Genome-wide analysis of poplar NF-YB gene family and identified PtNF-YB1 important in regulate flowering timing in transgenic plants

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Abstract

Background: Compared with annual herbaceous plants, woody perennials require a longer period of juvenile phase to flowering, and many traits can be only expressed in adulthood, which seriously makes the breeding efficiency of new varieties slower. For the study of poplar early flowering, the main focus is on the study Arabidopsis homologue gene CO/FT. Based on studies of Arabidopsis, rice and other plant species, some important research progress has been made on the regulation of flowering time by NF-Y subunits. However, little is known about the function of NF-Y regulating flowering in poplar.

Results: In the present study, we have identified PtNF-YB family members in poplar and focus on the function of the PtNF-YB1 regulate flowering timing using transgenic Arabidopsis and tomato. To understand this mechanisms, the expression levels of three known flowering genes (CO, FT and SOC1) were examined with RT-PCR in transgenic Arabidopsis. We used the Y2H and BiFC to assay the interactions between PtNF-YB1 and PtCO (PtCO1 and PtCO2) proteins. Finally, the potential molecular mechanism model in which PtNF-YB1 play a role in regulating flowering in poplar was discussed.

Conclusions: In this study, we have characterized the poplar NF-YB gene family and confirmed the function of the PtNF-YB1 regulate flowering timing. At the same time, we found that the function of PtNF-YB1 to improve early flowering can overcome species barriers. Therefore, PtNF-YB1 can be used as a potential candidate gene to improve early flowering by genetic transformation in poplar and other crops.

Keywords: Poplar, Genome-wide analysis, PtNF-YB1, Flowering time, Transgenic plant
processes, especially in embryogenesis [11, 12] and seed subunits in plants are involved in many important growth known about their biological function in plants. and their complexes in yeast and mammals, little is

10, 11 and 7, respectively [10]. Relative to the detailed

In rice, the genes encoding each NF-Y subunit are

mentioned in the same subfamily. indicating that there is a clear functional similarity be-

phosphorylation, and their complexes in yeast and mammals, little is

known about their biological function in plants.

Studies in recent years have shown that individual NF-Y subunits in plants are involved in many important growth processes, especially in embryogenesis [11, 12] and seed maturation [13–15], chloroplast synthesis [16–18], tissue division [19] and others processes. Simultaneously, the NF-Y subunit also plays an important role in response to stress, such as drought stress [20–25]. It is worth noting that more and more studies have found that the NF-Y subunit participates in the photoperiodic regulation of flowering induction pathways, and that different subunits function differently [26–34]. For example, Cai et al. found that the AtNF-YB2 promotes the flowering process by increasing expression of the flowering key genes FLOWER-ING LOCUS T (FT) and SUPPRESSOR OF OVER EXPRESSION OF CONSTANS1 (SOC1) [27]. Concurrently, AtNF-YB2 and AtNF-YB3 can interact with At-NF-YC3. 4, 9, which play important roles in the control of flowering time via the photoperiod pathway [33]. In addition, Hackenberg et al. demonstrated that AtNF-YC1 and AtNF-YC2 over-expression induce early flowering, and the transcript levels of FT genes in plants were significantly increased [30]. Interestingly, the regulation of flowering time by NF-Y in rice is exactly the opposite of Arabidopsis. Transcription factor NF-YB11 negatively regulates the flowering time by down-regulating the expression of flowering-related genes [35–37]. This also shows that the regulation mechanism of NF-Y in flowering time varies in different species.

Based on studies of Arabidopsis, rice and other plant species, some important research progress has been made on the regulation of flowering time by NF-Y subunits, and to unveiling the molecular mechanism. However, little is known about the function of NF-Y regulating flowering in poplar. In this study, we have characterized the poplar NF-YB gene family and confirmed the function of the NF-YB1 (PtNF-YB1) regulate flowering timing using transgenic Arabidopsis and to-

mato. Finally, the potential molecular mechanism model of PtNF-YB1 involved in flowering regulation was discussed.

Results and discussion

Identification of poplar PtNF-YBs

In order to identify poplar analogs of PtNF-YB proteins, amino acid sequences of Arabidopsis and rice NF-YBs sequences were used to search against the Phytozome database Populus trichocarpa V3.0 (https://phytozome. jgi.doe.gov/pz/portal.html). According to nomenclature of NF-YBs in Arabidopsis and rice, the genes were named as follows (Table 1).

The identified PtNF-YB genes in poplar encode proteins ranging from 143 to 295 amino acids in length with an average of 192 amino acids. The detailed information of PtNF-YB family genes in poplar, including sequence ID, chromosome location, amino acid length (aa), protein isoelectric point (PI) value and protein molecular weight (MW) (Da) was listed in Table 1.

To study the phylogenetic relationship between NF-YBs proteins in poplars, we constructed a unrooted tree based on the alignment of the NF-YBs full-length protein sequences (Additional file 1a). The phylogenetic tree was constructed using MEGA V5.5 by employing the Neighbor-Joining (NJ). As showed in the phylogenetic tree, it divided the PtNF-YBs family proteins into two distinct subgroups.

To better understand the functional prediction of PtNF-YBs, 10 conserved motifs were identified using MEME V4.12.0 (Additional file 1b). As expected, we found that most of the closely related members of the phylogenetic tree share a common motif composition, indicating that there is a clear functional similarity between the NF-YBs proteins in the same subfamily.

Analysis of the deduced amino acid sequence of PtNF-YB1

To investigate the evolutionary relationship, a phylogenetic analysis was made using the deduced amino acid sequence from poplar, Arabidopsis and rice based on the coding sequences of 21 PtNF-YBs, 13 AtNF-YBs and 11 OsNF-YBs (Fig. 1a). When compared with Arabidopsis and rice NF-YBs, it showed that PtNF-YB1 formed a close cluster with AtNF-YB2, and it has been associated with flowering time [27, 38] (Fig. 1a). The PtNF-YB1 gene encoded a predicted polypeptide with 167 amino acid residues, the protein molecular weight (MW) is 18, 193.2 Da and the protein isoelectric point (PI) value is 5.04 (Table 1). Just like Arabidopsis AtNF-YB2, amino acid sequence alignment showed that the poplar PtNF-YB1 contained the DNA binding domain, the NF-YC interaction and the NF-YA interaction domain [39–41].
The histone-fold motif (HFM) of the core histone H2B was also observed in PtNF-YB1 [42] (Fig. 1b).

**Temporal and spatial expression patterns of PtNF-YB1 gene**

To identify temporal and spatial expression patterns of PtNF-YB1 gene, semi-quantitative RT-PCRs were conducted. The results indicated that PtNF-YB1 was expressed in all five types of tissues: flowering (F), floral buds (FB), root (R), stem (S) and leaf (L). Among the five types of tissues, the flowering (F) and floral buds (FB) generated the higher level PtNF-YB1 transcripts, the root (R) generated the lowest level (Fig. 2a). The qRT–PCR was also performed to confirm the results. The results showed the similar trends with the semi-quantitative RT-PCRs (Fig. 2b). It suggests that PtNF-YB1 may be part of the regulation of flowering pathway, just like AtNF-YB2 [25, 39].

**Ectopic expression of PtNF-YB1 improves early flowering in transgenic Arabidopsis**

To determine the effects of poplar PtNF-YB1 gene on flowering time, we generated PtNF-YB1 over-expressing transgenic Arabidopsis plants. Consequently, more than 10 independent transgenic lines were obtained. Among them, 6 independent lines were used for further analysis (Fig. 3a). The T2 generation per line were grown in the long day conditions (LD, 16 h light/8 h dark) and their phenotypes were examined. The transgenic Arabidopsis lines (A2 and A4) were flowered significantly earlier than the wild-type (Col) (Fig. 3b, Additional file 2). For example, PtNF-YB1 transgenic line A4 flowered with 7.1 rosette leaves and 3.4 cauline leaves, while wild-type (Col) flowered with 12.5 rosette leaves and 5.1 cauline leaves (Additional file 2). The result showed that PtNF-YB1 ectopic expression noticeably improves early flowering in transgenic Arabidopsis.

**PtNF-YB1 ectopic expression verifies its functions in promote early flowering in tomato**

To verify PtNF-YB1 functions in promote early flowering, we also generated PtNF-YB1-overexpressing transgenic tomato. We obtained 8 independent transgenic lines and 5 independent lines were used for further analysis (Fig. 4a). The T2 generation were grown in the nursery soils pots and the greenhouse conditions at day
(25 °C) and night (20 °C). The PtNF-YB1-overexpressing tomato lines (T3 and T4) were also flowered significantly earlier than wild-type (WT) plants under same growth environment (Fig. 4b). For example, PtNF-YB1 transgenic line-T4 flowered with 50 days after transplanting, while wild-type (WT) plants flowered with 65 days after transplanting. Over-expression of PtNF-YB1 promoted early flowering in transgenic tomato, indicating that its ability to promote early flowering can cross species barriers. Therefore, the poplar PtNF-YB1 may serve as a potential candidate gene for improve early flowering of poplar and other crops through genetic transformation.

Regulation of flowering pathway genes in the transgenic Arabidopsis and the potential molecular mechanism model for how PtNF-YB1 expression can promote early flowering in poplar

How does PtNF-YB1 regulate the mechanism of early flowering in poplar? To understand this mechanisms, the expression levels of Arabidopsis three known flowering genes (CO, FT and SOC1) were examined with RT-PCR in the wild-type (Col) and transgenic plants (A2 and A4) (Fig. 5). Among of them, two genes were up-regulated, including Arabidopsis CONSTANS (CO) and FT. The CO is a key regulator of photoperiod-dependent flowering time in Arabidopsis [43]. The FT acts partially downstream of CO, which promotes flowering in plants [44, 45]. The SOC1 gene showed no difference between wild-type (Col) and transgenic plants. The SOC1 gene is a MADS transcription factor, a key integrator in photoperiod pathway [46]. This result was consistent with previous findings [38].

For the study of poplar early flowering, the main focus is on the study Arabidopsis homologue gene CO/FT [1, 2, 47–49]. Through the study of the model plant Arabidopsis, it was shown that AtNF-YB2 and AtCO interact to regulate FT and promote early flowering [27, 38]. In poplar, two CO-like genes PtCO1 (POPTR0017s14410.1) and PtCO2 (POPTR0004s10800.1) are the closest structural
orthologs of AtCO (At5g15840) (Additional file 3). The protein string interactions suggest a possible link between PtNF-YB1 and PtCO (PtCO1 and PtCO2) in poplar (Fig. 6a). We used the Y2H and BiFC assays to validate these hypotheses interactions between PtNF-YB1 and PtCO (PtCO1 and PtCO2) proteins in poplar (Fig. 6b, c). The poplar PtNF-YB1 promotion of flowering is achieved probably by interacting with PtCO1 and PtCO2 proteins (Fig. 7). We also generated PtNF-YB1 over-expressing transgenic poplar. So far, the transgenic poplar did not show the expected early flowering (Additional file 4). There may be at least three reasons to explain this phenomenon: (i) Epigenetic mechanism. Previous evidence supported that the NF-Y transcription factor as important modulators of epigenetic marks controlling flowering [50–53]. (ii) The multiple-year delay in onset of flowering of woody perennials. (iii) Whether PtNF-YA/PtNF-YC are involved in the formation of PtNF-Y complexes to regulate poplar flowering. Our future work is needed to analyze these questions through epigenetics and proteomics.

In summary, to elucidate the role of NF-Y transcription factor in poplar flowering induction and molecular regulation mechanism will be important for people to understand the role and function of NF-Y transcription factor family in woody plants, and provide important theoretical basis for regulating flowering time and shortening breeding cycle.

Conclusions
In the present study, we have identified the poplar NF-YB gene family and confirmed the function of the PtNF-YB1 regulate flowering timing using transgenic Arabidopsis and tomato. To understand this mechanisms, three known flowering genes (CO, FT and SOC1) were examined by RT-PCR in transgenic Arabidopsis. We also used the Y2H and BiFC to assay the interactions between poplar PtNF-YB1 and PtCO (PtCO1 and PtCO2) proteins. A potential molecular mechanism model in which PtNF-YB1 play a role in regulating flowering in poplar was discussed. Therefore, PtNF-YB1 can be used as a potential candidate gene to improve early flowering by genetic transformation in poplar and other crops.
Methods
Identification PtNF-YB family members in poplar
The *Arabidopsis* and rice NF-YB sequences were retrieved from the *Arabidopsis* TAIR database (https://www.arabidopsis.org) and rice OrygenesDB database (http://orygenesdb.cirad.fr/), respectively. The BLASTN program was used with an E-value cut-off of 1.0e−5 to identify predicted PtNF-YB sequences using Phytozome database *Populus trichocarpa* V3.0 (https://phytozone.jgi.doe.gov/pz/portal.html).

Phylogenetic trees and conserved motif analyses
The phylogenetic trees were constructed by the MEGA V5.5 Neighbor-Joining (NJ) method using conserved and amino acid sequences, and the parameters were p-distance model and 1000 bootstrap replicates. Multiple sequence alignments were implemented by Clustal X software. The conserved motifs of 21 poplar PtNF-YBs were analyzed using the Multiple Expectation Maximization for Motif Elicitation (MEME V4.12.0) (http://meme-suite.org/).

Fig. 5 The expression analysis of known flowering genes in transgenic *Arabidopsis*. a Expression analysis of flowering genes with qRT–PCR. b Expression analysis of flowering genes with semi-quantitative RT-RCRs. The three known flowering genes were *CO*, *FT* and *SOC1*. The RT-PCR reactions were repeated three times.

Fig. 6 PtNF-YB1 protein interactions with PtCO1 and PtCO2. a The potential PtNF-YB1 protein interactions with PtCO1 and PtCO2 were predicted using STRING software. b Y2H assays showing protein–protein interactions. c BiFC assay to detect the interactions of proteins.
tools/meme) by uploading the coding sequences according the instructions.

Plant material and growth conditions
The 6-year-old poplar 84K flowering (F), floral buds (FB), root (R), stem (S) and leaf (L) were collected from the Wei River planting base in Xi'an city (N33°42′44.37″; E107°39′36.62″; with altitude 500–550 m), Shannxi province, China. For transformation, wild-type Arabidopsis ecotype columbia (Col) were used. It was grown in the long day conditions (LD, 16 h light/8 h dark) at 20–22 °C.

For tomato genetic transformation, "Micro-Tom" tomato were used as the method described by Zhang and Blumwald [54]. It was grown in the nursery soils pots and the greenhouse conditions at day (25 °C) and night (20 °C).

PtNF-YB1 over-expressing vector construction
The open reading frame (ORF) of PtNF-YB1 gene were amplified by RT-PCR, and then was used to construct over-expression vector. The PtNF-YB1 gene was inserted into the vector pBI121 and under the 35 S promoter of the cauliflower mosaic virus (CaMV). The specific primers were shown in Additional file 5.

Arabidopsis and tomato transformation
The poplar PtNF-YB1 over-expressing constructs was introduced into Col with a floral dip method mediated with Agrobacterium strain GV3101 [55]. The seeds of positive transgenic plants carrying the PtNF-YB1 constructs were individually harvested. Homozygous transgenic lines were used for further investigation. "Micro-Tom" tomato cotyledons were transformed with the Agrobacterium strain LBA4404 containing the PtNF-YB1 over-expressing constructs as the method described by Zhang and Blumwald [54].

Yeast two-hybrid (Y2H) assay
According to the manufacturer’s instructions (Clontech, USA), we performed yeast two-hybrid (Y2H) experiments using a Gal4-based two-hybrid system. First, the poplar PtNF-YB1 gene ORF was inserted into the bait vector pGBK7. The resulting vector pGBK7-PtNF-YB1 was used as a bait. The ORFs of PtNF-CO1 and PtNF-CO2 genes were cloned into the vector pGAD7.

The specific primers are shown in Additional file 6. Then, co-transformation of pGAD7 with pGBK7-PtNF-YB1 was used as a control, the pGBK7-PtNF-YB1 construct was used together with pGAD7-PtNF-CO1 and pGAD7-PtNF-CO2 to co-transform the yeast strain AH109. Finally, positive colonies were selected using SD/-Trp-Leu-His-Ade medium and stained with β-galactosidase to confirm the positive colonies.

Bimolecular fluorescence complementation (BiFC) assay
We used the vectors pSPYNE-35S and pSPYCE-35S and the cotransfection vector 35S: P19 to construct a bimolecular fluorescent complementary (BiFC) plasmid vector. For the first time, the poplar PtNF-YB1 gene ORF was inserted into the vector pSPYNE-35S and the PtCO (PtCO1 and PtCO2) gene ORF were inserted into the vector pSPYCE-35S. Both the vectors contain the N- or C-terminus encoding the yellow fluorescent protein (YFP). The specific primers are shown in Additional file 7. Then, as described by Walter et al., we used the Agrobacterium-mediated infection method to introduce different combinations of gene vectors into onion epidermal cells [56]. Finally, the expression of YFP in onion epidermal cells was observed using a laser confocal microscope (Zeiss LSM510 Meta, Germany) after 48 h incubation at 24 °C. We use a wavelength of 488 nm and detection at 500–530 nm with a band-path filter for YFP.

Reverse transcription PCR (RT-PCR)
Semi-quantitative reverse transcription PCR (RT-PCR) was used to detect the expression level of PtNF-YB1 in poplar, Arabidopsis and tomato. Quantitative real-time reverse transcription PCR (RT-qPCR) were performed to confirm the results. The RT-qPCR reactions were performed in a Step One Plus Real-Time PCR System (Applied Biosystems, USA) using a Super Real Premix kit (SYBR Green) (Tiangen-biotech, China). The RNA relative expression of each gene was calculated according to the 2-ΔΔCT method, as reported previously in detail [57]. In RT-qPCR analysis, the 18S rRNA (poplar), AtACTIN (Arabidopsis) and T-Act (tomato) as the internal control gene. The RT-PCR reactions were repeated three times. The specific primers were shown in Additional files 8 and 9.
Additional files

Additional file 1: The phylogenetic tree and conserved motifs analysis of NF-YB families in poplar. a. PtNF-YBs phylogenetic tree, b. PtNF-YBs conserved motifs analysis. (TIF 2879 kb)

Additional file 2: Flowering time of Arabidopsis transgenic lines ectopically expressing PtNF-YB1. (DOC 29 kb)

Additional file 3: Analysis of the deduced amino acid sequence of poplar and Arabidopsis CO. a. The homology tree of poplar PtCO1, PtCO2 and AtCO. b. Multiple sequence alignments of the conserved domains PtCO1, PtCO2 and AtCO. The amino acid sequences were analyzed using DNAMAN software. (TIF 786 kb)

Additional file 4: Figure S3. Over-expressing PtNF-YB1 in transgenic poplar lines. a. The wild-type (WT) and transgenic tomato lines (PT1, PT3 and PT4) 45 days after transfer to the growth chamber. Bar = 10 cm. b. The wild-type (WT1 and WT2) and transgenic tomato lines (PTPT1 and PT3) 80 days after transfer to the growth chamber. Bar = 22 cm. (TIF 1503 kb)

Additional file 5: Primers for PtNF-YB1 gene cloning and over-expressing vector construction. (DOC 28 kb)

Additional file 6: Primers for yeast two-hybrid (Y2H) assay. (DOC 28 kb)

Additional file 7: Primers for bimolecular fluorescence complementation (BiFC) assay. (DOC 28 kb)

Additional file 8: Primers for expression analysis using semi-quantitative RT-PCR. (DOC 32 kb)

Additional file 9: Primers for expression analysis using qRT-PCR. (DOC 33 kb)

Abbreviations

BiFC: Bimolecular fluorescence complementation; CaMV: Cauliflower mosaic virus; HFM: Histone-fold motif; MW: Molecular weight; NF-Y: Nuclear factor Y; NJ: Neighbor-joining; ORF: Open reading frame; PI: Isoelectric point; RT-PCR: Semi-quantitative reverse transcription PCR; RT-qPCR: Quantitative real-time reverse transcription PCR; Y2H: Yeast two-hybrid

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Authors’ contributions

RW and LZ performed the experiments, analyzed the data, prepared figures and tables, reviewed drafts of the paper. YZ and JF reviewed drafts of the paper. LL conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials supporting the results of this study are included in the article and the additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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