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Genome-Wide Analysis of *NAC* Gene Family in *Betula pendula*

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Abstract: NACs (NAM, ATAF1/2, and CUC2) are plant-specific transcription factors that play diverse roles in various plant developmental processes. In this study, we identified the *NAC* gene family in birch (*Betula pendula*) and further analyzed the function of *BpNACs*. Phylogenetic analysis reveals that the 114 *BpNACs* can be divided into seven subfamilies. We investigated the expression levels of these *BpNACs* in different tissues of birch including roots, xylem, leaves, and flowers, and the results showed that the *BpNACs* seem to be expressed higher in xylem and roots than leaves and flowers. In addition to tissue-specific expression analysis, we investigated the expression of *BpNACs* under low-temperature stress. A total of 21 *BpNACs* were differentially expressed under low-temperature stress, of which 17 were up-regulated, and four were down-regulated. Using the gene expression data, we reconstructed the gene co-expression network for the 21 low-temperature-responsive *BpNACs*. In conclusion, our results provide insight into the evolution of *NAC* genes in the *B. pendula* genome, and provide a basis for understanding the molecular mechanism for *BpNAC*-mediated cold responses in birch.

Keywords: *Betula pendula*; *NAC*; gene duplication; phylogenetic relationships; expression analysis; abiotic stress; co-expression

1. Introduction

The genome sequence is the whole genetic information of an individual [1]. Functional analysis such as gene family identification, gene cloning, and reconstruction of gene regulatory networks largely depend on the genome references. In recent years, more plant genomes have been sequenced, and they have had a major impact on plant research and improvement in a short time [2]. However, most of the sequenced plant genomes are herbaceous [3–5], while whole genome sequences of woody trees [6,7] are still limited. Fortunately, the genome sequence of *Betula pendula* [8] has become available in the last few years, which can be exploited in numerous ways [9].

In molecular biology, a transcription factor (TF) [10] is a protein that controls the rate of transcription of genetic information from DNA to messenger RNA, by binding to a specific DNA sequence. In plant genomes, about 10% of the products of coding genes are TFs [11]. It is reported [12,13] that TFs are involved in all the aspects of the plant life cycle including growth, development, and stress tolerance. Research studies on TFs have been an important part of functional genomics analysis. Transcription factors in plants can be assigned to different families according to the conserved DNA binding domains. The well-studied TFs in plants include MIKC, C2H2, WRKY, bZIP, MYB, SBP, HB, AP2/EREBP, and NAC [11].

The gene family always arose from multiple ways including tandem duplication, duplicative transposition, and whole genome duplication (WGD), which was followed by mutation and divergence. The NAC (NAM, ATAF, and CUC) transcription factors are plant-specific TFs involved in multiple biological functions [14]. The name of NAC was originally derived from the abbreviation of three

proteins including no apical meristem (NAM), ATAF1-2, and CUC2 (cup-shaped cotyledon), which contain a similar DNA-binding domain [15,16]. It is reported that smaller numbers of NAC TFs are present in ancient land plants. The larger number of NAC TFs were considered to be the results of WGD (Whole Genome Duplication) and other small-scale duplication events [17]. Accumulating evidence indicated that a considerable portion of NAC transcription factors plays crucial roles in the processes of xylogenesis, fiber development, and wood formation in vascular plants. In *Arabidopsis thaliana*, NAC secondary wall thickening promoting factor 1 (NST1) and NAC secondary wall thickening promoting factor 3 (NST3) are key regulators of secondary wall formation in the woody tissue [18]. Secondary wall-associated NAC domain protein 1 (SND1) could upregulate the expression of multiple transcription factors, including one that is highly expressed in fibers during secondary wall synthesis [19]. Xylem NAC domain 1 (XND1) transcription factor negatively regulates lignocellulose synthesis and programmed cell death in the xylem [20]. A vascular related NAC-domain protein 7 (VND7) plays a pivotal role in regulating the differentiation of root protoxylem vessels [21]. In rice and maize, the secondary wall-associated *NACs* (namely *OsSWNs* and *ZmSWNs*) have been shown to be key transcriptional switches regulating secondary wall biosynthesis [22]. In *Eriobotrya japonica*, *EjNAC1* was associated with fruit lignification by activating genes involved in lignin biosynthesis [23]. In *Gossypium hirsutum*, *GhXND1* encodes an NAC transcription factor, and the cell wall-related genes were down-regulated in the *GhXND1* transgenic plants. Wang et al. [24] suggested that *GhXND1* may be involved in the negative regulation of plant xylem development. In *Populus*, *WND2B* and *WND6B* could effectively complement secondary wall defects in the *snd1/nst1* double mutant. They have been shown to be functional orthologs of *A. thaliana SND1*, and they could induce ectopic deposition of secondary walls when ectopically over-expressed in *A. thaliana* [25]. In addition, Hu et al. [14] identified a subset of more than 30 *NAC* genes that showed the highest level of transcript abundance in differentiating xylem and cambium tissues of *Populus*.

Numerous NAC domain proteins have been implicated in plant biotic stress and abiotic stress, such as fungi, bacteria, insects, drought, salinity, and cold and mechanical wounding [14,26]. The *AtNAC2* gene from *A. thaliana* is a transcription factor downstream of ethylene and auxin signaling pathways, which plays an important role in salt stress response [27]. Similarly, the expression of the *RD26* gene in *A. thaliana* was also induced by drought and high salinity stress [28]. Long vegetative phase 1 (LOV1) could regulate the response of plants to freezing temperature by controlling a subset of cold response genes via a CBF/DREB1-independent pathway [29]. In rice, transgenic plants overexpressing *ONAC045* showed enhanced tolerance to drought and salt treatments [30]. Banana fruit NAC transcription factor *MaNAC1* is a direct target of *MaICE1* and is involved in cold stress by interacting with *MaCBF1* [31]. In *Pyrus betulifolia, PbeNAC1* could confer cold and drought tolerance by interacting with *PbeDREBs* and activating the expression of stress-responsive genes [32].

Understanding the function of the NAC gene family will be instrumental in the study of molecular mechanisms of development and stress tolerance of plants. However, the research on the *NAC* gene mainly focuses on the model plants *A. thaliana* and *Oryza sativa* [33]. No systemic characterization of the *NAC* genes have been conducted in *B. pendula*. In this study, we used multiple bioinformatics methods to identify the *B. pendula NAC* gene family, and performed the comprehensive analysis including gene structure, conserved motifs, chromosomal location, phylogeny, tissue-specific expression profiles, and identification of low-temperature-responsive *BpNACs*. All the results laid the foundation for deeper research of the *BpNAC* genes.

2. Materials and Methods

2.1. Identification and Basic Information Statistics of BpNAC Gene Family

The *Betula pendula* genome [8] was downloaded and used for identifying *BpNACs*. We used NCBI (National Center for Biotechnology Information) Conserved Domain Database [34], InterProScan [35], PfamScan [36], and HMMER [37] to identify proteins that contain the NAC domain in the *B. pendula*

genome. We also downloaded all the annotated *NACs* proteins from the plant transcription factor database (PlantTFDB) [11] and used them as queries to identify candidates of *BpNACs* via BLASTP with a thread of e-value less than 1e-5. In addition, all the BpNAC proteins were further manually analyzed using the NCBI Conserved Domain Database [38] to confirm the presence of the NAC domain.

The basic statistics information of *BpNACs* was performed using the bio-python module [39]. Molecular weight and theoretical isoelectric point of the BpNAC protein sequences were analyzed by the ExPaSy Compute pI/Mw tool [40]. The genomic structure information (GFF) of the *B. pendula* genome was downloaded from the CoGe comparative genomics platform [41] and visualized by using TBtools [42]. The amino acid motif analysis of BpNAC proteins was performed using MEME 5.0.4 [43]. The motif discovery mode was selected as a classic mode and the site distribution was ZOOPS (Zero or One Occurrence Per Sequence). Other options have remained as defaults.

MCScanX [44] was used to identify the intra-genome collinear blocks of *B. pendula*. The results were displayed using Circos [45].

2.2. Phylogenetic Analysis based on the BpNAC Domain

To investigate the phylogenetic information of the *BpNACs* of *B. pendula*, an unrooted tree was constructed. Multiple sequence alignment was performed using MUSCLE [46] with the default parameters. Then, an unrooted phylogenetic tree was constructed using RAxML [47] with the maximum likelihood (ML) method. The parameters used for RAxML were "PROTGAMMALGX" and the bootstrap value was 1000.

2.3. Functional Analysis of the BpNAC Gene Family

The function of the *BpNAC* gene family was annotated by eggNOG-mapper [48]. GO, COG, and KEGG annotation were retrieved from the eggNOG [49] results. We also used BLAST (E-value < $1e^{-5}$) to find the *A. thaliana* homology of *BpNACs*. The homologous annotation of *A. thaliana* in TAIR [50] was used for functional annotation of *BpNACs*. For comparative analysis, the expression data of *A. thaliana* were downloaded from the TAIR abiotic stress database (https://www.arabidopsis.org/). The promoter sequences of *BpNACs* were analyzed using PlantCARE [51], which is a database of plant cis-acting regulatory elements and a useful promoter sequence analysis tool.

2.4. Differential Expression Profile of BpNAC Gene Family

Three biological replicates of roots, young leaves, and female inflorescences were collected from two-year-old birch planted in the experimental field of Northeast Forestry University (Harbin, China). Two-month-old birch were transplanted to the field from the greenhouse in the spring. Birch planted in the experimental field can bloom two years later with a good supply of water and fertilizer. Each biological replicate contains three individuals. We collected five leaves from each individual and mixed them together as one biological replicate. For xylem, we used the upper stem (about 10th nodes) of two-year old birch trees, and the tissue-specific sampling was performed, according to the Juan Alonso-Serra et al. [52] method. Sequential tangential cryosections were collected with two biological replicates per fraction and processed independently. Once a fraction was removed, a cross-section was observed under the microscope to localize the anatomical position. All the plants were grown under natural conditions with enough water and nutrition. Total RNA of the samples were extracted using the traditional CTAB (Cetyltrimethylammonium Bromide) method [53]. The quality and concentration of the isolated RNA were evaluated using agarose gel electrophoresis and UV-spectrophotometry. The cDNA libraries were constructed at Biomarker Technologies Corporation (Beijing, China), which was subjected to paired-end read sequencing using the Illumina HiSeq platform. The raw sequencing data were deposited into the NCBI SRA (Sequence Read Archive) database with an accession number of PRJNA535361.

To identify *BpNACs* in response to low-temperature stress, we designed a time series experiment including six time points, according to Yang's method [54]. Two-month-old *B. pendula* plants grown

in the greenhouse at a constant temperature of 25 °C with a photoperiod of 16 h of light and 8 h of dark were exposed to low temperatures (6 °C) for 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, and 3 h, respectively. After low-temperature exposure, all young leaves were harvested at the same time to avoid falsely differentially expressed genes caused by diurnal changes. Plants without low temperature treatment were used as the control for this study. We generated two biological replicates for time points of 1 h, 2 h, 2.5 h, 3 h, and CK (control check), and three biological replicates for time points of 0.5 h and 1.5 h, respectively. Total RNA of the samples were extracted using the traditional CTAB method [53]. The constructed cDNA libraries were sequenced using the Illumina HiSeq platform at Biomarker Technologies Corporation (Beijing, China). The raw sequencing data were deposited into the NCBI SRA database with an accession number of PRJNA532995.

After removing reads of low quality, each set of sequences was separately aligned to the *B. pendula* reference genome [8] using bowtie2 [55]. The RNA-Seq data were subsequently analyzed using the RSEM (RNA-Seq by Expectation-Maximization) pipeline [56] and the data were processed using a paired-end sequencing mode. The RSEM handle ambiguously mapping reads used the method of Li et al. [57], which treats mapping uncertainty in a statistically rigorous manner. This approach allows for accurate gene-level abundance estimates between both isoforms and genes using paired-end reads [56]. Compared to controls (plants without low-temperature treatment), differentially expressed genes (DEGs) at each time point were identified using edgeR [58] software with a threshold of FDR < 0.05 and $\log_2$FC > 1. The gene expression levels were normalized using the trimmed mean of the M-values (TMM) method. The fold changes of differentially expressed genes (DEGs) were the average TMM of each time point divided by the average TMM of the control group. The number of minimum repeat samples was 2, and the minimum value of count per million was 1 to filter out samples with a low expression.

2.5. Quantitative RT-PCR

The two-month-old plants of birch were treated with low-temperature stress for 3 h and 6 h. Three biological replicates were prepared for each time point. Total RNA of young leaves was then isolated for the qRT-PCR experiment. The resulting RNA was used as a template for first-strand cDNA synthesis with random hexamers, according to the manufacturer's instructions. Quantitative real-time RT-PCR (qPCR) was performed with an Applied Biosystems 7500 Real-time PCR System to examine the expression patterns of the selected genes. Primer pairs for real-time quantitative PCR (Table S1) were designed using A plasmid Editor v1.11. Each reaction contained 10 µL of THUNDERBIRD SYBR qPCR Mix (QPS-201, TOYOBO, Japan), 2 µL of cDNA, 1 µL of forward primer, 1 µL of reverse primer, and 6 µL of double-distilled water (ddH_2O). The PCR reactions were performed under the following conditions: 95 °C for 10 min, which was followed by 45 cycles of 95 °C for 30 s and 60 °C for 10 s. The $2^{-\Delta\Delta CT}$ method of analysis was used with the 18S RNA gene as the normalizing gene. Each sample was analyzed in triplicate.

2.6. Co-Expression Network Construction and GO Enrichment Analysis of BpNAC Gene Family

We used WGCNA (Weighted Correlation Network Analysis) to identify genes co-expressed with the low-temperature responsive *BpNACs* [59]. The co-expression network was visualized using Cytoscape [60]. The data used as input for the co-expression network construction are the gene expression data derived from the time series experiment of low-temperature stress. Only members with an expression greater than 10 in all samples are retained. The optimal β (soft thresholding power) value was determined to be 13 after 20 iterations. The Pearson algorithm is then used to calculate the correlation coefficient and the result is stored as a signed co-expression matrix. The "deepSplit" value was set to 2 during clustering, the "minModuleSize" value was set to 30, and the "mergeCutHeight" value was set to 0.15. To ensure the reliability and readability of the results, we filtered out the edges with weights below 0.1 and used Cytoscape to display genes directly related to TFs. The GO annotation was performed by EggNOG-mapper with the diamond model and visualized using WEGO 2.0 [61].

3. Results

3.1. Genome-Wide Identification of the BpNAC Gene Family

We first identified candidates of *BpNACs* by searching no apical meristem (NAM) domain (PF02365) in the *Betula pendula* genome using InterProScan [35], PfamScan [36], and HMMER [37]. A total of 108 putative *BpNACs* (Table 1) were identified in the *B. pendula* genome at this step. Further Conserved Domains Database (CDD) [34] analysis revealed that all the genes have a NAM or NAC conserved domain structure, which demonstrates the reliability of the results. The 108 genes were defined as *BpNAC001* to *BpNAC108*, and used for further analysis. In addition, we also identified six genes (Table S2) that were similar to the known *NACs*. Lastly, a total of 114 *BpNACs* were identified in the *B. pendula* genome. However, all the six genes may be truncated *BpNACs* and contain no conserved NAC domain. Thus, in this study, we did not use them for further analysis.

Table 1. Basic information of the *NAC* genes in *B. pendula*.

Gene Name	Gene Locus	CDS Length (bp)	AA [1]	pI [2]	MW (Da) [3]
BpNAC001	Bpev01.c0000.g0143	1056	351	4.58	39,004.80
BpNAC002	Bpev01.c0001.g0043	1158	385	6.40	43,779.76
BpNAC003	Bpev01.c0001.g0069	1512	503	6.56	57,125.50
BpNAC004	Bpev01.c0001.g0070	1542	513	6.02	58,179.28
BpNAC005	Bpev01.c0022.g0005	1185	394	5.14	45,193.43
BpNAC006	Bpev01.c0029.g0103	567	188	8.30	22,497.58
BpNAC007	Bpev01.c0039.g0006	1041	346	4.48	38,679.40
BpNAC008	Bpev01.c0039.g0015	1284	427	4.93	47,875.71
BpNAC009	Bpev01.c0044.g0097	1368	455	6.25	51,160.80
BpNAC010	Bpev01.c0050.g0126	804	267	5.73	30,466.32
BpNAC011	Bpev01.c0052.g0056	786	261	9.42	29,539.17
BpNAC012	Bpev01.c0052.g0182	1095	364	6.01	40,524.81
BpNAC013	Bpev01.c0055.g0027	1023	340	5.19	38,238.63
BpNAC014	Bpev01.c0080.g0077	474	157	7.92	17,308.59
BpNAC015	Bpev01.c0080.g0092	1086	361	7.65	41,283.20
BpNAC016	Bpev01.c0082.g0005	1047	348	8.47	39,276.47
BpNAC017	Bpev01.c0082.g0007	1092	363	6.27	40,947.88
BpNAC018	Bpev01.c0088.g0022	1314	437	5.02	49,013.50
BpNAC019	Bpev01.c0088.g0098	876	291	7.61	33,171.68
BpNAC020	Bpev01.c0101.g0069	1251	416	6.67	46,717.66
BpNAC021	Bpev01.c0129.g0029	918	305	4.64	34,313.49
BpNAC022	Bpev01.c0135.g0042	963	320	4.62	36,457.72
BpNAC023	Bpev01.c0135.g0072	921	306	4.67	34,460.78
BpNAC024	Bpev01.c0155.g0038	1059	352	8.34	38,836.59
BpNAC025	Bpev01.c0162.g0012	996	331	8.92	36,700.26
BpNAC026	Bpev01.c0162.g0014	1023	340	7.09	38,715.32
BpNAC027	Bpev01.c0167.g0004	813	270	8.73	30,315.34
BpNAC028	Bpev01.c0191.g0026	1359	452	4.72	50,997.03
BpNAC029	Bpev01.c0191.g0027	1551	516	5.53	56,743.10
BpNAC030	Bpev01.c0191.g0028	1914	637	4.71	70,689.54
BpNAC031	Bpev01.c0191.g0029	1398	465	5.82	51,879.04
BpNAC032	Bpev01.c0210.g0037	1743	580	4.91	63,826.53
BpNAC033	Bpev01.c0210.g0038	447	148	9.16	17,122.73
BpNAC034	Bpev01.c0210.g0039	1554	517	4.61	56,841.20
BpNAC035	Bpev01.c0210.g0040	1281	426	5.23	47,771.98
BpNAC036	Bpev01.c0210.g0041	1428	475	4.93	53,076.79
BpNAC037	Bpev01.c0210.g0042	1551	516	4.89	57,647.89
BpNAC038	Bpev01.c0210.g0043	3804	1268	5.02	139,552.01

Table 1. *Cont.*

Gene Name	Gene Locus	CDS Length (bp)	AA [1]	pI [2]	MW (Da) [3]
BpNAC039	Bpev01.c0210.g0048	657	218	4.28	23,950.57
BpNAC040	Bpev01.c0224.g0004	576	191	4.52	21,962.39
BpNAC041	Bpev01.c0245.g0072	1155	384	6.66	43,548.68
BpNAC042	Bpev01.c0261.g0059	684	227	9.65	25,959.43
BpNAC043	Bpev01.c0275.g0039	1005	334	4.72	37,105.64
BpNAC044	Bpev01.c0281.g0055	1200	399	6.13	44,787.28
BpNAC045	Bpev01.c0281.g0084	1023	340	6.57	38,567.56
BpNAC046	Bpev01.c0281.g0092	924	307	4.64	34,661.06
BpNAC047	Bpev01.c0330.g0003	915	304	5.51	34,776.84
BpNAC048	Bpev01.c0344.g0002	510	169	9.49	19,234.75
BpNAC049	Bpev01.c0344.g0004	3090	1029	5.37	117,765.80
BpNAC050	Bpev01.c0346.g0003	1065	354	8.18	39,093.88
BpNAC051	Bpev01.c0349.g0059	276	91	4.74	9914.98
BpNAC052	Bpev01.c0357.g0068	855	284	8.54	32,795.40
BpNAC053	Bpev01.c0376.g0004	483	160	7.92	17,263.55
BpNAC054	Bpev01.c0390.g0007	891	296	6.61	34,034.73
BpNAC055	Bpev01.c0404.g0003	1077	358	4.90	41,609.59
BpNAC056	Bpev01.c0404.g0007	966	321	5.94	37,486.94
BpNAC057	Bpev01.c0411.g0006	960	319	6.35	36,457.90
BpNAC058	Bpev01.c0428.g0005	1398	465	4.84	50,270.40
BpNAC059	Bpev01.c0450.g0023	987	328	5.78	37,235.70
BpNAC060	Bpev01.c0452.g0001	837	278	5.02	32,793.02
BpNAC061	Bpev01.c0452.g0002	717	238	8.14	28,149.23
BpNAC062	Bpev01.c0452.g0003	1047	348	4.64	40,257.88
BpNAC063	Bpev01.c0455.g0003	885	294	7.07	34,051.03
BpNAC064	Bpev01.c0457.g0009	1356	451	5.31	50,761.01
BpNAC065	Bpev01.c0477.g0025	891	296	4.87	33,263.97
BpNAC066	Bpev01.c0477.g0027	834	277	5.06	31,589.50
BpNAC067	Bpev01.c0480.g0034	1128	375	6.52	42,094.62
BpNAC068	Bpev01.c0483.g0016	1092	363	6.27	41,571.91
BpNAC069	Bpev01.c0514.g0006	756	251	4.61	28,414.49
BpNAC070	Bpev01.c0514.g0007	1575	524	5.08	57,993.34
BpNAC071	Bpev01.c0517.g0013	1074	357	8.23	40,150.47
BpNAC072	Bpev01.c0522.g0030	1281	426	6.16	48,038.53
BpNAC073	Bpev01.c0531.g0007	897	298	6.68	34,698.52
BpNAC074	Bpev01.c0555.g0002	906	301	5.99	33,702.72
BpNAC075	Bpev01.c0557.g0054	750	249	8.72	28,285.83
BpNAC076	Bpev01.c0568.g0011	726	241	4.85	27,749.94
BpNAC077	Bpev01.c0652.g0024	1692	563	4.80	63,738.88
BpNAC078	Bpev01.c0656.g0002	558	185	4.63	21,053.47
BpNAC079	Bpev01.c0726.g0004	1032	343	5.11	38,869.14
BpNAC080	Bpev01.c0727.g0012	1995	664	5.19	75,547.32
BpNAC081	Bpev01.c0751.g0007	858	285	8.60	32,919.09
BpNAC082	Bpev01.c0751.g0009	864	287	6.64	33,211.28
BpNAC083	Bpev01.c0755.g0001	942	313	8.72	35,486.93
BpNAC084	Bpev01.c0781.g0009	1152	383	5.29	42,218.92
BpNAC085	Bpev01.c0796.g0007	1236	411	6.02	46,275.75
BpNAC086	Bpev01.c0803.g0020	1395	464	4.86	51,157.26
BpNAC087	Bpev01.c0863.g0019	477	158	8.90	18,172.77
BpNAC088	Bpev01.c1058.g0003	1065	354	5.02	40,826.35
BpNAC089	Bpev01.c1071.g0002	564	187	6.18	20,761.41
BpNAC090	Bpev01.c1155.g0007	339	112	5.84	12,868.12
BpNAC091	Bpev01.c1185.g0017	2094	697	5.33	77,759.91
BpNAC092	Bpev01.c1189.g0015	744	247	6.34	28,484.74
BpNAC093	Bpev01.c1204.g0001	1089	362	5.74	40,418.56
BpNAC094	Bpev01.c1218.g0003	327	109	7.63	12,565.44

Table 1. *Cont.*

Gene Name	Gene Locus	CDS Length (bp)	AA [1]	pI [2]	MW (Da) [3]
BpNAC095	Bpev01.c1228.g0010	930	309	6.74	35,236.58
BpNAC096	Bpev01.c1250.g0009	1008	335	8.72	37,415.34
BpNAC097	Bpev01.c1272.g0001	783	260	6.55	29,666.12
BpNAC098	Bpev01.c1539.g0003	1191	396	5.88	46,313.98
BpNAC099	Bpev01.c1580.g0002	924	307	4.59	34,644.71
BpNAC100	Bpev01.c1670.g0008	735	244	8.72	28,528.46
BpNAC101	Bpev01.c1773.g0003	621	206	9.13	23,494.36
BpNAC102	Bpev01.c1773.g0004	582	193	9.48	22,043.09
BpNAC103	Bpev01.c1899.g0001	1014	337	7.07	38,269.85
BpNAC104	Bpev01.c1913.g0005	945	314	4.67	35,530.73
BpNAC105	Bpev01.c1933.g0004	675	224	8.54	26,398.37
BpNAC106	Bpev01.c2186.g0001	609	202	5.92	23,661.86
BpNAC107	Bpev01.c2413.g0002	591	196	4.88	22,210.41
BpNAC108	Bpev01.c2439.g0003	846	281	8.75	32,581.73

[1] Length of the amino acid sequence. [2] Isoelectric point of the BpNACs. [3] Molecular weight of the BpNACs.

The coding region lengths of the *BpNACs* were from 276 bp to 3804 bp. The deduced proteins range from 91 to 1268 amino acids in length with theoretical isoelectric points from 4.28 to 9.65. The predicted molecular weights of the BpNAC proteins range from 9.91 kDa to 139.55 kDa. The average predicted the isoelectric point of the encoded proteins, which was 6.30. The average molecular weight was 39.50 kDa. The detailed information is summarized in Table 1.

3.2. Sequence-Structure Features of BpNAC Gene Family

In a natural evolution, the diversity of genes is an important factor in promoting morphological diversity, and the morphological diversity allows plants to use natural resources efficiently or to adapt to the adverse environmental conditions [62]. To investigate the genomic structural features, we plotted the gene structural map of the *BpNAC* genes (Figure S1). *BpNAC038* contains 15 introns, which have the most introns in the *BpNAC* gene family, followed by *BpNAC064*, which contains nine introns. A large number of introns may be due to the complex evolutionary process of *B. pendula*. The number of exons in the *BpNAC* gene family is generally less than eight.

The conserved amino acid motifs of the BpNAC proteins were identified using MEME [43]. A total of eight amino acid conserved motifs of the BpNAC family were identified. As shown in Figure S2, about 50% of the BpNACs contain motif 1, 2, 3, 4, 5, 6, and 7. In the remaining sequence, motif 4 and motif 7 are replaced by motif 8. Motif 8 is the longest motif in BpNAC protein family including 29 amino acids. By comparison with the NR database [63], we found that this motif is the most similar with OMO69178 from *Corchorus capsularis*, which proved to be the NAM protein. Motif 1 has been found in *Glycine soja's* XP_028199933 and XP_028199935 protein, and they all belong to the NAC 59-like gene. Motif 1 was found in CUC2 of *Spinacia oleracea*, *Mucuna pruriens*, and *Chenopodium quinoa*. Kamiuchi et al. [64] reported that CUC2 promote carpel margin meristem formation during *A. thaliana* gynoecium development. Motif 2 was found in NAC48, and has similar structures in various species, such as *Dichanthelium oligosanthes*, *Oryza sativa*, *Apostasia shenzhenica*, *Panicum hallii*, *Ananas comosus*, and *Setaria italica*. NAC48 is involved in multicellular organismal development and regulation of transcription [65]. Motif 3 exhibits a similarity to NAC71 and NAC86, and has been found in *Capsella rubella*, *Arachis duranensis*, *Elaeis guineensis*, *Amborella trichopoda*, and *Solanum lycopersicum*. Motif 4 exhibits similarity to NAC40 and NAC86 and is widely distributed in a variety of plants, such as *Raphanus sativus*, *Brassica rapa*, *Arachis duranensis*, *Medicago truncatula*, and *Cicer arietinum*. Motif 6 exhibits similarity to the CUP-SHAPED COTYLEDON 3 in *Medicago truncatula*. Hibara et al. [66] reported that CUP-SHAPED COTYLEDON3 could regulate postembryonic shoot meristem and organ boundary formation. The other shorter motif sequences of motif 5 and motif 7 also show varying degrees of similarity in some NAC-related genes.

Based on the genomic structure results, we have a preliminary understanding of the characteristics of the *BpNACs*. The similar gene structures and conserved amino acid motifs of BpNACs may provide additional support to the phylogenetic analysis.

3.3. Chromosomal Location and Gene Duplication of the BpNAC Gene Family

We investigated the formation of the *BpNAC* family, according to the chromosomal location and intra-genome syntenic information. The analysis of chromosomal location revealed that the 97 *BpNAC* genes are distributed among 14 chromosomes (Chr). As shown in Figure S3, 13, 12, 11, and 10 *BpNACs* are located on Chr2, Chr12, Chr1, and Chr7, respectively. Seven *BpNACs* are located on Chr5 and Chr10, six on Chr3, Chr9, and Chr14, five on Chr4 and Chr13, four on Chr6 and Chr8, and the last one on Chr11.

According to the chromosomal location results, we found that some *BpNAC* genes were organized into duplicated blocks. A total of 35 *BpNACs* are distributed as clusters in the *B. pendula* chromosomes and may originate from duplicative transposition (Figure S3). For Chr1, Chr5, and Chr12, each chromosome contains eight, six, and seven tandem *BpNACs*, respectively. For Chr9 and Chr13, each chromosome contains four tandem *BpNACs*. For Chr2, Chr3, and Chr8, each chromosome contains two tandem *BpNACs*.

WGD events were the products of nondisjunction during meiosis [67], and can cause excessive gene duplication in a short period of time. It is reported that the *B. pendula* genome had experienced the paleohexaploid event [8]. To determine the possible relationship between the formation of the *BpNAC* family and the paleohexaploid event, we constructed 3538 chromosome segmental duplicated blocks for the *B. pendula* genome using MCScanX [44]. Then, the *BpNACs* were mapped to the duplicated blocks and the distributions were illustrated in Figure S4. According to the MCScanX results, 66 *BpNACs* were found to be in the syntenic blocks, and a total of 280 links were produced. Among them, chromosome 2 is most closely related to other chromosomes, and links with 40 *BpNACs* from 11 chromosomes.

3.4. Phylogenetic Analysis of the BpNAC Gene Family

In *A. thaliana*, rice, and soybean, the NAC family was divided into two, five, and 15 groups, respectively [68,69]. More recently, comprehensive phylogenetic analyses of numerous NAC proteins from a variety of plant species indicated that NAC proteins can be divided into eight subfamilies [70]. To investigate the phylogenetic relationship among the BpNAC proteins in the *B. pendula* genome, an unrooted phylogenetic tree was constructed (Figure S5) using BpNAC protein sequences by RAxML [47] with 1000 bootstrap values. According to the structure similarity and conserved motif information of branches on the tree, the 108 BpNAC proteins could be classified into seven clades: Clade I, Clade II, Clade III, Clade IV, Clade V, Clade VI, and Clade VII. Clade III is the largest branch including 30 *BpNACs*, which is followed by Clade I and includes 21 *BpNAC* members. Clade V and Clade VI include nine members, while Clade II and Clade IV contain 17 and 15 *BpNAC* members, respectively. *BpNAC002*, *BpNAC005*, *BpNAC015*, *BpNAC057*, *BpNAC072*, *BpNAC090*, and *BpNAC103* are not identical in structure similarity to the above categories, and were divided into Clade VII. In addition, we constructed a phylogenetic tree using proteins encoded by *BpNACs* and *AtNACs* (Figure 1), which facilitated us to better understand the functions of *BpNACs* via comparative analysis.

NAC proteins were considered to have undergone extensive expansion after the divergence of vascular plants [17]. We identified homologous genes of each *BpNAC* in *A. thaliana*, and then compared the results with the COG phylogenetic trees constructed by Jin et al. [69]. The results indicated that the *B. pendula* genome gained a total of 36 *NAC* genes by gene expansion. A total of 2, 1, 16, 3, 13, and 1 expanded *BpNACs* were distributed on clade I to VI of the COG phylogenetic trees, respectively. On the contrary, the *B. pendula* genome lost 26 *NAC* genes by gene contraction. A total of 11, 3, 10, and 2 *BpNACs* were lost on clade I, II, III, and V of the COG phylogenetic trees, respectively.

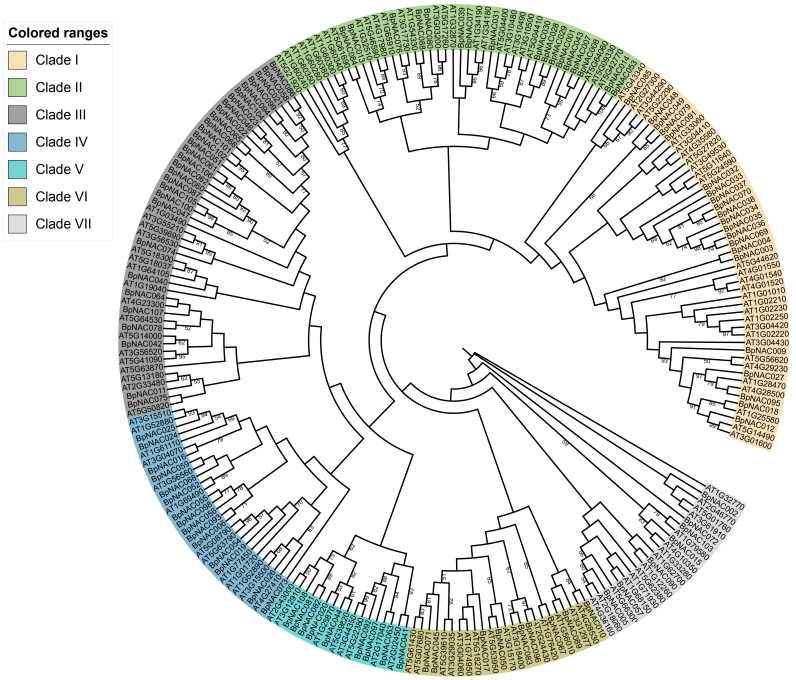

Figure 1. The phylogenetic tree of BpNACs and AtNACs made by RAxML using the maximum likelihood algorithm. Its confidence was evaluated with 1000 bootstraps.

3.5. Functional Analysis of the BpNAC Gene Family

Functional annotation of the *BpNAC* gene family was performed using eggNOG-mapper [48]. Of the 108 *BpNACs*, 80 were annotated with the GO database [71]. Of these, 28 *BpNACs* were annotated with GO terms of responses to stimuli, 14 were annotated to positive regulation of biological processes, and 18 were annotated to the impact on plant development processes. According to the COG database annotation [72], *BpNAC058* and *BpNAC086* may be involved in cell wall/membrane/envelope biogenesis. The annotation information is listed in Table S3.

Gene expression in eukaryotes is a highly coordinated process involving regulation at different levels. Promoter sequences of genes play a critical role in initiating the transcription of a gene. Identification and functional prediction of the promoter regulatory elements of the *BpNAC* gene family were performed using PlantCARE [51]. According to the PlantCARE result, a variety of motifs were distributed in the promoters of *BpNACs*, which indicates that *BpNACs* may be implicated in various biological processes of birch. All the promoter sequences of the *BpNACs* contain at least one light response motif (Table S4). In addition, many promoters of *BpNAC* contain motifs involved in methyl jasmonate, salicylic acid, and auxin responsiveness. The results indicated that *BpNACs* may be implicated in various aspects of plant development.

3.6. Tissue-Specific Expression Profiles of BpNACs

To further explore the function of *BpNACs* in birch development, we investigated the expression profiles of *BpNACs* in different tissues including roots, leaves, xylem, and flowers. We generated 11 samples from four tissues, with three biological replicates of flowers, leaves, and roots, and two biological replicates of xylem, which resulted in 435,991,087 reads. After removing reads of low quality and adaptor sequences, a total of 405,059,111 clean paired-end reads were obtained. The detailed information is summarized in Table S5.

We found that the *BpNACs* are widely expressed in *B. pendula* roots and xylem (Figure 2). *BpNAC053* is expressed at the highest level in all tissues when compared to other *BpNACs*. EggNOG-mapper predicts it to be the *BTF3*, which is considered to be the nascent polypeptide-associated complex *NAC*. *BpNAC017*, *BpNAC019*, *BpNAC052*, *BpNAC054*, *BpNAC071*, *BpNAC083*, and *BpNAC096* are

specifically expressed in roots when compared with other tissues. According to the eggNOG-mapper annotation, we found that these genes may play a role in promoting cell growth and cellulose synthesis. Combined with the annotations of the GO database, we speculated that these genes may be related to root development. In addition to root, the expression levels of *BpNACs* in xylem are higher than other tissues. The average TMM value of xylem is 24.63, which is much higher than 16.72 and 20.50 of flowers and leaves. This is slightly lower than 26.67 of roots. A total of 28 genes are expressed at the highest levels in xylem when compared to other tissues. Eight of these genes are specifically expressed in xylem (Table S6). Several *BpNACs*, i.e., *BpNAC002*, *BpNAC003*, *BpNAC004*, and *BpNAC072*, which are highly expressed in xylem, have been shown to be involved in the organic substance metabolic process. In addition, *BpNAC002* and *BpNAC072* also show the potential to synthesize the desired components of the cell wall.

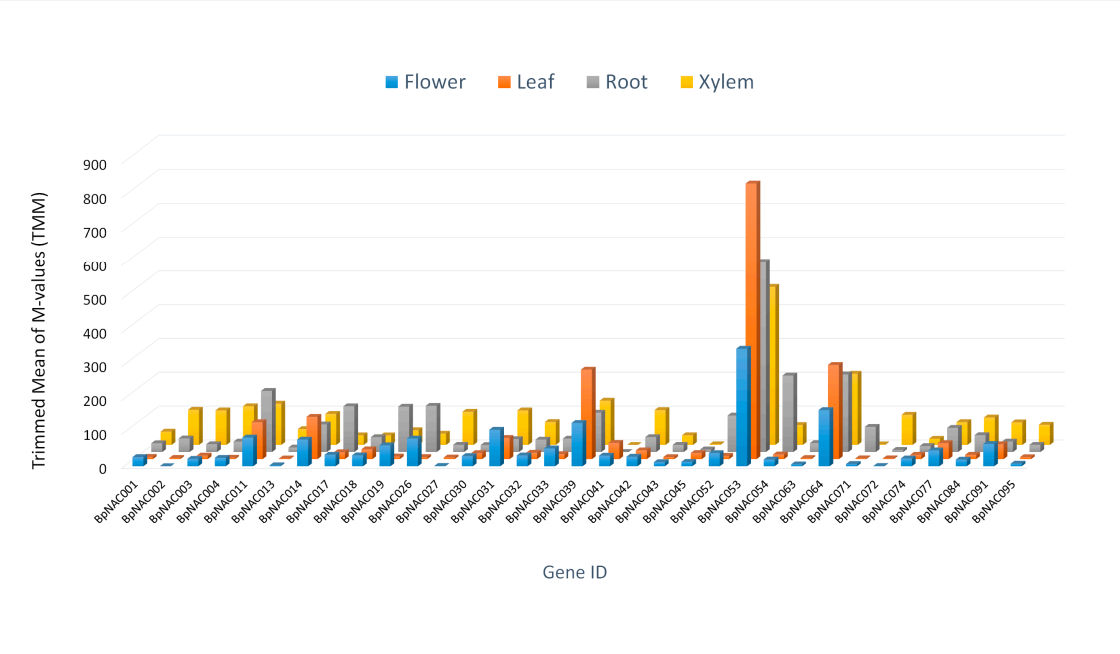

Figure 2. Tissue-specific expression profiles of *BpNACs*. Only genes with an average TMM value larger than 10 are shown.

3.7. Differential Expression Profiles of the BpNAC Gene Family under Cold Stress

Since birch is a cold-resistant species, we used RNA-Seq to analyze the expression profiles of *BpNACs* when exposed to a low temperature for 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, and 3 h (Table S7). We generated two biological replicates at 0 h, 1 h, 2 h, 2.5 h, 3 h, and three biological replicates at 0.5 h and 1.5 h. A total of 16 sets of data were obtained, including 212,356,980 reads. After removing reads of low quality, adaptor sequences, we got a total of 192,271,376 clean paired-end reads. The detailed information is summarized in Table S5.

A total of 21 *BpNACs* were differentially expressed in at least a one-time point under low-temperature stress (Table S7). Of the 21 low-temperature-responsive genes, 17 were up-regulated, and four were down-regulated by low-temperature stress. A total of 12 *BpNACs* were classified into Clade III, and half of them were significantly up-regulated by low-temperature treatment (FDR < 0.05), including *BpNAC016*, *BpNAC024*, *BpNAC025*, *BpNAC026*, *BpNAC052*, and *BpNAC107*. In addition, the other genes in this clade were slightly up-regulated (up-regulated, but FDR > 0.05). The results indicated that *BpNACs* in Clade III may be sensitive to low temperatures and may play a role during low-temperature treatment. In addition to Clade III, eight *BpNACs* in Clade V, including *BpNAC001*, *BpNAC004*, *BpNAC032*, *BpNAC035*, *BpNAC037*, *BpNAC043*, *BpNAC077*, and *BpNAC085*, were up-regulated by low-temperature treatment. On the contrary, no low-temperature response genes were found on Clade I.

We then analyzed the cis-acting elements in the promoter sequences of the 21 low-temperature-responsive *BpNACs* using PlantCARE [51]. The promoters of eight *BpNACs*, including *BpNAC016*, *BpNAC024*, *BpNAC043*, *BpNAC052*, *BpNAC063*, *BpNAC075*, *BpNAC077* and *BpNAC108*, contain low-temperature response-related motifs. In addition, the promoter of *BpNAC041* contains cis-acting elements involved in dehydration, a low temperature, and salt stress. All these results indicated that *BpNACs* may play important roles in cold resistance in birch.

3.8. Validation of DEGs Identified by RNA-seq Using qRT-PCR

We used qRT-PCR (Quantitative Real-time PCR) to validate the expression profiles of *BpNACs* after low temperature treatment. A total of 12 cold-responsive *BpNACs* were selected for qRT-PCR analysis. As shown in Figure 3, the qRT-PCR results indicated that all the 12 *BpNACs* were up-regulated by low-temperature stress, which was consistent with the results derived from the RNA-seq data. *BpNACs* including *BpNAC002*, *BpNAC004*, *BpNAC019*, *BpNAC025*, *BpNAC026*, *BpNAC032*, *BpNAC052*, *BpNAC054*, *BpNAC063*, and *BpNAC108* were up-regulated when exposed to a low temperature for 3 h and 6 h. *BpNAC003* and *BpNAC092* were up-regulated when exposed to a low temperature for 6 h. In general, this qRT-PCR result supports the reliability of the RNA-seq analysis.

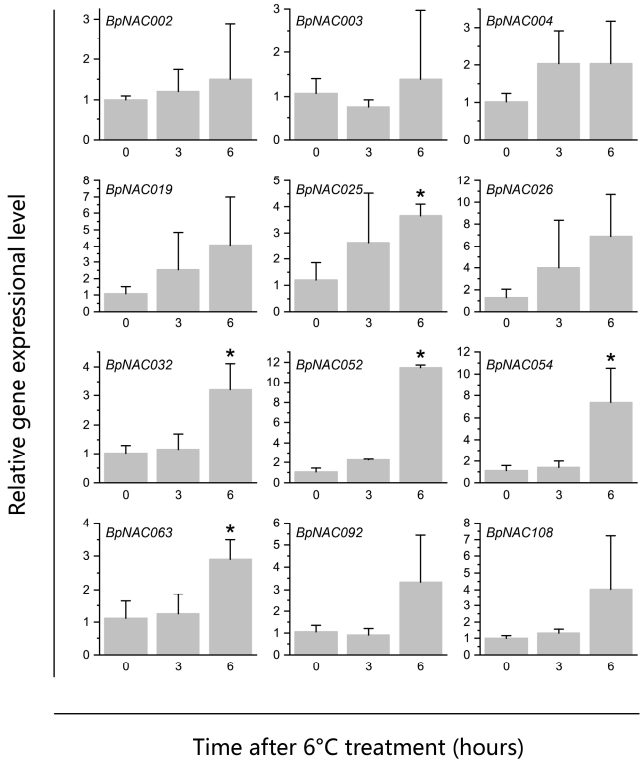

Figure 3. Expression analysis of selected *BpNACs* genes using quantitative real-time RT-PCR (RT-qPCR). Two-month old plants of birch were incubated at 6 °C for the indicated time. Data represent means ± SD in three replicates. Stars (*) indicate $p < 0.05$ (T-test).

3.9. Co-Expression of Low-Temperature Stress Genes in the BpNAC Gene Family

The gene co-expression network analysis is a system biology method for describing the correlation patterns among genes across RNA-Seq samples. We used WGCNA [73] to construct a TF-centered co-expression network for the 21 low-temperature response *BpNACs*. With a weight threshold of 0.1, we filtered out some branches with lower weights and only keep edges with strong connections. Lastly, 15 co-expression networks were constructed (Figure 4), of which seven networks contain less than 100 genes, and two contain more than 500 genes.

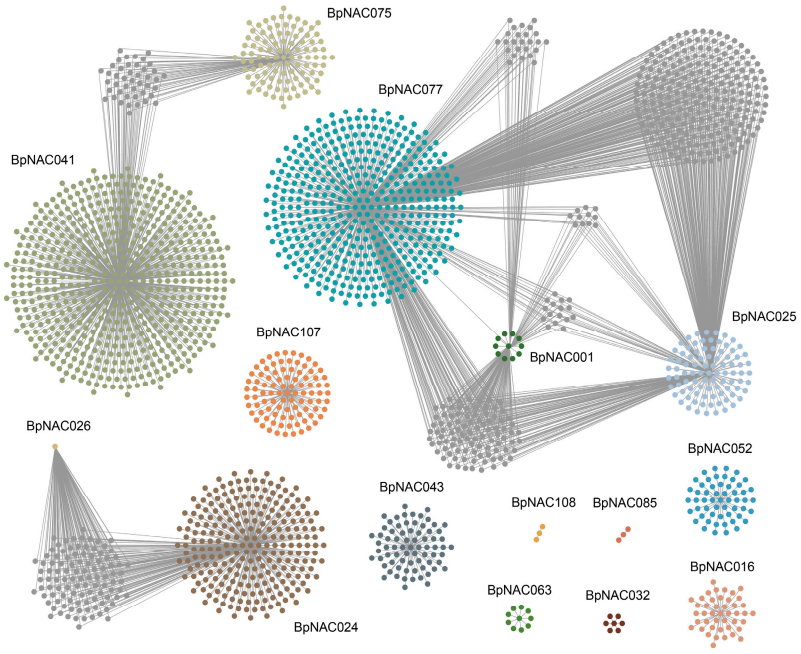

Figure 4. TF-centered co-expression network for the 15 low-temperature response *BpNACs*. Made by WGCNA and Cytoscape with a weight threshold of 0.1.

The largest co-expression network of *BpNAC077* contains 822 genes. By dissecting the cellular component of the co-expressed genes, we found that nearly 200 genes may be involved in membrane structure formation (Figure 5). The fluidity and stability of cell membranes are the basis for the survival of cells and even for the whole plants. It can effectively improve the cold resistance of plants by promoting the stability of membranes. The co-expressed network of *BpNAC041* contains 630 genes. GO annotation results indicated that one-third of the *BpNAC041* co-expressed genes are related to organelles, and they play a wide role in the growth and development of ribosomes, cell plates, spindles, the microtubule cytoskeleton, and other parts.

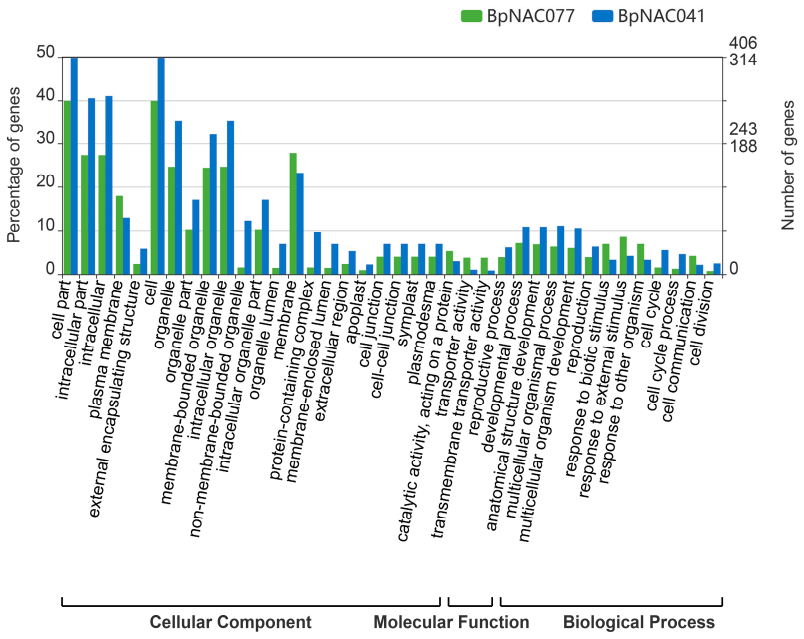

Figure 5. Statistical results of GO annotations co-expressing genes with *BpNAC077* and *BpNAC041*.

4. Discussion

The study of transcription factors is an important part of functional genomics research. Transcription factors control the expression of downstream genes and are key regulators of various biological processes [74]. The NAC family are plant-specific transcription factors. Previous studies [15,26,75] have shown that NACs are involved in plant growth and development, which pattern organs, biotic stress, and abiotic stress. However, current research on NAC transcription factors is mainly focused on model plants. In this study, we identified and investigated the function of *NACs* in a woody species, *Betula pendula*. Our study revealed that the *B. pendula* genome contains more *NAC* genes than some of the sequenced dicotyledonous angiosperms such as *Arabidopsis thaliana* [33], *Theobroma cacao* [76], and *Carica papaya* [77]. By examining the distribution of *BpNACs* on *B. pendula* chromosomes, we found that some of the *BpNACs* are clustered on the *B. pendula* genome. In addition, we found that some of the *BpNACs* are located on the intra-genome synthetic blocks. The results revealed that tandem duplication and the whole genome duplication have an important impact on the expansion of the *BpNAC* gene family.

Genetic analyses have revealed that *NACs* can affect the plant life cycle by regulating cell division [78]. In the *A. thaliana NTM1* mutant, a series of CDK (cyclin-dependent kinases) repressor genes are induced to express the inhibition of cell division, which results in delayed growth [79]. The expression of *AtNAP* is closely related to the senescence of *A. thaliana* leaves [80]. In our study, *BpNAC003*, *BpNAC004*, *BpNAC013*, *BpNAC032*, *BpNAC085*, and *BpNAC091* are homologous to *NTM1*/*AtNAP* of *A. thaliana* and are classified as Clade VI members. The results indicated that the Clade VI of *BpNACs* may be related to the growth of birch.

In this study, we identified *BpNACs* that were highly expressed in the xylem of birch, such as *BpNAC002*, *BpNAC003*, *BpNAC004*, *BpNAC013*, *BpNAC027*, *BpNAC042*, *BpNAC072*, and *BpNAC095*. These xylem-specific *BpNACs* may be involved in lignin biosynthesis. Compared to *A. thaliana*, we found that *BpNAC013* and *BpNAC042* are homologous to *AT4G17980* and *AT2G33480*, respectively. Pitaksaringkarn et al. [81] reported that *AT4G17980* (*ANAC071*) is involved in the development of inflorescence stems by regulating the expression of *XTH20* and *XTH19* in *A. thaliana*. Kim et al. reported that *AT2G33480* (*ANAC041*) directly regulates the expression of *CSLA9* [82], which belongs to one of the cellulose synthase-like genes [83]. It is interesting that *BpNAC002* and *BpNAC072* are homologous to *AT2G46770*, and *BpNAC027* and *BpNAC095* are homologous to *AT4G28500*, respectively. *AT2G46770* (*ANAC043*) is known as *NST1*, which regulates the secondary wall thickenings in anther walls and siliques, and an *NST1* promoter fusion was detected in various tissues in which lignified secondary walls develop [84]. *AT4G28500* (*ANAC073*), known as *SND2*, regulates genes involved in the development of secondary cell walls in *A. thaliana* fibers and increases the fiber cell area in Eucalyptus [85]. *BpNAC003* and *BpNAC004* are homologous to *AT4G35580* (*NTL9*), which encodes an NAC protein that contains a calmodulin-binding domain at the C-terminus of a protein. It is reported that *AT4G35580* functions as a calmodulin-regulated transcriptional repressor [86]. Wang et al. [87] found that *AT4G35580* may be a cell-wall related transcription factor based on a co-expression network analysis. *BpNAC005* is highly expressed in the xylem, which is homologous to *AT2G18060* (*VND1*). *VND1* could regulate the development of genes required for secondary cell wall biosynthesis with other members of this family. *BpNAC011* is highly expressed in all the four tissues, especially in the roots and xylem. Its homologous gene in *A. thaliana* is *AT5G13180* (*ANAC083*), which interacts with *VND7*. This acts as a master regulator of xylem vessel differentiation [88].

Plants are challenged by various environmental stresses. Low-temperature is one of the major factors that adversely impact plant development [89]. Plants have evolved multiple mechanisms to avoid cold stress [90]. Through a cold stress experiment, we identified 19 cold stress responsive *BpNACs*. By combining *A. thaliana* expression data [50], we found that *BpNAC026* and *BpNAC077* are homologous to *AT4G27410* (*ANAC072*) and *AT1G34190* (*ANAC017*), respectively, and they were up-regulated by cold stress in both *A. thaliana* and *B. pendula*. It is reported that *AT4G27410* acts as a transcriptional activator in the ABA-mediated dehydration response [91]. *AT1G34190* has been

shown to regulate mitochondrial retrograde responses and coordinate organelle function and a stress response [92].

Although the *NAC* genes are widely involved in growth and development as well as biotic and abiotic stress [75,93,94], the research on the complex regulatory network of *NAC* genes is still in its infancy. Given that only a few *BpNAC* genes have been well characterized in function, our results could help select candidate genes for further characterization.

5. Conclusions

This study aims to provide information on *Betula pendula NACs* regarding their potential physiological roles and the molecular mechanism associated. Using bioinformatics and phylogenetic analyses, we identified 114 *BpNACs* that were divided into seven subfamilies. The *BpNACs* gene structure, chromosome location, and BpNACs conserved protein motifs were identified. Using available *B. pendula* expression data, tissue expression profiles of the *BpNACs* were investigated and the results showed that the expression levels of *BpNACs* were higher in xylem and roots when compared to other tissues. In addition, RNA-seq analysis was used to determine the expression profiles of *BpNACs* when exposed to a low temperature. A total of 21 *BpNACs* were differentially expressed under a low temperature and associated gene co-expression networks were constructed. These analyses will help decipher the genetic information of the *NAC* genes in birch, and provide theoretical support for the screening and functional analysis of subsequent functional genes.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/9/741/s1. Figure S1: Exon-intron structures of *BpNACs*. The genomic structure information of the *BpNACs* was obtained from CoGe (https://genomevolution.org/coge/). Figure S2: Conserved amino acid motifs of the BpNAC proteins. Figure S3: Chromosomal distribution of *BpNAC* genes in *B. pendula*. Figure S4: Chromosomal distribution and gene duplications of the *BpNAC* genes. Figure S5: The phylogenetic tree of *BpNACs* made by RAxML. Its confidence was evaluated with 1000 bootstraps. Table S1: Primer pairs for real-time quantitative PCR. Table S2: Basic information of the putative truncated *BpNACs* without the NAC domain. Table S3: Functional annotation of the *BpNAC* gene family. Table S4: Promoter identification of the *BpNAC* gene family. Table S5: Summary of *B. pendula* RNA-Seq data. Table S6: Tissue-specific expression profiles of the *BpNAC* gene family. Table S7: Differential expression profiles of the *BpNAC* gene family under cold stress.

Author Contributions: Conceptualization, X.Z. and S.C. (Su Chen). Formal analysis, S.C. (Song Chen). Investigation, D.Z. Software, S.C. (Song Chen). Validation, X.L. and Q.L. Writing—original draft, S.C. (Song Chen) and S.C. (Su Chen). Writing—review & editing, X.Z. and S.C. (Su Chen).

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