Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2023.029350

ARTICLE

Genome-Wide Analysis of the *KANADI* Gene Family and Its Expression Patterns under Different Nitrogen Concentrations Treatments in *Populus trichocarpa*

Minghui Niu[1,#], Heng Zhang[1,#], Xiangyang Li[1], Zhibao Hu[1], Hongjiao Zhang[2,3], Zhiru Xu[2,3], Chunpu Qu[1,4,*] and Guanjun Liu[1,*]

[1]State Key Laboratory of Tree Genetics and Breeding (Northeast Forestry University), School of Forestry, Northeast Forestry University, Harbin, 150040, China

[2]Key Laboratory of Saline-Alkali Vegetation Ecology Restoration (Northeast Forestry University), Ministry of Education, Harbin, 150040, China

[3]College of Life Science, Northeast Forestry University, Harbin, 150040, China

[4]College of Forestry, Guizhou University, Guiyang, 550025, China

[*]Corresponding Authors: Chunpu Qu. Email: qcp_0451@163.com; Guanjun Liu. Email: liuguanjun2003@126.com

[#]The first two authors have contributed equally to this work

Received: 14 February 2023 Accepted: 22 March 2023 Published: 31 May 2023

ABSTRACT

KANADI (*KAN*) is a plant-specific gene that controlled the polarity development of lateral organs. It mainly acted on the abaxial characteristics of plants to make the lateral organs asymmetrical. However, it had been less identified in woody plants. In this study, the members of the *KAN* gene family in *Populus trichocarpa* were identified and analyzed using the bioinformatics method. The results showed that a total of 8 *KAN* family members were screened out, and each member contained the unique GARP domain and conserved region of the family proteins. Phylogenetic analysis and their gene structures revealed that all *KAN* genes from *P. trichocarpa*, *Arabidopsis thaliana*, and *Nicotiana benthamiana* could be divided into four subgroups, while the eight genes in *P. trichocarpa* were classified into three subgroups, respectively. The analysis of tissue-specific expression indicated that *PtKAN1* was highly expressed in young leaves, *PtKAN6* was highly expressed in young leaves and mature leaves, *PtKAN2*, *PtKAN5*, and *PtKAN7* were highly expressed in nodes and internodes, *PtKAN8* was highly expressed in roots, and *PtKAN3* and *PtKAN4* showed low expression levels in all tissues. Among them, *PtKAN2* and *PtKAN6*, and *PtKAN4* and *PtKAN5* might have functional redundancy. Under high nitrogen concentrations, *PtKAN2* and *PtKAN8* were highly expressed in mature stems and leaves, respectively, while *PtKAN4*, *PtKAN5*, and *PtKAN7* were highly expressed in roots. This study laid a theoretical foundation for further study of the *KAN* gene-mediated nitrogen effect on root development.

KEYWORDS

Bioinformatics analysis; *KANADI* gene family; nitrogen; *Populus trichocarpa*

1 Introduction

KANADI (*KAN*) genes, a subset of the GARP (Golden2, ARR-B, Psr1) family of transcription factors, are key regulators of abaxial identity in the polarity and development of lateral organs [1]. The lateral organs

of higher plants included the collateral branches, leaves, inflorescences, flowers, and other organs [2], which were developed from the apical meristem [3]. Almost all the lateral organs of higher plants show polarity development in the adaxial-abaxial fates [4], which eventually leads to the phenotype differences between the upper (adaxial) and the lower (abaxial) characteristics in the leaves and other organs [5]. The *KAN* gene was first identified in *Arabidopsis thaliana* in 2001 [1]. Later, members of the *KAN* family were found in rice [6] and maize [7]. According to the current reports, most of the related studies of this gene family were concentrated in herbaceous plants, and it found in gymnosperms such as ferns [8], few studies were conducted in other woody plants.

KAN was a transcription factor belonging to the GARP family and contained a DNA-binding domain of MYB class. In Arabidopsis, the *KAN* family included four members (*AtKAN1-4*) [1], and its nucleic acid sequence structure included 6 exons. In rice, the *KAN* family contained at least six members, among which, the *SLL1* (*SHALLOT-LIKE1*) gene was the closest to *AtKAN1*, which was a transcription factor encoding 377 amino acids [6]. In maize, the *MWP1* (*MILKWEED POD1*) gene had high homology with *SLL1*. *MWP1* also consisted of six exons, which encoded a predicted protein of 477 amino acids with an estimated molecular weight of 48.36 kD. The GARP domains of *MWP1* and *SLL1* were identical to those of *AtKAN1* and *AtKAN2* proteins, differing only by one amino acid residue [9]. All *KAN* genes contained a highly conserved GARP domain, which played an important role in chloroplast development, cytokinin signal transduction, phosphorus metabolism, organ polarity regulation, and other biological processes [10].

In plants, *KAN* genes had controlled embryogenesis, lateral root growth, leaf polarity, and integument formation, and were expressed in the phloem and distal developmental regions of lateral organs during early development [11,12]. In *A. thaliana*, Kerstetter et al. [1] found that the *KAN* gene was localized in the nucleus. RNA *in situ* hybridization showed that the *KAN* transcription factor was expressed in the distal plane of early globular embryos [13], which confirmed that this gene was involved in regulating the development of the abaxial characteristics of plants. Previous studies showed that *AtKAN1* expression was observed at the periphery of lateral root apices by the KAN::GUS fusion system, *AtKAN2* expression in lateral roots was consistent with *AtKAN1* expression, and *AtKAN4* expression was detected at the periphery of root cap and developing lateral root primordia [14]. These results suggested that *KAN* genes played an important role in the establishment of polarity in the distal surface of leaves and the development of lateral roots. At the same time, overexpression of the *KAN* gene could lead to defects in shoot apical meristem and vascular development in cotyledons in *Arabidopsis*. While the transgenic plants expressing *KAN* ectopic in the paraxial plane had narrow and unable to expand leaves, and the paraxial plane of cotyledons produced structural characteristics of the distal plane [15]. The gain-of-function *KAN* alleles result in a loss of cambium activity, while the cambium activity in the hypocotyls of seedlings of *KAN* loss-of-function mutants had increased [16]. In *Arabidopsis*, the four *KAN* genes displayed a complex pattern of genetic redundancy. *KAN1*, *KAN2*, and *KAN3* promote the establishment of leaf polarity, and *KAN1* and *KAN2* also could promote the establishment of floral organ polarity, including the outer integument. At the same time, single deficiency mutants have no or no obvious defective phenotype, none of the *kan1*, *kan2*, or *kan3* single mutants exhibited a dramatic loss of polarity, while all lateral organs had gross morphological defects in *kan1 kan2* plants, and in *kan1 kan2 kan3* leaves, the mature blade expanded in various planes giving rise to long narrow leaves with a fan-like blade at their distal end [15,17], which indicated that there were extensive redundancy relationships among *Arabidopsis KANADI* genes. In rice, *SHALLOT-LIKE1* (*SLL1*, closest to *AtKAN1* among the *Arabidopsis KAN* members) was crucial in polarity formation and help to direct the development of the leaf abaxial cell layer. *SLL1* deficiency suppresses the specification of sclerenchymatous cells in the abaxial mesophyll, while *SLL1* overexpression resulted in dwarf plants with twisted and abnormal inner rolled leaves, and the abaxial features of leaves following *SLL1* overexpression had been enhanced [6]. The maize MILKWEED POD1 (MWP1), the closest KAN protein to *SLL1*, was involved in the

abaxial-adaxial patterning of leaves, both *sll1* and *mwp1* show adaxialized sectors of cells in the sheath [9]. To sum up, *KAN* played an important role in the regulation of lateral organ polarity formation in plants. Previous studies had shown that this gene was regulated by nitrogen, but its mechanism in mediating nitrogen regulation of root development was still unclear.

Populus trichocarpa was a model species of woody plants, whose genome sequence had been widely used in genetic studies since it was sequenced and published in 2006 [18]. In this study, eight putative *KAN* genes of *P. trichocarpa* were analyzed by bioinformatics analysis methods. The chromosome distribution, phylogeny, gene structure, and motif composition of family genes were analyzed in detail. And qRT-PCR was used to analyze the tissue-specific expression of the *KAN* genes in *P. trichocarpa* under different exogenous nitrogen treatments. This study laid a theoretical foundation for speculating the role of this gene in the regulation of root development mediated by nitrogen.

2 Materials and Methods

2.1 Identification and Analysis of KAN Family Genes in P. trichocarpa

The protein sequences of *P. trichocarpa* were downloaded from phytozome v13.0 genome database (https://phytozome.jgi.doe.gov/pz/portal.html) to identify the KAN protein. The unique Myb DNA-binding (PF00249) domain of the *Arabidopsis KAN* family was downloaded from the Pfam 35.0 database (https://pfam.xfam.org/) [19]. The Hmmsearch command in HMMER (V3.1) software was used to search the poplar protein database with Myb DNA-binding [20]. The sequences had been integrated and identified, and candidate proteins in the *P. trichocarpa* protein database had been selected. In addition, the *Arabidopsis AtKAN1* sequence was used as a probe to search the *P. trichocarpa* protein database online using BlastP. Protein sequences obtained using these two methods were further screened. After screening and identification, eight PtKAN amino acid sequences were obtained. The online ExPASy program (https://www.expasy.org) [21] was used to analyze the physical and chemical properties of these amino acid sequences, including predicted molecular weight, isoelectric point, number of amino acids, aliphatic index, and grand average of hydropathicity (GRAVY). The subcellular localizations of PtKANs proteins were predicted by Wolfpsort (https://www.genscript.com/psort/wolf_psort.html) [22].

2.2 Gene Structure and Conserved Motifs Analysis

The organization of the exon-intron of the *PtKAN* gene family was predicted using Gene Structure Display Server (GSDS2.0, http://gsds.cbi.pku.edu.cn) [23]. The conserved motifs in PtKAN proteins were identified according to Multi Em for Motif Elicitation (MEME V5.0.5; http://meme-suite.org/tools/meme) [24]. Then Toolbox for Biologists (TBtools v1.09876) [25] was used for integrated analysis.

2.3 Multiple Sequence Alignment and Phylogenetic Analysis

The alignment of PtKAN amino acid sequences and conserved GARP domains was performed using the Clustal X program [26]. The *KAN* family protein sequences of *Nicotiana benthamiana* were downloaded from Solanaceae Genomics Network (http://solgenomics.net). Phylogenetic trees (No. of bootstrap replications = 1000) were constructed using MEGA V7.0.14 software according to Neighbor-Joining (NJ) method [27].

2.4 Chromosomal Mapping and Synteny Analysis

The chromosome locations of *PtKAN* gene family members were acquired and analyzed using Phytozome v13.0 and PopGenIE v2.0 (http://popgenie.org/chromosome-diagram) [28]. MG2C tools (MapGene2Chrom web, v2; http://mg2c.iask.in/mg2c_v2.0) was used to construct the chromosome distribution map of the *PtKAN* gene. *P. trichocarpa* and other species of chromosome gff3 files were obtained from Ensembl the Plants (https://plants.ensembl.org) [29]. Then, using the multicollinear

scanning toolkit (MCScanX; https://github.com/wyp1125/MCScanX) [30] to analyze the collinear relationship and gene replication events among *PtKAN* orthologous genes and other selected species orthologous genes.

2.5 Tissue-Specific Expression Patterns of Genes

The tissue-specific expression data of the *PtKAN* gene in mature leaves, young leaves, roots, nodes, and internodes were obtained from PopGenIE V2.0 (http://popgenie.org) and used to generate visual images.

2.6 Plant Materials, Growing Conditions, and Nitrogen Treatments

The experimental material *P. trichocarpa* was obtained from the State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University (Harbin). The homogeneous seedlings (about 15 cm) after rooting hydroponically were planted in vermiculite in the greenhouse under long-day conditions (16 h light/8 h dark) at 25°C ± 1°C. They were irrigated with a modified 1/2 Hoagland nutrient solution (ammonium nitrate concentration 1 mm) [31]. The nutrient solution had changed every 7 days. After cultivating 28 days, the seedlings were treated with nutrient solution supplemented with different concentrations of nitrogen (0.1, 1, 5, and 10 mM NH_4NO_3) for 28 days. The control group was cultured with 1 mM NH_4NO_3. The nutrient solution was updated every 7 days. The roots, leaves, upper stems (stems grown after treatment), and lower stems (stems grown before treatment) were sampled according to Sun et al. [32], frozen immediately with liquid nitrogen, and then stored at −80°C for future analysis. Each treatment had three biological replicates. Each sample also had three technical replicates.

2.7 RNA Extraction and qRT-PCR Analysis

Total RNA of different tissues was extracted using the Hexadecyl Trimethyl Ammonium Bromide (CTAB) method [33]. With the action of DNase I (Fermentas, Waltham, MA, USA), genomic DNA contamination was removed and then using PrimeScript™ RT reagent Kit with gDNA Eraser to reverse transcribed 1 μg of total RNA into cDNA (Takara Bio, Dalian, China). The qRT-PCR was performed using Power Green qPCR Mix reagent (Dongsheng Biotech Co., Ltd., Beijing, China) according to Zuo et al. [34]. UBQ7 gene was used as the reference gene [35], and the relative expression was calculated by the $2^{-\Delta\Delta CT}$ method [36]. The statistically significant differences ($p < 0.05$) among expression levels of *PtKANs* in different samples were tested by the Duncan test. TBtools V1.09876 [24] and GraphPad Prism 8.0.1 were used to generate gene expression maps.

3 Results

3.1 Identification and Sequence Analysis of PtKAN Gene

In this study, a total of eight *PtKAN* genes were obtained and named *PtKAN1-8* based on their positions on the chromosome, respectively. Their physical and chemical properties including amino acids, molecular weight, isoelectric points, GRAVY, aliphatic Index, chromosome location, and cellular localization were listed in Table 1. The length of encoded protein of PtKANs ranged from 341 (*PtKAN2*) to 485 (*PtKAN4*) amino acids. The maximum and minimum molecular weights were 53.87 kDa (*PtKAN4*) and 37.34 kDa (*PtKAN2*), respectively. The predicted pI ranged from 6.76 (*PtKNA6*) to 9.23 (*PtKAN1*), and the aliphatic index ranged from 56.89 (*PtKAN4*) to 75.03 (*PtKAN6*). They all were identified as hydrophilic proteins according to GRAVY scores. What's more, it was predicted that they were located in the cytoplasm and nucleus. The multiple sequence alignments of the eight PtKAN protein sequences showed that they all had typical GARP domains and conserved structures (Fig. 1).

Table 1: The physical and chemical properties of the eight identified *PtKAN* genes and deduced putatived polypeptides in *P. trichocarpa*

Gene name	Gene ID	Amino acids	Molecular weight kDa	Isoelectric points (pI)	GRAVY	Aliphatic index	Chromosome location	Cellular localization
PtKAN1	Potri.001G137600.1	378	42.76	9.23	−0.829	61.69	Chr01:11217021..11222607 (−)	Cytoplasm
PtKAN2	Potri.002G130200.1	341	37.34	9.13	−0.673	69.59	Chr02:9870762..9874363 (+)	Nucleus
PtKAN3	Potri.003G096300.1	378	42.66	8.32	−0.836	57.57	Chr03:12224273..12229729 (+)	Nucleus
PtKAN4	Potri.004G082400.1	485	53.87	8.91	−0.852	56.89	Chr04:6782881..6788780 (−)	Nucleus
PtKAN5	Potri.012G042100.1	436	47.61	7.74	−0.669	62.22	Chr12:3747826..3754998 (−)	Nucleus
PtKAN6	Potri.014G037200.1	342	37.51	6.76	−0.625	75.03	Chr14:2349028..2352687 (+)	Nucleus
PtKAN7	Potri.015G031600.1	443	48.36	8.22	−0.673	64.97	Chr15:2549049..2555571 (+)	Cytoplasm
PtKAN8	Potri.017G137600.1	482	53.24	8.42	−0.771	60.73	Chr17:13885990..13891467 (+)	Cytoplasm

3.2 Gene Structure and Phylogenetic Analysis of PtKAN Gene

According to the full-length sequence of eight PtKAN proteins, the phylogenetic tree was constructed and shown in Fig. 2. Phylogenetic tree analysis indicated that the *PtKAN* family was clustered into three subfamilies. The first group contained *PtKAN4*, *PtKAN5*, *PtKAN7*, and *PtKAN8*, and they had 10 motifs. The second group included *PtKAN1* and *PtKAN3*, while *PtKAN3* contained motifs and *PtKAN1* did not. *PtKAN2* and *PtKAN6* were the third groups, they both had 6 motifs. Meanwhile, the results of gene structure showed that all the family members had 6 exons, but the length of introns varied significantly.

In order to understand the evolutionary relationship among the *KAN* gene families of *P. trichocarpa*, *A. thaliana*, and *Nicotiana benthamiana*, MEGA7 software was used to compare their KAN amino acid sequences and constructed phylogenetic trees (Fig. 3). Phylogenetic analysis revealed that all *KAN* genes could be divided into four subgroups, while the eight genes in *P. trichocarpa* were classified into three subgroups, respectively. *PtKAN4*, *PtKAN5*, *PtKAN7*, and *PtKAN8* of *P. trichocarpa* were highly homologous to *AtKAN1*. *PtKAN1* and *PtKAN3* had similar homology to *NbKAN2*, while *PtKAN2* and *PtKAN6* had high homology to *AtKAN3*, *AtKAN4*, and *NbKAN4*.

3.3 Chromosomal Distribution and Synteny Analysis of PtKAN Gene

The chromosome distributions of these eight *PtKAN* genes were shown in Fig. 4. The results showed that the eight *PtKAN* genes were distributed on eight chromosomes, respectively. The MCScanX was used to analyze the segmental duplication events of the *PtKAN* genes (Fig. 5). Four pairs of segmental duplication events were found in this study, among which there were gene duplication events between *PtKAN1* (Chr01) and *PtKAN3* (Chr03), and between *PtKAN2* (Chr02) and *PtKAN6* (Chr14), and between *PtKAN4* (Chr04) and *PtKAN8* (Chr17), and between *PtKAN5* (Chr12) and *PtKAN7* (Chr15).

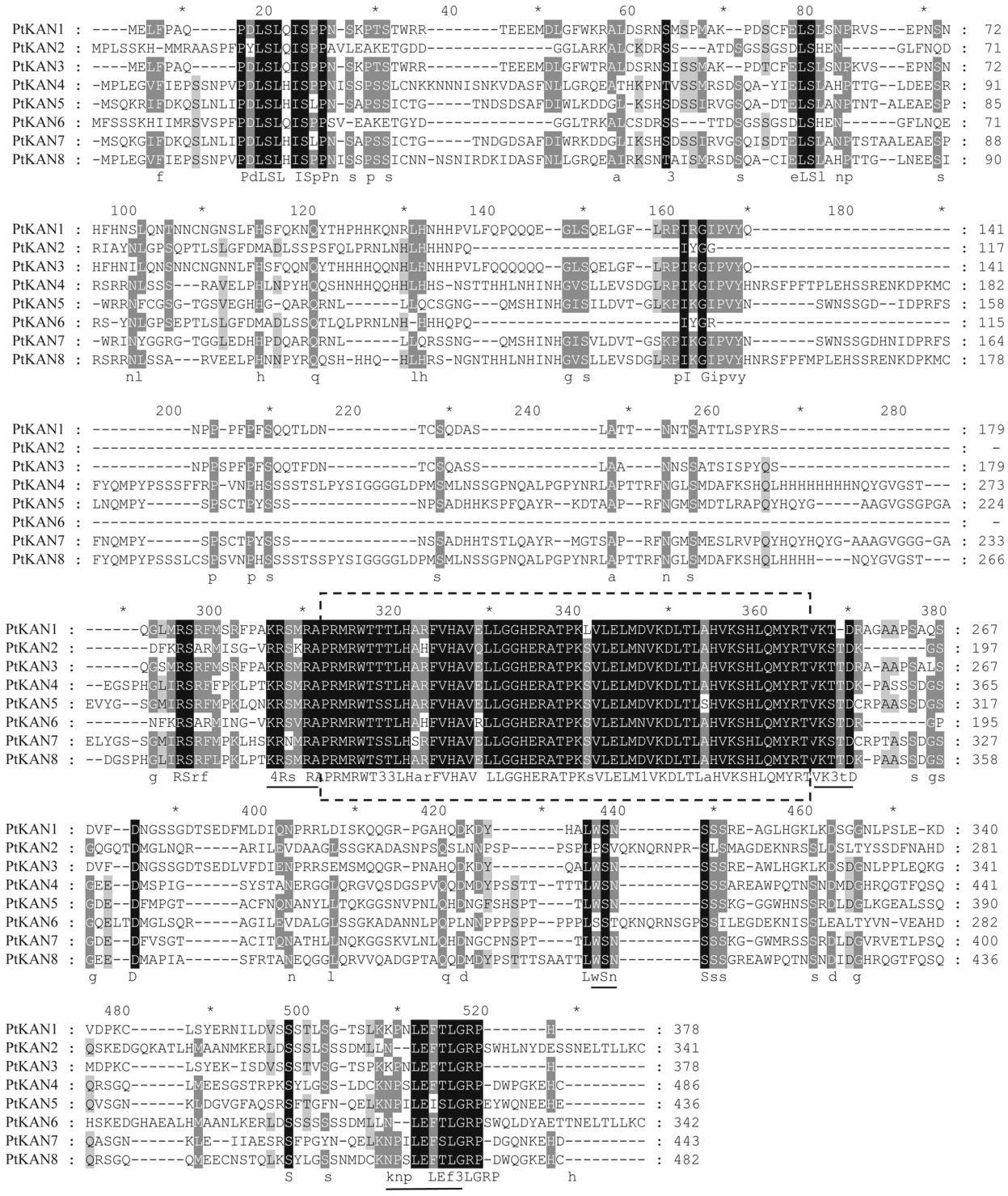

Figure 1: Multiple alignments of 8 putative PtKAN amino acid sequences. The GARP domain was in the box and the conserved region unique to the *PtKAN* gene family was underlined [37], where * represented the same amino acid

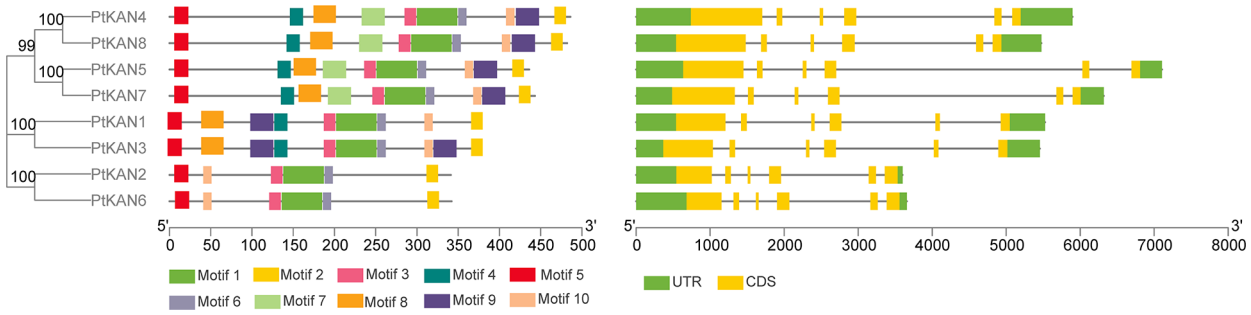

Figure 2: Phylogenetic tree and gene structural analysis of eight *PtKAN* genes. Phylogenetic trees (left) were constructed based on full-length protein sequences of PtKAN. The motifs (middle) were predicted using the MEME tool. The right was the structure of the corresponding *PtKAN* gene. Protein coding sequences (CDS) were shown in yellow. The green color indicated upstream/downstream sequences

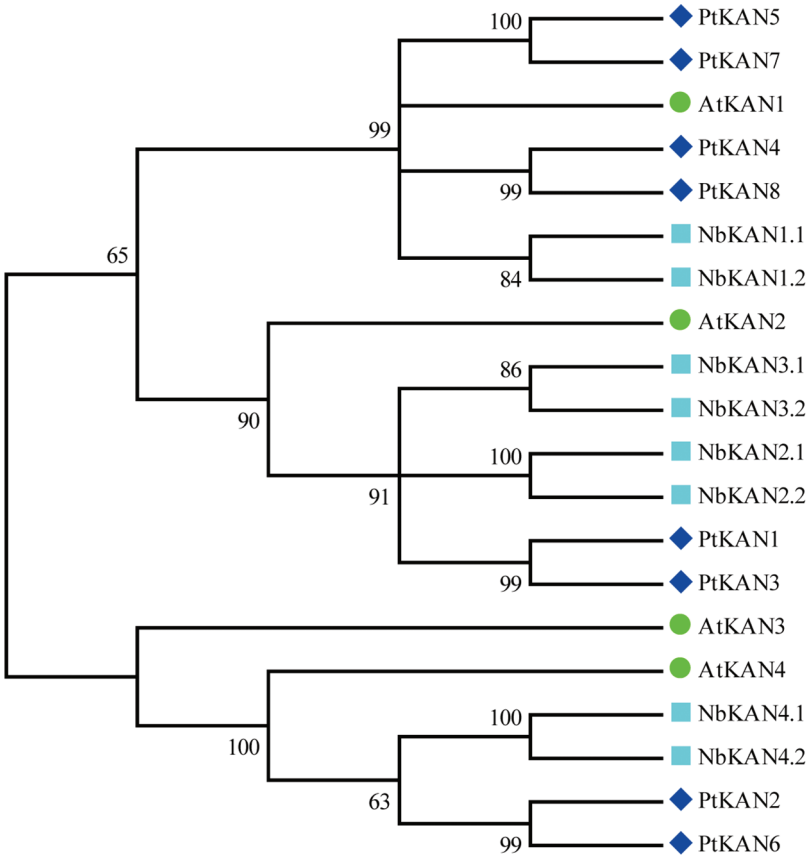

Figure 3: Phylogenetic trees of *KAN* gene families of *P. trichocarpa* (Pt), *Nicotiana benthamiana* (Nb), and *Arabidopsis thaliana* (At)

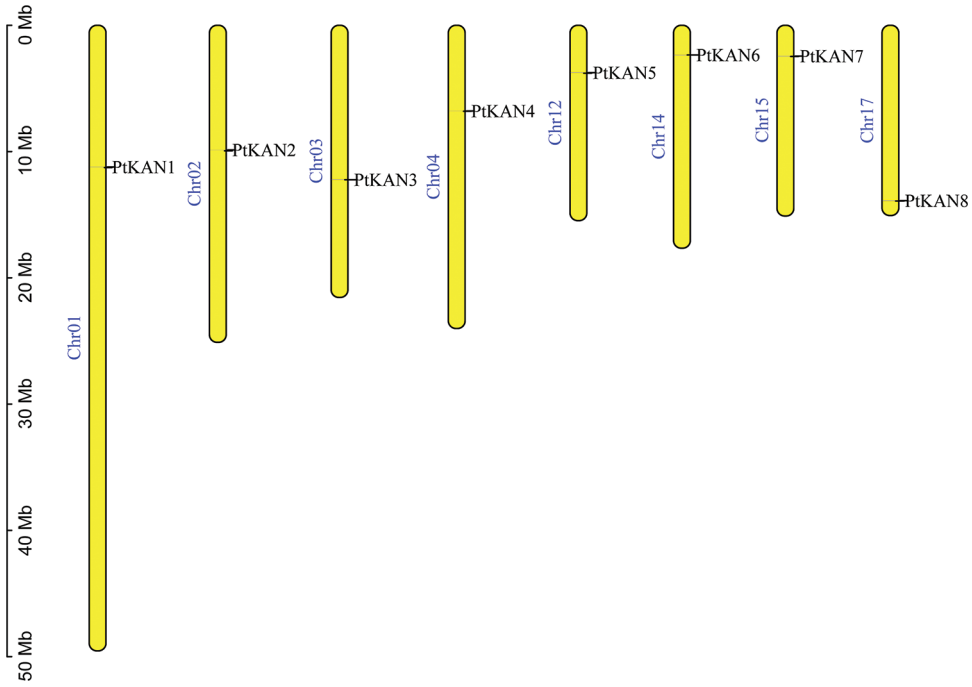

Figure 4: Chromosomal distribution of *KAN* gene family members in *P. trichocarpa*

To further understand the mechanism of gene replication among *PtKANs*, four comparative syntenic maps related to four representative species, including monocotyledon (*Oryza sativa*, Fig. 6A) and dicotyledons (*A. thaliana*, *Glycine max*, and *Medicago truncatula*, Fig. 6B) were constructed. The results showed that there were four *KAN* syntenic gene pairs between poplar and rice. Compared with *O. sativa*, *A. thaliana*, and *M. truncatula*, *PtKAN*s had the most syntenic gene pairs with *G. max*, with higher homology and closer kinship.

3.4 Tissue-Specific Expression Pattern of PtKAN Genes

The tissue-specific expression data of *PtKAN* genes in mature leaves, young leaves, roots, nodes, and internodes were obtained from PopGenIE (https://PlantGenIE.org) (Fig. 7). *PtKAN1* was highly expressed in young leaves. *PtKAN2*, *PtKAN 5*, and *PtKAN 7* were highly expressed in nodes and internodes. *PtKAN6* was highly expressed in young leaves and mature leaves. *PtKAN8* was highly expressed in roots. However, *PtKAN3* and *PtKAN4* showed low expression levels in all tissues.

3.5 Expression Pattern of PtKAN Genes under Different Nitrogen Treatment

The seedlings were treated with different concentrations (0.1, 1, 5, and 10 mM NH_4NO_3) of nitrogen, and the expression patterns of 8 *PtKAN*s in 4 tissues (upper stems, lower stems, leaves and roots) were determined (Fig. 8). 1 mM NH_4NO_3 was regarded as control group. In upper stems, the expression levels of *PtKAN2* and *PtKAN5* were significantly increased under low nitrogen treatment. The expressions of *PtKAN2* in upper stems were significantly upregulated under medium and high nitrate treatments, while the transcription levels of other genes were down-regulated. In lower stems, the expression of *PtKAN2* was significantly upregulated under low nitrogen treatment, while *PtKAN8* was significantly downregulated. *PtKAN2*, *PtKAN4*, *PtKAN6*, and *PtKAN8* were significantly downregulated under medium nitrogen treatment. However, *PtKAN2* was upregulated under high nitrogen treatment. In leaves, the transcription levels of *PtKAN1* and *PtKAN2* were significantly downregulated under low nitrogen

treatment. Almost all *PtKAN*s were significantly downregulated under medium and high nitrogen treatment. In roots, *PtKAN2* was significantly downregulated and *PtKAN7* was significantly upregulated under low nitrogen treatment. The relative expression levels of *PtKAN2*, *PtKAN4*, *PtKAN5*, and *PtKAN7* were significantly upregulated under medium nitrogen, and the expression levels of *PtKAN7* and *PtKAN8* were upregulated under high nitrogen treatment.

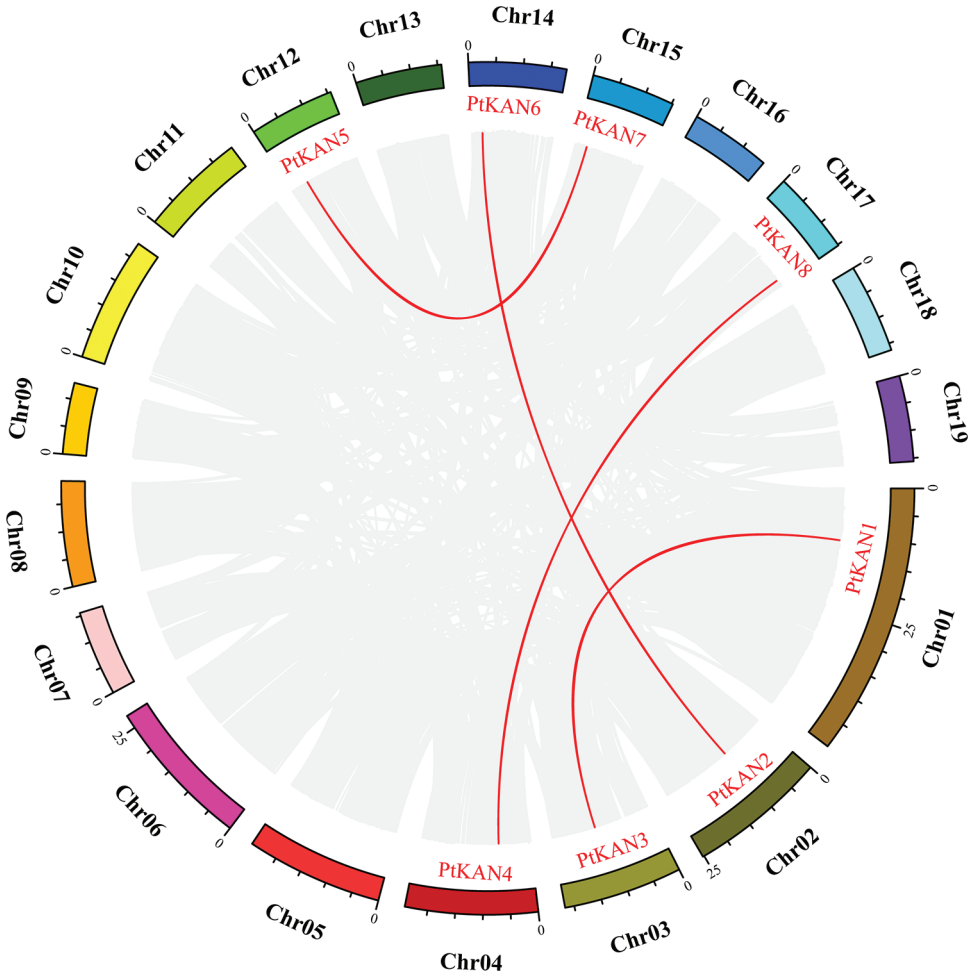

Figure 5: Schematic representations of segmental duplications of *PtKAN* gene. Gray lines indicate all syntenic blocks between each chromosome in the poplar genome; The red line showed the duplicated *PtKAN* gene pair. Gene names were shown in red. Chromosome numbers were shown in the middle of each chromosome. The scale of markers on each chromosome indicated chromosome length (Mb)

4 Discussion

KAN family genes played an important role in controlling the polar development of lateral organs in plants. In recent years, it had been confirmed that this gene family acted on the formation of leaf and flower polarities in *Arabidopsis* [38]. In previous studies, our team found that this gene was regulated by nitrogen and might play an important role in the nitrogen-induced root formation of woody plants (data not shown). However, the composition and functions of *KAN* family members in woody plants were less reported, and the gene family analysis was a necessary condition for further functional verification.

Therefore, in this study, eight members of the *KAN* gene family in *P. trichocarpa* were identified and analyzed using bioinformatics methods. Meanwhile, the expression of *KAN* family genes under nitrogen treatment was further studied. It could provide new insight into the response of the *KAN* gene to nitrogen and also lay an important foundation for exploring the function of *KAN* family members in the later stage.

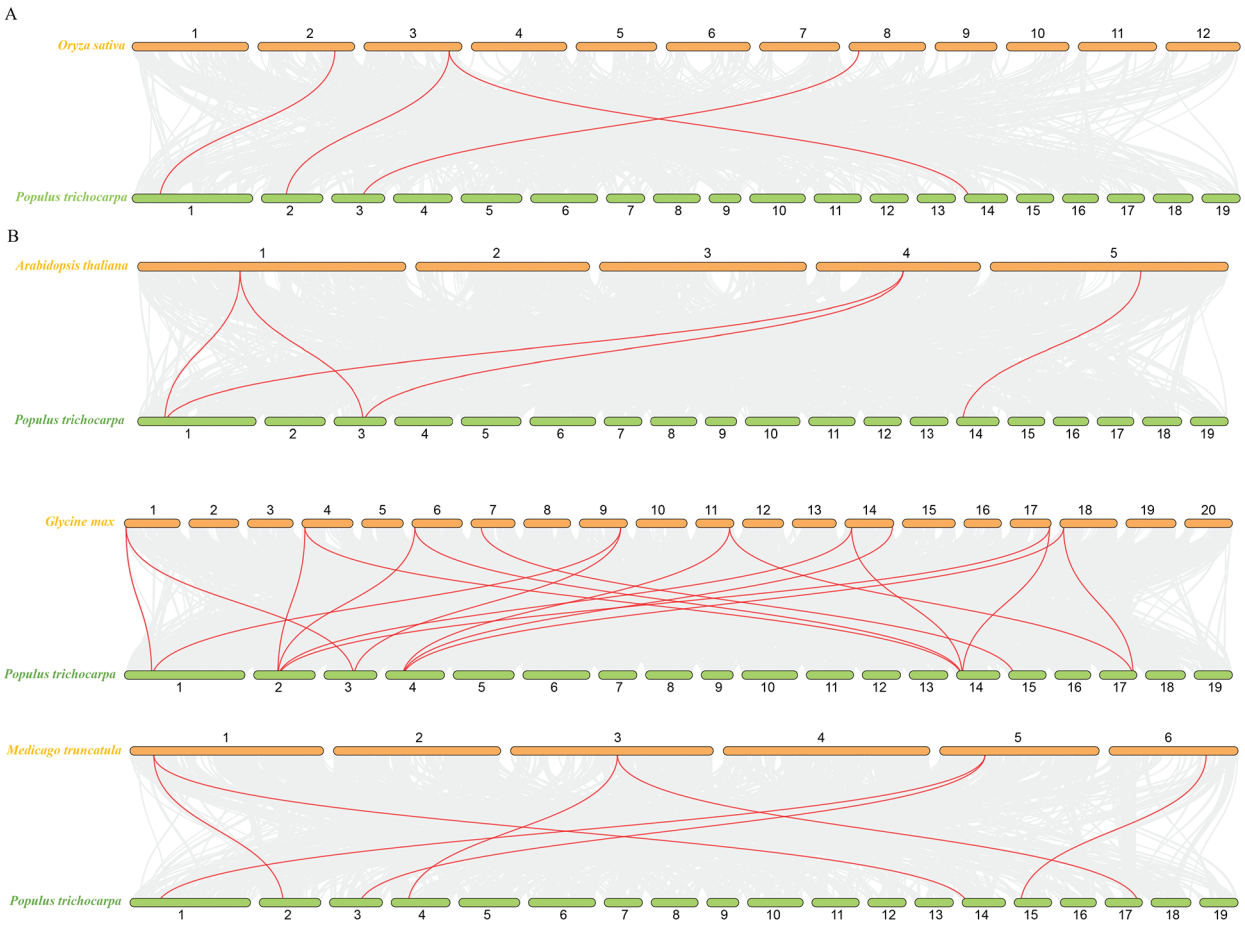

Figure 6: Synteny analysis of KAN genes between poplar and other plants. (A) was monocotyledon. (B) were dicotyledons. The red line showed the KAN syntenic gene pairs. Orange and green bars represented chromosomes, and whose numbers were at the top or bottom

In this study, the subcellular localization, protein conserved motifs analysis, and phylogenetic tree analysis of 8 *PtKAN* genes were conducted. The results showed that both *PtKAN2* and *PtKAN6* were located in the nucleus, and both of them had 6 identical motifs. Phylogenetic tree analysis showed that they were on the same branch, indicating that they had a high homology relationship. Therefore, it was speculated that there might be functional redundancy between them. At the same time, *PtKAN4* and *PtKAN5* also had similar characteristics, indicating that there might also be functional redundancy between them. At the same time, the phylogenetic analysis showed that *PtKAN2* and *PtKAN6* had similar evolutionary relationships with *AtKAN4*, while *PtKAN4* and *PtKAN5* had high homology with *AtKAN1*. Previous studies had shown that *Arabidopsis KAN1-4* were functionally redundant genes [1], so it was speculated that there was a similar situation of functional redundancy in *PtKAN* genes. However, whether this "redundancy" was real or not will be our future research direction.

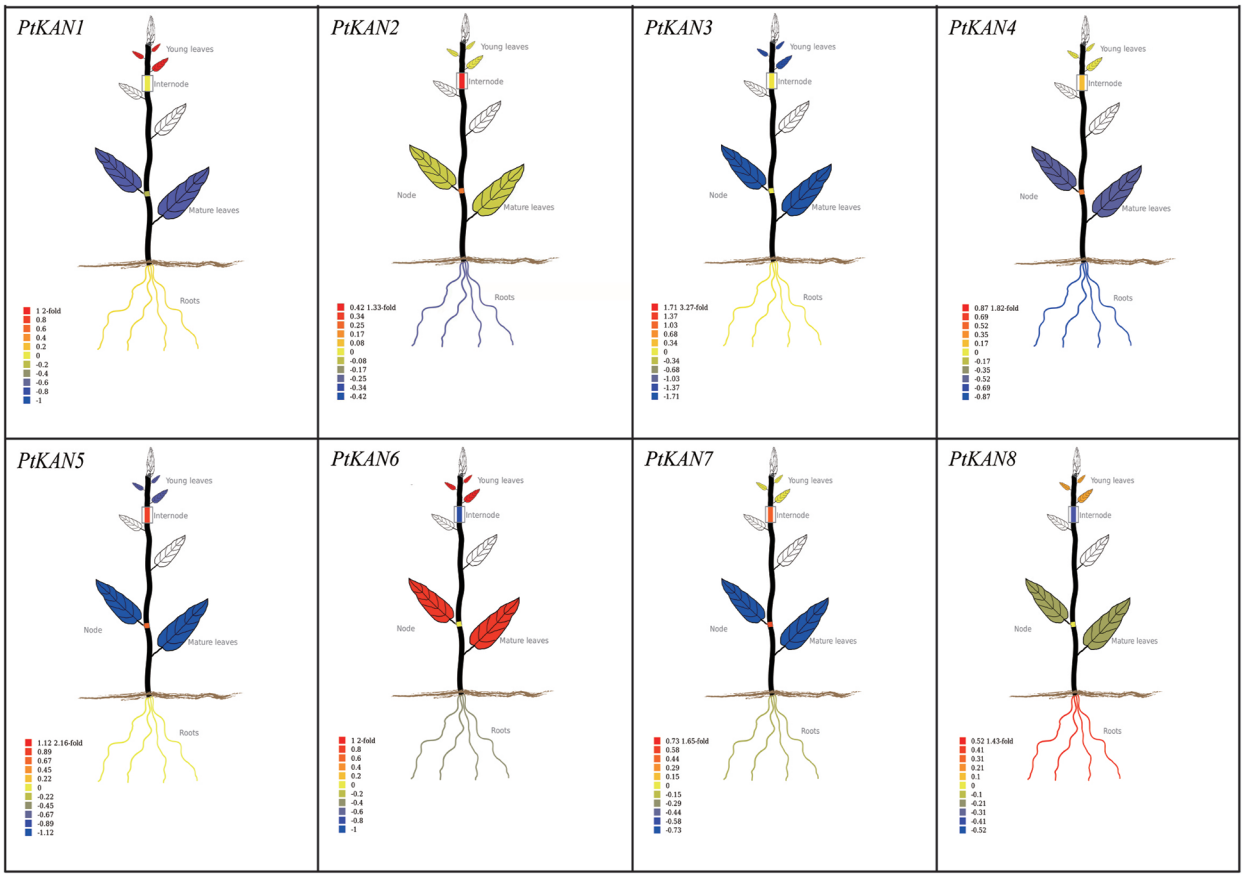

Figure 7: Tissue-specific expression pattern of *PtKAN* genes. Visual images of *PtKAN* gene in mature leaves, young leaves, roots, nodes, and internodes were generated using tissue-specific expression data from https://PlantGenIE.org

In this study, a total of 8 *PtKAN* genes were identified, and the chromosomal position of the genes was analyzed, and it was found that each chromosome contained one gene. MCScanX was used to analyze the segmental repeat events of the *PtKAN* genes, and a total of 4 pairs of duplicate genes were found (Fig. 5), namely *PtKAN1/3*, *PtKAN2/6*, *PtKAN4/8*, *PtKAN5/7*, and the *PtKAN*s repeat genes might have been formed during the whole genome doubling of poplars. The occurrence of multiple repeat events indicates that the *PtKAN* gene family continues to expand during the evolution of the poplar genome. Through collinear analysis, multiple *PtKAN* genes were homologous to *AtKAN*s and *OsKAN* were found (Fig. 6), which might be from the same ancestor as the *KAN* genes of *Arabidopsis thaliana* and rice, etc., and might have similar functions.

Previous studies had shown that the *KAN* gene family functions in the apical meristem and that the expression pattern of each gene varies in organs such as leaves, flowers, cotyledons, and embryos [15]. For example, *AtKAN1* could promote the development of abaxial leaf polarity [39]. *AtKAN1*, *AtKAN2*, and *AtKAN3* could promote the establishment of leaf polarity, while *AtKAN1* and *AtKAN2* also contributed to floral organ polarity [40]. *AtKAN4* was expressed in the root, but it had redundant activity with other *KAN* genes [14]. In this study, we found that *KAN* genes were expressed in various organs of *P. trichocarpa*, but their expression levels were different. For example, *PtKAN8* was highly expressed in

roots, indicating that the *KAN* gene might play an important regulatory function in the root development of *P. trichocarpa*.

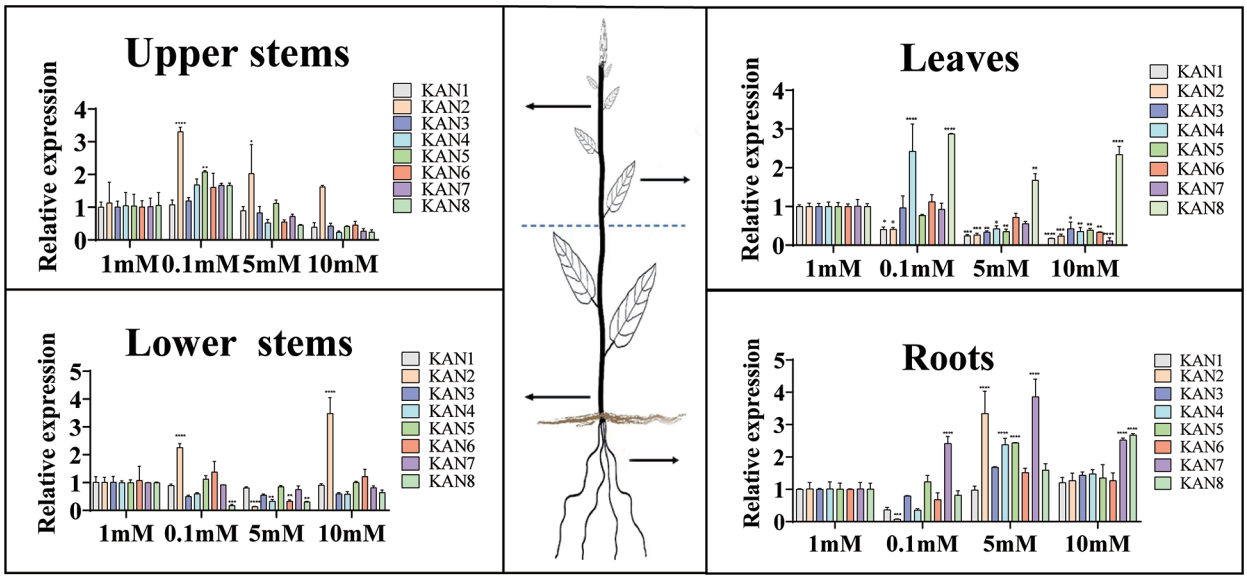

Figure 8: Expression patterns of *PtKAN* genes in different tissues (upper stems, lower stems, leaves, and roots) under different concentrations NH_4NO_3 treatments. Control: 1 mM. Low nitrogen: 0.1 mM. Medium nitrogen: 5 mM. High nitrogen: 10 mM. The relative expression of *PtKAN*s was calculated using $2^{-\Delta\Delta CT}$. The significance test indicated the statistical analysis of relative expression under different treatments and the gene expression under 1 mM nitrogen treatment. "*", "**" and "***" indicated significant differences at "$p < 0.05$", "$p < 0.01$" and "$p < 0.001$" levels, respectively

Nitrogen was an essential mineral nutrient element for tree growth and development, and it played an important role in improving yield [41]. Plant roots required N supply for growth and development. However, in terms of plasticity, lateral roots were more sensitive to N supply at different concentrations. Previous studies had shown that the *KAN* gene family was expressed in lateral root and acted in lateral root formation [14]. In this study, the expression characteristics of the *PtKAN* gene in response to nitrogen treatment with different concentrations were investigated. The results showed that the expression of *PtKAN2* in stems was significantly upregulated, while it was downregulated in leaves and roots under low nitrogen treatment. The expression of *PtKAN7* in roots was significantly upregulated. It was shown that *PtKAN2* and *PtKAN7* played important roles in stems and roots in the nitrogen starvation state, respectively. Other *PtKAN* genes were downregulated under low nitrogen treatment, while the *PtKAN* genes in the roots were upregulated under middle and high nitrogen treatments, which might be the *PtKAN* gene in roots was upregulated to adapt to high nitrogen environment. With higher nitrogen concentrations, the expression of most *KAN* genes was inhibited. However, the expression of *PtKAN8* was elevated under the conditions of nitrogen deficiency and high nitrogen concentration, indicating that *PtKAN8* was sensitive to nitrogen, and it might mainly play a role in lateral organs such as leaves and lateral roots. Therefore, it was possible that *PtKAN* family played an important role in mediating nitrogen response and root morphological differences. However, the *PtKAN* gene was down-regulated in stems and leaves to adapt to high nitrogen supply, which might be due to different regulatory mechanisms depending on different tissues in plants. The molecular mechanism by which the *PtKAN* family affected

root development under nitrogen treatment was still unclear. However, further studies needed to be carried out to confirm that.

5 Conclusion

In this study, the bioinformatics method was used to speculate *KAN* family members in *P. trichocarpa*, and their expression patterns under different nitrogen treatments had been analyzed. The results showed that a total of 8 *KAN* family members were screened out in *P. trichocarpa*, and each member contained the unique GARP domain and conserved region. Among them, *PtKAN2* and *PtKAN6*, and *PtKAN4* and *PtKAN5* might have functional redundancy, which needed to be further verified. This study also identified and analyzed the gene expression patterns under nitrogen treatment, aiming to provide an important theoretical basis for the future study of this gene in nitrogen-mediated root development.

Funding Statement: This research was funded by the Natural Science Foundation of Heilongjiang Province, China (ZD2020C004), the Fundamental Research Funds for the Central Universities (2572019CT02).

Author Contributions: Niu MH, Qu CP, and Liu GJ conceived and designed the study. Niu MH performed most of the experiments. Zhang HJ and Hu ZB conducted the sampling. Niu MH, Hu ZB, and Li XY performed bioinformatics analysis. Niu MH and Zhang H processed and analyzed the data, and Niu MH and Zhang H wrote the manuscript. Qu CP, Liu GJ and Xu ZR provided helpful comments in the draft and reviewed and edited the manuscript.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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