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

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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 potential role of Moso bamboo E2F/DP family has been limited.

Results

Here, we identified 24 E2F/DPs in the Moso bamboo genome including nine E2F, six DP, eight DEL and one gene with partial E2F domain. Estimation of the divergence time of paralogous gene pairs suggested that E2F/DP family expansion was mainly contributed by whole-genome duplication event. 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 played important roles in winter moso bamboo shoot growth. qRT-PCR results showed that PheE2F/DPs performed diverse expression patterns in response to drought and salt treatment and diurnal cycles.

Conclusion

Our findings provide novel insights into the Moso bamboo E2F/DP family and partial experimental evidence for further functional verification of the PheE2F/DPs.

Background

Moso bamboo (*Phyllostachys edulis*) generates nearly 5 billion US dollars in economic values. It is the most important non-timber forests for commercial purposes in the East Asia due to its fast growth rate [1, 2]. During Moso bamboo shoot growth, the height increment was contributed by simultaneous cell division and cell elongation. The previous one played critical roles in the winter and early growth period, while the next one was predominant in the late growth period.

The E2F/DP transcription factors in higher plants are categorized into E2F, DP, and DEL (DP-E2F-like) groups based on conserved domain [3]. The E2F group gene contains four functional domain including an RBR-binding domain, a 'marked box' domain, a DNA-binding domain, and a leucine zipper dimerization domain, while the members of DP group lacks RBR-binding domain, a 'marked box' domain by comparing with E2F. The DEL group genes are considered to be atypical E2F/DPs and act in monomeric form, the DEL genes only contain a duplicated DNA-binding domain [4].

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 via organization of their leucine zipper dimerization domains [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 is essential for cell growth and cell proliferation [9]. Studies on E2F in *Arabidopsis* have indicated that plants also have all the core regulators in the E2F signaling pathway, such as cyclin-dependent kinases inhibitors (CKIs), retinoblastoma (RBs), cyclins, and cyclin-dependent kinases (CDKs). In plants, E2F/DP binds to the E2FAT (TTTCCCGCC) motif in its target genes, playing a role in promoting transcription [10].

However, so far, no publications have addressed the *E2F/DP* genes as well as their expression pattern and potential function in Moso bamboo. In this study, the phylogenetic relationships, gene structures, and conserved motifs of 24 *PheE2F/DP* genes were analyzed. Promoter analysis indicated that various *cis*-acting elements including light response, hormone signaling, as well as many transcription factor binding sites were present in the promoter region of *PheE2F* genes. We surveyed potential gene promoters containing E2F/DP binding sites within the Moso bamboo genome and identified 580 E2F/DP target genes. Based on bioinformatic prediction, we further studied expression dynamics of the *PheE2F* genes during bamboo shoot growth and in response to stress stimuli and diurnal cycles. 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 provided new insights and some valuable information for the further functional verification of E2F/DPs in Moso bamboo.

Results

Identification and classification of the PheE2F/DP gene family

A total of 24 genes that were included in the PheE2F/DP family identified in the Moso bamboo genome. To determine the evolutionary relationship of E2F/DPs in Moso bamboo and other model plants, a NJ phylogenetic tree of E2F/DP proteins from Moso bamboo, *Oryza sativa*, *Brachypodium* and *Arabidopsis* was constructed. 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 did not fit in any group due to its incomplete E2F domain (Fig. 1A). In addition, phylogenetic analysis of E2F/DPs in four species indicated that PheE2F/DPs shared more sequence similarity with OsE2F/DPs and BdE2F/DPs than with AtE2F/DPs. 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

Gene structural analysis of E2F/DP family revealed that genes within the same group shared similar intron lengths and numbers (Fig. 1B). The quantity 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 quantity ranged from five to sixteen.

Eight conserved motifs were identified in the E2F/DPs using the MEME online program (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 were found exclusively in the E2F group, whereas motifs 3 and 8 appeared only 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 proteins involved in the same group shared several identical motifs outside the conserved domain mentioned above, indicating functional conservations within the same group.

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, *Oryza sativa*, *Brachypodium* and *Arabidopsis* were 11.57, 39.61, 49.08 and 25.24 mya (million years ago), respectively. The divergence time of most *PheE2F/DP* paralogous pairs (8 of 10) occurred approximately 7 to 12 mya, consistent with the Moso bamboo whole-genome duplicating time [11], and much later than the divergence times for the three model plants.

Regulatory network of *PheE2F/DP* in Moso bamboo

The *cis*-elements which located in the promoter region are essential to spatial, temporal, and tissue-specific control of gene expression under the external and internal environmental stimuli [12]. Thus, we screened the *cis*-element at the promoter regions (2000-bp upstream) of all the *PheE2F/DP* genes using the PlantCARE database. A large number of *cis*-elements including 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. MYB binding motifs were found in all 24 promoters of *PheE2F/DP* genes, and most *PheE2F/DP* promoters harbored MYC binding elements. Thirteen *PheE2F/DP* promoters contained P-box or GARE motifs that were responsive to gibberellin stimulation. The abscisic acid responsiveness (ABRE) sites was abundant in *PheE2F/DP* promoters, and this element was found in 21 promoters. In addition, TGACG motif and TCA element which responsive to methyl jasmonate (MeJA) and salicylic acid stimulation, appeared in most *PheE2F/DP* promoter regions. Nineteen promoters contained CAT box, a meristem expression related *cis*-element, 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/DP 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 explore the potential function of these genes regulated by *PheE2F/DP*, We performed Gene ontology (GO) enrichment analysis (Fig. 3). In category of biological processes, the groups with the highest abundance of genes included mitotic cell

cycle, mitotic cell cycle process, regulation of DNA metabolic process, regulation of DNA replication, and regulation of cell cycle.

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 ($R \geq 0.90$ or $R \leq -0.90$) 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 performed the highest accumulation 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).

In *Arabidopsis*, *E2F* can interact with *DP* to activate the cell cycle and cell proliferation-associated gene expression [8]. Thus, yeast two-hybrid experiment was adopted to examine whether *E2F-DP* complex also occurs in Moso bamboo. 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 positive control, negative control, and both two transformants tested grew well on SD/-Leu/-Trp medium (Fig. 7). The positive control and the

experimental group turned blue when the transformants had grown on the SD/-Trp/-Leu/-Ade/-His/X- α -Gal media for five days. In contrast, the negative control could not grow on that nutritional selection media which contained X- α -Gal. These experiment results indicated that PheE2Fs can interact with PheDPs in Moso bamboo.

Expression patterns of *PheE2F/DPs* under abiotic stress treatment and diurnal rhythms

Regulatory analysis revealed that most *PheE2F/DP* promoters harbored various kinds of hormones and light response elements. Hormone response elements, especially those of stress-related hormones such as abscisic acid, methyl limonate and salicylic acid, indicate that these genes might be induced or repressed by abiotic stress. To understand the expression changes of *PheE2F/DPs* under abiotic stress, the Moso bamboo seedlings were treated with drought (PEG) and salt treatments, respectively (Fig. 8). Under drought stress, the expression levels of *PH02Gene07950.t1*, *PH02Gene07670.t2*, *PH02Gene09693.t3*, *PH02Gene10996.t1*, *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.t1*, *PH02Gene20868.t1*, and *PH02Gene43494.t1* peaked at 12 h, 1 h, and 1 h, respectively. Under the salt stress, *PH02Gene30693.t2*, *PH02Gene31148.t1*, *PH02Gene34520.t1*, *PH02Gene43370.t2*, *PH02Gene34267.t1*, *PH02Gene26414.t1*, and *PH02Gene34267.t1* showed upregulated expression trends, the other *PheE2F/DPs* showed no significant differences in expression or downregulated expression trends.

To understand the transcriptional changes in *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*, *PH02Gene31148.t3*, *PH02Gene03595.t1*, and *PH02Gene26414.t1* peaked at 15:00, 09:00, 06:00, and 12:00, respectively. The transcriptional levels of *PH02Gene34520.t1*, *PH02Gene18408.t2* and *PH02Gene42115.t1* remained at the maximum values from 4:00 to 19:00 (daytime).

Discussion

The feature of Moso bamboo E2F/DP gene family.

E2F/DP controls the spatial and temporal expression of genes that are essential for multiple biological processes during the cell cycle, and their transcription level determined 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 that identified in rice (8), *Arabidopsis* (7), *Zea mays* (19), wheat (18) and *Brachypodium* (11) [13, 14, 15]. The Moso bamboo genome size (2,021 Mb) is comparable to that of *Zea 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 *Oryza sativa*, (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

PheE2F/DPs may be associated with genome duplication events. Previous reports suggested that Moso bamboo was tetraploid in origin, went through a long evolutionary progression from tetraploidy to diploidy, and carried two duplicates, similar to *Oryza sativa* gene model sets [11, 21]. A similar phenomenon was consistently observed in the E2F/DP family. Ten PheE2F/DP paralogous gene pairs were identified in Moso bamboo genome, far more than the quantities identified in *Arabidopsis*, rice, and *Brachypodium*, which harbored only three, four, and four paralogous pairs, respectively. In addition, most divergence times of PheE2F/DP paralogous pairs in Moso bamboo occurred 7-12 mya, consistent with the recent whole-genome duplicating time. These results suggested that the whole-genome duplication event played critical role in the PheE2F/DP family expansion.

PheE2F/DP proteins are involved in Moso bamboo shoot development

Recently, several studies suggested that PheE2F/DP participated in many important biological processes of plants, such as leaf growth [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 amount of genes that participated in cell cycle were regulated by the PheE2F/DP family, such as *CDKF*, *MRB1*, *SPR2* and *MCM2* (Fig. 5) [26], and most of these downstream genes also showed high expression levels in winter and the early growth period. Previous studies suggested that intercalary meristem cells grew and divided continuously and vigorously during the winter and early growth period (S1-S5), especially in the former, but the intercalary meristems growth activity decreased in the late period, and the continuous growth of the bamboo shoot was substituted with cell elongation [2]. In *Arabidopsis*, E2Fa and E2Fb interact with DPa to regulate downstream gene expression [8]. In this study, we used Y2H assay to detect the interaction between E2Fs and DPs, and three hub genes which showed highest accumulation levels in winter bamboo shoot were selected and further used to be examined. The experiment results showed that E2F-DP complex also occurs in Moso bamboo, which provides favourable evidence that PheE2F-PheDP complex may plays important roles in winter bamboo shoot growth. Thus, we concluded that the high abundance of E2F/DP genes in the winter 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 critical 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 of light response sites were identified in PheE2F/DP promoters. Expression analysis based on qRT-PCR 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 their expression, further accelerating light-mediated Moso bamboo shoot growth and development.

The identification of regulatory elements in the *PheE2F/DP* promoters indicated that most members harbored abscisic acid, MeJA and salicylic acid regulatory elements, indicating that the expression of these genes might be stimulated by some environmental cues. qRT-PCR analysis also provided evidence that several *PheE2F/DP* genes were induced by abiotic stress (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 *atrgha* 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.

The regulation of cell cycle might be interfered by drought and salt stress at the transcriptional level by repression of the expression of *E2Fs*. However, there is much new evidence 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 accumulation level of cell cycle regulators and salt stress. The expression of *OryzaCDKC1*, which is involved in 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 expression regulation of cell cycle genes [33]. Many *PheE2F/DPs* showed high

accumulation levels under abiotic stress, which may facilitate bamboo growth under harsh environment. Collectively, *PheE2F/DPs* participated in different biological processes in Moso bamboo.

Conclusion

In conclusion, a total of 24 *PheE2F/DPs* were identified in the Moso bamboo genome, including nine *E2F*, six *DP*, and eight *DEL* genes. *Cis*-acting elements involved in the photoperiodic response, hormone signaling, meristem growth, and many MYB and MYC2 binding sites were present in the *PheE2F/DP* promoters. Recent whole-genome duplication played important roles in the *E2F/DP* family expansion. Transcriptome sequencing and *insitu* hybridization analysis revealed that *E2F/DPs* play important roles in bamboo shoot growth, especially in the winter period. Expression profiles derived from qRT-PCR indicated that the expression of *E2F/DPs* are stimulated by various forms of abiotic stress and diurnal cycles. These results provide comprehensive insights into the Moso bamboo *E2F/DP* gene family and lay a solid foundation for further functional verification of the *PheE2F/DP* family.

Methods

Database searches

The *E2F/DP* sequences of *Arabidopsis thaliana* (*Arabidopsis*), *Oryza sativa* (rice), and *Brachypodium distachyon* (*Brachypodium*) were downloaded 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 used as queries in blastp searches against the local bamboo protein database with default parameters. The sequences were considered as predicted *E2F/DPs* if they had an E-value ≤ -10 . Finally, the Protein families database (Pfam) was further used to confirm each candidate *E2F/DP* sequence.

Plant materials

Moso bamboo shoots as well as other culm samples were collected in Jingxian County (E116°15'; N31°10'), Anhui Province, from January to August 2018 (Additional files1: Figure S2). The bamboo samples were frozen in liquid nitrogen and stored at -80 °C prior to RNA extraction. The permission of bamboo shoot and other culm samples collection for the experiments was obtained from forestry bureau of Jingxian County. Moso bamboo seeds were collected in Dajing County, Guilin (E110°179'; N25°049') in Guangxi Zhuang Autonomous Region from July to September, 2018 (Additional files1: Figure S2). The necessary permits were obtained for the field work from Guilin Forestry Bureau. The identification of these seeds was performed by Gao et al [37]. In addition, the sample collection work did not affect the ecology and did not involve protected species.

The expression experiment of circadian rhythm was conducted in artificial climate chambers with supplemental light from 4:00 to 19:00. Bamboo tissues 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 biological repeats were conducted for each sample.

For the abiotic stress treatment, bamboo seedlings were grown in artificial climate chambers with long-day conditions (15 h light/9 h dark). Drought stress test was conducted by irrigating the two month-old seedlings with 18% PEG6000 medium. Salt stress test was conducted by irrigating the two month-old seedlings 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 samples were frozen in liquid nitrogen and stored at -80 °C prior to use. Five individuals that showed similar growth patterns were pooled at each time-point and three biological repeats were conducted for each sample.

Gene structure, conserved motif and regulatory element analysis

Gene Structure Display Server program (GSDS) was used to investigate the gene structure of E2F/DP [38]. The conserved motifs in the identified E2F/DP sequences were identified by multiple em for motif elicitation program (MEME) [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 divergence time in paralogous pairs

Ka (nonsynonymous substitution), Ks (synonymous substitution) and the Ka/Ks ratio between the paralogous pairs were calculated using the DNA Sequence Polymorphism software (DnaSP). The formula $T = Ks/2\lambda$ was used to estimate the divergence time of the duplication event, with a divergence rate $\lambda = 6.5 \times 10^{-9}$ in monocotyledon and 1.5×10^{-8} in dicotyledon [41].

Multiple sequence alignment and phylogenetic tree construction

The ClustalX 2.1 program was executed for multiple sequence alignments using the full-length E2F/DP amino acids, and further generate phylogenetic tree with neighbor-joining (NJ) method using MEGA 7.0 software.

RNA extraction, reverse transcription and qRT-PCR analysis

Total RNA from each sample was isolated using the TRIzol reagent (Invitrogen, USA). First-strand cDNA was synthesized with 2 µg RNA using a PrimeScript™ RT Reagent Kit (TaKaRa, Japan) according to the manufacturer's instructions. qRT-PCR experiments carried out using SYBR Green chemistry (Roche, Mannheim, Germany) on a Light Cycler 480 instrument (Roche, Rotkreuz, Switzerland) following the manufacturer's directions. The Primer 3 online program was performed for gene-specific primers designing (Additional files1: Table S2) [42]. Three biological replicates and three technical replicates were

conducted for each experiment. The TIP41 was selected as an internal control [43]. The final relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ 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 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* were measured 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 gene expression correlation was calculated by Pearson correlation coefficient (PCC) using FPKM through R program. Only the transcription factor (TF) and target relationships which satisfied with PCC values $\geq +0.90$ or < -0.90 were remained for coexpression network construction [21]. The network was visualized using Cytoscape 3.7.0 software, finally.

In situ hybridization

Bamboo shoot tissues were fixed in 4% paraformaldehyde for one day at 4 °C, and then hybridized as described previously [2]. 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. Images were captured by an Olympus Nikon E600

Yeast two-hybrid

To attest whether E2F can interact with DP in Moso bamboo, 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 independently cloned into the pGBKT7 respectively, and the full length of *PH02Gene34520.t1* cDNAs was 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 AH109 yeast strain, respectively. The transformed yeast were cultured on SD/-Trp/-Leu for 4 days, and further transferred to SD/-Trp/-Leu/-Ade/-His/X- α -Gal plates for 5 days. The co-transformants with pGBKT7-53 + pGADT7-T and pGBKT7-Lam + pGADT7-T were selected as positive control and negative control, respectively.

Declarations

Ethics approval and consent to participate

The permission of bamboo shoot and other culm samples collection for the experiments was obtained from forestry bureau of Jingxian County. The permission of bamboo seeds collection for the experiments was obtained from Guilin Forestry Bureau.

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.

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Authors' Contributions

LL performed bioinformatics analyses, physiological experiments and drafted the manuscript. QQS helped in sample collection. JG 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.

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Abbreviations

ABA: abscisic acid

ABRE: abscisic acid responsiveness

CDC: cell division cycle

CDKC: [cyclin dependent kinase C](#)

CREs: cis-regulatory elements

DNnaSP: DNA Sequence Polymorphism

FPKM: fragments per kilobase of exon model per million mapped reads

GSDS: Gene Structure Display Server program

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

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Figures

Figure 1

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

Figure 2

The quantity of cis-regulatory elements (CREs) in the E2F/DP promoters. 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 patterns of E2F/DP genes in different Moso bamboo tissues. 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 target gene promoters. Larger circles in the network indicate gene edges with more connections. Edges or connections indicate the coexpression of genes with a Pearson correlation coefficient $\geq +0.90$ (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, co-transformation with the pGBKT7-53 and pGADT7-T; negative control, co-transformation with the pGBKT7-Lam and pGADT7-T.

Figure 8

Expression changes of PheE2F/DP genes under salinity stress and drought stress treatments. Seedlings irrigated with tap water were used as controls. TIP41 was selected as internal control. (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.

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