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Genome-wide identification and analysis of the NF-Y transcription factor family reveal its potential roles in tobacco (*Nicotiana tabacum* L.)

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ABSTRACT

Nuclear Factor Y (NF-Y) represents a group of transcription factors commonly present in higher eukaryotes, typically consisting of three subunits: NF-YA, NF-YB, and NF-YC. They play crucial roles in the embryonic development, photosynthesis, flowering, abiotic stress responses, and other essential processes in plants. To better understand the genome-wide NF-Y domain-containing proteins, the protein physicochemical properties, chromosomal localization, synteny, phylogenetic relationships, genomic structure, promoter *cis*-elements, and protein interaction network of NtNF-Ys in tobacco (*Nicotiana tabacum* L.) were systematically analyzed. In this study, we identified 58 NtNF-Ys in tobacco, respectively, and divided into three subfamilies corresponding to their phylogenetic relationships. Their tissue specificity and expression pattern analyses for leaf development, drought and saline-alkali stress, and ABA response were carried out using RNA-seq or qRT-PCR. These findings illuminate the role of NtNF-Ys in regulating plant leaf development, drought and saline-alkali stress tolerance, and ABA response. This study offers new insights to enhance our understanding of the roles of NtNF-Ys and identify potential genes involved in leaf development, as well as drought and saline-alkali stress tolerance of plants.

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Introduction

In nature, plants are constantly affected by various non-living factors, impacting their growth and development and causing significant losses in yield and biodiversity.[1,2] Abiotic stress refers to adverse environmental factors, such as drought, saline-alkali, extreme temperatures, and so on.[3] In the process of long-term evolution, plants have formed complex regulatory networks, including the process of signal perception, transduction, and amplification, in order to reduce the harm of extreme environments. With the development of plant genetics and crop breeding, the in-depth analysis of the molecular network of plant perception and response to drought, saline-alkali, and high/low temperature stresses is of great significance for improving crop breeding to cope with the ever-changing natural climate.

Nuclear factor Y is also known as CCAAT-binding factor family (NF-Y; CBF), which is a ubiquitous transcription factor in higher eukaryotes.[4–6] The nuclear factor Y family is mainly composed of three subunits: NF-YA (CBF-B; HAP2), NF-YB (CBF-A; HAP3), and NF-YC (CBF-C; HAP5). As that revealed in yeast and animals, the NF-YA, NF-YB, and NF-YC subunits can form a functional NF-Y TF, which can recognize and bind the CCAAT box promoter element to regulate downstream genes.[7] For example, the NF-YA2/YB3/YC10 complex plays a vital role in heat shock and drought stress responses in *Arabidopsis*.[8]

Scientific studies have shown that the NF-Y TFs are important regulators of plant growth and development,[9] including seed development,[10] embryonic development,[11] flowering time,[12] root development,[13] stress response,[14,15] and signaling pathways of phytohormones such as IAA, GA, and ABA.[16,17] Although several NF-Y proteins, such as AtNF-YB2, AtNF-YB3, ZmNF-YA3, and OsNF-YC5, have been reported to mediate abiotic stress responses in plants such as *Arabidopsis*, maize, and rice,[18–20] the potential biological functions and regulatory mechanisms of NF-Y proteins in the drought response need to be further explored.

Tobacco (*Nicotiana tabacum* L.) features highly efficient genetic transformation and regeneration systems, making it a pivotal model plant in the fields of plant biology research and biotechnology applications. In recent years, tobacco is widely used to study genes functional diversity and create new germplasms. Nevertheless, the potential biological function and molecular mechanism of NtNF-Ys in tobacco remain largely unknown and need further study.

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Materials and methods

Identification of NtNF-Ys

All tobacco protein sequences were extracted from the tobacco genome downloaded from http://lifenglab.hzau.edu.cn/Nicomics/.[21] We used the different screening strategies to correctly identify all members of the tobacco NF-Y family members. Firstly, we obtained all AtNF-Y protein sequences (AtNF-YAs, AtNF-YBs, and AtNF-YCs) through the *Arabidopsis* genome database (https://www.arabidopsis.org/), which were used as decoys to retrieve the tobacco genome database at the genome-wide level using BLASTP with an E-value ($\leq 1 \times 10^{-5}$) and an identity match (≥50%) as thresholds of TBtools.[22] Subsequently, we surveyed all tobacco NF-Y proteins via the Hidden Markov Model profiles (E value $\leq 1 \times 10^{-5}$) of the specific NF-Y conserved domain (PF02045 and PF00808), which were extracted from the Pfam database (http://pfam.xfam.org/).[23] Finally, all candidate genes were checked using SMART (http://smart.embl-heidelberg.de/) and Conserved Domains Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).[24]

Chromosomal location and identification of homologous genes

Based on the information of chromosomal locations provided by the tobacco genome database (http://lifenglab.hzau.edu.cn/Nicomics/.), TBtools-II software was employed to generate the chromosomal locations map and synteny analysis of the *NtNF-Ys*.[25]

Property prediction of NtNF-Ys

The information about each NtNF-Ys, including the molecular weight (MW), isoelectric point (pI), and the average value of hydrophilicity (GRAVY), was calculated using the ProtProm tool on the Expasy server (https://www.expasy.org/).[26] The Plant-mPLoc website was employed to predict the subcellular localization (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/).[27] Using the AlphaFold 3 model (https://alphafoldserver.com/) and PyMOL software to predict the three-dimensional structure of NtNF-Ys.[28]

Phylogenetic analysis of NF-Ys

The alignment of the NF-Y protein sequences of different species (https://www.ncbi.nlm.nih.gov/.),[29] including *Nicotiana tabacum*, *Arabidopsis thaliana*, and *Oryza sativa*, were carried out using the MUSCLE algorithm, and the results were imported into the MEGA 11 software for the construction of a phylogenetic tree using the Neighbor-Joining method with the Poission model with uniform rates. The phylogeny test was statistically supported by 1000 bootstrap replications to provide the probability of each branch's formation. The phylogenetic tree was further optimized through the iTOL online tool (https://itol.embl.de/) and Adobe Illustrator software.[30,31]

Domain identification and conserved motifs analysis of NtNF-Ys

The conserved motifs of NtNF-Ys were analyzed using the MEME online tool (https://meme-suite.org/meme/tools/meme).[32] The number of motifs was set to search for 20 motifs. In addition, using the Batch CD-Search tool, we identified the conserved domains of NtNF-Ys (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi).[33] The visual tools are TBtools-II and Adobe Illustrator software.

Motif and structural analyses of NtNF-Ys

The exon – intron structure was generated using TBtools-II software based on the CDS and genomic sequences of *NtNF-Ys*.[34] The *cis*-elements in the 2000 bp promoter regions of *NtNF-Ys* extracted from the tobacco genome were predicted using the online website PlantCARE and TBtools-II software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).[35]

Protein interaction network of NtNF-Ys

The protein interaction network of the NtNF-Ys was predicted using STRING (https://cn.string-db.org/) based on *Nicotiana tabacum* proteins.[36] The network map was built using Cytoscape 3.10.0 and Adobe Illustrator software.[37]

Transcriptome analysis

The RNA-seq data of *NtNF-Ys* in different tissue of tobacco were obtained from the NCBI SRA repository (PRJNA208209). In addition, the RNA-seq data used for expression analysis of *NtNF-Ys* in different developmental stages in tobacco were obtained at our laboratory. Finally, the RNA-seq data of *NtNF-Ys* in tobacco under 200 mm mannitol (PRJNA883680), 100 mm NaCl (PRJNA532660), 100 mm $NaHCO_3$ (PRJNA532660), and 0.1 mm ABA spraying (PRJNA684346) treatment were downloaded from the NCBI SRA repository.[38–40] The process of transcriptome analysis was conducted according to previous studies.[41,42]

qRT-PCR analysis of NtNF-Ys

The cultivated tobacco (*Nicotiana tabacum* L.) cultivar 'HongHuaDaJinYuan (HD)' was grown in a substrate consisting of a 1:1 mixture of peat soil and vermiculite and was cultivated in a greenhouse at the Tobacco Research Institute of the Chinese Academy of Agricultural Sciences, Qingdao, China. The greenhouse conditions included a light intensity of 400 μmol $m^{-2} \cdot s^{-1}$, a temperature regime of 25°C during the light period and 23°C during the dark period, a photoperiod of 16 h of light and 8 h of darkness, and a relative humidity of 65%. After 4 weeks of growth, healthy tobacco seedlings of the same size were grown on ½MS medium under 200 mm mannitol, 100 mm NaCl, 100 mm $NaHCO_3$, and 0.1 mm ABA spraying treatments for 0, 0.5, 1, 1.5, 3, 6, 12, 24, 48 h (h). Three independent biological replicates were taken. The total RNA of tobacco was extracted using the TRIzol Reagent

(CWBIO, Beijing, China). In order to synthesize cDNA, the RNA samples were used as templates with the M-MLV Reverse Transcriptase Kit (Takara Bio, Shiga, Japan). RT-qPCR was performed in a 20 μL reaction system containing 10 μL of 2 × UltraSYBR Mixture (CWBIO) and 0.4 μM of forward and reverse primers, to assess the expression of target genes. The reactions were incubated in a Rotor-Gene Q Machine (Qiagen) for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The tobacco *NtACTIN* gene served as an internal control (Table S2). Relative expression levels were measured using the $2^{-\triangle\triangle Ct}$ method.

Results

Identification and characterization of NtNF-Ys

In this study, to comprehensively and accurately identify all *NF-YA* genes in tobacco, three common strategies were employed based on the published genes in *Arabidopsis* (*AtNF-YAs*, *AtNF-YBs*, and *AtNF-YCs*). Then, a total of 58 *NtNF-Y* genes, including 23 *NtNF-YAs*, 25 *NtNF-YBs*, and 10 *NtNF-YCs*, were identified from the Tobacco Genome Database.[21] Furtherly, we analyzed their physicochemical properties (S 1). The genomic length of the 58 *NtNF-Ys* showed a wide distribution, ranging from 366 bp (Nta23g00810.1) to 17,715 bp (Nta24g11400.1). Meanwhile, the coding sequences (CDS) length of the 58 *NtNF-Ys* exhibited a wide distribution, ranging from 366 bp (Nta23g00810.1) to 1032 bp (Nta03g16330.1), encoding 133–344 amino acids, with the molecular weight (MW) varying from 13.4 kDa to 39.42 kDa. The isoelectric point (pI) of the 58 NtNF-Ys ranged from 4.62 (Nta01g13780.1) to 10.58 (Nta24g09230.1), and the grand average of hydropathicity (GRAVY) ranged from −1.096 (Nta01g26140.1) to −0.075 (Nta23g00810.1), suggesting that they are hydrophilic proteins. The findings of subcellular localization predictions reveal that most of NtNF-Ys are localized to the nucleus except four NtNF-Ys (Nta01g24520.1, Nta05g20020.1, Nta06g18140.1, and Nta12g17940.1), which were localized to the nucleus and cytoplasm (Table 1). The diversity of three-dimensional structure of NtNF-Ys indicated the NtNF-Ys might have functional redundancy and sub/neo-functionalization in tobacco (Fig. S1).

Chromosome location and synteny analysis of NtNF-Ys

Among the 58 NF-Y proteins in tobacco, 56 NF-Y genes exhibited a random distribution across 23 chromosomes, with each chromosome harboring between one and five genes, and the remaining two genes were located on 2 scaffolds. Specifically, chromosome 12 had the largest number of genes ($n = 5$) and Chromosomes 2, 3, 17, and 18 had four genes each (Figure 1a).

According to previous studies, segmental and tandem duplications are the two main ways of gene family expansion.[43,44] In order to understand the expansion mode of *NtNF-Ys* in tobacco, the synteny analysis of *NtNF-Ys* was performed. Using TBtools-II, the genome duplication events within *Nicotiana tabacum* were investigated. A total of 43 collinear pairs in Nt-Nt were identified (Figure 1b). Furtherly, the 43 gene pairs were analyzed by calculating the non-synonymous (*Ka*) and synonymous (*Ks*). These findings show that the substitution ratios (*Ka*/*Ks*) of all gene pairs are less than 1, demonstrating that these genes are purified during evolution (Table S1).

Phylogenetic relationship of NtNF-Ys

To further study the evolutionary relationship of NtNF-Ys in *Nicotiana tabacum*, *Arabidopsis thaliana*, and *Oryza sativa*, we used the MEGA 11 software to generate a phylogenetic tree for 128 NF-Ys from the four species (58 in *Nicotiana tabacum*, 36 in *Arabidopsis thaliana*, and 34 in *Oryza sativa*) based on their protein sequences. Based on the evolutionary divergence, the phylogenetic tree was categorized into three branches corresponding to three different subfamilies (NF-YAs, NF-YBs, and NF-YCs), respectively (Figure 2). All NF-Ys were grouped together with their respective orthologs, indicating that the evolutionary relationships of the NF-Ys have been relatively conserved across various species.

Motif and structural analyses of NtNF-Ys

To study *NtNF-Ys* structure, we analyzed the conserved motif by the MEME online website, and 20 conserved motifs were identified (Figure 3a; S2). The results indicate that different subfamilies (NtNF-YAs, NtNF-YBs, and NtNF-YCs) have different motif compositions, such NtNF-YA (containing motif 11, 6, 7, 5, 2, 17, 20, 10, 15, 18, 16, and 12), NtNF-YB (containing motif 19, 3, 1, 4, and 13), and NtNF-YC (containing motif 3, 1, 8, 9, 14, and 17).

To analyze the conserved domains in the different NtNF-Y subfamilies, we used the Batch CD-Search tool to identify the conserved domains of NtNF-Ys. The results were similar to conserved motifs, where there were different domains in different subfamilies with different functions (Figure 3b). For example, the NtNF-YAs have one domain for DNA binding and another domain for protein interacting with NF-YB/C.

The genomic distributions of *NtNF-Ys* were analyzed to better understand the evolution of the *NtNF-Y* family. The results included that the number of exons in *NtNF-YAs* was the most, followed by *NtNF-YCs*, and most of *NtNF-YBs* was the least (Figure 3c). The number of exons among each member exhibited differences, indicating that the function of *NtNF-Y* family genes might have become more intricate during the process of evolution.

Cis-acting elements in NtNF-Ys

Generally, the distribution of gene promoter elements correlates with gene function (plant development, hormone regulation, and stress response)[45,46] to explore the potential biological functions of *NtNF-Ys*, we analyzed the *cis*-element in the promoters of *NtNF-Ys*. Based on the PlantCARE website's predictions, the *cis*-elements in *NtNF-Y* promoter regions were systematically segmented into six distinct components: core/binding, abiotic/biotic, development, hormone, light, and other unknown elements (Figure 4). Most of *NtNF-Ys* contained the core/binding elements (CAAT-box and TATA-box) except six *NtNF-Y* genes

Table 1. Characterization of *NtNF-Ys* in tobacco.

Gene ID	Chr	Chr. Location	CDS /bp	Protein Size/aa	MW /kDa	PI	GRAVY	Subcellular Location
Nta01g13780.1	1	48578034–48578417	384	128	14.37	4.62	−0.747	Nucleus
Nta01g26140.1	1	137771583–137771990	408	136	15.43	5.09	−1.096	Nucleus
Nta01g24520.1	1	122199509–122204000	774	258	28.39	6.3	−0.465	Cytoplasm; Nucleus
Nta02g01570.1	2	6651507–6657474	750	250	27.55	9.28	−0.888	Nucleus
Nta02g04480.1	2	35428509–35436959	903	301	33.03	10.11	−0.538	Nucleus
Nta02g09650.1	2	62362715–62363197	483	161	18.2	6.54	−0.917	Nucleus
Nta02g26300.1	2	132673885–132674265	381	127	14.32	4.52	−0.76	Nucleus
Nta03g05210.1	3	43429041–43433205	477	159	18.05	9.41	−0.825	Nucleus
Nta03g22790.1	3	162598544–162606246	630	210	23.15	7.84	−0.893	Nucleus
Nta03g00250.1	3	541741–542244	504	168	19.05	6.13	−0.715	Nucleus
Nta03g16330.1	3	125420496–125428054	1032	344	39.42	4.65	−0.534	Nucleus
Nta04g27850.1	4	137521912–137526592	531	177	19.67	9.62	−0.813	Nucleus
Nta04g00730.1	4	2883918–2884737	528	176	19.85	7.04	−0.718	Nucleus
Nta05g20020.1	5	174888308–174895111	693	231	25.19	4.85	−0.44	Cytoplasm; Nucleus
Nta06g18140.1	6	115546516–115551580	693	231	25.18	4.86	−0.429	Cytoplasm; Nucleus
Nta07g04020.1	7	15983378–15991132	498	166	17.95	5.33	−0.66	Nucleus
Nta09g08870.1	9	43220518–43221138	621	207	22.93	7.5	−0.705	Nucleus
Nta09g11430.1	9	68087251–68103166	498	166	17.79	5.34	−0.603	Nucleus
Nta09g18860.1	9	143250798–143251526	729	243	27.18	6.92	−0.894	Nucleus
Nta10g06850.1	10	25581369–25581980	612	204	22.66	8.21	−0.748	Nucleus
Nta10g09130.1	10	38701161–38713741	492	164	17.56	5.98	−0.615	Nucleus
Nta10g15940.1	10	105030993–105031697	705	235	26.23	6.95	−0.833	Nucleus
Nta11g28600.1	11	202222882–202227783	756	252	28.12	9.62	−0.822	Nucleus
Nta11g27510.1	11	196738748–196739420	651	217	22.98	6.63	−0.611	Nucleus
Nta11g19510.1	11	127005401–127009034	792	264	29.06	6.63	−0.409	Nucleus
Nta12g21910.1	12	124064872–124072975	912	304	33.79	9.78	−1.031	Nucleus
Nta12g31310.1	12	193904280–193911737	933	311	33.99	9.63	−0.511	Nucleus
Nta12g34600.1	12	225870865–225877437	738	246	27.16	8.77	−0.827	Nucleus
Nta12g28040.1	12	168514871–168515356	486	162	18.17	8.31	−0.898	Nucleus
Nta12g17940.1	12	81908969–81913341	693	231	25.25	5.01	−0.464	Cytoplasm; Nucleus
Nta13g03220.1	13	15625116–15633998	909	303	33.05	7.09	−0.984	Nucleus
Nta14g03090.1	14	11169915–11178474	909	303	33.09	6.68	−1.019	Nucleus
Nta15g09830.1	15	50519983–50525273	978	326	35.66	9.71	−0.59	Nucleus
Nta16g09500.1	16	46967172–46973120	978	326	35.86	8.91	−0.629	Nucleus
Nta16g22470.1	16	140231206–140238793	498	166	18	5.08	−0.685	Nucleus
Nta16g24790.1	16	148921712–148922368	657	219	24.66	5.49	−0.525	Nucleus
Nta17g24960.1	17	167407584–167413004	945	315	34.85	9.59	−0.606	Nucleus
Nta17g02850.1	17	13538629–13544670	543	181	19.49	6.79	−0.817	Nucleus
Nta17g12300.1	17	88882955–88883512	558	186	20.67	6.63	−0.632	Nucleus
Nta17g12350.1	17	89370514–89371062	549	183	20.2	5.1	−0.714	Nucleus
Nta18g23510.1	18	129281303–129287100	954	318	35.14	9.5	−0.573	Nucleus
Nta18g02800.1	18	13329935–13336252	543	181	19.51	6.79	−0.821	Nucleus
Nta18g11380.1	18	76701524–76704296	669	223	24.92	6.97	−0.745	Nucleus
Nta18g11460.1	18	77028619–77029074	456	152	17.16	5.07	−0.668	Nucleus
Nta19g05550.1	19	26763773–26772389	984	328	36.78	8.44	−0.724	Nucleus
Nta20g05760.1	20	24403505–24408392	813	271	30.14	9.91	−0.794	Nucleus
Nta21g01930.1	21	7281641–7282585	945	315	34.77	9.72	−0.62	Nucleus
Nta21g14490.1	21	96670654–96675868	756	252	28	9.88	−0.855	Nucleus
Nta22g12560.1	22	87330597–87331004	408	136	15.23	5.07	−0.98	Nucleus
Nta22g10880.1	22	78991269–78996109	777	259	28.44	6.3	−0.462	Nucleus
Nta23g11340.1	23	85686686–85701607	813	271	30.06	9.33	−0.685	Nucleus
Nta23g00810.1	23	2746202–2746567	366	122	13.4	8.22	−0.075	Nucleus
Nta23g09720.1	23	70458937–70459353	417	139	15.36	10.4	−0.617	Nucleus
Nta24g11400.1	24	87181317–87199031	672	224	24.93	10.24	−0.838	Nucleus
Nta24g17500.1	24	119563090–119569354	846	282	31.62	8.31	−0.923	Nucleus
Nta24g09230.1	24	70610850–70611431	462	154	16.91	10.58	−0.346	Nucleus
Nta00g11500.1	50	352475–355783	723	241	26.54	5.33	−0.639	Nucleus
Nta00g03900.1	243	14888–19251	909	303	33.55	9.32	−0.968	Nucleus

50 and 243, scaffold50 and scaffold243; CDS, coding sequence; MW, molecular weight; pI, isoelectric point; GRAVY, grand average of hydropathicity.

(Nta02g09650.1, Nta12g31310.1, Nta12g34600.1, Nta13g03220.1, Nta24g11400.1, and Nta24g09230.1). In addition, the abiotic/biotic elements MYB, MYC, and STRE existed in most of the *NtNF-Ys* promoters,[47–49] meanwhile, a few of the *NtNF-Ys* promoters contained other abiotic/biotic elements (ARE, as-1, MBS, and so on). Most of the promoter regions of *NtNF-Ys* contained at least one development elements; however, no development-related elements were found in eight *NtNF-Ys*. Among the hormone-related elements, the number of abscisic acid response element (ABRE) was the most, suggesting most of the *NtNF-Ys* might be associated with ABA response.[50,51] Moreover, light-responsive elements such as the Box 4, G-box, and GT1-motif were found in the promoters of *NtNF-Ys* (Figure 4). The above results reveal that *NtNF-Ys* play key roles in plant development, hormone signaling, abiotic stress tolerance, and other essential processes in plants.

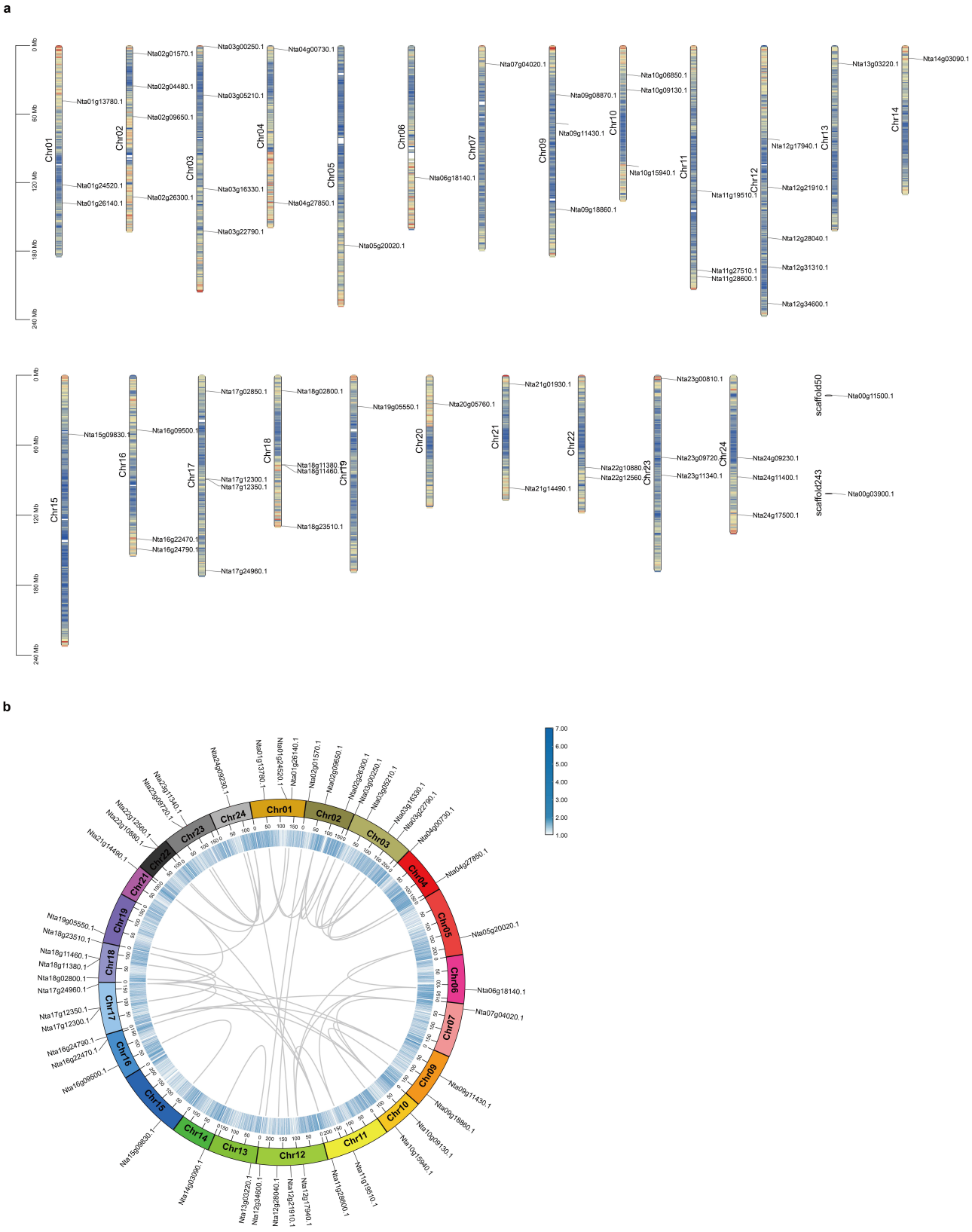

Figure 1. Chromosome location and synteny analysis of *NtNF-Ys*. (a) Chromosomal location of *NtNF-Ys*. The bars represent chromosome. The chromosome members are displayed on the left side, and the gene names are displayed on the right side. (b) Synteny analysis of *NtNF-Ys*. Different chromosomes are shown in different colors. The approximate positions of *NtNF-Ys* are marked with black lines on the chromosomes. Gray curves denote the syntenic relationships within *nicotiana tabacum*.

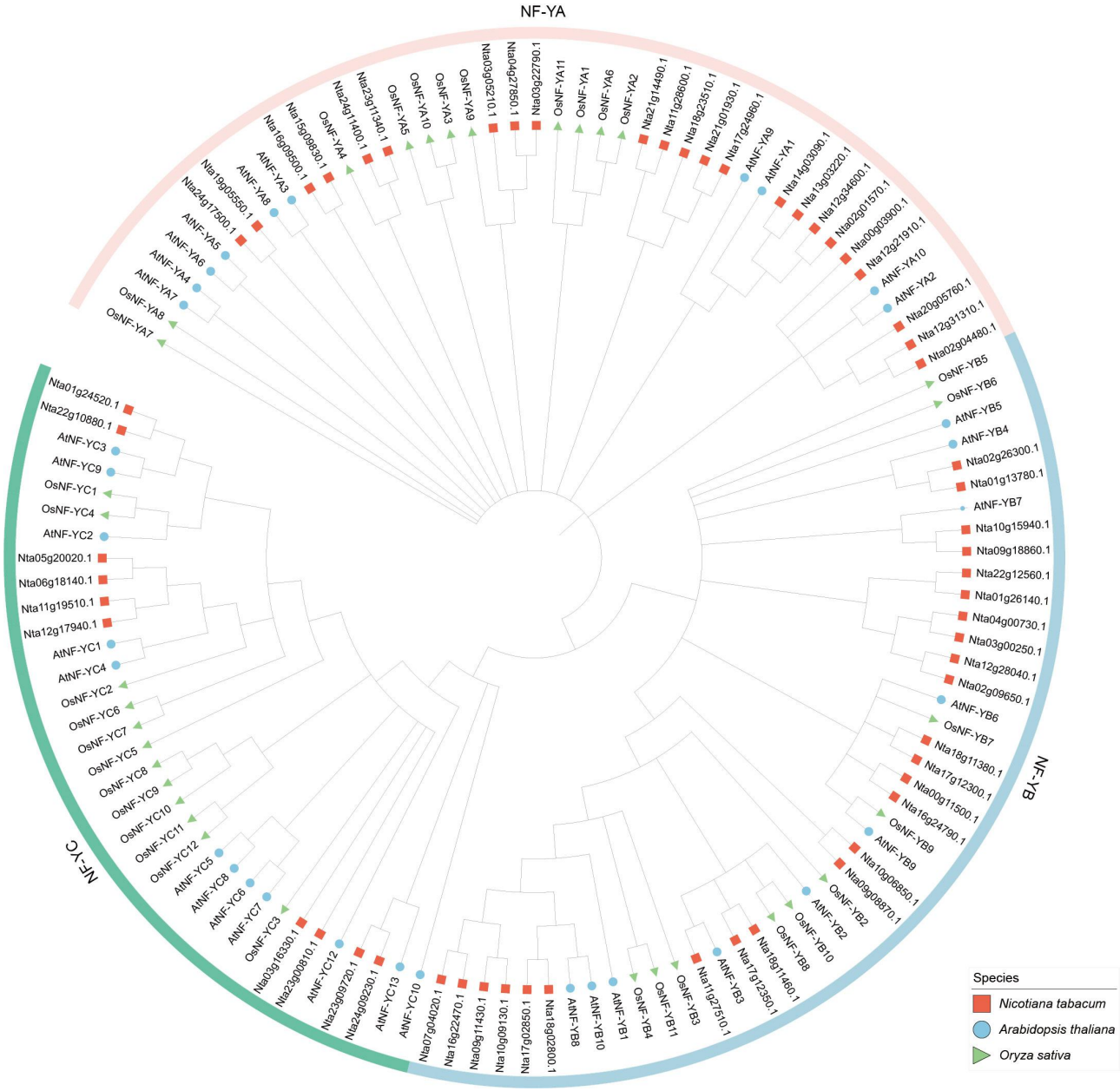

Figure 2. Phylogenetic analysis of the NF-Y proteins in *Nicotiana tabacum*, *Arabidopsis thaliana*, and *Oryza sativa*. NF-Ys were divided into three subfamilies (NF-YAs, NF-YBs, and NF-YCs) according to their subunits. The red squares, blue circles, and green triangle represent the NF-Ys in *Nicotiana tabacum*, *Arabidopsis thaliana*, and *Oryza sativa*, respectively.

Protein interaction network of NtNF-Ys

We constructed an interaction network for NtNF-Ys using *Nicotiana tabacum* proteins to investigate their potential regulatory network (Figure 5). NF-YA, NF-YB, and NF-YC usually modulate the expression of downstream genes through the formation of heterotrimeric complexes.[52] Obviously, NtNF-Ys could interact with other NF-Ys. Moreover, NtNF-Ys can interact with the master regulators of seed maturation (LEC2, FUS3, ABI3, and DPBF2)[53,54] embryogenesis-related proteins (AGL15 and RKD4)[55,56] stress response-associated proteins (PKL, bZIP28, and WRI1),[57–59] flower development-related protein RGL2,[60] grain quality-related protein bHLH144,[61,62] and so on (Figure 5). These findings suggest that NtNF-Ys are crucial in modulating growth, development, and stress tolerance in plants.

Tissue-specific expression analysis of NtNF-Ys

For the purpose of exploring the biological functions of *NtNF-Ys* in tobacco plants, the expression of *NtNF-Ys* was examined in nine representative tobacco tissues (i.e. root, stem, young, mature, and senescent leaves, young, mature, and senescent flowers, and dry caps) using RNA-seq data, which was extracted from the NCBI SRA repository. All members of the *NtNF-Ys*

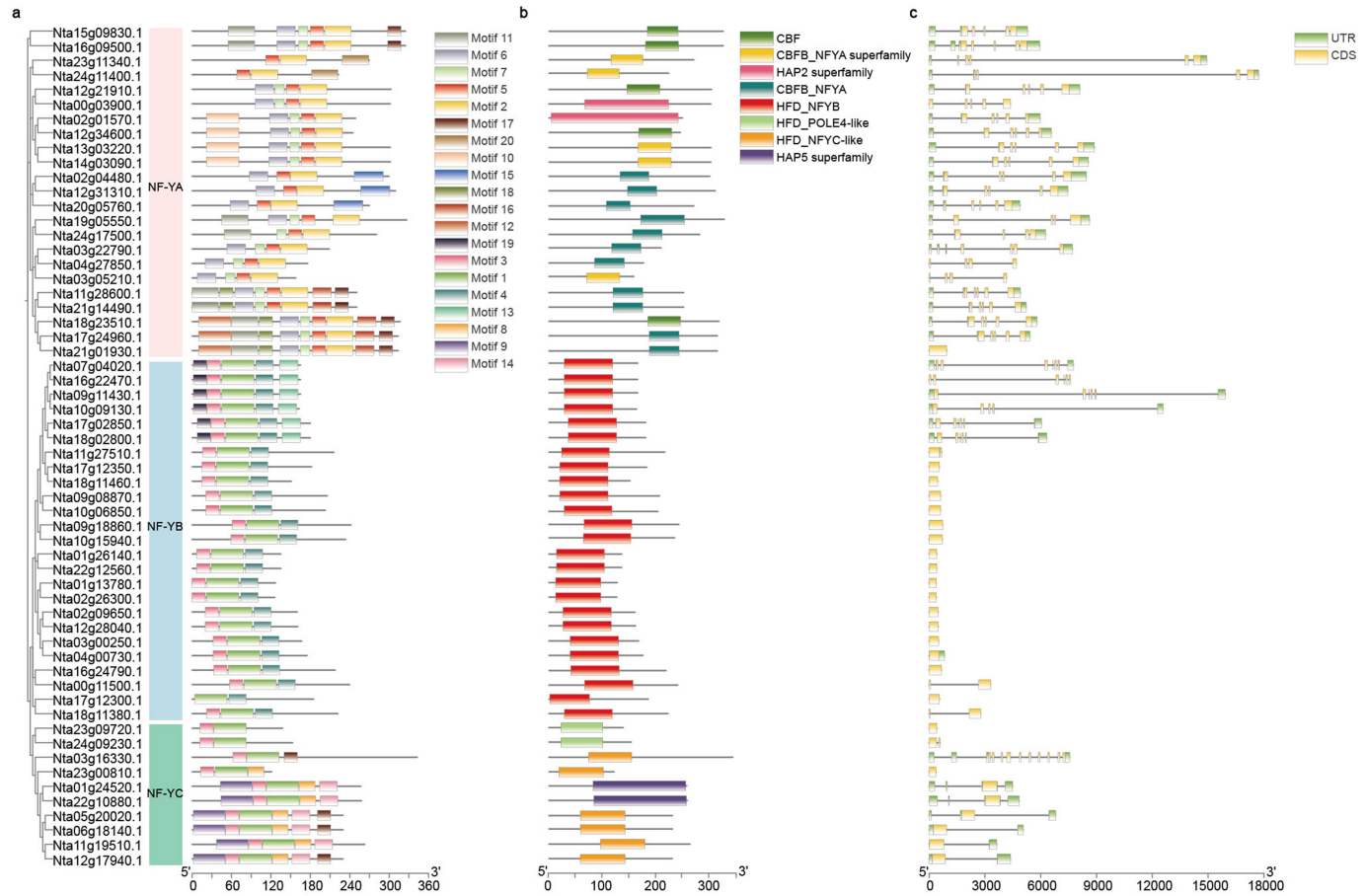

Figure 3. Conserved motifs and exon–intron structure analysis of NtNF-Ys. (a) The phylogenetic tree shows that the NtNF-Ys are distributed in three subfamilies (NF-YA, NF-YB, and NF-YC), which indicated by pink, blue, and green, respectively. Different colors boxes represent different motifs. (b) Conserved domain structures of *NtNF-Ys*. Different domains are shown using boxes with different colors. (c) Exon–intron structures of *NtNF-Ys*. The green and yellow boxes indicate UTR and CDS, respectively.

subfamily, except Nta03g00250.1 and Nta23g00810.1, were expressed at 1 to 9 in all tissues (Figure 6). Among all the *NtNF-Ys*, 10 *NtNF-Ys*, including Nta01g24520.1, Nta11g19510.1, Nta11g27510.1, Nta12g21910.1, Nta13g03220.1, Nta14g03090.1, Nta17g02850.1, Nta18g02800.1, Nta22g10880.1, and Nta24g17500.1, were highly expressed in all tissues, especially Nta01g24520.1, which was highly expressed by more than 5 (FPKM value) in all the tissues (Figure 6). Additionally, a few of the *NtNF-Ys* showed tissue-specific expression, such as Nta01g24520.1 (highly expressed in root and stem), Nta11g27510.1 (highly expressed in young and senescent leaf), and Nta18g02800.1 (highly expressed in root). Interestingly, all *NtNF-Ys* were expressed in root except 5 *NtNF-Ys* (Figure 6). Above all, these findings demonstrate that *NtNF-Ys* exhibit distinct expression patterns and are vital to plant development.

Expression analysis in different developmental stages of NtNF-Ys

In order to understand the roles of *NtNF-Ys* in regulating leaf development, we further analyzed the expression levels of *NtNF-Ys* at different developmental stages of leaves (prosperously growing, flower budding, and flower stage) using RNA-seq data. Most of *NtNF-Ys* were expressed in on or more stage, except few *NtNF-Ys* (Nta01g13780.1, Nta02g26300.1, Nta09g18860.1, Nta10g15940.1, Nta12g28040.1, Nta18g11380.1, Nta23g00810.1, and Nta00g11500.1) (Figure 7). Meanwhile, there were some *NtNF-Ys* (Nta01g24520.1, Nta04g27850.1, Nta11g27510.1, Nta12g21910.1, and Nta00g03900.1) highly expressed in all stage, suggesting these might play vital roles at all developmental stages of plant leaves (Figure 7).

Expression analysis under abiotic stress treatment of NtNF-Ys

Abiotic stress, especially drought and saline-alkali stress, constrains the growth and development of plants.[63,64] To study the potential regulators of *NtNF-Ys* in drought and saline-alkali stress responses, the expression patterns of *NtNF-Ys* were analyzed by using the RNA-seq data of tobacco under 200 mm mannitol, 100 mm NaCl, and 100 mm $NaHCO_3$ stress (Figure 8). Under 200 mm mannitol treatment, the expression of many *NtNF-Ys* was significantly upregulated or downregulated, such as Nta00g03900.1 belonging to NtNF-YA family by almost induced by almost 9.14-fold (at 8 h), Nta09g08870.1 belonging to NtNF-YB family by almost induced by almost 0.42-fold (at 1 h), and Nta01g24520.1 belonging to NtNF-YC family by almost induced by almost 2.41-fold (at 1 h) (Figure 8a). Under 100 mm NaCl or 100 mm $NaHCO_3$ treatment, the expression of most of the *NtNF-Ys* was significantly upregulated or downregulated, and there was no significant difference in the expression levels of 19 *NtNF-Ys*. It is interesting that the

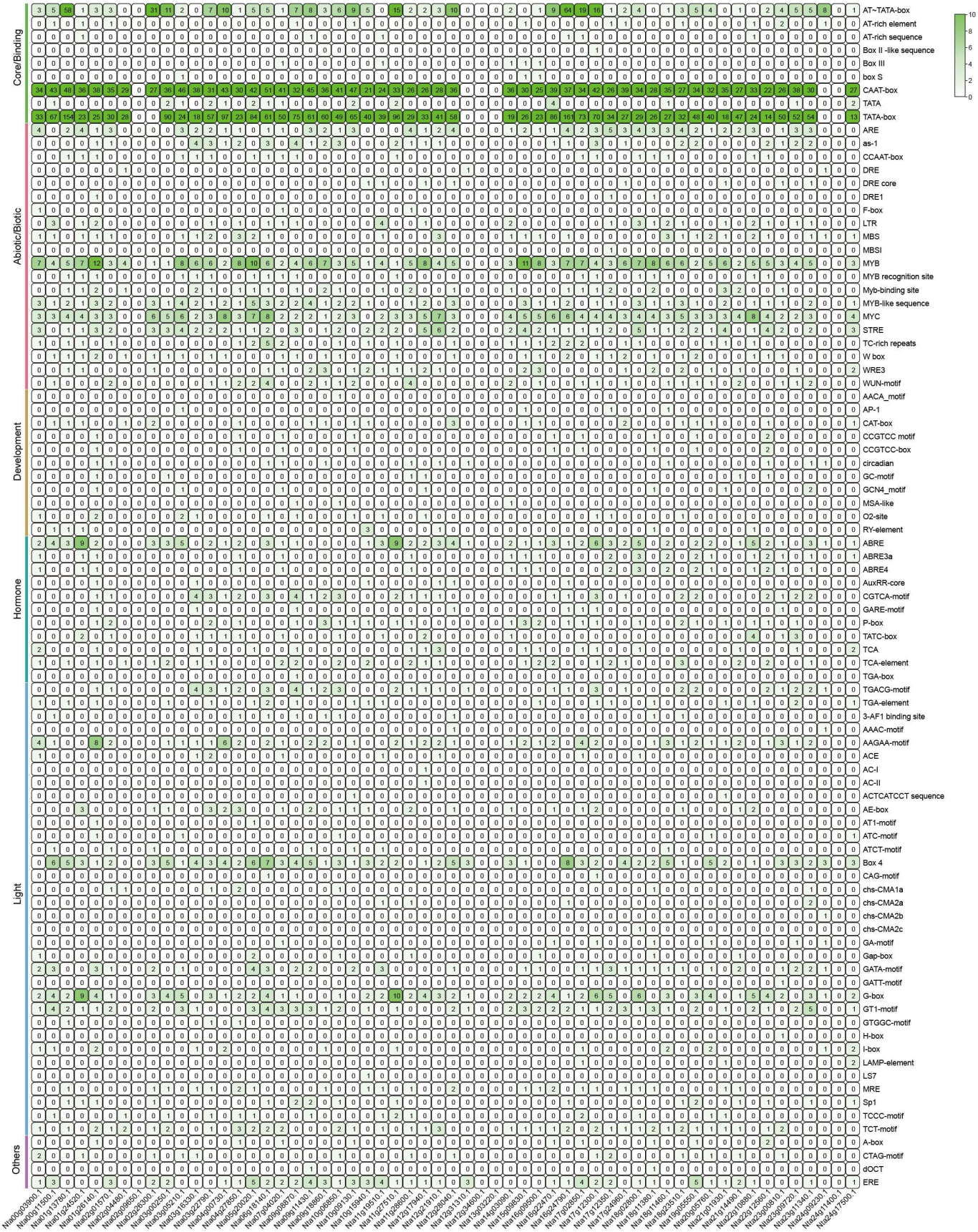

Figure 4. *Cis*-element analysis in the promoters of *NtNF-Ys*. The degree of green colors represents the number of *cis*-elements in the promoter of *NtNF-Ys*.

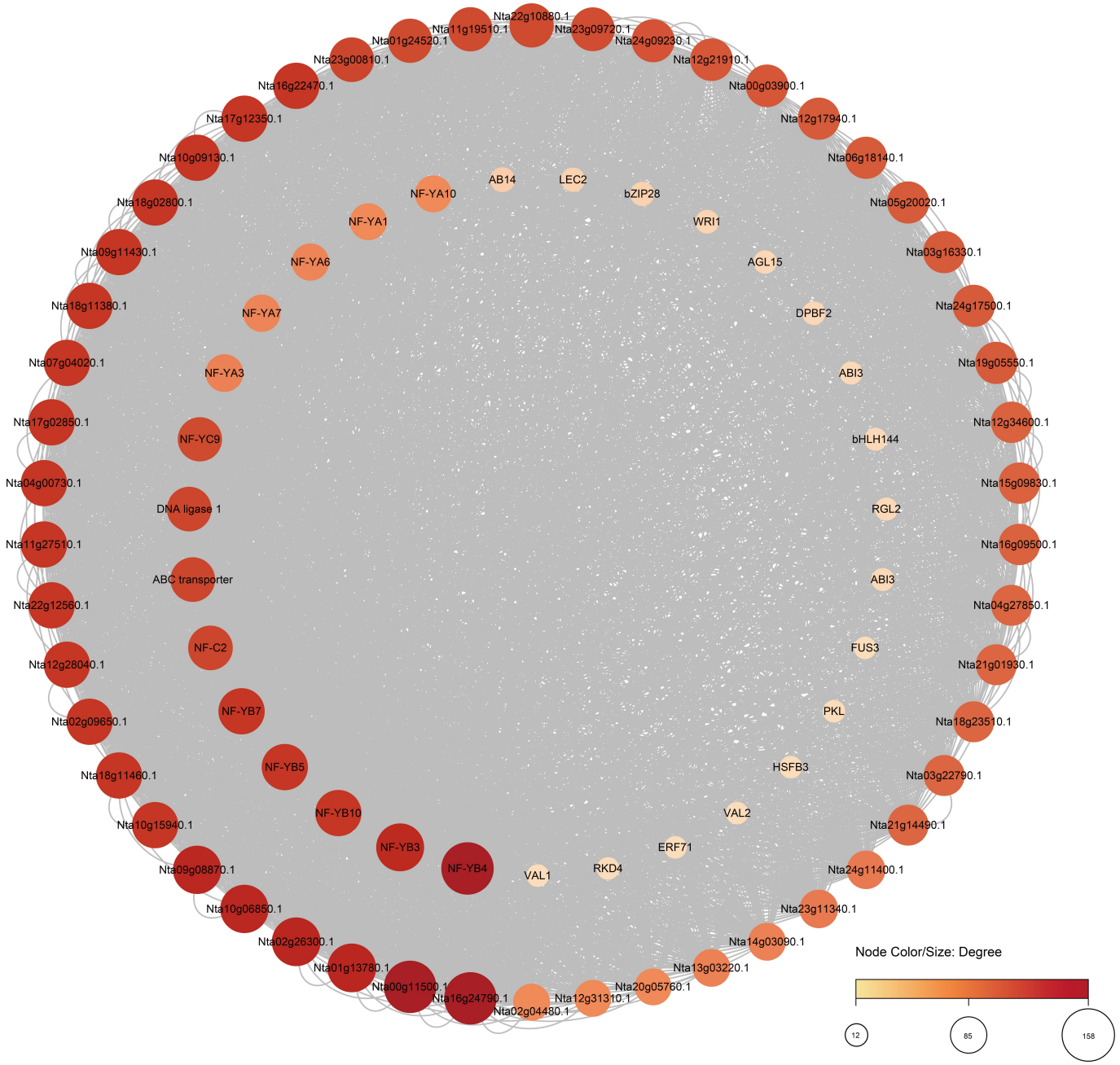

Figure 5. Functional interaction networks of NtNF-Ys. Network nodes represent proteins, and gray lines represent protein–protein associations. The node color/size represents the number of proteins that interact with each other.

expression trends of 11 *NtNF-Ys* were inconsistent under NaCl or $NaHCO_3$ treatment (Figure 8b). Abscisic acid (ABA) is a crucial phytohormone involved in plant responses to abiotic stress.[65,66] To investigate the functions of *NtNF-Ys* in ABA-mediated responses to abiotic stress, we conducted an analysis of *NtNF-Ys* expression patterns through RNA-seq data from tobacco subjected to ABA treatment. Comparatively to the control (CK), the expression levels of several *NtNF-Ys* were significantly induced following ABA treatment (Figure 8c). For example, Nta23g11340.1 was significantly upregulated at any point in time. The above results indicated that *NtNF-Ys* involved in the regulation of plant stress adaption (Figure 10).

It is amazing that the expression levels of some *NtNF-Ys* were significantly induced under all treatment, including Nta00g03900.1 from NtNF-YA family, Nta09g08870.1 from NtNF-YB family, and Nta01g24520.1 from NtNF-YC family, suggesting these *NtNF-Ys* were more important. To further verify the accuracy of transcriptome data, we selected a few *NtNF-Y* genes with significantly differential expression for expression analysis using qRT-PCR (Quantitative reverse transcription polymerase chain reaction). As shown in Figure 9, the expression profiles of the candidate genes were consistent with RNA-Seq results (Table S2). Therefore, the RNA-seq data are authentic.

Discussion

The NF-Y transcription factor family plays vital roles in various physiological processes of plant development. Furthermore, the presence of the NF-Y family has been documented in different plant species such as *Solanum tuberosum*,[67]

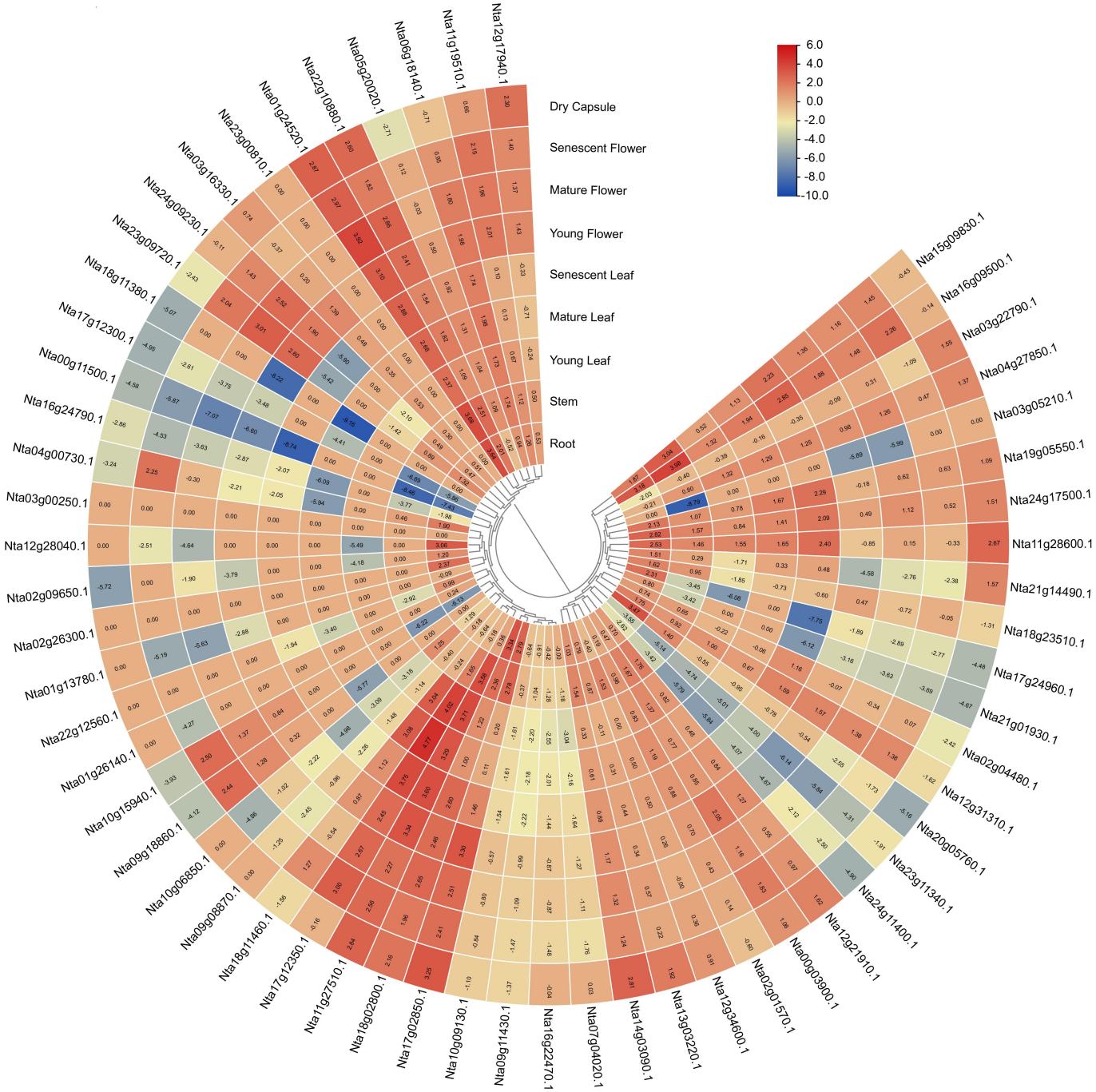

Figure 6. Expression analysis of *NtNF-Ys* in different tissues using RNA-seq. The $\log_2$ (FPKM) values are showed in boxes.

Medicago sativa,[29] *Populus*,[68] *Petunia hybrid*,[69] and others. Nevertheless, many NtNF-Ys remain largely unknown in terms of their biological functions and underlying regulatory mechanisms. Hence, it is essential to perform a comprehensive genome-wide identification and analysis of the *NF-Y* family genes in order to enhance our comprehension of their functions and the molecular mechanism of tobacco.

In 2024, Huazhong Agricultural University published the *N. tabacum* genome, which is the highest values among all published *N. tabacum* genome assembly.[21] In this study, we identified a total of 58 *NtNF-Ys* based on the *N. tabacum* genome (Table 1). Because the *N. tabacum* genome is as large as 4.3 Gb, the number of *NtNF-Ys* is more than most of the plants. Based on the chromosomal localization of NtNF-Ys, genes were uniformly distributed among all chromosomes (Figure 1a). We speculate that segmental and tandem duplications are the main cause of the copy number of *NtNF-Ys* increased, which might bring about functional redundancy and differentiation (Figure 1b). According to the phylogenetic relationship of *NtNF-Ys*, there are similar numbers and types of NtNF-Ys distributed in each group in *Arabidopsis thaliana* and *Oryza sativa*, revealing that the plant NF-Y transcription factor family is evolutionarily conserved (Figure 2).

The genomic structure is typically conserved in the process of plant evolution.[70] In our investigation, as Figure 3a shows, the *NtNF-YAs*, *NtNF-YBs*, and *NtNF-YCs* have their own

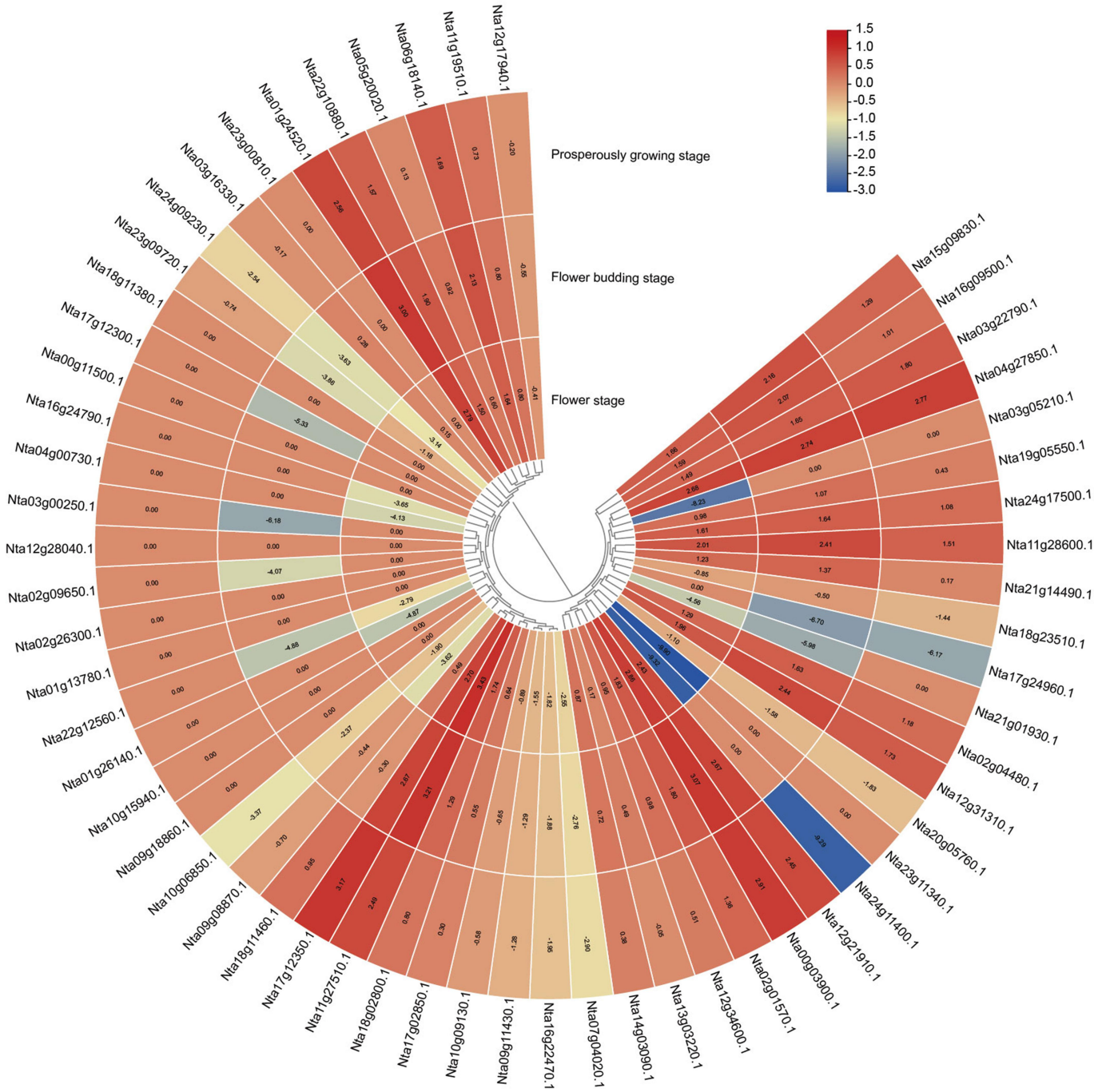

Figure 7. Expression analysis of *NtNF-Ys* in different developmental stages using RNA-seq. The $\log_2$ (FPKM) values are showed in boxes.

conserved motifs, respectively (Fig. S2). Meanwhile, the domains of *NtNF-YAs*, *NtNF-YBs*, and *NtNF-YCs* are also relatively conserved (Figure 3b). The majority of homologous *NtNF-Ys* possess same number of exons and introns in *NF-YAs*, *NF-YBs*, and *NF-YCs*, however, a few display variations in the exon–intron structure. For example, the exon–intron structure of Nta21g01930.1 belonging to *NtNF-YAs* is different from other *NtNF-YAs* (Figure 3c). Therefore, the change in the exon–intron structures of *NtNF-YAs*, *NtNF-YBs*, and *NtNF-YCs* could lead to more functional diversification.

According to the previous study, *NF-Ys* are key players in plant development.[71] In our study, there are many development-related *cis*-elements in the promoters of *NtNF-Ys*, suggesting *NtNF-Ys* may be involved in plant development (Figure 4). NtNF-Ys can interact with the master regulators of development-related proteins, further indicating that NtNF-Ys play crucial roles in plant development (Figure 5). We found many *NtNF-Ys* participate in regulation of leaf development by analyzing the leaf developmental transcriptome data of tobacco. For example, a few of *NtNF-Ys* (Nta01g24520.1, Nta02g01570.1, Nta02g04480.1, Nta03g22790.1, Nta04g27850.1, Nta06g18140.1, Nta11g27510.1, Nta11g28600.1, Nta12g21910.1, Nta15g09830.1, Nta16g09500.1, Nta17g12350.1,

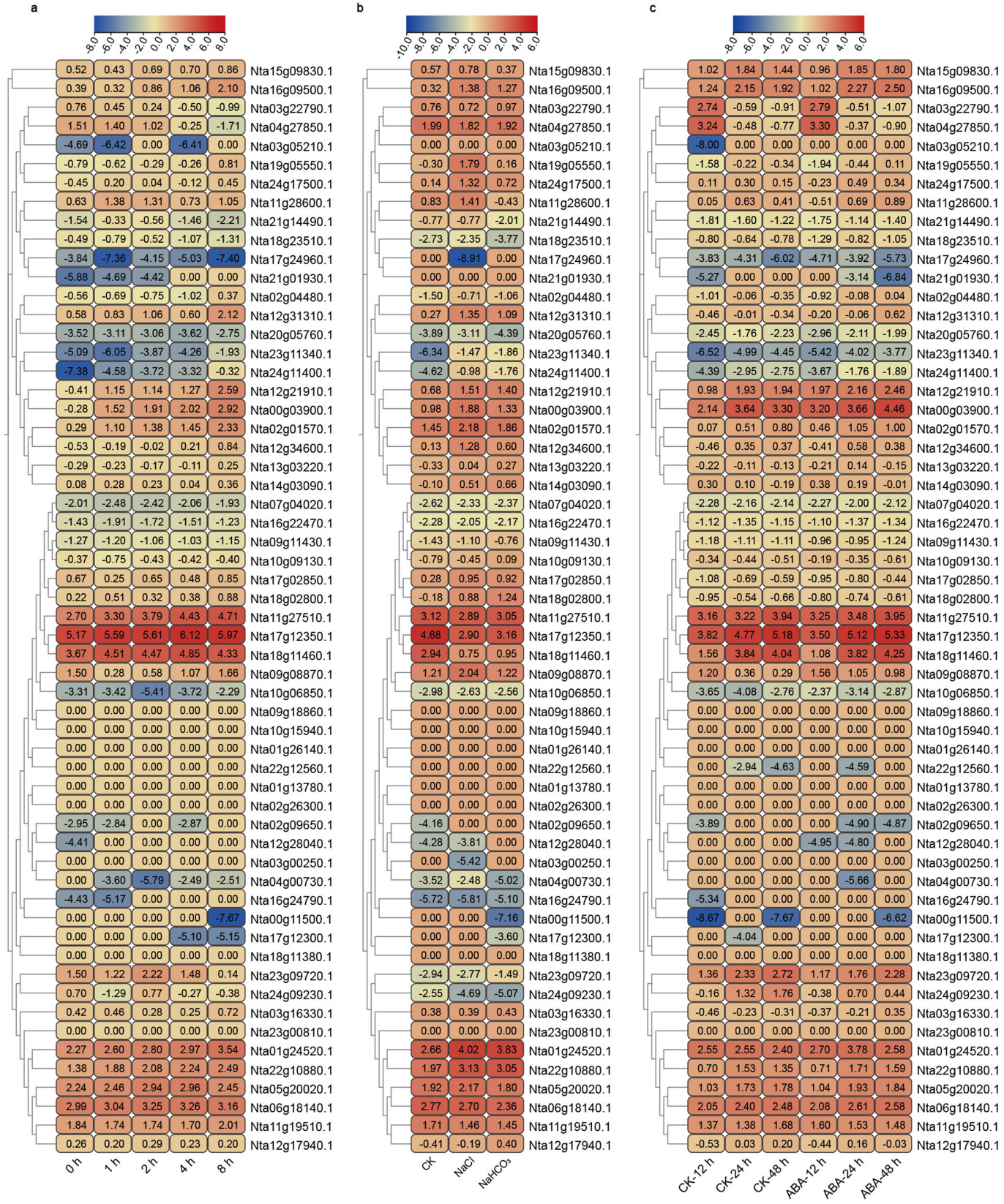

Figure 8. Expression analysis of *NtNF-Ys* under (a) mannitol, (b) NaCl, $NaHCO_3$, and (c) ABA treatments using RNA-seq. The log_2 (FPKM) values are showed in boxes.

Nta18g02800.1, Nta22g10880.1, Nta24g17500.1, and Nta00g03900.1) were highly expressed at all stages of leaf development (Figure 7). Combined with results of tissue-specific expression analysis (Figure 6), we speculate Nta01g24520.1, Nta04g27850.1, Nta06g18140.1, Nta11g 28600.1, Nta16g09500.1, Nta17g12350.1, Nta18g02800.1, Nta19g05550.1, and Nta24g17500.1 are vital regulators in plant leaf development (Figure 10).

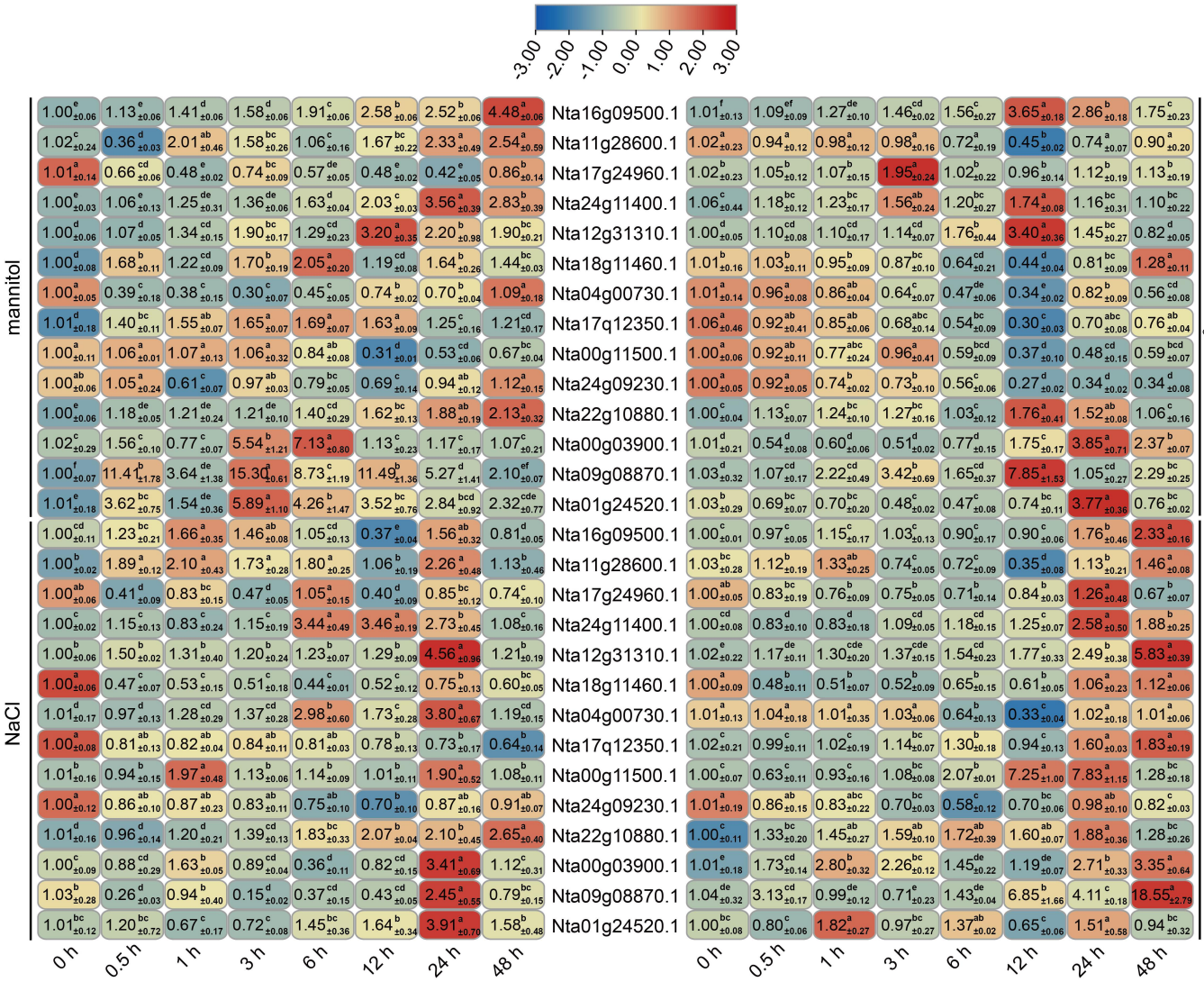

Figure 9. Expression analysis of *NtNF-Ys* under mannitol, NaCl, $NaHCO_3$, and ABA treatments using qRT-PCR. Data are shown as mean ± SD ($n = 3$). Different letters indicate statistically significant differences at $p < 0.05$ according to one-way ANOVA followed by *post-hoc* Tukey's test for each treatment. The tobacco *ACTIN* gene was used as a reference.

The NF-Ys are not only involved in plant development but also in plant abiotic stress.[29] Different NF-Ys members participate in stress responses in various plant tissues to adapt to external stress by mediating multiple signaling and plant hormone pathways, especially the ABA signaling pathway.[29,71] In this research, the promoters of *NtNF-Ys* contained many abiotic *cis*-elements, revealing *NtNF-Ys* were involved in abiotic stress responses (Figure 4). The abiotic related proteins (PKL, bZIP28, and WRI1) can interact with NtNF-Ys, furtherly verifying the significance of NtNF-Ys (Figure 5). The RNA-seq data indicated the expression of many *NtNF-Ys* was significantly upregulated or downregulated, including Nta01g24520.1, Nta02g01570.1, Nta02g04480.1, Nta04g27850.1, Nta11g27510.1, Nta12g 21910.1, Nta12g34600.1, Nta16g09500.1, Nta17g12350.1, Nta18g11460.1, Nta22g10880.1, Nta23g09720.1, and Nta00g03900.1 under 200 mm mannitol treatment (Figure 8a), Nta01g24520.1, Nta02g01570.1, Nta09g08870.1, Nta12g21910.1, Nta12g31310.1, Nta12g34600.1, Nta16g 09500.1, Nta17g12350.1, Nta18g11460.1, Nta19g05550.1, Nta22g10880.1, Nta24g17500.1, and Nta00g03900.1 under 100 mm NaCl or $NaHCO_3$ treatment (Figure 8b), and Nta01g24520.1, Nta09g08870.1, Nta10g06850.1, Nta11g 27510.1, Nta12g21910.1, Nta23g11340.1, and Nta00g03900.1 under ABA treatment (Figure 8c). It is important that the expression levels of some *NtNF-Ys* were significantly induced under all treatments, such as Nta00g03900.1 from NtNF-YA family, Nta09g08870.1 from NtNF-YB family, and Nta01g24520.1 from NtNF-YC family. These results suggest that *NtNF-Ys*, especially Nta00g03900.1, Nta09g08870.1, and Nta01g24520.1, may underlie the response to drought, and saline-alkaline stress in plant (Figure 9).

In summary, we provide genome-wide results of the tobacco *NF-Y* genes, the tissue specificity and expression pattern analyses for leaf development, drought and saline-alkali stresses, and ABA responses. The information described here could contribute to further studies investigating *NtNF-Y* family genes in the context of abiotic stress.

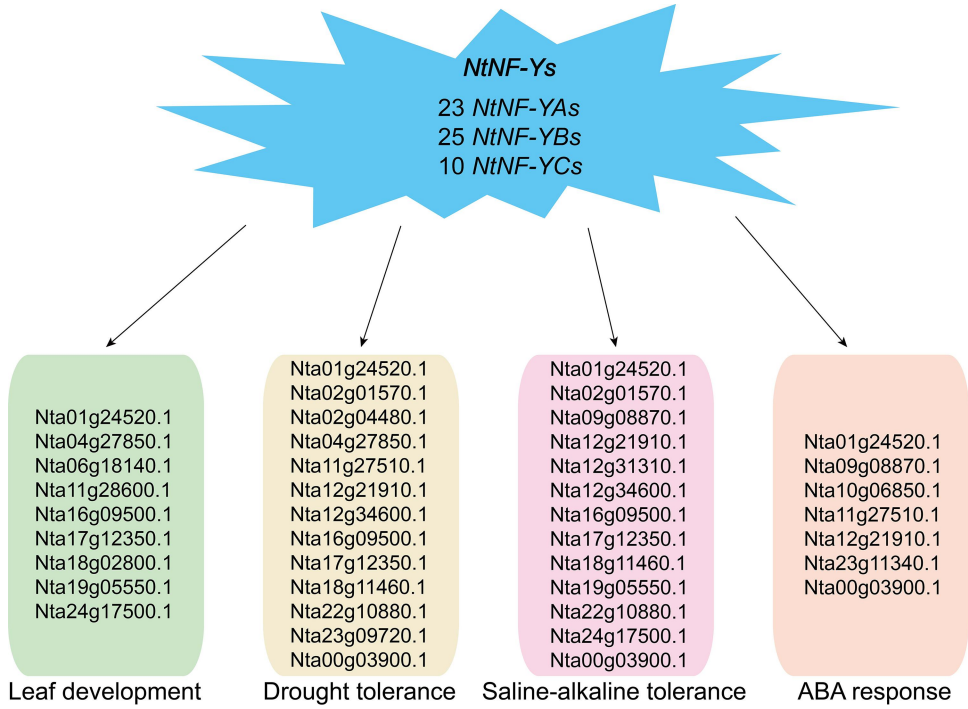

Figure 10. A conclusive graph of functions of *NF-Ys* in tobacco.

Conclusion

In this study, NtNF-Ys were identified from tobacco (*Nicotiana tabacum* L.), and then we systematically analyzed their basic information. Their expression pattern analyses under drought, saline-alkali, and ABA treatments were performed using RNA-seq. We conducted a screening for potential *NtNF-Ys* that may exert a regulatory function in enhancing abiotic stress tolerance. The purpose of this study was to offer novel insights for advancing our comprehension of the functions of *NtNF-Ys* and identifying potential genes linked to environmental stress tolerance in plants.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

S.L., Z.T., D.W., and J.C. conceived and designed the research. Z.T., L.X., J.F., and W.S. performed the experiments. Z.T., L.X., J.C., W.S., B.W., J.S., X.Y., F. C., and J.M. analyzed the data. Z.T. and L.X. wrote the paper. S.L., D.W., and J. C. revised the paper. All authors read and approved the final version of the paper.

Data availability statement

The transcriptome data were deposited at the NCBI database under accession numbers PRJNA883680, PRJNA532660, and PRJNA684346.

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