

Transcriptome-wide isolation and expression profiles of *NF-Y* gene family in male cone development and hormone treatment of Chinese pine (*Pinus tabuliformis*)

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Research article

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Abstract

Background

Conifers and angiosperms have difference in reproductive development, especially for flowering. It is known that NUCLEAR FACTOR Y (NF-Y) transcription factor play an important role in flowering, drought stress and GA, ABA signaling, but, little known in auxin, salicylic acid, jasmonic acid, etc. Moreover, the *NF-Y* genes family has been mainly analyzed in angiosperms, but it has not been comprehensively reported in conifers.

Results

In this study, we identified 9 *NF-YA*, 9 *NF-YB*, and 10 *NF-YC* genes in *Pinus tabuliformis* using *Arabidopsis* NF-Y protein sequences as queries. Besides, by comparing conserved regions and phylogenetic relationships of the PtNF-Ys, we found that the NF-Ys were both conserved and altered during evolution. *PtTFL2*, *PtCO*, *PtNF-YC1* and *PtNF-YC4* were exploited by expression profile in male cone development and the correlation analysis. In addition, NF-YC1/4 can interact with DPL by yeast two-hybrid assays and BiFC. The multiple types of phytohormones-responsive *cis*-elements (ABA, JA, IAA, SA) were present and many *NF-Y* genes responded positively to SA and as opposed to IAA and JA.

Conclusions

Twenty-eight *PtNF-Ys* were identified and bioinformatic characterization of *NF-Y* genes including conserved regions, phylogenetic relationships, gene-motifs, was carried out. Two candidate genes (*NF-YC1* and *NF-YC4*) were found to be involved in the regulation of conifer flowering and gibberellin signalling. The *cis*-elements and hormone transcriptome analysis revealed that the potential role of *NF-Ys* in conifers resistance. This study provides the basis for improved understanding of *NF-Y* genes function in conifers.

Background

There are about 630 conifer species on earth, and they dominate many terrestrial ecosystems, especially in the Northern Hemisphere [1]. At the same time, conifers have great economic and ecological value. Reproductive organ development is an essential feature in the whole life cycle of seed plants. Considerable progress has been made over the past decade in understanding the molecular mechanisms underlying flower development for angiosperms. However, there is still a considerable lack of molecular biology research on the development and regulation of conifers male and female cones. Chinese pine (*Pinus tabuliformis*) of Pinaceae is an indigenous conifer species and widely distributed in 14 provinces and autonomous regions in northern China, with an ecological area of 3 million square kilometers [2, 3]. Moreover, Chinese pine has many favored economic and ecological traits, such as wood corrosion

resistance, rich in resin and pollen as well as excellent tolerance to drought, wind, barren, pests and diseases, just like *Pinus sylvestris* var. *mongolica*, and *Pinus thunbergii*.

NUCLEAR FACTOR Y (NF-Y) transcription factors are almost found in all eukaryotes. NF-Y includes at least three subunits: NF-YA, NF-YB, and NF-YC, all of which can specifically bind to the CCAAT-box in eukaryotic promoters [4]. The core domain of NF-YA contains the NF-YB/C subdomain and the DNA binding domain. NF-YB and NF-YC which have the highly conserved histone-fold motif (HFM) are structurally similar to histone subunits H2B and H2A [5, 6]. NF-Ys should function in the form of a dimer or trimer. Initially, NF-YB and NF-YC dimerize in the cytoplasm, then move into the nucleus, recruit an NF-YA component, and subsequently bind DNA and affect transcription [7, 8].

The Nuclear Factor-Y (NF-Y) has been reported that play an important role in flowering time [9, 10]. NF-YA, in complex with NF-YB/NF-YC proteins, can directly bind the distal CCAAT box and a non-canonical NF-CO complex binding at a proximal CORE site of the *FT* promoter which are positive regulators of flowering [11–13]. Also, CmNFYB8 can influences flowering time through directly regulating the expression of *cmo-MIR156* in the aging pathway in chrysanthemum [14]. NF-Y proteins of *Triticum monococcum* can interact with VRN2 in the regulation of flowering initiation by the integration of the vernalization and photoperiod seasonal signals [15]. NF-Y mediates the effect of photoperiod and GA signaling on *SOC1* expression partly through H3K27me3 demethylation [16]. These results show that NF-Y can participate in regulating flowering time via the aging pathway, the photoperiod, the vernalization and GA pathway. Besides, *NF-Y* genes have involved in other growth and developmental processes, such as endosperm development [17], seed germination [18], pollen tube growth [19], hypocotyl elongation [20], starch biosynthesis [21].

In addition to plant growth and development, NF-Y participates in stress response and hormone signaling. According to the reports, we find studies on NF-Y mainly focus on drought resistance [22–24], ABA response [18, 22, 25] and salt stress [23, 26], a small part involving temperature [27], photoprotective [28, 29]. In brief, as an important transcription factor in response to drought stress, NF-Y is mainly regulated by miR169, ABA crossing signal and photosynthesis. However, related to biotic stress, such as pests and diseases and other hormones (auxin, salicylic acid, jasmonic acid, etc.) are also important research directions of plant life activities, and little is known about NF-Y. So, the role of NF-Y in other stresses, and other hormone signals, need to be further explored.

Now, the study of *NF-Y* genes mainly focus on angiosperms, only some studies have been reported on conifers. *PwHAP5* and *PwNF-YB3* (*Picea wilsonii*) can improve tolerance to salt and drought stress in Arabidopsis [30, 31], and *PwHAP5* interacts with *PwFKBP12* that play a role in pollen tube development and orientation regulation [19]. In addition, it has been discovered that *HAP3A* and *HAP3B* (*Picea abies* and *Pinus sylvestris*) are necessary to promote embryo maturation during conifer embryogenesis [32]. But, the study of *NF-Y* genes family in conifers has not been reported. Besides, one major difference between conifers and angiosperms is their reproductive development, and for example, FLOWERING LOCUS T (FT), the key activators of flowering, has no orthologues genes in conifers [1]. So, to clarify the

role of NF-Y in conifer flowering is of great significance to enrich the evolution of conifer reproductive development. In the research, we took Chinese pine (*Pinus tabuliformis*) as the experimental material to exploit the role of NF-Y in this type of conifer. In our study, we identified 28 *NF-Y* genes of *Pinus tabuliformis* and performed a relatively complete bioinformatics analysis, including conserved regions, phylogenetic relationships, gene-motifs analysis. Moreover, we found NF-YC1 and NF-YC4 participating in male cones development and GA signal. Also, *PtNF-Ys* responded to ABA, JA, IAA and SA signals by the *cis*-elements and transcriptome analysis. Taken together, the results obtained here provide a comprehensive profile to a more complete understanding of the function of the *NF-Y* genes in conifers.

Results

Isolation of the NF-Y family in *Pinus tabuliformis*

We used Arabidopsis NF-Y protein sequences as queries to search Chinese pine *NF-Y* genes [33]. Sixty-nine candidate *NF-Y* genes were identified in Chinese pine by BLAST (blast-2.6.0+) [34]. Forty-nine candidate genes were identified by HMMER [35]. The results of the two search methods were merged resulting in Sixty-nine candidate genes. By removing too long or too short or because they had improper domains and redundant sequences, 28 *NF-Y* genes were identified, including 9 *NF-YA*, 9 *NF-YB*, and 10 *NF-YC* genes (Table 1). The identified *PtNF-Y* genes encodes peptides ranging from 136 to 384 aa with the pI value varying from 4.55 to 9.76, and the molecular weight ranging from 14.88 to 41.86 KDa as estimated from ExPASy server (<http://WWW.expasy.org/>).

Table 1
NF-Y transcription factors identified in *Pinus tabulaeformis*

Gene name	Gene ID	Amino acid length (a.a)	Protein Mol. Wt.(kDa)	Protein PI value
PtNF-YA1	Pita_unigene60921	163	17.92 kDa	pH 8.25
PtNF-YA2	Pita_unigene63081	170	18.79 kDa	pH 9.76
PtNF-YA3	Pita_unigene43998	205	22.40 kDa	pH 7.29
PtNF-YA4	Pita_unigene12015	209	22.96 kDa	pH 8.79
PtNF-YA5	Pita_unigene11741	287	30.95 kDa	pH 8.97
PtNF-YA6	Pita_unigene12516	289	31.25 kDa	pH 9.40
PtNF-YA7	Pita_unigene42483	355	38.87 kDa	pH 9.51
PtNF-YA8	Pita_unigene41396	359	38.54 kDa	pH 8.11
PtNF-YA9	Pita_unigene40560	384	41.86 kDa	pH 8.58
PtNF-YB1	Pita_unigene46750	143	16.40 kDa	pH 7.91
PtNF-YB2	Pita_unigene17122	164	18.29 kDa	pH 6.12
PtNF-YB3	Pita_unigene39726	176	19.36 kDa	pH 8.70
PtNF-YB4	Pita_unigene6096	177	20.34 kDa	pH 5.31
PtNF-YB5	Pita_unigene27227	184	20.80 kDa	pH 4.93
PtNF-YB6	Pita_unigene42866	185	20.13 kDa	pH 7.37
PtNF-YB7	Pita_unigene2172	204	22.26 kDa	pH 7.62
PtNF-YB8	Pita_unigene41178	220	23.85 kDa	pH 8.20
PtNF-YB9	Pita_unigene62067	226	24.51 kDa	pH 6.52
PtNF-YC1	Pita_unigene43117	313	35.38 kDa	pH 6.93
PtNF-YC2	Pita_unigene59735	305	34.14 kDa	pH 4.87
PtNF-YC3	Pita_unigene5387	288	32.10 kDa	pH 4.55
PtNF-YC4	Pita_unigene12943	272	30.11 kDa	pH 6.40
PtNF-YC5	Pita_unigene43379	268	30.40 kDa	pH 7.53
PtNF-YC6	Pita_unigene1846	251	28.04 kDa	pH 6.64
PtNF-YC7	Pita_unigene44600	236	27.49 kDa	pH 6.56
PtNF-YC8	Pita_unigene15378	202	22.41 kDa	pH 6.68

Gene name	Gene ID	Amino acid length (a.a)	Protein Mol. Wt.(kDa)	Protein PI value
PtNF-YC9	Pita_unigene16781	142	15.81 kDa	pH 6.51
PtNF-YC10	Pita_unigene34094	136	14.88 kDa	pH 6.79

Conserved Regions And Phylogenetic Relationships Of The PtNF-Ys

To further investigate the conserved regions of PtNF-Ys, the protein sequences of 28 members were analyzed using MEGA7 [36]. Highly conserved domains were found among the members of each subunit as shown in Fig. 1. The NF-Y family should contain an interaction domain for interacting with other NF-Y subunits and a DNA binding domain for recognizing CCAAT binding sites [4]. The core conserved regions of the PtNF-YAs proteins were 55AAs but PtNF-YA4 (Fig. 1A). The NF-YB/C subdomain had 22 AAs and the DNA binding also had 22 AAs. The central domain of PtNF-YBs had 91AAs (Fig. 1B). Among PtNF-YBs, PtNF-YB1 had lower conserved domains than others. The central domain of PtNF-YCs contained 82 AAs for interactions between subunits, but PtNF-YC9 and PtNF-YC10 had no DNA binding domain (Fig. 1C). Meanwhile, PtNF-YC2, PtNF-YC3 and PtNF-YC6 were slightly different from other NF-YCs. The above results suggested that PtNF-YAs were more evolutionarily conservative than other types of PtNF-Y subunits. Furthermore, the distributions of conserved motifs were assessed by MEME software. The results showed that the three PtNF-Y subunits have a unique motif distribution (Fig. 2). Motif 2 and motif 5 were unique to the PtNF-YAs. Motif 8 and motif 9 were present only in PtNF-YBs. Motif 3 was widely distributed in PtNF-Ys.

To predict the functions of PtNF-Y proteins, phylogenetic trees using the NF-Y protein sequences of Arabidopsis and Chinese pine were created by MEGA7 software with the neighbor-joining (NJ) criteria. The phylogenetic analysis showed that the 28 PtNF-Ys were divided into three groups (Fig. 3). The phylogenetic tree suggested close relationships among the candidate NF-Ys within each of the three subfamilies. In the three group, we found that there was only a pair of NF-Y orthologue protein, PtNF-YC6 and AtNF-YC13, suggested the similarity biological functions. Moreover, seven pairs of paralogues were identified: PtNF-YB5 and PtNF-YB4; PtNF-B9 and PtNF-YB7; PtNF-A2 and PtNF-YA3; PtNF-A5 and PtNF-YA6; PtNF-A4 and PtNF-YA7; PtNF-C2 and PtNF-YC3; PtNF-C9 and PtNF-YC10. But, the vast majority of PtNF-Ys had lower identities with other members, implying their diversity during the evolutionary process.

Analysis of cis-elements in the PtNF-Y promoters

To explore the potential function and regulation of *PtNF-Y* genes, the 2000 bp upstream sequence of *PtNF-Y* promoters were analyzed. The results were shown except for the core *cis*-elements, a total of 55 type *cis*-elements were found, that includes 30 light responsive, 10 phytohormones responsive, 8 plant growth, and 7 stress responsive (Fig. 4). Among the *cis*-elements, light- responsive elements accounted for the largest proportion. G-box, box-4, GT1-motif and TCT-motif that were critical elements of light responsive. In plant growth module, the O₂-site related to zein metabolism, CAT-box related to meristem

expression and GCN4_motif related to endosperm expression were mainly detected. Besides, many stress-responsive elements and phytohormone-responsive were also present. For example, ARE related to anaerobic induction, MBS related to drought-inducibility and LTR related to low-temperature responsive were mainly detected. Analysis phytohormone-responsive elements found that AuxRR-core, TGA-element and TGA-box are involved in auxin-responsive, GARE-motif, TATC-box and P-box are involved in gibberellin-responsive, TGACG-motif and CGTCA-motif are involved in MeJA-responsive, ABRE is involved in ABA- responsive. In conclusion, the above results indicate the potential role of *PtNF-Y* genes in response to a variety of hormone regulation.

Expression patterns of *PtNF-Y*s in different development stages of male cones

The pollen in the male cone is allergenic and potentially harmful to the health of allergic people [37]. In addition, pollen also has economic value. So, it is of great significance to clarify the male cones development of conifers. Also, the NUCLEAR FACTOR-Y (NF-Y) families of transcription factors are important regulators in flowering time [10]. To investigate the potential functions of *PtNF-Y* genes, we examined their gene expression profiles (TPM) using RNA-seq of male cones sampled at six different developmental stages (M1–M6) and vegetative buds (VB). Cluster analysis showed that 4 *PtNF-Y* genes (i.e., *A1/B1/B3/C1/C4*) were highly expressed in male cones and 5 *PtNF-Y* genes (i.e., *B4/B5/C1/C7/C8*) were high expression in late stages of male. In contrast, 4 genes (*A4, A7, B8* and *C2*) were in low expression in male cones and 5 genes (*A5, A6, A8, A9* and *B6*) were low expressed in late stages of male (Fig. 5A) .

Furthermore, studies suggest that NF-Y regulation of *FT* is mediated through CONSTANS (CO) [38–40]. We selected *PtTFL2* (no *FT* homologs in conifer) and *PtCO* from the transcriptome sequencing data. The expressions of the *PtTFL2*, the *PtCO*, and the differentially expressed *PtNF-Y*s were determined by RNA-seq (Fig. 5A). The expression profile of *PtCO* was similar to that of *PtNF-YA1, PtNF-YB1, PtNF-YB3, PtNF-YC1* and *PtNF-YC4* in male cones (Fig. 5A, red black). However, the expression pattern of *PtCO* was the opposite of *PtTFL2*. Moreover, the correlation of *PtTFL2, PtCO* and *PtNF-Y*s in expression level are shown in Fig. 5B. *PtCO* showed positively correlation with *PtNF-YC1* ($r = 0.596$), *PtNF-YC4* ($r = 0.505$) and was negatively correlated with *PtTFL2* ($r = -0.881$). Also, *PtTFL2* showed negatively correlation with *PtNF-YC1* ($r = -0.64$) and *PtNF-YC4* ($r = -0.475$) in male cones. But other three *PtNF-Y*s showed no correlation with *PtTFL2* and *PtCO*. Overall, *PtTFL2, PtCO, PtNF-YC1* and *PtNF-YC4* may have regulatory role in conifers flowering that need to be tested.

Previous study has shown that NF-Y can regulate flowering by GA signalling, and DELLA proteins are the key transcriptional regulators that regulate plant development by GA mediating [16]. So transcriptome analysis and yeast two-hybrid assays were performed to confirm if there is a relationship between DPL (DELLA protein-like) and NF-YC1/4. Transcriptome analysis showed no significant changes in the expression of *PtNF-YC1* by gibberellins (GA₃ and GA₄) and paclobutrazol (PAC) treatments. But, *NF-YC4* was down-regulation by PAC treatment (Fig. 6A). However, we found NF-YC1/4 can interact with DPL via yeast two-hybrid assays (Fig. 6B). Also, Bimolecular fluorescence complementation (BiFC) assay showed

that DPL-cYFP and NF-YC1/4-nYFP interacted in the nuclei of living tobacco cells (Fig. 6C). These results support that NF-YC1/4 can interact with DPL. Therefore, we can use NF-YC1/4 as candidate genes to study strobil development and GA signal transduction.

Gene expression analysis of PtNF-Ys in response to hormone treatments

Except for involving in flowering time, the function of *NF-Y* genes were in response to stress response (drought, salt, light and temperature) and hormone treatments (GA and ABA). But, NF-Y in other stresses, such as pests, diseases, and other hormone signals, such as auxin, salicylic acid, jasmonic acid, etc need to be explored. Promoter analysis showed that *PtNF-Y* genes had the potential to participate in ABA, IAA, JA and SA signal transduction. Hence, expression of *PtNF-Y* genes was measured under these hormone treatments. The results showed that *PtNF-Y* genes response to ABA, IAA, JA and SA vary in strength (Fig. 7). The genes that respond positively to SA were in the majority, and in which the *NF-YC* genes had a large proportion, then ABA was followed by. However, a great part of *PtNF-Y* genes were negative response to IAA and JA, and the patterns were similar. Moreover, we found that most *NF-Ys* respond to salicylic acid in contrast to auxin and jasmonic acid. All this suggests that *PtNF-Y* genes were broadly involved in different hormone-responsive.

Discussion

Although NF-Y transcription factors have been broadly studied in several plant species, such as *Arabidopsis thaliana* [41], *Glycine max* [42], *Oryza sativa* [43], *Zea mays* [29], *Triticum aestivum* [44], *Populus tomentosa* [45], *Picea wilsoni* [19]. However, the identification and characterization of *NF-Y* gene family in conifers are still no reports. Here we use *Pinus tabulaeformis*, widely distributed in China, to study the characteristics of NF-Y in conifers. Based on the results obtained from NF-Y transcription factors in *A. thaliana* by using BLAST (blast-2.6.0+) and HMMER, we identified 28 *NF-Y* genes based on the Chinese pine reference transcriptome (Table 2). Compared with the numbers of *NF-Ys*, such as 36 in *Arabidopsis* [33], 25 in castor bean [46], 59 in tomato [47], 32 in grape [48], 33 in walnut [49] and 46 in *Populus trichocarpa* [45] harbored a comparable number of genes. We constructed the phylogenetic tree to analyse NF-Y proteins in Chinese pine and *Arabidopsis*, and some studies suggest that the *Arabidopsis* NF-Y family may not consist of AtNF-YB11/12/13 and AtNF-YC10/11/13 because they do not include the proper structure [41]. Also, *PtNF-YC6*, *PtNF-YC2* and *PtNF-YC3* have a distant evolutionary relationship with the three clusters of NF-YA/B/C which were similar to the six *Arabidopsis* NF-Ys mentioned above. Multiple alignments and our phylogenetic tree can support this opinion (Fig. 1 and Fig. 3).

Previous studies reported that the *Arabidopsis* NF-YB subunits can be divided into two classes, the LEC1-type and the non-LEC1-type [50]. The LEC1-type (AtNF-YB9 and AtNF-YB6) had Asp (D) residues where Lys (K) is found in mammals and most plants, and the aspartate at D55 site is necessary for LEC1 activity in embryogenesis [33]. In our study, *PtNF-YB5*|*Pita unigene27227* and *PtNF-YB4*|*Pita unigene6096* changed from Lys (K) to Asp (D) at this binding site (Fig. 1B). In addition, based on analysis of phylogenetic, *PtNF-YB5*|*Pita unigene27227* and *PtNF-YB4*|*Pita unigene6096* were most likely to be the

two LEC1-type orthologs of AtLEC1 (AtNF-YB9) and AtL1L (AtNF-YB6) that might share similar functions in regulating seed development and embryogenesis and like Arabidopsis (Fig. 3). According to the expression profiles of *NF-Y* genes during the developmental process of male cones, six different developmental stages of male from phenotypic recognition to maturation were collected (Table 2). *PtNF-YB4* and *PtNF-YB5* were only up-regulated in later development of male cones which suggested they may be involve in pollen maturation which tube growth and sperm delivery of conifers are fundamentally different from that of angiosperms [19]. The study found that two conifer LEC1-type *HAP3* genes, *HAP3A* and *HAP3B*, from *Picea abies* and *Pinus sylvestris* were high expression during early embryo development, but decreased during late embryogeny [32]. Also, we found that the sequence of *PtNF-YB4* had high homology (the similarity > 77%) by blast with *PsHAP3A* and *PaHAP3A* and *PtNF-YB5* was the same gene with *PsHAP3A* (Additional file 3) which suggested *PtNF-YB4* and *PtNF-YB5* may also participate embryo development. So, these results gave us the direction to study LEC1-type of NF-YBs in conifers.

NF-Y complexes can bind to CO proteins to activate the photoperiodic pathway and regulate *FT* expression in Arabidopsis [10]. CO accumulates during the day and its expression peaks at dusk; then CO can replace the NF-YA subunit of Arabidopsis to form a CO/NF-YB/NF-YC trimer (NF-CO) complex that promote the *FT* peak expression at dusk [11, 12, 51, 52]. We observed that *PtCO* expression was positively correlated with *PtNF-YC1* and *PtNF-YC4* in male cones, but was negatively correlated with *PtTFL2* (Fig. 5). Because there are no *FT* orthologous gene members in conifer, and the *FT/TFL 1-like* members are functionally repressors, meanwhile perennial characteristics of conifers [53]. The *PtCO*, NF-Ys (*PtNF-YC1* and *PtNF-YC4*) and *PtTFL2* may be have different regulatory mechanisms that compared with Arabidopsis. In addition, the discovery of the Arabidopsis NF-Y complex that can regulate flowering time by developmental signals, such as gibberellin pathway. So, we want to know *PtNF-YC1* and *PtNF-YC4*, that participating in flowering, whether respond to gibberellin. Via yeast two-hybrid assays and bimolecular fluorescence complementation assay, we found that NF-YC1/4 can interact with DPL. But, *PtNF-YC1* was no significant changes by gibberellins (GA₃ and GA₄) and paclobutrazol (PAC) treatments, and maybe it depends on the sampling time. In a word, we found *PtNF-YC1* and *PtNF-YC4* participate in flowering and gibberellin pathway, but further research is needed. Moreover, it provides a good direction whether NF-YC1/4 can regulate flowering time by gibberellin pathway in conifers to be tested.

NF-Y genes are not only involved in flowering time, early seedling development but also have roles in stress responses and hormone signaling [54]. The AtNF-YC (3/4/9) [55], and *CdtNF-YC1* (*Cynodon dactylon* × *Cynodon transvaalensis*) [23], *SINF-YA-L1* (*Solanum pimpinellifolium*) [26] and *ZmNF-YA3* (*Zea mays*) [29] can result in enhanced drought tolerance. Over-expressing *AtNF-YA2* or *AtNF-YC1* displayed enhance tolerance against freezing stress [27, 56]. According to our analysis of *cis*-elements in the *PtNF-Y* promoters (Fig. 4), the promoter regions containing MBS related to drought-inducibility and LTR related to low-temperature responsive implied that *PtNF-Ys* can be involved in the drought and low-temperature pathway. Moreover, we found multiple types of phytohormones-responsive *cis*-elements (ABA, JA, IAA, SA) suggested that *PtNF-Ys* might be involved in drought tolerance, pathogen and pest resistance and it

conformed to the resistance characteristics of some conifers, such as *Pinus tabuliformis*, *Pinus sylvestris* var. *mongolica*, *Pinus thunbergii*. Besides, researches show that salicylic acid can inhibit pathogen growth through repression of the auxin signaling pathway [57], while JA and SA interact with each other in an antagonistic manner [58]. Based on an analysis of gene expression under hormone treatments, *NF-Y* genes can be in response to salicylic acid (SA), abscisic acid (ABA) and jasmonic acid (JA) treatment which were consistent with our analysis of *cis*-elements inference (Fig. 7). Also, many *NF-Y* genes responded positively to salicylic acid, as opposed to auxin and jasmonic acid. Our data suggest that PtNF-Y may control disease resistance by dynamically regulating SA, IAA and JA signaling. This study provided the possibility for further study of novel resistant pathways related to *NF-Y* genes in *Pinus tabuliformis* even for kinds of conifers.

Conclusions

NF-Y transcription factors have been extensively recognized and classified in several plants. Although there have been some studies on NF-Y in conifer trees, most studies have focused on single genes and most family studies have focused on angiosperms. So, this research trend of NF-Y should be extended to conifer trees. Our study, carried out in *Pinus tabuliformis*, a conifer widely distributed in China can pay close attention to current research priorities. 28 *PtNF-Ys* were first identified in conifer trees and their evolutionary, structural were analyzed. Comparison of NF-Ys in Chinese pine and *Arabidopsis* can provide rudimentary understanding on the function between less studied *PtNF-Ys* and its known homologs. Moreover, by analyzing transcriptome data of male development and experimental verification, two candidate genes (*NF-YC1* and *NF-YC4*) were found to be involved in the regulation of conifer flowering and gibberellin. Furthermore, analysis the *cis*-elements combined with the hormone treatment transcriptome indicated the potential role of NF-Y in a class of conifer resistance. According these results, we found some special scientific problems that may contribute to further functional investigation of NF-Y family in conifers.

Methods

Identification of NF-Y family members in *Pinus tabuliformis*

The protein sequences of *NF-Y* genes (10 *NF-YA* genes, 13 *NF-YB* genes, and 13 *NF-YC* genes) in *A.thaliana* were retrieved from the TAIR (<http://www.arabidopsis.org/>) (Additional file 1). These sequences were used to search our *Pinus tabuliformis* transcriptome database (unpublished) with the blastx program in BLAST (blast-2.6.0+) and the E-value cut-off was set as 1e-10. In addition, hidden Markov model (HMM) for the NF-Y genes was constructed using HMMER package version 3.0. The results of the BLAST and HMMER searchers were merged, resulting in 69 candidate *NF-Y* genes in *Pinus tabuliformis*. The incomplete and redundant sequences were omitted. Finally, 28 unigenes were identified (Additional file 2).

Multiple Alignments And Phylogenetic Analysis

Multiple sequence alignments of identified *NF-Ys* in *Pinus tabuliformis* were constructed using ClustalX. The Neighbor-Joining tree was constructed using MEGA7.0.21 software with 1000 bootstrap replications. The phylogenetic tree constructed by MEGA was uploaded to iTOL (<http://itol.embl.de/>) for further editing. Motifs were predicted using MEME software (<http://meme-suite.org/tools/meme>).

Cis-elements of the *PtNF-Y* promoter

The promoter sequences (length, 2 kb) of *PtNF-Ys* were collected from the Genome Database of *Pinus tabuliformis* (has yet to genome annotation and obtained the promoter sequences based on the CDS alignment by blast). The *cis*-elements were analyzed in the PlantCARE program (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Transcriptome Plant Materials And Data Analysis

The seeds of *Pinus tabuliformis* were obtained from a primary clonal seed orchard located in Pingquan City, Hebei Province, China (40°99' N, 118°45' E, 560 m above sea level). The seeds of *Pinus tabuliformis* were sown on sphagnum moss soaked with water and then germinated for 14 days in a growth chamber under conditions of 22 °C 14 h light/10 h dark photoperiod. Then transferred in plastic pots and irrigated weekly according to the hormone types and concentrations in Table 2. After 50 days of treatment, needles were collected, liquid nitrogen quick-frozen, and stored at -80 °C. Furthermore, the male cones development samples for RNA-Seq analysis were collected from individual trees at the botanical gardens in Beijing, China (116°33.91160'E, 40°00.08610'N and 44 m above sea level) and datas were deposited in the NCBI Sequence Read Archive (SRA) under the accession number SRA 056887. The RNA-seq datas (Additional file 4) were shown as heat map by TBtools toolkit [59]. Hierarchical clustering displays the expression profiles and the color scale indicating Normalize expression values. Also all the samples used for RNA-Seq analysis is this study are provided in Table 2.

Table 2
Pinus tabulaeformis sampling for gene expression analysis

Developmental stages			
	Analysis	Time point	Repetitions
Vegetative buds(VB)	RNA-seq	26 September 2012	3
Male cones(M)	RNA-seq	M1 (26 September 2012); M2–M6 (16 March 2013- every 9 d)	3
Hormone treatments			
	Analysis	Hormone concentration	Repetitions
Needles	RNA-seq	Water + ethyl alcohol (CK)	6
	RNA-seq	Abscissic acid (ABA)	10 μ M
	RNA-seq	Auxin (IAA)	10 μ M
	RNA-seq	Jasmonic acid (JA)	30 μ M
	RNA-seq	Salicylic acid (SA)	100 μ M

Yeast Two-hybrid Assay

The coding regions of *NF-YC1*, *NF-YC4* and *DPL* were amplified and cloned into pGBKT7 and pGADT7 (Clontech). Yeast two-hybrid assays were performed using the Yeastmaker Yeast Transformation System 2 (Clontech). Yeast AH109 cells were co-transformed with the specific bait and prey constructs. All yeast transformants were grown on SD/-Trp/-Leu or SD/-Trp/-Leu/-His/-Ade medium for selection or interaction test.

BiFC Analysis

For the bimolecular fluorescence complementation (BiFC) assay, *DPL* gene was cloned into the pSPYCE vector and *NF-YC1/4* were cloned into the pSPYNE [60]. All expression vectors were introduced into *A. tumefaciens* LBA4404. Agrobacteria were incubated, harvested, and resuspended in agroinfiltration buffer (0.2 mM acetosyringone, 10 mM MgCl₂, and 10 mM MES). Agroinfiltration buffer was mixed with an equal volume of the protein mixture and injected into tobacco leaves using a syringe. Seventy two hours after infiltration, images were taken using a Leica TCS SP8 confocal microscope.

Statistical Analysis

The data are statistically described as mean $\pm$ standard deviation ($\pm$ SD) and visualization through GraphPad Prism 7.0. The correlation were analyzed by R 3.6.2.

Abbreviations

ABA: abscisic acid; IAA: auxin; JA: jasmonic acid; SA: salicylic acid; CO: CONSTANS; TFL2: TERMINAL FLOWER2; HMMs: Hidden Markov Models

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data analyzed during this study are included in this published article and its additional files.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

YTG analyzed the data and wrote the manuscript, SHN collected data and samples in the field, WL modified the manuscript. All authors have read and agreed to the published version of the manuscript.

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Additional Files

Additional file 1: Full length sequences of the Arabidopsis.

Additional file 2: CDS sequences and translated amino acid sequences of 28 Chinese pine NF-Ys.

Additional file 3: Sequence alignment of PtNF-YB4, PtNF-YB5, PsHAP3A and PaHAP3A proteins.

Additional file 4: Transcriptome data (TPM) of *PtNF-Ys* in different development stages of male cones and hormone treatments.

Figures

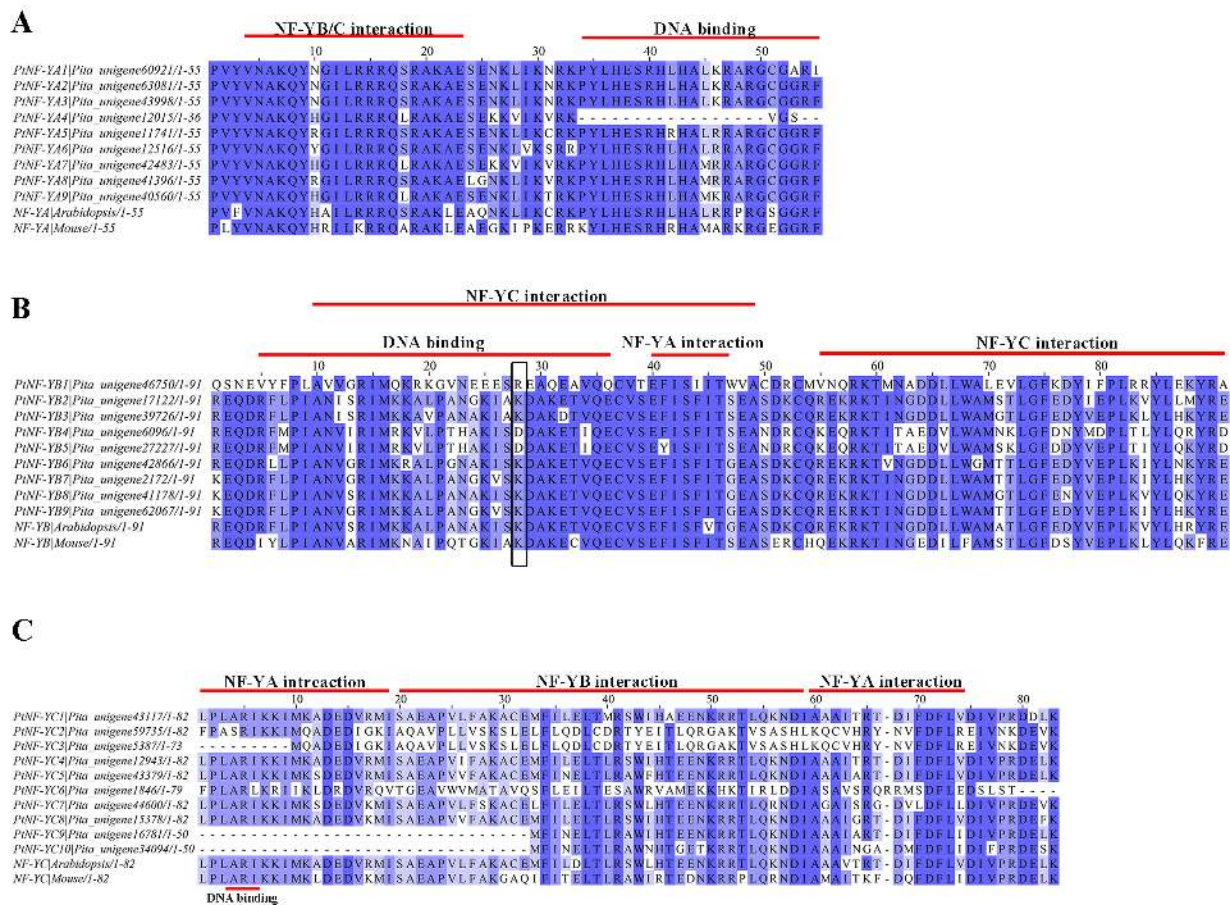


Figure 1

Multiple alignments of *Pinus tabuliformis* 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 are indicated by black box.

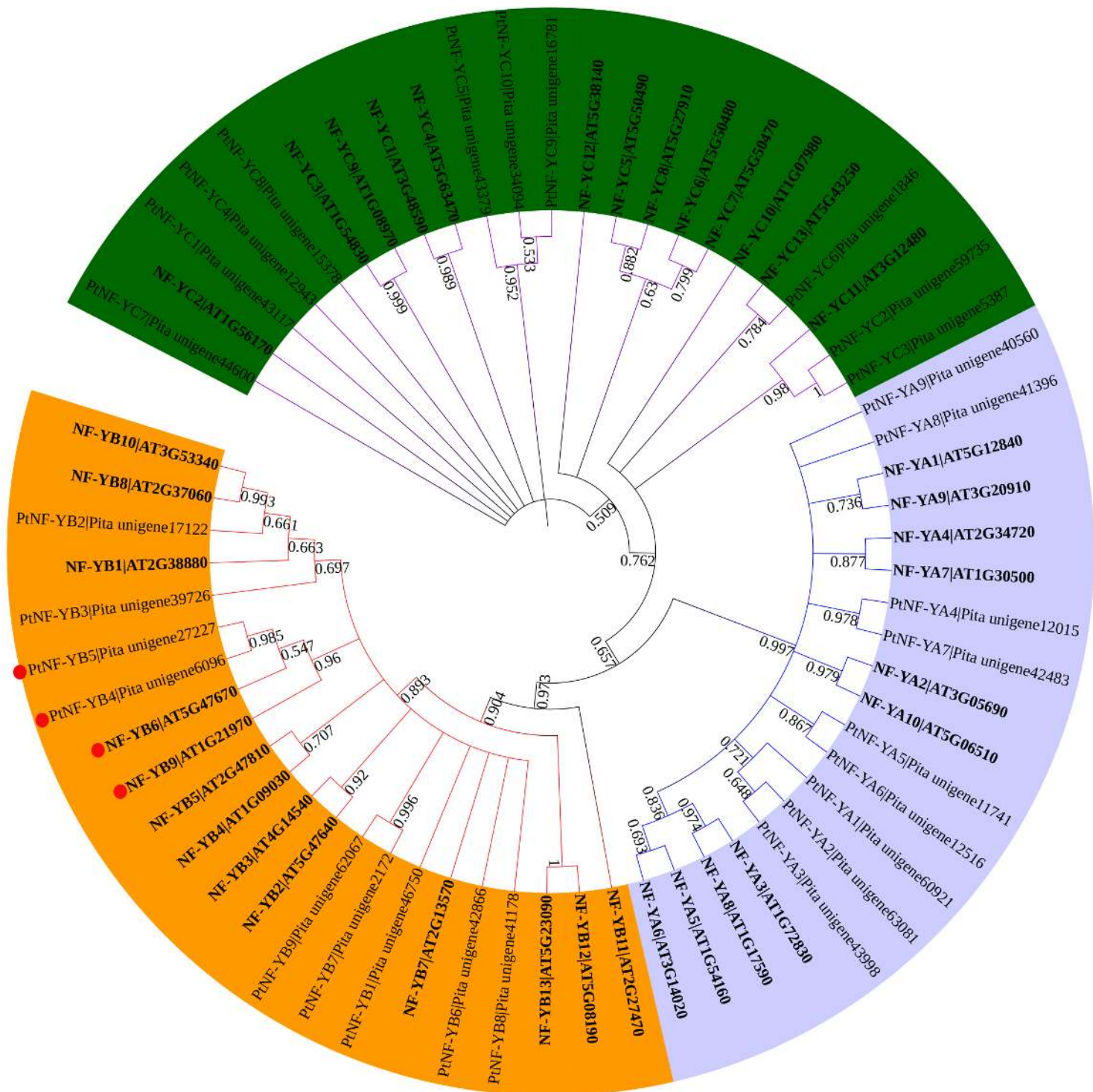


Figure 2

Motif distributions of NF-YA, NF-YB, NF-YC subfamilies in Chinese pine proteins. Ten motifs were identified through MEME tool search and indicated with different colors.

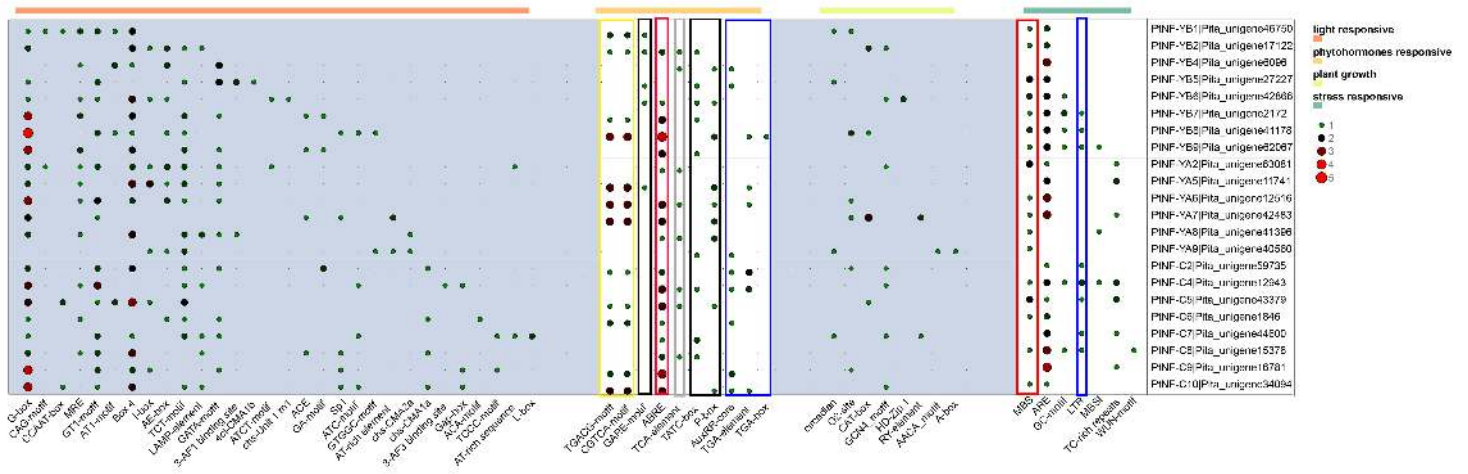


Figure 4

Heat map of cis-elements in the promoter region of PtNF-Ys. Color bars and circle sizes indicate the numbers of cis-elements. Yellow box indicates MeJA-responsive, black box indicates gibberellin-responsive, red box indicates ABA-responsive, gray box indicates SA-responsive and blue box indicates auxin-responsive in phytohormone-responsive module. Red box indicates drought-responsive and blue box indicates low temperature-responsive in stress-responsive module.

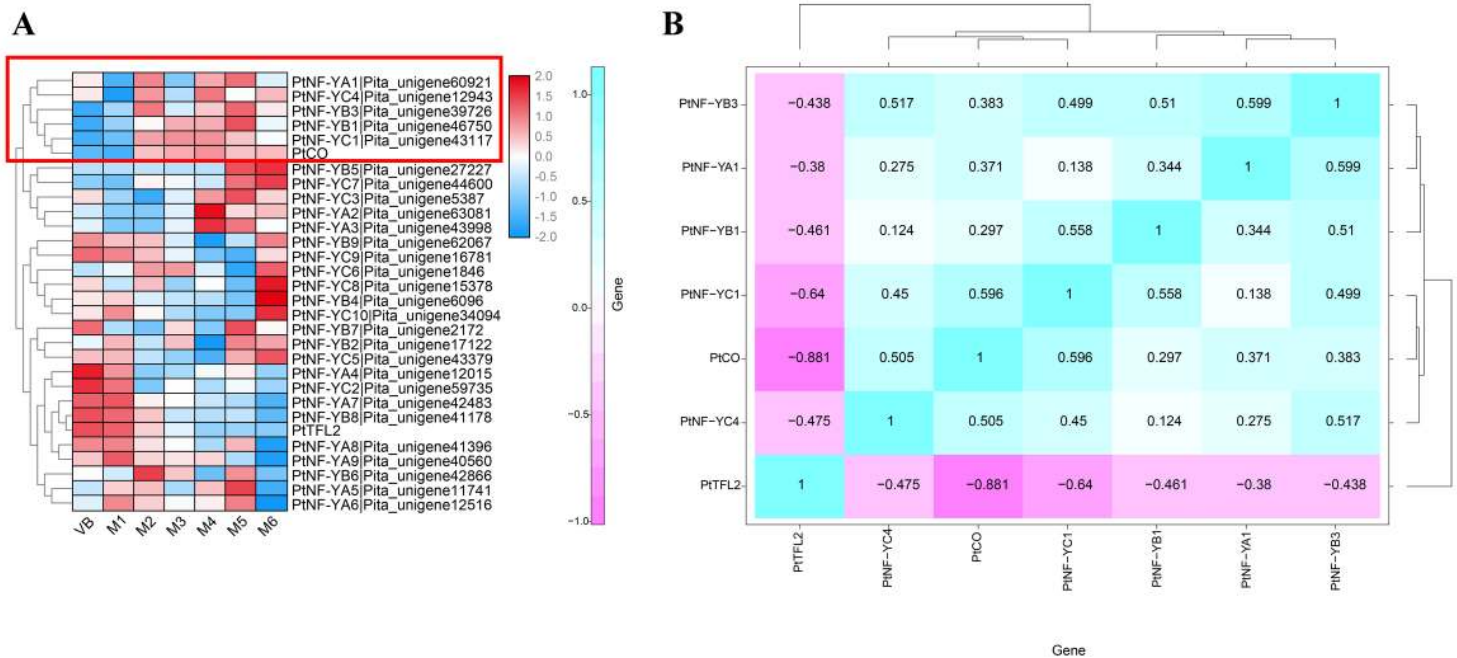


Figure 5

Expression patterns of PtNF-Ys and correlations in expression levels between PtCO, PtTFL2 with PtNF-Ys in different development stages of male cones. (A) Expression patterns of PtNF-Ys. (B) Correlations in expression levels. VB represents vegetative bud, M1-M6 represent male cones sampled at consequential developmental stages. The color scale indicates fold-change values (normalize expression values of

TPM) with red representing increased transcript abundance and blue indicating decreased transcript abundance.

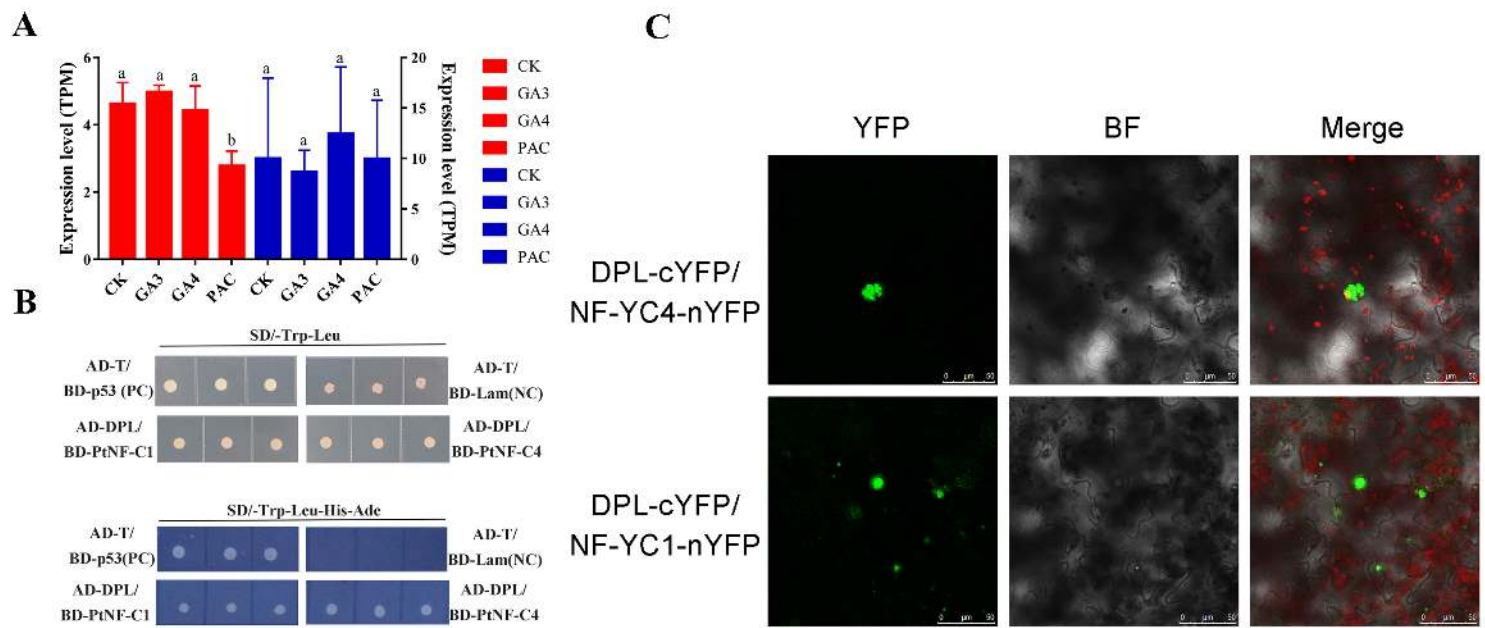


Figure 6

NF-YC1/4 physically interact with DPL in vivo. (A) Transcriptomic analysis of NF-YC1/4 in response to GA and PAC. Red histogram represents NF-YC4 expression level, and blue histogram represents NF-YC1 expression level. (B) Yeast two-hybrid assays show the interactions between NF-YC1/4 and DPL. Transformed yeast cells were grown on SD/-Trp/-Leu/-His/-Ade and SD/-Trp/-Leu medium. (C) BiFC analysis of the interactions a between NF-YC1/4 and DPL in tobacco epidermal cells. YFP, fluorescence of yellow fluorescent protein; BF, bright field; Merge, merge of YFP and bright field. Scale bar, 50 μ m.

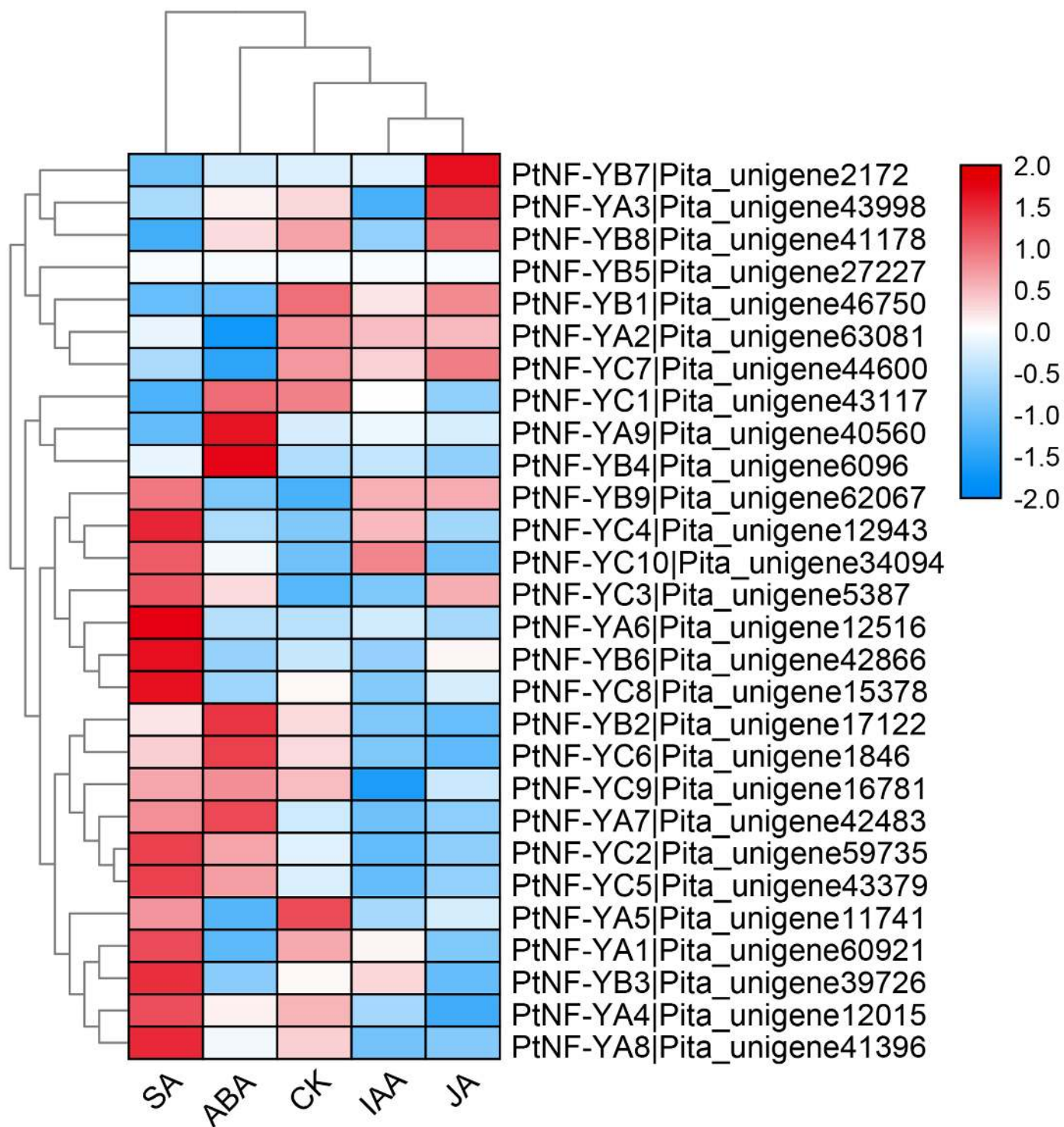


Figure 7

Expression patterns of PtNF-Ys in response to hormone treatments. ABA, IAA, JA, SA treatments of needles in Chinese pine are shown. The color scale indicates fold-change values (normalized expression values of TPM) with red representing increased transcript abundance and blue indicating decreased transcript abundance.

Supplementary Files

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