on the seeds of three wheat varieties, 'ZM22', 'BN207' and 'BN607', under the waterlogging and control treatments. A total of 18 samples (C72_ZM22, C72_BN207, C72_BN607, W72_ZM22, W72_BN207, W72_BN607, each treatment in triplicate) were first obtained by extracting total RNA from samples using the TRIzol kit (Invitrogen, CA, USA). To avoid contamination of genomic DNA, total RNA was removed with RNase-FreeDNaseI (Takara, Tokyo, Japan). To ensure that transcriptome sequencing was performed using high-quality samples, a NanoDrop, a Qubit 2.0, and an Agilent 2100 were used to determine the purity, concentration, and integrity of RNA samples, respectively. First, the mRNA was enriched with oligo (dT) magnetic beads, and fragmentation buffer was added to randomly disrupt the mRNA. The first cDNA strand was synthesized using mRNA as a template and a six-base random primer (random hexamers). To synthesize the second cDNA strand, the first cDNA strand was combined with buffer, dNTPs, RNase H, and DNA polymerase I. The purified double-stranded cDNA was subjected to end repair, A tailed and ligated to the sequencing adapter. Finally, fragment size selection was performed using AMPure XP beads, and a cDNA library was enriched by PCR. After the library was constructed, the concentration of the library and the insert size were detected using a Qubit 2.0 and an Agilent 2100, respectively. The average insert size for the paired-end libraries was 150 bp (±50 bp). Transcriptome sequencing was performed using an Illumina NovaSeq™ 6000 (LC Sciences, San Diego, CA, USA) after library quality inspection.

Normalization and annotation of sequencing data

To ensure accurate and credible analysis results, we preprocessed the raw data, including the removal of sequencing adapters and low-quality sequencing data. The valid data were aligned to the reference genome of the wheat species (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v1.0/), while statistics were performed based on gene location information specified by genome annotation files (GTF and GFF).

Sequencing sequence statistics and quality control

Raw data generated by sequencing needs to be preprocessed. We used cutadapt to filter out unqualified sequences and obtain clean data [45]. The specific processing steps were as follows: (1) removing reads with adapter; (2) removing reads with the content ratio of N (N represents a base information cannot be determined) is greater than 5% of the reads; (3) removing low mass reads (mass value of $Q \leq 10$ base accounting for more than 20% of the entire reads); and (4) analysing the amount of raw sequencing, an effective amount of sequencing, Q20, Q30, and GC content.

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

The reads aligned for each sample were assembled using StringTie. The transcriptomes of all samples were then reused to construct a comprehensive transcript with a Perl script. After the final generation of transcripts, StringTie and DegeR were used to estimate the expression levels of all transcripts. The expression levels of genes were characterized by calculating FPKM values (Fragments Per Kilobase of exon model per Million mapped reads)[46], which eliminated the influences of gene length and sequencing level during the calculation of gene expression. Screening of differentially expressed genes (DEGs) was conducted at the waterlogging treatment compared to the control treatment in 'ZM22', 'BN207' and 'BN607', respectively. The differentially expressed mRNAs were selected with log2 (fold change) > 1 or log2 (fold change) < − 1 and with statistical significance (p-value < 0.05) by R package Ballgown [47]. The fold change represents the ratio of expression between two samples under the waterlogging and control treatments. The expression level dominance analysis was performed by using EdgeR software [48]. Cluster analysis was performed on DEGs using software cluster and Java Treeview. Gene functional annotations were based on the pear genome database and mapped onto gene ontology (GO) terms. GO enrichment analysis was carried out using WEGO. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified according to p-values and adjusted q values using a BLAST search against the KEGG database and were then mapped onto KEGG pathways [49-54].

Total RNA extraction, reverse transcription and qRT-PCR assays

Based on the target gene sequences, 10 gene-specific primer pairs were designed (Table S1). The RNA samples used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis were aliquots of the samples used in the RNA-Seq experiments. qRT-PCR assays were performed using the Primer Script RT Reagent Kit (Takara, Dalian, China) and the reference gene 18S (Gene ID: AJ272181.1) using cDNA from transcriptome samples as templates. The total reaction volume for each qRT-PCR was 20 μL, which comprised 10 μL SYBR Green PCR SuperMix (Vazyme Biotech Co., Ltd. Nanjing, China), 0.4 μL of each primer, 2 μL of cDNA, 0.4 μL passive reference dye and 6.8 μL double-distilled water. The PCR conditions were as follows: 95 ℃ for 10 s and 40 cycles of 95°C for 5 s followed by 60 ℃ for 30 s. qRT-PCR reactions were performed using an ABI Step One Plus. The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method [55].

Statistical analysis

All statistical analyses were performed with SPSS 18.0 (SPSS Inc., Chicago, IL, USA) and Microsoft excel (Microsoft Corporation, Redmond, WA, USA). Data were analysed by one-way analysis of variance (ANOVA). Mean separations were performed by Duncan's multiple range tests. OriginPro 8.1 (Origin Inc., Chicago, IL, USA) was used to draw the figures. Differences at $p < 0.05$ were considered significant.

Abbreviations

ZM22
Zhoumai 22; BN207:Bainong 207; BN607:Bainong 607; DEGs:Differentially Expressed Genes; ADH:Alcohol Dehydrogenase; GO:Gene Ontology; mRNA:Messenger RNA; KEGG:Kyoto Encyclopedia of Genes and Genomes; PCA:Principal Component Analysis; qRT-PCR:Quantitative real-time PCR; FPKM:Fragments Per Kilobase Per Million Mapped Reads; NCBI:National Center for Biotechnology Information; PDC:Pyruvate Decarboxylase; LDH:Lactate Dehydrogenase; GR:Germination Rates; CH:Coleoptile Height; FAA:38% Formaldehyde- Acetic acid- 70% Alcohol

Declarations

Acknowledgement:

Not applicable.

Authors' contributions:

CS, JY, XR and XO conceived, designed and supervised the experiment; CS and JP wrote the manuscript; CS, HQ, ZW, and YL performed the experiment; CS and JY provided support in lab experiment and data analysis. CS and JY analyzed the data. All authors read and approved the manuscript.

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Availability of data and materials

All raw sequence reads have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE144554 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE144554).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

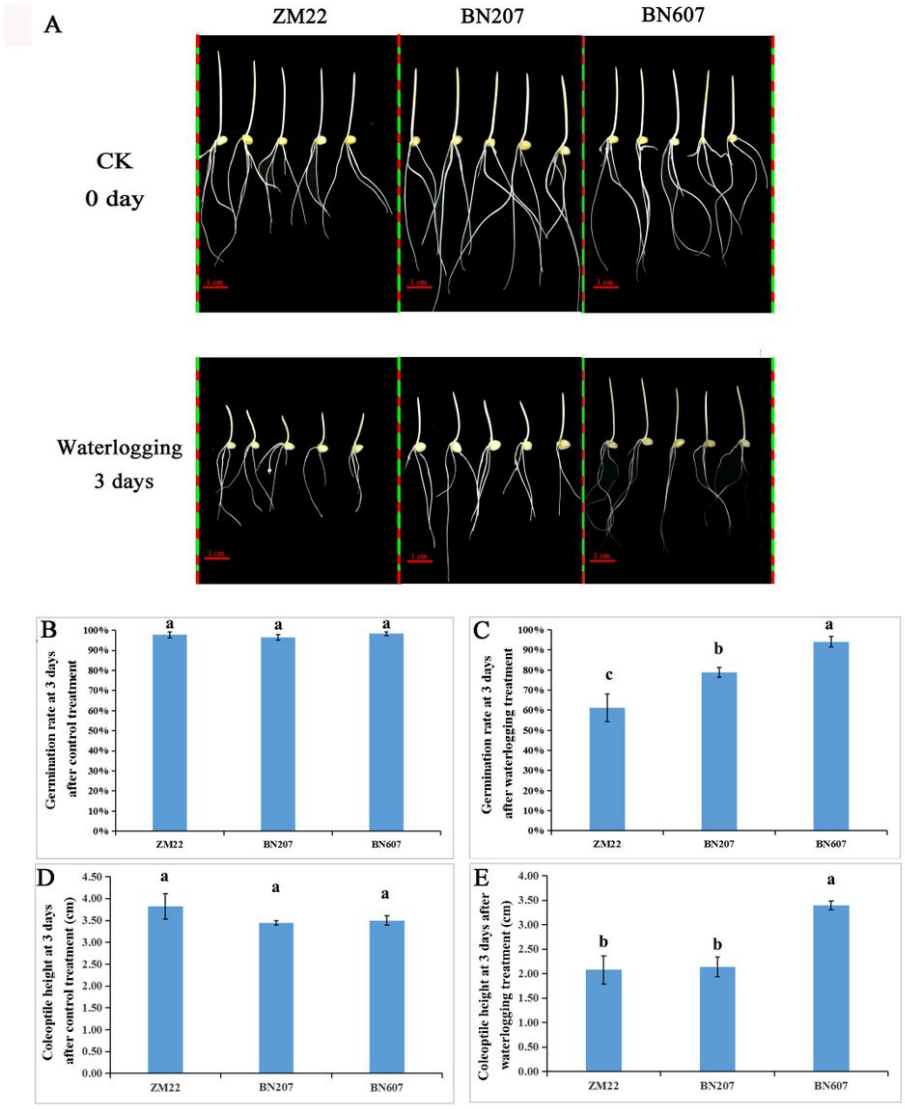

Figure 1

Phenotypic analysis of different wheat varieties after 3 days of germination under the control and waterlogging treatments. A, Phenotypes of ZM22, BN207 and BN607 seeds after 3 days of germination under the control and waterlogging treatments. Bars: 1 cm. B, Germination rate at 3 days after control treatment for ZM22, BN207 and BN607. C, Germination rate at 3 days after waterlogging treatment for ZM22, BN207 and BN607. D, Coleoptile height at 3 days after control treatment for ZM22, BN207 and BN607. E, Coleoptile height at 3 days after waterlogging treatment for ZM22, BN207 and BN607. Each point represents the average of five samples. Error bars represent SD. Statistical analysis was performed by LSD test at $p < 0.05$.

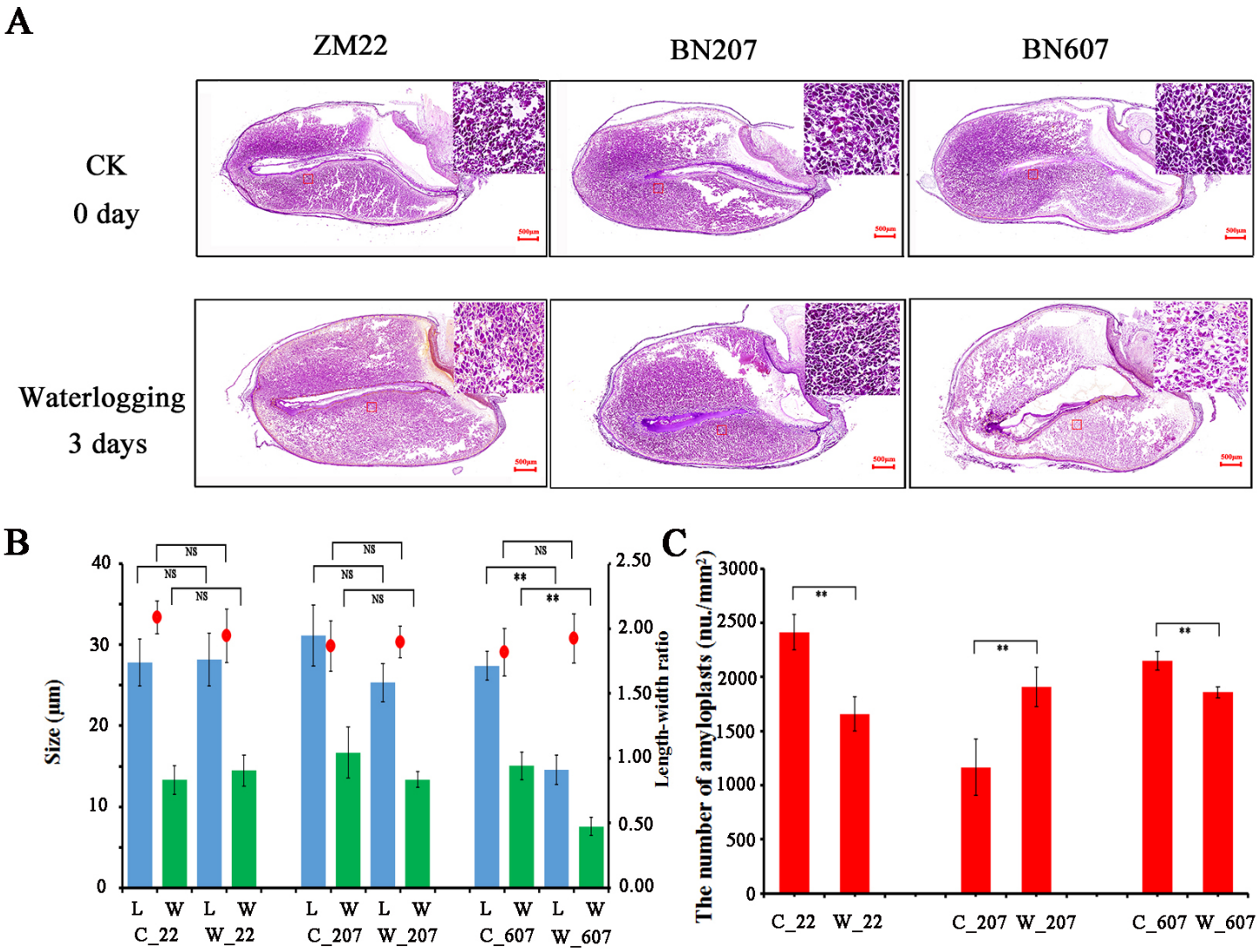

Figure 2

Structural anatomy of endosperm of three wheat varieties after 3 days of germination under the control and waterlogging treatments. A, Structural anatomy of endosperm of ZM22, BN207 and BN607 under the control and waterlogging treatments, respectively. Bars:500μm. B, Length, width and ratio of length to width of anyloplast in ZM22, BN207 and BN607. L, the length of anyloplast; W, the width of anyloplast. C, The number of anyloplast in ZM22, BN207 and BN607. ZM22: Zhoumai 22, BN207: Bainong 207, BN607: Bainong 607; C_22, ZM22 for 72 hours after germination under the control treatment; W_22: ZM22 for 72 hours after germination under the waterlogging treatment; C_207, BN207 for 72 hours after germination under the control treatment; W_207, BN207 for 72 hours after germination under the waterlogging treatment; C_607, BN607 for 72 hours after germination under the control treatment; W_607, BN607 for 72 hours after germination under the waterlogging treatment. Each point represents the average of three samples. Error bars represent SD. ∗ means the significant level at $p<0.05$, ∗∗ means the extremely significant level at $p<0.01$.

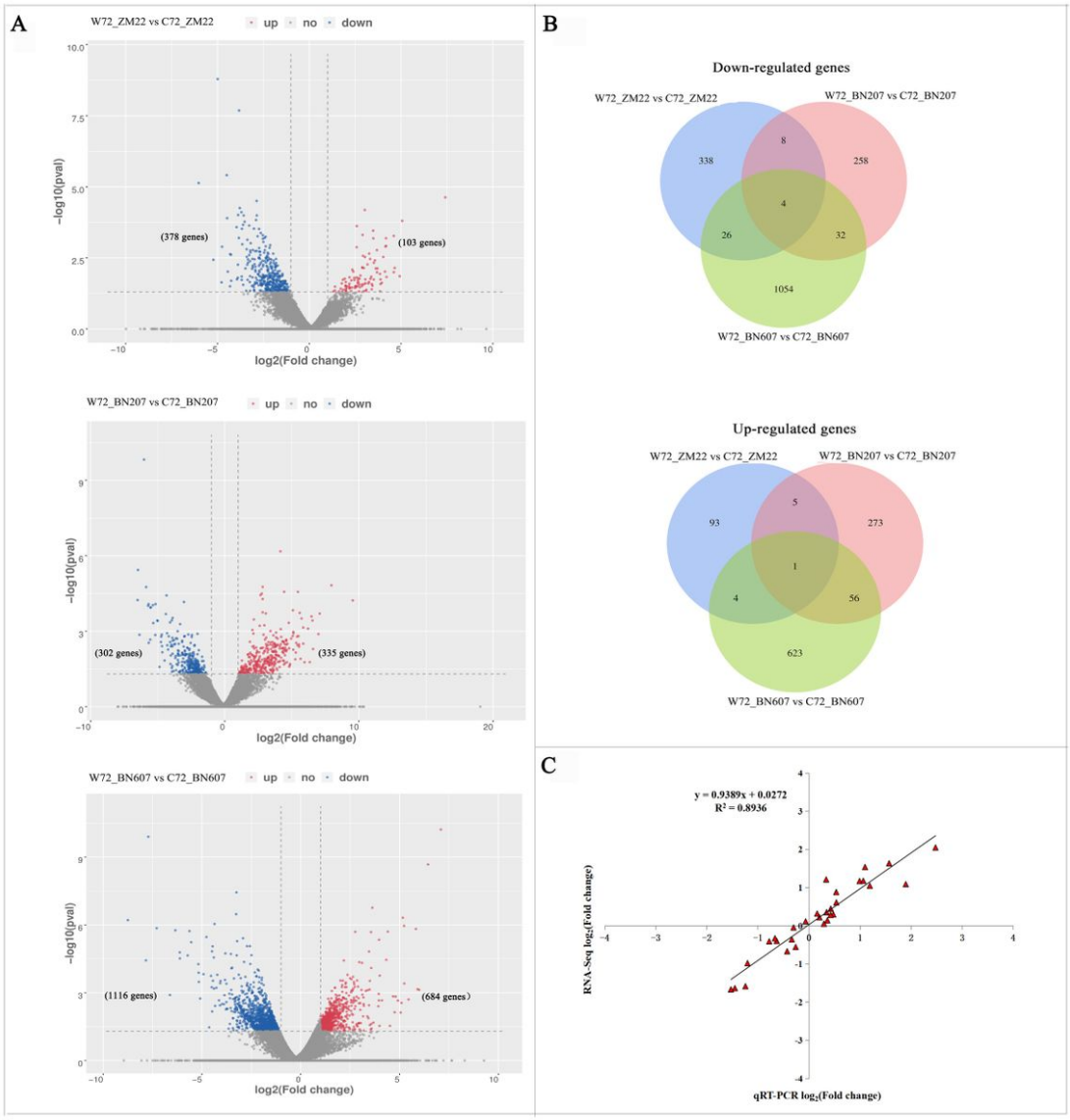

Figure 3

Analysis of differentially expressed genes (DEGs) in three wheat cultivars under waterlogging stress. A. Volcano plot analysis of DEGs in ZM22, BN206 and BN607. Take log 2(foldchange) as the abscissa and -log2(p-value) as the ordinate. Red represents up-regulated significantly DEGs, blue represents down-regulated significantly DEGs, and gray represents non-significant DEGs. B. Venn diagram of down-regulated and up-regulated genes in ZM22, BN206 and BN607. C. Correlation analysis of qRT-PCR data and RNA sequencing results. W72_ZM22, W72_BN207 and W72_BN607 were the sample names of ZM22, BN207 and BN607 under the waterlogging treatment, respectively. C72_ZM22, C72_BN207 and C72_BN607 were the sample names of ZM22, BN207 and BN607 under the control treatment, respectively.

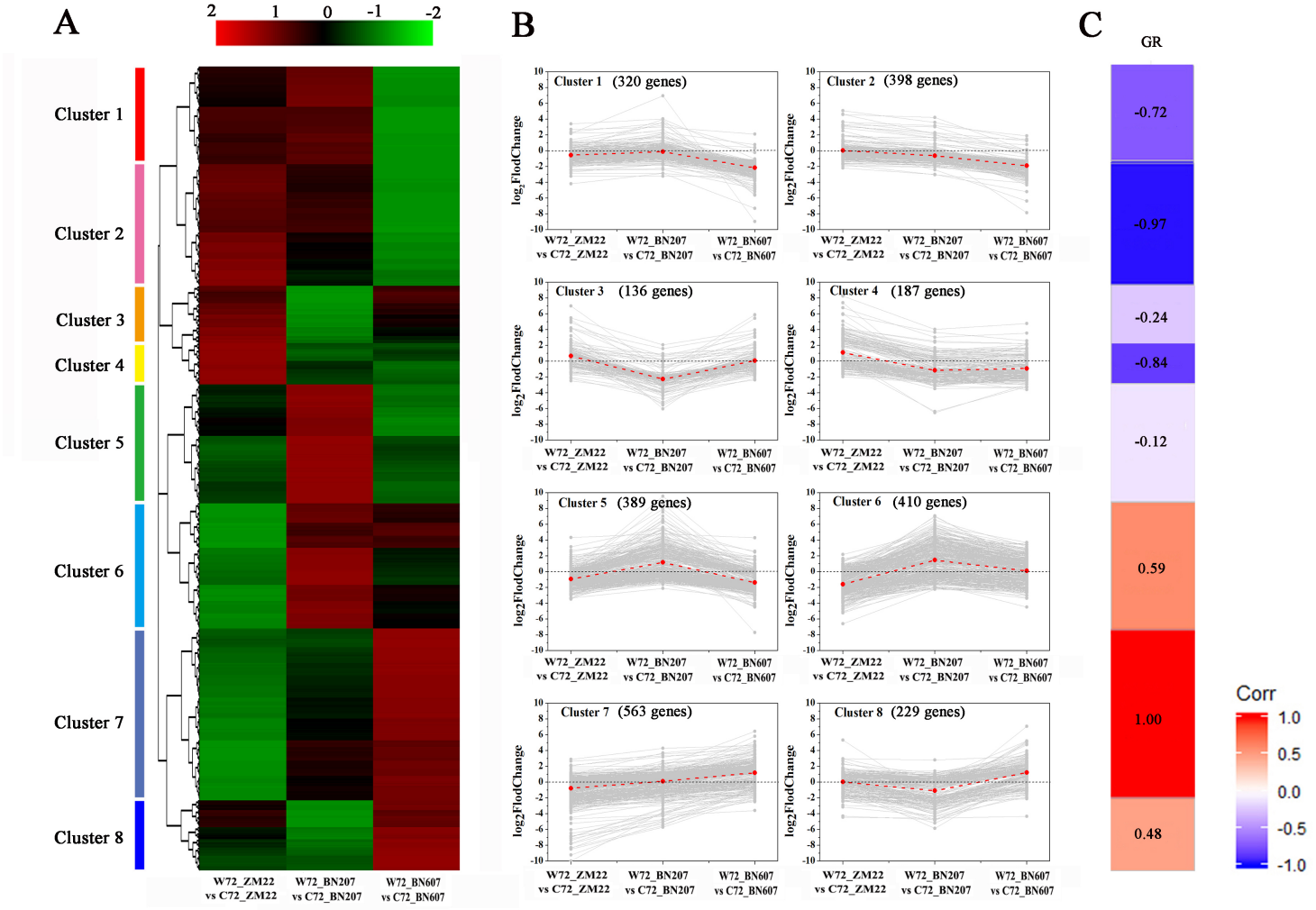

Figure 4

Cluster analysis of differentially expressed genes (DEGs) in ZM22, BN207 and BN607. A. The DEGs with the similar expression pattern were grouped into the same cluster. B. The ordinate means the relative expression of DEGs, standardized by log2flodchange, and the abscissa means ZM22, BN207 and BN607. C. Correlation analysis between FPKM values of all differentially significant genes from eight clusters and germination percentage of three wheat cultivars under the waterlogging treatment. The color of the heat map from top to bottom corresponds to Cluster1- Cluster8. GR: Germination rate. W72_ZM22, W72_BN207 and W72_BN607 were the sample names of ZM22, BN207 and BN607 under the waterlogging treatment, respectively. C72_ZM22, C72_BN207 and C72_BN607 were the sample names of ZM22, BN207 and BN607 under the control treatment, respectively.

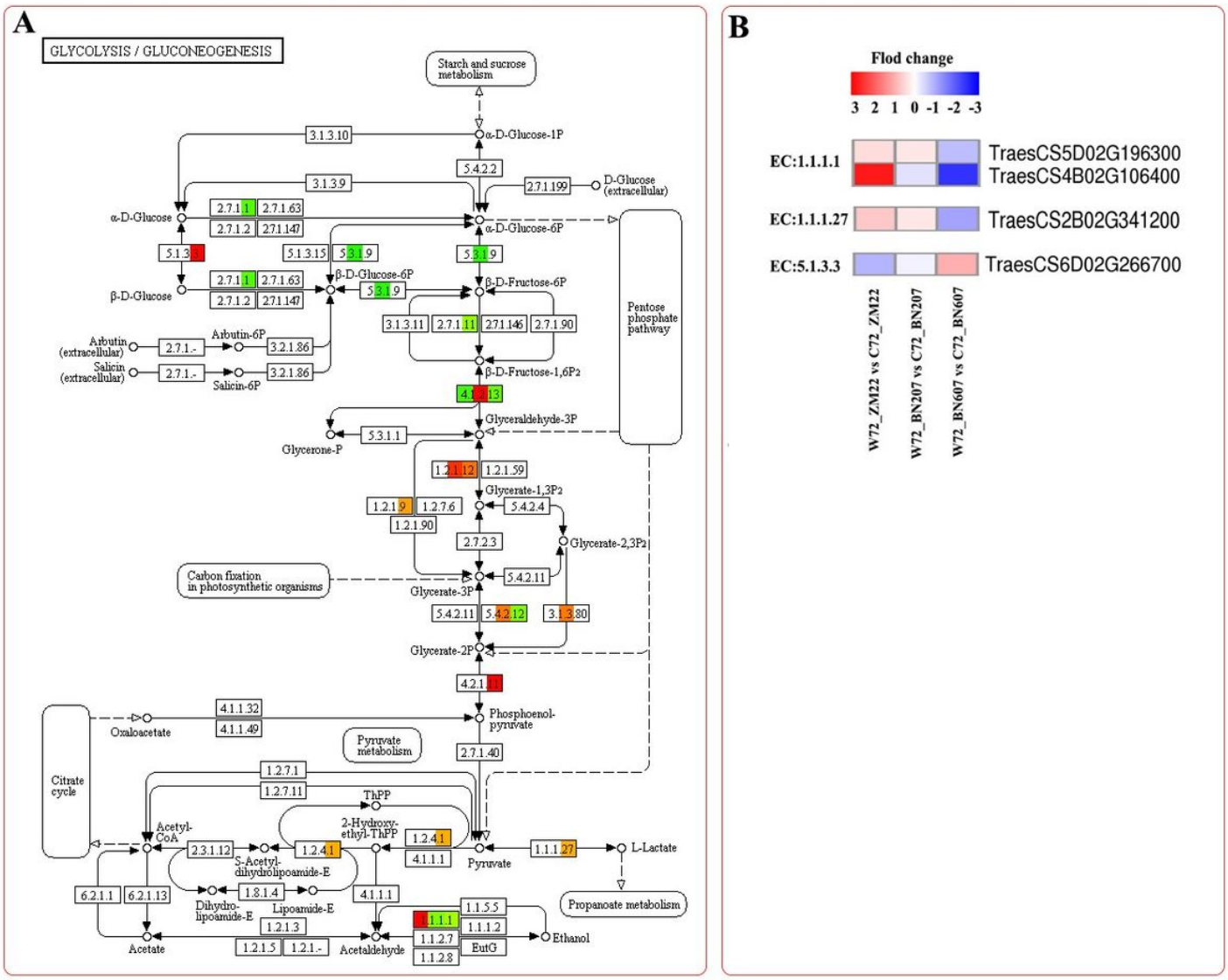

Figure 5

Analysis of differentially expressed genes (DEGs) involved in glycolysis pathway in ZM22, BN207 and BN607. A, The pattern diagram of glycolysis pathway. B, The expression profiles of DEGs involved in glycolysis pathway. The metabolism of each previously selected DEGs was analyzed using the heatmap. Red squares indicate up-regulated genes, green squares indicate down-regulated genes, and orange squares indicate both up-regulated and down-regulated genes.

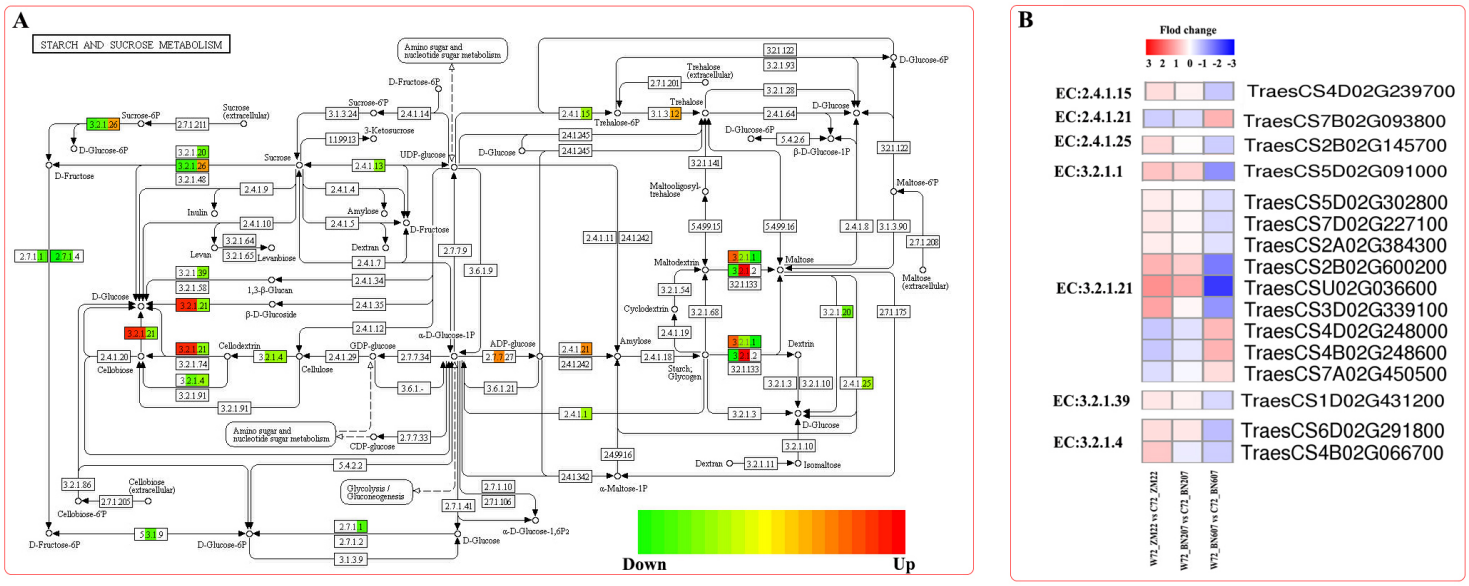

Figure 6

Analysis of differentially expressed genes (DEGs) involved in starch and sucrose metabolic pathway in ZM22, BN207 and BN607. A, The pattern diagram of starch and sucrose metabolic pathway. B, The expression profiles of DEGs involved in starch and sucrose metabolic pathway. The metabolism of each previously selected DEGs was analyzed using the heatmap. Red squares indicate up-regulated genes, green squares indicate down-regulated genes, and orange squares indicate both up-regulated and down-regulated genes.

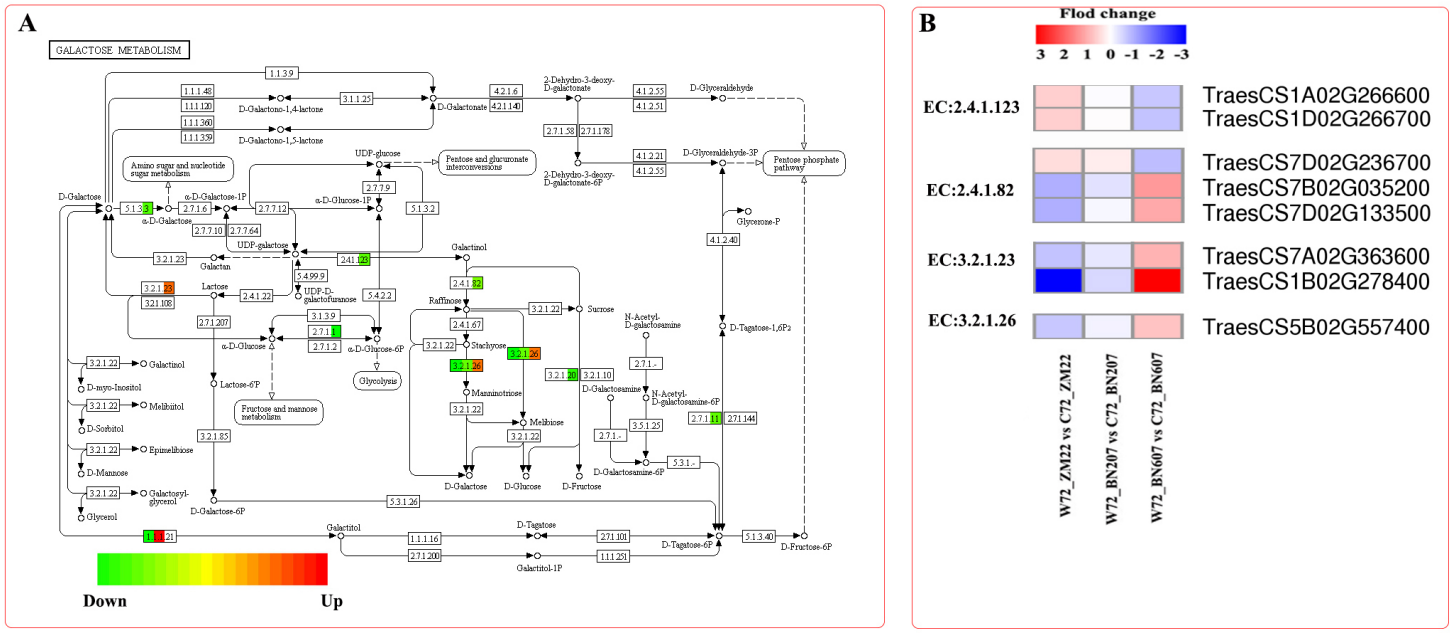

Figure 7

Analysis of differentially expressed genes (DEGs) involved in lactose metabolic pathway in ZM22, BN207 and BN607. A, The pattern diagram of lactose metabolic pathway. B, The expression profiles of DEGs involved in lactose metabolic pathway. The metabolism of each previously selected DEGs was analyzed using the heatmap. Red squares indicate up-regulated genes, green squares indicate down-regulated genes, and orange squares indicate both up-regulated and down-regulated genes.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS3.xls
- Fig.S3.tif
- Fig.S2.tif
- Fig.S1.tif
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- TableS1.xlsx
- TableS2.xlsx
- TableS6.xls
- TableS4.xls