BMC Plant Biology

RESEARCH

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Diurnal dynamics of different circadian transcription modules in Chinese pine needles and roots during dormancy induction

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Abstract

Winter dormancy ensures that trees in temperate zones respond appropriately to environmental variations, thereby enhancing their adaptability and resilience. In the northern hemisphere, the dormancy of conifers is induced by short-day and cold temperature. Previous studies have revealed that *TFL2* is a key regulator involved in conifers' bud set and growth cessation during the dormancy-induced phase. Based on the annual expression profile analysis of *PtTFL2* in Chinese pine (*Pinus tabuliformis* Carr.), we identified key time nodes for dormancy initiation in autumn. To provide insight of the diurnal transcriptome dynamic in needles and roots during dormancy introduction, RNA-seq was performed at 12 consecutive time points in 24 h under natural environment in *P. tabuliformis*. Interestingly, we found that both needles and roots have rhythmic oscillatory genes, even though the roots could not receive light signals directly. We applied weighted gene co-expression network analysis (WGCNA) to integrate differentially expressed genes between needles and roots at different time points into highly correlated gene modules. Although the two modules are subject to different transcriptional controls during dormancy, both contain 35 identical transcriptional regulators. Some transcriptional factors with functional similarities and synergistic effects were found to play a role in the regulatory pathway, which provided some data support for mining gene functions and analyzing related regulatory pathways. Our results provide new insights into the molecular regulatory mechanisms involved in pine dormancy.

Keywords Chinese pine (*Pinus tabuliformis* Carr.), Dormancy, Circadian rhythm, Needles, Roots

Introduction

Dormancy serves as a crucial adaptive feature for the survival and growth of tree species that are distributed in the temperate and sub-frigid climatic zones of the Northern Hemisphere. Specifically, conifers, which constitute the majority of the northern hemisphere forests, short days and temperature are required for successful dormancy induction [1]. Trees employ a dormant period during the winter as a strategy to endure adverse environmental conditions [2]. Dormancy serves as a critical adaptation mechanism for plants to cope with seasonal climate changes, and it is not a uniform state but rather a series of states. Consequently, most studies rely on visual

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observation, such as completion of bud set, percentage of bud burst and time required for 50% bud burst to assess the state of quiescence. Plants initiate quiescence by detecting changes in light and temperature, as there are some species which only require one of these factors to enter dormancy [3–5].

Plant circadian clock regulators play a crucial role in regulating long-term developmental processes, such as the transition from vegetative to reproductive development and from growing to dormant stages, in response to long-period circannual changes in environmental factors [6, 7]. The circadian clock regulators in plants are typically divided into three components: the input pathway, central oscillator and output pathway. These circadian clock components interact to form a complex network, consisting of a central feedback loop and two ancillary feedback loops [8, 9]. Plants can sense external environmental information such as temperature, light, and nutrition and transfer these signals to central oscillator, which generat a rhythm of the output genes. Previous studies have found a large number of components functioning in input pathway, central oscillator or output pathway in Arabidopsis [10–13]. Studies have reported that 2 to 11% of *Arabidopsis* genes are regulated by the circadian rhythm [14–17]. In *Arabidopsis*, the feedback regulatory loop consists of three genes: *LATE ELONGATED HYPOCOTYL* (*LHY*), *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), and *TIMING OF CABEXPRESSION 1* (*TOC1*). Where *LHY* binds to *CCA1* to negatively regulate *TOC1* expression, while *TOC1* positively regulates the expression of *LHY* and *CCA1* [18] 。*PSEUDO-RESPONSE REGULATOR 5/7/9* (*PRR5/7/9*), *EARLY FLOWERING 3* (*ELF3*), *EARLY FLOWERING 4* (*ELF4*), *GIGANTEA* (*GI*), and *LUX ARRHYTHMO* (*LUX*) are involved in the additional interlocked feedback loops comprising the central oscillator [10–13, 19–22]. Furthermore, during the daytime in the presence of blue light, circadian clock-controlled genes such as *FLAVIN-BINDING*, *KELCH REPEAT*, *F-BOX1* (*FKF1*), and *GIGANTEA* (*GI*) form a protein complex that degrades the transcriptional repressor *CYCLING OF DOF FACTOR1*(*CDF1*) and results in *CONSTANS* (*CO*) expression [18, 19, 23–25]. Previous studies have demonstrated that in trees, *CO* and *FT* regulate flowering time, the short-day-induced growth cessation, and bud set occurring in the fall, suggesting their potential key role in the regulation of dormancy in seasonal growth [6].

Numerous physiological and biological processes in conifers are influenced by environmental circadian rhythms [26]. Currently, patterns of cyclic gene expression have been examined in a limited number of coniferous species, such as Norway spruce (*Picea abies*) [27], Japanese cedar (*Cryptomeria japonica*) [26] and Douglas-fir (*Pseudotsuga menziesii*) [28]) to identify the 'core clock' genes. Comparative studies have revealed a sinificant conservation of crucial circadian clock genes among monocotyledons and dicotyledons [29–34]. However, there are fundamental differences in the biological clock function between gymnosperms and other plant taxa [27]. This difference may be due to the adaptive adjustment of different plant groups to the environment in the evolutionary process, which makes them form their own unique strategies in the regulation of biological clock. This finding is of great significance for us to further analyze the differences in biological clock function among different plant groups.

To date, research has revealed the existence of core clock genes and diurnal rhythms of the transcriptome in summer in Japanese cedar [26]. Due to seasonal changes, the circadian rhythm of related genes is weakened in winter. The data also revealed genes that showed different expression patterns compared to angiosperms, suggesting a unique circadian clock gene regulatory network in conifers. Circadian clock genes may adjust their expression patterns to adapt to short light and low-temperature environments. Some studies have found that in winter or cold regions, the expression levels of certain circadian clock genes in gymnosperms decrease as photoperiod shortens [27]. In summer or tropical regions, circadian clock genes may maintain or enhance their expression to support efficient photosynthesis and growth of trees under sufficient light and water conditions [35]. This indicates that gymnosperms may have genes that show different expression patterns under different climatic conditions as compared to angiosperms, suggesting the existence of unique gene regulatory networks in conifers.

Research has shown that *CO/FT* regulatory modules and circadian clocks play important roles in controlling plant growth and dormancy in angiosperms [36]. The poplar ortholog of *AtFT*, *Populus trichocarpa FT* (*PtFT1*), plays a role in regulating the multi-year delay in flowering time, as well as contributing to growth cessation and bud set in the fall [6]. It is likely that the perception and integration of dormancy signals during dormancy may vary between coniferous and deciduous tree species [28]. In conifers, *FT* orthologs are absent, but only undifferentiated *FT/TFL1-like* subgene families are present [37]. *PtTFL2* is thought to have a significant role in regulating growth rhythms [38], bud set, and dormancy [39, 40]. Despite the involvement of *FT/TFL1-like* in pine dormancy, the molecular mechanisms underlying needle dormancy are largely unknown.

Leaves, shoots, and roots are essential organs that sense and integrate external stimuli. Although long-distance signals have been identified as originating from bud cells, the existence, role, and pathways of circadian clock signals from roots and needles remain unknown. Studies have demonstrated that for coherent and synchronized

circadian oscillations in plants, plant circadian clocks must communicate circadian information between cells and tissues [41]. Diurnal cycles of temperate trees are superimposed on longer annual cycles that encompass transitions between active growth periods, where light energy is harnessed and transformed into biomass, and dormancy stages, during which growth potential is suspended to safeguard cellular integrity from the seasonal stresses of cold temperatures and freezing [10–12]. The transitions between growth and dormancy is of critical importance for the ability of organisms to adapt to variable environments [13]. It is well established that photoperiod and temperature are important cues for initiating seasonal growth rhythms and establishing the onset of dormancy for many trees. It is anticipated that pathways involved in light capture and photoperception will demonstrate annual rhythmic variation [10–12]. As dormancy is established, trees also perceive and respond to cold temperatures by increasing their cold hardiness resistance [13].

Due to the complexity of circadian processes regulated by multiple genes, thousands of genes are differentially expressed between needles and roots in different biological pathways. In this study, we focused on the main mechanisms of circadian rhythms in needles and roots during dormancy. We applied weighted WGCNA [42] to integrate differentially expressed genes between needles and roots at different time points into highly correlated gene modules. Weighted gene co-expression network analysis clusters genes with similar expression patterns, providing a powerful tool for interpreting transcriptional patterns biologically [43–45]. It represents a co-expression relationship between genes in the same module, allowing unknown genes to participate in a specific biological function [46]. We further elucidated the expression differences and relationships between related genes in needles and roots during dormancy. This study is expected to provide essential information for understanding the molecular mechanisms underlying the dormancy diurnal cycle of gymnosperms and to offer a new perspective for studying the dormancy mechanism in gymnosperms in response to cold.

Materials and methods

Plant material and sample collection

Tissue samples of *P. tabuliformis* were collected from the Green Forest located on Beijing Forestry University(Beijing, China (116°34.535′ E, 40°0.0135′ N, 43.5 m a.s.l.)). campus for the annual transcriptome sequencing cycle. Current year needles were collected every 15 days from July 1, 2017 to July 1, 2019 at approximately 12 P.M. A total of 147 samples (49 time points × 3 biological replicates) were analyzed to determine the expression patterns throughout the annual cycle. Additionally, The daily cycle samples were collected every 2 h on March 25, June 25, July 25, August 25, September 25, and December 25, 2020, from 8 a.m. to 6 a.m. the next day, and a total of 216 samples (72 time points × 3 biological replicates) were collected at 12 time points in 24 h.

The 24 h daily cycle samples were collected from 36 15-year-old *P. tabuliformis* during dormancy in the artificial forest of Beijing Hot Spring Nursery (116°33.91160E, 40°00.08610 N, altitude 44 m). 2 g of needles and roots each were collected every 2 h between 6 am on 16 October 2021 and 4 am on 17 October 2021. Three trees were randomly selected for sampling, and sampled trees were closely planted and experienced similar photoperiodic conditions. Needles were obtained from recently developed mature shoots (see Figure S1 available as Supplementary Data at BMC Plant Biology Online). Fine roots with a diameter of 1 mm were sampled (see Figure S1 available as Supplementary Data at BMC Plant Biology Online), rinsed with distilled water, and blotted dry. Each sampling time point was replicated using the needles from five seedlings, three of which also had their roots sampled. The samples were collected on dry ice and stored at -80 °C for further processing.

RNA extraction and sequencing

After the tissues were frozen in liquid nitrogen and ground into powder, total RNA was extracted from the tissues according to the standard procedure of RNAprep Pure polysaccharide polyphenol plant total RNA extraction kit of Tiangen Biochemical Technology Co., LTD. (Beijing, China). For the total RNA obtained, the RNA purity was determined by Nano Photometer spectrophotometer (Implen, Westlake Village, CA, USA), and the RNA concentration was accurately determined by Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). The integrity of RNA was assessed using the RNA Nano 6000 Assay kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). The mRNA was fragmented into small pieces using divalent cations at high temperatures. The final complementary DNA (cDNA) library was created using cleaved RNA fragments which were reverse-transcribed according to the protocol provided by the mRNA-Seq sample preparation kit (Illumina, Inc., San Diego, CA, USA). The average insert size for the paired-end libraries was 200–300 base pairs (bp). The paired-end module was used to sequence the pooled libraries on the Illumina HiSeq×Ten platform (2 × 150 bp) using the paired-end module. The clean reads were mapped to the *P. tabuliformis* genome [47] and the transcript abundances were estimated using the Kallisto (0.44) software application.

Differential gene expression analysis and functional analysis

Differential analysis was performed using the DESeq2 R package [49]. The differentially expressed genes (DEGs) were determined using the threshold of|fold change (FC)|>2 and adjusted p-value (Padj) < 0.01, which was adjusted using the Benjamini-Hochberg Procedure to control the false discovery rate (FDR). Differentially expressed genes were heat mapped using TM4:MeV software [50]. Data were homogenized by the Log Transformations module of TM4:MeV software, and differential genes were clustered by Hierarchical Cluster (HCL) method [51]. The candidate response genes were queried against the RefSeq non-redundant proteins (NR) database, Swiss-Prot database, Gene Ontology (GO) database, and Kyoto Encyclopedia of Genes and Genomes (KEGG). Information on candidate response genes was obtained through Blast comparison in the DEG Dynamic GO enrichment analysis were performed using Omicshare online tool (https://www.omicshare.com/tools/) library to predict the functional annotations and pathways associated with these candidate response genes.

Construction of co-expression network

For network construction we performed a WGCNA on all of the samples using the standard method [42, 52]. We produced a cluster dendrogram by evaluating the average linkage hierarchical clustering of genes on the basis of topological overlap. The brown modules which were significantly related to circadian rhythm were selected as the basis of the co-expression network from the dormant stage of *P. tabuliformis*. We further constructed a collaborative network(s) based on all of the expressed genes in the roots and needles [in at least one sample group, transcripts per million (TPM) > 1] rather than only differentially expressed genes.

Results

Expression of *PtTFL2* is tightly correlated with the dormancy establishment in *P. tabuliformis*

In late September, the photoperiod decreases and *P. tabuliformis* enters dormancy period. Over the two consecutive years, from early July to early January, *PtTFL2* was significantly activated under short-day conditions concomitantly with a decrease in temperatures (Fig. 1A). The diurnal cycle expression of *PtTFL2* was relatively flat in March, June, July and December. From August, *PtTFL2* was induced during the day. Notably, at the end of September, the sunshine period changed from long to short, and *P. tabuliformis* entered the resting period, when *PtTFL2* was significantly activated(Fig. 1B). In the diurnal cycle, *PtTFL2* first increased and then decreased during the day, and its expression was low at night, indicating that *PtTFL2* has a certain circadian rhythm. According to the changes of annual cycle expression, *PtTFL2* expression reached its peak on October 15 for the two consecutive years and then decreased (Fig. 1A), showing obvious seasonal characteristics. *PtTFL2* may be a key factor regulating dormancy and is activated only in the autumn responding to diurnal changes. It is speculated that *PtTFL2* in *P. tabuliformis* under short day conditions responds to low temperature and light, and exhibits a certain circadian rhythm in the dormant period. Therefore, we determined that the second half of Octoberis a key period for the response of dormancy genes to the circadian rhythm.

Diurnal dynamics of transcriptomes in the *P. tabuliformis* natural environment

As daylight hours decrease, *P. tabuliformis* enters dormancy and is gradually exposed to low temperature conditions in both needles and roots, resulting in significant transcriptional changes in both tissues. There were 2,579 and 2,107 circadian rhythm genes in needles and roots, respectively (Pmean < 0.01) (Fig. 2A). Principal component analysis (PCA) was used to classify the 36 samples into four sets: ND(coniferous leaf samples collected at 6 AM, 8 AM, 10 AM, 12 AM, 14 PM)、NN(leaf samples collected at 18 PM, 20 PM, 22 PM, 24 PM, 2 AM, and 4 AM at night)、RD(root samples taken at 6 AM, 8 AM, 10 AM, 12 AM, 2 PM, and 4 PM during the day)、RN (root samples taken during the night period during which root samples were taken at 18 PM, 20 PM, 22 PM, 24 PM, 2 AM, and 4 AM)(Fig. 2C). The four sets were sampled in two phases, day and night, corresponding to needles and roots, respectively. Interestingly, roots also have rhythmic oscillatory genes, even though they do not receive light signals directly. In both day and night samples, PC1 clearly distinguished needles samples from root samples. On PC2 and PC3, all four sets were basically on the same side. The overall group separation pattern indicated that there were significant differences in the transcriptomic profiles of needles and roots, suggesting that *P. tabuliformis* needles and roots use different specific transcriptional mechanisms. The increase of DEGs expression in needles at night was higher than that in the daytime(Fig. 2B), and DEGs expression in needles at night increased with the decrease of temperature (Fig. 3A). Many of these DEGs are cold resistance genes, suggesting that related cold resistance genes may increase in expression as temperature drops. The number of DEGs in the root is less than the number of changes in the daytime and the night, which may be due to the influence of soil as a temperature buffer in the daytime and night (Fig. 2B).

The air and soil temperatures corresponding to the needles and roots collected during the dormancy of *P. tabuliformis* showed a gradual increase throughout

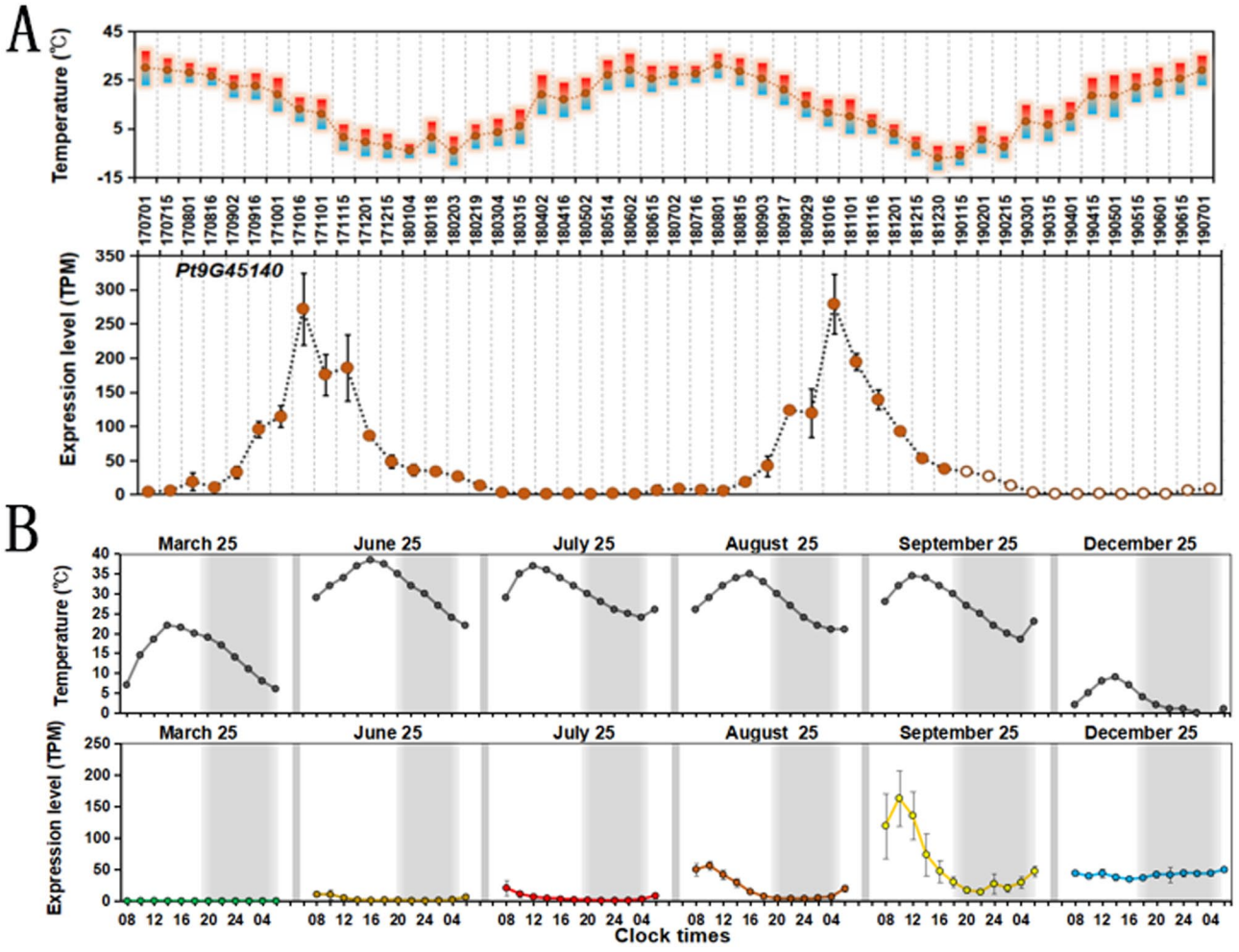

Fig. 1 The expression of *PtTFL2*(*Pt9G45140*) during the annual and diurnal cycles of *P. tabuliformis*. (**A**) Annual cycle sample sampling time and temperature of *P. tabuliformis*, where the X-axis represents the specific year, month and day of sampling. Monitoring lasts for two years, from July 1, 2017 to July 1, 2019. (red square represents the highest temperature of the day, blue square represents the lowest temperature of the day) (**B**) The expression of *PtTFL2* at 8:00, 12:00, 16:00, 20:00, 24:00 and 4:00 in March 25, June 25, July 25, August 25, September 25, and December 25. Error bars represented variability of three independent replicates

the day (From 6am on October 16, 2021 to 16pm on October 16), and a gradual decrease as the night (From 18pm on October 16, 2021 to 4am on October 17, 2021) (Fig. 3A). We clustered these genes according to their expression patterns and found four sets. (Fig. 3B-E). The results showed that the expression levels of 77 genes in the needles decreased with the increase of temperature during the day (Fig. 3B), while the expression levels of 245 genes increased with the decrease of temperature at night (Fig. 3C). With the decrease of temperature, the expression of 131 genes in the root gradually decreased during the day (Fig. 3D), but gradually increased at night (Fig. 3E). At night, significant induction of rhythmical genes occurred in both needles and roots, suggesting that the regulatory mechanisms controlling the dormant period are influenced not only by light, but also by temperature.

Diurnal rhythms in transcription of clock-related genes

Studies have reported that 2 to 11% of *Arabidopsis* genes are regulated by the circadian rhythm [14–17]. To explore the circadian rhythm of needles and roots during dormancy, we analyzed the expression patterns of these genes. Then found that the transcriptional expression patterns of PtCCA1, PtPRR4, PtGI, PtTCP42, and PtLOB94, which are related to circadian rhythm and photoreceptors, were significantly oscillated in both needles (Fig. 4A) and roots (Fig. 4B). The expression pattern of *PtGI* in needles and roots was different. The expression level of *PtGI* in needles was lower during the day, but increased abruptly with the fall of night. On the contrary, in the root, the expression of *PtGI* was higher during the day, and decreased sharply with the onset of night. Although there are genes of circadian rhythm in needles and roots, they have different regulatory mechanisms.

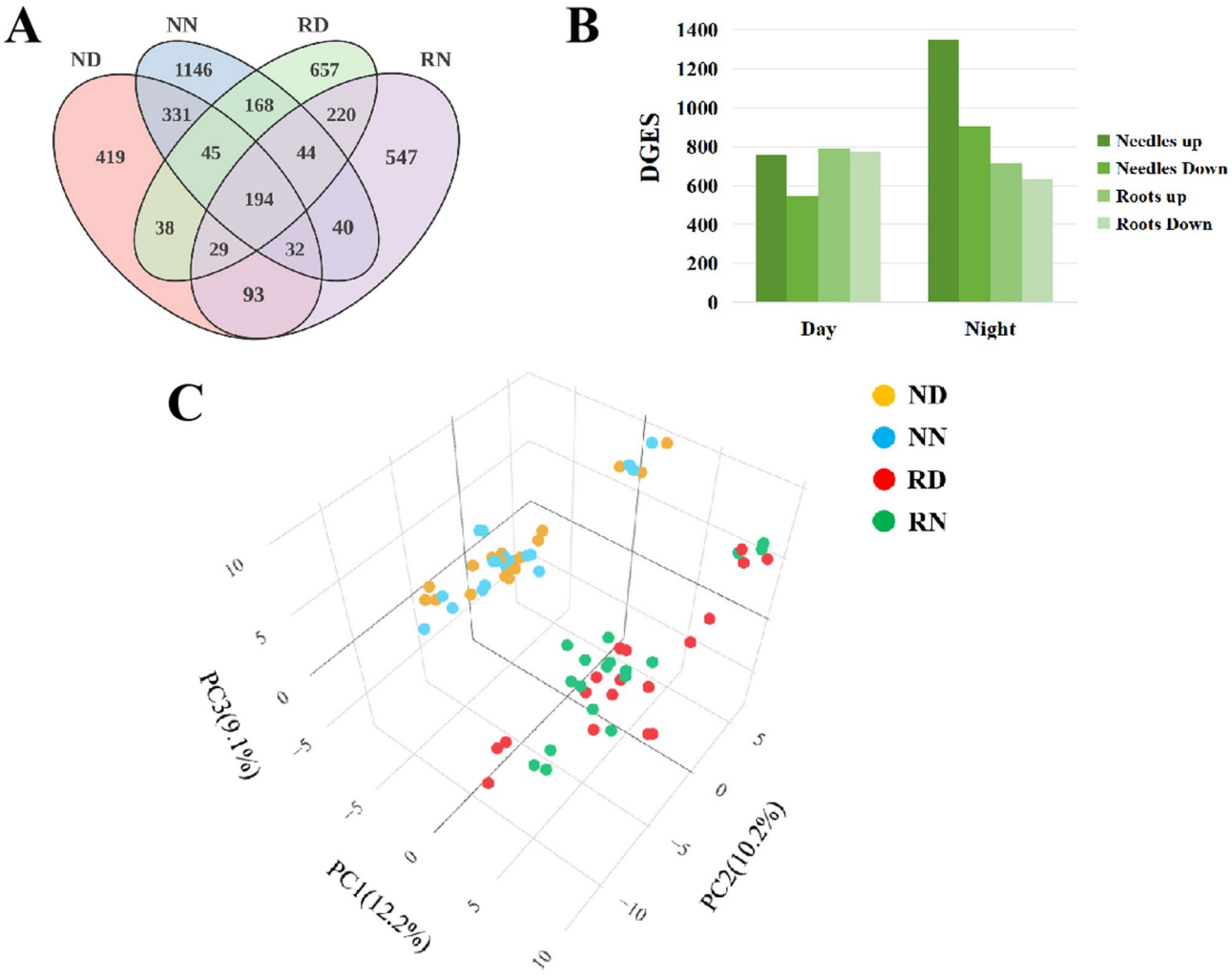

Fig. 2 Transcriptome analysis of needles and roots during dormancy. (**A**) Venn diagram of DEG distribution for each set of sampling times.ND represents coniferous leaf samples collected at 6 AM, 8 AM, 10 AM, 12 AM, 14 PM, and 16 PM during the day; NN represents needle leaf samples collected at 18 PM, 20 PM, 22 PM, 24 PM, 2 AM, and 4 AM at night; RD represents root samples taken at 6 AM, 8 AM, 10 AM, 12 AM, 2 PM, and 4 PM during the day; RN represents root samples taken during the night period during which root samples were taken at 18 PM, 20 PM, 22 PM, 24 PM, 2 AM, and 4 AM. (**B**) The numbers of DEGs (|Log2 fold change| > 1, FDR < 0.05) arising from four set of sampling. (**C**) Principal component analysis (PCA) of transcriptomic profile of four set of sampling

In order to explore the expression of genes of circadian rhythm under different diurnal cycles, we selected three days representative of months with long day and short day conditions (March, June, July, August, September and December) and analyzed the diurnal cycle expression profiles by RNA-seq (Fig. 4). The results revealed that the expression of *PtTCP42* and *PtLOB94* on March 25, June 25, July 25, August 25, and September 25 exhibited significant circadian rhythms, except for December 25. In contrast, *PtCCA1*, *PtPRR4*, and *PtGI* displayed significant circadian rhythms on all six time points. The transcriptional level of *PtCCA1* peaked at 8:00 on March 25, and then decreased. Transcription gradually increased on June 25, July 25, August 25, and September 25, and gradually decreased towards dawn, reaching its lowest levels at 12:00. However, on December 25, the expression of *PtCCA1* began to gradually decrease slightly from 16:00, suggesting that low temperature may have affected its diurnal rhythm. The expression of *PtPRR4* increased first and then decreased on March 25, June 25, July 25, August 25 and September 25, and this trend was particularly obvious on June 25, July 25, August 25 and September 25, and all reached its lowest levels at 2AM. The expression of *PtGI* fluctuated on March 25, June 25, July 25, August 25, and September 25, initially increasing before decreasing, with a peak observed at 4 PM followed by a decline. Conversely, on December 25, the expression of *PtGI* mirrored that of *PtPRR4*, reaching a minimum at 20 PM before increasing. Notably, *PtTCP42* exhibits a diurnal expression pattern, displaying a pronounced circadian rhythm [53]. *PtLOB94* demonstrated a significant circadian rhythm on August 25 and September 25,

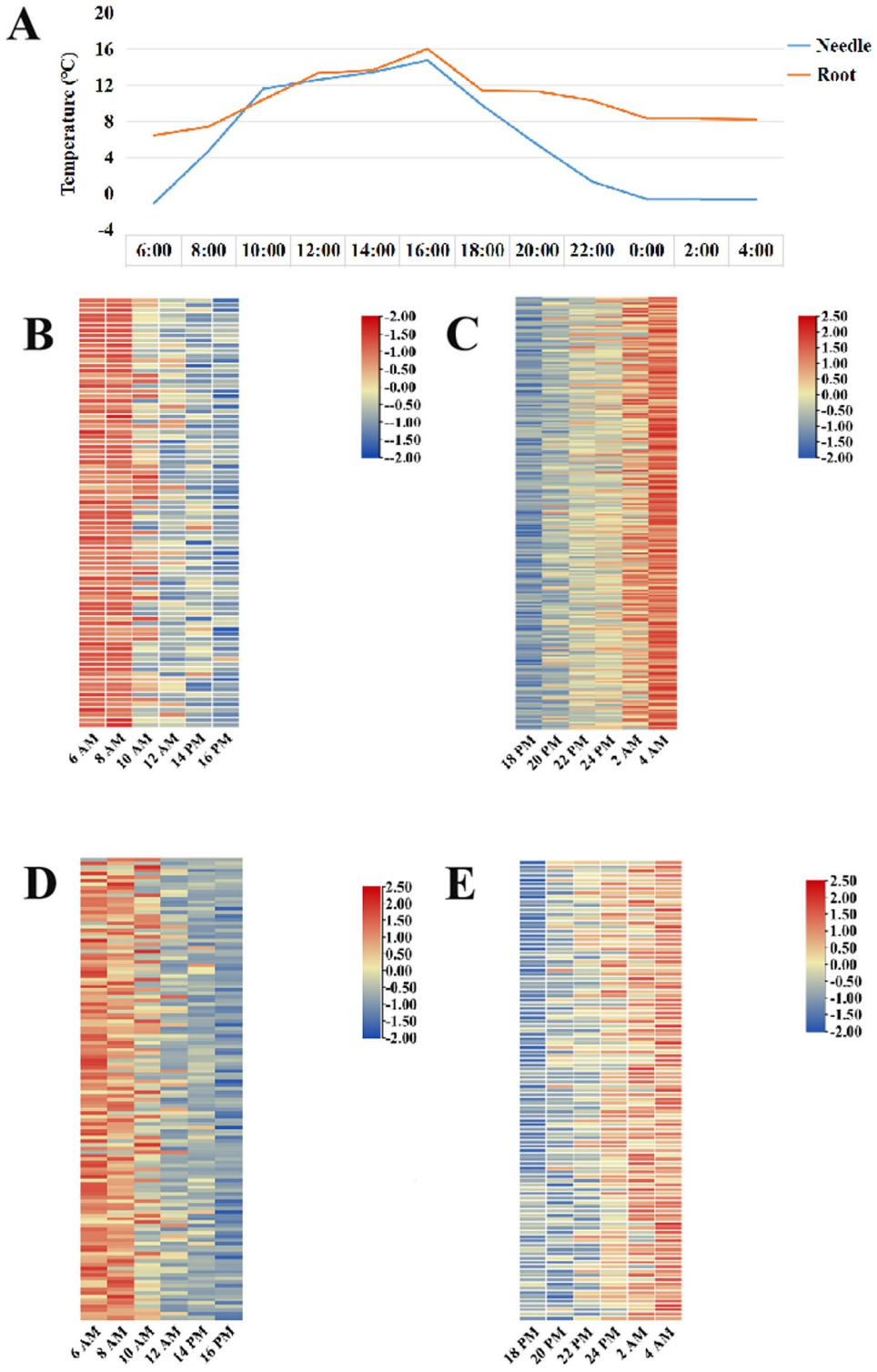

Fig. 3 Hierarchical clustering analysis of four sets genes with circadian rhythm and corresponding low temperature. (**A**) The corresponding ambient temperature of needles and root samples collected during the dormancy. (**B**-**E**) Hierarchical clustering analysis of decreased expression of circadian rhythm genes with increasing temperature in ND、NN、RD and RN set. Each datapoint consists of the expressions (FPKM) of three biological replicates. Red indicates high relative gene expression and blue indicates low relative gene expression

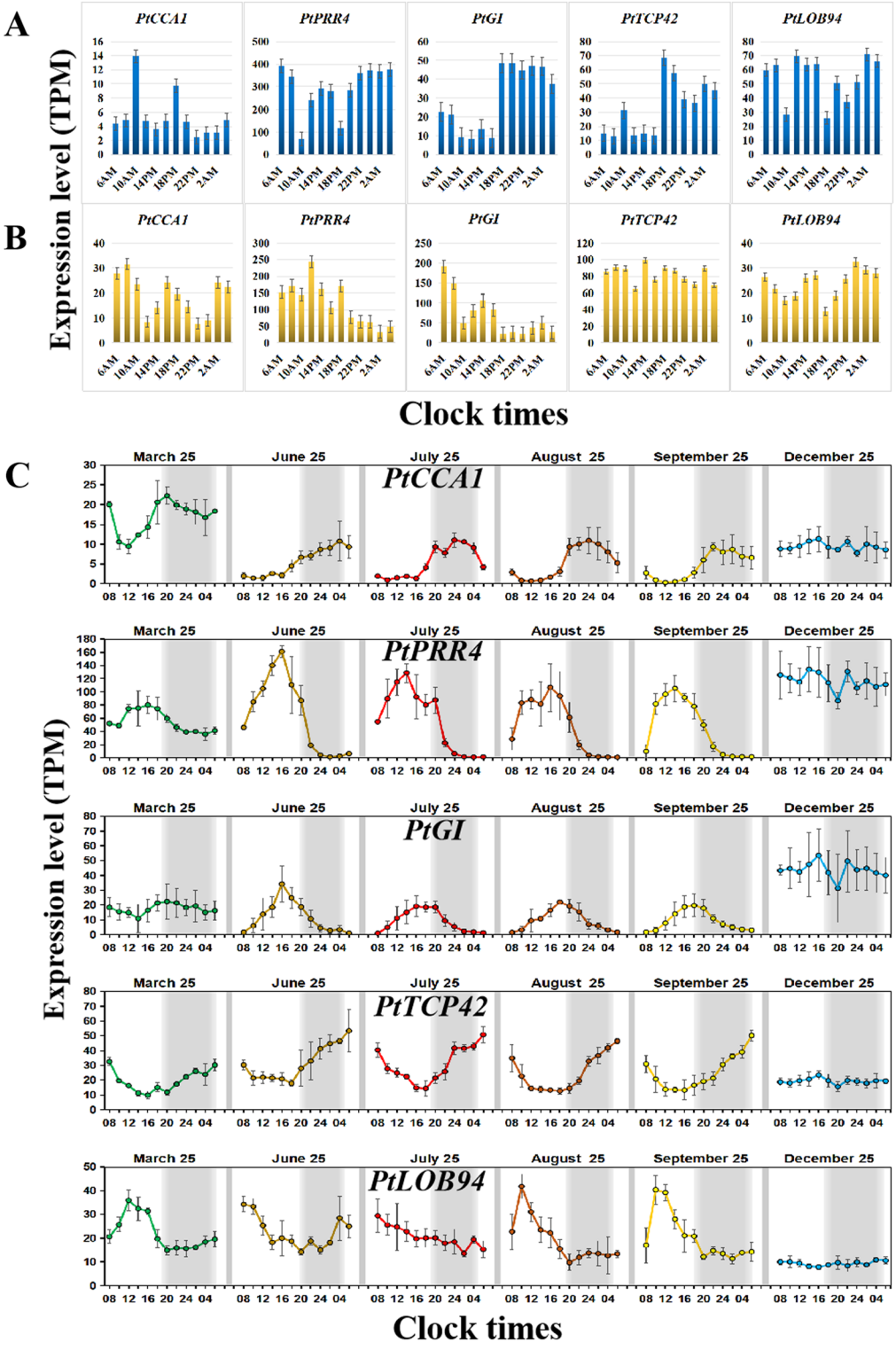

Fig. 4 Diurnal rhythms in transcription of clock-related genes. (**A**) Expression trend of clock-related genes in needles of Chinese pine at dormancy stage. (**B**) Expression trend of clock-related genes in roots of Chinese pine at dormancy stage. (**C**) The expression of clock-related genes at 8:00, 12:00, 16:00, 20:00, 24:00 and 4:00 in March 25, June 25, July 25, August 25, September 25 and December 25. Error bars represented variability of three independent replicates

reaching a minimum at 20 PM before increasing. However, on December 25, the expression of *PtLOB94* did not exhibit a significant circadian rhythm, suggesting that low temperature may impact its regulation. These observations reinforce the critical role of *PtCCA1*, *PtPRR4*, *PtGI*, *PtTCP42*, and *PtLOB94* in modulating the biological clock of *P. tabuliformis*.

Characterizing responses in needles and roots to dormancy

The rhythmic regulation of transcript levels is a crucial aspect of the circadian system. During the dormancy period of needles and roots, genes with circadian rhythms play a unique and complementary role in coordination. To gain a more detailed understanding of the function of rhythm genes in the needles and roots of *P. tabuliformis* in the four groups of ND, NN, RD, and RN, we conducted a DEG-enrichment analysis using GO categories(Fig. 5A). GO categorizes 'biological process', 'molecular function', and 'cellular component'. Our study aimed to investigate the conservation and specificity of the transcriptional reaction between the needles and roots of *P. tabuliformis*. The results of the GO enrichment analysis demonstrated that while most circadian genes were shared between needles and roots, there were significantly more enriched classes in the needles (Fig. 5A). Genes related to solute and ion transport across membranes were found to be abundant in both DEGs and needle-specific genes across both tissues. This suggests that regulating solute and ion transport across membranes is an important response to cold stress in both tissues, particularly at night when roots are exposed to colder soil temperatures. However, the relatively higher temperature experienced by needles may explain why they exhibit greater responsiveness and diversity towards cold stress during the night.

The stronger response by needles to exposure to cold is further demonstrated by the finding that 'membrane part' and 'binding' categories were only enriched in needle-specific DEGs. Additionally, at night, the needles had significantly more genes involved in responding to circadian rhythms and 'nucleic acid-binding transcription factor activity' than the other three groups. This reflects the increased expression of transcription factors (TFs) such as *bHLH*, *bZIP*, *MYB*, *NAC*, and *AP2/ERF* superfamily in needles exposed to freezing temperatures (Fig. 5H), which can resist low temperatures. Interestingly, genes in the root system that respond to circadian rhythms are mostly enriched during the day, even though the root system cannot directly receive light signals, the expression of genes in the root system that respond to circadian rhythms is higher when exposed to light (Fig. 5A). These DEGs were enriched in 'cellular process', 'developmental process', 'single-organism process', 'metabolic process', 'multicellular organismal process', 'multi-organism process', 'response to stimulus', 'regulation of biological process', and 'transporter activity'. These results indicate that genes in the root play an important role in responding to stress and environmental stimuli.

Finally, KEGG was used to analyze the biological functions of genes that have circadian rhythms in needles and roots during the day and night(Fig. 5B-G). Both day and night, needles and roots have a similar number of genes involved in transport and catabolism of cellular components. In terms of 'environmental information processing', there is a relatively high abundance of genes involved in the 'plant hormone signal transduction' pathway, with most being expressed during nighttime.

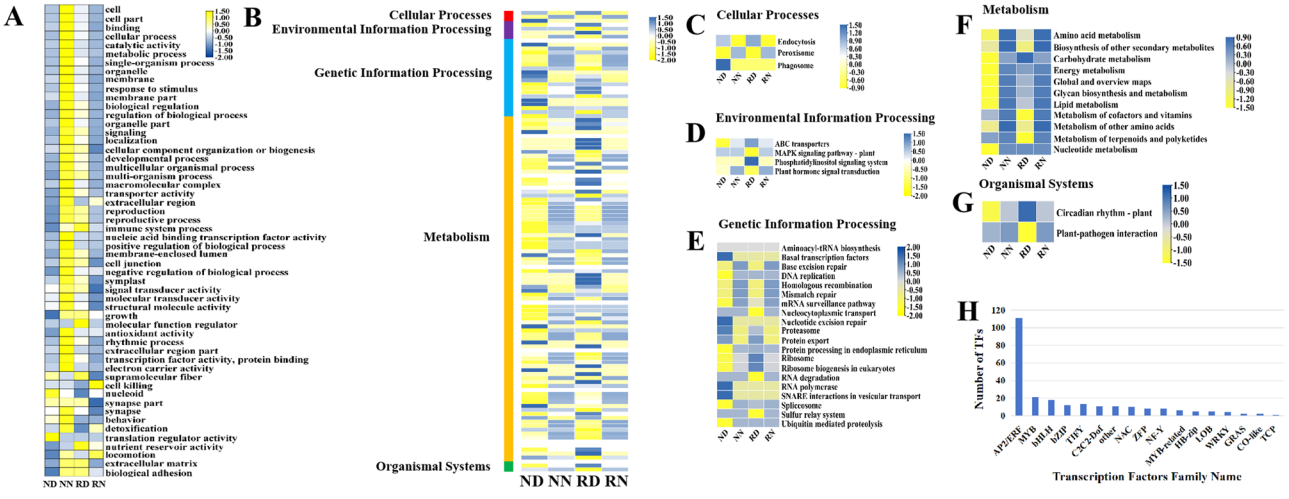

Fig. 5 Enrichment analysis and KEGG analysis of four group genes with circadian rhythm. (**A**) Enrichment analysis of four group genes with circadian rhythm.(**B**) KEGG pathways of four group genes with circadian rhythm.(**C**-**G**) Four groups of genes with circadian rhythms are involved in cellular processes、environmental information processing、genetic information processing、metabolism and organismal systems. (**H**) Four groups of transcription factors with circadian rhythms

The 'genetic information processing' pathway contains a relatively small number of genes, primarily distributed in 'folding, sorting and degradation' as well as 'translation'. The Metabolism module encompasses 74% of all genes, with 'metabolic pathways' and 'biosynthesis of secondary metabolites' accounting for nearly 50%. Within the environmental adaptation of 'organismal systems', 'circadian rhythm plants' exhibit enriched gene expression across all groups.

The patterns of hormone-related genes representing hormone biosynthesis and signaling pathways through the hormone signal-related genes listed in the KEGG database. We found that 23 genes may be related to growth hormone showed significant circadian rhythms. Among them, the expression levels of *Pt5G04180*, *Pt1G09210*, *Pt1G09200* and *Pt2G47630* gradually increased in needles at night, and suddenly increased from the same time (22PM), indicating that these four genes may be related in the resting period of *P. tabuliformis* (Fig. 7). These four genes, *Pt5G04180*, *Pt1G09210*, *Pt1G09200* and *Pt2G47630*, tended to be expressed only in night needles. The homolog of *Pt5G04180* in *Arabidopsis* is *AT5G53160*, which is a regulatory component of ABA receptor and interacts with protein phosphatase 2Cs ABI1 and ABI2 to stimulate ABA signaling [54]. *Pt2G47630* homologue in *Arabidopsis* is *AT4G01026*, which encodes a member of the *PYR/PYL/RCAR* family proteins with 14 members. These proteins function as abscisic acid sensors and mediate ABA-dependent regulation of protein phosphatase 2Cs ABI1 and ABI2 [55]. Low temperature stress has been shown to induce an increase in plant hormone ABA content [56]. It has been further indicated that ABA, functioning as an important 'anti-stress hormone' in plants, can also promote plant dormancy and enhance plant adaptation to stressed environments.

WGCNA associates specific co-expression modules with diurnal dynamics during dormancy in needles and roots of *P. tabuliformis*

To obtain an overview of the complex dataset and identify gene hubs of roots and needles regulatory networks under the diurnal time regulation during the dormancy process of *P. tabuliformis*, a total of 2784 and 2580 genes that were differentially expressed ($P < 0.05$) between the needles and roots samples for at least one time point were analysed using WGCNA based on the expression profiles of 72 samples from needles and 72 samples from roots. Genes in the roots (Fig. 6A) and needles (Fig. 6B) were clustered by expression patterns, as indicated by the dendrogram, and modules are indicated in different colours. We found eight modules in the needles that contained more than 60% of the 1,663 circadian rhythm genes. The nine modules in the root had more than 59% of the 1,614 circadian rhythm genes.

Among these, the brown module in the root contains 177 genes with a total of 31 transcription factors. The other eight modules, black, blue, green, gray, pink, red, purple, and yellow, contain only 6, 16, 9, 15, 13, 10, 17, 13 transcription factors. Moreover, brown module include the major clock genes *PtCCA1* and *PtCOL10*, which are associated with circadian rhythms. We constructed the gene regulatory network of the brown module based on the correlations between genes in the samples (Fig. 6C). However, in needles, the brown module still had the largest number of transcription factors, 42 in total, including the major clock genes *PtCCA1*, *PtCOL10*, *PtMYB270* and *PtTCP42* related to circadian rhythm. The other seven modules, black, blue, green, gray, red, purple, and yllow, contain only 6, 22, 18, 0, 7, 38 and 8 transcription factors. In addition, we constructed a gene regulatory network of the brown module based on the correlation between these 42 genes in the needles (Fig. 6D).

The brown module in the needles and roots contains 35 identical genes(highlighted in Fig. 4C-D) and 12 identical transcription factors. These include the *v-myb avian myeloblastosis viral oncogene homolog* (*MYB*)*-related* transcription factor *PtCCA1* and *PtMYB270*, as well as clock genes that respond to circadian rhythm, cold stress-related *APETALA2/ethylene response factor* (*AP2/ERF*) members *PtERF111* and *PtERF112*. *Abiotic stress-related nascent polypeptide-associated complex* (*NAC*) transcription factor member *PtNAC16*, *DNA-binding one zinc finger* (*Dof*) transcription factors members *PtDof1*, *PtDof2*, *PtDof3*, and *C2C2-CO-like zinc finger* transcription factor *PtCOL10*, which responds to diurnal rhythm. Although common TFs were significantly induced in both tissues, the majority exhibited distinct regulation between the two tissues. When this was considered alongside the evidence for tissue-specific TFs clusters, it suggested that needles and roots exhibited different transcriptional control in dormancy.

Discussion

Woody plants growing in temperate or cold regions rely on a survival mechanism known as winter dormancy to acclimate to such environments during the cold season [56]. Conifers are the primary source of wood globally [47], and in these long-lived forest species, the length of dormancy limits the growing season, thereby affecting wood production and quality [57]. Consequently, the timing of dormancy onset is a crucial ecological variable. Understanding the core regulatory factors of conifers' dormant period is of great significance for their genetic improvement. This study analyzed the expression of *PtTFL2*, a key regulatory for growth arrest in conifers during the dormancy induction stage, for two

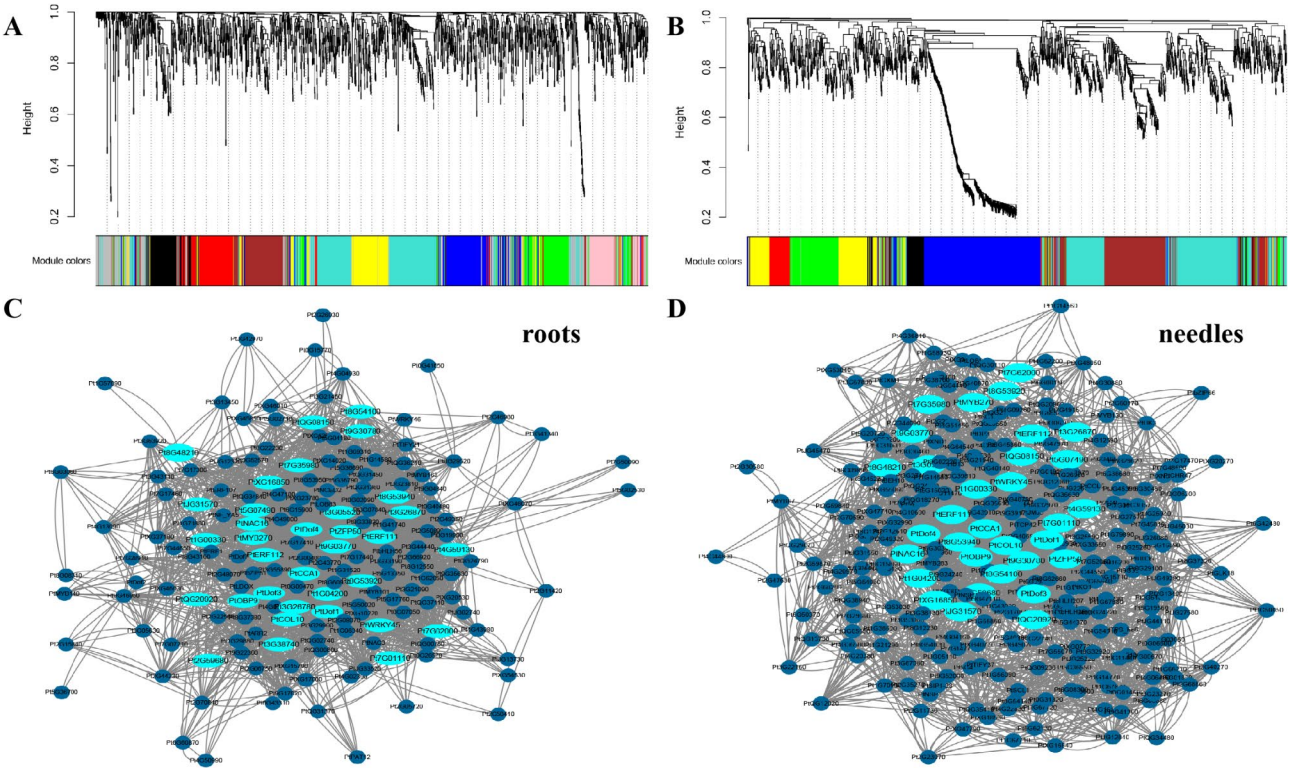

Fig. 6 Weighted gene co-expression network analysis (WGCNA) of expression patterns of differentially expressed genes between roots and needles identifies distinct modules of co-expressed genes. (**A**-**B**) Using the expression profiles of all 84 samples, WGCNA was performed on 2784 and 2580 differentially expressed ($P < 0.01$) genes between root and needle samples at each time point, respectively. On the basis of topological overlap, the average linkage hierarchical clustering of genes is carried out to obtain the clustering tree. (**C**-**D**) They are the regulatory networks of the conifer and root brown module genes. Nodes (genes) are connected by mutual expression relationships. The light blue ovals represent genes that differ significantly between needles and roots at each point in time

consecutive years, and determined the key timing for dormancy initiation in autumn. A systematic analysis of the diurnal dynamics of different circadian transcription modules in *P. tabuliformis* needles and roots during dormancy introduction was also conducted.

Winter dormancy is a continuous process in which plants undergo a series of complex and finely regulated physiological state transitions, a process that profoundly reflects plant response and adaptation to environmental signals.The depth of dormancy and the speed of the process depend to a large extent on the external environmental factors that induce dormancy, in particular the continuous effect of changes in sunlight duration and temperature. In the *Populus trichocarpa*, *PtFT1* is a key determinant of the timing of growth arrest and bud formation in response to short sunlight [6]. When the photoperiod changes from long to short and the temperature drops at the end of September, *P. tabuliformis PtTFL2* (homologue of *PtFT1*) is also significantly activated. This phenomenon has occurred steadily in two consecutive years of observations, showing a sharp peak around 15 October, displaying a distinct seasonal pattern of change [37]. The dormancy process in both angiosperms and gymnosperms is co-regulated by a series of core genes and signalling pathways that form a complex network within the plant in response to changes in the external environment. There is a close and complex interaction between light signalling and *abscisic acid* (*ABA*) signalling pathway, and this interaction mechanism plays a crucial role in the continuous regulation of cold acclimatisation and dormancy in *Populus trichocarpa* [58]. Covington et al. investigated circadian microarray data in Arabidopsis and found that plant hormones and various stress response pathways are affected by the biological clock, further emphasizing the importance of the biological clock in plant physiological regulation [13]. As a key phytohormone, ABA is widely recognised in angiosperms as an important component of the crosstalk phenomenon in the stress signalling pathway, and its role in plant response to environmental changes cannot be ignored. In gymnosperms, ABA also plays an important role in stress response, although the specific mechanism of action may be different from that of angiosperms. ABA is not only involved in the regulatory process of cold domestication, but also more directly involved in the photoperiodic control mechanism of cold domestication, and this

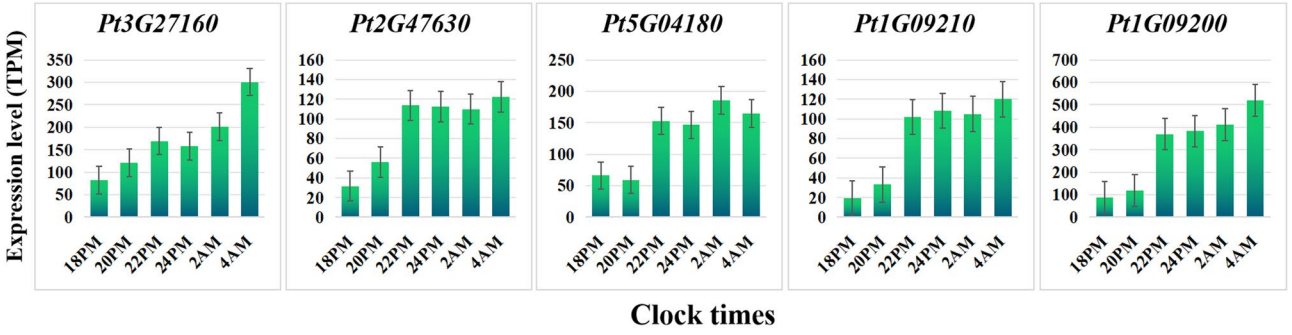

Fig. 7 The expression of circadian rhythm genes in different time periods. Error bars represented variability of three independent replicates

finding provides a new perspective for us to understand how woody plants adjust their physiological state according to changes in the external environment [59]. As an important stage in the plant life cycle, the occurrence and development of winter dormancy are jointly influenced by a variety of internal and external factors. The regulation of dormancy and circadian rhythms in angiosperms and gymnosperms reflects the adaptability and diversity of plants to the environment during the evolutionary process.

Through screening analysis of Differentially Expressed Genes (DEG), it was found that both tissues produced a progressive transcriptional response to cold, exhibiting the most common cold adaptation response [60]. This was done to identify potentially key conserved transcription factors mediating cold acclimation during the dormancy phase of *P. tabuliformis*. GO analysis of all DEGs induced in *P. tabuliformis* (Fig. 5) revealed that, regardless of the tissue, common genes that induce cold responses were overrepresented in core stress categories such as 'transporter activity', 'response to stimulus' and 'nucleic acid binding transcription factor activity'. It was observed that among the genes that induce cold responses, there are relatively many genes in the 'response to stimulus' category and the response of the two tissues indeed differs, as shown in the GO diagram. The GO analysis revealed that root-specific induction genes were enriched in categories such as 'cell', 'cell part', 'binding', 'cellular process', and 'metabolic process'. In contrast, needle-specific induced DEGs were enriched in categories such as 'single-organism process', 'organelle', 'membrane', and 'response to stimulus' when compared with roots. The contrast responses observed suggest that during the dormant period, needles are exposed to extremely cold and dry environments, which require membrane recombination and protection. It also shows that in order to keep the evergreen foliage alive throughout the winter, the needles have to acquire extreme low-temperature tolerance and maintain a more active metabolism. Relevant studies have demonstrated that the accumulation of carbohydrate and lipid metabolism plays a crucial role in the survival of perennial evergreen needles at extremely low temperatures [61–63]. Similar to angiosperms, changes in carbohydrates, accumulation of low molecular weight cryoprotective metabolites and cryoprotective proteins (e.g. dehydrins) contribute to cold hardiness in conifers. Furthermore, when exposed to low temperatures, both needles and roots increased cold adaptability, as measured by electrolyte leakage [64, 65]. *Xyloglucosyltransferase/hydrolase* (*XTH*) is a cell wall modifying enzyme that regulates plant growth and development and stress response, playing an important role in cell growth [66]. *Pt3G27160* is the homologue of *AT2G36870*, which in Arabidopis encodes for *XTH*. The expression of *Pt3G27160* in the roots of *P. tabuliformis* increased with the decrease of temperature at night, suggesting that *Pt3G27160* may respond to low temperature stress during dormancy and participate in the regulation of root growth and development [67](Fig. 7).

When day lengths become shorter and temperatures drop, temperate trees go dormant in order to survive the cold of winter. In autumn, photoreceptors cooperate with a biological clock system that senses the shortening of the photoperiod, leading to growth cessation and dormancy progression to protect against the cold. The adaptive mechanisms by which trees enter dormancy differ depending on their tissue type. For example, bark, lamina and leaf cells can tolerate freezing-induced dehydration through extracellular freezing, whereas xylem thin-walled cells can avoid intracellular freezing by deep subcooling [68]. In this study, through WGCNA analysis of circadian genes in needles and roots of *P. tabuliformis* during dormancy, we found the key modules responding to circadian rhythms and resolved the co-expression network of core genes in needles and roots. The homologs of clock genes have been identified and are highly conserved in conifers [26]. The photoperiodic pathway is one of the most conserved pathways during the evolution of seed plants, as evidenced by the *P. tabuliformis* genome project [69]. The present study demonstrated that the clock genes *PtCCA1*, *PtTCP42* and *PtMYB270* from *P. tabuliformis* exhibited significant circadian rhythm, thereby

confirming the conservation of these genes in conifers. In *Populus*, the high and sustained expression of the clock gene *Populus tremula* × *Populus tremuloides* (*Ptt*) *LATE ELONGATED HYPOCOTYL* is necessary for the regulation of cold biological clock during dormancy.

In our study, key regulators also included members of the *AP2-ERF*, *MYB*, *NAC*, *Dof* families, which exhibited increased expression at low temperatures. Moreover, some studies have validated that these transcription factors are involved in cold responses [70]. These types of TFs, are well known as hub regulators in complex stress-responsive regulatory networks [71, 72]. Studies have shown that increased tolerance of plants to abiotic stress is linked to enhanced nutrient uptake, altered hormonal balance, increased activity of reactive oxygen scavenging systems, and synthesis of osmoregulatory substances. On the other hand, the circadian clock plays a role in regulating plant nutrient homeostasis, hormone synthesis and signaling, redox reactions, and changes in the concentration of some major osmoregulatory substances [26, 73–76]. This shows that the circadian rhythm system of plants plays an important role in the face of abiotic adversity.

The *AP2/ERF* gene family has been extensively studied in relation to cold stress during the dormant period [77, 78]. Notably, this gene family also exhibited the most significant circadian rhythm in this study. AP2/ERF proteins are involved in various signal transduction pathways, with some family members playing a role in the stress signaling crossover pathway [79]. The *ERF* subfamily serves as a crucial regulatory factor in plant responses to various stress factors, including hormones, pathogens, low temperature, drought, and high salt. Additionally, the *ERF* subfamily plays a significant role in ABA and other signal transduction pathways [80]. Previous studies have demonstrated that the product of chrysanthemum *CmERF110* functions as a flower activator, primarily via the photoperiodic pathway. The transcription of *CmERF110* exhibits a similar circadian rhythm, suggesting that *CmERF110* is a clock gene that predominantly regulates flowering through the photoperiodic pathway [79]. Previous studies have demonstrated that the high expression levels of several *AP2/ERF* genes in dormant flower buds of *Prunus pseudocerasus* occur in different dormancy stages. This suggests that these genes are involved in the dormancy transition of flower buds and may play a role in cold-mediated dormancy transition [77]. In addition, relevant research shows that *Dof* genes have been identified as playing a role in the development of dormancy and the transitional stage in *Prunus persica* and *Fagus sylvatica* [78, 81]. In grape, the *Dof* gene plays a role in initiating the dormancy process when the plant is subjected to low temperature stress [82]. Apple tree (*Malus* × *domestica Borkh.*) development is regulated by chilling temperatures, which are required for dormancy progression. Some studies have shown that, *NAC* is involved in the establishment and maintenance of dormancy [83]. This finding further enriches our understanding of plant adaptation strategies to low temperature environments. This not only deepens our understanding of plant dormancy and stress response mechanisms, but also provides a valuable theoretical basis and practical guidance for future genetic engineering to improve tree resilience and forestry productivity.

Our study provides a comprehensive overview of the transcriptome associated with the dormancy of *P. tabuliformis*. This study reveals the core regulatory modules and key regulatory factors with significant circadian rhythm in needles and roots during the dormant period of *P. tabuliformis* Which provided basic data for understanding the circadian regulation mechanism during the resting period of conifers. While our findings provide new insights into the molecular regulatory mechanisms of dormancy in conifers, it should be acknowledged that there are inherent limitations in our study. The lack of suitable conifer genetic transformation tools currently prevents us from directly validating these findings in the conifer system. Future work directly in conifers, including the generation of transgenic lines and the application of gene editing technologies, can be utilized for directional breeding, such as the efficient production of cold-stress resistant trees, enhancing the ecological adaptability of trees and helping the forestry industry to meet the challenges posed by future climate change.

Conclusion

Most studies of diurnal mechanisms in the annual model species Arabidopsis, which does not exist in Arabidopsis. This study determined the key time node for the onset of autumn dormancy in *P. tabuliformis* based on the annual expression profile of *PtTFL2*. Microarray analysis revealed differences in transcriptome dynamics between needles and roots. Interestingly, both needles and roots were found to have rhythmic oscillatory genes, even though the roots could not receive light signals directly. We applied WGCNA to integrate differentially expressed genes between needles and roots at different time points into highly correlated gene modules, some transcriptional factors with functional similarities and synergistic effects were found to play a role in the regulatory pathway, providing data support for mining gene functions and analyzing related regulatory pathways. Regulatory network analysis also identified key TFs associated with dormancy and cold acclimation in both tissues. These results provide new insights into the molecular regulatory mechanisms involved in pine dormancy.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06365-5.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Author contributions

JHY collected samples, analyzed the data and wrote the manuscript. KQ and HLW collected samples in the field. WL, YAE conceived the study and revised the manuscript. All authors have read and approved the manuscript.

Funding

This work was supported by "National Key R&D Program of China (2022 YFD2200304)".

Data availability

The sequenced raw reads generated during the current study have been submitted to the National Center for Biotechnology Information (NCBI) with BioProject ID: PRJNA1072300 (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1072300.). Any additional information required to reanalyze the data reported in this work paper is available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 29 January 2024 / Accepted: 7 March 2025
Published online: 02 April 2025

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