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Article

Genome-Wide Characterization of the *Brassica juncea NDPK* Gene Family and Functional Analysis in Root Development Under Drought Stress

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Abstract: Nucleoside diphosphate kinase (NDPK) is a multifunctional enzyme family widely distributed in both prokaryotic and eukaryotic organisms, playing crucial roles in energy metabolism, stress perception, signal transduction, and reactive oxygen species (ROS) regulation. However, this gene family has not been systematically identified and functionally characterized in *Brassica juncea*. This study conducted a genome-wide identification and comprehensive analysis of the *BjNDPK* gene family in *Brassica juncea*, integrating bioinformatics and experimental validation based on the *Brassica juncea* genome data. A total of 18 *BjNDPK* family members were identified. Their encoded proteins retain high sequence and structural conservation within the NDPK family while exhibiting structural features adapted to diverse functions. Analysis of cis-acting elements revealed that multiple members possess promoters rich in drought-response-related elements. Colinearity analysis indicated that *BjNDPK* genes underwent complex subgenomic rearrangements and selective retention during the formation of *Brassica juncea* heterotetraploidy. Expression profiling showed that most BjNDPK genes responded significantly during different tissue development stages and under drought stress, with some members highly expressed in seed-related tissues. Furthermore, internal gene stability assessment confirmed *qBjActin* exhibits outstanding expression stability in this study system. Physiological experiments further demonstrated that drought stress induces oxidative stress in *Brassica juncea*, while the AtNDPK2 mutant in *Arabidopsis thaliana* exhibits enhanced root growth capacity under osmotic stress. This study systematically analyzed the structure, evolution, and expression characteristics of the *BjNDPK* gene family in rapeseed, laying a theoretical foundation for investigating its functional mechanisms in abiotic stress responses and providing important candidate gene resources.

Keywords: *Brassica juncea*; *NDPKs*; gene family; drought stress; qRT-PCR

1. Introduction

Plants frequently encounter various abiotic stresses during growth and development, such as salinity, drought, low temperatures, and high temperatures. These adverse environmental factors not only disrupt cellular homeostasis but also significantly reduce crop yield and quality, posing major challenges to sustainable agricultural development [1-3]. To cope with stress, plants have evolved a complex and finely tuned molecular regulatory network over long-term evolution. Relevant genes play crucial roles in stress perception, signal transduction, and response processes [4, 5].

Nucleoside diphosphate kinase represents a multifunctional enzyme family widely distributed across both prokaryotic and eukaryotic organisms [6]. NDPK proteins universally contain a highly conserved catalytic domain (PF00334) and exhibit substantial homology across different species [7]. Their classical function is to catalyze phosphate transfer between nucleoside triphosphates (NTPs) and nucleoside diphosphates (NDPs), thereby maintaining intracellular nucleotide homeostasis and energy metabolism [8]. Recent studies reveal that *NDPKs* not only participates in basal metabolism but also plays vital roles in cellular signaling, antioxidant regulation, and stress adaptation, making it an indispensable factor for sustaining normal cellular physiological activities [9].

In animals, *NDPKs* are closely associated with processes such as cell proliferation, differentiation, apoptosis, and tumor suppression. In plants, this gene family has undergone expansion and functional diversification, forming multiple subfamilies. Beyond maintaining energy metabolism, these proteins participate in regulating photosynthesis, responding to hormone signals (such as ABA, IAA, SA, MeJA, etc.) , and mediating responses to various abiotic stresses including salt, drought, low temperature, and mechanical injury. For example, in *Arabidopsis thaliana*, three representative *NDPK* members (*AtNDPK1*, *AtNDPK2*, and *AtNDPK3*) have been reported. *AtNDPK1* is primarily localized in the cytoplasm and nucleus, where it regulates reactive oxygen species (ROS) scavenging and signal transduction, and has been demonstrated to be closely associated with salt and drought stress tolerance [10]. *AtNDPK2* is primarily localized to chloroplasts, where it interacts with photosystem II associated proteins, playing a crucial role in photosynthetic electron transport and antioxidant defense. Additionally, *AtNDPK2* exhibits regulatory functions in ABA and drought response pathways, positioning it as a core factor in stress responses [11, 12]. *AtNDPK3* localizes to the chloroplast stroma and mitochondrial inner membrane, where it is associated with energy metabolism and redox balance. It is hypothesized to be crucial for maintaining mitochondrial function under cold and oxidative stress [13]. In rice, *NDPK* family members have been shown to be involved in salt and drought stress tolerance as well as root growth [14, 15]. In maize, *ZmNDPK1* specifically recognizes and binds G-quadruplex structures within gene promoter regions, stabilizing this non-B-type DNA conformation to regulate downstream gene transcription [16]. Furthermore, this class of factors has been extensively studied across diverse plant species, including *Solanum tuberosum* L., *Nicotiana tabacum* L. *Brassica napus* and *P. sativum* [17-20]. These studies indicated that the *NDPK* gene family not only possesses conserved catalytic functions but has also undergone subfunctionalization during plant evolution, gradually forming diverse stress adaptation mechanisms. It serves as a crucial molecular network linking plant energy metabolism and stress adaptation, and its systematic analysis holds significant importance for elucidating plant stress resistance mechanisms and improving crop stress tolerance.

Brassica juncea is a vital oilseed, vegetable, and forage crop extensively cultivated in China, India, and Southeast Asia, playing a significant role in global food and oil production and multipurpose utilization [20]. However, its growth and yield are highly susceptible to abiotic stresses such as salinity and drought, posing severe challenges to breeding and industrial development. Therefore, identifying key gene families closely associated with stress responses in *Brassica juncea* is crucial for understanding molecular regulatory mechanisms and advancing stress-tolerant breeding. Yet, systematic identification, evolutionary characteristics, and functional analysis of these families in *Brassica juncea* remain unexplored.This study conducted a genome-wide analysis of the *BjNDPK* gene family in rapeseed "T84-66" using bioinformatics tools and qRT-PCR experiments. The objectives were: (1) to determine the chromosomal localization, phylogenetic relationships, and intraspecific or interspecific collinearity of family members; (2) to analyze gene structures, protein physicochemical properties, secondary/tertiary structures, and

conserved domains; (3) to reveal their expression patterns in different tissues and under drought stress; and (4) to validate the functional conservation of *NDPK* genes in root development and stress response through phenotypic analysis of the *Arabidopsis* AtNDPK2 mutant. This study provides a theoretical foundation and candidate gene resources for deepening the understanding of the biological functions of the *BjNDPK* family and their role in adaptation to abiotic stress.

2. Materials and Methods

2.1 Identification of BjNDPK family members

To identify *BjNDPK* gene family members, we first downloaded the genome sequences of *Brassica juncea* (T84-66.V2.0), *Brassica rapa* (CCA.V1.0), and *Brassica nigra* (C2.V1.0) along with their corresponding GFF3 annotation files from the BjuIR database (http://yanglab.hzau.edu.cn/BjuIR) [21]. Simultaneously, we obtained protein sequences of reported *NDPK* genes from *Arabidopsis thaliana* in the TAIR database (https://www.arabidopsis.org) as references for homology searches [22]. Additionally, we downloaded the HMM file (PF00334) for the conserved NDPK domain from the Pfam database (http://pfam.xfam.org) [23]. During candidate gene screening, homologous searches were first performed using the BLASTP tool integrated in the BjuIR platform (E-value ≤ 1e−5, identity ≥ 40%, coverage ≥ 70%) against the protein sequences. Subsequently, domain searches were conducted using HMMER 3.0 (http://hmmer.org) based on the PF00334 HMM model[24]. The results from both methods were intersected, and candidate genes underwent manual verification to remove redundant transcripts and sequences lacking conserved domains. This process ultimately identified non-redundant members of the *BjNDPK* gene family.

2.2 Chromosomal localization, protein physicochemical properties, and structure prediction of NDPKs

To gain deeper insights into the distribution of *NDPK* genes within the *Brassica juncea* genome, we *extracted* the chromosomal location information for each *NDPK* gene from the *Brassica juncea* GFF3 file provided by BjuIR. This data was then visualized using the "Gene Location Visualize" module within the TBtools-II software (v2.330) [25]. A distribution map of *BjNDPK* genes was generated based on the chromosomal order of the *Brassica juncea* T84-66 v2.0 genome. Concurrently, physicochemical properties of 18 NDPK proteins were predicted using the ExPASy ProtParam tool (https://web.expasy.org/protparam/) [26]. Secondary structure analysis and tertiary structure homology modeling of family members were performed using the SOPMA (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and SWISS-MODEL online servers (https://swissmodel.expasy.org) [27, 28]. Furthermore, the subcellular localization of BjNDPK proteins was predicted using the WoLF PSORT online tool (https://wolfpsort.hgc.jp/) [29].

2.3. Phylogenetic analysis

To investigate the evolutionary relationships among NDPK proteins, we first performed multiple sequence alignment of the identified BjNDPK protein sequences using the MUSCLE program within MEGA11 software. Based on the alignment results, a phylogenetic tree was constructed using the Neighbor-Joining (NJ) method. Branch confidence was assessed through 1000 bootstrap replicates, with all other parameters set to default values. Finally, the phylogenetic tree was visualized and enhanced using iTOL (https://itol.embl.de/) to more clearly illustrate the evolutionary relationships among *NDPK* gene family members [30].

2.4 Gene structure, conserved motif, and cis-element analysis

To reveal the conserved motif features and domain distribution of NDPK proteins, we first analyzed the full-length protein sequences of identified *NDPK* gene family members using the online tool MEME (https://meme-suite.org) [31]. Subsequently, NCBI CD-Search (https://www.ncbi.nlm.nih.gov/Structure/cdd) was employed for conserved domain identification [32], confirming the presence of the typical NDPK catalytic domain. To analyze cis-acting elements in the promoter regions of the *BjNDPK* gene family, we extracted 2000 bp upstream sequences for each gene using TBtools and submitted them to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/) for element identification [33], focusing on elements associated with stress responses (e.g., drought, cold, wounding), light regulation, and tissue-specific expression. Trans-acting elements on each *BjNDPK* gene promoter were statistically analyzed and categorized. Finally, the gene structures and trans-acting elements of *BjNDPK* genes were visualized using the Gene Structure Visualization plugin in TBtools.

2.5 Synteny analysis of BjNDPK genes

To investigate the colinearity of *NDPK* genes in the genomes of *Brassica rapa*, *Brassica juncea* and *Brassica nigra* genomes, we performed interspecies protein homology alignment using Diamond [34] interspecific and intraspecific synteny analysis with MCScanX v1.0.0 [35]. Results were visualized using TBtools ' Multiple Synteny Plot and Advanced Circos '. To assess selective pressures on these genes across species, we calculated Ka/Ks values using Ka/Ks_Calculator 3.0 [36].

2.6 Interactions between microRNAs and BjNDPK target genes

To investigate potential relationships between *BjNDPKs* and microRNAs, we employed the online tool psRNAtarget. This tool was used to predict target relationships between the *BjNDPK* coding sequences and microRNAs from rapeseed, *Brassica nigra*, and *Brassica rapa* downloaded from the mibase database. We selected miRNA-target gene combinations with an expectation value ≤3 [37]. Finally, data visualization was performed using the online tool WeBio (https://www.bioinformatics.com.cn/plot_basic_alluvial_plot_017).

2.6 Tissue expression pattern analysis

To investigate the expression characteristics of *BjNDPKs* in different tissues of *Brassica juncea*, we analyzed the expression profiles across 16 tissues (including leaves, seeds, seed coats, callus, roots, stems, pods, pod wall, flower bud, whole seedling, seedling, shoot, nectary, ovule, embryo, and flower) and generated a heatmap of *BjNDPK* tissue expression patterns.

2.9 Expression analysis of BjNDPKs under drought stress

The experimental material ‘23-443-1’ is a *Brassica juncea* germplasm resource provided by Professor Zhengjie Wan’s research group, College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan, China. At sowing, seeds were pre-germinated in germination trays lined with gauze over Hoagland's nutrient solution to maintain humidity. Cultivation was conducted under a photoperiod of 20°C, 16 h light/8 h dark until day 10. When seedlings reached the two-leaf-one-heart stage, they were subjected to 20% (w/v) PEG 6000 stress treatment for 12 h.Samples were collected before treatment (CK) and after treatment (20% PEG), harvesting the aboveground plant tissues. Each treatment included three biological replicates. The obtained samples were used for subsequent qPCR analysis and determination of H_2O_2 and CAT content.

Total RNA was extracted from leaves using the Vazyme Total RNA Kit (Nanjing , China). Concentration and purity were assessed using a NanoDrop micro-volume spectrophotometer. First-strand cDNA was synthesized using the TransScript One-Step

gDNA Removal and cDNA Synthesis SuperMix Reverse Transcription Kit (Beijing, China). Real-time quantitative PCR (qPCR) was performed on the QuantStudio 5 system using the Vazyme ChamQ Universal SYBR qPCR Master Mix Kit (Nanjing, China). All primers were designed using the Primer 3 online tool [38]. Based on previous studies, *qBjActin*, *Bj18sRNA*, and *BjuTiP41* were selected as candidate reference genes (Table S1) [39]. Experiments were performed with three biological replicates and three technical replicates. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

2.10. Analysis of H_2O_2 and CAT content in Brassica juncea under drought stress

To determine H_2O_2 and CAT content in *Brassica juncea* leaves, enzyme solutions were extracted via homogenization in PBS (w/v = 1 g : 9 ml). Absorbance values were measured at 450 nm using the Plant H_2O_2 and CAT ELISA Kit (Jiangsu, China). H_2O_2 and CAT contents were calculated from standard curves and expressed as μg/g (fresh weight). Each treatment comprised three biological replicates and three technical replicates.

2.11. Root Phenotype Analysis of AtNDPK2 Mutant Arabidopsis

The *Arabidopsis* AtNDPK2 mutant (SN22241) lines were purchased from Arshare Biotechnology Co., Ltd (Fujian, China). Experiments employed 1/2 MS medium as the normal growth condition (CK), while 1/2 MS medium supplemented with 250 mM mannitol simulated drought stress.

3. Results

3.1. Identification and chromosomal localization of the BjNDPK gene family

To identify the *NDPK* gene family in *Brassica juncea*, we screened the *Brassica juncea* reference genome (T84-66.V2.0) provided by the BjuIR database. This was done based on the reported AtNDPK protein sequences from *Arabidopsis thaliana*, combined with BLASTP homology alignment and HMMER domain searches. Redundant transcripts and sequences lacking conserved domains were excluded. Ultimately, 18 non-redundant *BjNDPK* genes were identified (Table S1). Based on chromosome localization information from the genome annotation file, these genes were sequentially named *BjNDPK1* to *BjNDPK18*, and a chromosomal distribution map of *BjNDPK* genes was constructed (Figure 1). Results show these 18 *BjNDPK* genes are distributed across 12 chromosomes in the A and B subgenomes. The A subgenome contains 9 genes located on AA_Chr01, AA_Chr02, AA_Chr03 (2 genes), AA_Chr06, AA_Chr08 (2 genes), and AA_Chr09 (3 genes); Subgenome B contained 9 genes located on BB_Chr03, BB_Chr04 (2 genes), BB_Chr05 (3 genes), BB_Chr06, BB_Chr07 (2 genes), and BB_Chr08.Notably, multiple *BjNDPK* genes cluster on certain chromosomes such as AA_Chr09 and BB_Chr05, while no *BjNDPK* members were detected on chromosomes like Chr10.

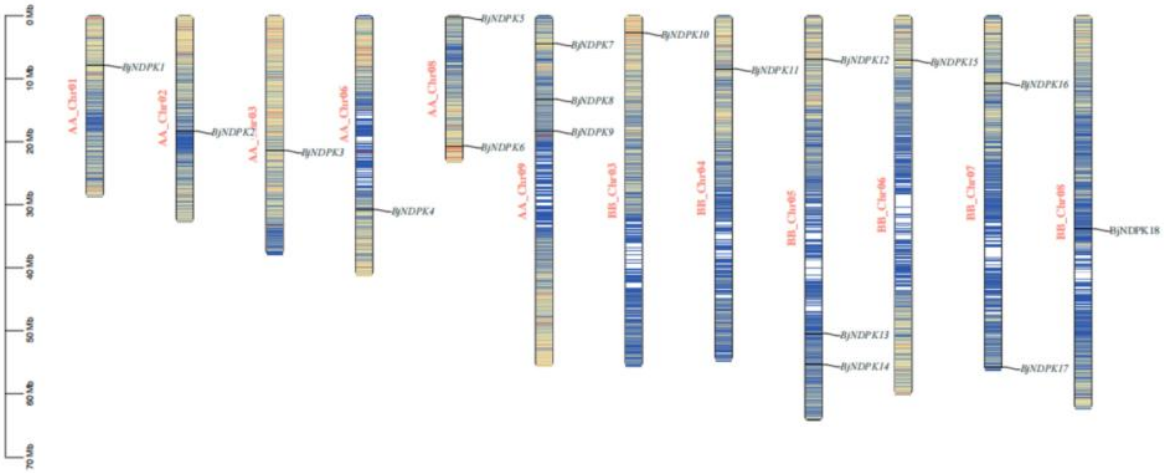

Figure 1. Chromosomal location of the *BjNDPK* in the *B. juncea* genome

3.2 Phylogenetic analysis of the BjNDPK proteins

To investigate the phylogenetic relationships among NDPK proteins across different crops, we classified all NDPK proteins into five groups based on the genetic relationships among 18 BjNDPK proteins, 9 BrNDPK proteins, 8 BnNDPK proteins, and 5 characterized AtNDPK proteins, combined with the position of AtNDPK proteins in the phylogenetic tree: Group I, Group II, Group III, Group IV, and Group V (Figure 2). Five proteins (BjNDPK3, BjNDPK5, BjNDPK8, etc.) clustered in Group I. Five proteins (BjNDPK4, BjNDPK14, etc.) clustered in Group II. Group III contained BjNDPK2, BjNDPK9, BjNDPK18, while the fourth (BjNDPK1 and BjNDPK12) and fifth (BjNDPK6 and BjNDPK10) groups each contain two proteins. Notably, within each branch, most proteins from *Brassica juncea*, *Brassica nigra*, and *Brassica rapa* form tightly clustered subclades, exhibiting extremely close phylogenetic relationships. For example, BjNDPK17 first clusters with BnNDPK6 and then forms a separate branch with BrNDPK5. This suggests that *BjNDPK* genes have maintained high evolutionary conservation.

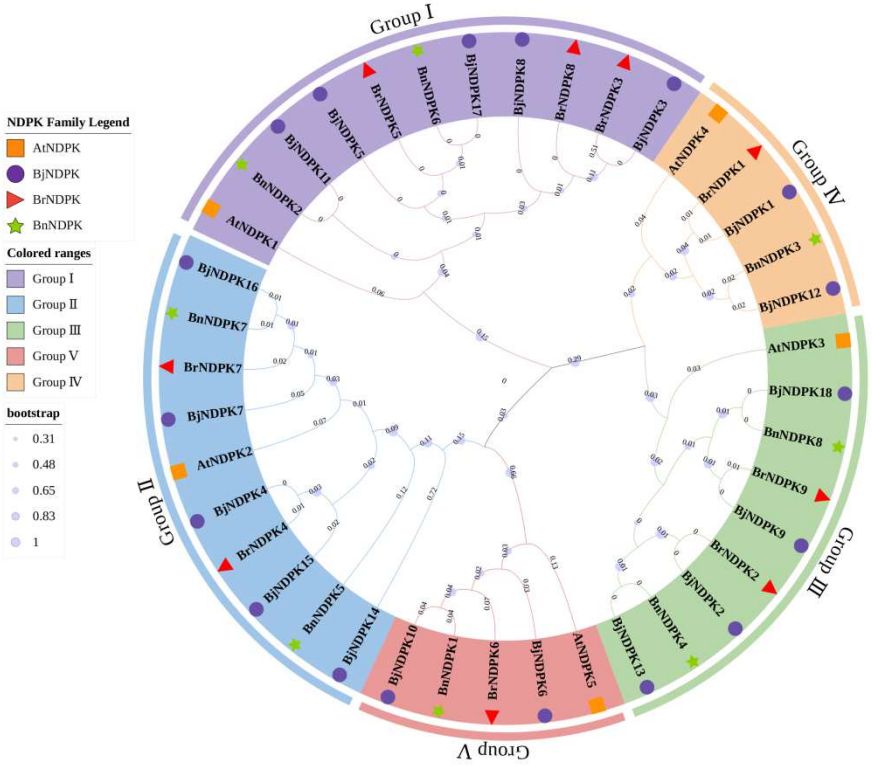

Figure 2. Phylogenetic tree of NDPK proteins in *Arabidopsis thaliana*, *Brassica junce*, *Brassica nigra* and *Brassica rapa*.

3.3 Physicochemical properties and subcellular localization prediction of BjNDPK proteins

Protein physicochemical properties revealed that BjNDPK protein amino acid sequences range from 111 to 237 residues, with molecular weights distributed between 12.07 and 25.95 kDa. Theoretical isoelectric points fluctuate between 5.61 and 9.66. Most stability indices are below 40, with some members like BjNDPK4 exceeding 40, suggesting overall stability. Lipophilicity indices range from 76.58 to 91.55, while average hydrophilicity values are all below zero, indicating strong lipophilicity in BjNDPKs. Predictions from the WoLF PSORT online tool indicate that group 1 contains five genes, all localized to the cytoskeleton. In Group 2, four genes are targeted to the chloroplast, with *BjNDPK14* additionally localized to the cytoskeleton. All six genes in groups 3 and 4 are localized to the mitochondria, while the two genes in group 5 are localized to the endo-

plasmic reticulum (Table S3).This subcellular localization pattern suggests that the *BjNDPK* gene family plays important roles in cellular energy production and metabolism, potentially coordinating energy-related signaling across multiple organelles.

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3.4 Structural prediction of NDPK proteins

Secondary structure analysis revealed (Figure 3): α-helix content ranged from 34.47% to 54.08%; β-sheet content ranged from 4.59% to 14.89%; and disordered coils accounted for 37.84% to 54.43%. Among these, BjNDPK14 exhibits the highest α-helix proportion (54.08%), while BjNDPK16 shows the lowest α-helix content (34.47%) and the highest β-sheet proportion (14.89%).Most proteins contained over 40% disordered coils , followed by α-helix, with β-sheet being the least abundant. This distribution may correlate with structural stability relevant to their functions. SWISS MODEL three-dimensional homology modeling revealed (Figure S1). Sequence identity between all models and their templates generally exceeded 85%, reaching up to 100% in some cases. Analysis indicates that the predicted oligomeric state for most proteins is a homomeric hexamer. The crystal structures of their templates (e.g., PDB: 1u8w, 1s59, 1w7w) clearly demonstrate the typical hexameric assembly of NDPK proteins: two ring-shaped trimers face each other and interlock to form a flat cylinder with a central channel. Notably, AtNDPK5, BjNDPK6, BjNDPK10, and BjNDPK14 were predicted to exist as monomers (Table S3 & Figure S1). Despite minor variations among members, the overall structure exhibits significant conservation, reflecting the maintenance of a similar structural framework throughout the evolutionary history of the NDPK family proteins and supporting their potential functional homology.

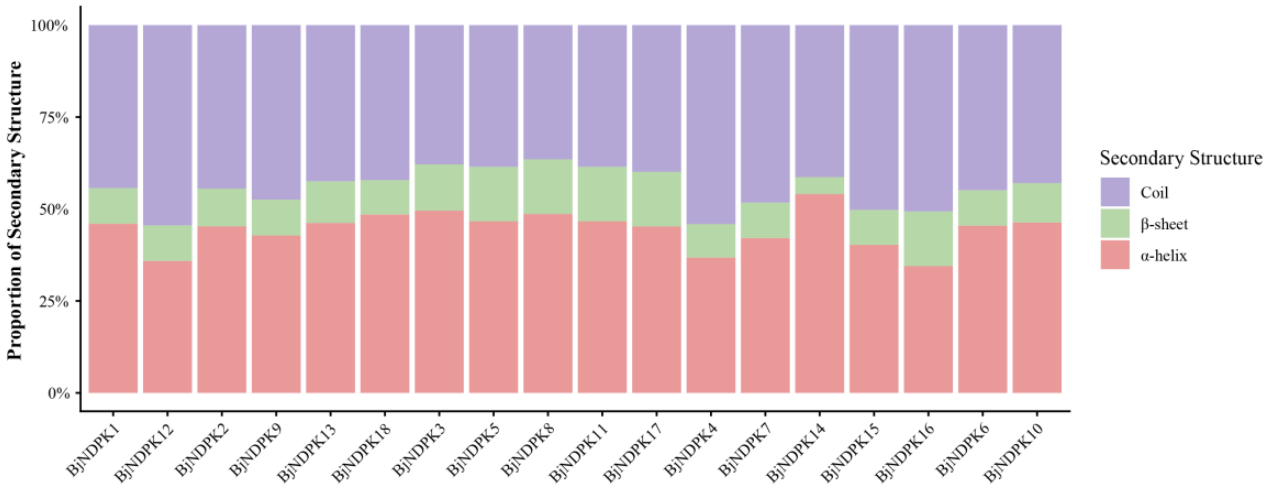

Figure 3. Secondary structure prediction of NDPK protein in *Brassica juncea*.

3.4 Analysis of BjNDPKs structure, conserved motifs and promoter cis-acting elements

The distribution patterns of conserved motifs were analyzed across the protein sequences of 18 BjNDPK family members (Figure 4A). A total of 10 conserved motifs were identified. Motif 1 was present in all BjNDPK proteins, while motif 3 was found in 17 members, suggesting the critical roles of these two motifs within the *BjNDPK* gene family. Additionally, distinct differences were observed among different branches: motif 9 was specific to Groups I and V, motif 8 was specific to Group II, motif 5 was specific to Group I, motif 10 was specific to Group V, and motif 6 was absent only in Group V.These group-specific distribution patterns of motifs suggest functional diversification among BjNDPK family members during evolution. Analysis of Pfam domain composition (Fig-

ure 4B) indicates that Groups I and III possess the typical NDPK_1 domain, while Groups II and IV contain the NDPK-associated PLN02619 domain. Notably, BjNDPK14 possesses an NDPK superfamily domain. In summary, the BjNDPK proteins not only retains highly conserved core components but also develops branch-specific molecular features through domain and motif acquisition or loss, potentially closely related to its involvement in diverse biological functions.

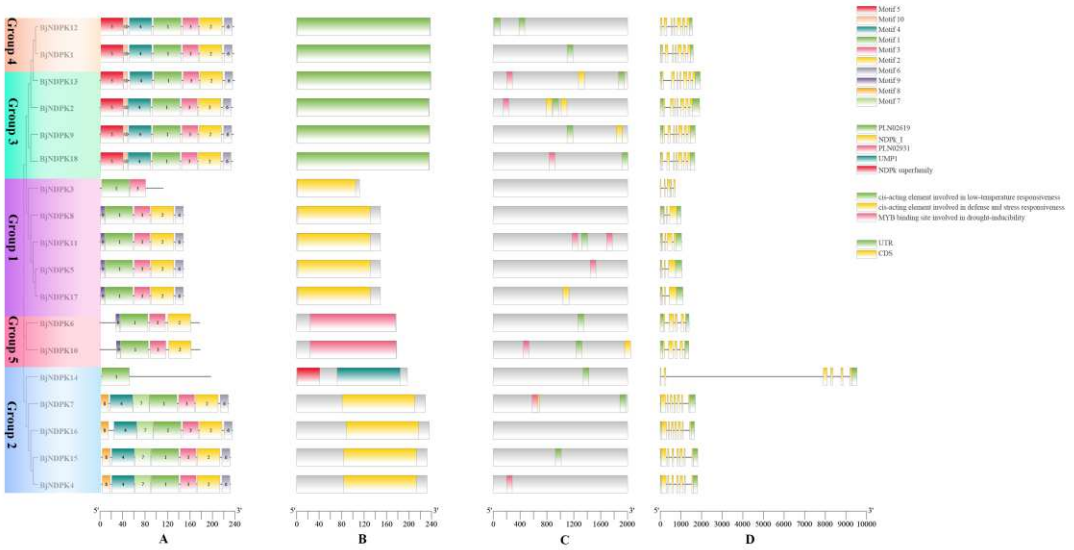

Figure 4. Gene structure analysis of NDPKs family in *Brassica juncea* (A) Conserved motifs of BjNDPK family proteins. (B) Pfam structure of BjNDPK family proteins. (C) Promoter cis-acting element of BjNDPKs. (D) The mRNA structure encoded by the BjNDPKs

Intron-exon structure analysis (Figure 4D) revealed significant structural differences among members of the *BjNDPK* gene family. All members exhibited a marked 3' end bias in their CDS . Furthermore , within the same branch , the 3' CDS length was highly conserved among members, while the 5' CDS length showed considerable variation, indicating a specific spatial distribution pattern of conservation and variability in the coding region. Promoter cis-acting element analysis revealed that 6 genes (including *BjNDPK2* and *BjNDPK13*) contain cis-acting elements involved in defense and stress responses; 9 genes (such as *BjNDPK2* and *BjNDPK10*) carry drought-induced MYB binding sites, distributed in Groups I, II, III, and V; and 12 genes (including *BjNDPK1* and *BjNDPK2*) contain elements related to low-temperature responses, which are present in all phylogenetic groups. These cis-acting elements exhibit specific distribution patterns across different evolutionary branches, suggesting that the *BjNDPK* family may play a functionally differentiated role in various abiotic stress responses (Figure 4C) . To systematically compare the compositional characteristics of cis-acting elements in the promoter regions of *BjNDPK* genes and identify element types specifically enriched in particular evolutionary branches, we performed hierarchical cluster analysis on all identified elements and their quantities, and constructed a visualization heatmap based on this (Figure 5A) . The analysis showed that light-responsive elements are widely present in the family. Among them, G-Box elements are distributed in the promoter regions of most genes , especially significantly enriched in the evolutionary branches containing *BjNDPK2* , *BjNDPK13* , *BjNDPK9* and *BjNDPK18*. In contrast , *BjNDPK1* and *6* not only lack G-Box elements, but also have extremely low overall light-responsive element content. Among stress-related elements, anaerobic response elements (AREs) dominate the distribution. Furthermore, the abscisic acid response element ABRE, associated with plant hormones, is ubiquitous throughout the family. Interestingly, ABRE and the

light-responsive element G-box show a high degree of similarity in their distribution across all *BjNDPK* gene promoters, suggesting that they may synergistically play a role in the cross-regulation of light and ABA signaling pathways. We also identified several cis-regulatory elements related to plant growth and development, including AT-rich elements and Box III associated with chromatin structure and organization, circadian elements regulating biological rhythms, and O_2-sites specifically regulating the expression of seed storage protein genes. Analysis of the four types of elements (Figure 5B) revealed that light-responsive elements were the most numerous, followed by hormone-responsive elements, while growth and development-related elements were the fewest. These results indicate that the *BjNDPK* family not only participates in environmental stress responses but may also play a finely regulated role in various developmental processes.

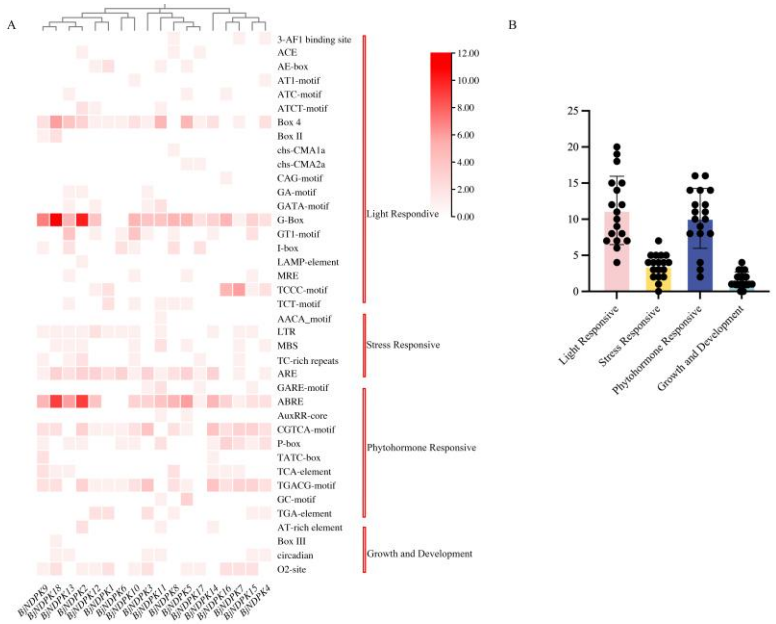

Figure 5. Distribution and enrichment analysis of cis-acting elements in the promoters of *BjNDPK* genes.(A) Heatmap showing the presence of cis-elements across *BjNDPK* members; (B) Quantitative distribution of cis-elements categorized by functional groups.

3.5 Synteny analysis of the BjNDPK gene family

To further investigate the expansion mechanism of the *BjNDPK* gene family in *Brassica juncea* and its evolutionary homology with the genomes of *Brassica rapa* and *Brassica nigra* , we conducted a systematic collinearity analysis using MCScanX software . The interspecific collinearity analysis results (Figure 6) showed 18 pairs of *NDPK* homologous genes between *Brassica nigra* and *Brassica juncea*; and 19 pairs of *NDPK* homologous genes between *Brassica rapa* and *Brassica juncea*. Notably , the *BjNDPK* genes in the A subgenome of *Brassica juncea* showed collinearity not only with the *Brassica rapa* (AA) genome but also with the *Brassica nigra* (BB) genome. Genes in the B subgenome also showed similar collinearity . This supports the hypothesis that *Brassica juncea* originated as an allotetraploid (AABB) formed by heteropolyploidization of *Brassica rapa* and *Brassica nigra*, and also demonstrates the high conservation of the *NDPK* family. Intraspecific collinearity analysis identified 24 collinear gene pairs. These collinear relationships not only existed within the same subgenome but were also widely distributed between subgenomes, indicating that the *BjNDPK* family underwent complex inter-subgenome rearrangement and retention after the whole-genome duplication (WGD) event in *Brassica juncea*. Notably, no collinearity was detected in *BjNDPK3*, possibly due to recent tandem duplication or transposition events causing it to deviate from its ancestral collinearity

region. To further investigate the evolutionary selection pressure on *BjNDPK* collinear gene pairs, we calculated the ratio of nonsynonymous substitution rate (Ka) to synonymous substitution rate (Ks) (Ka/Ks) . Analysis of the 29 collinear gene pairs involved showed that the Ka/Ks values were significantly less than 1 (0.027-0.246 , Table S5) , indicating that this gene family has undergone strong purifying selection during evolution.

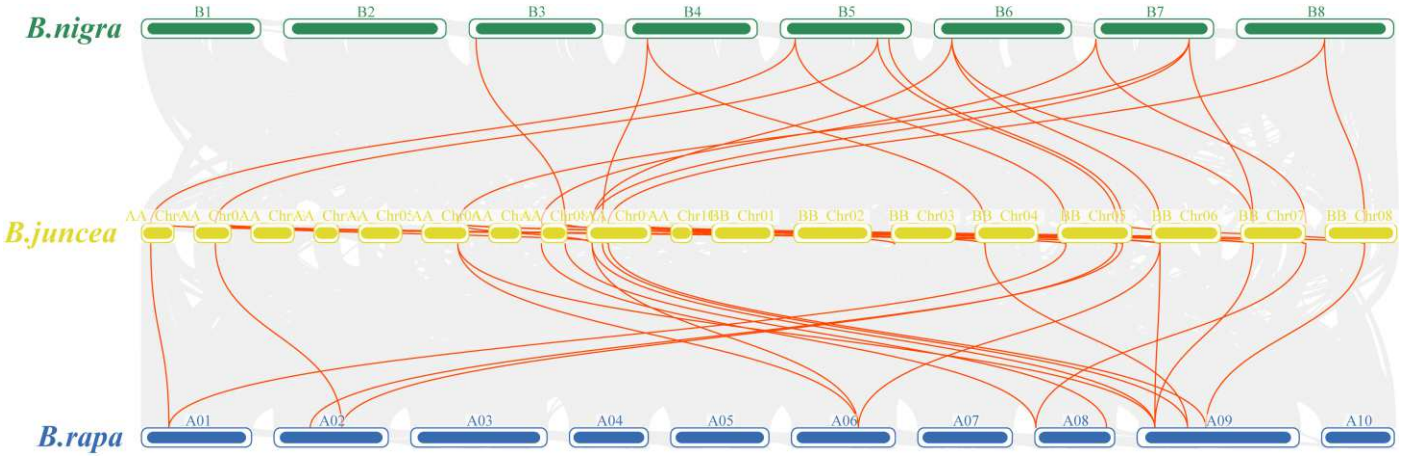

Figure 6. Collinearity of *NDPK* genes in *Brassica junce* (yellow) , *Brassica nigra* (green) and *Brassica rapa* (blue) .

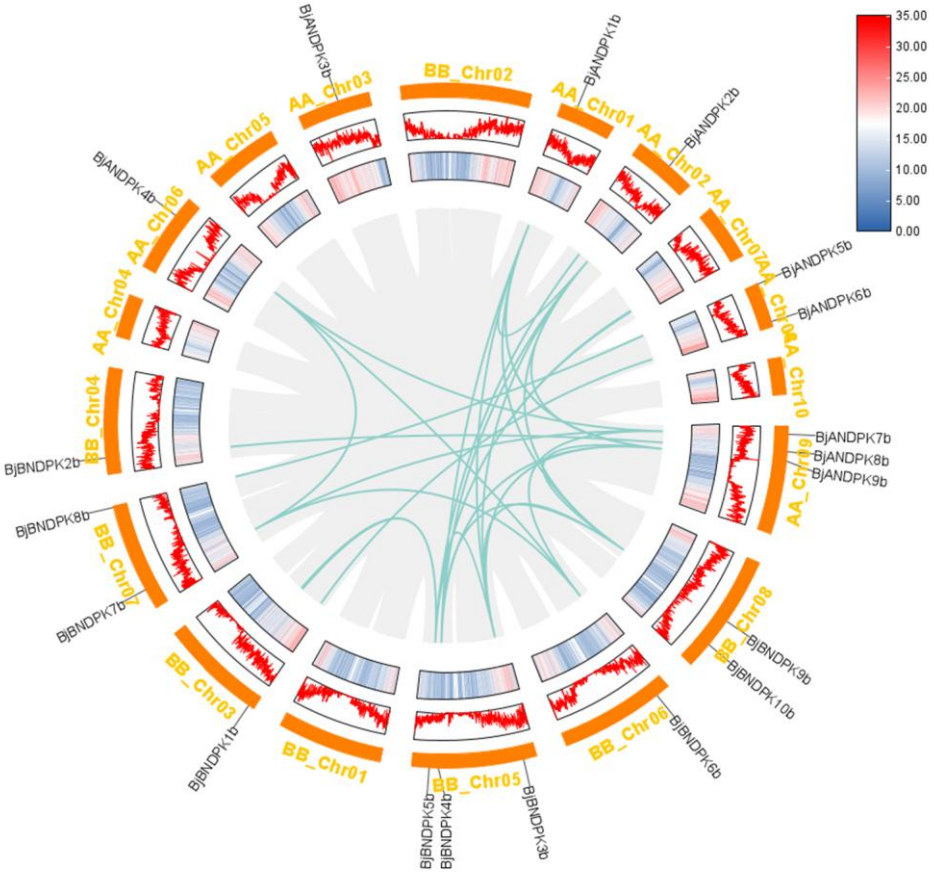

Figure 7. Collinearity of *BjNDPKs* . The circles in the figure from inside to outside represent the unknown base gene density, GC ratio and chromosome length of the *Brassica juncea* genome .

3.7 Analysis of miRNA–BjNDPK target gene interactions

To investigate the post-transcriptional regulatory mechanism of *BjNDPK* genes, we performed a predictive analysis of miRNA targeting relationships among *BjNDPK* family members based on the miRNA sequences of its close relative (*Brassica rapa*), and visualized the complex interactions using impact maps. A total of 7 different miRNAs were identified that can target and regulate 10 *BjNDPK* genes, forming 16 specific regulatory pairs. The results showed that there are two main interaction relationships between miRNAs and genes, and two regulatory modes: 'one-to-many' and 'many-to-one'. Specifically, *bra-miR9563b-3p* can regulate up to 7 target genes, while *BjNDPK1* and *BjNDPK12* can be synergistically regulated by 3 miRNAs. This suggests that *BjNDPKs* may be finely regulated at the post-transcriptional level.

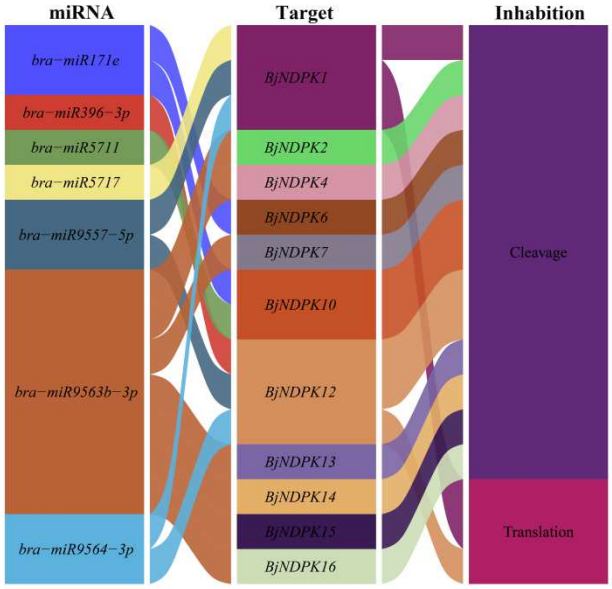

Figure 8. Analysis of the interaction between microRNA and *BjNDPKs.*

3.8 Organizational expression pattern analysis

To investigate the expression patterns of *BjNDPKs* in different tissues of the *Brassica juncea* variety T84-66, we integrated transcriptome data (expressed as TPM values) from 15 tissue types of this variety and constructed a gene expression heatmap after normalization ($\log_{10}$ (TPM + 1)) (Figure 9). The results showed that members of the *BjNDPK* family were expressed in multiple tissues, exhibiting certain tissue specificity and functional differentiation. Specifically, *BjNDPK1* , *BjNDPK13* , *BjNDPK3* , and *BjNDPK11* showed extremely low or undetectable expression levels in most tissues, suggesting they may have specific regulatory mechanisms or limited function. *BjNDPK17*, *BjNDPK6*, *BjNDPK10*, and *BjNDPK7* showed relatively uniform expression across tissues but low overall abundance. In contrast, five genes, *BjNDPK12*, *BjNDPK2*, and *BjNDPK18*, showed high expression levels in most tissues, with particularly significant enrichment in seed coat , embryo , and ovule, suggesting these genes may play important roles in seed development and reproduction.

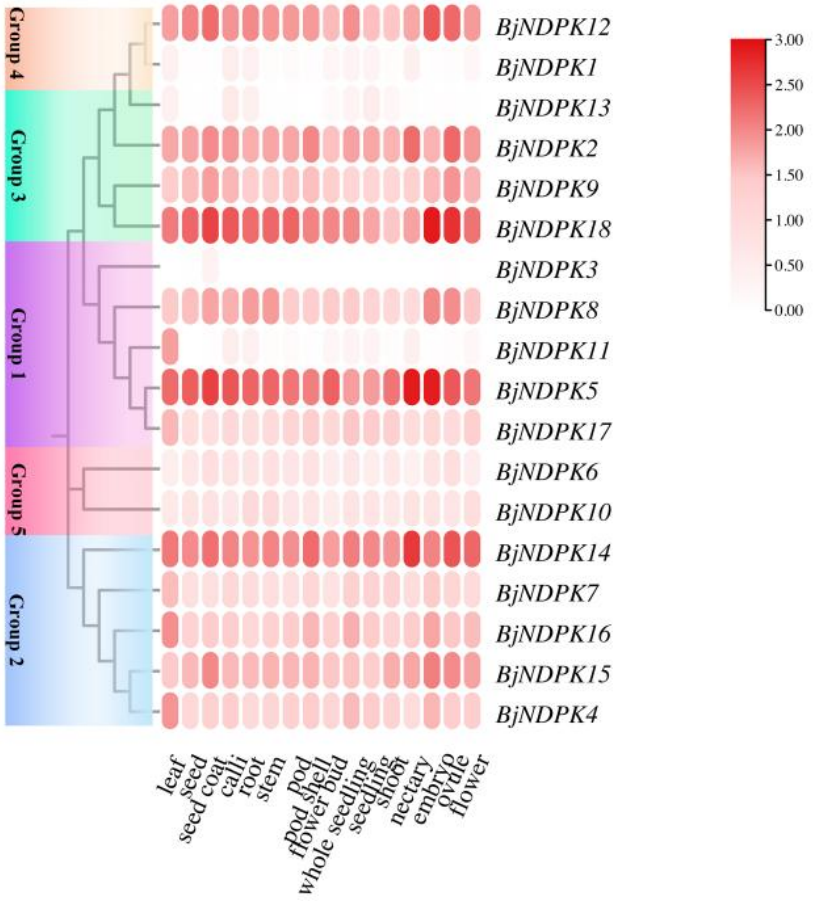

Figure 9. The expression levels of *BjNDPK* genes in 16 tissues (leaf, seed, seed coat, calli, root, stem, pod, pod shell, flower bud, whole seedling, shoot, nectary, embryo, ovule, flower).

3.9.Expression analysis of the BjNDPK gene family in response to drought stress based on stable internal references

To investigate the expression of the *BjNDPK* gene family under abiotic stress , we used qRT-PCR to analyze the expression changes of each gene in *Brassica juncea* seedlings under drought stress. To ensure the accuracy of the qPCR data, we evaluated the stability of three selected candidate internal control genes (*qBjActin*, *Bj18sRNA*, and *BjuTiP41*) using geNorm, NormFinder, and BestKeeper software. The internal control stability matrix showed that *qBjActin* exhibited the most stable expression under different stress treatments and was ultimately selected for subsequent standardized calculations of target gene expression levels (Figure 10 & Table S6).

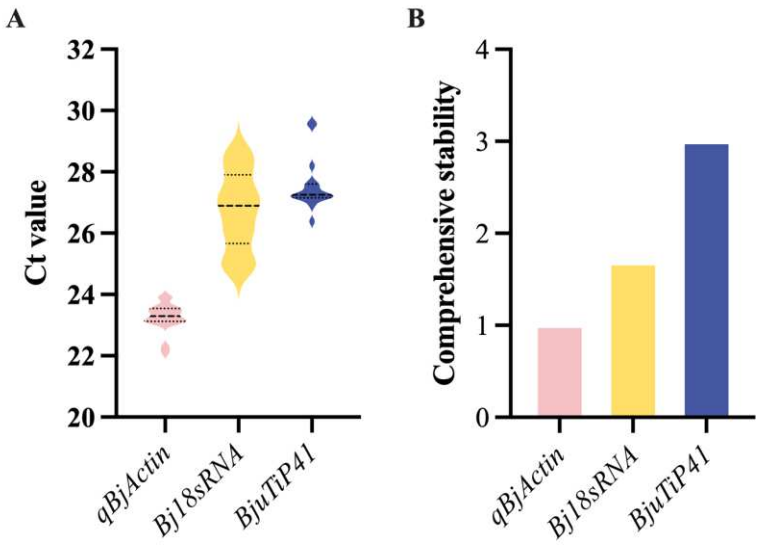

Figure 10. Evaluation of reference gene stability in *Brassica juncea* based on Ct values and comprehensive stability scores.(A) Distribution of Ct values for candidate reference genes; (B) Comprehensive stability scores calculated using multiple algorithms.

We analyzed the expression levels of 18 members of the *BjNDPK* family in *Brassica juncea* leaves under drought stress (Figure 11). The results showed that the expression levels of 10 genes (including *BjNDPK1*, *BjNDPK4*, *BjNDPK5* and *BjNDPK6*) were significantly higher than those of the control group (P≤0.01); the expression levels of *BjNDPK16* and *BjNDPK18* were significantly increased (P≤0.05), while the expression levels of *BjNDPK2* and *BjNDPK3* decreased significantly (P≤0.05). No significant changes were observed in the four genes (*BjNDPK9* , *BjNDPK12* , *BjNDPK14* and *BjNDPK17*).

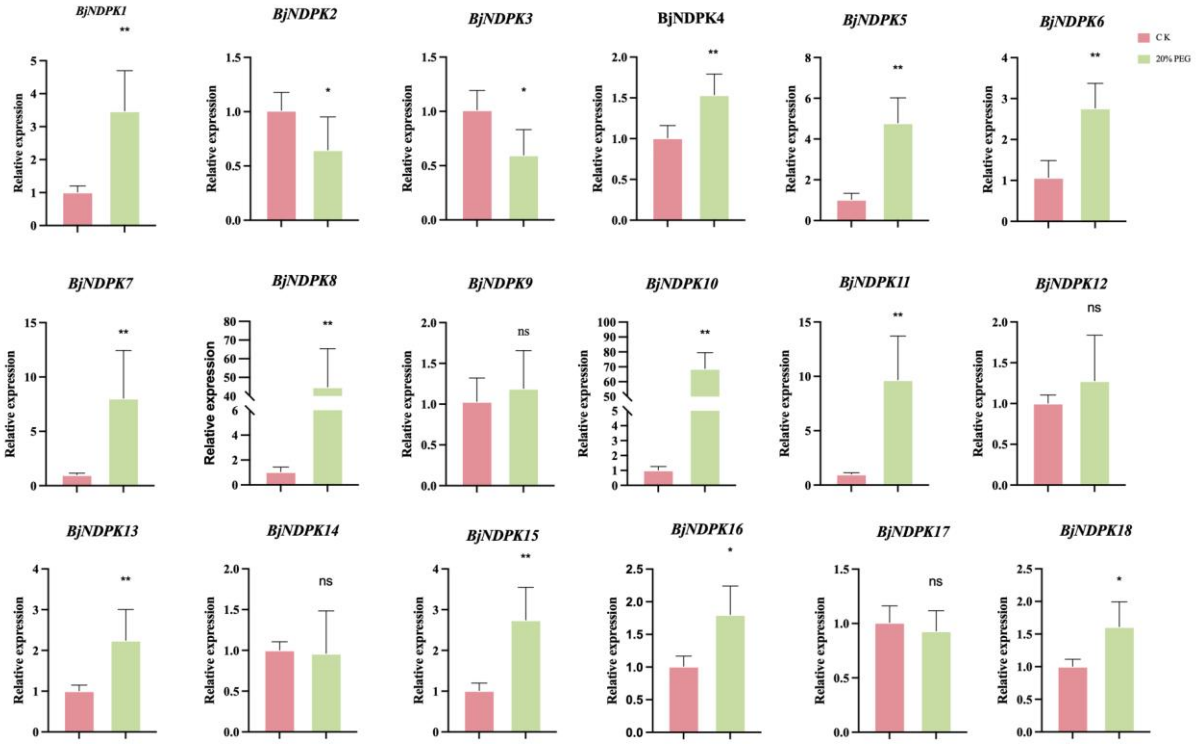

Figure 11. The relative expression of *BjNDPKs* in 20% (w/v) PEG 6000 after 0 h, 6 h. Data represent the mean ± standard error for three biological experiments. Statistical differences between treat-

ment groups were determined using T-test. ns : no significience. *: significant differences between treatments at $p \leq 0.05$. **: significant differences between treatments at $p \leq 0.01$.

3.11. Analysis of changes in hydrogen peroxide and catalase activity under drought stress

We first constructed standard concentration curves for H_2O_2 and CAT , with correlation coefficients greater than 0.99 for both. Physiological parameters showed that abiotic stress effectively induced an oxidative burst in the plants. Compared to the control, H_2O_2 content increased significantly under drought stress. However, drought stress treatment did not cause a significant change in CAT (Figure 12).

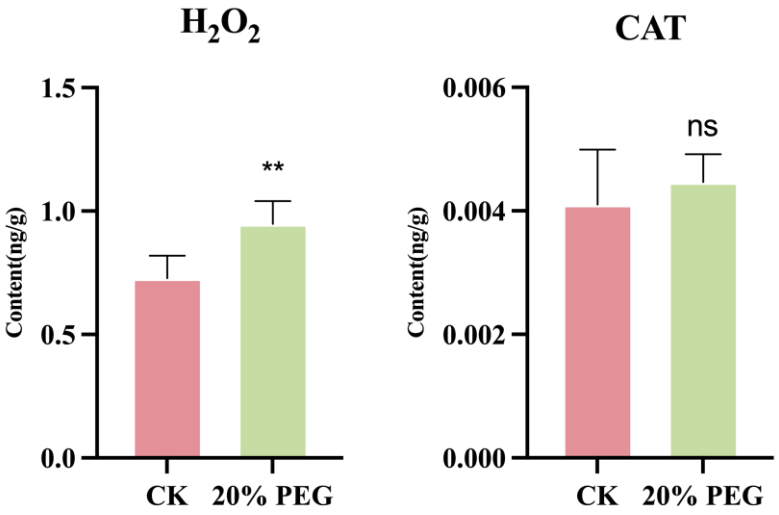

Figure 12. Changes in H_2O_2 and CAT content in *Brassica juncea* leaves under CK and 20% PEG-induced drought stress.

3.12. *Root phenotype analysis of the AtNDPK2 mutant Arabidopsis*

The WoLF PSORT online database predicted that the AtNDPK2 protein is located in chloroplasts, and an AtNDPK2 mutant system was constructed in a wild-type *Arabidopsis* background. Phylogenetic analysis showed that this mutant gene is most recently related to *BjNDPK7* in *Brassica juncea*. To investigate the role of *AtNDPK2* in root development and drought stress response, we compared the root length phenotype of the mutant (AtNDPK2) and the wild type (WT) under normal growth conditions (1/2 MS medium) and drought stress conditions (1/2 MS + 250 mM Mannitol) (Figure 13A). In the 1/2 MS , there was no significant difference in root length between the mutant and wild-type plants: the average root length of the wild-type was 60.93±3.58 mm , while that of the mutant was 58.64±3.21 mm. Under simulated drought stress, the root length of the wild-type was significantly shortened to 22.07 ± 1.85 mm, while the root length of the mutant remained at 34.08 ± 2.13 mm, which was significantly higher than that of the WT (Figure 13B) .

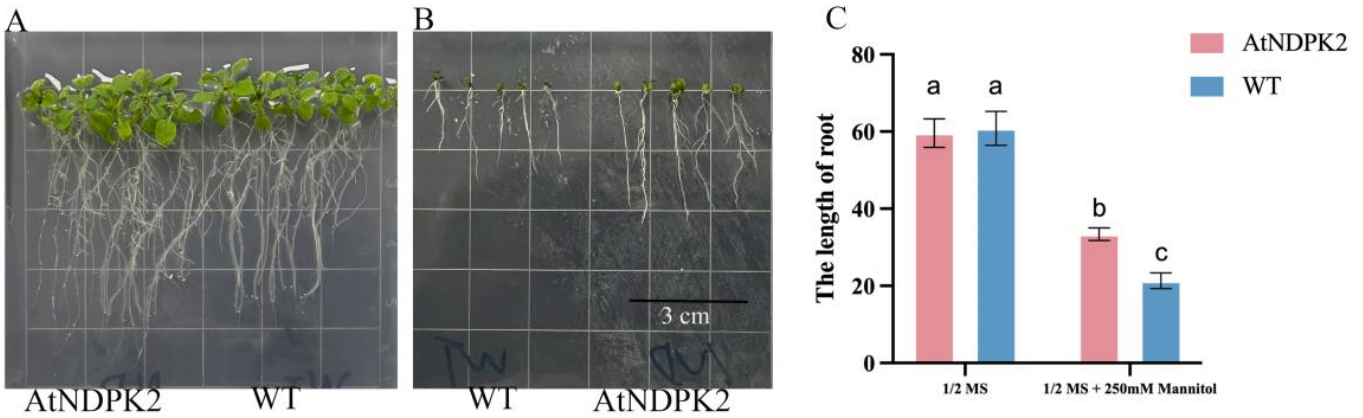

Figure 13. Phenotypic analysis of the AtNDPK2 mutant in *Arabidopsis thaliana*. (A) Phenotype of the AtNDPK mutant in 1/2 MS(CK), (B) Phenotype of the AtNDPK mutant in 1/2 MS+250 mM mannitol(Drought), (C) Root length of the AtNDPK2 mutant at 15 days old. The letters above the columns indicate significant differences ($p < 0.05$).

4. Discussion

Brassica juncea (36, AABB), an important crop used for oil, vegetables, and forage, is an allotetraploid formed by the doubling of hybrids between *Brassica nigra* (AA) and Brassica rapa (BB) approximately 6000 years ago[40, 41]. In this study , we systematically identified the *NDPK* gene family in *Brassica juncea*. Most of the 18 *BjNDPK* genes identified could be found in both *Brassica rapa* and *Brassica juncea*, showing high conservation, indicating that a large number of genes were preserved during evolution. However, some genes did not show detectable collinearity with their ancestral species, suggesting that they may have undergone independent evolutionary trajectories after speciation through chromosomal rearrangement or new functionalization [42-44]. In addition, subgenomic cross-collinearity was detected in some genes , which may be related to complex intergenomic rearrangement and selective conservation during evolution . Further Ka/Ks (both less than 1) analysis showed that this family was mainly constrained by strong purifying selection to maintain its conserved core functions.

Protein structure prediction results showed that all BjNDPK proteins were mainly α-helical, followed by α-helix , with the lowest β-sheet content, which is consistent with the folding characteristics of typical NDPK proteins. More importantly, most proteins assembled in the form of homohexamers, which has been confirmed in multiple NDPK crystal structure templates (such as PDB: 1u8w, 1s59, 1w7w). This highly conserved tertiary structure and oligomeric form [45]. further supports the fundamental role of this family in energy metabolism and signal transduction. In addition, we divided the 18 proteins of the *BjNDPK* family into 5 groups. Group III and group Ⅳ contains the PLN02619 structural domain ; Group V contains the PLN02931 domain. These two domains are closely related to the NDPK core domain. PLN02619 retains the NDPK core domain; PLN02931 is a domain formed after the key catalytic residues of the NDPK core domain undergo mutations; this may cause group Ⅴ to lose kinase activity and instead participate in protein-protein interactions or regulatory functions. This is consistent with the tertiary structure prediction results of group Ⅴ (Figure S1). It has been reported that some plant NDPKs, after losing or weakening their catalytic function, can participate in signal transduction, ROS dynamic balance and stress response through protein interaction [46]. Therefore, these members may no longer mainly undertake the classical nucleotide transfer function, but instead turn to the role of regulation. This situation also exists in rapeseed. It reveals that while maintaining basic energy metabolism function, the

NDPK gene family also meets the adaptive needs of plants in complex environments through the functional differentiation or even new functionalization of some members.

Analysis of cis-acting elements revealed that G-box and ABRE, two types of cis-acting elements, exhibit a highly consistent distribution pattern in the promoter regions of most members. This feature has not only been verified in Brassica spp., but is also consistent with the research conclusions of model plants such as *Arabidopsis thaliana* [47]. G-box is a key binding site for phytochromes and bZIP-type transcription factors in the light signaling pathway, while ABRE is the core regulatory element of ABA signaling response. The interaction between light and ABA plays an important role in plant stress response (such as drought and high salinity), which may represent a conserved regulatory feature formed by the NDPK family in long-term evolution. This distribution pattern not only reveals the multiple functions of NDPK in energy metabolism and stress tolerance, but also indicates that some members may have obtained new regulatory functions beyond traditional energy metabolism functions through deep coupling with light signaling and hormone pathways.

This study revealed the expression profiles of *BjNDPK* gene family members in *Brassica juncea* under different tissues and drought stress . Under drought stress, most genes (such as *BjNDPK1*, *BjNDPK4*, *BjNDPK5*, and *BjNDPK6*) were induced to express, while some members (such as *BjNDPK2* and *BjNDPK3*) were suppressed, suggesting that different members may be involved in different stress signaling pathways. This expression differentiation likely stems from the differences in the distribution of cis-regulatory elements in their promoter regions, thereby mediating the specific responses of genes to drought signals. Simultaneously, drought stress triggered a significant increase in H_2O_2 content, indicating a typical oxidative burst . Upregulated *BjNDPK* genes (such as *BjNDPK4* , *BjNDPK6* , *BjNDPK7* , *BjNDPK8* , and *BjNDPK11*) may be involved in ROS-related stress tolerance pathways, potentially acting as signal amplifiers to activate downstream defense responses and indirectly enhancing antioxidant capacity. Downregulated members (such as *BjNDPK3* and *BjNDPK15*) may play a negative feedback regulatory role to prevent overreaction. It is worth noting that although the H_2O_2 level rose sharply, the CAT enzyme activity did not change significantly, which may be related to the timeliness of the antioxidant enzyme system response [48], subcellular localization specificity or regulatory hierarchy differences. In addition, it is also related to its fine regulation by miRNAs, such as *bra-miR9563b-3p* targeting multiple members. In summary, the *BjNDPK* gene family mainly responds to abiotic stress by regulating ROS signal balance and downstream expression networks. Its role goes beyond the traditional maintenance of energy metabolism. It is also the core regulatory node that integrates redox signals and stress response. This dual function is likely the key mechanism by which it endows plants with stress resistance.

Under normal growth conditions, there was no significant difference in root length between the AtNDPK2 mutant and WT; however, under drought stress, the root length of the mutant was significantly higher than that of WT ($P < 0.01$) , indicating that loss of *AtNDPK2* function can enhance the root growth capacity of *Arabidopsis* under osmotic stress. This phenotype may be due to its regulatory role in key physiological processes such as cell turgor pressure, hormone signal transduction or ion homeostasis, thereby alleviating the inhibitory effect of drought on root elongation. It is worth noting that this functional conservation may be related to natural genetic variation among members of the NDPK family. For example, in the *Brassica juncea* homolog *BjNDPK9* , there are two single nucleotide polymorphism (SNP) subtypes, T and C, at position 265,638. The distribution of germplasm resources of the T subtype is highly correlated with the drought environment of the six continents of the world (Figure 14), suggesting that this position

may play an important role in plant adaptation to drought stress. This has been studied in wheat [49].

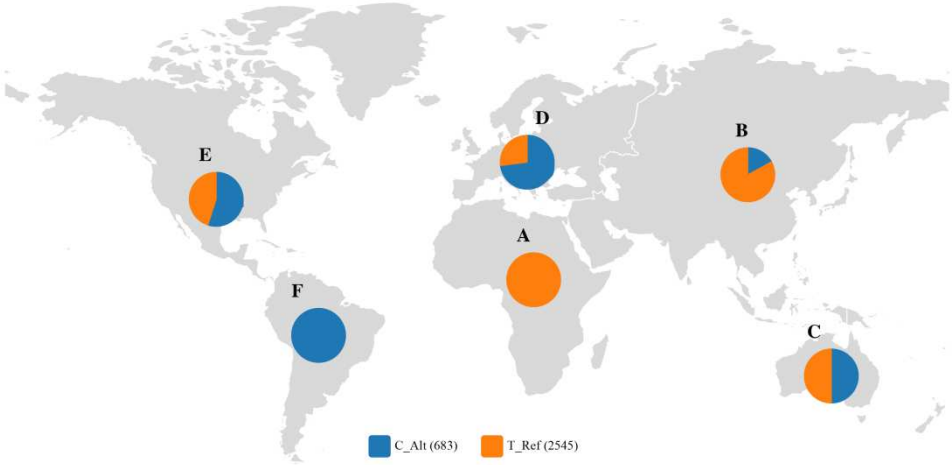

Figure 14. Global distribution of the T/C SNP at position 265,638 in BjNDPK9 and its association with arid environments across six continents. A - F respectively represent Africa, Asia, Oceania, Europe, North America and South America.

5.Conclusions

This study systematically identified 18 *BjNDPKs* in *Brassica juncea*, revealing their conservation and functional differentiation in structure, evolution, and expression. Collinearity analysis showed that this family underwent subgenomic rearrangement during tetraploidization in *Brassica juncea* and was subject to strong purifying selection. Promoter and miRNA analysis revealed their involvement in the regulation of stress responses to light, ABA, and drought. Expression profiling confirmed that several members showed significant responses to tissue development and drought stress, while the *Arabidopsis* AtNDPK2 mutant exhibited enhanced root growth under osmotic stress, validating the functional conservation of NDPK in stress resistance (Figure 15). This study provides an important theoretical basis and candidate genes for elucidating the role of the *BjNDPK* family in abiotic stress and for *Brassica juncea* stress-tolerant breeding.

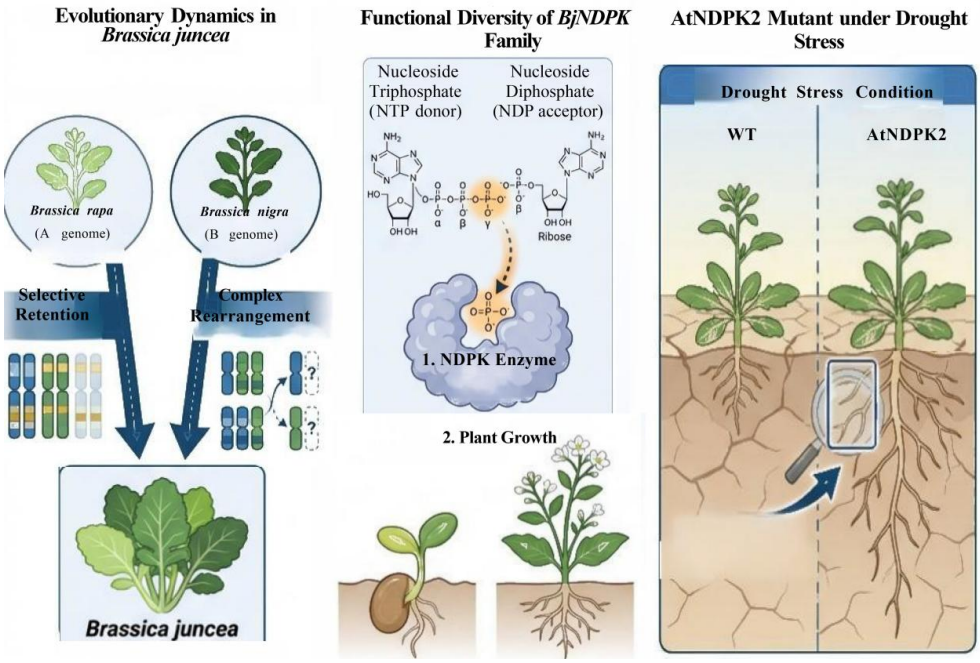

Figure 15. Systematic analysis of *BjNDPK* genes reveals their structural conservation, regulatory complexity, and potential involvement in drought tolerance.

Supplementary Materials: The following supporting information can be downloaded at: https://doi.org/10.1186/s12870-024-05316-w, Table S1. Sequence information of *BjNDPKs*; Table S2. Primer sequences used for qPCR analysis of *BjNDPK* gene family members and reference genes. Table S3. Physicochemical properties and subcellular localization prediction of BjNDPK proteins; Table S4. Tertiary structure *modeling information of* NDPK protein; Table S5. KaKs value of *Brassica juncea;* Table S6. The stability of 3 different candidate reference genes; Figure S1. Prediction of the tertiary structure of the NDPK protein.

Author Contributions: Data curation, H.H. and H.Z.; formal analysis, X.Z, Z.L., X.W., X.X. and Q.L.; writing—original draft preparation, H.H. and H.Z.; writing—review and editing, Z.L., X.X., Q.L. and H.W.; funding acquisition, H.Z. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All the data generated or analyzed during this study are included in this published article and its supplementary information files.

The original datasets generated and analyzed during this study are available in the BjuIR repository, BjuIR, *Brassica juncea* multi-omics database (information resource).

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Ethics declarations

Ethics approval and consent to participate

Study complied with local and national regulations for using plants.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Clinical trial number

Not applicable

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