Novel insights into the effect of drought stress on the development of root and caryopsis in barley

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Abstract

Drought is a common natural disaster in barley production, which restricts the growth and development of barley root and caryopsis seriously, thereby decreasing yield and deteriorating grain quality. However, mechanisms for how drought stress affects barley caryopsis and root development ~~under drought stress~~ (DS) is not clear. In this paper, the morphological and structural changes in root growth and caryopsis development of barley under DS were investigated. DS increased root/~~-~~shoot ratio and eventually led to the reduction of ear weight and 1000-grain weight by affecting the biomass accumulation of root and caryopsis. The barley root under DS had more lateral roots while the vessel number and volume of root decreased. Meanwhile, DS accelerated the maturation of caryopsis, resulting in a decrease in the accumulation of starch but a significant increase of protein accumulation in barley endosperm. There was a significantly positive correlation between the area of root vessel and the relative area of protein in endosperm cells and drought increased the correlation coefficient. Transcriptome analysis indicated that DS induced differential expressions of genes in caryopsis were mainly involved in encoding storage proteins and protein synthesis pathways. In general, DS caused changes in the morphology and structure of barley root, and the root ~~transported~~conveyed stress signals to caryopsis, inducing differential expression of genes related to protein biosynthesis, thus ultimately led to the increase in the accumulation of endosperm protein. The results ~~can~~ not only deepen the study on drought mechanism of barley, but also provide theoretical basis for molecular breeding, high-yield cultivation and quality improvement in barley.

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Introduction

Barley (*Hordeum vulgare* L.) is an important food, feed and cash crop~~s~~, and its planting area ranks fourth all around the world (Lü, Wu & Fu 2015). Drought stress ~~(DS)~~ is a common natural disaster in agricultural production, which seriously restricts the root growth, caryopsis development and final yield of barley. Previous studies have found that the root of barley can absorb inorganic salt and water and transport them to the aboveground parts (Varney & Canny, 1993; Xiong et al., 2006), so the root morphological characteristics can be used as a key index for drought tolerance evaluation (Chloupek et al., 2010). Seniors pointed that DS reduced the number of tillers, plant height and grains per panicle of barley, resulting in a significant decrease in thousand kernel weight (TKW) and yield of the main panicle (Samarah, 2005; Samarah et al., 2009). The contents of protein and starch were also affected by DS. Studies showed that under DS, protein content in grains increased, starch content and size changed, while starch structure did not change significantly (Nezhadahmadi, Prodhan & Faruq, 2013; Gous, Gilbert & Fox, 2015; Yu et al., 2017). From the genetic lays, scholars have confirmed that drought tolerance ~~indexs~~indexes can be used as efficient criterion for screening drought-resistant and sensitive genotypes of barley (Sharafi et al., 2015). The discovery of drought-tolerant genes and quantitative trait loci is of great significance to the breeding and quality improvement of barley (Nevo & Chen, 2010).

For plant, survival in adverse conditions need substantial changes in the metabolism, which is reflected in extensive transcriptome reconstruction upon the occurrence of stress (Janiak et al., 2018). The transcriptome analysis can furnish ~~with~~ information about regulation of gene expression at transcriptional levels and provide an insight into the mechanisms underlying stress responses. Since the draft genome of barley has been available for years, researches on transcriptome analysis of barley under DS has increased rapidly. In previous studies, the root hair morphology and transcriptional characteristics of two contrasting Tibetan wild barley genotypes and drought-tolerant cultivar were investigated and then the full length cDNA of a novel β-expansin gene (*HvEXPB7*) was cloned, which is the unique root hair development related gene (He et al., 2015; Kokáš, Vojta & Galuszka, 2016). Additionally, Abebe et al. (2015) compared response of the transcriptome of the lemma, ~~palea~~pale, awn, and seed to drought and found that transcript abundance followed the water status of the spike organs. While, Vojta et al. (2016) conducted a detailed transcriptomic analysis on leaves of transgenic plants subjected to re-watering after drought stress and revealed that the up-regulated expression of genes encoding putative enzymes involved to production of jasmonates and other volatile compounds caused a faster tendency of return to initial photochemical activities compared to wild-type.

The effects of DS on the growth of barley are reflected in the underground and aboveground organs. Previous studies about drought effect of barley have focused on the root morphology and yield traits (Barnabás, Jäger & Fehér, 2008; Afsharibehbahanizadeh et al., 2014; Tyagi et al., 2014; Haddadin, 2015; Hannah et al., 2018). However, few studies have been reported on root structure characteristics and caryopsis development in barley under DS and their relationships is unclear. In the present study, Suluomai1 (SLM1) was subjected to DS from flowering to

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caryopsis mature stage, and the morphology and microstructural changes in root and caryopsis were observed. Meanwhile, transcriptome analysis was carried out to investigate the possible mechanism underlying the barley caryopsis development responding to DS. The results may provide valuable information for revealing the relationship between barley root and caryopsis development under DS, and provide a theoretical foundation for high-yield cultivation and quality improvement in barley.

Materials & Methods

Plant materials and DS treatment

The barley variety selected in this study was SLM1, which was provided by the Agricultural College of Yangzhou University, Jiangsu Province, China. Seeds were sown in plastic pots (30 cm×30 cm, 10 seeds per pot), which were placed in rainproof shelters under DS simulation in the experimental field of Key Laboratory of Crop Genetics and Physiology in Yangzhou University from September 2017 to May 2018. The soil was sandy loam containing organic matterial (2.45%), available nitrogen (106 mg • kg^{-1}), available phosphorus (33.8 mg • kg^{-1}), and available potassium (66.4 mg • kg^{-1}). Plants were thinned to eight plants per pot 2 weeks after sowing. A minupressure soil hygrometer (SP-11, Institute of Soil Science in Nanjing, China) was inserted into the soil at a depth of 15 cm to detect the soil water potential. Barley plants were accurately irrigated from flowering to caryopsis mature stage to maintain the water potential at −20 and −60 kPa, which reflected the optimum level of control condition (CC) and DS, respectively. Each treatment contained 30 pots. During barley flowering stage, the flowering ears were tagged to label the anthesis date, and the root and caryopsis of at different development stage were collected and tested.

Roots and caryopsis morphology observation and growth indexes determination

Barley root and caryopsis samples were collected at 10, 20 and 30 days post anthesis (DPA), and their morphologiesy were observed and photographed. The fresh weight of roots, canopy structure and 1000-grain of caryopsis were conductedweighed. Then, the samples were placed in a fan-forced oven at 105 ℃ for 1 h, and then baked at 42 ℃ to attain constant weights for dry weight determination. The root-shoot ratio and water content were calculated. Meanwhile, the ripe barley ears were collected to measure the indexes such as ear length, ear weight, grain number per ear and 1000-thousand grain weight.

Microstructure observation of root and caryopsis

Barely caryopses and roots at 10, 20 and 30 DPA were acquired and cut transversely into 2 mm slices from the middle using a razor blade. The caryopsis slices were soaked in 2.5% glutaraldehyde fixative [25% glutaraldehyde diluted 10 times at pH 7.2 phosphate buffered solution (PBS)] at 4℃ for 48 h immediately. The fixed samples were subsequently rinsed thrice with PBS and dehydrated in a graded ethanol series [20, 40, 60, 80, 90, 95, and 100% (thrice)], followed by propylene oxide replacement. Afterward, the samples were infiltrated and embedded in low-viscosity Spurr's resin and polymerized at 70 ℃ for 12 h. The samples were cut into 1 μm slices using an ultramicrotome (Ultracut R, Leica, Germany) and pasted onto glass slides. Then,

the slices were stained with 0.5% methyl violet or toluidine blue, rinsed, dried, and observed under a light microscope (DMLS, Leica, Germany). Photographs were captured using a CCD camera (Truechrome II, Truechrome, China) attached to the light microscope.

Structural characteristics analysis of root and caryopsis

Image-Pro Plus (ver. 6.0, Media Cybernetics, USA) and Photoshop (ver. CC 2017, Adobe, USA) were used to ~~analysis~~ analyze structural characteristics of root and caryopsis based on microphotographs as previously described (Yu et al., 2015). The roots were photographed at 100X ~~×~~ and the transversal section areas of root vessel were measured. Meanwhile, two main endosperm regions, including abdominal and dorsal endosperm were photographed at 200X ~~×~~. The number of starch granules, areas of starch granules and protein bodies along with their corresponding endosperm cells were measured. The ratios of starch granules and protein body~~ies~~ areas to corresponding endosperm cell~~s~~ areas were calculated, defining as the relative areas of starch granules and protein bodies in endosperm respectively. Each sample selected ten microphotographs and each treatment conducted three replicate samples.

RNA extraction and sequencing

Barely caryopses under CC and DS were collected at 10 DPA and frozen immediately in liquid nitrogen. Total RNA was extracted ~~in~~ for two biological replicates using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. Genomic DNA in the samples was removed using RNase-free DNase (Promega, USA). The mRNA samples were enriched by using oligo (dT)-magnetic beads and then cut into fragments with fragmentation buffer. The cDNA was synthesized by reverse transcription using the fragments as templates and added with double-stranded DNA poly A and adaptor sequences after purification and terminal repair. Subsequently, cDNA libraries were constructed by polymerase chain reaction (PCR) amplification after selecting for fragment size and undergoing a quality check with an Agilent 2100 Bioanalyzer system. Finally, four qualified libraries were sequenced with an Illumina HiSeq 2500 system by Oe Biomedical Science and Technology Co., Ltd. (Shanghai, China).

Bioinformatics analysis of RNA-Seq

Clean reads were obtained from the raw reads after filtering out low-quality reads (quality threshold < 20, length threshold < 35 bp). The clean reads were blast to the *H. vulgare* cDNA database from Ensembl (ftp://ftp.ensemblgenomes.org/pub/plants/release-44/fasta/hordeum_vulgare/cdna/Hordeum_vulgare.IBSC_v2.cdna.all.fa.gz) using Tophat/bowtie2 software. Transcript expression was carried out and differentially expressed genes (DEGs) were screened using a 2-fold change at the $P < 0.05$ level (Trapnell et al., 2010). The functional annotation of DEGs ~~were~~ was performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Statistical analysis

Statistical analysis was performed using Microsoft Excel (ver. 2016, Microsoft Corp., USA) and SPSS (ver. 19.0, SPSS Inc., USA). The means were compared using t-test at a probability significance level of $P < 0.05$. Figure production was using Photoshop and Origin (ver. 2017, Origin Lab., USA).

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Results

Changes in root growth and caryopsis development of barley under DS

As we can see from Fig. 1, the fresh and dry weight of barley root increased first and then decreased under DS, which ~~and the fresh and dry weight of root under DS~~ was significantly higher than that under CC at 10 and 20 DPA. However, the water content of root had a tendency of decrease as the growth of root under both CC and DS and DS decreased the water content of root (Fig. 1A). The fresh weight of caryopsis increased first and then decreased but the dry weight of caryopsis continually increased during the development of caryopsis. Before 20 DPA, the fresh and dry weight of caryopsis under DS were higher than that under CC. But DS decreased the fresh and dry weight of caryopsis at 30 DPA. The water content decreased with the caryopsis development and fluctuated around the control level when treated with drought (Fig. 1B). Additionally, DS significantly decreased the root-shoot ratio during whole development stage of barley (Fig. 1C). Moreover, DS not only effected the biomass accumulation of plant but also result in the changes of ear characteristics with shorter ear length, smaller number of grains per ear, lower ear weight and 1000-grians weight (Table 1).

Changes in morphology and structure of barley root under DS

The morphology of barley root changed when subjected to DS. The barley had a longer, larger and more complex root system with more lateral roots under DS, comparing with the root under CC (Fig. 2A, B). From the microstructure of the barley root, the cortex and xylem vessel were observed. As the growth of root, the number of vessels decreased under CC while the number of vessels in root under DS increased (Fig. 2C-H). This indicated that DS decreased the vessel numbers in barley root. Meanwhile, DS also significantly reduced the area of root vessel transversal section at 10 and 20 DPA according to the statistical data (Fig. 2I).

Changes in morphology and structure of barley caryopsis under DS

The morphology and microstructure of barley caryopsis at 10, 20 and 30 DPA were observed by resin semi-thin slicing, and the number of starch granules along with relative areas of starch granules and protein bodies in endosperm were also counted using Image-Pro Plus software (Fig. 3). During the development of barley caryopsis, DS affected its morphology and endosperm substance accumulation. When barley treated with DS, the epidermis of caryopsis turned to yellow and was shrunken at the earlier stage compared to CC (Fig. 3A). This indicated that DS promoted the early mature of caryopsis. At 10 DPA, a number of starch granules and protein bodies were observed in dorsal and abdominal endosperm under DS while few were observed under CC (Fig. 3B-E). The statistical data showed that DS significantly increased the relative areas of protein bodies in dorsal endosperm and the relative areas and number of starch granules in both dorsal and abdominal endosperm (Fig. 3N-P). At 20 DPA, the number and volume of starch granules in endosperm increased along with the accumulation of protein bodies under both CC and DS and some small starch granules began to occur (Fig. 3F-H). However, several large protein aggregations consisting of many small protein bodies units assembled in abdominal endosperm (Fig. 3I). Meanwhile, there was no significant difference in the accumulation of

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starch granules in dorsal and abdominal endosperm between CC and DS but a significant increase of protein bodies accumulation in dorsal endosperm under DS (Fig. 3N-P). At 30 DPA, endosperm cells were almost occupied by starch. Starch granules squeezed to deformed and protein bodies filled into the gap between starch granules. More small starch granules in endosperm under DS were observed compared to CC, but the number and relative areas of starch granules in endosperm under DS were lower than that under CC (Fig. 3J-M, O-P). Additionally, DS significantly increased the relative areas of protein bodies in dorsal and abdominal endosperm (Fig. 3N).

According to the results above, it can be concluded that DS promoted the precocity and shortened the development process of caryopsis. Moreover, DS affected the substance accumulation of endosperm, which showed an increase in protein accumulation and a decrease in final starch accumulation.

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Correlation analysis of root and caryopsis structure under DS

To investigate the relationship between barley root and endosperm substance accumulation under DS the correlation analysis on the structure of root and caryopsis was conducted. The correlation coefficient between the area of root vessel transversal section and the relative areas of starch granules in endosperm cells was 0.44, indicating significantly moderate correlation, and DS reduced its correlation coefficient to 0.36. However, the area of root vessel transversal section and the relative areas of protein bodies in endosperm cells had a strong correlation (0.76) and DS increased the correlation coefficient to 0.81. Therefore, the area of root vessel transversal section was more closely correlated with the relative areas of protein bodies compared to the relative areas of starch granules and the correlation increased after treating with drought. The results indicated that changes of root structure under DS had a greater influence on the accumulation of endosperm protein bodies.

Analysis of gene differential expression

In total, 258 billion clean reads were obtained in four samples after filtering, with approximate 64 billion reads on average from each sample. The results of sample to sample clustering analysis showed that the distance between ~~treatment of~~ CC and DS treatments was long while the similarity of gene expression pattern in two replicates was high (Fig. 4A). This indicated that the repeatability of samples in this study was reliable. DEGs were screened out at the threshold of 2-fold change as a basis of $P < 0.05$ ~~and showed in~~(-Fig. 4B). A total of 2,642 DEGs in barley caryopsis were identified between CC and DS. Among these DEGs, 1,192 genes were upregulated and 1,450 genes were downregulated (Fig. 4C).

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DEGs involved in caryopsis storage protein synthesis

The information of functional annotations for all DEGs was obtained from various database. A total of 30 DEGs involved in encoding caryopsis storage protein were screened out and specific information was shown in Table 2. Among these DEGs, nine of them showed up-regulated and 21 showed down-regulated expression under DS. Specifically, three genes encoding gliadin and nine genes encoding glutenin showed down-regulated expression under DS. Among DEGs encoding albumin, eight genes were up-regulated and seven were down-regulated. Meanwhile,

there were three genes encoding 11S seed storage protein, one of which expressed with up regulation and two showed down-regulated expression.

To gain more insights about DEGs, GO enrichment and KEGG pathway analysis were also implemented. Functional annotation based on GO and KEGG databases revealed that DEGs had various functions, involving biological processes, molecular genetics and other aspects. To investigate the effects of DS on protein synthesis in barley caryopsis, 14 GO terms that might be involved in protein biosynthesis were selected ~~out~~, including protein folding, protein binding, protein transport, intracellular protein transport and so on. Among them, the DEGs annotated to protein binding entry was the most (198 DEGs), followed by protein heterodimerization activity entry (96 DEGs) (Fig 5A). Similarly, five possible KEGG pathways related to protein biosynthesis were also screened ~~out~~. They are protein processing in endoplasmic reticulum (ER), protein export, ribosome biogenesis in eukaryotes, various types of N-glycan biosynthesis, aminoacyl-tRNA biosynthesis. The number of DEGs enriched in the pathway of protein processing in ER was the largest and six DEGs were enriched in aminoacyl-tRNA biosynthesis pathway, which took the second place (Fig. 5B).

The pathway of protein processing in ER was ~~picked out~~chosen to further investigate the possible mechanism underlying the protein biosynthesis in barley caryopsis under DS. The up-regulated and down-regulated DEGs enriched in the pathway were shown in Fig. 6. The newly synthesized peptide chain entered the ER through the translocation of the Sec61 pore, followed by N-glycosylation. The correctly folded proteins were packaged into the vesicle II (COPII) transport vesicles, which were then transported into the Golgi apparatus. The unfolded or misfolded proteins were remained in the ER and eventually entered the ubiquitin-mediated proteasome degradation process. During this process, the expression of certain genes altered under DS. For example, *SEC61A* and *BIP* showed the expression of down regulation, which both encoded protein transport protein subunit. *MBTPS1* encoded membrane-bound transcription factor site-1 protease in COPII and was down-regulated under DS. The results indicated that DS altered the expression of some certain genes during the process of protein export and COPII transport vesicle formation in protein processing in ER pathway.

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Discussion

The growth of root is related to plant growth stage, genotype, arbuscular mycorrhizal colonization rate in soil and can be affected by environmental factors such as drought (Akman & Topal, 2016; Sendek et al., 2019). Generally, the root of plants firstly sensed the stress signal when subjected to DS. Then the morphology and structure of root changed to help the plant to absorb water more efficiently, thus adapting to drought. The vessel in root xylem is the channel for water and inorganic salt transportation, which is crucial for coping with water changes and deficit. In this study, DS reduced the area of root vessel transversal section in barley, which can be explained from the aspect of water flow conductivity. The water flow conductance of root is affected by radial and axial resistance. When the diameter of vessel is too large, the bubbles in the vessel will form embolism more easily, which will increase the radial and axial hydraulic resistance and reduce the root water flow conductivity. Therefore, the root water absorption is

limited and it is not conducive to adapt to drought for plant growth (Thorsten & Wieland, 2011; Comas et al., 2013; Vadez, 2014; Bartlett et al., 2016; Niu et al., 2016). While, the narrower xylem vessels could overcome this phenomenon, which was consistent to the results in wheat (Comas et al., 2013).

Barley caryopsis is an important organ for nutrient storage and its development is also limited by DS. Previous studies found that drought caused the early senescence of plants and affected the grain filling by shortening grain filling time and reducing grain filling rate (Shi et al., 2016). This was consistent with our study, which found the pre-mature of barley caryopsis under DS. Meanwhile, DS significantly decreased the accumulation of starch granules but increased the protein ~~bodies~~body's accumulation in endosperm of barley caryopsis. Transcriptome analysis showed that DS induced differential expression of genes were mainly related to storage proteins and protein synthesis pathways, thus regulating protein biosynthesis and eventually leading to the increase of protein accumulation in barley caryopsis.

As the important organ for water and nutrient absorption in barley, the morphological and structural characteristics of root are closely related to the development of caryopsis (Ramireddy et al., 2018). In this study, DS caused changes in root and caryopsis weight and increased root/~~-~~shoot ratio, which eventually led to a decrease in the spike weight and 1000-grain weight. Similar results were also reported in previous papers (Samarah, 2005; Samarah et al., 2009). Additionally, the correlation analysis between root structural characteristics and endosperm substance accumulation was conducted under CC and DS. It was found that the area of root vessel had a strong correlation with the area of protein in endosperm cells and drought increased the correlation coefficient. This indicated that the changes of root structure caused by DS had a greater influence on the accumulation of endosperm protein compared to endosperm starch accumulation. In general, the morphology and structure of barley root changed under DS and the root transported stress signals to caryopsis. The drought signal induced differential expression of genes related to protein biosynthesis in caryopsis and ultimately resulted in the increase of endosperm protein accumulation.

Conclusions

In this study, the morphological and structural changes in root growth and caryopsis development of barley under DS were investigated. Under DS, the root of barley had more lateral roots with a narrower vessel structure. Meanwhile, DS promoted the pre-mature of caryopsis and affected the substance accumulation in caryopsis with a decrease in endosperm starch but an increase in endosperm protein. DS induced changes in root and caryopsis weight led to the increase of root/~~-~~shoot ratio but a decrease in 1000-grain weight. While, the changes of root structure caused by DS had a greater influence on the accumulation of endosperm protein. Transcriptome analysis indicated that DS induced differential expression of genes were mainly related to protein biosynthesis in caryopsis. In conclusion, the root of barley firstly sensed the stress signals when subjected to DS, which led to the changes in root morphology and structure. ~~Thus~~ Then the water and nutrient absorption was affected, resulting in changes of caryopsis

development and substance accumulation, especially endosperm protein accumulation. At the same time, the stress signals from root to caryopsis induced the differential expression of genes involved in encoding storage proteins and protein biosynthesis pathways, ultimately leading to the increase in endosperm protein accumulation in barley caryopsis. These results can provide novel insight into the development of barley root and caryopsis under DS.

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