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Metabolomics, phytohormone and transcriptomics strategies to reveal the mechanism of barley heading date regulation to responds different photoperiod

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Abstract

Background The correlation between heading date and flowering time significantly regulates grain filling and seed formation in barley and other crops, ultimately determining crop productivity. In this study, the transcriptome, hormone content detection, and metabolome analysis were performed systematically to analyze the regulatory mechanism of heading time in highland barley under different light conditions. The heading date of D18 (winter highland barley variety, Dongqing18) was later than that of K13 (vernal highland barley variety) under normal growth conditions or long-day (LD) treatment, while this situation will reverse with short-day (SD) treatment.

Results The circadian rhythm plant, plant hormone signaling transduction, starch and sucrose metabolism, and photosynthesis-related pathways are significantly enriched in barley under SD and LD to influence heading time. In the plant circadian rhythm pathway, the key genes *GI* (*Gigantea*), *PRR* (*Pesudoresponseregulator*), *FKF1* (*Flavin-binding kelch pepeat F-Box 1*), and *FT* (*Flowering locus T*) are identified as highly expressed in D18SD3 and K13SD2, while they are significantly down-regulated in K13SD3. These genes play an important role in regulating the heading date of D18 earlier than that of K13 under SD conditions. In photosynthesis-related pathways, a-b binding protein and RBS were highly expressed in K13LD3, while NADP-dependent malic enzyme, phosphoenolpyruvate carboxylase, fructose-bisphosphate aldolase, and triosephosphate isomerase were significantly expressed in D18SD3. In the starch and sucrose metabolism pathway, 41 DEGs (differentially expressed genes) and related metabolites were identified as highly expressed and accumulated in D18SD3. The DEGs *SAUR* (*Small auxin-up RNA*), *ARF* (*Auxin response factor*), *TIR1* (*Transport inhibitor response 1*), *EIN3* (*Ethylene-insensitive 3*), *ERS1* (*Ethylene receptor gene*), and *JAZ1* (*Jasmonate ZIM-domain*) in the plant hormone pathway were significantly up-regulated in D18SD3. Compared with D18LD3, the content of N6-isopentenyladenine, indole-3-carboxylic acid, 1-aminocyclopropanecarboxylic acid, trans-zeatin, indole-3-carboxaldehyde, 1-O-indol-3-ylacetylglucose, and salicylic acid in D18SD3 also increased. The expression levels of vernalization genes (*HvVRN1*, *HvVRN2*, and *HvVRN3*), photoperiod genes (*PPD*), and *PPDK* (*Pyruvate phosphate dikinase*) that affect photosynthetic efficiency in barley are also analyzed, which play important regulatory roles in barley heading date. The WGCNA analysis of the metabolome data and circadian regulatory genes identified the key metabolites and candidate genes to regulate the heading time of barley in response to the photoperiod.

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Conclusion These studies will provide a reference for the regulation mechanism of flowering and the heading date of highland barley.

Keywords Highland barley, Vernalization, Photoperiod

Background

Hordeum vulgare L. (Barley) ranks as the fourth most important cereal crop in yield production worldwide and is widely used for animal feed, human food, the brewing industry, and medicinal health food products [1, 2]. Barley has two subspecies: hull-less barley (*Hordeum vulgare* L.) and naked barley (*Hordeum vulgare* var. nudum Hooker f.) [3]. *Hordeum vulgare* L. var. nudum (Qingke), as it is more often called in Chinese, is a naked caryopsis barley that accounts for more than 70% of Tibet's grain production. It is mostly grown on the Tibetan Plateau, which is situated between 1400 and 4700 m above sea level [4]. Cultivated on the Tibetan Plateau approximately 3600 years ago, Tibetan hull-less barley is one of the oldest crops known to humans and is mostly farmed there [5]. Barley is a crop with a short maturation time that is highly resilient to harsh weather circumstances and has strong cold tolerance characteristics. The only grain that can grow and mature organically in alpine regions above 4200 m above sea level is Tibetan hull-less barley [4]. Given that Qingke, or highland barley, is one of the most important industrial crops grown on the Tibetan plateau, further advancements in Qingke will have a major positive impact on the local economy.

The exact timing of head sprouting (heading date) associated with the flowering time for grain filling and seed formation determines crop productivity. Barley cultivars' planting area range is determined by their heading date (anthesis), which also influences how adaptable they are to different seasons. In order for it to occur, plants must sense when a favorable season is approaching in order to promptly begin the reproductive phase [6]. Numerous paths have been identified by researchers to control the timing of flowers, and these pathways' signals are combined with one another by floral integrators to create a particular pathway for the commencement of blooming [7]. There are five known routes in Arabidopsis that regulate flowering: gibberellin, photoperiod, autonomous, aging, and vernalization [8]. Three factors—the need for vernalization, photoperiod sensitivity, and narrow-sense earliness (earliness per se)—affect when to head temperate cereals like barley and wheat [9]. In variational environments, vernalization-related genes (HvVRN1, HvVRN2, and HvVRN3) and photoperiod-related genes (PPD-H1 and PPD-H2) had different interactions, which finally made barley bloom at the most suitable time, and the light-temperature interaction greatly affected the yield of barley. [10, 11]. Therefore, light and temperature are important factors in regulating the adaptive growth of barley, such as flowering (or heading).

With the completion of barley genome sequencing, multi-omics technology has been widely used to explore the genes and regulatory mechanisms regulating important agronomic traits (such as waxy traits and stress resistance traits, etc.) in barley [12], but the key regulatory genes and mechanisms related to heading date (or flowering) traits are rarely reported. Therefore, the purpose of this study was to (1) analyze the influence of different vernalization times on the heading date of barley; (2) explore the key metabolic pathways and regulatory genes regulating barley heading through long- and short-day treatments; and (3) try to analyze the regulatory mechanisms of vernalization and photoperiod on the barley heading date, so as to provide important gene resources and a theoretical basis for barley adaptive breeding.

Methods

Plant materials and treatment

Two barley varieties, winter variety Dongqing18 (D18) and vernal highland barley variety K13, were vernalized at 4 °C at 0 d, 7 d, 14 d, 21 d, 28 d, and 35 d, respectively. The influence of D18 and K13 on heading dates under different lighting and vernalization conditions is significant, which is representative. The effects of six different vernalization conditions on each developmental stage, such as the seedling stage, elongation stage, heading period, and mature period of two barley varieties, were observed in the Lhasa experimental base, and the appropriate vernalization time was selected. Among them, the 35-day vernalization condition allows D18 and K13 to fully vernalize and be able to heading and fructification normally. Then, after being vernalized at 4 °C for 35 days, two barley varieties were placed in an artificial climate incubator under long-day (LD) and short-day (SD) growth conditions. The LD is 16 h/8 h light/dark, 22 °C; the SD is 8 h/16 h light/dark, 22 °C. Barley tissues (12 in total) were taken at the seedling stage, elongation stage, and heading period, respectively, with three replicates per tissue. Among them, sampling of seedlings is at the two-leaf stage of barley. The sampling tissues of barley at the seedling stage and elongation stage were mixed samples of leaves and stems. Sampling at the heading stage was a mixed sampling of the young ear and stem.

Transcriptome sequencing (RNA-Seq), metabolome (UPLC-MS/MS), and plant hormone content (absolute quantitative) detection were performed on a total of 36 barley samples.

Transcriptome sequencing

Using Oligo (dT) (MetWare Biotechnology), the polyA-containing mRNA was isolated from the total RNA and fragmented into smaller pieces with the addition of fragmentation buffer. The first strand of cDNA was synthesized with random primers using a small piece of RNA as a template, and the second strand was created using the template from the first strand. Double-stranded cDNA was purified using AMPure XP beads; the end was then repaired, the tail was added, and the head was linked; fragments were then chosen using AMPure XP beads, and the final cDNA library was produced by PCR enrichment. The Illumina HiSeq sequencing platform carried out the sequencing, which Wuhan MetWare Biotechnology Co., Ltd. finished. Using Tophat 2 software and the barley genome as a reference genome, sequence alignment of the filtered clean data was performed to obtain mapped data, and differential expression gene analysis and gene functional enrichment analysis were performed combined with gene expression levels in different barley samples [13].

Metabolome detection

In a mixer mill (MM 400, Retsch), the barley samples were crushed with a zirconia bead (30 Hz, 1.5 min). Before LC–MS analysis, 100 mg of powder was extracted overnight at 4 °C in 1.2 mL of 70% aqueous methanol (v/v). The liquid supernatant was retained and filtered using a Millipore filtering system (SCAA-104, 0.22 μm; ANPEL) after 10,000 g centrifugation was performed for 10 min. The extract samples were assessed using ultra-performance liquid chromatography (Shim-pack UFLC SHIMADZU CBM30A system) and tandem mass spectrometry (Applied Biosystems 6500 Q TRAP) systems (UPLC-MS/MS). For the HPLC column, the analytical conditions specified by Liu et al. (2023) were followed [13]. Acetonitrile and ultrapure water together provide 0.04% acetic acid to the solvent system. The temperature is 40 °C, the injection volume is 2 μl, and the flow rate is 0.40 mL/min. LIT and triple quadrupole (QQQ) scans were generated using an API 6500 Q TRAP LC–MS/MS system equipped with an ESI Turbo Ion-Spray interface. This triple quadrupole-linear ion trap mass spectrometer (Q-TRAP) was utilized for this purpose. It can function in both positive and negative ion modes and is controlled by the Analyst 1.6 program (AB SCIEX). In QQQ and LIT modes, the device was calibrated and adjusted using solutions of 10 and 100 μmol/L polypropylene glycol, respectively. A particular set of MRM ion pairs was investigated based on the metabolites eluted during each period.

Plant hormone content detection

Using the AB Sciex QTRAP 6500 LC–MS/MS platform, MetWare (http://www.metware.cn/) assessed the phytohormone content of barley. Every sample was prepared, extracted, and analyzed using LC–MS/MS in accordance with the preceding section. A triple quadrupole linear ion trap mass spectrometer (QTRAP), the QTRAP 6500 + LC–MS/MS system, fitted with an ESI Turbo ion spray interface and capable of working in both positive and negative ion modes, is used for linear ion trap and triple quadrupole scanning. The software Analyst 1.6.3 is in charge of it. To examine phytohormones, multiple response monitoring (MRM) was employed. The Multiquant 3.0.3 program was used to quantify each metabolite. Based on metabolite elution throughout each interval, a particular set of MRM transformations was observed.

Weighted Gene Correlation Network Analysis (WGCNA)

Using the WGCNA R software package and a soft thresholding power of 10, co-expression networks were built. The barley-assembled genes were chosen for the WGCNA unsigned co-expression network analysis based on FPKM > 5. The key metabolite content identified by previous analysis was used as a phenotype. Every other module parameter was left at its default setting. For each gene, the following connection measures were determined: total connectivity, intramodular connectivity (also known as function soft connectivity), kME (for modular membership, or eigengene-based connectivity), and kME-*p*-values. High-connectivity genes were identified as hub genes with potentially significant activities.

qRT-PCR detection

The SYBR Green PCR kit (Qiagen, 204,054) was used in qRT-PCR to confirm the applicability of the RNA-Seq profile of gene expression data. For the creation of gene-specific primer pairs, primer 4.0 was employed. And using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Lithuania) in accordance with the manufacturer's instructions, the first strand cDNA of barley was obtained. In this study, there are three technical repeats for every gene. Every barley leaf sample was replicated three times. The $2^{-\Delta\Delta ct}$ technique was utilized to calculate the gene expression level, with GAPDH serving as the internal reference gene.

Statistical analysis of the data

Utilizing SPSS 20.0 and Analyst 1.6.1 software, all metabolite data from barley samples were examined. These data were used to determine the means and SD. Tukey's significant difference test was performed after one-way ANOVA, and p-values of 0.05 were deemed significant. To create an orthogonal partial least squares-discriminant analysis (OPLS-DA) model, several supervision techniques were applied. To determine each metabolite's relative importance in the OPLS-DA model, the parameter variable effect on projection (VIP) was employed. The metabolites between each sample were analyzed using hierarchical clustering using the R programming language (www.r-project.org). According to Liu's description, the screening criteria for metabolites that had accumulated differentially were |log2(fold change) |≥1 and VIP≥1 [13].

Results

Phenotypic changes of barley treated with different photoperiod

The heading rate of the winter barley variety increased with the increase in vernalization time. In this experiment, the percentage of completed headings of barley greater than 80% is regarded as the standard for the variety to complete vernalization. The spring highland barley variety (K13) can produce normal heading without low temperature vernalization (Fig. 1), while the winter variety of Damai 18 (D18) in Tibet needs at least 35 days to complete vernalization under the low temperature vernalization condition (4 °C) at the seedling stage. The results showed that the heading time of K13 was shorter than that of D18 at the uniform vernalization level of 35 days. Under the condition of a different vernalization time, the heading date of D18 was later than that of K13. Therefore, this experiment determined 35d as the standard of vernalization for follow-up experimental analysis. Then, the heading date of D18 was observed under different lighting conditions. The results showed that under LD treatment, the heading date of D18 was later than K13, while under SD treatment, the heading date of D18 was earlier than K13. These results indicate that vernalization and photoperiod are the main factors affecting the heading time of barley crops.

Transcriptome data statistics and differentially expressed gene analysis

In order to find the internal factors of the difference between the head times of D18 and K13 under different light cycles, the transcriptome sequencing of 36 samples of D18 and K13 at different developmental stages under LD and SD conditions was conducted by the Illumina platform. Including the seedling stage (LD1, SD1), jointing stage (LD2, SD2), and heading stage (LD3, SD3). Raw reads of each sample were obtained. Clean reads were obtained after raw data filtering, sequencing error

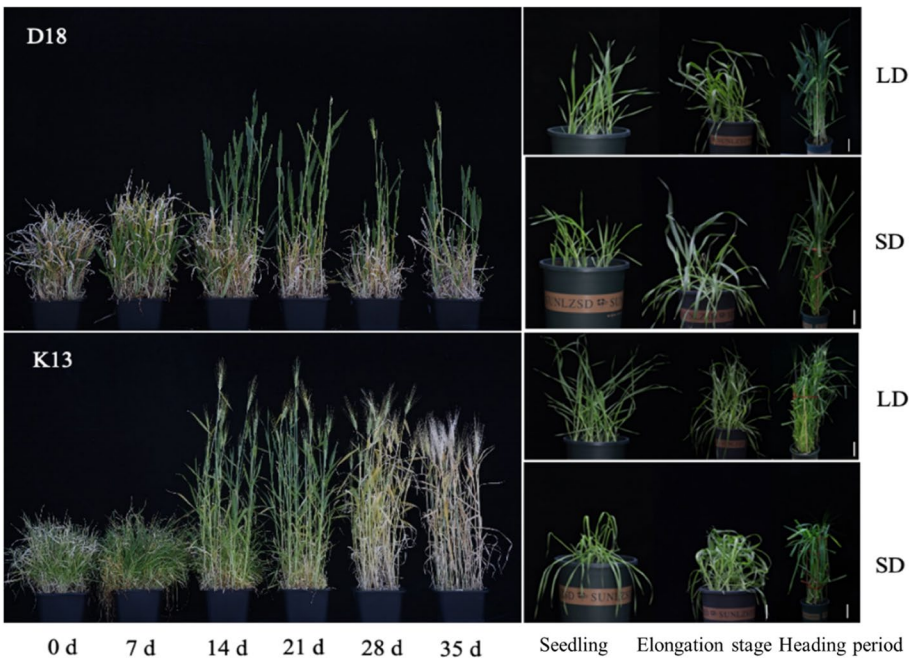

Fig. 1 The phenotypic changes of winter barley variety D18 and spring highland barley variety K13 treated with different vernalization times and photoperiod condition at seedling stage, jointing stage and heading stage. LD represents long-day and SD represents short-day

rate detection, and GC content distribution detection. The base quality test found that the base percentage Q20 value of each sample was greater than 96% and the Q30 value was greater than 91%, which indicated that the sequencing quality met the experimental requirements and could be used for subsequent analysis. We used Hordeum_vulgare.MorexV3 as the reference genome for sequence comparison, and the ratio of reads in all samples was greater than 92% (Table S1). We first conducted global PCA analysis and sample correlation analysis, and the results showed that no matter D18 or K13, the light cycle had the greatest influence on the heading date (Figures S1A and S1B). Moreover, we also found that barley heading was more affected by SD treatment than LD, especially D18, which was most significantly affected by SD3.

The results of differentially expressed gene screening in each comparison group showed that 870 DEGs (395 up-regulated, 475 down-regulated) were screened in D18LD1_vs_D18SD1. 2076 DEGs were identified in D18LD2_vs_D18SD2 (983 down-regulated, 1093 up-regulated). 5713 DEGs were screened in D18LD3_vs_D18SD3 (2586 down-regulated, 3127 up-regulated). A total of 2662, 2716, and 5137 DEG were identified from K13 at the seedling stage, joining stage, and heading stage, respectively (Figure S1C). The quantity of DEG indirectly reflected the greatest influence of long and short sunshine on the barley heading stage. Compared with D18, the light cycle seems to have a greater effect on K13 at the seedling stage. A total of 3961 DEG were screened in the comparison groups D18 and K13 at the heading stage under long-day conditions, while 6972 DEG were screened under short-day conditions. This result is also consistent with the results of PCA and sample correlation, which suggested that short days have the greatest influence on barley heading time compared with long days.

KEGG enrichment analysis of photoperiod regulatory pathways in barley

Firstly, KEGG enrichment analysis was performed on the DEGs selected from the D18LD3_vs_D18SD3 and K13LD3_vs_K13SD3 comparison groups (Fig. 2). The results showed that D18LD3_vs_D18SD3 was significantly enriched in starch and sucrose metabolism, circadian rhythm plant, photosynthesis-related pathways (carbon fixation in photosynthetic organisms, photosynthesis—antenna proteins, etc.) and lipid-related metabolism (glycerophospholipid metabolism, phosphatidylinositol signaling system, inositol phosphate metabolism, alpha-Linolenic acid metabolism, etc.). In K13LD3_vs_K13SD3, circadian rhythm plant, photosynthesis-related pathways (carbon fixation in photosynthetic organisms, photosynthesis—antenna proteins, etc.) were also significantly enriched. However, different from D18LD3_vs_D18SD3, carotenoid biosynthesis and terpenoid backbone biosynthesis were significantly enriched in K13LD3_vs_K13SD3. The results speculated that these pathways may play a related role in the regulation of barley heading.

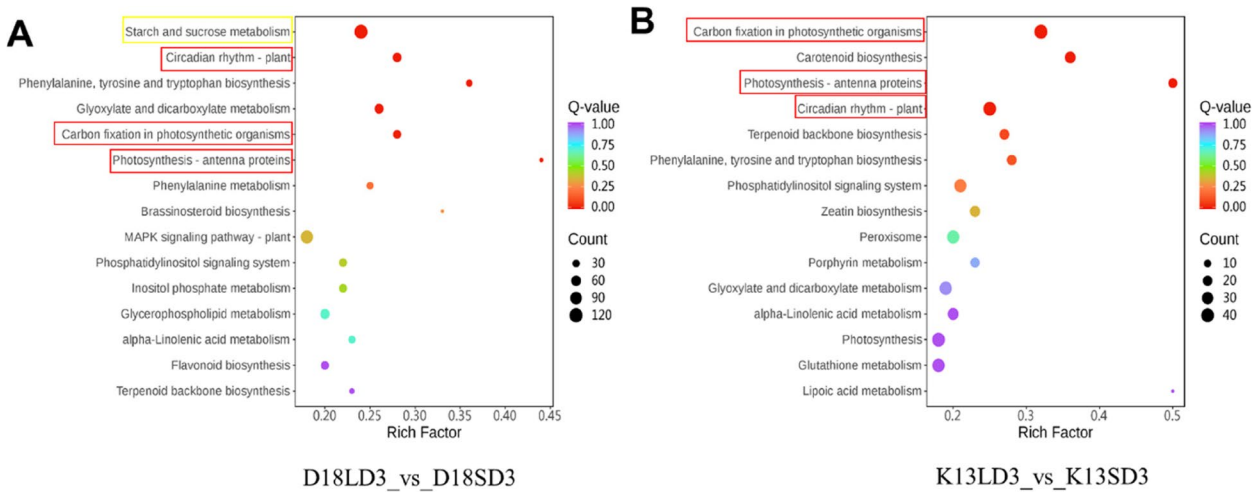

Fig. 2 Differentially expression gene enrichment analysis of two barley varieties under LD and SD conditions. KEGG enrichment analysis of differentially expressed genes in D18LD3 VS D18SD3 (**A**) and K13LD3 VS K13SD3 (**B**) comparison groups correlated with regulatory pathways of photoperiod

Key regulation DEG analysis of plant circadian rhythm pathway

We obtained 45 genes from D18LD3_vs_D18SD3 enriched into the circadian rhythm—plant pathway (17 DEG up-regulated and 28 DEG down-regulated). 40 DEGs were screened in K13LD3_vs_K13SD3 (12 DEGs up-regulated and 28 DEGs down-regulated). Among them, 21 DEGs were common to the two comparison groups, and detailed analysis of them showed that these 20 DEGs were highly expressed in D18SD3, and showed an opposite trend in D18 and K13 (Fig. 3). Further, 13 genes were identified, including *GI* (*HORVU. MOREX. R3.3 HG0238250*), *PRR73* (*novel.10112*), *CONSTANS-like CO3* (*HORVU. MOREX. R3.6 HG0569830*), *FKF1* (*HORVU. MOREX. R3.4 HG0369880*), *PRR95* (*HORVU. MOREX. R3.6 HG0596460*), *CRY2* (*HORVU. MOREX. R3.6 HG0596460*), *HY5* (*HORVU. MOREX. R3.7 HG0723770*), *FT* (*HORVU. MOREX. R3.3 HG0244930*) (Table S2).

The expression levels of these genes at the seedling stage and jointing stage of D18 and K13 were also analyzed. The results showed that the above genes were specifically highly expressed in D18SD3, while in K13, these genes were highly expressed in K13SD2 and significantly

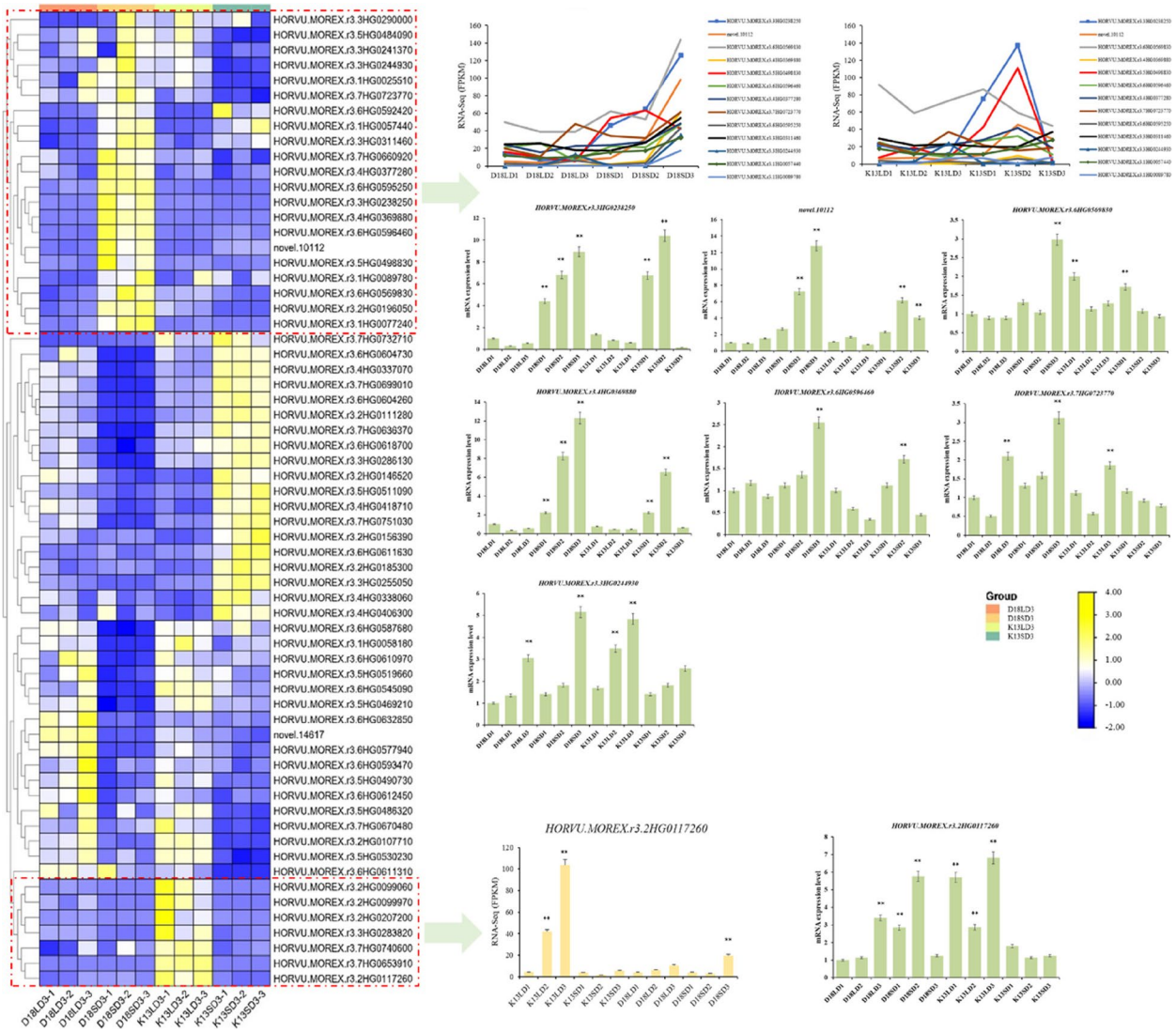

Fig. 3 The expression pattern of DEGs in plant circadian rhythm pathway and key regulated genes expression level in D18 and K13 under LD and SD conditions. The line chart and yellow bar chart in the figure show the RNA-Seq expression levels (FPKM values) of these key genes, respectively. The green bar graph shows the expression levels (fold change) of these key DEGs after RT-PCR detection. The ** on the column indicates the significance of the difference compared with the control ($p < 0.01$)

down-regulated in K13SD3 (Fig. 3). Therefore, we speculate that these are sensitive genes in barley to regulate photoperiod under short-day conditions, and combined with phenotypic results, the heading time of D18 is earlier than that of K13 under SD conditions, indicating that these genes play an important role in regulating the heading date of D18. At the same time, we screened the genes that were significantly highly expressed in K13LD3, and a total of 6 DEGs were obtained. Combine them in D18 and K13, change expression levels in different periods, and finally select a high-specificity expressed gene in K13LD3, namely the *FT* (*HORVU. MOREX. R3.2 HG0117260*). This result suggested that these genes play an important role in the regulation of K13 heading under LD condition.

Analysis of pathways related to photosynthesis and starch and sucrose metabolism

A total of 79 DEGs were screened in photosynthesis-related pathways, and most DEGs were highly expressed in LD, especially in K13 (Fig. 4A). Among them, 33 DEGs were highly expressed in K13LD3, which mainly includes a-b binding protein (*novel.14939*, *novel.9925*, *novel.16515*, *novel.2297*). *RBS* (Ribulose bisphosphate carboxylase small subunit; *HORVU. MOREX. R3.2 HG0104740*, *HORVU MOREX. R3.2 HG0104700*, *HORVU. MOREX. R3.2 HG0104730*). At the same time, we also found that 12 DEGs in D18SD3 specificity high expression, including *NADP-dependent malic enzyme* (*HORVU. MOREX. R3.1 HG0029730*), *phosphoenolpyruvate carboxylase* (*HORVU. MOREX. R3.5 HG0472960*), *fructose—bisphosphate aldolase* (*HORVU. MOREX. R3.3 HG0304680*, *HORVU. MOREX. R3.3 HG0304710*), *triosephosphate isomerase* (*HORVU. MOREX. R3.2 HG0183890*) (Table S2). The results indirectly indicated that these genes were involved in light treatment response during barley heading.

In addition, we found that the starch and sucrose metabolism pathway is also specifically enriched in D18LD3_vs_D18SD3. A total of 122 DEGs were screened to show significant expression changes in D18, and more than half of them were specifically expressed in D18SD3 (Fig. 4B). We screened 41 highly expressed genes, including *glucan endo-1,3-beta-glucosidase* (*HORVU.MOREX.r3.7HG0720980*),

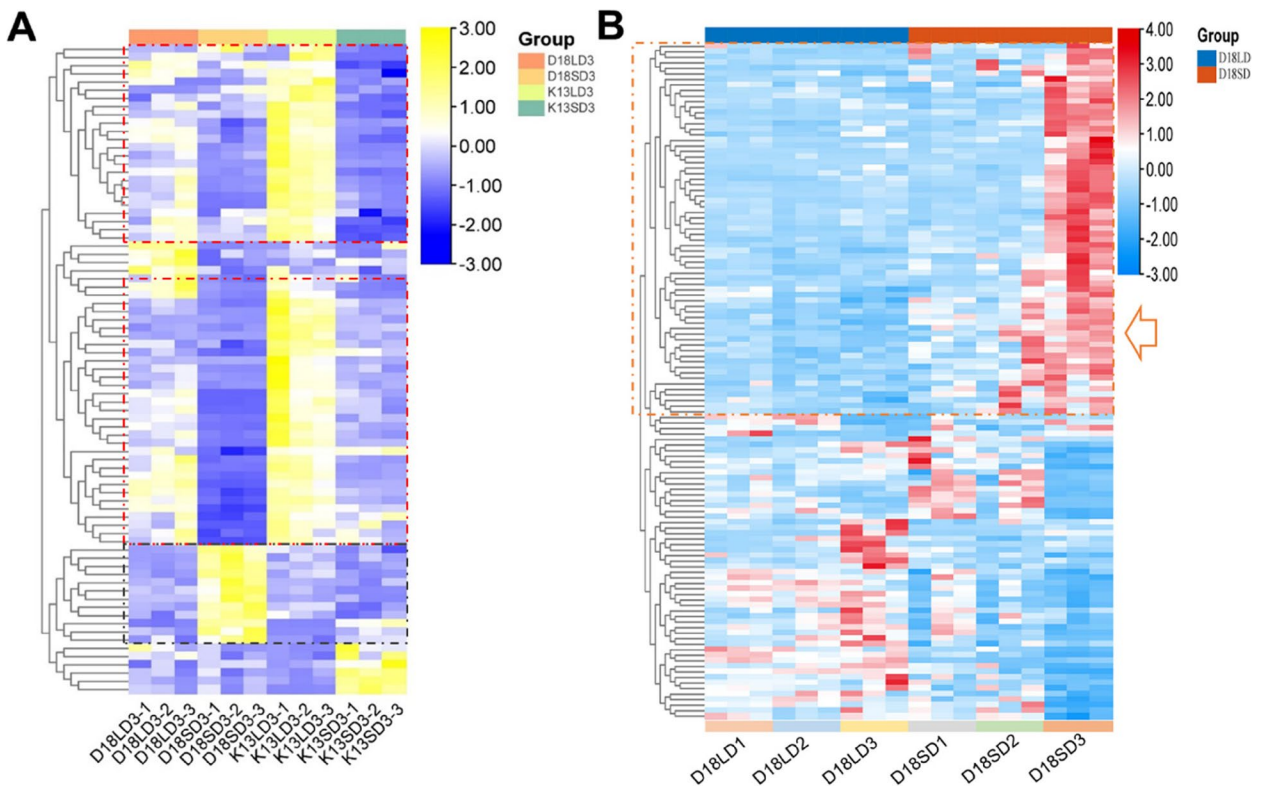

Fig. 4 The expression pattern of DEGs in photosynthesis-related pathways (**A**), and the starch and sucrose metabolism pathway (**B**) in D18 and K13 under LD and SD conditions. The red dotted boxes and arrows in the figure are the key genes whose expression levels trend changes significantly in the pathway. In Figure A, the yellow color shows significantly up-regulated gene expression levels, while the blue color shows down-regulated gene expression. In Figure B, red color indicates significantly up-regulated gene expression levels, while blue color indicates significantly down-regulated gene expression

sucrose synthase 1 (*HORVU.MOREX.r3.7HG0661420*), *alpha-amylase* (*HORVU.MOREX.r3.7HG0680870*), *endoglucanase* (*HORVU.MOREX.r3.2HG0184930*, *HORVU.MOREX.r3.2HG0178520*). At the same time, we also analyzed the changes of these genes in K13, which the results show in K13 (Table S3). Therefore, we speculate that these genes may play a certain role in the regulation of D18 early heading under SD, and the metabolism of starch and sucrose is extremely active at this time, providing important energy substances for D18 heading.

Analysis of plant hormone related genes

Plant hormones play a very important role in regulating the process of flowering and heading of crops. Therefore, we analyzed the relevant differentially expressed genes in the plant hormone pathway in detail (Fig. 5A). A total of 181 DEGs were screened, which were classified from different stages, seedling stage (D18LD1 vs D18SD1, K13LD1 vs K13SD1), joining stage (D18LD2 vs D18SD2, K13LD2 vs K13SD2), and heading stage (D18LD3 vs D18SD3, K13LD2 vs K13SD2), K13LD3 vs K13SD3). We found that these DEGs are mainly concentrated in the heading stage. The analysis results showed that some DEGs (78) were significantly up-regulated in D18SD3, including auxin genes, *SAUR* (*HORVU.MOREX. R3.7 HG0721320*, *HORVU. MOREX. R3.5 HG0502220*, *will be. 21,690*), *ARF* (*HORVU. MOREX. R3.7 HG0735280*, *HORVU. MOREX. R3.1 HG0086460*), *TIR1* (*HORVU. MOREX. R3.6 HG0616790*, *HORVU. MOREX. R3.2 HG0163780*, *HORVU. MOREX. R3.7 HG0664490*), ethylene related genes, *EIN3* (*HORVU. MOREX. R3.7 HG0720010*), *ERS1* (*HORVU. MOREX. R3.4 HG0336040*), salicylic acid related genes, *PR1* (*HORVU. MOREX. R3.5 HG0519270*, *HORVU. MOREX. R3.7 HG0668900*, *HORVU MOREX. R3.5 HG0473580*) and jasmonic acid related genes, *JAZ1* (*HORVU. MOREX.*

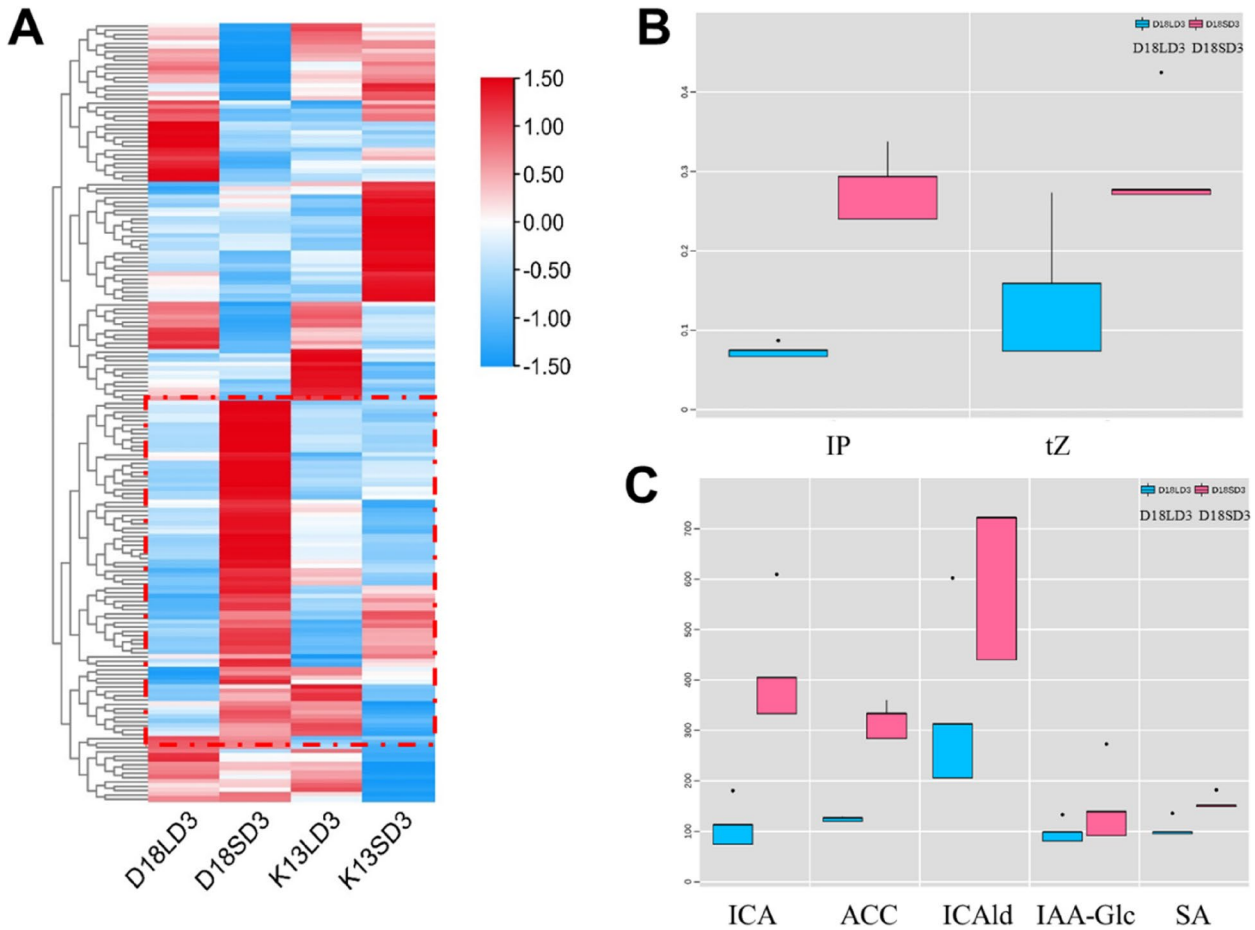

Fig. 5 The expression pattern of DEGs in plant hormone related pathways (**A**) in D18 and K13 under LD and SD conditions. The content of N6-isopentenyladenine (IP) and trans-zeatin (tZ) in D18 under LD (D18LD3) and SD (D18SD3) conditions (**B**). The content of indole-3-carboxylic acid (ICA), 1-aminocyclopropanecarboxylic acid (ACC), indole-3-carboxaldehyde (ICAld), 1-O-indol-3-ylacetylglucose (IAA-Glc), and salicylic acid (SA) in D18 under LD (D18LD3) and SD (D18SD3) conditions (**C**). The colors in the heat map show that the expression level of genes expression; the red color is significantly up-regulated, and the blue color is significantly down-regulated

R3.5 HG0479120, *HORVU. MOREX. R3.5 HG0480600*, *HORVU. MOREX. R3.4 HG0405240*). The results indicated that D18 not only activated the synthesis of growth-regulating hormones, but also activated stress hormones under short day conditions, which may play an important role in regulating the early heading process of D18.

We used the metabolome to detect changes of hormone levels in each sample. The results showed that compared with D18LD3, N6-isopentenyladenine (IP), indole-3-carboxylic acid (ICA), 1—aminocyclopropane-carboxylic acid (ACC) in D18SD3 raised 3.8, 3.6, 2.6 times, respectively. Moreover, trans-zeatin (tZ), indole-3-carboxaldehyde (ICAld), 1-O-indol-3-ylacetylglucose (IAA-Glc), salicylic acid (SA) were increased by 1.9, 1.7, 1.6, 1.6 times, respectively (Fig. 5B, and C). The contents of ICA, ACC and ICAld in D18SD3 reached 449, 325 and 628 ng/g, respectively. At the same time, we compared the differences of these hormones in K13SD3 and D18SD3, and the results showed that the contents of these hormones were significantly higher in D18SD3 than in K13SD3. These results were also consistent with the transcriptome results, which confirmed the accuracy of the experimental results.

Expression profile analysis of genes related to vernalization and photoperiod in barley

We screened the known vernalization genes (*HvVRN1*, *HvVRN2*, *HvVRN3*), photoperiod genes (PPD), and PPDK that affect photosynthetic efficiency in barley, and analyzed the expression levels of these genes under different treatments (LD and SD) in D18 and K13. Including *HvVRN1* (*HORVU.MOREX.r3.5HG0511210*), *HvVRN3* (*HORVU.MOREX.r3.2HG0157030*), *PPDK* (*HORVU.MOREX.r3.7HG0677380*), *PPD1* (*HORVU.MOREX.r3.5HG0443280*), *PPD2* (*HORVU.MOREX.r3.2HG0169260*), *PPD6* (*novel.8770*). We found that under LD conditions, the expression level of *HvVRN1* in D18 was lower than that in K13 (Fig. 6A). Under SD conditions, the expression levels of *HvVRN3* and *PPDK* were significantly higher in D18SD3 than in K13 (Fig. 6B, and C). However, photoperiod related genes *PPD1*, *PPD2* and *PPD6* were greatly affected by short day in both K13 and D18, and their expression levels decreased under long day conditions, but were higher under K13LD3 than D18. On the whole, the expression levels of these three genes decreased during heading period (Fig. 6D, E, and F). These results suggest that genes related to vernalization and photosynthetic efficiency were play important regulatory roles in barley heading.

Metabolome analysis of barley after different light treatments

Metabolome was used to detect the changes of metabolite accumulation in D18 and K13 at seedling stage (LD1, SD1), jointing stage (LD2, SD2) and heading stage (LD3, SD3) under LD and SD conditions. A total of 1170 substances were detected, and after passing the quality control, we performed PCA analysis (Figure S2A). The

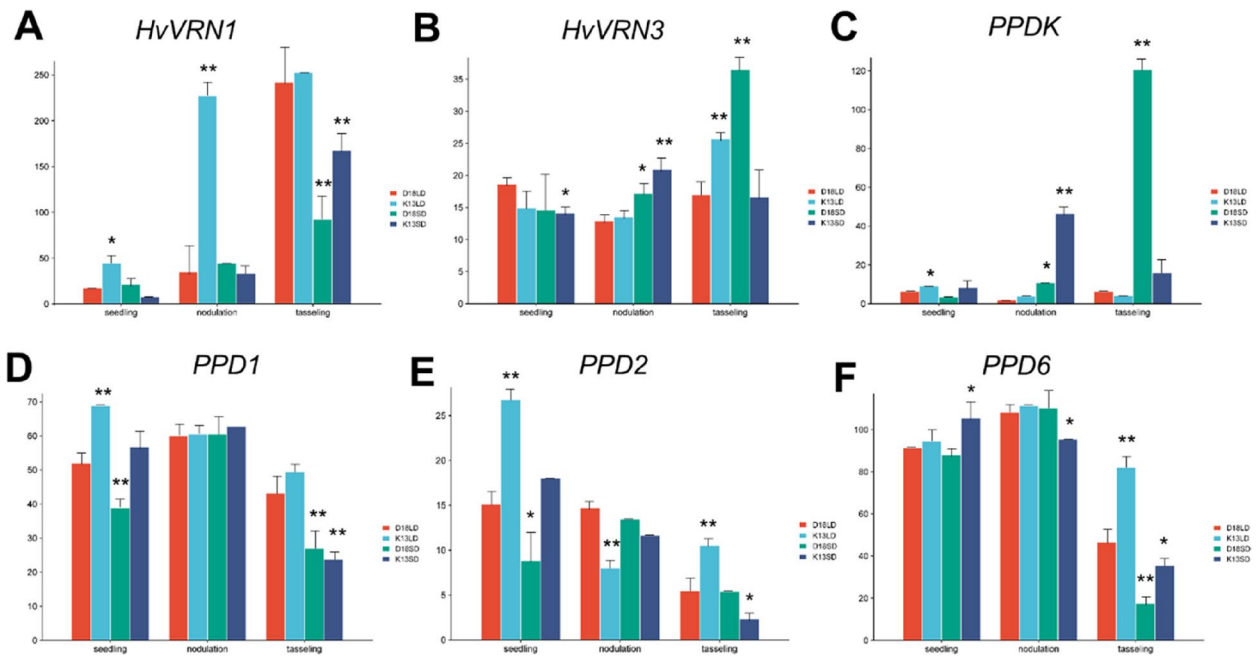

Fig. 6 Analysis of expression levels of vernalization and photoperiod related genes in barley. *HvVRN1* (**A**), *HvVRN3* (**B**), *HvPPDK* (**C**), *HvPPD1* (**D**), *HvPPD2* (**E**), *HvPPD6* (**F**). In the column chart, * indicates the significant difference ($p < 0.05$), **($p < 0.01$)

analysis results showed that in both D18 and K13, the length of sunshine had the greatest influence on heading time, and the samples were obviously separated, to consistent with the transcriptome results. At the same time, we also found from the results that the metabolite spectrum of heading stage was obviously separated from that of seedling stage and jointing stage, indicating that their metabolite basis was quite different. We compared differentially accumulated metabolite (DAM) in K13LD3 vs K13SD3 and D18LD3 vs D18SD3. A total of 308 DAMs (up 117, down 191) were screened from K13LD3 vs K13SD3, and 305 DAMs (up 62, down 243) were screened from D18LD3 vs D18SD3 (Figure S2B). In terms of the number of DAMs, there is almost no difference between K13 and D18. However, from the change of accumulation content (up-regulated or down-regulated), it can be seen that D18 seems to be more sensitive to short days, and most substances are down-regulated.

Then, we focused on comparing the differences in the accumulation of metabolic compounds between D18SD3 and K13SD3 to explain the phenomenon that the heading time of D18 was earlier than that of K13 under SD conditions. A total of 177 DAMs were screened, 106 of which were significantly accumulated in D18SD3. Detailed analysis showed that there were 18 sugars with differentially accumulation, 17 of which were significantly accumulated in D18SD3, including gluconic acid, D-maltose, D-trehalose, D-sucrose, rutinose, etc. At the same time, we also found that most of the flavonoids (71/82) accumulated in D18SD3. Combined with transcriptome data analysis, this study speculated that the early heading of D18 was related to carbohydrate accumulation, and flavonoids also played a key regulatory role.

Analysis of co-expression regulation of genes and metabolites in barley

Finally, we performed WGCNA analysis of metabolome data and circadian regulatory genes. These differentially accumulated metabolites were divided into 12 modules (Fig. 7). The results showed that the red module and *FKF1* (*HORVU. MOREX. R3.4 HG0369880*) has significant correlation ($p < 0.05$). At the same time, the red module also with *PRR73* (*will. 10,112*), *CONSTANS—like CO3* (*HORVU. MOREX. R3.6 HG0569830*) also correlation ($p < 0.05$). This module has a total of 82 DAMs, including phenolic acids and alkaloids (Table S3), and suggesting that these substances play an important regulatory role in the early heading of D18 under SD condition.

Discussion

As one of the oldest cereal crops, barley has been grown for around 10,000 years in a region that includes southern Turkey, Israel, China, Lebanon, Jordan, and Syria, as well as the Nile River in Egypt and the Tigris River in Iraq [14]. China has a long history of barley cultivation, and barley germplasm resources are rich, with certain polymorphisms among each other, and can adapt to the planting needs of different ecological conditions. Different varieties respond differently to light. For barley, the photoperiod cycle has a greater impact on heading date, and the heading date is related to flowering time, which will regulate the grain filling and yield traits. Previous study through the differences in light exposure of different varieties, important regulatory genes and pathways were found, which played an important role in promoting the breeding of wide-fit barley varieties. In this study, D18 needed at least 35 days to complete vernalization, while K13 could produce a normal heading without low-temperature treatment. Combined transcriptome and metabolome analysis of D18 and K13 barley at seedling stage, jointing stage, and heading stage was performed under different light duration conditions to elucidate the photoperiod regulatory pathways and mechanisms in barley response to heading. The results showed that the number of differentially expressed genes of D18 and K13 at each developmental stage changed significantly under LD and SD conditions, and the functional pathways of enrichment were also different. This result suggests that different barley varieties may regulate heading time through different genes.

Barley's Circadian rhythm-plant pathway was considerably enhanced in response to varying light treatment durations (LD and SD). The orthologues of Arabidopsis' circadian clock genes have been found to be potential genes in barley and wheat that contribute to the early flowering phenotype [15, 16]. In the present study, D18SD3 exhibits strong expression of *PRR73*, *FKF1*, *PRR95*, *FT*, *CONSTANS-like CO3*, *CRY2*, *HY5*, and other important regulatory genes in this pathway, while K13 exhibits considerable down-regulation of these genes. The findings indicated that under short day conditions, these genes are sensitive to controlling photoperiod in barley. F-BOX 1 (FKF1) and GIGANTEA (GI) are two proteins that regulate Arabidopsis thaliana blooming, according to earlier research [17]. Flavin mononucleotide is used by the LOV (light, oxygen, or voltage) domain of FKF1 to sense light. Consequently, D18's heading time was earlier than K13's under SD conditions [18]. It is also known that the GI influences CO expression. Moreover, it has been demonstrated that FKF1 binds to GI in a blue light-dependent way [19]. The *CONSTANS* (*CO*) gene primarily controls the production of the FLOWERING LOCUS T (FT) protein, which in turn controls Arabidopsis thaliana flowering [20]. *Ppd-1*, the homologue of Arabidopsis *PRR7* and *PRR3*, is thought to be the primary regulator of photoperiod sensitivity in wheat and barley [21]. These results suggested that *FKT1*, *GI*, *FT*, *CONSTANS-like CO*, *PRR* and

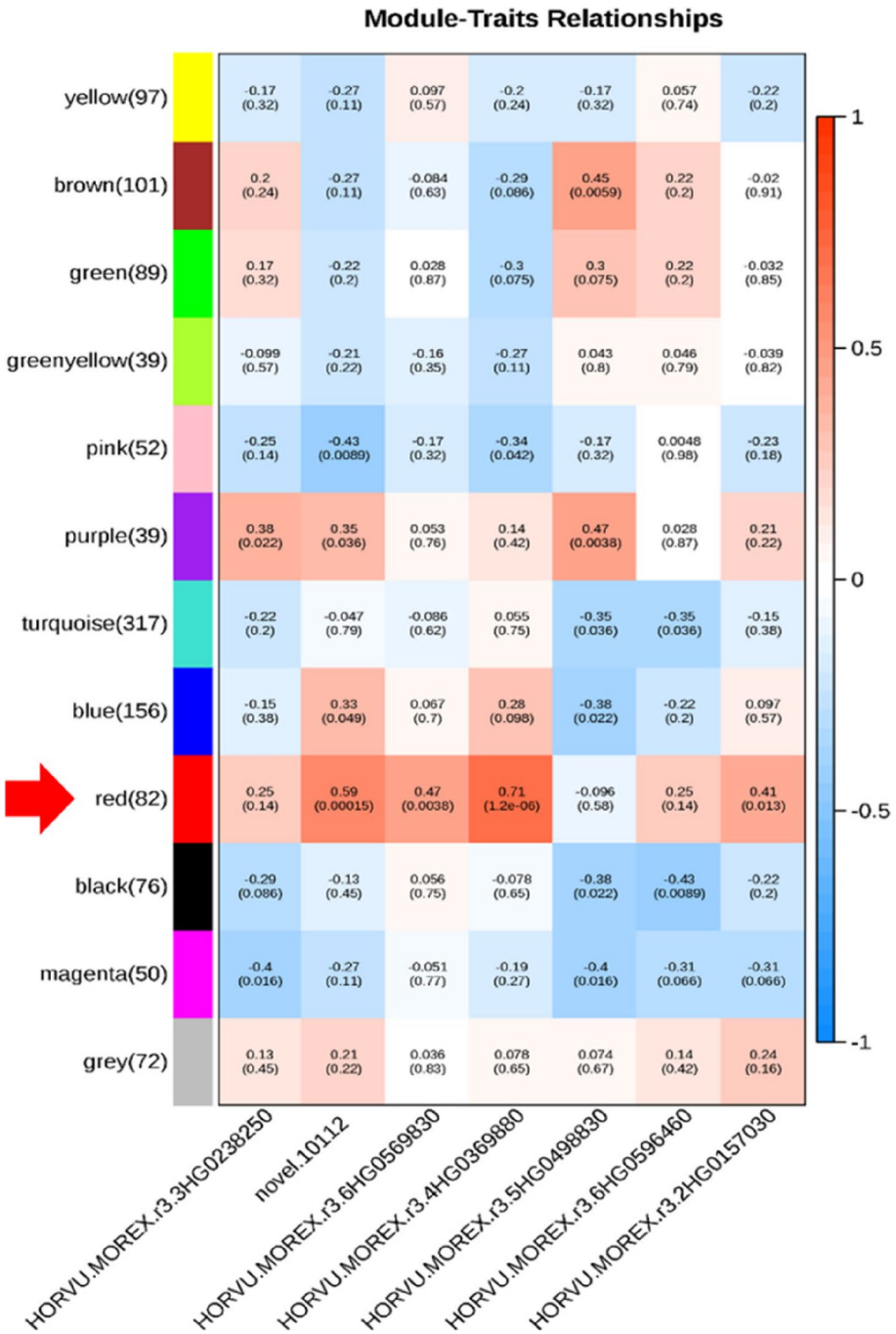

Fig. 7 Weighted gene co-expression network analysis (WGCNA) between metabolic compound content and gene expression levels in barley after different light treatments (LD and SD)

other key genes were affected by light, which caused the gene expression changes or interactions, thus accelerating the growth and development cycle of barley and shortening the heading time.

The primary genetic variables influencing adaptation are genes related to earliness per se, photoperiod, and vernalization sensitivity (*Vrn* and *Ppd*) [22]. In the current work, the expression levels of the genes responsible for photoperiodic response and vernalization in barley were examined. Three genes, *VERNALIZATION 1* (*Vrn-1*), *VERNALIZATION 2* (*Vrn-2*), and *VERNALIZATION 3* (*Vrn-3*), regulate the vernalization need [23]. In this

experiment, K13's *HvVRN1* expression level was substantially higher than D18's under the circumstances of LD. An *APETALA1/FRUITFUL-like* (*AP1/FUL-like*) *MADS-box* transcription factor is encoded by *Vrn-1* [24]. Whereas one or more dominant alleles at *Vrn-1* homoeoloci result in a spring growth habit (vernalization insensitive), recessive alleles at all *Vrn-1* homoeoloci give a winter growth habit (vernalization sensitive). *Vrn-1* expression levels in leaves are correlated with earliness, indicating that *Vrn-1* controls the timing of blooming [25]. In this experiment, K13's *HvVRN3* expression level was considerably lower than D18's under SD conditions. A protein that resembles Arabidopsis *FT* and is comparable to RAF kinase inhibitor is encoded by the *Vrn-3* gene. Strong flowering promoter *Vrn-3* is demonstrated by the extra-early flowering phenotype of transgenic wheat plants overexpressing it, without the need for vernalization [26].

Ppd-1 photoperiod insensitive alleles were found for each homoeolocus on common wheat chromosomes 2A, 2B, and 2D, in that order [27]. The cultivars with these *Ppd-1a* alleles exhibited aberrant circadian rhythms for Ppd-1 expression in addition to elevated expression levels. Early flowering traits are correlated with enhanced *TaFT* (*WFT*) expression level in wheat plants carrying at least one *Ppd-1a* allele [27]. In this experiment, the expression levels of *PPD1*, *PPD2* and *PPD6* genes decreased overall during barley heading period, and their expression levels were more affected by short day conditions, and their gene expression levels significantly decreased compared with long day conditions. Under LD treatment, the expression of these genes in K13LD3 was higher than that in D18. As seen in rice and Arabidopsis, *FT1* expression is thought to travel through the phloem to the shoot apical meristem in barley as a consequence of light-induced transcriptional activation of *PPD1* in the leaves [28, 29]. In order to bind to the promoter of the *MADS-box* transcription factor *VRN1*, which is essential to initiating the transition of the shoot apical meristem from the vegetative to the reproductive phase, FT1 interacts with the proteins 14–3-3 and FDL to form a florigen activation complex [30]. In these abundant barley germplasm resources, the genes related to vernalization and photoperiod pathways have abundant alleles, and mining excellent alleles from them will help promote the effective utilization of barley germplasm resources and accelerate the breeding of barley wide suitability varieties.

The present study also found that carbon fixation in photosynthetic organisms, starch and sucrose metabolism, and photosynthesis protein pathways related to photosynthesis and energy metabolism have also been significantly enriched in barley in response to LD and SD treatment. Among them, a chlorophyll-a-bbinding protein, RBS, is highly expressed in K13LD3 to enhance their photosynthetic capacity [31]. The phosphoenolpyruvate carboxylase (PEPC), NADP-dependent malic enzyme, fructose-bisphosphate aldolase, and triosephosphate isomerase are highly expressed specifically in D18SD3. It has been observed that the PEPC is phylogenetically informative in several flowering plant groups and exists in one or a small number of copies [32]. The NADP-dependent malic enzyme, which also includes *PEPC* and *PPDK*, is linked to the fixation of carbon dioxide during photosynthesis [33]. The primary C4 photosynthetic enzyme genes that have been extensively studied, D18 and K13, have substantially different *PPDK* expression levels in this experiment under the condition of SD [34]. In addition, the important regulatory genes, including trehalose 6-phosphate phosphatase, glucan endo-1,3-beta-glucosidase, sucrose synthase, endoglucanase, alpha-amylase in the glucose metabolism pathway were also found to be specifically highly expressed in D18SD3. Metabolome test results also showed that gluconic acid, D-maltose, D-trehalose, D-sucrose, rutinose, etc., were also significantly accumulated in D18SD3, which was consistent with the results of transcriptome analysis.

Plant hormones are always crucial for controlling a plant's ability to blossom. The routes are determined by environmental factors based on photoperiod and vernalization. The gibberellin-dependent pathway is made up of a group of genes involved in signal transduction and gibberellin production [35]. Conversely, the autonomous route is reliant on endogenous growth-related components. In this study, it was found that plant hormone-related genes, SAUR, ARF, TIR1, EIN3, ERS1, PR1, and JAZ1, were significantly up-regulated in D18SD3. These hormone content levels (ICA, IP, ACC, tZ, ICAld, IAA-Glc, and SA) were significantly higher in D18SD3 than in K13SD3. In summary, we used the transcriptome, hormone content detection, and metabolome analysis systematically to understand the reasons for the difference in the heading time of D18 and K13 highland barley under different light conditions. We found that the growth and development process involved the comprehensive effects of circadian rhythm, glucose metabolism, photosynthesis, and hormone-related pathways, which provided a reference for the regulation mechanism of flowering and the heading date of highland barley.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10788-z.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

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Authors' contributions
Z.G. and Z.S. performed the study designed, analyzed the data, wrote and revised the main manuscripts. L.Y.G., X.R.Q.Z and W.M. provided technical support for the bioinformatic analysis data and image analysis. Z.S. conceived and designed the study experiments idea and had also contribution in processing data and image analysis. P.P.Z.M., X.N.T.B., J.G.C., D.W.D., and L.N. have collected all plant samples and modified manuscript. All authors reviewed the manuscript.

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Data availability
The data and supporting sample-specific information discussed in this publication is available on National Center for Biotechnology Information (NCBI) dataset accession number (PRJNA1122922).

Availability of data and materials
The data and supporting sample-specific information discussed in this publication is available on National Center for Biotechnology Information (NCBI) dataset accession number (PRJNA1122922). We confirm that carried out all materials and experiments in this publication were accordance with the relevant guidelines and regulations.

Declarations

Ethics approval and consent to participate
The plant materials used in this article do not involve disputes. The collection of barley varieties used in this experiment was carried out with the permission of Research Institute of Agriculture, Tibet Academy of Agriculture and Animal Husbandry Sciences and passed Professor Zha Sang from the State Key Laboratory of Hulless Barley and Yak Germplasm Resources and Genetic Improvement authenticated the plant. We confirm that all experiments were carried out in accordance with the relevant guidelines and regulations and the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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