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Genome-Wide Identification and Expression Analysis of the NAC Gene Family in *Kandelia obovata*, a Typical Mangrove Plant

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Abstract: The NAC (*NAM*, *ATAF1/2*, and *CUC2*) gene family, one of the largest transcription factor families in plants, acts as positive or negative regulators in plant response and adaption to various environmental stresses, including cold stress. Multiple reports on the functional characterization of NAC genes in *Arabidopsis thaliana* and other plants are available. However, the function of the NAC genes in the typical woody mangrove (*Kandelia obovata*) remains poorly understood. Here, a comprehensive analysis of NAC genes in K. obovata was performed with a pluri-disciplinary approach including bioinformatic and molecular analyses. We retrieved a contracted NAC family with 68 genes from the *K. obovata* genome, which were unevenly distributed in the chromosomes and classified into ten classes. These *Ko*NAC genes were differentially and preferentially expressed in different organs, among which, twelve up-regulated and one down-regulated *Ko*NAC genes were identified. Several stress-related cis-regulatory elements, such as LTR (low-temperature response), STRE (stress response element), ABRE (abscisic acid response element), and WUN (wound-responsive element), were identified in the promoter regions of these 13 *Ko*NAC genes. The expression patterns of five selected *Ko*NAC genes (*Ko*NAC6, *Ko*NAC15, *Ko*NAC20, *Ko*NAC38, and *Ko*NAC51) were confirmed by qRT-PCR under cold treatment. These results strongly implied the putative important roles of *Ko*NAC genes in response to chilling and other stresses. Collectively, our findings provide valuable information for further investigations on the function of *Ko*NAC genes.

Keywords: abiotic stress; cold stress; *Kandelia obovata*; mangrove; NAC transcription factor

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1. Introduction

Transcription factors (TFs) are of immense importance due to their crucial impact on controlling the transcription rate by binding to the cis-regulatory elements, resulting in activation or inhibition of the transcription level of target genes [1]. There are numerous types of TF families in plants, among which the NAC (*NAM*, *ATAF1/2*, and *CUC2*) family serves as one of the largest plant-specific TF families and is named after the *Petunia hybrida* E. Vilm. *NO APICAL MERISTEM* (NAM) [2] and *Arabidopsis thaliana* (L.) Heynh. genes *ATAF1/2* and *CUP-SHAPED COTYLEDON 2* (*CUC2*) [3]. A typical NAC protein contains an N-terminal conserved NAC domain for DNA binding and nuclear localization and a variable C-terminal region with transcriptional regulatory activity [4].

As a complex plant-specific family, the NAC genes with considerable quantities are present in a wide range of species. A large number of NAC TFs have been identified in various plants, including *A. thaliana* [5], *Actinidia eriantha* Benth. [6], *Asparagus officinalis* L. [7], *Betula pendula* Roth [8], *Hylocereus undatus* (Haw.) Britton & Rose [9], *Juglans mandshurica*

Maxim. [10], *Medicago sativa* L. [11], *Miscanthus sinensis* Andersson [12], banana (*Musa acuminata* Colla) [13], *Oryza sativa* L. [5], *Populus trichocarpa* Torr. & A. Gray ex Hook. [14], *Salix psammophila* C. Wang & Chang Y. Yang [15], *Solanum lycopersicum* L. [16], *Zanthoxylum bungeanum* Maxim. [17], and *Zea mays* L. [18]. Multiple lines of evidence illustrate that NAC genes act as positive or negative regulators involved in diverse biological processes, including plant response and adaptation to cold and other abiotic stresses [19,20]. *Ma*NAC1, one banana NAC TF, acts as a downstream target of MaICE1 and interacts with the C-repeat binding factor *Ma*CBF1, conferring fruit cold tolerance [21]. Two overexpressed NAC genes from *H. undatus*, *Hu*NAC20 and *Hu*NAC25, confer enhanced cold tolerance of transgenic *A. thaliana* plants [9]. Overexpression of *Mb*NAC25 from *Malus baccata* (L.) Borkh. improves the resistance against chilling stress through enhanced scavenging capability of reactive oxygen species (ROS) in transgenic *A. thaliana* plants [22]. The tomato NAC gene *NAM3* and its upstream regulator *miR164a* positively modulates cold tolerance by inducing ethylene synthesis in tomato plants [23]. *CaNAC035*, a novel NAC gene from *Capsicum annuum*, was shown to positively regulate cold stress in company with its upstream TF gene *Cab*HLH79 [24]. *Ca*NAC064, another NAC gene from *C. annuum*, is strongly induced by chilling stress and positively modulates cold stress tolerance via interacting with low temperature-induced haplo-proteinase proteins [25]. Additionally, NAC TFs also function as negative regulators in response to low temperature. Overexpression of *Md*NAC029, an apple NAC gene, reduces cold tolerance in apple and *A. thaliana* via a CBF-dependent pathway [26]. *Ma*NAC25 and *Ma*NAC28, two NAC genes from banana, negatively regulate cold tolerance in fruits by upregulating the expression levels of phospholipid degradation genes [27].

Mangroves are a dominant halophytic vegetation with significant ecological value in various tropical and subtropical coastal wetlands and are well-adapted to these highly stressful intertidal regions [28,29]. Among them, *Kandelia obovata* Sheue C.R., H.Y. Liu & J.W.H. Yong is regarded as a typical true mangrove due to its highest natural distribution latitude, indicating that *K. obovata* possesses stronger resistance against low temperature in contrast to other mangroves [30,31]. Various physiological evidences have shown that *K. obovata* displays better performance when exposed to chilling stress than other mangrove plants [32–34]. However, the underlying molecular mechanisms of cold response and adaptation in *K. obovata* are largely unknown. Here, we describe the genome-wide identification and expression analysis of *K. obovata* NAC (*Ko*NAC) genes in response to low temperature based on its available chromosome-level reference genome [35] with a pluri-disciplinary approach including bioinformatic and molecular analyses, hopefully providing valuable insights into the function of NAC genes in cold response and breeding for cold resistance.

2. Materials and Methods

2.1. Identification and Chromosomal Distribution of NAC TFs in K. obovata

The *K. obovata* chromosome-scale genome (2n = 2x= 36) was obtained from Genome Warehouse (https://bigd.big.ac.cn/gwh) (accessed on 8 March 2022) under accession number GWHACBH00000000 [35]. The Hidden Markov Model (HMM) file for NAM domain (PF02365) was downloaded from Pfam database (https://pfam.xfam.org/) [36] (accessed on 8 March 2022), and was used to retrieve the NAC proteins with a cut-off value of 0.001 by HMMER 3.3.2 (http://hmmer.org/download.html) [37] (accessed on 8 March 2022). BLASTP (basic local alignment search tool for proteins) against *K. obovata* genome data with *A. thaliana* NAC protein sequences (Table S1) retrieved from The *Arabidopsis* Information Resource (TAIR, https://www.arabidopsis.org/) [38] (accessed on 10 March 2022) was implemented (*e*-value = 0.001). Taking these two results together, the final members of the *Ko*NAC genes were acquired and verified by Pfam-Scan (*e*-value = 0.001, https://www.ebi.ac.uk/Tools/pfa/pfamscan/) (accessed on 12 March 2022) [39] and NCBI's conserved domain database (NCBI-CDD) (*e*-value = 0.001, https://www.ncbi.nlm.nih.gov/cdd/) (accessed on 12 March 2022) [40]. The basic in-

formation for *Ko*NAC gene, including chromosome localization, intron number, average intron length, protein length, and isoelectric point (*pI*) values was determined based on the genome database. The chromosomal distribution map of *Ko*NAC genes was drawn using MapChart 2.32 (https://www.wur.nl/en/show/Mapchart.htm) (accessed on 15 March 2022) [41].

2.2. Phylogenetic Analysis of NAC Proteins

The amino acid sequences of the NAC members of *K. obovata* and *A. thaliana* were aligned using Clustal X, and a neighbor-joining unrooted phylogenetic tree with 1000 bootstrap replications was constructed by MEGA 7.0 (www.megasoftware.net) (accessed on 15 March 2022) [42]. Finally, the tree was further modified by iTOL v6.5.8 (https://itol.embl.de/) (accessed on 15 March 2022) [43].

2.3. Gene Structure, Motif Identification, and Collinearity Analysis

The intron/exon structure of *Ko*NAC genes was determined with the online gene structure display server (http://gsds.gao-lab.org/) (accessed on 18 March 2022) [44]. The conserved motifs in *Ko*NAC proteins were identified by MEME suite v5.4.1 (http://meme-suite.org/) (accessed on 18 March 2022) [45]. The collinearity relationship of the *K. obovata* NAC genes between *A. thaliana* [5] and *P. trichocarpa* [14] were analyzed by MCScanX (http://chibba.pgml.uga.edu/mcscan2/) (accessed on 18 March 2022) [46]. These results were presented and visualized using TBtools (https://github.com/CJ-Chen/Tbtools) (accessed on 18 March 2022) [47].

2.4. Expression Analysis of KoNAC Genes Based on Public RNA-Seq Data

Two previously released RNA-seq data sets of *K. obovata* were introduced here to analyze the expression profiles of *Ko*NAC genes. The expression patterns of *Ko*NAC genes in eight organs (root, stem, leaf, flower, pistil, stamen, sepal, and fruit) were obtained according to the previously published transcriptomic data under the NCBI BioProject accession number PRJNA416402 (https://www.ncbi.nlm.nih.gov/bioproject) (accessed on 31 March 2022) [31]. The expression levels of *Ko*NAC genes in response to cold stress were determined based on the publicly released data from the NCBI BioProject under accession number PRJNA678025. These two RNA-seq data were remapped back to the *K. obovata* genome used here [35]. All expression data were normalized as fragments per kilobase of transcript per million fragments mapped (FPKM) values [48]. The differentially expressed genes (DEGs) related to chilling stress were defined under the criteria of fold change (FC) $\geq$ 1.5. The expression profiles of *Ko*NAC genes were visualized as heatmaps using TBtools [47].

2.5. Plant Materials and Treatment

The healthy mature propagules of the typical viviparous mangrove plant *K. obovata* were sampled from Guangxi Maoweihai Mangrove Nature Reserve, Qinzhou, China (21°37′23″ N, 108°44′13″ E) and cultured in the Mangrove Germplasm Resources Center (MGRC) of Guangxi Forestry Research Institute (GFRI) (Figure S1). The seedlings were grown in plastic pots containing sand and cultivated in a growth chamber at 28 °C and 75% humidity with a photoperiod of 14 h light/10 h darkness, and watered weekly with half-strength Hoagland's nutrient solution [49]. At the eight-leaf stage, the seedlings were treated under low temperature (4 °C) for 0 h, 6 h, 12 h, and 24 h, respectively. All treatments were performed with three replicates. The leaves were harvested, immediately frozen in liquid nitrogen, and stored at −80 °C for RNA extraction.

2.6. Cis-Regulatory Element Analysis of the KoNAC Genes

The upstream 1500 bp promoter sequences from the ATG start codon of the *Ko*NAC genes were retrieved from the *K. obovata* genome, and the cis-regulatory elements in the

promoter regions were predicted using Plant CARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (accessed on 31 March 2022) [50] and displayed by TBtools [47].

2.7. Quantitative Real-Time PCR Assays

Total RNA was extracted from the sampled leaves mentioned above using TRIzol (Invitrogen, http://www.invitrogen.com) (accessed on 20 April 2022). Quantitative real-time PCR (qRT-PCR) assays were conducted, as described previously [51], using an ABI PRISM 7500 Real-time PCR System (Applied Biosystem) with $2^{-\Delta\Delta CT}$ method [52]. The specific primers of *Ko*NAC genes used here are listed in Table S2. The actin gene (GWHTACBH010383.1) was used as an internal control. Student's *t*-test in statistical analysis was performed using Graphpad Prism 9.0.0 (https://www.graphpad-prism.cn/) (accessed on 28 April 2022).

3. Results

3.1. Genome-Wide Identification of the K. obovata NAC Genes

Two independent strategies for retrieval of *Ko*NAC genes from the *K. obovata* genome, HMM search and BLASTP, were used here. Taken together, 68 putative *Ko*NAC genes were identified and confirmed by PfamScan and NCBI-CDD. Based on their chromosome location, these *Ko*NAC genes were named *Ko*NAC1 to *Ko*NAC68 and unevenly distributed on 17 chromosomes (Chrs), with no *Ko*NAC gene present on Chr18 (Table 1, Figure 1). Detail-wise, nine *Ko*NAC genes were located on Chr08, six genes were located on both Chr03 and Chr12, and five *Ko*NAC genes each were located on Chr02, Chr04, Chr06, Chr09, and Chr17, while only one *Ko*NAC gene each was found on Chr07, Chr14, and Chr16.

Table 1. Basic information of *K. obovata* NAC genes.

Name	Gene ID	Class	Chromosome Position	Intron Number	Average Intron Length (bp)	Protein Length (aa)	*pI*
KoNAC1	GWHPACBH000260.1	VII	Chr01: 1952420-1954549	2	210	375	8.33
KoNAC2	GWHPACBH000261.1	X	Chr01: 1966355-1968293	2	213	381	6.38
KoNAC3	GWHPACBH001011.1	IV	Chr01: 9324213-9325936	2	179	268	9.76
KoNAC4	GWHPACBH001737.1	IX	Chr02: 545874-547928	2	540	314	4.72
KoNAC5	GWHPACBH002133.1	VI	Chr02: 5018997-5022392	2	627	414	4.56
KoNAC6	GWHPACBH002150.1	VII	Chr02: 5630019-5632845	2	557	291	6.26
KoNAC7	GWHPACBH002927.1	X	Chr02: 12147292-12149791	2	695	308	9.72
KoNAC8	GWHPACBH002942.1	VII	Chr02: 12271496-12273886	2	265	397	6.91
KoNAC9	GWHPACBH003351.1	VII	Chr03: 1575855-1576587	1	152	170	10.01
KoNAC10	GWHPACBH003542.1	X	Chr03: 2845984-2847971	3	82	358	8.29
KoNAC11	GWHPACBH003714.1	IV	Chr03: 4257070-4258248	2	188	215	10.07
KoNAC12	GWHPACBH004035.1	VI	Chr03: 8571932-8575841	7	267	573	4.35
KoNAC13	GWHPACBH004037.1	VI	Chr03: 8580877-8585584	3	922	432	5.87
KoNAC14	GWHPACBH004257.1	III	Chr03: 10268576-10273325	6	484	340	8.05
KoNAC15	GWHPACBH005193.1	VII	Chr04: 4833704-4835719	2	237	301	6.63
KoNAC16	GWHPACBH005487.1	II	Chr04: 8899908-8901721	2	518	259	7.96
KoNAC17	GWHPACBH005488.1	II	Chr04: 8902766-8904022	1	590	220	8.48
KoNAC18	GWHPACBH005795.1	VII	Chr04: 10981103-10983284	2	229	317	9.64
KoNAC19	GWHPACBH005903.1	III	Chr04: 11744358-11747114	4	148	304	5.55
KoNAC20	GWHPACBH005980.1	VII	Chr05: 171989-173574	2	182	285	8.57
KoNAC21	GWHPACBH006496.1	II	Chr05: 3585985-3588070	2	179	576	5.03
KoNAC22	GWHPACBH006945.1	VII	Chr05: 8286321-8289550	2	626	425	7.89
KoNAC23	GWHPACBH007161.1	VII	Chr05: 10223818-10225996	2	171	372	8.40
KoNAC24	GWHPACBH007671.1	IX	Chr06: 2167096-2168677	2	300	327	6.13
KoNAC25	GWHPACBH007697.1	VI	Chr06: 2347486-2351319	4	385	591	4.47
KoNAC26	GWHPACBH007806.1	VII	Chr06: 3191353-3193417	2	295	303	6.78
KoNAC27	GWHPACBH007934.1	II	Chr06: 4507683-4512207	5	551	365	5.29
KoNAC28	GWHPACBH008265.1	X	Chr06: 8671993-8674297	2	374	441	6.35
KoNAC29	GWHPACBH009271.1	VI	Chr07: 7943036-7946752	5	272	592	4.37
KoNAC30	GWHPACBH009626.1	X	Chr08: 595220-599512	2	1624	300	7.03
KoNAC31	GWHPACBH009686.1	VII	Chr08: 1067480-1068977	2	114	343	9.60
KoNAC32	GWHPACBH009687.1	VII	Chr08: 1074862-1076943	2	171	347	8.70
KoNAC33	GWHPACBH009711.1	X	Chr08: 1347371-1350316	2	636	357	7.67
KoNAC34	GWHPACBH009970.1	X	Chr08: 3208565-3211847	2	1096	286	7.10
KoNAC35	GWHPACBH010230.1	V	Chr08: 6804416-6807401	4	274	345	5.82
KoNAC36	GWHPACBH010231.1	V	Chr08: 6808948-6810099	3	123	261	4.47
KoNAC37	GWHPACBH010248.1	IX	Chr08: 6936960-6938647	2	191	366	7.37
KoNAC38	GWHPACBH010352.1	VII	Chr08: 7704131-7705968	2	435	305	6.78
KoNAC39	GWHPACBH010982.1	VII	Chr09: 1762983-1763731	1	160	176	9.98

Table 1. *Cont.*

Name	Gene ID	Class	Chromosome Position	Intron Number	Average Intron Length (bp)	Protein Length (aa)	*pI*
KoNAC40	GWHPACBH011181.1	II	Chr09: 3089069-3090025	1	112	236	6.50
KoNAC41	GWHPACBH011224.1	X	Chr09: 3378802-3380434	2	115	356	7.92
KoNAC42	GWHPACBH011287.1	VIII	Chr09: 3895422-3899009	2	1261	243	4.62
KoNAC43	GWHPACBH011356.1	X	Chr09: 4526977-4529037	2	553	318	7.21
KoNAC44	GWHPACBH012132.1	III	Chr10: 2632918-2637871	5	584	373	7.08
KoNAC45	GWHPACBH012473.1	VI	Chr10: 5929512-5932298	3	255	574	4.20
KoNAC46	GWHPACBH012540.1	X	Chr10: 7425092-7428318	3	579	333	9.19
KoNAC47	GWHPACBH012930.1	VIII	Chr11: 4340404-4343638	4	342	326	5.37
KoNAC48	GWHPACBH013040.1	IX	Chr11: 5432389-5434082	2	285	354	6.19
KoNAC49	GWHPACBH013264.1	IX	Chr11: 7036403-7038056	3	165	331	6.75
KoNAC50	GWHPACBH013622.1	VII	Chr12: 1361271-1363117	2	215	294	9.80
KoNAC51	GWHPACBH013650.1	V	Chr12: 1584267-1587516	4	219	534	4.43
KoNAC52	GWHPACBH013651.1	V	Chr12: 1588784-1591086	4	165	336	6.58
KoNAC53	GWHPACBH013779.1	X	Chr12: 3635952-3638945	2	591	354	8.79
KoNAC54	GWHPACBH013961.1	X	Chr12: 5551002-5553482	3	571	256	9.26
KoNAC55	GWHPACBH014293.1	III	Chr12: 7828847-7830888	2	494	296	8.61
KoNAC56	GWHPACBH014606.1	VII	Chr13: 304056-306202	2	230	255	9.29
KoNAC57	GWHPACBH014849.1	VII	Chr13: 2243283-2244839	2	109	265	7.90
KoNAC58	GWHPACBH015137.1	III	Chr13: 8039942-8044242	5	466	461	4.62
KoNAC59	GWHPACBH015463.1	VII	Chr14: 3448051-3450062	2	222	255	9.64
KoNAC60	GWHPACBH015782.1	V	Chr15: 43374-47367	5	324	638	4.57
KoNAC61	GWHPACBH015958.1	IX	Chr15: 1512975-1513703	1	125	162	9.55
KoNAC62	GWHPACBH016068.1	VIII	Chr15: 2698343-2700471	4	261	306	9.33
KoNAC63	GWHPACBH016810.1	III	Chr16: 4714504-4716464	2	516	304	9.25
KoNAC64	GWHPACBH017115.1	VII	Chr17: 1665396-1666616	2	119	245	8.31
KoNAC65	GWHPACBH017251.1	IV	Chr17: 2901510-2902567	1	103	314	7.77
KoNAC66	GWHPACBH017590.1	VII	Chr17: 5655463-5657073	2	147	358	8.88
KoNAC67	GWHPACBH017594.1	VII	Chr17: 5688319-5689928	2	146	358	8.88
KoNAC68	GWHPACBH017666.1	III	Chr17: 6866174-6868665	2	727	325	7.67

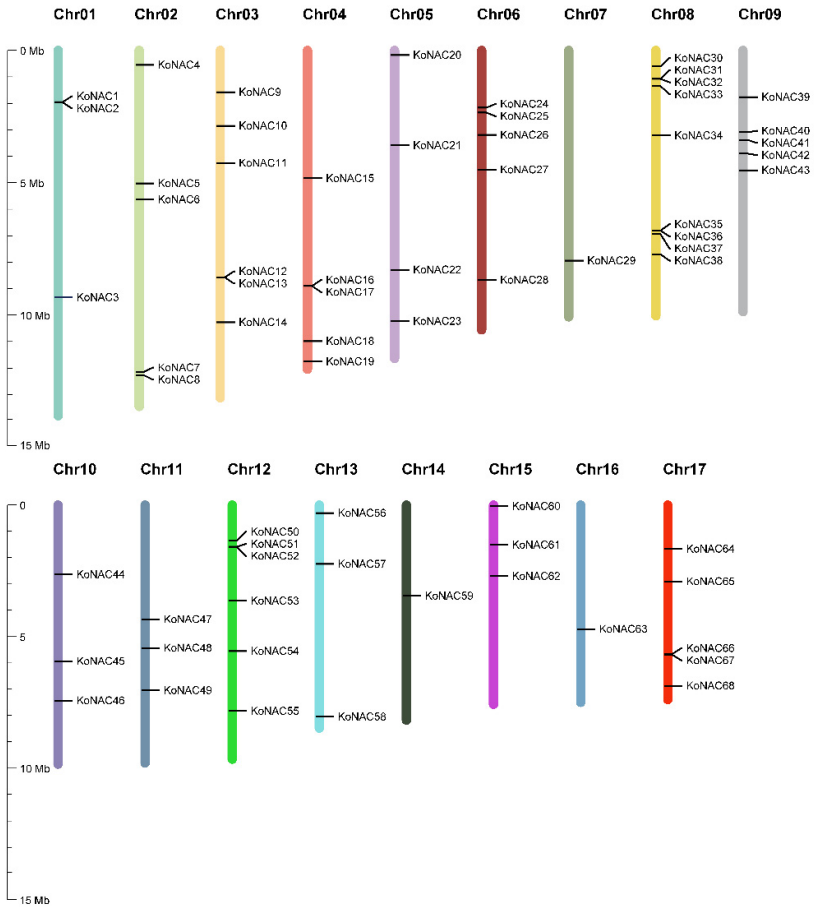

Figure 1. Distribution of *Ko*NAC genes in *K. obovata* genome. The 68 *Ko*NAC genes were unevenly distributed on 17 chromosomes (Chr01–Chr17) denoted in different colors, while no *Ko*NAC gene was found on Chr18. Values on the y-axis indicate the chromosome length and gene position.

Moreover, every *Ko*NAC gene contained one or more introns with an average length of 371 bp, while the proteins encoded by *Ko*NAC genes ranged from 162 amino acid (aa) residues (*Ko*NAC61) to 638 aa (*Ko*NAC60) in length, with an average length of 344 aa. The pI values varied from 4.20 (*Ko*NAC45) to 10.07 (*Ko*NAC11), over half of the members (39/68) exhibiting $pI > 7$ (Table 1).

3.2. Phylogenetic Analysis and Classification of KoNAC Proteins

To illustrate the phylogenetic relationship among *K. obovata* and *Arbidopsis thaliana* NAC proteins, a neighbor-joining phylogenetic tree was constructed with 68 *Ko*NAC proteins and 105 *At*NAC proteins (Table S1). The result showed that the 173 NAC proteins could be classified into ten classes, namely, Class I to Class X (Figure 2). Obviously, Class VII, with 21 *Ko*NACs and 24 *A*NACs was the largest class, followed by Class X with 12 *Ko*NACs and 14 *A*NACs. Other classes contained no more than 10 *Ko*NACs each. Specially, no *Ko*NAC belonged to Class I (Figure 3).

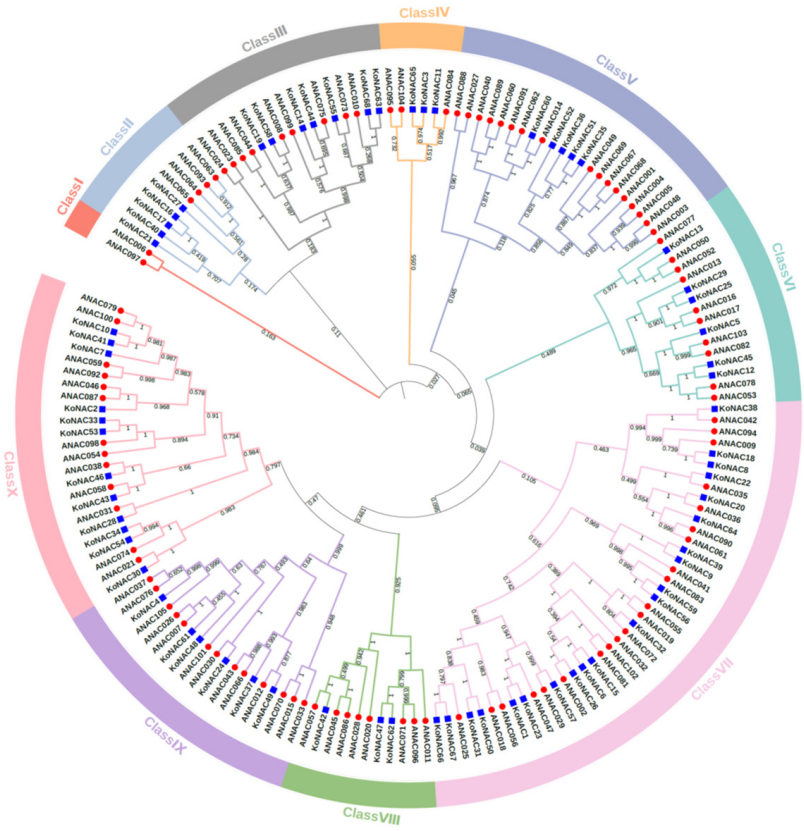

Figure 2. Phylogenetic analysis of the NAC proteins from *K. obovata* and *A. thaliana*. The unrooted tree was constructed by MEGA 7.0 based on the neighbor-joining (NJ) method with 1000 bootstrap replications. The NAC proteins of *K. obovata* (*Ko*NAC) in blue squares, and *A. thaliana* (*At*NAC) in red circles were classified into ten classes represented with different colors.

To better understand the phylogenetic relationship and classification of *Ko*NAC genes, the gene structure and motif organization of the 68 *Ko*NAC genes were analyzed. Each *Ko*NAC gene had one or more introns and contained no more than six exons, while over half of the *Ko*NAC genes (41/68) contained three exons (Figure 4c). Additionally, a total of 10 conserved motifs were queried within all *K. obovata* NAC proteins. Most motifs were located within the N-terminal region (Figure 4b), and motif 1, motif 2, motif 4, and motif 5 were the common elements in *Ko*NAC genes. Clearly, these results showed that the *Ko*NAC genes in the same phylogenetic cluster harbored similar gene structures and motif compositions (Figure 4), which further supported the evolutionary relationship of *Ko*NAC genes demonstrated above.

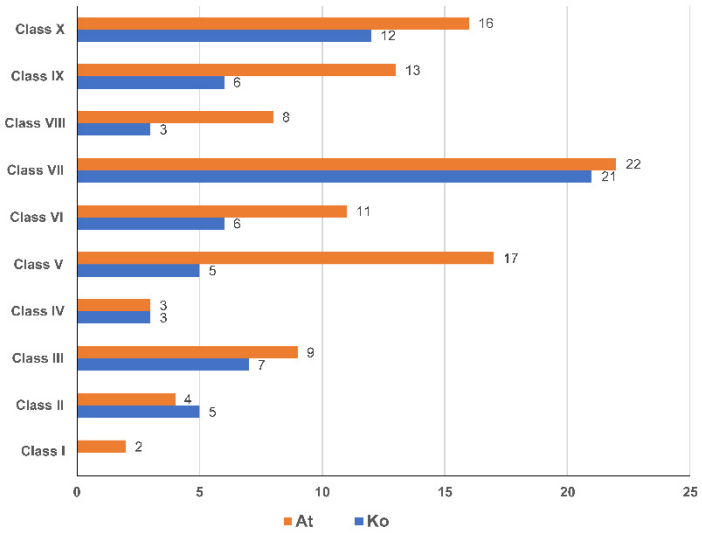

Figure 3. Classification comparison between *K. obovata* (*Ko*) and *A. thaliana* (*At*) NAC proteins.

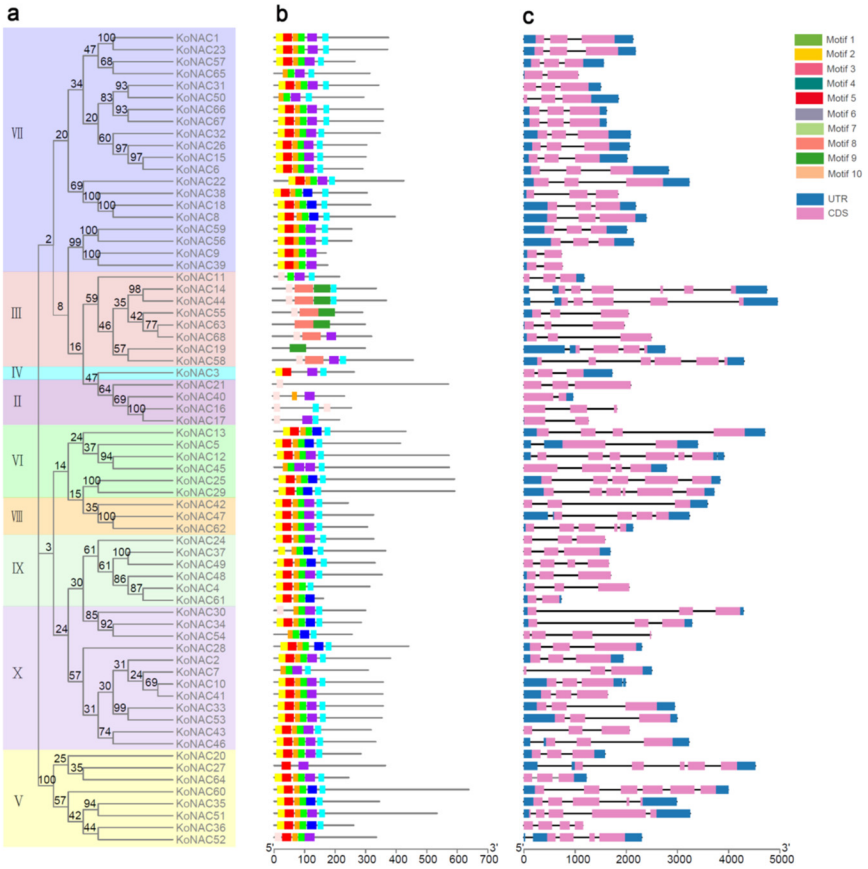

Figure 4. Gene structure and motif organization of the *Ko*NAC genes. (**a**) The unrooted NJ tree of *Ko*NAC proteins was constructed using MEGA 7.0, while different classes were represented in different colors. Bootstrap values from 1000 replicates are shown on the nodes. (**b**) Ten different conserved motifs of *Ko*NAC proteins were identified using MEME software. Different colored boxes indicate different motifs. (**c**) The structures of *Ko*NAC genes are shown, including UTR regions (blue box), exons (grey boxes), and introns (black lines). UTR, untranslated region; CDS, coding sequence of a gene.

3.3. Collinearity Analysis of KoNAC Genes

It is well-known that *P. trichocarpa* is a typical model plant for functional genomics and molecular studies in woody species. Moreover, *K. obovata* (Rhizophoraceae) and *P. trichocarpa* (Salicaceae) belong to the same order, Malpighiales (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) (accessed on 18 March 2022). Therefore, to better investigate the evolutionary relationship of NAC genes, the collinearity analysis was performed based on the genomes of *A. thaliana* and *P. trichocarpa* (Figure 5). There were 16,355 collinear gene pairs between *K. obovata* and *A. thaliana* identified, among which 52 orthologous gene pairs between *Ko*NACs and *At*NACs were obtained (Figure 5a, blue lines). Meanwhile, a total of 26,594 collinear gene pairs between *K. obovata* and *P. trichocarpa* were available, among which 54 orthologous gene pairs between *KoNAC*s and *Pt*NACs were determined (Figure 5b, purple lines). Taken together, 49 common *Ko*NAC genes shared homologous relationships with both *A. thaliana* and *P. trichocarpa* NAC genes (Table S3), implying these genes might function in a similar manner. In the meantime, there were 11 non-orthologous *Ko*NAC genes (*Ko*NAC13, *Ko*NAC16, *Ko*NAC17, *Ko*NAC21, *Ko*NAC40, *Ko*NAC50, *Ko*NAC53, *Ko*NAC63, *Ko*NAC65, *Ko*NAC67, and *Ko*NAC68) compared to NAC genes of *A. thaliana* and *P. trichocarpa*. These genes displayed different structure (Figure 4), among which four genes (*Ko*NAC16, *Ko*NAC17, *Ko*NAC21, *Ko*NAC40) clustered as the subgroup of Class II (Figure 2).

(**a**) (**b**)

Figure 5. Collinearity analysis of *Ko*NAC genes. (**a**) Genes in 5 chromosomes of *A. thaliana* (*At*Chrs), in orange, and 18 chromosomes of *K. obovata* (*Ko*Chrs), in cyan, are introduced here. The orthologous pairs between *Ko*NACs and *A*NACs are highlighted in blue. The gene pairs among *A*NACs are colored in yellow, while the gene pairs among *KoNAC*s are colored in red. (**b**) Genes in 19 chromosomes of *P. trichocarpa* (*Pt*Chrs), in pink, and 18 chromosomes of *K. obovata* (*Ko*Chrs), in cyan, are introduced here. The orthologous pairs between *Ko*NACs and *Pt*NACs are highlighted in purple. The gene pairs among *Pt*NACs are colored in green, while the gene pairs among *Ko*NACs are colored in red.

3.4. Expression Patterns of KoNAC Genes in Different Organs

To gain an insight into the function of NAC genes in *K. obovata*, the expression levels of all *Ko*NAC genes in various organs, including root, stem, leaf, flower, pistil, stamen, sepal, and fruit were determined based on previously published RNA-seq data of *K. obovata*. Noticeably, the expression patterns of *Ko*NAC genes were not in a constitutive mode, whereas they were differentially and preferentially expressed in different organs (Figure 6, Table S4). For example, 28 out of 68 *Ko*NAC genes were highly expressed in roots, while 18 *Ko*NAC genes were preferentially expressed in leaves, and 13 *Ko*NAC genes were mainly expressed in fruits.

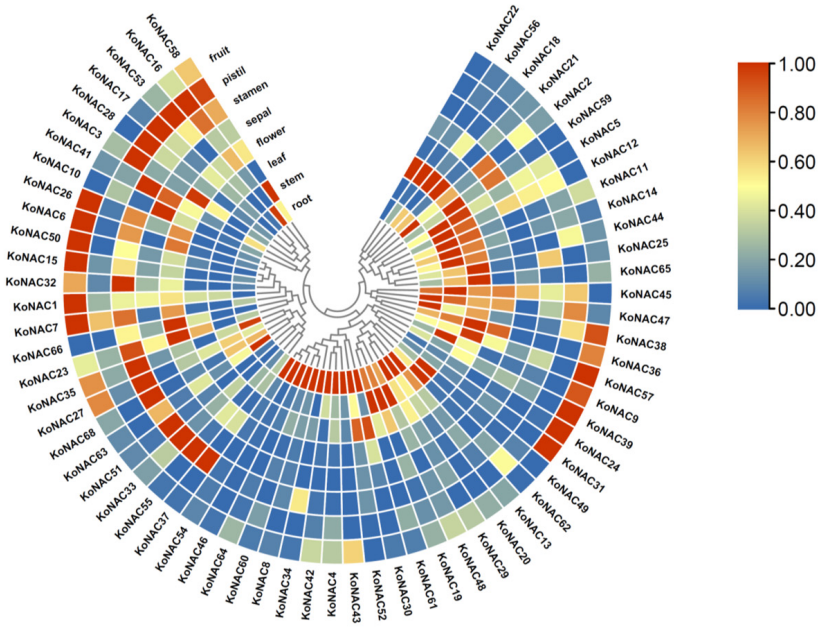

Figure 6. Expression patterns of *Ko*NAC genes in different organs. The transcript levels of the *Ko*NAC genes in eight organs of *K. obovata* were determined based on published transcriptomic data (NCBI BioProject: PRJNA416402). The color scale indicates increasing expression levels from blue to red. Deeper red colors represent higher expression levels, while darker blue colors indicate lower values.

3.5. Expression Analysis of KoNAC Genes under Cold Treatment

To gain more insight into the function of *Ko*NAC genes, the expression profiles of these genes under cold treatment were detected based on the public transcriptomic data of *K. obovata*. There were 13 *Ko*NAC genes differentially expressed in response to chilling stress, among which one down-regulated *Ko*NAC gene (*Ko*NAC51) and 12 up-regulated *Ko*NACs (*Ko*NAC6, *Ko*NAC11, *Ko*NAC15, *Ko*NAC20, *Ko*NAC24, *Ko*NAC26, *Ko*NAC32, *Ko*NAC35, *Ko*NAC38, *Ko*NAC41, *Ko*NAC62, and *Ko*NAC68) were available (Figure 7a, Table S5). Specifically, four different genes, *Ko*NAC6, *Ko*NAC15, *Ko*NAC20, and *Ko*NAC38, were largely upregulated with higher and more significant values after treatment. The expression levels of these four up-regulated and one down-regulated *Ko*NACs were confirmed by qRT-PCR (Figure 7c,d), implying that these *Ko*NAC genes might act as positive or negative regulators in response to chilling stress.

3.6. Stress-Related Cis-Regulatory Elements Identified in KoNAC Genes

To obtain more evidence for the differentially expressed *Ko*NAC genes on stress responses, the cis-regulatory elements in the promoter regions of these 13 *Ko*NAC genes were predicated. Consequently, 8 well-known stress-related elements were available (Figure 7b, Table S6). LTR (low-temperature response; CCGAAA), a core cis-acting element involved in cold stress response, was present in the majority of the detected *Ko*NAC genes. STRE (stress response element; AGGGG) and ARE (antioxidant response element; AAACCA) were two types of regulatory elements in rapid response to anaerobic stress and environmental stimuli. ABRE (abscisic acid response element; ACGTG), ERE (ethylene response element; ATTTTAAA), and TGACG-motifs were responsible for stress induction by three major stress-related hormones, ABA, ethylene, and methyl jasmonate (MeJA), respectively. Additionally, two biotic stress-responsive elements, WRE3 (wound-response element 3; CCACCT) and WUN-motif (wound-responsive element; AAATTACT) were found in several detected promoters, as well.

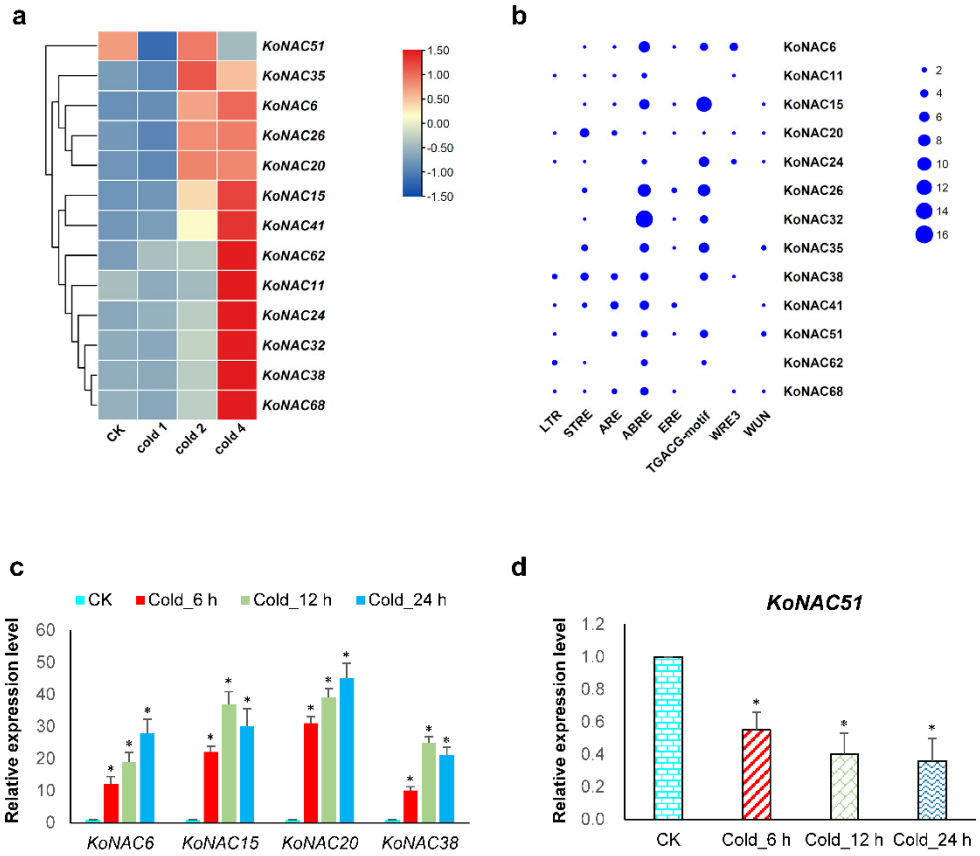

Figure 7. Expression analysis of *Ko*NAC genes under cold treatment. (**a**) The transcript levels of the *Ko*NAC genes in response to cold were determined based on publicly available RNA-seq data (NCBI BioProject: PRJNA678025). Deeper red colors represent higher expression levels of up-regulated *Ko*NAC genes, while darker blue colors indicate higher values of down-regulated *Ko*NAC genes. Cold 1, first-time cold treatment; Cold 2, second-time cold treatment; Cold 4, fourth-time cold treatment. (**b**) The cis-regulatory elements in the promoters of the 13 *Ko*NAC genes were predicated by PlantCARE. Eight well-known stress-related elements were identified. The size of the blue ball indicates the number of the elements in the *Ko*NAC promoters. Expression levels of four upregulated *Ko*NAC genes (**c**) and one down-regulated *Ko*NAC (**d**) under cold treatment were confirmed by qRT-PCR. Three independent experiments were performed. The actin gene in *K. obovata* acted as the internal control. Asterisks indicate significant differences compared with CK by Student's *t*-test. *, $p < 0.05$.

4. Discussion

The NAC gene family, one of the largest TF families in plants, was used as positive or negative regulators in response to environmental stimuli including cold stress [19]. Multiple investigations on the functional characterization of NAC genes were reported for *A. thaliana* [5,53,54], *P. trichocarpa* [14], and other plants [19]. However, the function of the NAC genes in the typical woody mangrove *K. obovata* responding to abiotic stresses remains largely unknown. Here, we identified a contracted NAC gene family with 68 members from the *K. obovata* genome. These *Ko*NAC genes were differentially and preferentially expressed in various organs, and 13 *Ko*NAC genes were differentially expressed under cold treatment based on the publicly available RNA-seq data.

*Ko*NAC proteins, in company with *A. thaliana* NAC proteins were categorized into 10 classes according to phylogenetic analysis. Obviously, the *K. obovata* NAC family exhibited a significant contraction in number compared to the NAC families in *A. thaliana* [5], *P. trichocarpa* [14], and other plants [15,16], and the decreased *Ko*NAC genes in class V, class VIII, and class IX mainly contributed to the contraction (Figure 3). These results are consistent with the previous findings [35], and the contraction might relate to the evolu-

tionary adaption to the intertidal zones. Moreover, 49 genes from the contracted *Ko*NAC family shared orthologous relationships with the NAC genes of *A. thaliana* and *P. trichocarpa*, implying these genes might have similar functions [55]. Additionally, compared to *At*NAC and *Ko*NAC genes, there existed 11 non-orthologous *Ko*NAC genes, among which, four genes (*Ko*NAC16, *Ko*NAC17, *Ko*NAC21, and *Ko*NAC40) clustered in class II (Figure 2). Another non-orthologous gene, *Ko*NAC68, was induced under cold treatment, implying it might potentially function in response to cold stress in *K. obovata* (Figure 7a). More attention should be paid to these non-orthologous genes, and functional investigations of these genes will provide valuable knowledge about mangrove species.

To explore the function of NAC genes in *K. obovata*, the expression patterns of all *KoNAC*s in various organs were determined. In contrast to the constitutive expression patterns of other gene families [56–58], *KoNAC* genes were differentially and preferentially expressed in different organs. For instance, there were 28 *Ko*NAC genes expressed highly in roots, 18 *Ko*NAC genes expressed preferentially in leaves, and 13 *Ko*NAC genes expressed mainly in fruits. Referentially, the root-expressed gene *Os*NAC2 modulated root development in rice by involving the crosstalk of auxin and cytokinin pathways [59]. The *A. thaliana* rosette-expressed gene, *ANAC087*, positively regulated rosette development and leaf senescence [60]. *FaRIF*, a strawberry NAC gene, was reported as one key regulator controlling fruit ripening [61]. This evidence implied that these organ-specific *Ko*NAC genes might function as key regulators in organ development. Moreover, the organ expression patterns of NAC genes in *K. obovata* were not similar to that in *A. thaliana* and other plants. For instance, *Ko*NAC46 and *Ko*NAC54 were primarily expressed in roots (Figure 6), however, *A*NAC048 and *A*NAC074, the closest orthologues of these two *Ko*NAC genes (Figure 2), respectively, were expressed in different organs. *A*NAC048 was involved in vascular development [62], and *A*NAC074 positively regulated programmed cell death of stigmatic tissue in *A. thaliana* [63]. Therefore, functional characterization of the *Ko*NAC genes primarily expressed in roots distinct from other plants should be deeply covered in the future.

To better understand the roles of *Ko*NAC genes, the expression analysis under chilling stress was performed based on the public transcriptomic data. In total, 13 out of 68 *Ko*NAC genes were differentially expressed under cold treatment. Among them, *Ko*NAC51 was the only down-regulated gene, whereas its closest homologue *Ko*NAC35 was up-regulated after treatment, implying these two class V genes might function oppositely in response to cold stress. Half of the up-regulated genes (*Ko*NAC6, *Ko*NAC15, *Ko*NAC20, *Ko*NAC26, *Ko*NAC32, and *Ko*NAC38) belonged to the class VII subgroup (Figures 2 and 7a). Particularly, *Ko*NAC6, *Ko*NAC15, and *Ko*NAC26 clustered together and shared high sequence similarity to their closest orthologs *A*NAC002, *A*NAC081, and *A*NAC102 in *A. thaliana*. *A*NAC002 (*ATAF1*) was reported to serve as dual regulators responsive to abiotic and biotic stresses [64–66]. *A*NAC081 (*ATAF2*) was rapidly induced by pathogen attack and involved in plant defense [67,68], while *A*NAC102 was responsive to low-oxygen and high-light stresses [69,70]. Overexpression of *Ml*NAC5, another closest ortholog of *Ko*NAC26 and *A*NAC002 from *Miscanthus lutarioriparius* L.Liu ex S.L.Chen & Renvoize, led to enhanced tolerance to cold and drought stresses in *A. thaliana* [71]. Additionally, three closest orthologs of *Ko*NAC32, *A*NAC019, *A*NAC055 and *A*NAC072, were required for drought tolerance in *A. thaliana* [72], among which *A*NAC019 and *A*NAC055 displayed a dual function in regulating ABA response and jasmonate response [73,74]. *A. thaliana A*NAC042, the closest ortholog of *Ko*NAC38, conferred stress tolerance through regulating phytohormone metabolism and signaling [75–77]. Moreover, various stress-related cis-regulatory elements were identified from the promoters of these *Ko*NAC genes (Figure 7b). The LTR element is an indispensable cis-acting element in plant response to low temperature [78,79]. Deletion of the LTR element will result in complete loss of promoter activity under cold stress [79]. STRE is a common cis-regulatory element in eukaryotes, and involved in response to multiple environmental stimuli [80]. ARE is an antioxidant response element in rapid response to anaerobic stress [81]. Meanwhile, ABRE, ERE, and TGACG motifs are three major types of elements related to plant hormones (ABA, ethylene, and MeJA) [82–84]. Among them, ABRE

and TGACG motifs are enriched in the majority of the detected *Ko*NAC genes, implying these *Ko*NAC genes might respond to stresses via hormone-mediated pathways [85]. Additionally, both WRE3 and WUN motifs are biotic stress-responsive elements and present in several detected promoters as well, implying the *Ko*NAC genes might function in response to biotic stresses [86,87]. Taken together, these findings suggest that these *Ko*NAC genes may be involved responses to other abiotic or biotic stresses in addition to the cold response, providing auxiliary evidence for these *Ko*NAC genes in response to abiotic and biotic stresses. To know more about the function of *Ko*NAC genes, further investigation and more proof are required.

5. Conclusions

In the present study, a pluri-disciplinary work concerning comprehensive analysis of the *Ko*NAC gene family was performed. We identified a contracted NAC TF family containing 68 genes from the genome of the typical mangrove plant *K. obovata* based on bioinformatic analysis. These *Ko*NAC genes were unevenly distributed in 17 chromosomes of *K. obovata*. The NAC genes of *K. obovata* and *A. thaliana* were classified into ten classes, while no *Ko*NAC gene belonged to class I. Obviously, the decreased members of class V, class VIII, and class IX mainly contributed to the contraction of the *Ko*NAC family. *Ko*NAC genes were differentially and preferentially expressed in different organs. Among them, 13 *Ko*NAC genes were rapidly induced by chilling stress. The expression patterns of five selected *Ko*NAC genes (*Ko*NAC6, *Ko*NAC15, *Ko*NAC20, *Ko*NAC38, and *Ko*NAC51) were confirmed by qRT-PCR. Additionally, several stress-related cis-acting elements were detected in the promoter regions of these *Ko*NAC genes, implying *Ko*NAC genes might participate in multiple stress responses. Summarily, our findings will provide positive references for further investigations on functional characterization of *Ko*NAC genes in stress responses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb44110381/s1, Figure S1: Morphological features of *K. obovata*; Table S1: The basic information of *A. thaliana* NAC genes; Table S2: The primers of *Ko*NAC genes in this study; Table S3: Common orthologous gene pairs of *K. obovata* between *A. thaliana* and *P. trichocarpa*; Table S4: The FPKM values of *Ko*NAC genes expressed in different organs; Table S5: The FPKM values of differentially expressed *Ko*NAC genes under cold treatment; Table S6: Number of stress-related cis-regulatory elements in the promoter regions of the differentially expressed *Ko*NAC genes.

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31 March 2022). The RNA-seq data related to cold treatment in *K. obovata* was obtained from NCBI BioProject under accession number PRJNA678025.

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