Transcriptome Analysis of Photosynthetic Characteristics was Induced by Low Temperature Stress in *Brassica napus* L.

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Abstract

Background RNA Sequencing (RNA-Seq) technique could be utilized to compare the transcription groups of two different cold-resistant rapeseed leaves responding to low temperature at the seedling stage, analyze the photosynthetic characteristics of rapeseed subjected to low temperature stress, and identify the related genes for low temperature induction in rapeseed leaves. Results Using cold-tolerant variety 17NS and sensitive variety NF24 as experimental materials, carrying out RNA-Seq analysis by photosynthetic parameter determination and Illumina HiSeq™ platform. and screen out the KEGG significant enrichment pathway related to photosynthetic characteristics under low temperature stress. Differential Expressed Genes (DEGs) were used for real-time PCR to verify the reliability of RNA-Seq results. The results showed that the response of *Brassica napus* L. to low temperature stress mainly was achieved by inhibiting photosynthesis, the cold-tolerant variety 17NS had a strong ability to maintain membrane system stability and structural integrity after 24 h of low temperature stress, while the sensitive variety NF24 photosynthesis was significantly inhibited. Two pathways of Photosynthesis and Photosynthesis-antennas, which were significantly correlated with photosynthetic characteristics and low temperature stress were screened by KEGG enrichment. The results of DEGs indicated that 64 differentially expressed genes in these two pathways were induced by low temperature stress, and 8 of them were up-regulated expression and 56 of them were down-regulated expression. The expression pattern of DEGs was consistent with the results of RNA-Seq analysis by qRT-PCR detection and confirmed the reliability of RNA-Seq results. Conclusion Our study analysis and identified 17 low-temperature-induced photosynthetic-related candidate genes in *Brassica napus* L., and the GO and KEGG metabolic pathways clarified the molecular function of differentially expressed genes.

Background

Brassica napus, as one of the main oilseed crops in China, is widely distributed and highly adaptable. With the northward movement of cabbage winter rape, the planting range of *Brassica napus* is also expanding day by day. The quality, yield and lodging resistance of the *Brassica napus* are better than those of the *Brassica rapa*, but the cold resistance is weak and is greatly affected by the low temperature stress [1]. Low temperature will cause the delay of sowing date and growth period of soybean, corn, rice and other crops[2], which is the main factor leading to reduce of crop yield loss [3, 4]. Rape as the overwintering crop second only to winter wheat, is also deeply hurt by low temperature[5]. *Brassica napus* L., one of China's major oil crops, is also deeply damaged by low temperature stress. The cold-tolerant ability of *Brassica napus* L. is far less than that of cabbage-type winter rape. The middle and lower reaches of Yangtze river, extreme low temperature was frequently occurrence during the flowering stage of *Brassica napus* L. in the spring, the photosynthesis was significantly inhibited and the supply of photosynthetic products was insufficient, resulting in the loss of flower and fruit, and the yield was seriously reduced[6]. Therefore, the low temperature-induced photosynthetic characteristics of *Brassica napus* L. was studied, and the potential genes in response to low temperature and regulating photosynthesis were explored. It is important to clarify the molecular functions and metabolic pathways

of these genes. Previous studies have found that plants themselves can form a series of defense mechanisms during evolution in order to adapt to low temperature stress[7]. Low temperature mainly causes damage to plant leaves, roots, growth points and flowers[8], causing physiological and biochemical changes such as penetrating substances, photosynthetic parameters[9, 10], photosynthesis, respiration and antioxidant enzyme systems, which will cause plant development poor, metabolic and molecular dysfunction and even death[11-13]. Low temperature inhibits plant photosynthetic pigment content and physiological processes such as photosynthesis[14-17]. Photosynthesis is the main pathway for plant vegetative organs, which can use light energy to synthesize organic compounds from carbon dioxide and water, from light-dependent parts (photoreaction) and light independence part (dark reaction, carbon fixation) composition. Low temperature stress can cause a decrease in photosynthetic parameters such as agronomic traits, photosynthetic rate, transpiration rate, leaf stomatal conductance and intercellular CO₂ concentration[18, 19]. Chloroplasts, as the main organelles for photosynthesis of plants, are also the most sensitive target organs for stress such as low temperature[20]. After chilling stress, the morphological structure of chloroplasts changes significantly. The chlorophyll content is reduced to reduce the light-capturing ability of leaves [21], and the grainy lamella are reduced or blurring, the number of them were reduced, the number of hungry granules increased and the starch granule content decreases or even disappears[22-25]. In recent years, with the continuous improvement of molecular biotechnology, predecessors have successfully isolated many low temperature stress response genes[26-28] and antifreeze proteins[29, 30] through genomics research. At present, the research of omics analysis on *Brassica napus* L. is also relatively mature. Du Chunfang found that after treatment of *Brassica napus* L. at low temperature for 24h, transcriptomics analysis got 17824 significantly different genes by high-throughput sequencing analyzed, and 573 differential protein [31] were identified with proteomics analysis by iTRAQ sequencing. There are few studies on photosynthetic regulation mechanisms based on transcriptomics analysis of low temperature stress response. Therefore, based on high-throughput RNA-Seq technology, combined with leaf ultra-microstructure and photosynthetic gas parameters, the photosynthesis response genes of *Brassica napus* L. leaves under low temperature stress and the photosynthetic regulation mechanism of *Brassica napus* L. under low temperature stress was revealed were analyzed.

Results

Responses of plant morphology and leaf development to low temperature stress

Observation on the morphological development of plants under low temperature stress

The *Brassica napus* L. was treated at low temperature for 24 h at 4 °C (Fig.1). The morphological changes of the plants showed that the leaves of 17NS and NF24 had wilting and sagging, of which the

old leaves of sensitive NF24 were obviously wilted, the new were leaves slightly wilted, and the old leaves of cold tolerant 17NS were wilted while the new leaves barely changed. The two varieties after low temperature treatment were restored at room temperature for 24 hours. It was found that the old leaves of the sensitive variety NF24 all died, the new leaves returned to life, and the cold-tolerant variety 17NS recovered after 24 hours, the new leaves and the old leaves are green and revitalized. The results showed that the new leaves showed strong tolerance and adaptability in response to low temperature stress and the leaves of the cold-resistant variety 17NS have strong frost resistance under low temperature stress.

Observation chloroplast morphological under low temperature stress by transmission electron microscopy

Transmission electron microscopy observation of rapeseed leaves under low temperature stress showed that low temperature stress had a great influence on the structure and quantity of organelles in leaf cells, which were manifested in the morphological structure of chloroplasts, the number of hypersalotic particles and starch granules were changed. The varieties with different cold resistance have different degrees of changes in organelles. After 24 hours of cold tolerance of 17NS cold-resistant varieties, the granum were dispersed, the chloroplasts shrank, the volume became smaller, and the starch granules became smaller (Fig.2 -A1). Compared with the control (Fig.2-B1), the number of starch granules increased. There are up to 3 starch granules in the chloroplast, and the number of starvation granules increases (Fig.2-B2), the number and morphology of chloroplasts are not obvious (Fig.2-B3). In the sensitive variety NF24, the control (Fig. 2-C1) starch granules were faintly visible, disappeared after low temperature stress, and the thylakoid layer was significantly reduced (Fig.22-D1). The chloroplast changes from a spindle shape close to the cell wall to a paramecium shape far away from the cell wall, the number of mitochondria increased significantly (Fig.2-D2), and the number of chloroplasts in individual cells decreased (Fig.2-D3). It indicated that the varieties with different cold resistance had different responses to chloroplast photosynthesis and respiration after low temperature stress.

Response of Physiology and Biochemistry of Brassica napus L. to Low Temperature Stress

Response of cell membrane of Brassica napus L. to low temperature stress

When the plant tissue is damaged by the stress, the low temperature stress firstly damages the membrane system of the cell, the membrane function is impaired or the structure is destroyed, and the

permeability is increased, the water-soluble substances in the cells, including the electrolyte, will have different degrees of extravasation. The relative conductivity measurement showed that the relative conductivity of the cold-tolerant variety 17NS and the sensitive variety NF24 was almost the same before the low temperature stress. After the low temperature stress, the relative conductivity increased significantly (Fig.3). The relative conductivity of the sensitive variety NF24 increased sharply. It is 0.869 ms/cm³, which is larger than the rising value of 17NS (0.760 ms/cm³), indicating that the electrolyte of the cell membrane of *Brassica napus* L. has a large amount of extravasation after low temperature damaging. Sensitive varieties have large electrolyte exosmosis, poor cold tolerance and adaptability while cold resistant variety have strong ability to maintain the relative stability and structural integrity of the cell membrane system, and better resistance to low temperature damage.

Effects of low temperature stress on photosynthetic parameter

The photosynthetic parameters of 0~24h under low temperature stress showed that the low temperature significantly reduced the net photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (Tr), and increased the intercellular CO₂ concentration (Ci). Pn, Gs, Tr and Ci between different varieties were significantly different with the treatment time (Fig. 4). Before the low temperature stress, the Pn, Gs and Tr of the cold-tolerant variety 17NS were lower than the cold sensitive variety NF24, but the Ci was higher than the cold sensitive variety NF24. After 24 h of low temperature stress, the Tr, Gs and Pn of the cold-tolerant 17NS leaves were significantly lower than the control, with the reductions being 30%, 60% and 72%, respectively. During the low temperature stress of NF24, the Pn, Gs and Tr of the leaves increased first and then decreased, however, it was significantly higher than the control group after 24h of stress, Pn decreased by 66.13% compared with the control, and Tr and Gs increased by 205.01% and 280% compared with the control. The variation range of cold sensitive cultivars was more unstable than that of cold resistant cultivars at the low temperature stress. The Ci of the cold-tolerant variety 17NS and the cold-sensitive variety NF24 were first decreased and then increased. After 24 hours, the Ci increased by 16.29% and 20.01%, respectively. Therefore, low temperature significantly affects photosynthesis and stomatal gas exchange, and has a more greater impact on Tr, Gs and Pn of cold sensitive varieties.

Effect of low temperature on chlorophyll content of *Brassica napus* L

It can be seen from Fig.5 that the chlorophyll a and chlorophyll b contents showed a significant downward trend after 24 h of low temperature stress in the functional leaves of different *Brassica napus*

L., and the chlorophyll a and chlorophyll b contents of sensitive NF24 sharp fell compared with the cold resistant variety 17NS, which was 0.184 mg/ g FW and 0.387 mg/g FW. The carotenoid content increased with the decrease of temperature, and the cold resistant variety 17NS increased faster, reaching 0.337 mg/g FW. It can be seen that the content of chlorophyll a and chlorophyll b in *Brassica napus* L. decreased with low temperature stress, and the content of carotenoids increased, and the decrease of chlorophyll content of cold resistant varieties was smaller than that of sensitive varieties, indicating that the cold resistant varieties had stronger resistance after low temperature stress. The strong photosynthetic capacity of the cold resistant variety is conducive to maintaining the normal growth of seedlings for adversity injury.

Screening of differentially expressed genes in Brassica napus L. under low temperature stress

According to the transcriptome data of *Brassica napus* L. under low temperature stress, the difference gene expression was analyzed by using edgeR software. The differential gene was screened by FDR and log2FC. The screening criteria were $FDR < 0.05$ and $|\log_2FC| > 1$. Among the different cold cultivars, NF24t0-17NSSt0 was paired to obtain 18978 genetic difference genes (Table 1.). NF24t-17NSSt paired to obtain 22019 differential genes, and the genetic background difference was removed to obtain significant differences in low temperature induction. A total of 3041 genes were expressed (739 differential genes up-regulated expression and 2302 differential genes down-regulated expression).

KEGG pathway analysis

By KEGG Pathway analysis of differential genes, the pathway with $Qvalue \leq 0.05$ was chosen as the pathway for differential gene enrichment. The results showed that in all differentially expressed genes, after 24 h of low temperature stress, 4229 differentially expressed genes of NF24t0-17NSSt0 and 4675 differentially expressed genes of NF24t-17NSSt were enriched in 131 and 132 pathways, respectively. Twenty of the most significant pathways were screened by $P < 0.05$ (Fig.6), and Ribosome and Metabolic pathway were the most significant pathways before and after low temperature stress, respectively.

Light and regulation related differential expression gene analysis

The analysis of pathways related to photosynthetic characteristics after low temperature stress revealed that Photosynthesis and Photosynthesis-antenna proteins are significant difference in energy metabolism, of which Photosynthesis-antennas were ranked in the top 20 in the NF24 t-vs-17NS t comparison group (Fig.6). Using Venny online software (<http://bioinfogp.cnb.csic.es/tools/venny/>) to analyze the DEGs(Differentially Expressed Genes) of different varieties under low temperature stress (Fig. 7), it was found that the significantly DEGs of Photosynthesis have 50 and the significantly DEGs of Photosynthesis-antennas have 12 in the NF24t0-17NSt0 comparison group, and the significantly DEGs Photosynthesis have 69 and the significantly DEGs of Photosynthesis-antennas have 30 in the NF24t-17NSt comparison group.

Analysis of photosynthesis regulation pathway induced by low temperature stress

After low temperature stress, in the photosynthesis and photosynthesis-antenna proteins related to photosynthetic regulation, DEGs repeated with NF24t0-vs-17NSt0 were removed from the DEGs of NF24t-vs-17NSt. 64 significant DEGs were obtained (As shown in the blue part of Fig.8). There were 38 DEGs in the Photosynthesis pathway, of which 7 DEGs up-regulated expression and 31 DEGs down-regulated expression, 26 DEGs in the Photosynthesis-antenna proteins pathway, of which one DEGs up-regulated expression and 25 DEGs down-regulated expression (Fig.9 and Fig.10).

Go function classification of specific expression genes

Through the function annotation (Fig.11) of Go function, it is found that 64 specific DEGs related to photosynthetic regulation in the differentially expressed genes under low temperature stress are mostly annotated into cell component processes and biological processes, and the number of differentially expressed genes are least in molecular function. It indicated that low temperature stress affected the growth and development of *Brassica napus* L. Among them are mainly cellular processes, metabolic processes, cells, cellular components, organelles, binding, catalytic activity and transport activity.

qRT-PCR validation of specific expression genes

The 64 low-temperature-induced specific DEGs (Table 2), mainly with photosynthetic system I, photosynthetic system II, oxygen-releasing protein, iron Oxygen-reducing protein-NADP reductase,

chlorophyll protein, and ATP synthase are related—most of the genes of sensitive NF24 and cold-tolerant cultivar 17NS were down-regulated after low temperature stress. From Photosynthesis pathway screening one up-regulated DEGs and 7 up-regulated DEGs, from Photosynthesis - antenna proteins pathway screening one gene up-regulated DEGs and 8 down-regulated performed with qRT-PCR. The results (Fig. 12) of qRT-PCR indicate that were consistent with the results of RNA-Seq analysis—the expression of 17 DEGs in qRT-PCR and RNA-Seq showed the same trend, confirming the reliable results of RNA-Seq analysis.

Low temperature stress inhibited the expression of ferredoxin-coenzyme ii reductase, photosystem I reaction, oxygen-releasing protein, ATP synthase, photosystem II repair protein PSB27-H1, photosystem I chlorophyll a/b binding protein, chlorophyll a-b binding protein and so on, and induces expression of ferredoxin and chlorophyll a-b binding protein CP26. It is indicated that the inhibition of photosynthesis by low temperature mainly occurs in chloroplasts, and photosynthesis is inhibited by inhibiting photosynthetic pigments and photoreaction systems.

Analysis of cold resistance of Brassica napus L

To promote the breeding process of Brassica napus L. were used, the existing with the aid of Brassica genome information, analysis identified 1173 flowering related genes of Brassica napus L.[34], Two QTLs related to the content of fatty glycosides in seeds [35] and Twenty abiotic drought-related genes[36], There were 1651 up-regulated genes and 405 down-regulated genes in tea trees after low temperature stress and are closely related to photosynthesis regulation[37, 38]. At present, there are many researches on the analytical mechanism of plant cold tolerance, for example, *RbohB* gene[26], *COLD1*[39] and chitinase gene (*BnCHB4*)[28], COR[40], ICE[41], HSF[42], CBF[43] and other antifreeze proteins. This study found that low temperature stress could cause to the chloroplast number of Brassica napus L. reduce were used, the volume contraction of the chloroplast, grana lamella fuzzy, addicted to hungry particle number increases, chlorophyll a, chlorophyll b content decreased significantly, the photosynthetic rate (Pn), transpiration rate (Tr) and stomatal conductance (Gs) and intercellular CO₂ concentration (Ci) go down and then it goes up. It indicates that plants can alleviate the damage of low temperature by adding osmotic regulatory substances, and the varieties with stronger cold resistance are hindered in CO₂ supply and CO₂ assimilation capacity [44-46], which eventually leads to decreased photosynthetic capacity and affects photosynthetic response [47-49], which is consistent with previous research results[22, 24, 25, 50].

Transcriptome analysis under low temperature stress

Through the transcriptome analysis, get two significantly photosynthesis regulation pathways with Photosynthesis and Photosynthesis-antenna proteins. NF24t-v-17NSt0 for comparison, NF24t-v-17NSt for processing, 7 DEGs has up-regulated and 31 DEGs has down-regulated in Photosynthesis pathway, 1 DEGs has up-regulated and 25 DEGs has down-regulated in Photosynthesis-antenna proteins pathways, a total of 64 specific gene expression. Analyzed found that the low temperature stress lead to PS I and PS II be suppressed[51], was mainly related to PS I, PS II, increasing oxygen protein, Ferriox-NADP reductase put oxygen reductase, phylochlorin, ATP synthesis enzyme and so on, partially restore ferredoxin is used for by PETH reduction NADP +, which is used for producing NADPH carbon assimilation and other biosynthetic pathway, and can also be mediated electron flow to protect leaf light system [38]. Genetic variations of top up or down expression by qRT - PCR analysis, test and verify the reliability of the RNA - Seq results, and access to 17 stable expression of photosynthetic regulation candidate genes, expression of 15 genes, 2 raised expressed genes, confirmed that the low temperature stress on the stress of *Brassica napus* L. were used mainly by inhibition of photosynthesis and light reaction system. Further research is needed on the molecular mechanism of candidate genes.

Conclusions

Through the analysis of the photosynthetic parameters of *Brassica napus* L. under low temperature, the results showed that the photosynthesis was inhibition at low temperature stress, and the strong cold resistance varieties is suppressed to a lesser degree. By RNA - Seq analysis found that the response of low temperature stress significantly photosynthesis pathway for Photosynthesis and Photosynthesis-antenna proteins, got 64 significantly DEGs. By analyzing qPCR, verified the reliability of the transcriptome and determine the 17 low temperature response of photosynthesis regulation genes.

Methods

Materials

17 NS, a strong cold winter variety of *Brassica napus* strains and professor Liu of Gansu Agricultural University breeding from "Tiangan" of *Brassica napus* L. as the female parent and "Longyou 7" of *Brassica rapa* L. as the male parent to selected homozygous line, and the cold sensitive material NF24 is an inbred line of *Brassica napus* L. which both have been undertook the formal identification by professor Liu in my study.

The aim

In order to reveal the molecular mechanism of rapeseed's adaptation to low temperature stress, RNA Sequencing (RNA-seq) technique is utilized to compare the transcription groups of two different cold-

resistant rapeseed leaves responding to low temperature at the seedling stage, analyze the photosynthetic characteristics of rapeseed subjected to low temperature stress, and identify the related genes for low temperature induction in rapeseed leaves, by the determination of photosynthetic parameters and the RNS-seq analysis of Illumina HiSeq™ platform, the KEGG enrichment pathway associated with photosynthetic characteristics was identified under low temperature stress, and the (DEGs) in the significant enrichment pathway were Expressed by real-time quantitative PCR, to verify the reliability of the RNA-Seq results.

The design

By pot experiments, the seeds with the same clean and uniform size were placed in a petri dish with two layers of filter paper as the germination bed, cultured in an artificial climate chamber (6000 lux light 25 ° C / 14 h and 20 ° C / 10 h) , and then sown on mix nutrient matrix and vermiculite in a 3:1 pot after seed germination, artificial light incubator culture, light 25 ° C / 14 h and 20 ° C / 10 h, relative humidity 40%. When the seedlings grow to 5~6 leaf stage, the low temperature -4 °C light incubator starts low temperature treatment for 0h and 24h, two pots of which are used for photographing, two pots are used for photometry, and the other two are used for sampling. Wearing a mask and sterile enzyme-free gloves collects photographs and measures photosynthetic parameter, and liquid nitrogen quick freeze the newly-fully-expanded leaves after rinsing with sterilized up-water and stored in a -70°C ultra-low temperature freezer for determination of various indicators.

Determination of photosynthesis index

Six pieces leaves without shade, no damage, no pests, and good growth of the fourth functional leaf were selected for each material, and the small-label was placed. According to the method of Liu Zigang[32], the LI-6400 portable photosynthetic apparatus produced in the United States was used. The changes of photosynthetic gas exchange parameters (G_s , C_i , T_r , P_n) of new leaves that were fully developed at 0h, 12h and 24h after low temperature treatment were repeated three times, setting the light intensity to $1200\mu\text{mol}/\text{m}^2$, and the indoor temperature of the leaves was 20°C. .

Determination of chlorophyll content and relative conductivit

The chlorophyll content and relative conductivity were measured according to the scholar[33].

Transmission electron microscope

On the new leaves which were completely unfolded at 0 h and 24 h after low temperature treatment, a rectangular blade of 2 mm² was cut out with a blade carefully, and placed in a fixing solution containing 2 ml of 3% glutaraldehyde. According to the scanning procedure of electron microscopy, PB was rinsed three times, 15 min/time, hungry acid was fixed at 4 °C for 2 h, and then rinsed with PB three times, 15 min/time, according to 50%, 70%, 80%, 90%, 100%, acetone I and Acetone II gradient dehydration for 10 min each time. Acetone and resin embedding agent were embedded in an oven at 35 °C for 12 h, and then transferred to an oven at 60 °C until the material was completely polymerized. With the ultramicrotome, cutting into slices and fixing on a copper grid with 2% uranyl acetate and lead citrate for double staining and photographs were taken with a Hitachi JEM-1230 projection electron microscope.

Analysis of transcriptome data

The transcriptome analysis was carried out using cold-tolerant variety 17NS and low-temperature sensitive variety NF24. The low temperature stress of -4 °C was used as the control, and the low temperature stress of -4 °C was used as the treatment for 24 hours. Two varieties were treated at two different times, a total of four samples. Total RNA was extracted from the sample by the company named GENE DENOVO, the mRNA was enriched with magnetic beads with Oligo (dT), and the fragmentation buffer was added to the obtained mRNA to make a fragment. As a short fragment, the first strand of cDNA was synthesized with random hexamers using the mRNA after fragmentation, and the second strand of cDNA was synthesized by adding buffer, dNTPs, RNase H and DNA polymerase I, after QiaQuick The PCR kit was purified and eluted with EB buffer, the end of the repair, base A was added, the sequencing linker was added, and the fragment of interest was recovered by agarose gel electrophoresis, and PCR amplification was performed to complete the whole library preparation work. A good library was sequenced using Illumina HiSeqTM. After filtering the off-data, the clean data is compared, the reads are aligned to the reference genome, and the transcripts are assembled using Cufflinks to obtain known transcripts and new transcripts. Then, the obtained gene was analyzed and statistically analyzed, and differential expression analysis and functional enrichment analysis were performed. In addition, structural analysis of genes, including genetic structure optimization, variable shear, and the like.

Total RNA extraction from leaves

RNA extraction The total RNA of 17NS-0h, 17NS-24h, NF24-0h and NF24-24h was extracted according to the instructions of Tiangen kit (DP419). The reverse transcription was reversed according to the TaKaRa cDNA First-strand Synthesis Kit (RR036A). Single-stranded cDNA was obtained by transcription, and the concentration was measured by Bio Mater 5 ultra-micro UV spectrophotometer, and then stored in a refrigerator at -80 °C for use.

Real time PCR

Using the PrimeScript RT reagent Kit (Dalian Bioengineering Co., Ltd., Dalian), reverse transcription kit to reverse transcribe the RNA extracted from the previous step to synthesize the first strand of cDNA. According to the transcriptome data, the reverse transcription product was diluted 100-fold as a template, primers were designed with Primer Premier 5.0 (Table 3), the internal reference was Bnactin, and the primer was synthesized by Shanghai Biotech Co., Ltd., using SYBR Premix Ex Taq (Bao Bioengineering Co., Ltd., Dalian) Quantitative kit, two-step amplification, each reaction is set to 3 repeats, the reaction system and procedures are shown in Table 4, according to the amplification efficiency E of the gene, the correction $2^{-\Delta\Delta C_t}$ is calculated as $(1+E)^{-\Delta\Delta C_t}$ relative quantification. As a result, statistical analysis was performed using Excel 2010 and SPSS 22.

Thermal cycle program: 95°C 30 sec predegeneration

PCR amplification: 95°C 5sec degeneration, 60°C 30sec annealing, Total 40 cycles,

solubility curve: 95°C 15sec, 60°C 30 sec, 95°C 15sec

Abbreviations

DEGs : Differential Expressed Genes

Pn: Photosynthetic rate)

Gs: Stomatal conductance

Tr: Transpiration rate

Ci: Increased the intercellular CO₂ concentration

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Author Contributions

JJ interpreted conceptualization, data curation, writing – original draft and editing. LZ made formal analysis, done funding acquisition and supervision. ZY, MW, XM, XC, and JJ participated in test design and operation. LX, SW, WJ, FY, and LL provided methodology and software. All authors read and approved the final manuscript.

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References

1. Li, M.A., et al., *Expression of cold resistance genes from winter rapeseed of Brassica rapa and B.napus under chilling stress*. Chinese Journal of Oil Crop Sciences, 2016(In Chinese).
2. Li-Xia, J., et al., *Characteristics of Low Temperature in Spring and its Effect on Crops Seeding Dates in 2013 in Heilongjiang Province*. Chinese Journal of Agrometeorology, 2019(In Chinese).
3. Hao, J., L. Zhang, and F. Xie, *EFFECTS OF THE LOW TEMPERATURE ON DEFENSE ENZYME ACTIVITIES OF DIFFERENT CHILLING-TOLERANT SOYBEAN CULTIVARS DURING THE GERMINATION*. Soybean Science, 2007(In Chinese).
4. Cai, Y., et al., *Effects of low temperature conditioning on chilling injury and quality of cold-stored juicy peach fruit*. Transactions of the Chinese Society of Agricultural Engineering, 2010. **26**(6): p. 334-338(In Chinese).

5. Yıldız, M. and H. Terzi, *Proteomic analysis of chromium stress and sulfur deficiency responses in leaves of two canola (Brassica napus L.) cultivars differing in Cr(VI) tolerance*. Ecotoxicol Environ Saf, 2016. **124**: p. 255-266.
6. Yu-Qin, T.U. and X.L. Dai, *Effects of continuous low temperature overcast and rainy weather on yield and oil content of Brassica napus during flowering stage*. Chinese Journal of Oil Crop Sciences, 2011(In Chinese).
7. Kun, L.U., et al., *Identification of Drought Stress-Responsive Genes in Leaves of Brassica napus by RNA Sequencing*. Scientia Agricultura Sinica, 2015(In Chinese).
8. Chen, J., et al., *Effects of low temperature storage on chilling injury and energy status in peach fruit*. Transactions of the Chinese Society of Agricultural Engineering, 2012. **28**(4): p. 275-281(In Chinese).
9. Wang, X.E., et al., *Effects of low temperature treatment on physiological-biochemical characteristics of six seedlings*. Journal of Nanjing Forestry University, 2009. **33**(3): p. 139-142(In Chinese).
10. Han, M., et al., *Effects of Rootstock and Scion Interaction on Photosynthesis and Nitrogen Metabolism of Grafted Tomato Seedlings Leaves Under Low Temperature Stress*. Acta Horticulturae Sinica, 2018(In Chinese).
11. Öquist, G., *Effects of low temperature on photosynthesis*. Plant Cell & Environment, 2010. **6**(4): p. 281-300.
12. Luxova, M., *The effect of low temperature on root respiration in maize*. Biologia, 1999. **54**(4): p. 453-458.
13. Dong, H.L. and C.B. Lee, *Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays*. Plant Science, 2000. **159**(1): p. 75-85.
14. Zhang, S.Q., Z.H. Cheng, and G.D. Geng, *Effect of Low Temperature on Eggplant-Seedling Physiological Characteristics*. Journal of Hunan Agricultural University, 2006(In Chinese).
15. Yang, Z., et al., *Changes in photosynthetic parameters and antioxidant enzymatic activity of four tea varieties during a cold wave*. Acta Ecologica Sinica, 2016. **36**(3): p. 629-641(In Chinese).
16. Xie, J.M., et al., *Changes of Three Osmotic Regulatory Metabolites in Leaves of Pepper under Low Temperature and Poor Light Stress and Relations between Its and Varietal Tolerance*. Acta Botanica Boreali-Occidentalia Sinica, 2009(In Chinese).
17. Jian, L.I., G. Geng, and Z. Cheng, *Effects of chilling stress on chill-resistance physiological and biochemical indexes of eggplant seedlings*. Journal of Northwest Sci-Tech University of Agriculture and Forestry, 2003(In Chinese).
18. WOLF and MILLATINER, *Effect of tobacco mosaic virus movement protein on photosynthesis in transgenic tobacco plants*. Journal of Plant Physiology, 2000. **156**(2): p. 253-258.
19. Shao, Y., et al., *Effects of low temperature stress on physiological-biochemical indexes and photosynthetic characteristics of seedlings of four plant species*. Acta Ecologica Sinica, 2013. **33**(14): p. 4237-4247(In Chinese).

20. Liu, Z.G., et al., *Effects of Low Nocturnal Temperature on Photosynthetic Apparatus of Winter Rapeseed(Brassica campestris L.)*. Scientia Agricultura Sinica, 2015(In Chinese).
21. Xiaojing, L., *Research Progress on the Physiological Response of Plants to Environmental Stress*. Anhui Agricultural Science Bulletin, 2018.
22. Zheng, G.H., H.Y. Zhang, and X.R. Zhong, *Changes in cell ultra-structure, membrane permeability and protective enzyme activity in Eriobotrya japonica Lindl. leaves under cold stress*. Chinese Journal of Eco-Agriculture, 2009. **17**(4): p. 739-745(In Chinese).
23. Wang, N., Y.Y. Dong, and S.U. Jin-Le, *Comparison of leaf blade ultra-structures of two camphor varieties under cold stress*. Journal of Northwest A & F University, 2013(In Chinese).
24. Tian, J.H., et al., *Effects of chilling stress on antioxidant system and ultrastructure of walnut cultivars*. Chinese Journal of Applied Ecology, 2015. **26**(5): p. 1320(In Chinese).
25. Chen, Y., et al., *Physiological Response to Cold Stress and Cell Ultra-structure Changes in Passiflora edulis Leaves*. Acta Botanica Boreali-Occidentalia Sinica, 2012(In Chinese).
26. Zhang, T.G., et al., *Cloning and Expression Analysis of RbohB Gene in Brassica campestris L*. Bulletin of Botanical Research, 2017(In Chinese).
27. Xiao-Chen, H.U., et al., *Development and Application of a Functional Marker for the Cold Stress Responsive Gene OsSADMC*. Chinese Journal of Rice Science, 2015(In Chinese).
28. Fang, Y., et al., *SDS-PAGE Electrophoresis Analysis on Apoplast Antifreeze Protein in Winter and Spring Turnip Rape(Brassica rape L.)and Their First-filial Generation*. Southwest China Journal of Agricultural Sciences, 2017(In Chinese).
29. Zhang, T., et al., *Isolation and Characterization of a CBF Gene from Brassica campestris*. Acta Botanica Boreali-Occidentalia Sinica, 2015(In Chinese).
30. Wang, Z., et al., *Response to environmental cues of BnCHB4 from Brassica napus*. Journal of Biology, 2012(In Chinese).
31. Koeslinfindelee, F., et al., *Transcriptomic analysis of nitrogen starvation- and cultivar-specific leaf senescence in winter oilseed rape (Brassica napus L.)*. Plant Science, 2015. **233**: p. 174-185.
32. Liu, Z.G., et al., *Comparison of Winter Rapeseed Varieties(Lines) with Different Cold Resistance Planted in the Northern-Extending Regions in China under Low Temperature before Winter*. Acta Agronomica Sinica, 2014. **40**(2): p. 346(In Chinese).
33. Macdougall, D.T., *Experimental plant physiology*. 1974.
34. Harper, A.L., et al., *Associative transcriptomics of traits in the polyploid crop species Brassica napus*. Nature Biotechnology, 2012. **30**(8): p. 798-802.
35. Cheng, H., et al., *Genomic identification, characterization and differential expression analysis ofSBP-boxgene family inBrassica napus*. BMC Plant Biology, 2016. **16**(1): p. 196.
36. Bing, W., et al., *Discovery of Drought-tolerant Genes in Brassica napus by Transcriptome Analysis*. Genomics and Applied Biology, 2018(In Chinese).

37. Zheng, C., et al., *Integrated RNA-Seq and sRNA-Seq Analysis Identifies Chilling and Freezing Responsive Key Molecular Players and Pathways in Tea Plant (Camellia sinensis)*. Plos One, 2015. **10**(4): p. e0125031.
38. Li, N.N., et al., *Transcriptome sequencing dissection of the mechanisms underlying differential cold sensitivity in young and mature leaves of the tea plant (Camellia sinensis)*. Journal of Plant Physiology, 2018. **224-225**: p. 144-155.
39. Ma, Y., et al., *COLD1 confers chilling tolerance in rice*. Cell, 2015. **160**(6): p. 1209-1221.
40. Horvath, D.P., B.K. Mclarney, and M.F. Thomashow, *Regulation of Arabidopsis thaliana L. (Heyn) cor78 in response to low temperature*. Plant Physiology, 1993. **103**(4): p. 1047-1053.
41. Zhang, Z., et al., *OsMAPK3 Phosphorylates OsbHLH002/OsICE1 and Inhibits Its Ubiquitination to Activate OsTPP1 and Enhances Rice Chilling Tolerance*. Developmental Cell, 2017. **43**(6): p(In Chinese).
42. Chen, S., et al., *The expressions of CsHSFs gene in relation to chilling tolerance of cucumber fruits*. Journal of South China Agricultural University, 2015(In Chinese).
43. Talanova, V.V. and I.E. Malysheva, *Effect of Stress Factors on Expression of the Gene Encoding a CBF Transcription Factor in Cucumber Plants*. Doklady Biological Sciences, 2008. **423**(1): p. 419-421.
44. Songyou, Z. *Effects of Different LED Light Qualities on Photosynthetic Characteristics of Basil*. Chinese Journal of Tropical Crops, 2017(In Chinese).
45. Farquhar, G.D. and T.D. Sharkey, *Stomatal conductance and photosynthesis*. Annual review of plant physiology, 1982. **33**(1): p. 317-345.
46. Ensminger, I., F. Busch, and N.P. Huner, *Photostasis and cold acclimation: sensing low temperature through photosynthesis*. Physiologia Plantarum, 2006. **126**(1): p. 28-44.
47. Zhang, Z.G. and Q.M. Shang, *Effect of combined low temperature, poor light and salt stress on photosynthetic characteristics of pepper seedling leaf*. Chinese Journal of Eco-Agriculture, 2010. **18**(1): p. 77-82(In Chinese).
48. Perera, N.H., E. Hartmann, and A.S. Holaday, *Regulation of cotton photosynthesis during moderate chilling*. Plant Science, 1995. **111**(2): p. 133-143.
49. Huner, N.P.A., G. Öquist, and A. Melis, *Photostasis in Plants, Green Algae and Cyanobacteria: The Role of Light Harvesting Antenna Complexes*. 2003.
50. Zhang, G. and J. Yu, *Effects of low temperature on photosynthetic characteristics and starting time of pepper seedlings under weak light*. Acta Botanica Boreali-Occidentalia Sinica, 2006(In Chinese).
51. Zhang, Z.S., et al., *The higher sensitivity of PSI to ROS results in lower chilling–light tolerance of photosystems in young leaves of cucumber*. Journal of Photochemistry & Photobiology B Biology, 2014. **137**(8): p. 127-134.

Tables

Table 1 All significant differentially expressed genes identified by RNA-Seq

Paired comparison	Significantly up-regulated differential genes	Significantly down-regulated differential genes	Significantly different number of genes
NF24 0h-NF24 24h	12583	24414	36997
17NS 0h-17NS 24h	11710	23141	34851
NF24 0h-17NS 0h	10708	8270	18978
NF24 24h-17NS 24h	11447	10572	22019

Table 2 Genetic information of 17 differentially expressed genes

Gene ID	Description
ncbi_106366341	ferredoxin--NADP reductase, leaf isozyme 1, chloroplastic-like
ncbi_106360470	photosystem I reaction center subunit VI, chloroplastic-like
ncbi_106400646	oxygen-evolving enhancer protein 2, chloroplastic
ncbi_106430073	ATP synthase gamma chain 1, chloroplastic-like
ncbi_106381576	ATP synthase gamma chain 1, chloroplastic-like
ncbi_106405529	photosystem I reaction center subunit VI, chloroplastic-like
ncbi_106401683	photosystem II repair protein PSB27-H1, chloroplastic
ncbi_106350097	ferredoxin-1, chloroplastic
ncbi_106371874	photosystem I chlorophyll a/b-binding protein 5, chloroplastic-like
ncbi_106382137	chlorophyll a-b binding protein 2.4, chloroplastic-like
ncbi_106439795	chlorophyll a-b binding protein 2.1, chloroplastic
ncbi_106349237	chlorophyll a-b binding protein 2.4, chloroplastic-like
ncbi_106396209	chlorophyll a-b binding protein 1, chloroplastic
ncbi_106447496	chlorophyll a-b binding protein 1, chloroplastic
ncbi_106447497	chlorophyll a-b binding protein 1, chloroplastic-like
ncbi_106421061	photosystem I chlorophyll a/b-binding protein 3-1, chloroplastic
ncbi_106440450	chlorophyll a-b binding protein CP26, chloroplastic-like

Table 3 qRT-PCR identification primers used in this study

KEGG pathway	Gene ID	Sequence of primer (5-3')	Amplification length (bp)	Amplification efficiency
Photosynthesis	ncbi_106366341	AGATCCTAATGCCACCATCATC (F) GTAGTCCTCATGCTCCTCAAAG (R)	100	101.80%
	ncbi_106360470	CTCCTTACAACCCACTTCAGAG (F) GAGCCACCTCCAAGAATCAA (R)	100	96.80%
	ncbi_106400646	CTACCGACAAGAAGTCCATCAC (F) AGGCAGTCTCACCGAAGTA (R)	102	88.28%
	ncbi_106430073	GCAGAGGCAAGGATCAAAGA (F) GTAAGGACGACGGAGGAAATAAG (R)	99	93.16%
	ncbi_106381576	GCAGAGGCAAGGATCAAAGA (F) GTAAGGACGACGGAGGAAATAAG (R)	99	100.09%
	ncbi_106401683	CCGAGATTTGCTCTCCTTATCC (F) CCGACGTATCTTTCACGTACTC (R)	119	103.61%
	ncbi_106405529	CTCCTTACAACCCACTTCAGAG (F) GAGCCTCCTCCAAGAATCAAA (R)	100	95.07%
	ncbi_106350097	CATAACACCAGATGGAGAGAAGG (F) CAACCGAGCCAGAGACTATTT (R)	149	102.17%
	ncbi_106371874	TAGCTGGAGATTACGGGTTTG (F) GGTACGAAGTAGGTTCGGTTAAG (R)	143	122.90%
	ncbi_106382137	CTCGGAAACCCTAACTTGATCC (F) CACCTCCAATTCTGTACCCTTC (R)	100	101.97%
Photosynthesis-antenna proteins	ncbi_106439795	AACCGTGAGCTCGAAGTAATC (F) CTCCGAATTTGACTCCGTTCT (R)	100	98.06%
	ncbi_106349237	AACCGTGAGCTCGAAGTAATC (F) ATCTGAGAACCTGCCTTGAAC (R)	131	102.84%
	ncbi_106396209	GAAGGTGGGCTCGACTATTT (F) CGACTCTGTAACCCTCAACAG (R)	112	94.26%
	ncbi_106447496	GAAGGAGGGCTTGACTACTTG (F) CTCAACAGCTCCCATGAGAAT (R)	99	103.51%
	ncbi_106447497	AAACGGAAGGTTGGCTATGT (F) GCCAAATGGTCAGCAAGATTC (R)	96	100.88%
	ncbi_106421061	CTGTCAAGCAAGGAGCAAAC (F)	102	97.35%

		CAAGAGGGTCTGAATCCATAGTC (R)		
	ncbi_106440450	AACCTTGTTCTGGCTGTAGTT (F)	98	93.57%
		GGGTGTAGCTTGTCTCTCAA (R)		
Reference gene	Bnactin-F	TCCATCCATCGTCCACAG		106.80%
	Bnactin-R	GCATCATCACAAGCATCCTT		

F: Former primer R: Reverse primer.

Table 4 qPCR reaction system

Reagent	Volume
SYBR ^R Premix Ex Taq TM II	10µl
Forward primer	0.8µl
Reverse primer	0.8µl
ROX Reference Dye II	0.4µl
Template cDNA	2µl
ddH ₂ O	6µl

Figures

(a)

(b)

(c)

(d)

(e)

(f)

Figure 1

Morphological were observed of *Brassica napus* L. under low temperature stress. (a)17NS: -4°C-Treatment-0h; (b)17NS: -4°C-Treatment-24h; (c)17NS: -4°C-Treatment-24h-Recovery-24h; (d)NF24: -4°C-Treatment-0h; (e)NF24: -4°C-Treatment-24h; (f)NF24: -4°C-Treatment-24h-Recovery-24h

Figure 2

Transmission electron microscopic view of *Brassica napus* L. under low temperature stress

Figure 3

The influence of low temperature to conductivity in rapeseed leaves. Note: The figure with $P < 0.01$ as highly significant, marking "A, B or C", as the same below.

Figure 4

Changes in photosynthetic characteristics of different cold-tolerant varieties under low temperature stress. (a): Intercellular CO_2 concentration ($\mu\text{mol/mol}$); (b): Transpiration rate ($\text{mol/m}^2/\text{s}$); (c): Stomatal conductance ($\text{mmol/m}^2/\text{s}$); (d): Photosynthetic rate ($\mu\text{mol/m}^2/\text{s}$).

Figure 5

The influence of low temperature to Chlorophyll content in rapeseed leaves

Figure 6

Figure 9

Heat map of specific expression genes in the photosynthetically related significant enrichment pathway of *Brassica napus* L.

Figure 10

Number of differentially expressed genes in photosynthetic pathways significantly enriched in *Brassica napus* under low temperature stress

(a)

(b)

Figure 11

Go classification of differentially expressed genes in cold stress response of *Brassica napus* L. (a): Photosynthesis; (b): Photosynthesis - antenna proteins

Figure 12

Validaton of 17 differentially expressed genes by qRT-PCR