Genome-Wide Identification and Functional Characterization of the PheE2F/DP Gene Family in Moso Bamboo

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Research article

Keywords: E2F/DPs, Moso bamboo, expression, cell cycle, bamboo shoot

Posted Date: August 1st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-45428/v1

Version of Record: A version of this preprint was published at BMC Plant Biology on March 29th, 2021.
See the published version at https://doi.org/10.1186/s12870-021-02924-8.

Abstract

Background

E2F/DP proteins have been shown to regulate genes implicated in cell cycle control and DNA repair. However, to date, research into the E2F/DP family and its functional role in Moso bamboo has been limited.

Results

Here, we identified 24 *E2F/DP* genes in the Moso bamboo genome, including nine E2F, six DP, and eight DEL genes. Simulation of the divergence time of paralogous gene pairs revealed an important role of whole-genome duplication in the expansion of the E2F/DP family. The regulatory element and coexpression network analysis indicated that *E2F/DP* regulated the expression of cell cycle-related genes. Yeast two hybrid assay and expression analysis based on transcriptome data and *in situ* hybridization indicated that PheE2F-PheDP complex play important roles in winter moso bamboo shoot growth. Expressional analysis based on qRT-PCR performed diverse expression patterns of PheE2F/DPs in response to both various abiotic stimuli and diurnal cycles.

Conclusion

These findings provide insights into the E2F/DP family members in Moso bamboo and experimental evidence for further functional verification of the E2F/DP family.

Background

Moso bamboo (*Phyllostachys edulis*) is one of the most important nontimber forest products in the world. Indeed, the bamboo shoot can grow as long as 1 m within 24 h and reaches a final height of 15–20 m in just one to two months in suitable spring conditions. Due to its remarkable growth rate and unique strength, Moso bamboo generates an equivalent of 5 billion US dollars, including both the timber and food industries [1, 2]. During Moso bamboo shoot growth, simultaneous cell division and cell elongation affect internode elongation. Cell division is predominant in the winter growth period and early growth period, while cell elongation is predominant in the late growth period, leading to height increments in bamboo shoots.

The E2F/DP transcription factors in higher plants consist of three subgroups: E2F, DP, and DEL (DP-E2F-like) [3]. Generally, the members of the E2F group contain a DNA-binding domain, an Rb-binding domain, a ‘marked box’ domain, and a leucine zipper dimerization domain, while the DP group member contains a leucine zipper dimerization domain and a DNA-binding domain. The DEL group genes, which contain only

a duplicated DNA-binding domain, were considered to be atypical E2F/DPs and act in monomeric form [4].

The domain organization controls the interaction of the E2F and DP proteins via their leucine zipper dimerization domains. The E2F/DP family has eight members in *Arabidopsis*. It has been reported that during cell proliferation, the canonical AtE2Fs play an antagonistic role, as E2Fc is a negative regulator, whereas E2Fa and E2Fb are positive regulators [5, 6, 7]. E2Fa and E2Fb interact with DPa to activate the cell cycle and cell proliferation-associated gene expression [8].

It has been found that the E2F member might perform distinct roles in the control of cell fate determination [3]. In mammals, the E2F signaling pathway plays a key role in cell growth and cell proliferation [9]. Studies on E2F in *Arabidopsis* have indicated that plants also have orthologs of all the core regulators in the E2F signaling pathway, including CDK inhibitors (CKIs), retinoblastoma (RB), cyclins, and cyclin-dependent kinases (CDKs). In plants, E2F binds to the E2FAT (TTTCCCGCC) motif in its target genes, playing a role in promoting transcription [10].

However, to date, no studies have addressed the *E2F* gene family and its function in Moso bamboo. Here, for the first time, the phylogenetic relationships, gene structures, and conserved motifs of 24 *PheE2F* genes were analyzed. Promoter analysis indicated that various *cis*-acting elements involved in the photoperiodic response, hormone signaling, and meristem growth as well as many MYB and MYC2 binding sites were present in the promoter region of *PheE2F* genes. We surveyed potential genes containing E2F binding sites in their putative promoters within the Moso bamboo genome and identified 580 E2F/DP target genes. Based on bioinformatic prediction, we further studied changes in the expression of the *PheE2F* genes during Moso bamboo shoot growth and in response to diurnal cycles and abiotic stress (PEG and NaCl). Yeast two hybrid assay and expression analysis were conducted in order to investigate the potential roles of PheE2F/DPs involved in moso bamboo shoot growth. Our study should inform the characterization of PheE2F/DP genes and provide new insights and valuable information for the further identification of this versatile gene family in Moso bamboo.

Results

Identification and classification of the PheE2F/DP gene family

A total of 24 *PheE2F/DP* genes that contained complete E2F domains were identified in the Moso bamboo genome. To determine the evolutionary relationship of these genes in Moso bamboo and other species, we constructed a NJ phylogenetic tree of E2F/DP proteins from Moso bamboo, rice, *Brachypodium* and *Arabidopsis*. For the 24 identified PheE2F/DP genes of Moso bamboo, the proteins could be classified into three functional groups: E2F, DP, and DEL [3]. Nine of these proteins were classified into the E2F group, six into the DP group and eight into the DEL group. PH02Gene25981.t1 contained only partial E2F domains and did not fit in any group (Fig. 1A). In addition, phylogenetic analysis of E2F/DPs in four species indicated a divergence between monocotyledons and dicotyledons. PheE2F/DP shared more sequence similarity with OsE2F/DP and BdE2F/DP than with AtE2F/DP. For

example, AT5G14960.1, AT3G01330.1, and AT3G486160.1 clustered together in the DEL group, showing a distant relationship with *Brachypodium*, rice, and Moso bamboo.

Characterization of the PheE2F/DP gene family

Structural analysis of the genes revealed that E2F/DP gene family members within the same group shared similar gene structures in terms of their intron numbers and intron length (Fig. 1B). The quality of introns in the DP group and DEL group ranged from seven to nine and three to nine, respectively. The average numbers of introns in the E2F group were much higher than those in the other two groups, and the intron quality ranged from five to sixteen.

Eight conserved motifs in the E2F/DP proteins were identified using the MEME tool (Fig. 1C and Additional files1: Fig. S1). Motifs 1 and 6 represent the E2F_TD domain, and motif 2 and motif 3 represent the E2F_DD domain and DP domain, respectively. Motifs 2, 4 and 7 appeared only in the E2F group, whereas motifs 3 and 8 were found exclusively in the DP group. Most DEL members shared motifs 1 and 5 with most E2F protein sequences. PH02Gene25981.t1, which contained only partial E2F domains, harbored only motif 3. Motif analysis indicated that most members of the same subgroup shared one or more identical motifs outside the conserved domain mentioned above, and closely related members were found to exhibit common motif compositions, suggesting functional similarities within the same subgroup.

Of the 24 sequences, we identified 10 putative paralogous pairs of *PheE2F/DP* genes, whereas only three paralogous pairs were identified in *Arabidopsis.* The four paralogous pairs were detected in rice and *Brachypodium* (Additional files1: Table S1). The mean values of the divergence time for Moso bamboo, rice, *Brachypodium* and *Arabidopsis* were 11.57, 39.61, 49.08 and 25.24 mya (million years ago), respectively. The divergence of most *PheE2F/DP* gene pairs (8 of 10) occurred approximately 7 to 12 mya, consistent with the time of the Moso bamboo whole-genome duplication event (7-12 mya) [11] and much later than the times for the three model plants.

Regulatory network of PheE2F in Moso bamboo

Regulatory elements in the promoter region are essential to temporal, spatial, and cell type-specific control of gene expression [12]. Thus, we searched the 2000-bp upstream promoter regions of all the *PheE2F/DP* genes in the PlantCARE database. A large number of *cis*-acting elements related to light response sites, hormone response sites, transcription factor binding sites and meristem-related motifs were found (Fig. 2). All 24 *PheE2F/DP* promoters contained at least one light response motif. All MYB binding motifs and most MYC binding elements were found in 24 promoters of *PheE2F* genes. Thirteen *PheE2F/DP* promoters contained P-box or GARE motifs that were responsive to gibberellin stimulation. A *cis*-acting element involved in abscisic acid responsiveness (ABRE) was abundant in *PheE2F* gene promoters, and this element was found in 21 promoters. In addition, a *cis*-acting regulatory element involved in methyl jasmonate (MeJA) responsiveness (TGACG motif) and a *cis*-acting element involved in salicylic acid responsiveness (TCA element) appeared in most *PheE2F* promoter regions. Nineteen

promoters containing a *cis*-acting regulatory element were related to meristem expression (CAT box), indicating the importance of *PheE2Fs* involved in cell division and proliferation. In addition, no significant difference was observed between different groups by comparison of *cis*-acting elements.

A previous study reported that E2F binds to the E2FAT (TTTCCCGCC) motif of its target genes and regulates their transcription [10]. Thus, we screened the binding sites in the promoter regions of 51,074 protein-coding genes in the Moso bamboo genome using PlantCARE. Finally, 580 genes that contained E2F/DP binding sites in their promoter regions were identified. To gain further insight into the potential function of these genes, Gene ontology (GO) enrichment analysis was performed (Fig. 3). Within the biological category, the groups with the highest abundance of genes included mitotic cell cycle (GO:0000278), mitotic cell cycle process (GO:1903047), regulation of DNA metabolic process (GO:0051052), regulation of DNA replication (GO:0006275), and regulation of cell cycle (GO:0051726).

The transcriptome data generated from 13 different culm tissue samples were used to investigate the expression patterns of *PheE2F/DPs.* Most E2F/DPs showed relatively high expression levels in winter bamboo shoots (S1) and spring bamboo shoots, especially during the early growth period (S2-S5), compared with the other growing culms, such as the rhizome (R) and seedling stems (SS1 and SS2). Of the 18 genes that were expressed in at least one tissue, 11 showed the highest expression level in winter bamboo shoots (S1) (Fig. 4). *PH02Gene01086.t1*, *PH02Gene07670.t2*, *PH02Gene34520.t1* and *PH02Gene43370.t2* were highly expressed in 50-cm-tall bamboo shoots (S2), while *PH02Gene46765.t1* exhibited the highest accumulation level in the outward rhizome (O). *PH02Gene05248.t2* and *PH02Gene10996.t1* were highly expressed in S4 (300-cm-tall bamboo shoots) and S5 (600-cm-tall bamboo shoots), respectively. Expression analysis results revealed that *PheE2F/DPs* played important roles in the growth of bamboo shoots, especially in the winter period.

To understand the regulatory network of PheE2F/DPs implicated in the different types of culm growth, we carried out regulation tests between quantitative changes of transcripts in the 13 different culm tissue samples. Based on the regulatory relationship obtained by prediction of regulatory elements in promoter regions and PCC (R≥0.90 or R≤-0.90) screening, a total of 58 genes had a strong correlation with at least one PheE2F/DP gene (Fig. 5). In the coexpression network, the main hub gene PH02Gene26414.t1 with the highest connecting times positively regulated the expression of *PH02Gene35099.t1* (*LSD1*), *PH02Gene44324.t1* (*ATXR3*), *PH02Gene09249.t1* (*HOP*), etc. In addition, PH02Gene05248.t2 and PH02Gene01086.t1 had the second and third highest connecting times. All three hub genes showed the highest expression level in winter bamboo shoots, and all of them were regulated by PH02Gene08546.t1 (MYB), PH02Gene20517.t1 (MYB) and PH02Gene25898.t2 (MYB). In addition, all three genes regulated the expression of cell cycle-associated genes, including *PH02Gene32333.t1* (*CDKF*), *PH02Gene29974.t1* (*MRB1*), *PH02Gene36158.t1* (*SPR2*), and *PH02Gene33706.t1* (*MCM2*), as well as DNA replication-associated genes, including *PH02Gene32448.t3* (*POLD3*) and *PH02Gene37503.t2* (*POLA2*). Furthermore, many genes involved in RNA processing or environmental stress response showed high correlation with many E2F/DP members. The *in situ* hybridization data showed that the mRNAs of the hub genes

PH02Gene01086.t1 and *PH02Gene26414.t1* were highly expressed in ground tissues of winter bamboo shoots but showed low expression levels in vascular bundles (Fig. 6).

Previous studies have reported that E2F can interact with DP in *Arabidopsis* [8]. In order to attest whether this interaction also occurs in moso bamboo, yeast two-hybrid experiment was adopted to examine the interaction between E2Fs and DPs. The three hub genes, *PH02Gene34520.t1* (*E2F*), *PH02Gene26414.t1* (*DP*), *PH02Gene34005.t1* (*DP*) which showed the highest accumulation level in winter bamboo shoot were selected and were further to be tested. The combination of pGBKT7-PH02Gene26414.t1 + pGADT7-PH02Gene34520.t1 and pGBKT7-PH02Gene34005.t1 + pGADT7-PH02Gene34520.t1 were co-transformed into yeast strain AH109, respectively. Y2H results showed that both two transformants tested grew well on SD/-Leu/-Trp medium (Fig. 7). When transferred onto SD/-Trp/-Leu/-Ade/-His/X-α-Gal plates for 5 days, both the positive control and the experimental group turned blue. In contrast, the negative control displayed no α-galactosidase activity. The results suggested that PheE2Fs can interact with PheDPs in vitro.

Expression profiles of PheE2F genes under abiotic stress treatment and diurnal rhythms

Regulatory analysis revealed that most *PheE2F* gene upstream sequences carry a variety of hormones and light response elements. Hormone response elements, especially those of stress-related hormones such as abscisic acid, MeJA and salicylic acid, indicate that these genes might be induced or repressed by abiotic stress. To understand the transcriptional changes in *PheE2F* genes under abiotic stress, the seedlings were subjected to drought (PEG) and salt treatments (Fig. 8). Under drought stress, the expression levels of *PH02Gene07950.t1*, *PH02Gene07670.t2*, *PH02Gene09693.t3*, *PH02Gene10996.t*1, *PH02Gene26414.t1*, *PH02Gene34005.t1*, *PH02Gene01086.t1*, *PH02Gene05248.t2*, and *PH02Gene18408.t2* were downregulated, while those of *PH02Gene34520.t1*, *PH02Gene34267.t1,* and *PH02Gene42115.t1* were upregulated. The expression of *PH02Gene03595.t*1, *PH02Gene20868.t1,* and *PH02Gene43494.t1* peaked at 12 h, 1 h, and 1 h, respectively. For the salt treatment, while *PH02Gene30693.t2*, *PH02Gene31148.t1*, *PH02Gene34520.t1*, *PH02Gene43370.t2,* and *PH02Gene34267.t1*showed upregulated expression trends, the other *PheE2F/DPs* showed no significant differences in expression or downregulated expression trends.

To analyze the expression pattern of *PheE2F/DPs* during diurnal cycles, we investigated the expression profiles of the bamboo shoots at 4 h intervals over a 24 h period (Fig. 9). qRT-PCR analysis indicated that most *PheE2FDPs* were regulated by diurnal cycles. The transcription levels of *PH02Gene07950.t1*, *PH02Gene09693.t3*, and *PH02Gene03595.t*1 peaked at 15:00, 09:00 and 06:00, respectively. The transcriptional levels of *PH02Gene34520.t1*, *PH02Gene18408.t2* and *PH02Gene42115.t1* remained at the maximum values from 05:00 to 20:00 (daytime).

Discussion

The E2F/DP gene family in Moso bamboo.

E2F/DP controls the temporal expression of genes that are essential for multiple biological processes during the cell cycle, and their transcription level is important for cell proliferation [7]. In a genome-wide screening, 24 E2F/DP genes harboring conserved domains were identified in Moso bamboo, much higher than the number in *Arabidopsis* (7), rice (8), *Zea mays* (19), wheat (18) and *Brachypodium* (11) [13, 14, 15]. The genome size of Moso bamboo (2,021 Mb) is comparable to that of *Z. mays* (2,300 Mb) [16], much smaller than that of wheat (*Triticeae*) (17 Gb) [17], and much larger than that of *Brachypodium* (300 Mb), *Arabidopsis* (164 Mb), and rice (441 Mb) [18, 19, 20]. Rather than the genome size, the much higher number of *PheE2F/DPs* in Moso bamboo indicated that the abundance of *E2F/DP* genes in Moso bamboo may be related to genome duplication events. Previous reports suggested that Moso bamboo was tetraploid in origin, experienced a long progression from tetraploidy to diploidy, and carried two duplicates, similar to rice gene model sets [11, 21]. A similar phenomenon was consistently observed in the E2F/DP family. Although Moso bamboo suffered a large-scale gene loss event after whole-genome duplication, 10 putative paralogous pairs of PheE2F/DP genes remained, far exceeding the quantities in *Arabidopsis*, rice, and *Brachypodium,* which harbor only three, four, and four paralogous pairs, respectively. In addition, most duplication events of PheE2F/DP paralogous pairs in Moso bamboo occurred 7-12 mya, which is consistent with the time of the recent whole-genome duplication event and much later than those of the three model plants. All the results suggest that the recent whole-genome duplication, which was likely linked to polyploidy events, played an important role in the expansion of PheE2F/DP.

PheE2F/DP proteins are involved in Moso bamboo shoot development

Recently, extensive studies have been conducted on the roles of PheE2F/DP in regulating important biological processes of plants, such as leaf development [22, 23], root growth [8], maintenance of genome integrity and viability [24], and the environmental stress response [25]. Expression analysis showed that more than half of the *PheE2F/DPs,* including five *E2Fs* and four *DPs,* showed the highest expression in winter bamboo shoots (Fig. 4). The screening of the binding sites at the promoter regions suggested that a large number of cell cycle genes were regulated by the PheE2F/DP family, such as *CDKF*, *MRB1*, *SPR2* and *MCM2* (Fig. 5), and most of these downstream genes also showed high expression levels in winter and the early growth period [26]. Previous studies suggested that intercalary meristem cells grew and divided vigorously during the winter growth period (S1) and early growth period (S2-S5), especially in the former, but in the late growth period, mitosis in the intercalary meristems decreased, and the continuous growth of the bamboo shoot was substituted with cell elongation [2]. In *Arabidopsis*, E2Fa and E2Fb combine with DPa to activate downstream gene expression [8]. In this study, we used yeast two-hybrid analysis to detect the interaction between E2Fs and DPs, and we selected three hub genes which showed highest accumulation levels in winter bamboo shoot and to be tested. Our results showed that PheE2Fs can interact with PheDPs (Fig. 7), which provides further favourable evidence that PheE2F-PheDP complex may plays important roles in winter moso bamboo shoot growth. Thus, we concluded that the high abundance of E2F/DP genes in the winter growth period and early growth period was essential for E2F-DP complex formation and that the E2F-DP complex promoted meristem cell growth vigorously by activating the expression of cell cycle genes.

Coexpression network analysis suggested that *PH02Gene2641.t1*, *PH02Gene05248.t2* and *PH02Gene01086.t1* were regulated by PH02Gene08546.t1 (MYB), PH02Gene20517.t1 (MYB) and PH02Gene25898.t2 (MYB). In addition, several MYB genes, such as *PH02Gene26882.t1* and *PH02Gene09101.t1,* repressed the expression of *E2F/DP* genes (Fig. 5). In tobacco, MybA1 and MybA2 mRNAs fluctuated and peaked at mitotic period, and regulated the expression of cell cycle genes. In transient expression assays, MybA1 and MybA2 activated the MSA-containing (M-specific activator) promoters, whereas MybB repressed these promoters [27]. In the current study, all 24 promoters contained MYB binding sites, and most *E2F/DP* genes showed positive or negative correlations with *MYBs.* Thus, under the control of MYBs, the *E2F/DP* genes played important roles in regulating the expression of cell cycle genes during Moso bamboo shoot growth and development.

PheE2F/DP proteins are involved in stress response and light response

Based on the regulatory element analysis, a large number pression analysis based on qRT-CPR also confirmed that most *PheE2F/DPs* were photoresponse genes. The transcriptional levels of *PH02Gene18408.t2* and *PH02Gene42115.t1* remained at the maximum value during the daytime in Moso bamboo shoots, while *PH02Gene30693.t2* and *PH02Gene07670.t2* were highly expressed at night (Fig. 9). In *Arabidopsis*, light alters the balance of E2FC and E2FB, with opposing functions in the regulation of cell proliferation. Light increases the expression of E2FB protein levels and further induces the expression of cell cycle genes [28]. Thus, we speculated that *PH02Gene30693.t2* and *PH02Gene07670.t2,* which showed high accumulation levels at night, might act as negative regulators of the cell cycle and cell proliferation, and their high abundance at night might repress the expression of cell cycle genes. In the daytime, light increased the abundance of *PH02Gene18408.t2* and *PH02Gene42115.t1* and activated the expression of cell cycle genes, further accelerating light-mediated Moso bamboo shoot growth and development.

The identification of regulatory elements in the promoter regions of *PheE2F/DPs* indicated that most members harbored abscisic acid, MeJA and salicylic acid regulatory elements, indicating that the expression of these genes was regulated by some environmental cues. qRT-PCR analysis also provided evidence that several *PheE2F/DP* genes were induced under drought and salt stress treatments (Fig. 8). Coexpression network analysis suggested that many environmental stress response genes were regulated by E2F/DPs. *PH02Gene44788.t1* (*AtRGGA*), which showed high correlation with *PH02Gene41928.t1* (*E2F/DP*) and PH02Gene26414.t1 (*E2F/DP*), is involved in stress responsiveness. In *Arabidopsis*, *AtRGGA* is induced by salt and osmotic stress, and *atrgga* mutants exhibit increased sensitivity to abiotic stress [29]. In addition, *PH02Gene25752.t1* (an ABA-responsive gene) and *PH02Gene37465.t1* (a plant viral response gene) were also regulated by E2F/DPs in the network [30]. The high accumulation level of several *PheE2F/DPs* under abiotic stress might be essential for plant resistance to numerous adverse environments.

Cell cycle regulation might be disturbed by drought and salt stress at the transcriptional level by repression of the expression of *E2Fs*. However, there is much new evidence that suggests that drought

and salt stress can stimulate the expression of cell cycle genes. For the *Medicago truncatulaE2Fb* gene, an upregulated trend was reported under high-concentration salt treatment [3, 25]. In addition, many studies have also revealed the relationship between the expression of some cell cycle regulators and salt stress. The expression of *OryzaCDKC1,* which is involved in different aspects of cell biology, such as gene transcription, cell proliferation and differentiation, was triggered by NaCl treatment through the ABA signaling pathway [31]. In *Arabidopsis*, *CDC2aAt*, a cyclin-dependent kinase-coding gene and two mitotic cyclin genes, namely, *Arath;CycB1;1* and *Arath;CycA2;1,* were monitored during salt stress [32]. In mammals, many E2F members act as key factors in DNA damage-dependent transcriptional regulation of cell cycle genes [33]. Many PheE2F/DPs showed high accumulation levels under abiotic stress, which may facilitate bamboo growth under harsh environments through a direct or indirect effect. Collectively, *PheE2F/DP* genes play varied roles in different biological processes in Moso bamboo.

Conclusion

In conclusion, 24 *E2F/DP* genes were identified in the Moso bamboo genome, including nine E2F, six DP, and eight DEL genes. Promoter analysis indicated that various *cis*-acting elements involved in the photoperiodic response, hormone signaling, and meristem growth and many MYB and MYC2 binding sites were present in the promoter region of *PheE2F* genes. Recent whole-genome duplication played important roles in the expansion of the E2F/DP family. Expression analysis based on transcriptome data and *in situ* hybridization analysis revealed that *E2F/DPs* play important roles in bamboo shoot growth, especially in the winter period. Expression profiles derived from qRT-PCR analyses indicated that *E2F/DPs* are involved in various forms of abiotic stress and diurnal cycles. These findings provide comprehensive insights into the E2F/DP family members in Moso bamboo and some experimental evidence for further functional verification of the E2F/DP family.

Methods

Database searches

The annotated *E2F/DP* genes of *Arabidopsis thaliana* (*Arabidopsis*), *Oryza sativa* (rice), and *Brachypodium distachyon* (*Brachypodium*) were obtained from The Arabidopsis Information Resource (TAIR) [34], the rice genome annotation project [35], and the *Brachypodium distachyon* genome database [36], respectively. Then, published *AtE2F/DP* and *OsE2F/DP* sequences were retrieved and used as queries in blastn searches against the local bamboo CDS database, and sequences were selected as candidate genes if they had an E-value ≤ −10. The Pfam database was further used to confirm each predicted *E2F/DP* sequence.

Plant materials

Moso bamboo shoots as well as other culm samples were harvested in Jingxian County (116°10'; 31°12'), Anhui Province, from January to August 2018 (Additional files1: Figure S2). The plant materials were

snap frozen in liquid nitrogen and stored at -80 °C prior to use. Shoot tissues were sampled from sites that were suitable for Moso bamboo growth and free of insect pests and artificial destruction. The permission of bamboo shoot collection for the experiments was obtained from forestry bureau of Jingxian County. Moso bamboo seeds were collected in Dajing County, Guilin (E110u179-110u479; N25u049-25u489) in Guangxi Zhuang Autonomous Region from April to August, 2018 (Additional files1: Figure S2). The necessary permits of bamboo seed were obtained for the field studies from Guangxi Provincial Academy of Forestry and Guilin Forestry Bureau, Dajing County in Guangxi Provence. The identification of these seeds was performed by Gao et al [37]. In addition, the field work conducted for sampling did not affect the local ecology and did not involve endangered or protected species.

The expression experiment of circadian rhythms was conducted under long-day (15 h: 9 h, light: dark) conditions with samples collected every 3 h during a 48-h period from one month old bamboo seedlings. Five individuals that showed similar growth patterns were pooled at each time-point and three biologically independent replications were performed for each sample.

For the abiotic stress treatment, the one month old seedlings were grown in an artificial climate chamber with long-day conditions (15 h light/9 h dark). Drought stress was created by irrigating the plants with medium containing 18% (v/v) PEG6000. Salt stress was created by irrigating the plants with medium containing 250 mM NaCl. The leaves were harvested at 1, 3, 12 and 24 h after abiotic stress treatment. Seedlings irrigated with tap water were used as the control (CK). The plant materials were snap frozen in liquid nitrogen and stored at -80 °C prior to use. For all experiments, quintuplicate independent individuals showing similar growth states were combined to form one sample, and three biological repeats were performed for each sample.

Gene structure, conserved motif and regulatory element analysis

The gene structure based on full-length mRNA alignments with relevant genomic sequences was investigated using the online program of the Gene Structure Display Server [38]. MEME was used to identify conserved motifs in the identified E2F/DP sequences [39]. The regulatory elements in the promoter region of *PheE2F/DPs* as well as the other 51,050 Moso bamboo genes (from -2000 bp to the transcription start site) were analyzed using the PlantCARE online program [12]. The promoters of genes that contained E2F/DP binding sites were further used for Gene Ontology (GO) enrichment analysis via TBtools [40].

Estimation of the duplication time in paralogous pairs

The nonsynonymous substitution (Ka) and synonymous substitution (Ks) values between the paralogous pairs were calculated by the DNASP program. The formula $T = Ks/2\lambda$ was used to calculate the divergence time of the duplication event, with a divergence rate $\lambda = 6.5 \times 10^{-9}$ in Moso bamboo, rice and *Brachypodium* and 1.5×10^{-8} in *Arabidopsis* [41].

Multiple sequence alignment and phylogenetic tree analysis

Sequence alignment was executed using the full-length amino acids of *Arabidopsis*, rice, *Brachypodium* and Moso bamboo E2F/DPs in the program ClustalX 2.1. A phylogenetic tree based on the neighbor-joining (NJ) method was constructed using MEGA 7.0 with the following parameters: pairwise deletion, Poisson model, and 1000 bootstrap replicates.

RNA isolation, reverse transcription and gene expression analysis

Total RNA from each sample was extracted using the TRIzol reagent method (Invitrogen, USA). First-strand cDNA synthesis was conducted with approximately 2 μg of RNA using AMV (Promega, Madison, Wisconsin, USA). qRT-PCR was performed using SYBR Green chemistry (Roche, Mannheim, Germany) on a Light Cycler 480 instrument (Roche, Rotreuz, Switzerland) according to the manufacturer's directions. Primers were designed using the Primer 3 online program (Additional files1: Table S2) [42]. All technical and biological reactions were performed in triplicate. All samples were normalized to the reference gene *TIP41* to analyze the target gene expression level [43]. The final relative expression levels were calculated using the $2^{-\triangle\triangle}$Ct method.

Transcriptome data analysis

The transcriptome data of *PheE2F/DPs* from developing rhizome-root systems and bamboo shoots have been previously generated and processed (GSE90517, PRJNA604634) [2, 44]. These transcriptome data were generated from different types of growing culms, including 1.5-cm-tall seedling stem (SS1), 8-cm-tall seedling stem (SS2), lateral bud (L), rhizome (R), outward rhizome (O), and bamboo shoots at different growth stages (winter bamboo shoot and 50-, 100-, 300-, 600-, 900- and 1200-cm-tall shoots, designated S1-S7, respectively (Additional files1: Figure S2). Culms after leaf expansion were selected in accordance with bamboo developmental stages labeled as CK. The four growing culms (S1, O, SS1 and R) were sampled when the culm length reached one-tenth of the final length. The expression abundance of *PheE2F/DPs* was calculated as fragments per kilobase of exon model per million mapped reads (FPKM). The heatmap was generated using R.

Regulation network of PheE2Fs

Transcription factor-target gene (TF-TG) interactions were predicted based on regulatory elements in the promoters of target genes. The expression correlation of the genes was calculated by determination of the Pearson correlation coefficient (PCC) using gene expression values from the high-throughput transcriptome data in R. Expression correlation data were used for the correlation network, and coexpressed gene pairs were filtered with PCC values $\geq +0.90$ or < -0.90 as previously described [21]. The network was further visualized using Cytoscape 3.7.0 software.

*Insitu*hybridization

Bamboo shoot tissues were fixed in 4% paraformaldehyde for one day at 4 °C, dehydrated in an ethanol series, infiltrated with xylene, and embedded in paraffin. The embedded tissues were then sectioned at a

12-μm thickness using a microtome (Leica, Weztlar, Germany). The slides were dehydrated and baked, followed by dewaxing with dimethylbenzene. The specific probes of *PH02Gene01086.t1* and *PH02Gene26414.t1* were amplified by PCR using gene-specific primers with T7 and SP6 RNA polymerase-binding sites. Antidigoxigenin antibodies coupled with NBT/BCIP solution were used to detect hybridization signals. Images were captured by an Olympus Nikon E600

Yeast two-hybrid

To confirm the interaction between E2Fs and DPs, the Matchmaker GAL4 two-hybrid system (Clontech, Palo Alto, CA) was carried out. Based on in-fusion cloning method (Clontech, Palo Alto, CA), the full length of PH02Gene26414.t1 and PH02Gene34005.t1 cDNAs were cloned into the pGBKT7 bait vector respectively, and the full length of PH02Gene34520.t1 were cloned into pGADT7 (Additional files1: Table S3). Next, the combination of pGBKT7-PH02Gene26414.t1 + pGADT7-PH02Gene34520.t1 and pGBKT7-PH02Gene34005.t1 + pGADT7-PH02Gene34520.t1 were co-transformed into yeast strain AH109, respectively. The transformed yeast cells were selected on SD/-Trp/-Leu and SD/-Trp/-Leu/-Ade/-His/X-α-Gal plates at 30 °C for 5 days to verified the protein-protein interaction. We used the co-transformants with pGBKT7-53 and pGADT7-T as a positive control, while the co-transformants with pGBKT7-Lam and pGADT7-T were used as negative controls in the Y2H experiments.

Abbreviations

ABA
abscisic acid
ABRE
abscisic acid responsiveness
CDC
cell division cycle
CDKC
cyclin dependent kinase C
CREs
cis-regulatory elements
DNAsp
DNA Sequence Polymorphism
FPKM
fragments per kilobase of exon model per million mapped reads
GO
Gene ontology
Ka
nonsynonymous substitution
Ks
synonymous substitution

MEGA
molecular evolution genetics analysis
MeJA
methyl jasmonate
MEME
multiple em for motif elicitation
MYA
Million years ago
NJ
neighbor-joining
PCC
pearson correlation coefficient
Pfam
Protein families database
PEG
Polyethylene glycol
qRT-PCR
Quantitative real-time PCR
TAIR
Arabidopsis Information Resource
TF-TG
transcription factor-target gene
Y2H
Yeast two hybrid

Declarations

Consent for publication

Not applicable.

Availability of data and materials

All the RNA-Seq raw data are available at NCBI under accession number GSE90517, PRJNA604634.

Competing interests

All the authors have declared no conflict of interest.

Funding

This work was supported by the National Key point Research and Invention Program of the 13th Five-Year (grant No. 2018YFD0600101), the Shaanxi Provincial Natural Science Foundation (Grant No. 2019JQ-402), the Fundamental Research Funds of ICBR (Grant No. 1632018006) and Scientific Startup foundation for Doctor of Northwest A&F University (grant No. Z109021715).

Contributions

Long Li performed bioinformatics analyses, physiological experiments and drafted the manuscript. Qianqian Shi helped in sample collection. Jian Gao designed the experiments and conceived the project, provided overall supervision of the study and revised the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

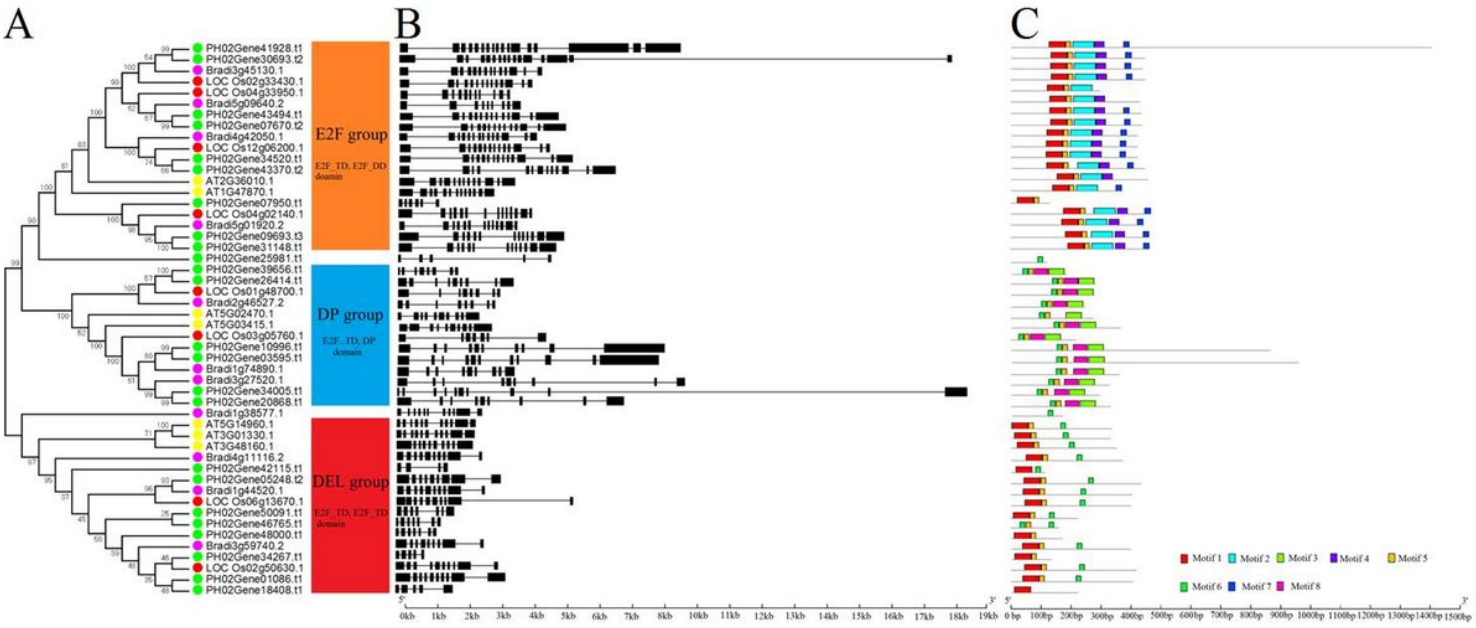

Figure 1

Phylogenetic relationship, gene structure and motif compositions of the PheE2F/DP families. (A) Phylogenetic analysis of the E2F/DP family. A. thaliana, O. sativa, B. distachyon, and Moso bamboo proteins are indicated by yellow, red, purple, and green dots, respectively. (B) Gene structures of E2F/DP genes. The exons and introns are represented by black boxes and black lines, respectively. The scale bar represents 1.0 kb. (C) Distribution of conserved motifs within each group. The scale bar represents 100 amino acids.

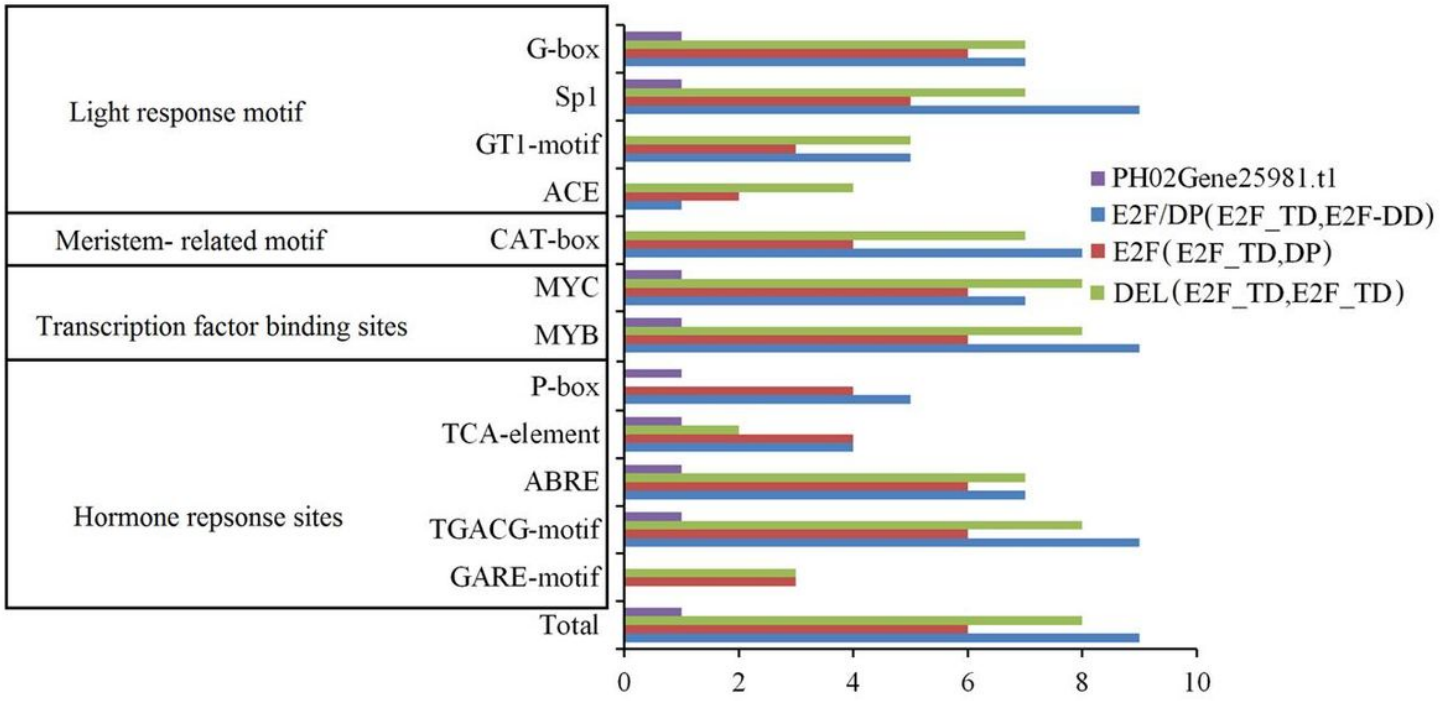

Figure 2

Number of cis-regulatory elements (CREs) in the putative promoter regions of E2F/DP genes. Only identified elements with quality values greater than five were retained.

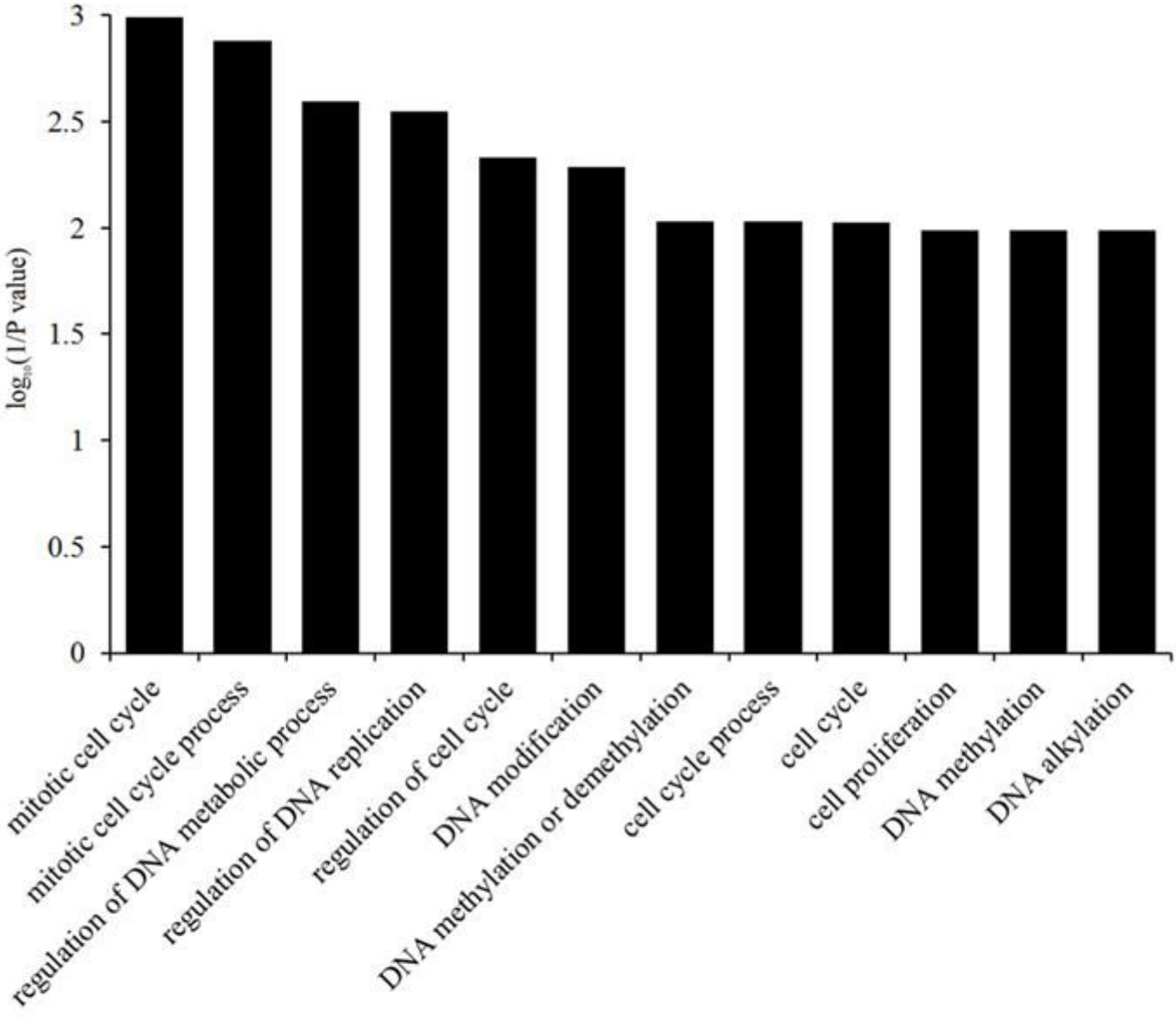

Figure 3

GO enrichment (biological processes) of PheE2F/DP-regulated genes (FDR <0.05).

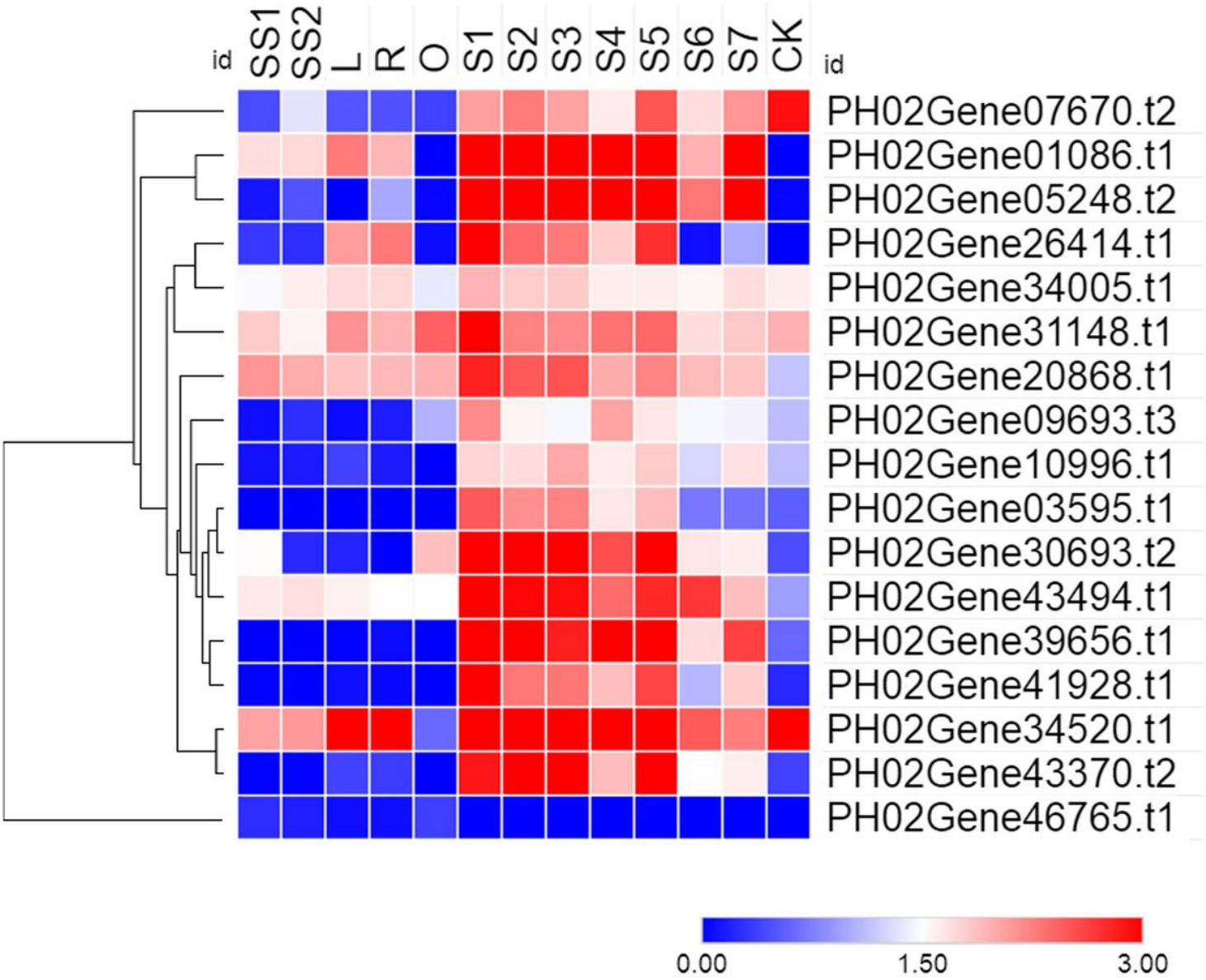

Figure 4

Expression profiles of E2F/DP genes in different Moso bamboo tissues. Expression was visualized using heat maps. Blocks with colors indicate high accumulation levels (red) or low accumulation levels (blue). S1-S7 and CK represent winter bamboo shoots; 50-, 100-, 300-, 600-, 900-, and 1200-cm height bamboo shoots; and one-year-old mature culms, respectively. SS1, SS2, L, R and O represent the 1.5-cm-tall seedling stem, 8-cm-tall height seedling stem, lateral bud, rhizome and outward rhizome, respectively.

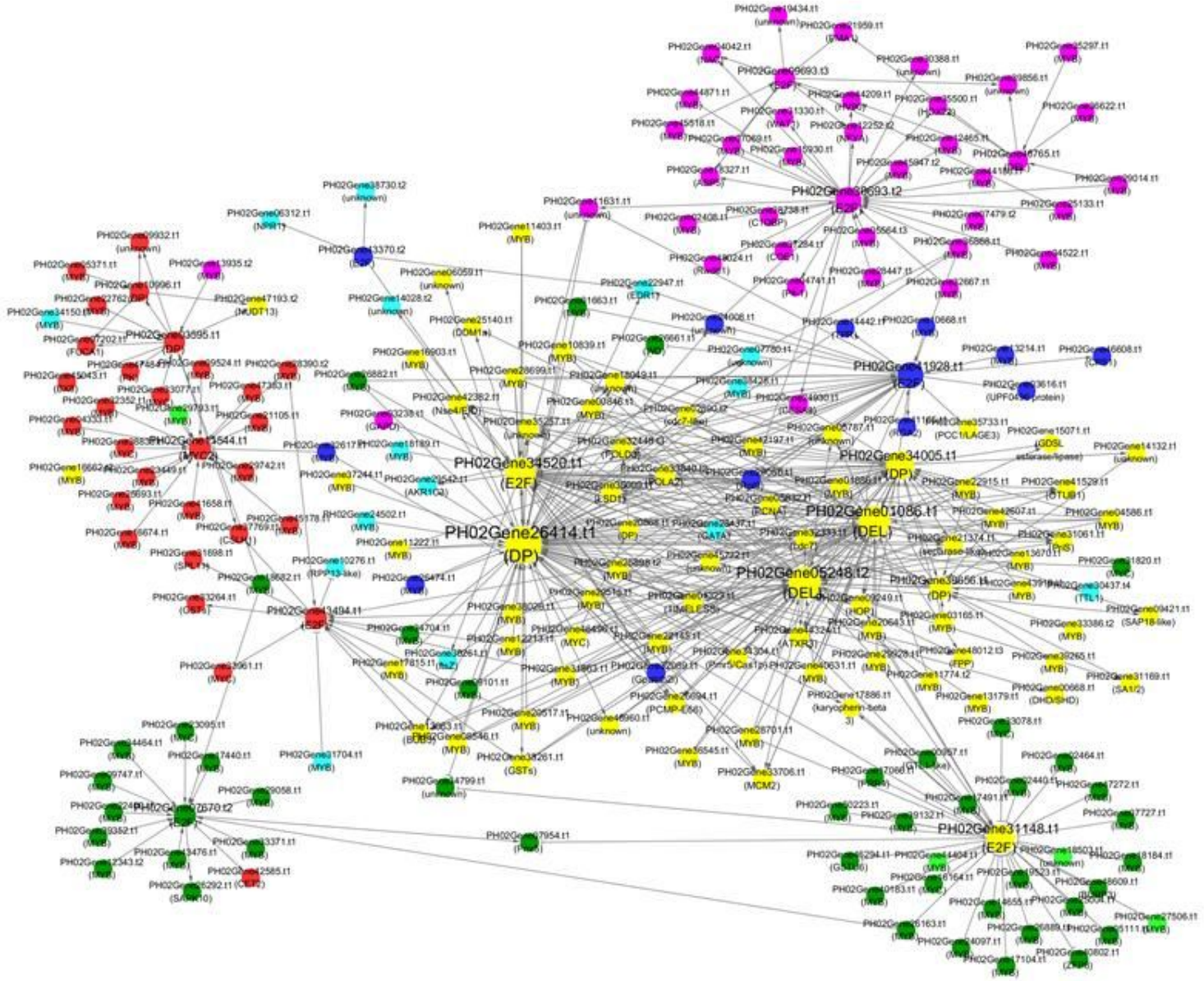

Figure 5

Interaction network describing transcription factor-target gene (TF-TG) interactions. TF-TG interactions were predicted based on regulatory elements in the promoters of target genes. Larger circles in the network indicate gene edges with more connections. Edges or connections indicate the coexpression of genes with a Pearson correlation coefficient ≥ +0.90 (arrows) or ≤ -0.90 (T-type arrow). Yellow, red, purple light green, dark green, light blue and dark blue circles represent the genes with the highest expression levels in winter bamboo shoots, 0.5-m-tall bamboo shoots, outward rhizomes, 1.5-cm-tall seedling stems,8-cm-tall seedling stems, lateral bud and rhizome, respectively.

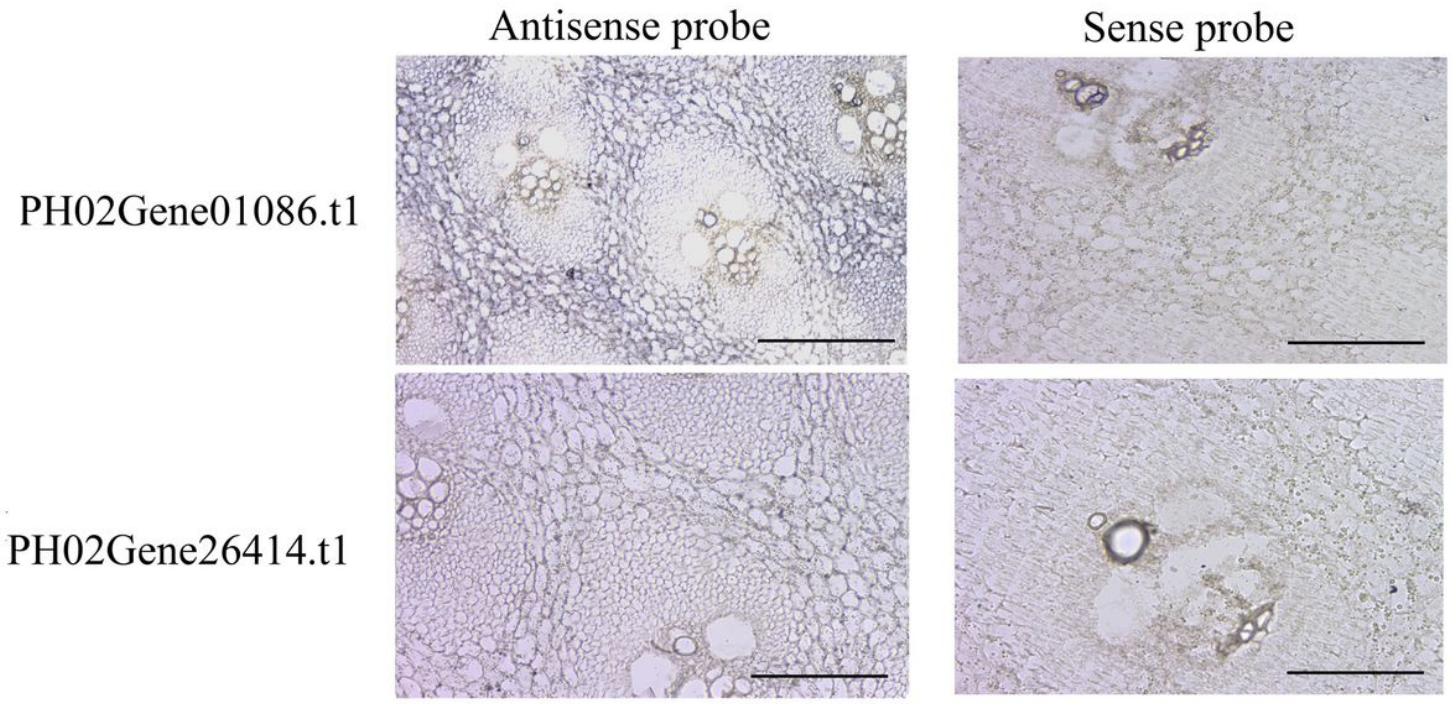

Figure 6

In situ hybridization of PH02Gene01086.t1 and PH02Gene20868.t1 in winter Moso bamboo shoots.

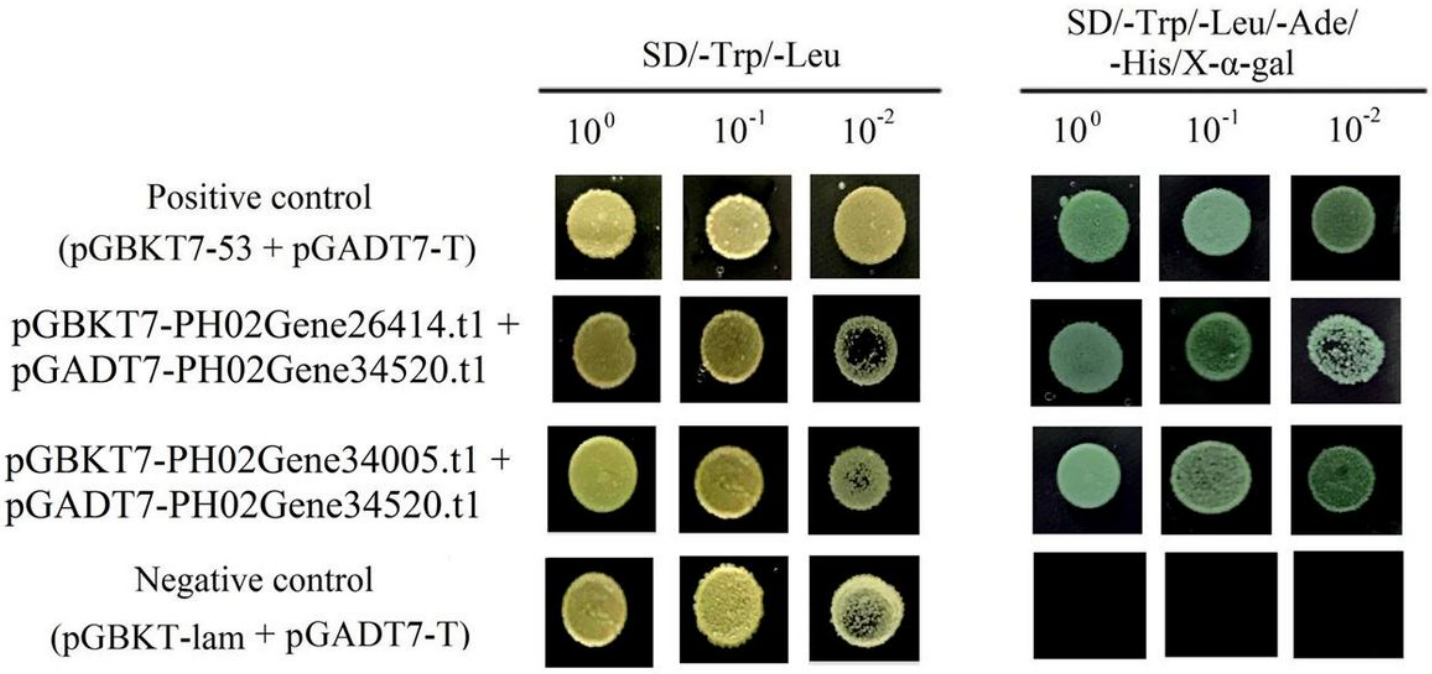

Figure 7

The interaction between PheE2Fs and PheDPs. Positive control, pGBKT7-53 + pGADT7-T; negative control, pGBKT7-Lam + pGADT7-T.

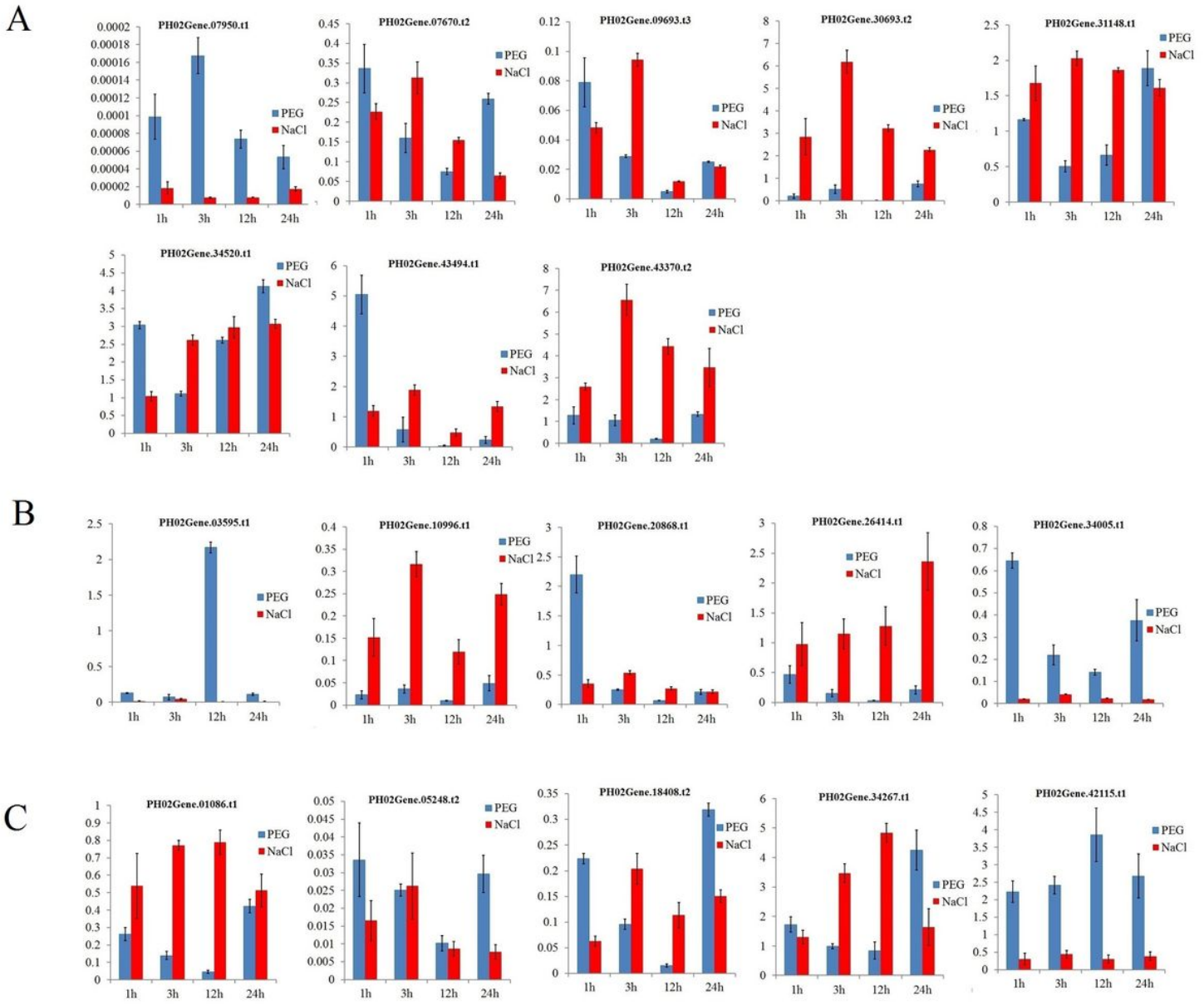

Figure 8

Expression analysis of PheE2F/DP genes under salinity stress and drought stress treatments. Seedlings irrigated with tap water were used as controls. TIP41 was used to normalize the expression data. (A) E2F group, (B) DP group, (C) DEL group.

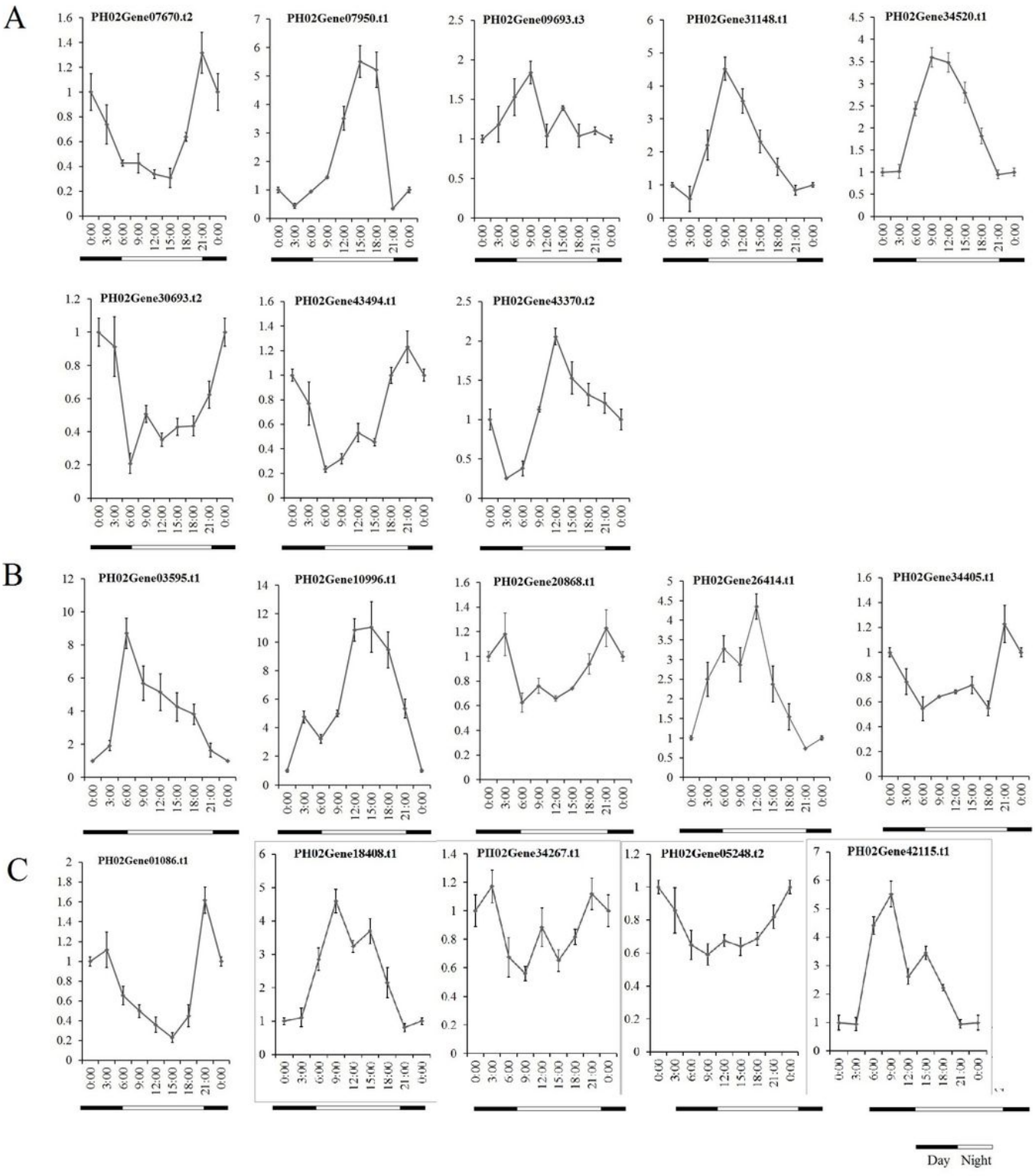

Figure 9

Diurnal rhythms of PheE2F/DP genes. TIP41 was used to normalize the expression data. (A) E2F group, (B) DP group, (C) DEL group. The expression of genes at each time-point was averaged by two days data.

Supplementary Files

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