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ORIGINAL ARTICLE

Genome-wide analysis of the poplar *NF-Y* gene family and its expression in floral bud development of *Populus tomentosa*

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Abstract

Key message **The *NF-Y* gene family was identified and characterized in *Populus trichocarpa* by genome-wide analysis. Expression of *PtoNF-Y* was analyzed during floral bud development in *Populus tomentosa*.**

Abstract Nuclear factor Y (NF-Y) is a transcription factor widely found in eukaryotes. It is composed of three subunits: NF-YA, NF-YB, and NF-YC. NF-Y has been identified as a key regulator of multiple pathways that control responses to developmental, biotic, and abiotic factors. Although the *NF-Y* gene has been reported in many plants, it has not been comprehensively analyzed in poplar. In this study, a total of 46 *NF-Y* gene family members in the *P. trichocarpa* genome were accessed using NF-Y amino acid sequences from *Arabidopsis thaliana* as a probe. In addition, bioinformatic characterization of *NF-Y* gene family members in *P. trichocarpa*, including gene structure, chromosome location, and phylogenetic relationships, was conducted. The results of chromosome distribution showed that the 46 *PtNF-Y* genes were distributed among 16 chromosomes of *P. trichocarpa* at varying frequencies. Gene-structure analysis showed that seven members have no introns. Phylogenetic analysis indicated that the NF-Y protein may have similar functions in *Arabidopsis* and *P. trichocarpa*. We used transcriptome data from different tissues to analyze the expression of the *NF-Y* genes in *Populus tomentosa,* and verified the results by qRT-PCR. The results indicated that some *PtoNF-Y* genes play significant roles in the floral bud development of *P. tomentosa*.

Keywords Poplar · NF-Y · Genome-wide analysis · Expression profiles

Introduction

Transcription factors (TF) play vital roles in numerous biological activities by binding to specific DNA elements of their targeted genes. Nuclear factor Y (NF-Y), as a transcription factor, is composed of three subunits: NF-YA (HAP2 or CBF-B), NF-YB (HAP3 or CBF-A), and NF-YC (HAP5 or CBF-C) (Bucher and Trifonov 1988). NF-Y is widely found in fungi, animals, and plants. It specifically binds to the cis-element CCAAT-box in eukaryotic promoters (Nardini et al. 2013). Subunits NF-YB and NF-YC form dimers in the cytoplasm and then bind to the NF-YA protein and form a trimer in the nucleus (Tom et al. 2013).

In many mammals and yeasts, a single gene encodes each of the three NF-Y subunits (Li et al. 1992). For example, in mice and humans, only one *NF-Y* gene encodes each NF-Y subunit. However, in plants, each NF-Y subunit gene is represented by multiple orthologs (Zhao et al. 2016). For example, in *Arabidopsis thaliana*, the NF-YA, NF-YB, and NF-YC subunit families have 10, 13, and 13 members, respectively

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Juan Li and Kai Gao have contributed equally to this work.

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(Siefers et al. 2009). In rice (*Oryza sativa*), the NF-YA subfamily is encoded by 10 *NF-YA* genes, NF-YB by 11 *NF-YB* genes, and NF-YC by 7 *NF-YC* genes (Thirumurugan et al. 2008). Likewise, in soybeans (*Glycine max*), the number of identified members of the three subfamilies has reached 21, 32, and 15, respectively (Quach et al. 2015). In addition, the *NF-Y* genes of citrus (Pereira et al. 2018), walnut (Quan et al. 2018), tomato (Li et al. 2016), foxtail millet (Feng et al. 2015), sorghum (Malviya et al. 2016), and maize (Zhang et al. 2016) have previously been studied, and their numbers of members have increased in plants. This expansion of *NF-Y* gene number might contribute to the formation the well-established TF network and regulate plant growth and development (Calvenzani et al. 2012; Petroni et al. 2012).

Recent studies have revealed that *NF-Y* genes play a key role in plant response to various abiotic and biotic stresses. Overexpression of *ATNF-YA5* and *ATNF-YB1* in plants generally increases their growth under drought stress (Li et al. 2008). However, *nfya5* knockout plants have the opposite response to overexpression of *AtNF-YA5*, becoming more sensitive to drought stress. In castor bean, cold stress significantly up-regulated the expression level of *RcNF-YC6* (Wang et al. 2018). In addition, in soybean, the expression level of *GmNF-YA3* was induced by various stress treatments (Ni et al. 2013). These studies have indicated that the *NF-Y* gene family participates in various regulatory processes in response to abiotic stress in plants, but with functional differences among members.

Many *NF-Y* genes have been reported to be involved in growth and developmental processes, such as embryogenesis (Fornari et al. 2013), seed germination (Liu et al. 2016), photoperiod or age-dependent flowering (Kumimoto et al. 2010; Wei et al. 2017), and fruit maturation (Li et al. 2016). *NF-Y* genes are also involved in plant physiological processes, including photomorphogenesis (Myers et al. 2016), regulation of nodulation during nitrogen fixation (Quach et al. 2015), and stress responses of the endoplasmic reticulum (ER) (Liu and Howell 2010).

Numerous studies have reported that *NF-Y* genes are involved in the regulation of flowering timing and flower differentiation, implying that *NF-Y* genes play a particularly important role in flower development (Su et al. 2018; Wei et al. 2017). Poplar is an economically important tree that is highly valued for bioenergy and timber production purposes (Ji et al. 2013). Extensive research has focused on traditional breeding. Due to rapid growth and ease of transformation, *P. trichocarpa* is considered an ideal tree species for basic and applied research (Yan et al. 2012). Taking advantage of functional genomics studies of the plant *NF-Y* gene and current genomic data of *P. trichocarpa*, we attempted to analyze the *NF-Y* gene family of *P. trichocarpa*. In addition, the urban environmental pollution issues (such as causing traffic accident, fire risk, as well as spreading bacteria and virus) caused by female catkins of poplar during spring have attracted much attention by researchers. Meanwhile, the pollen in male floral buds has allergenic properties and poses potential health hazards to allergic people (An et al. 2011). Therefore, clarifying the flower development in poplar is of great importance. In this study, we identified 46 *NF-Y* genes from the *P. trichocarpa* genome and performed a relatively complete bioinformatics analysis, including conserved regions, phylogenetic relationships, gene-structure analysis, and chromosome localization. Moreover, we analyzed *NF-Y* genes in terms of their expression levels among five different tissues and seven different flower developmental stages using transcriptome data and validated these expression levels by qRT-PCR, which is particularly important for identifying candidate genes involved in regulation of the growth and flowering of *P. tomentosa*. Taken together, our results contribute to a more complete understanding of the function of the *NF-Y* genes in poplar.

Materials and methods

Identification of *PtNF-Y* gene family members

Using the classification criteria of NF-Y TFs in *A. thaliana* (Petroni et al. 2012), we obtained 36 AtNF-Y amino acid (AA) sequences from The *Arabidopsis* Information Resource (Swarbreck et al. 2008). These sequences included 10 NF-YA, 13 NF-YB, and 13 NF-YC subunits. To anchor the *P. trichocarpa* NF-Y family members, AA sequences of the *Arabidopsis* NF-Y members were used as queries to search for NF-Y family members of *P. trichocarpa* in Phytozomev12.1 (Goodstein et al. 2012). We selected all sequences with e value $< 10^{-10}$ for subsequent analysis. In addition, SMART tool (http://smart.embl-heidelberg.de/) (Zhang et al. 2019) was utilized to identify NF-Y protein domains. We obtained the NF-Y candidate members of *P. trichocarpa* based on this comprehensive screening for use in subsequent analysis.

Chromosomal distribution and structure analysis of *PtNF-Y*

The *PtNF-Y* genes were mapped to the chromosomes using the MapInspect software (Zhang et al. 2019). The gene structures of *PtNF-Y* members were analyzed using the Gene-Structure Display Serve (http://gsds.cbi.pku.edu.cn/) (Hu et al. 2015).

NF-Y protein alignment and phylogenetic prediction

We obtained the genomic sequences, coding sequences (CDS) and protein sequences of PtNF-Y members using

Phytozome v12.1. Multiple sequence alignments analysis of NF-Y proteins was performed using Clustal X2.1 (Larkin et al. 2007). Additionally, based on the alignments obtained, genetic distance matrices were obtained, and an unrooted phylogenetic tree was generated in MEGA7 using the neighbor-joining (NJ) method based on the PtNF-Y protein sequences of *P. trichocarpa* and *Arabidopsis*. Their evolutionary relationships were estimated with 1000 bootstrap replications (Kumar et al. 2016).

Plant material

Adult *P. tomentosa* trees were grown under natural conditions in the Beijing Forestry University greenhouse (Beijing, China). Roots, stems, and leave tissue were obtained from 6-month-old cultured plantlets. Dormant and germinating leaf buds were collected during the flower transition period in 2017. Male and female floral buds of *P. tomentosa* were collected from June 2017 to February 2018 at Beijing Forestry University. These flower buds covered seven stages of flower development, from flower induction to flower initiation and organ differentiation. In addition, all collected samples were immediately frozen in liquid nitrogen and then stored at − 80 °C until use.

Transcriptome sequencing and de novo assembly

RNA-seq libraries were generated using the Illumina kit according to the manufacturer's protocol (Illumina, San Diego, CA, USA). Equal amount of RNA for each sample were pooled, and sequenced separately on the Illumina HiSeqTM 2000 platform using paired-end technology. The assembly method used was described previously (Chen et al. 2018). The expression levels of *PtoNF-Ys* were calculated as fragments per kilobase per million (FPKM) (Cole et al. 2010) reads values to determine differences in expression in various organs and tissues among *NF-Y* members, and heat maps were constructed using TBool software. Hierarchical cluster analysis was conducted to visualize the expression pattern of NF-Y genes in different *P. tomentosa* organs and tissues.

RNA isolation and qRT-PCR analysis

Total RNAs were isolated from each tissue at various developmental stages of *P. tomentosa*. We used Trizol Total RNA Extraction Kit (Promega, Madison, WI, USA) and RQ1 DNase to remove genomic DNA (Promega) and incubated the extracts at 65 °C for 10 min to inactivate the DNase. The RNA was detected by 1.0% agar gel electrophoresis and a NanoDrop 2000 spectrophotometer (IMPLEN, CA, USA). The total RNA was reverse-transcribed into first strand cDNA using the Reverse Transcription System (Promega). The resulting cDNA was diluted 1:10 with ddH_2O and used as the template for qRT-PCR amplification.

For qRT-PCR, we first designed primers by reference to the CDS sequence of *P. trichocarpa NF-Y* to amplify this sequence. The amplified DNA fragments were then cloned into the pMD-18T vector (TaKaRa, Otsu, Japan) for sequencing compared with the *P. trichocarpa* CDS (Fig. S1). Then, qRT-PCR primers were designed based on the CDS sequence of *P. tomentosa* using primer 5.0 software. Finally, primers qRT-PCR was identified by PCR (Fig. S1). The *PtoACTIN* (GenBank accession: AY261523.1) (An et al. 2011; Zhang et al. 2008; Zheng et al. 2009) was selected as an internal control gene for normalization according to the $2^{-\Delta\Delta Ct}$ method. All primers are listed in Table S1. Real-time quantitative PCR was performed using SYBR ® Premix Ex Taq ™ (TaKaRa) on the ABI PRISM 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA), The PCR program was as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 20 s, and then, a final elongation step of 72 °C for 7 min. The plates were read at 0.2 °C intervals for 1 s from 70 to 95 °C to generate melting curves and thus verify the specificity of the amplified product. The expression values of these genes were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All analyses were performed with three technical and three biological replicates.

Results

Isolation of the NF-Y family in *P. trichocarpa*

To obtain information on the *NF-Y* genes of *P. trichocarpa*, *Arabidopsis* NF-Y protein sequences were used to search for NF-Y using the most recent *P. trichocarpa* genome assembly in Phytozome v12.1 and the ExPASy server (Table 1). A total of 46 *NF-Y* genes were identified in the *P. trichocarpa* genome: 11 PtNF-YA, 21 PtNF-YB, and 14 PtNF-YC. The 46 predicted *P. trichocarpa NF-Y* genes were named PtNF-YA1 to 11, PtNF-YB1 to 21 and PtNF-YC1 to 14, respectively. The bioinformatics data of all gene members were analyzed, including genomic DNA sequence length, transcript length, CDS length, number of AAs, theoretical molecular weight (MM), and theoretical isoelectric point (pI). The identified *PtNF-Y* genes encodes peptides ranging from 143 to 348 AA, with pI values ranging from 4.85 to 9.46, and molecular weights ranging from 12.81 to 41.42 kDa, as estimated using the ExPASy server.

Genomic structure and locations of *PtNF-Y* gene family members

Gene-structure analysis can provide insight into the evolution of families of genes. Therefore, we determined the

Table 1 Nuclear factor Y (NF-Y) identified in *P. trichocarpa*

Name	Transcript name	*A. thaliana* ortholog	DNA length	CDS length	Peptide residue	MW (Kd)	pI	Locus
NF-YA								
PtNF-YA1	Potri.016G068200.1	AtNF-YA2,10	4645	966	321	30.39	9.34	Chr16
PtNF-YA2	Potri.018G064700.1	AtNF-YA3,8	5595	1014	337	37.37	8.94	Chr18
PtNF-YA3	Potri.006G145100.1	AtNF-YA3,8	5756	1026	341	37.4	9.22	Chr06
PtNF-YA4	Potri.011G098400.1	AtNF-YA4,7	4801	627	208	22.77	9.17	Chr11
PtNF-YA5	Potri.001G372100.1	AtNF-YA4,7	6404	672	223	24.53	6.49	Chr01
PtNF-YA6	Potri.006G053500.1	AtNF-YA1,9	4270	900	299	33.33	9.39	Chr06
PtNF-YA7	Potri.001G257600.1	AtNF-YA1,9	5175	1047	348	38.45	6.77	Chr01
PtNF-YA8	Potri.001G266000.1	AtNF-YA1,9	5627	942	313	34.49	7.30	Chr01
PtNF-YA9	Potri.009G060600.1	AtNF-YA1,9	6063	1134	377	41.42	7.04	Chr09
PtNF-YA10	Potri.009G052900.1	AtNF-YA1,9	5028	1044	347	38.19	5.97	Chr09
PtNF-YA11	Potri.006G201900.1	AtNF-YA2,10	4989	975	324	34.97	9.19	Chr06
NF-YB								
PtNF-YB1	Potri.016G006100.1	AtNF-YB2	1035	594	197	21.19	5.75	Chr16
PtNF-YB2	Potri.016G085000.1	AtNF-YB8,10	3228	546	181	20.05	8.38	Chr16
PtNF-YB3	Potri.016G005600.1	AtNF-YB6,9	1706	696	231	26	6.45	Chr16
PtNF-YB4	Potri.006G005500.1	AtNF-YB2	940	645	214	23.24	5.69	Chr06
PtNF-YB5	Potri.006G005000.1	AtNF-YB6,9	1426	687	228	25.61	6.45	Chr06
PtNF-YB6	Potri.007G082200.1	AtNF-YB3	1107	849	282	31.4	9.19	Chr07
PtNF-YB7	Potri.008G044800.1	AtNF-YB1	4834	531	176	19.12	6.14	Chr08
PtNF-YB8	Potri.008G210300.1	AtNF-YB4,5	668	453	150	16.54	5.64	Chr08
PtNF-YB9	Potri.008G217900.1	AtNF-YB4,5	453	453	150	16.57	5.64	Chr08
PtNF-YB10	Potri.005G065300.1	AtNF-YB7	636	636	211	24.03	5.87	Chr05
PtNF-YB11	Potri.005G083400.1	AtNF-YB3	1365	888	295	32.95	7.02	Chr05
PtNF-YB12	Potri.010G216600.1	AtNF-YB1	4684	621	206	22.51	7.03	Chr10
PtNF-YB13	Potri.001G367500.1	AtNF-YB3	1292	504	167	18.2	5.04	Chr01
PtNF-YB14	Potri.014G167800.1	AtNF-YB3	2345	588	195	20.29	5.97	Chr14
PtNF-YB15	Potri.014G132600.1	AtNF-YB4,5	828	531	176	19.93	6.33	Chr14
PtNF-YB16	Potri.005G027400.1	AtNF-YB4,5	450	450	149	16.79	5.62	Chr05
PtNF-YB17	Potri.013G019600.1	AtNF-YB4,5	450	450	149	16.86	5.14	Chr13
PtNF-YB18	Potri.013G019500.1	AtNF-YB4,5	432	432	143	16.47	5.45	Chr13
PtNF-YB19	Potri.009G163500.1	AtNF-YB11	3013	546	181	20.28	9.46	Chr09
PtNF-YB20	Potri.015G052800.1	AtNF-YB12,13	4320	471	156	17.33	4.9	Chr15
PtNF-YB21	Potri.012G058200.1	AtNF-YB12,13	4494	591	196	21.56	4.85	Chr12
NF-YC								
PtNF-YC1	Potri.015G097400.1	AtNF-YC1,4	5281	765	254	27.58	5.23	Chr15
PtNF-YC2	Potri.012G098500.1	AtNF-YC1,4	4856	702	233	25.36	5.47	Chr12
PtNF-YC3	Potri.007G070900.1	AtNF-YC2	3781	777	258	29.02	5.61	Chr07
PtNF-YC4	Potri.005G094900.1	AtNF-YC2	3517	798	265	29.81	5.72	Chr05
PtNF-YC5	Potri.005G035800.1	AtNF-YC3,9	2870	753	250	28.08	5.90	Chr05
PtNF-YC6	Potri.013G025000.1	AtNF-YC3,9	2842	711	236	26.73	5.72	Chr13
PtNF-YC7	Potri.008G203500.1	AtNF-YC3,9	2197	681	226	25.52	5.77	Chr08
PtNF-YC8	Potri.019G047000.1	AtNF-YC3,9	2474	777	258	28.79	5.77	Chr19
PtNF-YC9	Potri.003G124500.1	AtNF-YC1,4	354	354	117	12.81	7.90	Chr03
PtNF-YC10	Potri.001G106900.1	AtNF-YC1,4	357	357	118	12.84	9.26	Chr01
PtNF-YC11	Potri.001G033200.1	AtNF-YC11	5552	939	312	33.78	5.07	Chr01
PtNF-YC12	Potri.001G225400.1	AtNF-YC11	4874	930	309	34.42	5.51	Chr01
PtNF-YC13	Potri.003G192100.1	AtNF-YC11	5743	951	316	34.86	5.60	Chr03
PtNF-YC14	Potri.001G055000.1	AtNF-YC13	708	438	145	16.16	8.89	Chr01

numbers and positions of exons and introns of the *PtNF-Y* genes using genomic DNA sequences in the Phytozome v12.1 database (Fig. 1). All *PtNF-YA* genes were separated by introns. *PtNF-YA* genes had four or five exons, except *PtNF-YA6*, which had three introns. Among the *PtNF-YB* genes, 11 had no introns and the others had 1–5 introns. Of the *PtNF-YC* genes, 10 of 14 genes were intronless, whereas *PtNF-YC13* had six introns. In addition, we found that most homologs in the *PtNF-Y* subfamily, such as *NF-YA6/A7*, *NF-YA4/A5*, *NF-YB7/B12*, *NF-YB6/B11*, and *NF-YC1/C2*, had different gene structures. These results indicate that the intron/exon distribution in *PtNF-Y* genes is highly variable.

We used the current *P. trichocarpa* genome database in Phytozome v12.1 to analyze the positions of *PtNF-Y* genes on *P. trichocarpa* chromosomes. The 46 *PtNF-Y* genes were found to be distributed among 16 of the 19 chromosome scaffolds of *P. trichocarpa* (Table 1, Fig. 2).

Multiple alignments analyses of the *PtNF-Y* genes

For each PtNF-Y subunit, multiple alignments performed using ClustalX 2.0 software revealed conserved DNA binding domains in PtNF-YA, PtNF-YB, and PtNF-YC proteins (Fig. 3). The results illustrate that PtNF-Y proteins contain

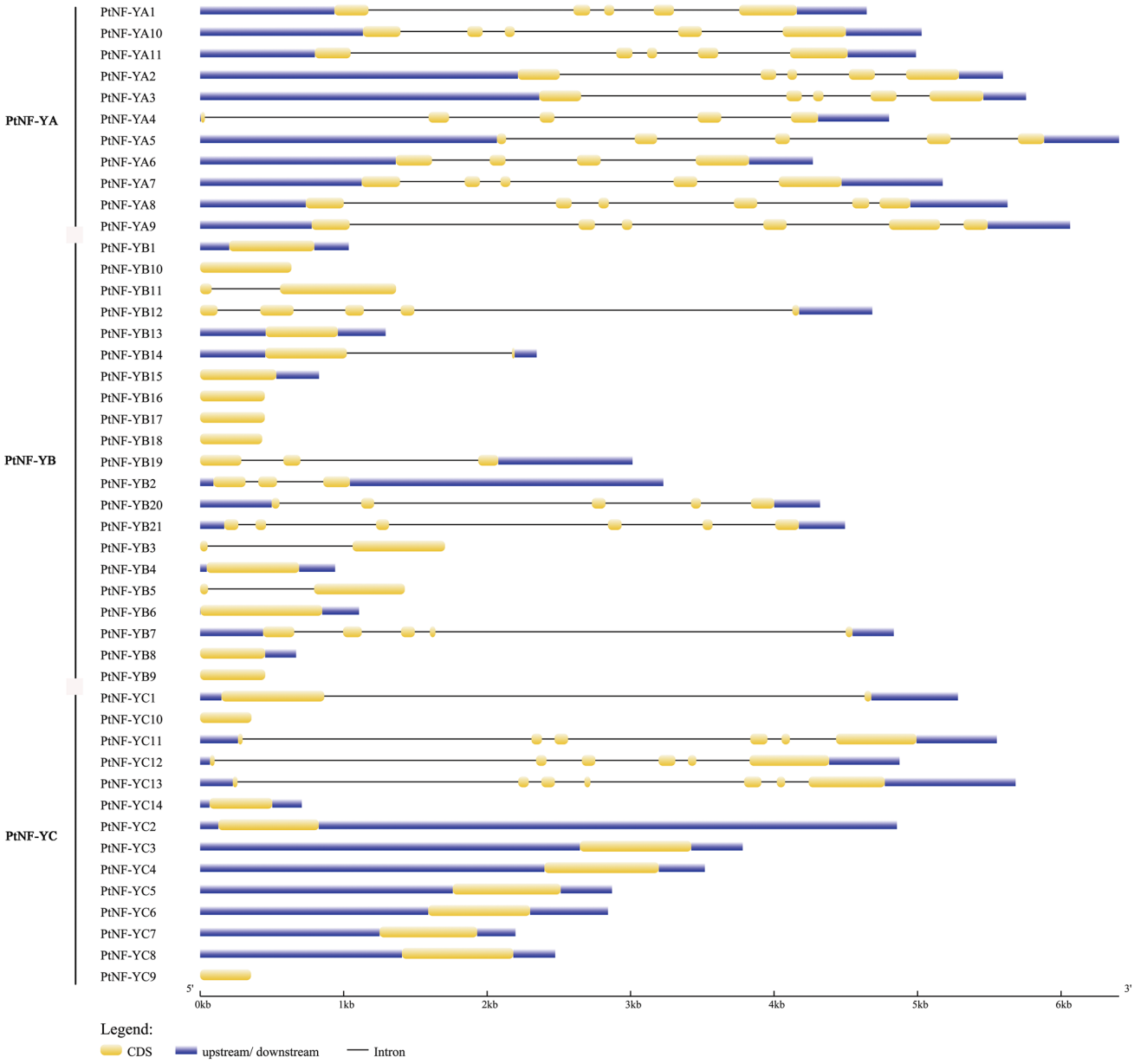


Fig. 1 Gene structure of the *PtNF-Y* gene family. Yellow boxes and black lines represent exons and introns, respectively. Blue box indicates the 5′ and 3′ non-coding regions. The length represents the size of exon and intron

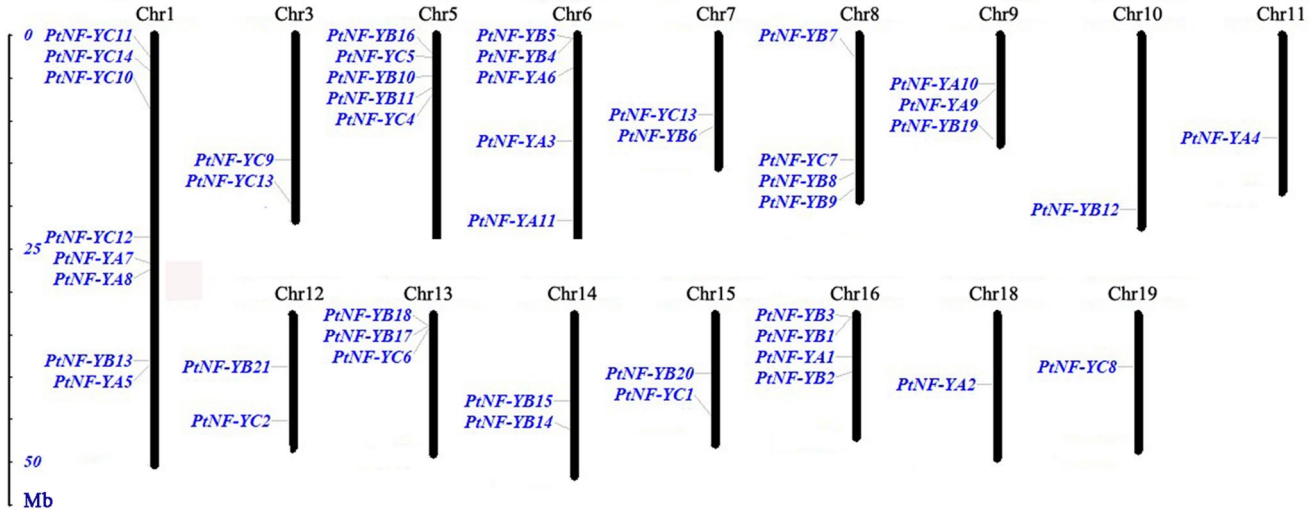


Fig. 2 Positions of *NF-Y* gene family members on *P. trichocarpa* chromosomes. Scaffold numbers are indicated at the top of each scaffold. Chromosome size is indicated by the vertical scale. Apart from chromosome 2 and 4, other gene members are distributed on each chromosome

evolutionarily conserved domains and more variable N- or C-terminal transcriptional regulation regions. The conserved domain of the PtNF-YA protein core contains 53 AAs, including two highly conserved domains: NF-YB/C interaction domain α1 and DNA contact domain α2. The relatively conserved linker of 21 AAs separates the alpha1 and alpha2 subdomains (Fig. 3a). The conserved core region of PtNF-YB was 86 AAs in length, which is similar to the lengths reported in other species. In addition, it also contains a central domain similar to the histidine folding domain (HFM) in the core histone H2B and plays a key role in DNA binding and protein–protein interactions (Fig. 3b). PtNF-YC subunits were also found to consist of a core histone-like sequence with a central domain about 79 AAs in length (Fig. 3c).

Phylogenetic relationships of *PtNF-Y* gene family

To explore the evolutionary relationships among different PtNF-Y family members, an unrooted phylogenetic tree of 46 PtNF-Y proteins was constructed (Fig. 4a). The results showed that all PtNF-Y members formed three major clusters: I (PtNF-YA), II (PtNF-YB), and III (PtNF-YC). Closely related members showed the most similar physical and chemical properties, such as MM and pI (Table 1). In addition, the result also indicated that the PtNF-YB proteins are more closely related to PtNF-YC proteins than they are to PtNF-YA proteins, implying that *PtNF-YB* and *PtNF-YC* genes may share a more recent ancestor than *PtNF-YA*.

To investigate and elucidate the phylogenetic relationships among PtNF-Y proteins and assist with functional predictions, we constructed a comprehensive phylogenetic tree using the full-length protein sequences of all the NF-Y proteins of *Arabidopsis* and *P. trichocarpa* (Fig. 4b). The phylogenetic tree shows close relationships among the proteins, all of which, except AtNF-YC10, were grouped into three subfamilies (A, B, and C). The phylogenetic tree indicated close relationships among the PtNF-Ys within each of the three subfamilies. Furthermore, in Fig. 4b, we found that PtNF-YB3, PtNF-YB5 and AtNF-YB9 (AtLEC1), AtNF-YB6 (AtL1L) were clustered in a separate subgroup. However, protein multiple alignment analysis revealed that PtNF-YB3 and PtNF-YB5 were more diverse compared to the others, due to an amino acid change from lysine (K) to aspartic acid (D) (Fig. 3, Fig S2). Therefore, based on this phylogenetic tree, we further predicted that the structures and functions of some PtNF-Y members may be similar to those of *Arabidopsis*.

Expression of the *PtoNF-Y* gene family different tissues determined by transcriptome and qRT-PCR analyses

To identify the potential functions of *PtoNF-Ys* in the development of *P. tomentosa*, we compared the relative expression levels of the *PtoNF-Y* genes in root, stem, leaf, leaf bud, and male and female flower buds at seven different developmental stages. The results indicated that 34 of the 46 *PtoNF-Ys* were expressed in all tissues, while the other 12 *PtoNF-Y* gene members were not detected (Fig. 5). The transcription levels of the 34 expressed *PtoNF-Y* genes varied among tissues. We observed four main clusters of expression among the vegetative organs (see Fig. 5, clusters I–IV). Six of the genes are clustered together (clusters I: A3, A8, B4, B14, B21, C6) have relatively high expression except in the germinating leaf bud.

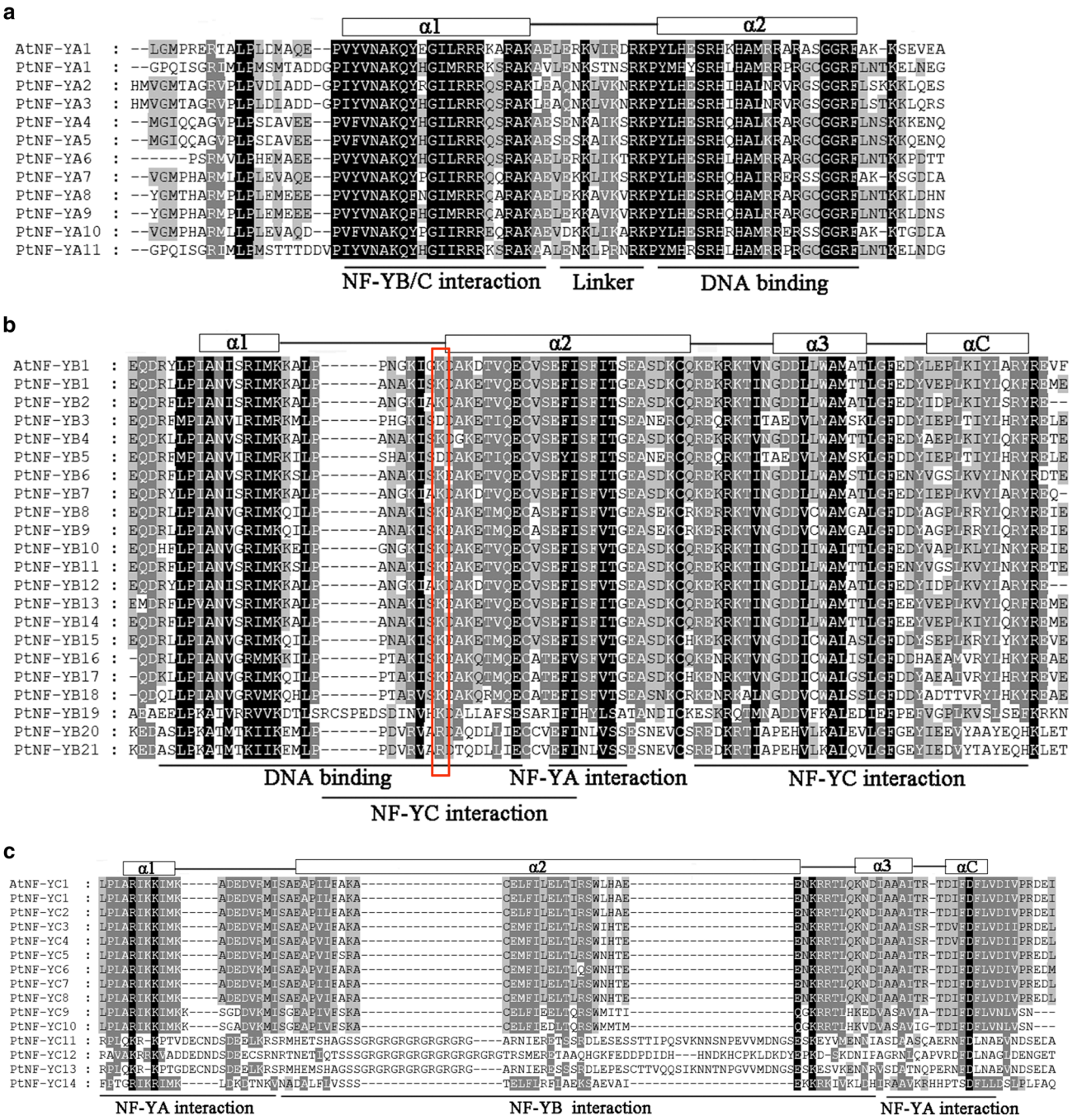


Fig. 3 Multiple alignments of *P. trichocarpa* NF-Y family members. Multiple alignment of (**a**) PtNF-YA proteins, (**b**) PtNF-YB proteins, and (**c**) PtNF-YC proteins. Amino acids critical for distinguishing between LEC1 and non-LEC1 were indicated by red box

Among the reproductive organs, we analyzed the expression patterns of male and female floral buds at seven different developmental stages by RNA-seq and qRT-PCR (Fig. 6, these genes are highly expressed in fig S3). The results showed that about 90% of the genes appear to have similar RNA-seq and qRT-PCR patterns. In addition, the result analysis found that some genes show similar expression patterns across the male and female floral bud stages. Such as, in male flower buds and female flower buds, *PtoNF-YB4* and *PtoNF-YB21* peak in early and late stages, respectively. However, *PtoNF-YB15*, *PtoNF-YC2*, and *PtoNF-YC7* peak in the middle stages. Moreover, the patterns also showed that the same gene can exhibit contrasting peak times between male and female floral buds. Such as, in the later stages, the expression level of *PtoNF-YC5* is completely opposite in male flower buds and female flower buds. The peak of

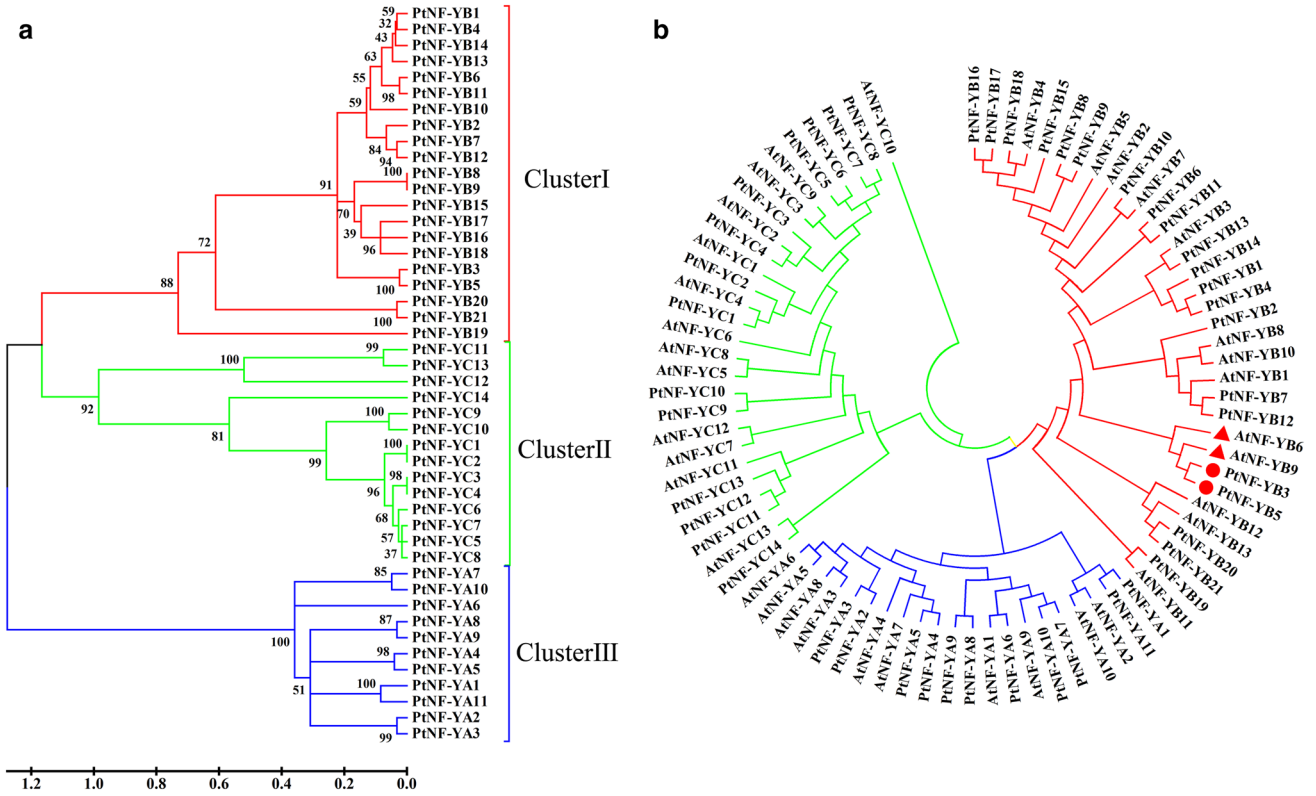


Fig. 4 Phylogenetic analysis of *P. trichocarpa* NF-Y proteins. (**a**) Combined phylogenetic tree for 11 PtNF-YA, 21 PtNF-YB, and 14 PtNF-YC proteins constructed by the neighbor-joining method in MEGA 7.0 software with 1000 bootstrap reiterations. (**b**) The genes encoding different subunits formed three separate branches. Different colors indicate different subfamilies. Blue, red, and green represent NF-YA, NF-YB, and NF-YC, respectively. Proteins prefixed by the term 'At' and 'Pt' indicate NF-Y from *Arabidopsis thaliana* and *Populus trichocarpa*, respectively. Among, AtNF-YB6 and AtNF-YB9, which are marked by red triangles, represent AtL1L and AtLEC1, respectively. Numbers on branches show bootstrap values > 50%

PtoNF-YC14 appears in the late stage of male flower buds and the early stage of female flower buds. The above phenomenon indicated that some genes evolved different functional roles in the two floral bud types.

Discussion

In this study, based on the currently available *P. trichocarpa* genome sequences and the updated classification criteria of NF-Y TFs in *A. thaliana* (Petroni et al. 2012), 46 *PtNF-Y* genes were identified in *P. trichocarpa* (11 PtNF-YA, 21 PtNF-YB, and 14 PtNF-YC) (Table 1). Previous studies have reported 33 NF-Y members in *Arabidopsis* (Siefers et al. 2009), 28 in rice (Thirumurugan et al. 2008), and 33 in walnuts (Quan et al. 2018). The high number of NF-Y members in *P. trichocarpa* may be due to the expansion of this gene family in poplars. In contrast, soybean and tomato contain 66 and 59 NF-Y members, respectively. Compared with the numbers of NF-Y genes in these species, fewer *PtNF-Y* genes were identified in our study. These differences in gene numbers may be related to differences in classification criteria. The NF-Y family member classification criteria for maize and tomato include several NC2 and Dpb3/4 members (Li et al. 2016; Zhang et al. 2016).

Gene-structure alterations might lead to changes in gene or protein function. In this study, compared with the exon–intron structures of *PtNF-YB* and *PtNF-YC*, most *PtNF-YA* members showed a more variable and complicated exon–intron organization pattern. This result is also consistent with the *NF-Y* gene structures previously reported in *Arabidopsis*, *Brassica napus,* and *Solanum lycopersicum*. Moreover, exon–intron structure analysis provides new insight into the evolutionary relationships among genes or organisms. We analyzed the exon–intron structure of 46 *PtNF-Y* genes (Fig. 1), of which 26 lacked introns. However, introns are considered to be essential components of structural eukaryotic genes. Previous studies have reported that introns have many functions, such as alternative splicing and regulation of the evolution rate of genes (Lee et al. 2003; Roy and Gilbert 2006). Their

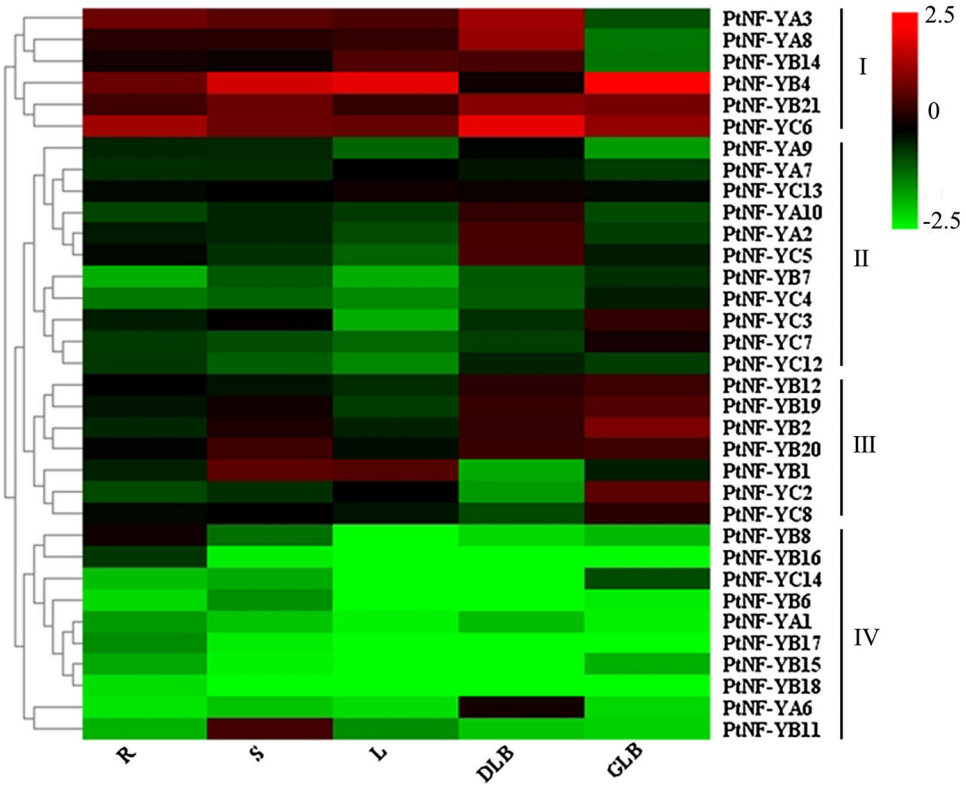


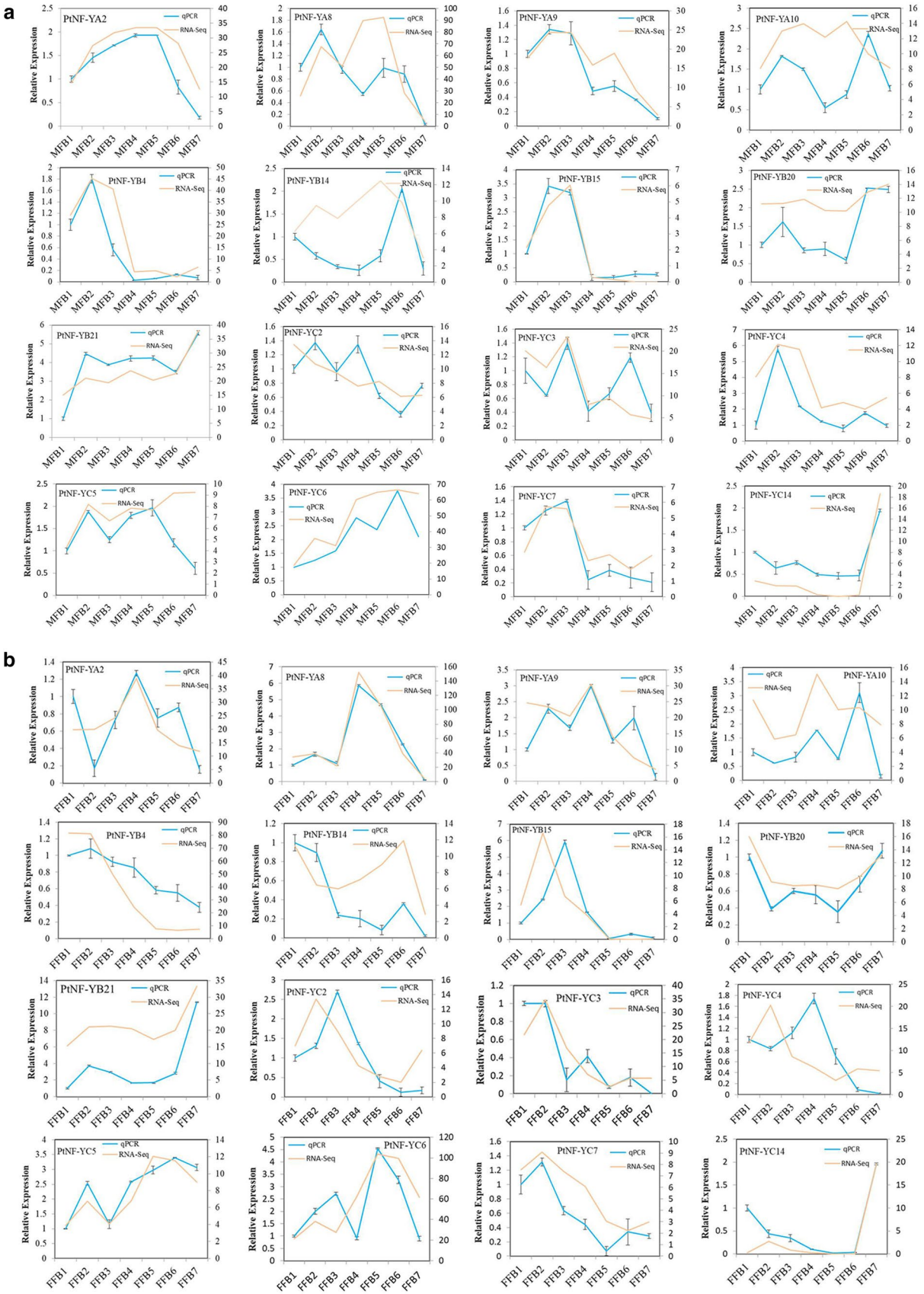
Fig. 5 Heat map of *PtoNF-Y* predicted expression in different tissues and organs of *P. tomentosa*. The abbreviations R, S, L, DLB, and GLB represent roots, stems, leaves, dormant leaf buds and germinated leaf buds. Expression potential from high to low is represented by square colors from red to green and the black is medium. FPKM value was used to create the heat map. The scale represents the relative expression level intensity of FPKM values. In this study, de novo assembly was done using pooled samples

deletion or alteration might lead to structural diversity and complexity, in turn affecting the evolution of the gene family.

In general, the conserved domain is located at the C-terminus of mammalian proteins; the phenomenon is more apparent in plant NF-YAs. Previous studies have shown that three histidine (H) and three arginine (R) residues are essential for DNA binding (Xing et al. 1993). The results of the current study show that these six sites were also highly conserved among all 11 PtNF-YA proteins (Fig. 3a). This finding is consistent with the previously observed residues in *Arabidopsis*, *Oryza sativa,* and *Brachypodium* (Cao et al. 2011; Siefers et al. 2009; Thirumurugan et al. 2008). Previous studies reported that the aspartate at D55 site was considered a critical protein interaction site of the AtNF-YB subfamily, and the *Arabidopsis* NF-YB subunit based on the 55th Asp in its domain divided into two classes: LEC1 and non-LEC1 (Lee et al. 2003). LEC1 has been identified to play a key role in *Arabidopsis* embryogenesis and the development of castor seeds (Wang et al. 2018). However, in our study, PtNF-YB3 and PtNF-YB5 changed from lysine (K) to aspartic acid (D) at this binding site. (Figure 3, Fig S2). In addition, phylogenetic relationship analysis also showed that PtNF-YB3, PtNF-YB5 and AtNF-YB9 (AtLEC1), AtNF-YB6 (AtL1L) were clustered in a separated subgroup (Fig. 4b). Therefore, we speculate that these two members are possibly orthologs of AtLEC1 and AtL1L, and propose that PtNF-YB3 and PtNF-YB5 may have functions similar to those of AtLEC1 and AtL1L.

Previous studies have reported that *NF-Y* genes play important roles in regulating plant growth and development (Li et al. 2016; Potkar et al. 2013; Wang et al. 2018; Wei et al. 2017). In this study, the expression levels of *PtoNF-Y* genes (except for 12 *NF-Y* genes that had no expression data) were observed in five tissues and organs, indicating that *PtoNF-Y* is involved in regulating the growth and development of *P. tomentosa* (Figs. 5, 6, S3). It has been reported that *AtNF-YB2* and *3* and *AtNF-YC3*, *4,* and *9* are critical regulators of flowering time (Kumimoto et al. 2010; Wenkel et al. 2006). Here, we detected expression of their orthologs, *PtoNF-YB4*, *PtoNF-YB14,* and *PtoNF-YB5-7*, during the early stages of floral bud development, but the specific regulatory mechanism of this expression remains unclear. Therefore, determining how these genes regulate floral bud development and affect the growth and development of *P. tomentosa* are interesting questions for future research.

Comprehensive bioinformatics analysis of the NF-Y family in *P. trichocarpa* identified 46 genes as putative *PtNF-Y* genes, and the expression of *PtoNF-Y* genes in various *P. tomentosa* tissues and organs was evaluated using transcriptome data. The results suggest that the expression and transcription levels of 34 of 46 members in various tissues and organs vary among family members. The results of this study provide a basis for further studies on the functions of


a
PtNF-YA2
PtNF-YA8
PtNF-YA9
PtNF-YA10
PtNF-YB4
PtNF-YB14
PtNF-YB15
PtNF-YB20
PtNF-YB21
PtNF-YC2
PtNF-YC3
PtNF-YC4
PtNF-YC5
PtNF-YC6
PtNF-YC7
PtNF-YC14
qPCR
RNA-Seq
Relative Expression
MFB1 MFB2 MFB3 MFB4 MFB5 MFB6 MFB7
b
PtNF-YA2
PtNF-YA8
PtNF-YA9
PtNF-YA10
PtNF-YB4
PtNF-YB14
PtNF-YB15
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PtNF-YC6
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PtNF-YC14
qPCR
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FFB1 FFB2 FFB3 FFB4 FFB5 FFB6 FFB7

◂**Fig. 6** Expression profiles of the *PtoNF-Y* genes in male floral buds and female floral buds determined by qRT-PCR. (**a**) qRT-PCR validation of NF-Y gene expression in the male floral of *P. tomentosa*. (**b**) qRT-PCR validation of the expression levels of PtoNF-Y genes in female floral buds. The blue and orange lines represent values obtained by qRT-PCR and RNA-seq, respectively. MFB1–MFB7 represents the seven stages (from June 2017 to February 2018) of male floral bud and female floral buds development. The data represent mean ± SD. The error bars indicate the standard deviation

the *PtNF-Y* gene family. However, the specific functions of these genes in *P. tomentosa* must be further verified experimentally, and the regulatory mechanisms must also be determined in future studies.

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Author contribution statement Juan Li, Kai Gao, and Xinmin An designed the experiments and wrote the manuscript. Juan Li, Wasif Ullah Khan, Tianyun Zhao, and Xiong Yang performed the experiments. Juan Li, Xiaoyu Yang, and Zhong Chen analyzed the data. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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