OPEN ACCESS Freely available online

Journal of Plant Biochemistry & Physiology

ISSN: 2329-9029

Review Article

A Transcriptomic Analysis of Vigna Radiate Revealed a Role of Uniconazole in Response to Chilling Stress

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ABSTRACT

BACKGROUND

Chilling injury of Mungbean (Vigna radiate L.) is a common problem which is encountered in the early spring of cultivation. Uniconazole (UNZ) can alleviate a variety of abiotic stresses such as low temperature. There has been no systematic investigation on the genes underlying the response to Uniconazole-induced tolerance of mungbean to chilling stress in mungbean. RNA sequencing was used to compare different transcriptome of mungbean plant with or without UNZ prior chilling stress.

RESULTS

The pod number per plant, particle number per plant and yield of the two mungbean varieties were significantly lower than those of control after chilling treatment at outset of flowering stage (R1). Spraying UNZ before chilling stress at R1 stage could effectively alleviate the decline of mungbean yield. UNZ can effectively delay the decrease of chlorophyll content of leaves under chilling stress at R1 stage and accelerate the increase of chlorophyll content in leaves during the recovery period. Eighteen separate RNA-Seq libraries were generated from the RNA samples of leaves from six different treatments, including mungbean plant with or without UNZ prior chilling stress for 1 d and 4 d. The expression levels of numerous different genes in mungbean seedlings were affected by the chilling stress and UNZ. GO assignments showed that photosynthesis in the biological process category and in cellular component category enriched obviously by chilling stress and application of UNZ. KEGG pathway enrichment analysis proved that the duration of chilling stress and application of UNZ obviously altered the pathway of cutin, suberine and wax biosynthesis, plant- pathogen interaction, photosynthesis, ribosome and Porphyrin and chlorophyll metabolism. Chilling stress down-regulated gene expressions of the 4 KEGG pathways except plant-pathogen interaction. UNZ treatment could effectively prevent to further down-regulate the expression level of the 4 Kegg pathways. Ten differentially expressed genes (DEGs) were validated using quantitative RT-PCR.

Conclusions

Our results provided a preliminarily understanding of the molecular basis of chilling stress response in mungbean, and establish a foundation for the future genetic improvement of cold sensitivity in mungbean by transferring genes for cold tolerance.

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Received date: February 11, 2021; **Accepted date:** August 30, 2021; **Published date:** September 15, 2021

Citation: Zheng D 2020) A Transcriptomic Analysis of Vigna Radiate Revealed a Role of Uniconazole in Response to Chilling Stress. J Plant Biochem Physiol 9: p029

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BACKGROUND

Mungbean (Vigna radiate L. Wilczek) is an important legume crop for its valuable nutritional and health benefits. Mungbean seeds and its sprouts contain high levels of proteins rich in essential amino acids and various phytochemicals with beneficial activities, such as antioxidant, antimicrobial, anti-inflammatory, antidiabetic, antihypertensive, lipid metabolism accommodation and antitumor effects [1]. According to Food and Agriculture Organization of the United Nations, worldwide production of mungbean increased fourfold to 21.4 million tons between 1990 and 2014, with the majority produced in Asia [2]. Mungbean is one of the most important legume crops in China, is mainly produced in Huang Huai River Basin and Northeast China [3]. Northeast China belongs to the cold zone, the effective accumulated temperature is insufficient, and cold damage frequently occurs in Heilongjiang province [4]. Cold stress can be classified as chilling (0–15 °C) and freezing (<0 °C) stresses. Cold stress limits plant growth and causes significant crop loss. Cold stress responses are influenced by duration of exposure [5], species [6] and the stage of plant development [7]. Exposure of mungbean seedlings to chilling resulted in irreversible cellular electrolyte leakage [8]. Chilling susceptibility mungbean varieties were related with differentially expressed cold related genes [2, 9].

It has been well documented that plant growth regulators (PGRs) play important roles in crop production and resisting environmental stresses. Uniconazole (S-3307, UNZ), a plant growth retardant was increasingly applied in crops to increase output and quality of crops [10-12]. This chemical could alleviate adverse stresses and UNZ-induced tolerance of water deficit, waterlogging and low-temperature which was related to the changes of photosynthesis [13-15].

The genome of mungbean has been recently sequenced [16], which provides a valuable resource for molecular-based investigations for stress tolerance in plants. RNA sequencing (RNA-seq) analysis is an efficient and powerful method for transcriptome analysis, the technology has increasingly been used to characterize transcriptome profiling to chilling/cold stress for many legumes species[12, 17-20]. Transcriptome analysis have been conducted to analyze desiccation tolerance, adventitious rooting and bruchid resistance in mungbean [21-23], however, the effect of UNZ on the growth and on the transcriptome under chilling stress in mungbean has not been investigated yet. In this work, the physiological parameters and relevant transcriptome of mung bean in response to UNZ under low-temperature stress were studied. Thus, this study aimed to find the correlation between the physiological parameters and differentially expressed genes (DEGs) via the transcriptome analysis, which would be useful for the application of UNZ in the production of mungbean. At the same time, it provided some references for the mungbean cultivation in Heilongjiang and other cold regions.

RESULTS

EFFECTS OF UNZ ON YIELD AND YIELD COMPONENTS OF MUNGBEAN SEEDLINGS UNDER LOW-TEMPERATURE STRESS

Pod number per plant and particle number per plant are two yield components. The effect of UNZ on yield and yield components in mungbean under chilling stress at R1stage in 2017 was shown in table 1, the yields of the two mungbean varieties were significantly lower than those of CK after chilling treatment at stage R1. The order of pod number per plant, particle number per plant and yield was the same in 2 varieties, which was CK >D1 > D2 > D3 > D4. The yield loss of Lvfeng 2 was more serious which indicated the different genotype of mungbean had different tolerance to chilling stress. The yield loss in 2017 was similar as the results in 2016 (data not shown). However, spraying UNZ at R1 stage could effectively alleviate the decline of mungbean yield.

EFFECTS OF UNZ ON PHOTOSYNTHETIC PARAMETERS IN MUNGBEAN UNDER CHILLING STRESS AT R1 STAGE

Chlorophylls and carotenoids are the main photosynthetic pigment in plants. Photosynthetic pigments are involved in the process of absorbing and transferring energy in photosynthesis of crops. The change of its content can reflect the degree of chilling stress. The contents of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl (a + b) and total carotenoids (Car) of lvfeng 2 （L2） in D and D+S treatments were significantly lower than those in control compared with the control. The contents of Chl a, Chl b, Chl (a + b) and Car of lvfeng 2 in D treatments were significantly lower than those in D +S treatment. The contents of Chl a, Chl b, Chl (a + b) and total Car in leaves of Lvfeng2 decreased gradually with the extension of chilling stress and increased with the extension of recovery time, but always lower than control (Fig. 1). The contents change of Chl a, Chl b, total CHL and Car in Lvfeng5 in D and D+S are similar with those in Lvfeng2 (data not shown). UNZ can effectively delay the damage of photosynthetic pigment content of leaves under chilling stress at R1 stage and accelerate the increase of photosynthetic pigment content in leaves during the recovery period after exogenous application UNZ.

RNA-SEQ AND TRANSCRIPTOME PROFILES OF UNZ IN RESPONSE TO LOW-TEMPERATURE STRESS FOR MUNGBEAN LEAVES

An overview of the RNA-Seq reads derived from the eighteen libraries was presented in Table 2. After the low-quality reads were removed,133.08 Gb clean reads were obtained with an average of 22.18 Gb reads for each sample, and the proportion of Q30 was greater than 94.28% and the distribution of GC content was uniform and stable, which indicated that the sequencing results were highly accurate.

The clean reads of each library was compared with the reference genome sequence of mungbean by top hat 2 software, the mapped ratio of the libraries were 92.22% -93.28 % while the unique mapped ratio of the libraries were 87.24-90.50%. The data meet the needs of subsequent analysis. The correlation

coefficient of the three biological repeats in each treatment of RNA-seq was more than 0.9 (Fig. S1).

Wilcoxon signed-rank test (FDR < 0.01 and fold change ≥ 1) showed the results of numbers of DEGs of different combinations (Table S2). The number of DEGs was 4 905 in D1+S-CK1 while the number of DEGs in D1-CK1was 4 025. The number of DEGs decreased to 3 266 in D4+S-CK4 compared to the number of DEGs 4 023 in D4-CK4. These results indicated that the expression levels of numerous different genes in mungbean seedlings were affected by the low temperature and UNZ.

GO ENRICHMENT ANALYSIS OF DEGS IN MUNGBEAN LEAVES UNDER UNZ AND CHILLING STRESS AT RL STAGE

GO assignments system was used to classify the possible functions of mung bean genes. GO functional enrichment indicated that 1170, 1184, 2017, 1385 DEGs could be classified into three categories of biological process (BP), cellular component (CC), and molecular function (MF) in D1-CK1, D1+S-CK1, D4-Ck4 and D4 +S-CK4, respectively. The significantly enriched GO categories in CC, BP and MF were showed in Table 3. Only the Go terms in MF category was significantly enriched in D1-CK1 and D1+S-CK1 among the three categories. The number of the enriched GO terms in the BP category was more in D1+S-CK1 than D1-CK1, which implies that application of UNZ plays an important role in regulating BP gene expression under chilling stress. In the D4-CK4 and D4+S-CK4 there were Go terms enriched significantly in each three categories, in which the number of Go terms of CC category was the most among the 3 categories. It is obvious that the cell components of photosynthesis were most influenced by chilling stress.

KEGG Enrichment Analysis of DEGs in Mungbean Leaves under UNZ and chilling stress at Rl stage

The KEGG database was used to understand further the biological functions and pathways of DEGs. For the KEGG pathway enrichment analysis, pathways displaying significant changes (Q value ≤0.05) in response to chilling stress with or without UNZ treatment were identified. The top 20 enriched KEGG pathways compared are presented in Figure 2. The significantly enriched pathways of DEGs were cutin, suberine and wax biosynthesis (Vra00073) in D1-CK1, Photosynthesis (Vra00195) in D4-CK4, cutin, suberine and wax biosynthesis (Vra00073) and Plant- pathogen interaction (Vra04626) in D1+S-CK1 and Photosynthesis (Vra00195), Ribosome (Vra03010) and Porphyrin and chlorophyll metabolism (Vra00860) in D4+S-CK4.

Differential Expressions of Plant- pathogen interaction pathway genes

The expressions of the all plant-pathogen interaction genes except calcium-dependent protein kinase SK5 were up-regulated after chilling stress with or without UNZ in all the four treatments. These genes are important Ca2+ signal genes, including 7 calcium-binding protein, 3 calcium-dependent protein kinase and 5 calmodulin calmodulin-like genes. Two heat shock protein, heat shock cognate protein and endoplasmin were up-regulated. One mitogen-activated protein kinase, pathogenesis-related genes transcriptional activator, respiratory burst oxidase homolog protein, somatic embryogenesis receptor kinase and WRKY transcription factor were also up-regulated.

The log2 FC of most Calmodulin and calmodulin-like protein were positive in D1+S- D1 and D1+S- D1, especially calmodulin in D1+S-D1 and calmodulin-like protein 11 in D1+S-D1 and D4+S-D4 were notably up-regulated (log2 FC >1), meant that UNZ treatment before chilling stress up-regulated the expression of some Ca2+ signal genes (Table 4).

Differential Expressions of ribosome proteins

The ribosome is a large assembly of proteins and ribosomal RNAs (rRNAs) that functions to translate messenger RNAs (mRNAs) into proteins. Ribosome is a significantly enriched pathway in the D1+S treatment. The expressions of DEGs in this pathway were shown in table S3. The expressions of all ribosome genes were notably down-regulated (log2 FC>1) after chilling stress with or without UNZ except 40S ribosomal protein S3a and 60S ribosomal protein L10-like were notably up-regulated. The log2 FC of 23 in 26 genes were positive in D1+S-D1 meant that UNZ treatment could effectively prevent to further down-regulate the expression of ribosomal genes, while the log2 FC of only 11 in 26 genes were positive in D4+S- D4 meant that UNZ treatment could not effectively prevent ribosomal genes to further down-regulate with the extension of chilling stress.

Differential Expressions of cutin, suberine and wax biosynthesis

Cutin, suberin and wax which are involved in cell wall structure have the principal function of setting the boundaries between the cell and the environment [32]. The expressions of all cutin, suberin and wax genes were obviously down-regulated except cytochrome P450 86A8 was up-regulated in 4 treatments, the results meant that the synthesis of cutin, suberin and wax decreased (Table S4). Only peroxygenase 4 in D1+S-D1 and ECERIFERUM 1in D4+S-D4 were obviously down-regulated meant that UNZ treatment could effectively prevent these two genes.

Differential Expression of Chlorophyll and photosynthesis biosynthesis

Chlorophyll is an important photosynthetic pigment in the chloroplast of plants, and the metabolism of chlorophyll is an important factor in determining crop yield. The contents of Chl a, Chl b, total CHL and Car in leaves were found to increase after UNZ application. To investigate whether chlorophyll synthesis-related genes were involved in the increase in chlorophyll content, we further studied expression patterns of regulatory enzymes involved in chlorophyll biosynthesis. One significantly enriched pathway of DEGs was porphyrin and chlorophyll metabolism. The genes involved in this pathway and their expression were listed in (Table 5). There are 17 enzymes required for chlorophyll biosynthesis from glutamyl-tRNA to chlorophyll b [33,34]. Ten genes were differentially expressed, in

which 8 genes were down-regulated in the 4 treatments. Staygreen protein and chlorophyll b reductase which are associated with chlorophyll degradation were found up-regulated (Horie et al., 2009; Park et al., 2007). The log2 FC of 8 and 9 genes in ten genes were positive in D1+S- D1 and D4+S- D4 means that UNZ treatment could prevent to further down-regulate the expression of chlorophyll biosynthesis genes.

Efficient photosynthesis involves photosynthetic pigments and photosystems, the electron transport system, CO2 fixation pathways, and glycolic metabolism. The majority of photosynthetic energy is harnessed via linear electron flow involving light-stimulated electron transfer between two reaction centers, PSI and PSII [35]. According to the results of transcriptome sequencing, 31 genes which were differentially regulated in the 4 treatments were annotated to photosystem, and their expression level were listed Table 6. Nine genes were detected for PSI, eleven genes including 3 Oxygen-evolving enhancer protein were detected for PSII; nine genes including cytochrome b6-f complex iron-sulfur subunit, plastocyanin and ferredoxin-NADP, ferredoxin reductase, photosynthetic NDH subunit of luminal location and ATP synthase were detected for photosynthetic electron transfer. The log2 FC of 13 and 27 genes in 31 were positive in D1+S- D1 and D4+S- D4 respectively meant that UNZ treatment could prevent to further down-regulate the expression of the photosynthesis genes under the chilling stress and improve the cold tolerance of mungbean.

Quantitative Real-time PCR Verification

To verify the reliability and accuracy of our transcriptome data, qRT-PCR were performed on 10 randomly selected unigenes, all of which are predicted to be associated with cold tolerance.The functional annotations of these unigenes are listed in Table S1. The expression patterns of selected genes were determined and further compared with those of in RNA-seq assay. Nearly all of these genes displayed similar expression trend in both techniques. Moreover, the correlation between qRT-PCR and RNA-seq was measured by scatter plotting log2-fold changes (Figure 3), which showed a positive correlation coefficient (Pearson coefficient R2 = 0.9119).

DISCUSSION

EFFECTS OF UNZ ON YIELD AND PHOTOSYNTHESIS OF MUNGBEAN UNDER LOW-TEMPERATURE STRESS

Low temperature stress can cause changes in membrane phase and membrane permeability in plants, and it can destroy enzyme systems, cause metabolic disorders, reduce energy supplies, inhibit photosynthesis, promote accumulation of toxic substances, and affect plant growth and biomass production [36]. Different mungbean varieties differed in their tolerance to cold stress [2,8]. UNZ was increasingly applied in crops to increase output and quality of crops [10-12, 37]. Application UNZ could alleviate adverse stresses and UNZ-induced tolerance of stress was related to the changes of photosynthesis[13-5]. Two mainly cultivated varieties Lvfeng 2 and Lvfeng 5 were selected to identify the influence of chilling stress and application of UNZ to mungbean. Our results proved that chilling stress of 1 day and 4 days decreased the yields and chlorophyll content significantly in two mungbean varieties although different genotypes of mungbean had different tolerance to chilling stress. Application UNZ before the chilling stress increased the yields and chlorophyll content significantly in two mungbean varieties compared to the chilling stress treatments. The results were consistent with the findings in other plants [11,13,37-39]. In mungbean the yield and photosynthesis of mungbean were also found to increase after application of UNZ before waterlogging [15]. These results proved that application UNZ could alleviate chilling stress and UNZ-induced tolerance of stress was related to the changes of photosynthesis and thus increase the yield of mungbean.

TRANSCRIPTION PATTERNS OF PLANT-PATHOGEN INTERACTION IN RESPONSE TO LOW TEMPERATURE AND UNZ

Early research had found that chilling susceptibility of mungbean varieties were related with differentially expressed cold related genes[2,9], but these researches did not conduct at global transcriptomics level. The use of transcriptomics to quantify the nearly complete set of cellular transcripts allows quantitative and qualitative differences in genes expression to be determined for a specific developmental stage or stress condition [40]. In this study, RNA-seq was used to study the molecular mechanisms underlying UNZ-induced low-temperature tolerance in mungbean. The significantly enriched pathways were cutin, suberine and wax biosynthesis, plant- pathogen interaction, ribosome, porphyrin and chlorophyll metabolism and photosynthesis.

Abiotic and biotic stresses responses of plants are regulated by multiple signaling pathways and the patterns of gene expression that are induced in plants in response to different stresses over lapped[41-44]. It is well known that Ca2+ acts as a key messenger in regulating growth and developmental processes and plays a crucial role in cold stress signaling, a variety of signaling pathways is triggered, including reactive oxygen species (ROS), Ca2+-dependent protein kinases (CDPKs), mitogen-activated protein kinase (MAPK) cascades and the activation of transcription factors (TFs), all of which promote the production of cold-responsive (COR) proteins [45]. Here the expressions of the all plant-pathogen interaction genes except calcium-dependent protein kinase SK5 were found up-regulated after chilling stress with or without UNZ, seven genes are calcium-binding protein, three genes are calcium-dependent protein kinase and five are calmodulin-like genes among 23 genes in plant pathogen interaction pathway. Ca2+ signal genes including calmodulin and calcium binding protein genes were significantly up-regulated were also found up-regulated in other plants exposed to low temperature [12, 46, 47]. Calcium-binding protein and calmodulin-related protein were down-regulated after Brassinosteroids treatment under chilling stress [48]. The reasons that the results were different from ours maybe that Brassinosteroids are plant growth-promoting compounds while UNZs are plant growth retardants. The activation of Ca2+

channels might represent a signaling link that is common to chilling stress and plant pathogen interaction in mungbean.

One mitogen-activated protein kinase kinase kinase (MAPKKK) genes were higher up-regulated in D1 and D1+S than D4 and D4+S respectively, the results showed that MAPK cascade, especially MAPKKK genes, participated in the plant's response to chilling stress in the early stage. WRKY, one of the five major transcription factor classes was related to plant cold stress [48]. One WRKY was up-regulated in four treatments which suggested that the WRKY gene played an important role in the whole process of chilling response. The respiratory burst oxidase homolog (Rboh) gene family encodes the key enzymatic subunit of the plant NADPH oxidase, ROS are generated via the NADPH oxidase [50]. One Rboh was highly up-regulated in all the four treatments which suggested that the Rboh gene also played essential roles in the whole process of chilling response.

The expression of COR genes has been shown to be critical in plants for chilling tolerance, the expressions of heat shock protein (HSP) and pathogen-related proteins (PR) are induced by cold in plants [51-53]. Two heat shock protein, heat shock cognate protein and endoplasmin were up-regulated, one pathogenesis-related genes transcriptional activator which promote the expression of PR genes and one somatic embryogenesis receptor kinase which is a central regulator of innate immunity in plants were also up-regulated [54], the results proved that some proteins which were important in plant disease response also played vital role in chilling stress. UNZ treatment before chilling stress up-regulated the expression of some Ca2+ signal genes and promote the tolerance of mungbean to chilling stress.

TRANSCRIPTION PATTERNS OF CHLOROPHYLL AND PHOTOSYNTHESIS IN RESPONSE TO LOW TEMPERATURE AND UNZ

Chlorophyll is an important photosynthetic pigment in the chloroplast of plants that performs the essential processes of harvesting light energy in the antenna systems [55]. The metabolism of chlorophyll is an important factor in determining the photosynthetic rate and affects crop yield [56]. To date, a few studies have focused on UNZ involved in photosynthesis [11-15], but the expression of chlorophyll synthesis genes after application of UNZ was only in duckweed [11]. In this study, a genome-wide transcriptomic analysis method was used to investigate the metabolism of key enzymes involved in the chlorophyll biosynthesis pathway in mungbean. Eight genes of ten DEGs required for chlorophyll biosynthesis from glutamyl-tRNA to chlorophyllb [33,34] were seriously down-regulated in the 4 treatments, two chlorophyll degradation genes were up-regulated [57,58]. The log2 FC of most genes of chlorophyll biosynthesis were positive in D1+S- D1 and D4+S- D4 means that UNZ treatment could prevent to further down-regulate the expression of chlorophyll biosynthesis genes by chilling stress. UNZ enhanced the chlorophyll content, up-regulated the expression of key enzymes involved in the chlorophyll biosynthesis pathway. These results were consistent with that chlorophyll biosynthesis genes were up-regulated after UNZ [11, 59].

Photosynthesis is among the primary processes in plants that are often affected by chilling stress [59,60]. In the present study, two photosynthesis-related pathways were among the 6 most reliable significantly enriched pathways of DEGs. The high ratio of "Photosynthesis" pathway implied that genes involved in the photosynthesis perform key functions at the flower developmental stage under UNZ treatment. At the flowering stage, in addition to the "Photosynthesis" pathway, the three photosynthesis-related KEGG pathways "Antenna protein", "Porphyrin and chlorophyll metabolism" and "Carbon fixation in photosynthetic organisms" were also enriched. Twenty-eight genes which were down-regulated in D1+S-CK1 and D4+S-CK4, respectively were annotated to photosystem, eight genes were detected for PSI, eleven genes including 3 Oxygen-evolving enhancer proteins were detected for PSII, nine genes including cytochrome b6-f complex iron-sulfur subunit, plastocyanin and ferredoxin-NADP, ferredoxin reductase, photosynthetic NDH subunit of lumenal location and ATP synthase were detected for photosynthetic electron transfer. Chilling stress down-regulated the expression of chlorophyll biosynthesis and photosynthesis genes, UNZ treatment before chilling stress could prevent to further down-regulate the expression photosynthesis genes under the chilling stress. Our results were consistent with that UNZ treatment up-regulated genes expression involved in the chlorophyll biosynthesis and photosynthesis [11,38]. Interestingly both Brassinosteroid, a plant growth regulator, and UNZ, a plant growth retardant up-regulated genes expression involved in the chlorophyll biosynthesis and photosynthesis under chilling stress conditions [48].

TRANSCRIPTION PATTERNS OF CUTIN, SUBERIN AND WAX AND RIBOSOME IN RESPONSE TO LOW TEMPERATURE AND UNZ

The plant cuticle, covering terrestrial plants, is the first protective barrier against environmental stress. The cuticle is composed of a cutin polymer matrix or related polymer suberin and waxes. Cold stress increases wax load in Arabidopsis [61]. Most cuticle-associated genes including cuticle lipid synthesis, export of cuticular lipids, and regulation of plant cuticle development were up-regulated under the low temperature treatment [62]. Interestingly, the DEGs in significantly enriched cutin, suberin and wax pathway were all cuticle lipid synthesis genes in our experiment [63-66]. Most genes were down-regulated after chilling stress with or without UNZ, and UNZ treatment could effectively prevent to further down-regulate the expression of these genes. Our results were obvious different from these in Thellungiella salsuginea [62]. The reason maybe the plants were treated by different temperature and different stage, Thellungiella salsuginea was stressed under cold conditions of 4 °C, however the mungbean was treated under cold condition of 15°C at the stage of flowering.

The expressions of all ribosome genes were notably down-regulated after chilling stress with or without UNZ except 40S ribosomal protein S3a and 60S ribosomal protein L10-like were notably up-regulated. UNZ treatment could effectively prevent to further down-regulate the expression of ribosomal genes. Some ribosomal proteins in eukaryotes and prokaryotes exhibit extra ribosomal functions, serves both as a structural component of

the ribosome and as a regulatory protein controlling the expression of itself and other genes in the operon. Conditional over synthesis of individual ribosomal proteins demonstrated that excessive accumulation of a single key regulatory protein from an regulatory protein operon repressed expression of all genes in that operon[67,68]. 40S ribosomal protein S3a and 60S ribosomal protein L10-like maybe 2 regulatory proteins, their conditional over synthesis repressed expression of other ribosomal genes.

CONCLUSIONS

An overview of the many changes to the Vigna radiate transcriptome induced by chilling stress and UNZ has been provided. Five pathways were significantly enriched, cutin, suberine and wax biosynthesis, photosynthesis, ribosome and porphyrin and chlorophyll metabolism were down-regulated while plant- pathogen interaction was up-regulated. UNZ treatment could effectively increase the expressions of these genes in significantly down-regulated pathways and increased mungbean tolerance of chilling stress.

PLANT MATERIALS AND UNZ TREATMENTS

Mungbean "Lvfeng2" and "Lvfeng5" were planted in pots and grown in a greenhouse of Heilongjiang Academy of Agricultural Sciences in 2017. The seedlings were divided into three groups for treatment at outset of flowering stage (R1): one group (CK) was sprayed with water and leaved in nature temperature to grow for 4 days; The second group (D) was sprayed with water for 36 h, the plants were leaved in 15 ℃ to grow for 1 d, 2 d, 3 d and 4 d and then transferred to nature temperature to grow for 4 days for recovery respectively; the third group (D+S) was sprayed with 50 mg•L-1 of UNZ (5% WP) for 36 h, the plants were leaved in 15 ℃ to grow for 1 d, 2 d, 3 d and 4 d and then transferred to nature temperature to grow for 4 days for recovery respectively [13]. The plants of the 3 treatments were grown under nature conditions to maturity. The lowest external temperature of each day during the 4 days of chilling stress was above 20°C monitored by EM50 micro meteorological monitoring system.

YIELD COMPONENTS AND CHLOROPHYLL MEASUREMENT

After mungbean was grown to maturity, the pod number per plant, particle number per plant and yield were measured at maturity stage (R8). Each treatment consists of 4 replications with 10 plants per replicate.

The mungbean leaves of different treatments were taken as the materials to determine the chlorophyll content from day 1 chilling stress to day 4 of recovery. 0.1g fresh leaf was immersed 10ml alcohol and kept at room temperature for 24 h avoid light at the 1d after chilling stress. The leaf chlorophyll a, chlorophyll b and carotenoid concentrations in the supernatant of the solution were measured using a spectrophotometer at 663 and 645 nm and 470 nm, respectively [24, 25].

RNA EXTRACTION, CDNA LIBRARY CONSTRUCTION AND SEQUENCING

Lvfeng 2 was used as experimental material. There were 6 treatments in the experiment. CK1 and CK4 plants grew in natural environment for 1 d and 4 d after spraying with water for 36 h at R1 stage respectively; D1 and D4 plants grew in 15 ℃ for 1 d and 4 d after spraying with water for 36 h at R1 stage respectively; D1 +S and D4+S plants grew in 15 ℃ for 1 d and 4 d after spraying with 50 mg•L-1 of UNZ for 36 h at R1 stage respectively. Each treatment consists of 3 replications. Collection of samples were carried out at 8:00-9:00 a.m. Mixed samples of three leaves in the treatment of CK, D and D+S were used for RNA extraction (Invitrogen Trizol Reagent of RNA extraction kit 15596018). Eighteen libraries were constructed using a NEBNext Ultra RNA Library PrepKit for Illumina (NEB, USA) and sequenced using an Illumina HiSeqTM2000 (Beijing Biomarker Technologies Co.).

ASSEMBLY AND FUNCTIONAL ANNOTATION

The raw sequencing reads were cleaned by removing adaptors and low-quality reads as previously described [26]. After filtering, Bowtie software (version 2.2.5) was used to map the clean reads to the mungbean (VC1973A) reference genome (463.638 Mbp) [27], and then processed with RSEM [28] to estimate the expression levels with the FPKM value. To identify DEGs, the criteria applied were an FDR (false discovery rate) less than 0.01 and an absolute value of log2 ratio of at least 1. The screened DEGs were mainly analyzed by GO function and KEGG pathway enrichment. GO annotations of DEGs, mapped GO function to the corresponding secondary features based on unigene's GO annotation were extracted and drew the histogram [29]. The KEGG pathway enrichment analysis was implemented via KOBAS2.0 (http://kobas.cbi.pku.edu.cn/home.do)[30].

QUANTITATIVE REAL-TIME PCR VERIFICATION

Quantitative real-time PCR (qRT-PCR) were performed toexamine the expression patterns shown by the RNA-seq analysis. Ten genes were selected on the basis of their potential functions in RNA-seq. The actin gene of mungbean was used as the internal control in this study. The sequences of each primer are shown in the Table S1. RNA (1 μg) of each treatment was treated with DNAse I (Invitrogen), translated into first-strand cDNA, which was synthesized with TransScript One-Step gDNA Removal (Super Script) and cDNA Synthesis SuperMix (TransGene Biotech), and then stored at −20°C for subsequent analysis. Each PCR reaction contained 20 μl mixture, consisting of 2 μl cDNA, 10 μl of 2× TransStart Top Green qPCR SuperMix, and 0.4 μl of the forward and reverse primers. All qRT-PCRs were performed in three technical replicates in Bio-Rad CFX96 and performed in two steps: predenaturation for 2 min at 94°C, 45 cycles of denaturation for 2 s at 94°C, and annealing/extension for 15 s at 60°C. The housekeeping gene actin was used as internal standard to calculate the relative expression level by the 2−ΔΔCt method [31]. Each measurement included three biological and three technical repetitions.

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