

Article

Genome-Wide Identification of Peanut Pyruvate Kinase Gene Family and Their Potential Roles in Seed Germination and Drought Stress Responses

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Abstract: Pyruvate kinase (PK), a pivotal enzyme in glycolysis, serves as a multifunctional regulator of plant growth, development, and stress adaptation. Despite its significance, the functional roles of PKs in peanut remain largely unexplored. Here, we performed a genome-wide identification and systematic characterization of PK genes in cultivated peanut, identifying 21 *AhPK* genes (*AhPK1–AhPK21*). Phylogenetic classification divided these genes into two subfamilies: PKc (comprising PKc-1 and PKc-2 subgroups) and PKp (comprising PKp- α and PKp- β subgroups). *AhPK* members within the same subfamily shared similar motif composition patterns, while genes from different subgroups showed significantly different exon–intron organizations. Collinearity analysis indicated that segmental duplication events and purifying selection predominantly drove the expansion and evolution of the *AhPK* family. Evolutionary analysis further indicated closer evolutionary relationships between peanut PKs and those of Arabidopsis than with rice. Predicted protein interaction networks suggested that *AhPKs* can form polymeric protein complexes (e.g., PKp- α and PKp- β) or interact with some important proteins, including FBA4, F14O13.7, APY, DLD, and T16L4.190. Promoter analysis identified abundant *cis*-regulatory elements associated with light responses, stress responses, hormone responses, and development. Expression pattern analysis demonstrated the significant induction of multiple *AhPK* genes during seed germination and under polyethylene glycol (PEG)-induced drought stress or abscisic acid (ABA) treatment. Collectively, these findings provide critical insights into the functional roles of *AhPK* genes in seed germination and drought stress responses, establishing a foundation for future mechanistic studies.

Keywords: peanut; PK gene family; expression analysis; seed germination; drought stress



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1. Introduction

Glycolysis, a central metabolic pathway, is ubiquitous in eukaryotes. Pyruvate kinase (PK; EC 2.7.1.40) is an essential enzyme that catalyzes the final step of glycolysis, where it drives the transformation of phosphoenolpyruvate (PEP) into pyruvate and adenosine triphosphate (ATP) by transferring a phosphate group to adenosine diphosphate (ADP) through an irreversible reaction [1–3]. As one of the three critical rate-limiting enzymes in

glycolysis, PK regulates carbon flux from glucose to pyruvate [4], a central metabolite interfacing with the tricarboxylic acid cycle, lipid biosynthesis, and amino acid metabolism [5]. Its pivotal role underscores its influence on cellular energy metabolism [6].

In plants, PK exists in the form of two isozyme types: cytoplasmic PK (PKc) and plastid PK (PKp) [7]. These isoforms exhibit distinct physical, kinetic, immunological, and regulatory properties [7–11]. The PK gene family varies significantly across species in terms of subunit composition and gene number. For example, *Arabidopsis* harbors 14 PK genes (10 PKc and 4 PKp) [12], rice contains 10 (6 PKc and 4 PKp) [13], and soybean has 27 (16 PKc and 11 PKp) [14]. PK enzymes typically form homotetramers, though they can also exist as monomers, homodimers, heterodimers, heterotetramers, or heterohexamers, enabling functional versatility [15–18]. Collectively, PK genes comprise a functionally diverse family integral to plant metabolism and development [19,20].

The PKc and PKp isoforms play significant roles in plant growth, development, and stress adaptation. In *Arabidopsis*, *PKp1* and *PKp2* genes have demonstrated roles in seed oil biosynthesis, embryo development, and seed germination [19,20]. Reduced PKc activity in transgenic potato led to decreased pyruvate pools associated with the citric acid cycle, thereby altering carbon allocation and respiratory processes [21]. The cotton PKc *GhPK6* modulated fiber elongation via a reactive oxygen species-dependent inhibition mechanism [22]. In rice, PKc *OsPK1* influenced key morphological traits, especially plant height [23]. Multiple rice PK genes, including *OsPK1*, *OsPK2*, *OsPK3*, *OsPK4*, *OsPK5*, *OsPK6*, *OsPK7*, *OsPK8*, and *OsPK10*, have been identified as critical factors affecting grain quality and yield [13,24–26], with some also linked to seed germination [13,24,27]. Additionally, the soybean PKc *GmPK21* negatively influenced salt stress resistance in transgenic *Arabidopsis* at the seedling stage [14].

Cultivated peanut (*Arachis hypogaea* L.) is an allotetraploid crop with an AABB genome [28]. The recently released whole genome sequence of the cultivated peanut variety Shitoqi [28] enables the genome-wide identification and examination of gene families. In this study, we characterized the peanut PK gene family by examining phylogenetic relationships, gene structure, conserved motifs, gene duplication, protein–protein interaction networks, and *cis*-acting elements. Previous studies have highlighted the significant roles of PK in plant growth, development, and stress responses [13,14]. However, the expression pattern and the functional roles of peanut PK genes in seed germination and stress responses, including drought and ABA, remain poorly understood. To address this, we investigated the tissue-specific expression and stress-responsive profiles of peanut PK genes. Our primary objectives were to understand the evolution and diversity of peanut PK genes, as well as their potential contributions to plant development and stress responses. This work provides a robust foundation for future functional studies on the PK gene family in peanuts.

2. Materials and Methods

2.1. Identification of the AhPK Gene Family

Protein sequences of the *Arabidopsis* pyruvate kinase (*AtPK*) gene family were retrieved from the *Arabidopsis* Information Resource (TAIR; <https://www.arabidopsis.org/>, accessed on 7 March 2024). Genomic information and GFF3 annotations for *A. hypogaea* were sourced from the Peanut Genome Resources database (<http://peanutgr.fafu.edu.cn/index.php>, accessed on 7 March 2024) [28]. *AtPK* protein sequences served as queries for a BLASTP search against the local protein database of *A. hypogaea*, employing an e-value cutoff of 10^{-7} . Additionally, the PK and PK_C domains (PF00224 and PF02887) from the PFAM database (<http://pfam.xfam.org/>, accessed on 7 March 2024) were used as the queries to pick out AhPK via the Simple HMM Search pro-

gram within TBtools v2.154 software [29]. Candidate AhPK proteins identified through BLAST and HMM searches were merged, manually curated to remove redundancies, and validated for conserved domains using Pfam (<http://pfam.xfam.org/>, accessed on 8 March 2024), SMART (<http://smart.embl-heidelberg.de/>, accessed on 8 March 2024), and NCBI-CDD (<https://www.ncbi.nlm.nih.gov/cdd/>, accessed on 8 March 2024). The predicted AhPK genes were named based on their chromosomal positions. Physicochemical properties, including molecular weight (MW), theoretical isoelectric point (pI), instability index (II), grand average of hydropathicity (GRAVY), and aliphatic index (AI), were calculated using the ProtParam Tool (<https://web.expasy.org/protparam/>, accessed on 9 March 2024). Subcellular localization was predicted using Plant-mPLoc (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>, accessed on 9 March 2024).

2.2. Phylogenetic Analysis of AhPK Proteins

PK protein sequences from peanut, Arabidopsis, and rice were aligned using the MUSCLE algorithm implemented in MEGA 11.0 software [30]. A phylogenetic tree was then constructed using the neighbor-joining approach with 1000 bootstrap replicates. Visualization and optimization were conducted using Evolview version 3 (<http://www.evolgenius.info/evolview>, accessed on 12 March 2024) [31].

2.3. Gene Structure, Conserved Motif, and Synteny Analysis

Conserved motifs in AhPK proteins were predicted using the MEME tool (<https://meme-suite.org/meme/>, accessed on 17 March 2024) with default parameters (maximum 10 motifs). Exon–intron structures of the AhPK genes were annotated and visualized using TBtools v2.154 software [29]. Gene duplication events and collinearity were analyzed using MCScanX in TBtools v2.154 software [29]. The values of Ka (nonsynonymous), Ks (synonymous), and Ka/Ks of each AhPK gene pair were calculated using the Simple Ka/Ks Calculator method implemented in TBtools v2.154 software [29].

2.4. Prediction of cis-Acting Elements in Promoter Sequences

Promoter regions (2000 bp upstream of transcription start sites) of the identified AhPK genes were analyzed for cis-acting elements using the PlantCARE online program (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 17 March 2024). The results were visualized with TBtools v2.154 software [29].

2.5. Protein–Protein Interaction Network Analysis

Possible interactions of AhPKs were predicted by constructing their protein–protein interaction network based on their AtPK homologs in Arabidopsis using the bioinformatic webserver STRING (<http://www.string-db.org/>, accessed on 17 March 2024).

2.6. Plant Growth and Treatment

The peanut cultivar “Silihong” was used, with seeds obtained from the Shandong Institute of Peanuts (Qingdao, China). Plants were grown in the experimental field at Zhongkai University of Agriculture and Engineering (Guangzhou, China). Tissues (root, leaf, seed, shell, root nodule) were collected at specified developmental stages, as follows: root, leaf, and root nodule at 40 days after sowing; seed and shell at 80 days after sowing. To collect embryo samples, sterilized seeds were placed on filter paper moistened with sterile distilled water in a seed germination box and incubated at 28 °C. Peanut embryos were isolated from dry seeds (0 h) or seeds at two imbibition stages (4 h and 8 h after imbibition). For stress treatments, ten-day-old seedlings (greenhouse-grown at 28 °C, 16 h light/8 h dark) grown in 1/2 Hoagland solution were treated with PEG-induced drought stress (15% (*w/v*) PEG-6000) or 0.5 µM ABA. Root samples were collected at various time

points (0, 2, 4, and 8 h post-treatment). All samples were immediately frozen in liquid nitrogen after collection and stored at -80°C for RNA extraction.

2.7. RNA Extraction and qRT-PCR

Total RNA was isolated using the RaPure Plant RNA Kit (Magen Biotechnology Co., Ltd., Guangzhou, China) following the manufacturer's instructions. First-strand cDNA was synthesized using the Vazyme HiScript II 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd., Nanjing, China). Real-time quantitative PCR (qRT-PCR) assays were performed with a Bio-Rad CFX96 machine (Bio-Rad Laboratories Inc., Hercules, CA, USA) using SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China). The *AhActin* gene (AH15G32190) served as an endogenous internal standard to normalize the expression of the tested genes. Relative expression levels were calculated using the comparative CT method with the formula $2^{-\Delta\Delta C_t}$ [32]. Gene-specific primers for qRT-PCR are listed in Table S1. Three biological replicates were conducted.

2.8. Statistical Analysis

Experimental data were analyzed using Microsoft Excel 2019 (Microsoft, Redmond, WA, USA) and SPSS software (IBM SPSS Statistics version 27, Chicago, IL, USA). Data are means and standard deviations (SD) from three biological replicates conducted in triplicate. Significant differences were determined by Student's *t*-test and Duncan-test.

3. Results

3.1. Identification and Characterization of AhPKs Gene Family

A total of 21 *AhPK* genes were identified in the cultivated peanut genome through BLAST searches and conserved domains confirmation. Based on their chromosomal locations, these genes were designated *AhPK1* through *AhPK21*. These genes exhibited uneven chromosomal distribution (Figure 1A), with 20 localized to 13 chromosomes and 0 detected on chromosomes 1, 2, 7, 8, 10, 11, 13, 17, 18, or 20. *AhPK21* remained unassigned to a chromosomal location. The amino acid lengths of *AhPK* proteins ranged from 507 to 578, and their molecular weights varied from 55.04 kDa to 63.82 kDa. The isoelectric point (pI) values ranged from 5.52 to 8.18, with six *AhPK* members having $pI > 7$. The analysis of the instability index suggested that 8 of the proteins may be unstable (with instability index > 40), whereas 13 proteins were likely stable (with instability index ranging from 28.33 to 37.17). The Grand Average of Hydropathicity (GRAVY) values for most *AhPKs* were below 0, indicating that these proteins are relatively hydrophilic. The aliphatic index ranged from 83.27 to 100.57, suggesting a high content of aliphatic amino acids and stability over a wide temperature range. Most members of the PK family were predicted to localize to either chloroplast or cytoplasm (Table S2).

To investigate the evolutionary relationships between divergent plant lineages (monocot vs. eudicot) and classify the PK family, we constructed a phylogenetic tree by analyzing the amino acid sequences of PK proteins from peanut, *Arabidopsis* (eudicot model plant), and rice (model monocot plant). Based on the phylogenetic analysis and prior studies [12,14], all PK proteins were classified into two subfamilies—PKc and PKp. The PKc subfamily was further divided into two subgroups, PKc-1 and PKc-2, while the PKp subfamily was divided into two subgroups, PKp- α and PKp- β . As illustrated in Figure 1B, the PKc subfamily comprised 11 *AhPKs*, with 4 belonging to the PKc-1 subgroup and 7 to the PKc-2 subgroup. The PKp subfamily consisted of 10 *AhPKs*, equally distributed between the PKp- α and PKp- β subgroups.

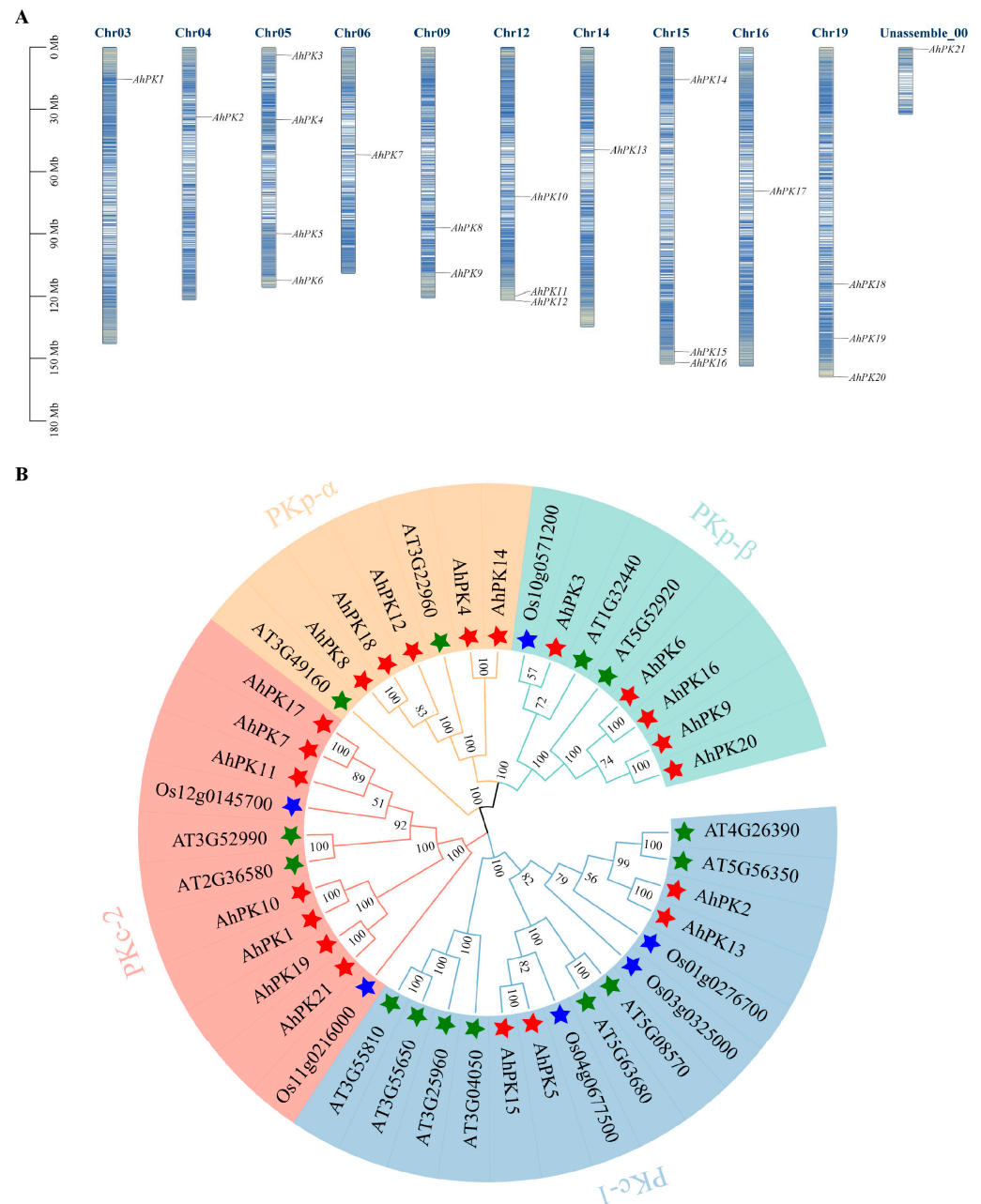


Figure 1. Chromosomal distribution and phylogenetic analysis *AhPK* genes. **(A)** Chromosomal distribution of *AhPK* genes. The chromosome number is labeled at the top of each chromosome, and the scale is in mega bases (Mb). The gene name on the right side of each chromosome corresponds to the approximate locations of each *AhPK* gene. Colors represent the density of genes, from low density in blue to high density in yellow. Unassemble_00 indicates the sequence had not been assembled into chromosomes in peanut. **(B)** Phylogenetic relationships of PK proteins in peanut, Arabidopsis and rice. The red asterisk represents PK in peanut; the blue asterisk represents PK in rice; the green asterisk represents PK in Arabidopsis.

3.2. Structure Analysis of *AhPKs*

To further investigate the diverse functions of *AhPK* proteins, we analyzed their conserved motifs using the Multiple Em for Motif Elicitation (MEME) suite, identifying ten distinct motifs labeled Motifs 1–10. Proteins within the same subfamily exhibited similar motif types and distributions (Figure 2A,B). Members of the PKp subfamily consistently contained Motifs 1–4 and 6–9, while members of the PKc subfamily consistently contained all ten motifs. Notably, Motif 5 was exclusive to the PKc subfamily, reinforcing the accuracy

of the subfamily classification. The conserved domains of the AhPK proteins were further analyzed using NCBI-CDD, confirming that each family member contained both a PK domain and a PK_C domain (Figure 2B).

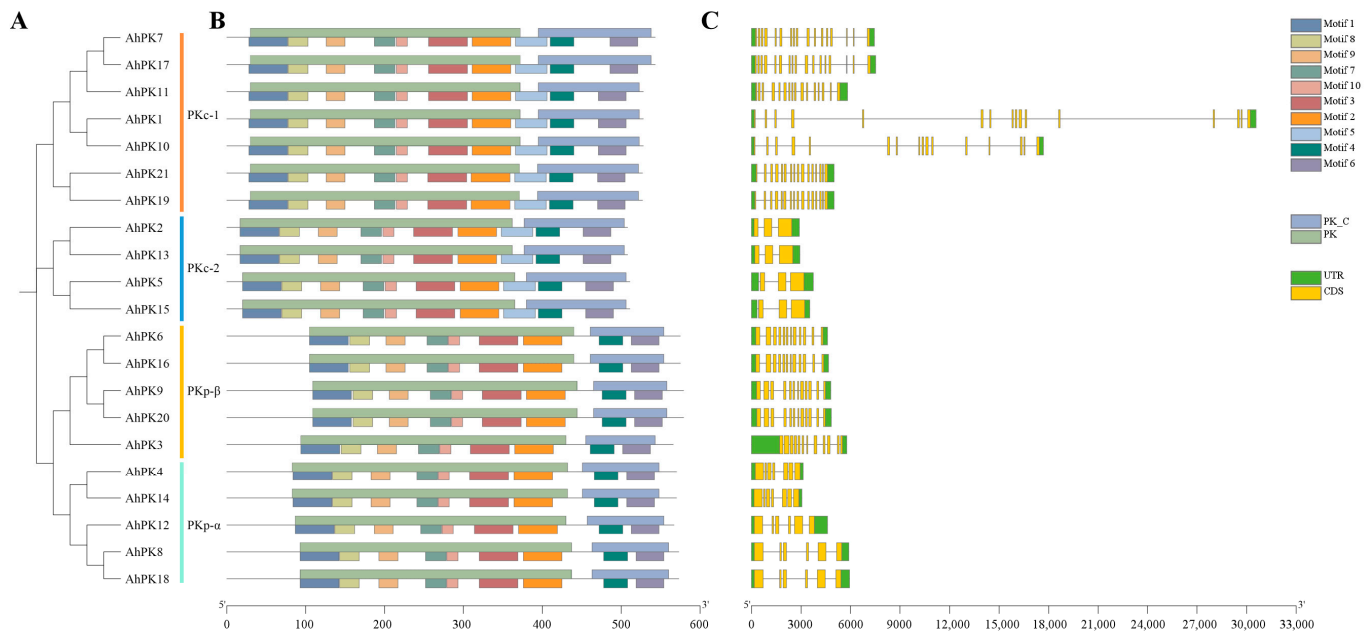


Figure 2. Phylogenetic relationships (A), conserved motifs and domains (B), and exon/intron structures (C) of PK in peanut. UTR, untranslated regions; CDS, coding sequences.

To further investigate the structural characteristics of the PK gene family in peanut, we analyzed the exon–intron structures of the 21 *AhPK* genes. Figure 2A,C illustrate that the number of exons varied significantly among subgroups within the same subfamily. For example, the PKc-2 subgroup had 16 or 17 exons, whereas PKc-1 subgroup members contained only three exons. Within the PKp subfamily, PKp-α had 6 or 7 exons, in contrast to PKp-β, which had 12 exons.

3.3. Gene Duplication of the *AhPK* Family

Tandem and segmental duplications significantly contribute to the expansion of gene families in plants. To understand the expansion process of the *AhPK* gene family, we examined the duplication events of peanut PK genes. Our analysis revealed that 14 pairs, comprising 18 *AhPK* genes, were segmental duplications, with no tandem duplications detected (Figure 3A; Table S3). The calculated Ka/Ks ratios for all 14 pairs were less than 1 (Figure 3B; Table S3), indicating that the peanut PK gene family has undergone strong purifying selection during evolution. To explore the synteny of *AhPK* genes, we performed a collinearity analysis between peanut and other organisms (*Arabidopsis* and rice). This analysis revealed that eight peanut PK genes had syntenic relationships with *Arabidopsis* PK genes, while only one peanut PK gene had a syntenic relationship with the rice PK gene (Figure 3C). These findings suggest that peanut PK genes exhibit greater evolutionary divergence compared to monocotyledonous plants.

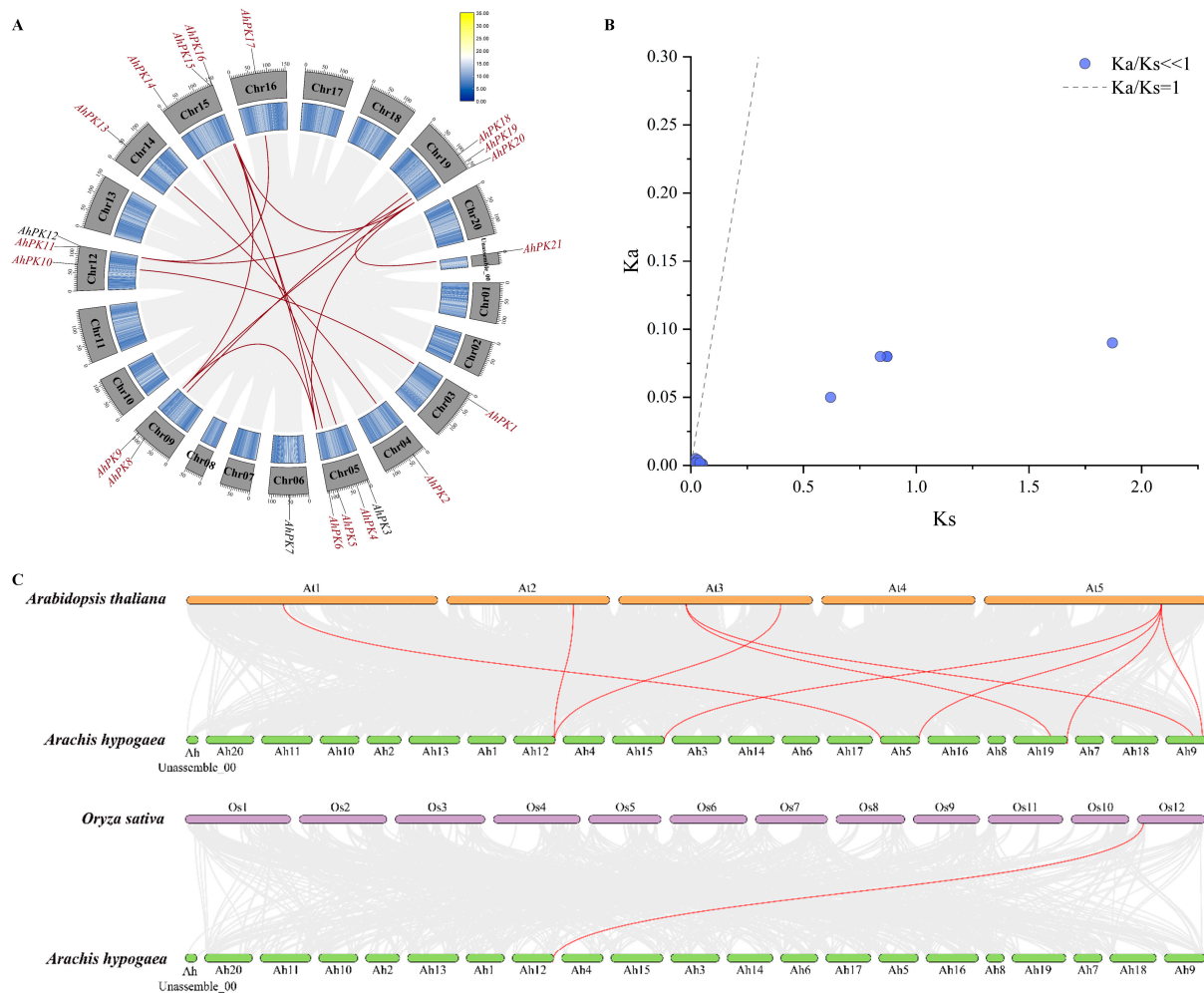


Figure 3. Synteny analysis of *PK* genes. (A) Collinear analysis of the *AhPK* gene family in peanut. The grey lines in the central background represent all gene duplication events within the genome, and the red lines in the center represent duplication events of *AhPK* genes. The outer circle represents the location of *AhPK* genes on the chromosome, and the inner circle represents gene density. (B) K_a/K_s ratios of duplicated pairs of *AhPK* genes. The dotted line indicates $K_a/K_s = 1$. (C) Synteny analysis of *PK* genes between peanut and Arabidopsis/rice. The grey lines in the background represent all gene duplication events between peanut and Arabidopsis/rice genomes. The red lines represent duplication events of *PK* genes between peanut and Arabidopsis/rice genomes.

3.4. Interaction Network of *PK* Between Peanut and Arabidopsis

To elucidate the putative functions of *AhPKs*, we performed a protein interaction network analysis using orthologs of *PKs* in Arabidopsis from the STRING database. In this network, nodes represent proteins, and edges of varying colors indicate protein–protein interactions (Figure 4). The predicted interaction network indicated that numerous *AhPKs* are functionally related to Arabidopsis proteins, such as FBA4 (Fructose-bisphosphate aldolase 4), F14O13.7 (F-box/kelch-repeat protein), APYs (Apyrases), DLD (D-lactate dehydrogenase), and T16L4.190 (Alkaline-phosphatase-like family protein). Notably, the interactions between five *PKp-α* and four *PKp-β* members suggest that peanut *PKs* may form polymeric protein complexes composed of various subunits. These findings imply that the interacting proteins may collectively exert specific biological functions in peanut.

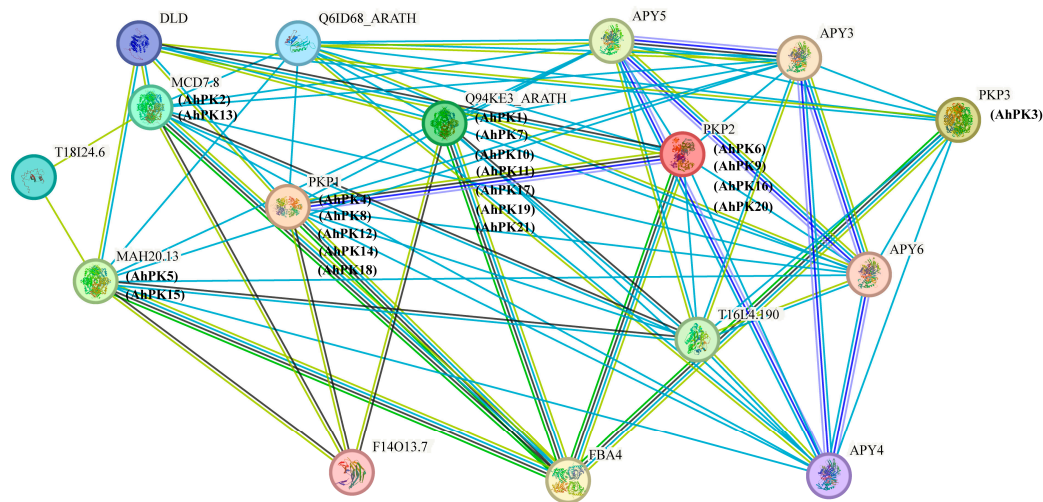


Figure 4. The predicted protein–protein interaction network of AhPKs based on Arabidopsis orthologs. The proteins are represented by circular nodes and protein–protein relationships are represented as edges (lines).

3.5. Analysis of *cis*-Acting Elements in the Promoters of the AhPKs

Analyzing transcription factor binding sites, termed *cis*-regulatory elements, is essential for functional genomic research. To predict the potential functions of *AhPKs*, we examined 2 kb upstream regions of each PK gene to identify regulatory sequences (Figure 5). In addition to general promoter elements, these important *cis*-elements were categorized into four functional groups: development-related, hormone-responsive, light-responsive, and stress-responsive elements. The primary light-responsive elements identified included Box 4, G-box, GT1-motif, and TCT-motif. Hormone-responsive elements primarily consisted of ABRE, CGTCA-motif, and TGACG-motif. Stress-responsive elements were dominated by TC-rich repeats, ARE, LTR, and MBS, while development-related elements were primarily A-box and O2-site. The number of *cis*-elements varied among *AhPK* members, ranging from 13 to 36, with seven genes harboring over 30 elements. These findings highlight the functional diversity of *AhPK* promoters, suggesting their critical roles in regulating growth, developmental processes, and stress adaptation in peanut.

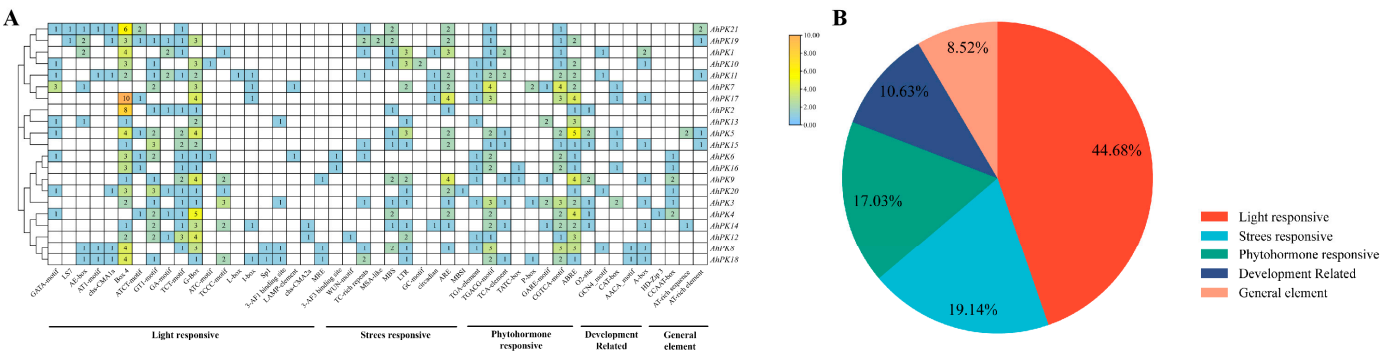


Figure 5. The *cis*-acting regulatory elements identified within in the promoters of *AhPKs*. (A) Distribution of *cis*-acting elements in the promoters of *AhPKs*. The numbers and gradient colors indicate the number of *cis*-acting elements in *AhPKs*. (B) Pie chart showing the frequency of various functional *cis*-acting elements in *AhPKs*.

3.6. Expression Pattern Analysis of *AhPK* Genes Using qRT-PCR

To evaluate the potential functions of peanut *AhPK* genes, we conducted a qRT-PCR analysis on 10 randomly selected genes from different subgroups, including *AhPK2*, *AhPK4*, *AhPK5*, *AhPK6*, *AhPK11*, *AhPK13*, *AhPK15*, *AhPK16*, *AhPK19*, and *AhPK21*.

We analyzed the expression patterns of these genes in five tissues (root, leaf, seed, shell, and root nodule) to generate hypotheses regarding their functions (Figure 6). Notably, *AhPK2*, *AhPK4*, *AhPK5*, *AhPK11*, *AhPK13*, and *AhPK15* were predominantly expressed in the shell, suggesting their likely involvement in pod development. *AhPK6* and *AhPK16* exhibited high expression levels in the seed, suggesting a role in seed development, while *AhPK19* and *AhPK21* had significantly higher expressions in the root compared to other tissues, indicating their potential involvement in root growth.

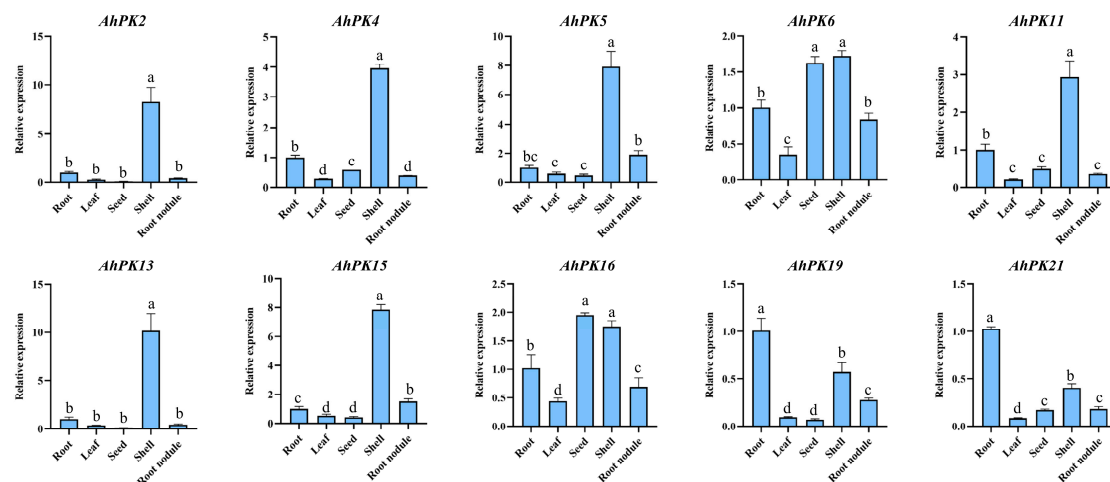


Figure 6. Expression patterns of 10 selected *AhPK* genes in different tissues. Root, leaf, and root nodule were collected at 40 days after sowing, and seed and shell were collected at 80 days after sowing. Data are presented as mean $\pm$ SD. The relative expression level was measured by qRT-PCR and normalized to *AhActin* gene. Different letters in columns indicate significant differences (Duncan test, $p < 0.05$).

Previous studies have highlighted the critical role of PKs in seed germination in *Arabidopsis* and rice [13,19,20,24,27]. To further investigate the potential functions of *AhPKs* in seed germination, we monitored the expressions of these genes during the early stages of imbibition in embryos (imbibition for 0, 4 and 8 h; Figure 7). The analysis showed a gradual decrease in the expressions of *AhPK2*, *AhPK4*, and *AhPK6* during imbibition, while *AhPK5*, *AhPK11*, and *AhPK15* exhibited a dynamic increase. Notably, *AhPK5*, *AhPK11*, *AhPK15*, *AhPK19* and *AhPK21* showed significantly higher expression levels at the 8 h imbibition stage compared to the dry embryo (0 h), indicating their potential regulatory role in peanut seed germination.

Moreover, previous studies have highlighted the prominent role of PKs in responding to various abiotic stresses [13,14,33,34]. To further explore the functions of *AhPKs* under abiotic stress conditions, we examined the expressions of these genes in seedling roots subjected to 15% (w/v) PEG (Figure 8A) and 0.5 μ M ABA treatments (Figure 8B) at various time points (0, 2, 4, and 8 h). Under PEG treatment, all analyzed genes exhibited significant upregulation at least at one time point compared to the control (0 h). Notably, the expressions of *AhPK5*, *AhPK15*, *AhPK19*, and *AhPK21* displayed the highest expression levels at 4 h, with increases of 43-, 72-, 115-, and 122-fold, respectively. In response to ABA treatment, nearly all tested genes were induced to express except for *AhPK16*. Among these genes, *AhPK2*, *AhPK4*, *AhPK5*, *AhPK6*, *AhPK11*, *AhPK13* and *AhPK15* had the highest expression levels at 4 h, while *AhPK19* and *AhPK21* exhibited the highest expression levels

at 2 h. Notably, the expression of *AhPK5*, *AhPK15*, *AhPK19*, and *AhPK21* showed the most significant changes after ABA treatment (greater than 4-fold). These findings suggest that peanut PK genes may play a significant role in stress responses.

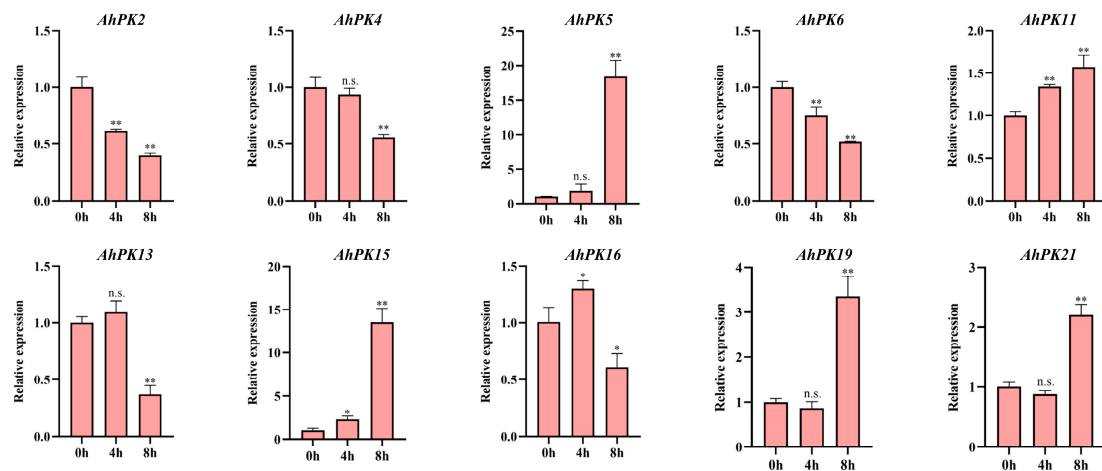


Figure 7. Expression patterns of 10 selected *AhPK* genes in the embryo during imbibition. Data are presented as mean $\pm$ SD. The relative expression level was measured by qRT-PCR and normalized to *AhActin* gene. * and ** indicate significant differences compared to the 0 h imbibition stage (dry embryo) at $p < 0.05$ and $p < 0.01$, respectively. n.s. indicates not statistically significant compared to the 0 h imbibition stage (dry embryo).

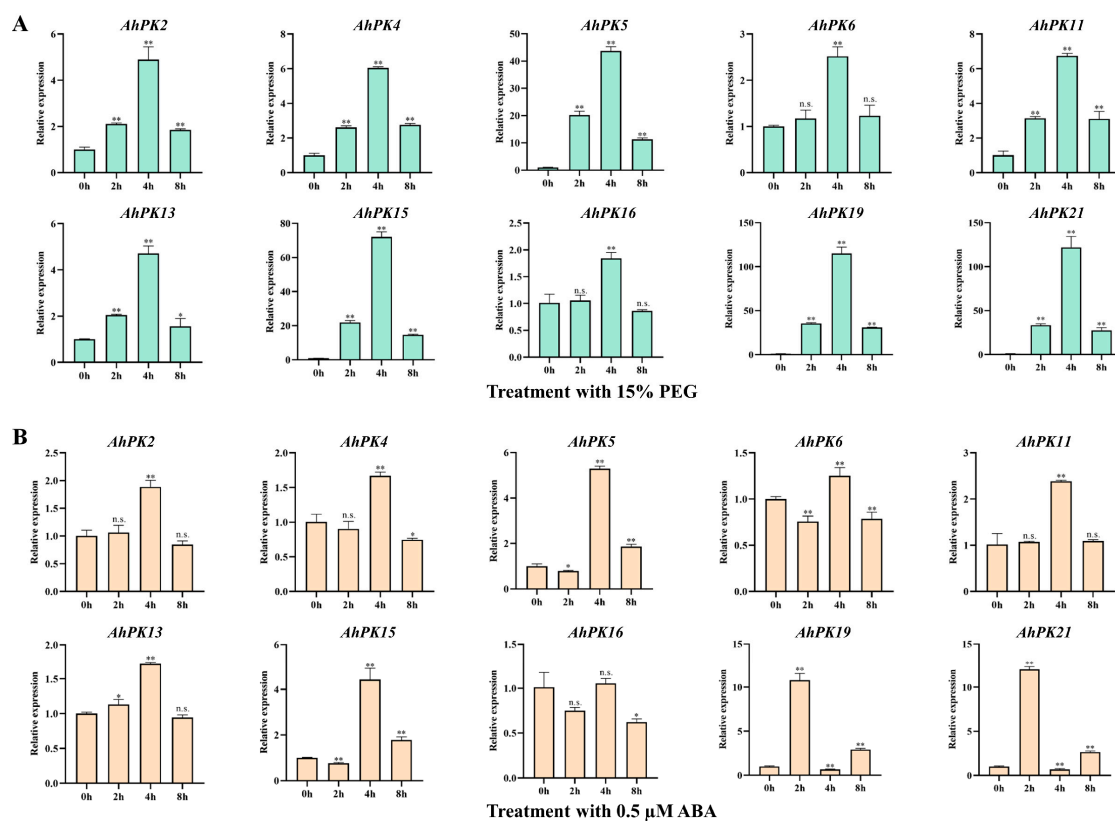


Figure 8. Expression patterns of 10 selected *AhPK* genes under PEG-induced drought stress and ABA treatments. (A) PEG. (B) ABA. Data are presented as mean $\pm$ SD. The relative expression level was measured by qRT-PCR and normalized to *AhActin* gene. * and ** indicate significant differences compared to the control condition (0 h) at $p < 0.05$ and $p < 0.01$, respectively. n.s. indicates not statistically significant compared to control condition (0 h).

4. Discussion

Pyruvate kinase, which catalyzes a key regulatory step in glycolysis, plays a significant role in plant growth and development [35]. Biotechnological advancements have facilitated the genome-wide identification of PK genes in several species, including *Arabidopsis* [12], rice [13], soybean [14], potato [21], and cotton [22]. Peanut is an economically important oilseed crop cultivated worldwide [36]. The availability of cultivated peanut genomes allows for the comprehensive identification and characterization of its multigene family [28,37,38]. However, little is known about the characteristics and evolution of the PK gene family in peanut. A comprehensive characterization of the pyruvate kinase gene family in cultivated peanut can provide a reference for advancing research on the functions of PK genes in peanut.

In this study, we identified 21 *AhPK* genes in the Shitoqi peanut genome using BLAST and HMM methods, guided by *Arabidopsis AtPK* gene sequences and conserved PK domains. The number of PK genes in peanut was greater than that in *Arabidopsis* ($n = 14$), rice ($n = 10$) and potato ($n = 11$), but fewer than that in cotton ($n = 33$) and soybean ($n = 27$) [12–14,21,22]. This variation may result from gene duplication events or differences in genome size [39,40]. The *AhPKs* were distributed across 10 of the 20 peanut chromosomes, and their uneven distribution suggests possible evolutionary adaptations. Distinct protein characteristics were observed among *AhPKs*, implying that they may have unique physiological roles in plant metabolism.

Phylogenetic analysis has demonstrated that the 21 *AhPK* genes can be categorized into two subfamilies, PKc and PKp, with each further divided into two subgroups. These findings align with classifications observed in *Arabidopsis* and rice [12,13], underscoring evolutionary conservation across species. All *AhPK* proteins were predicted to possess both PK and PK_C domains, with domain positions consistent with those reported in a previous study [13]. The motif compositions displayed a certain regularity among different PK subfamilies, implying functional similarities among *AhPK* members within the same subfamily. However, the gene structures within the same subfamily exhibited considerable diversity. The PKc-2 genes had more exons and introns than PKc-1, while PKp- β genes had more exons and introns than PKp- α , mirroring findings in rice and soybean [13,14]. These results indicate that peanut PKs have evolved their gene structures in response to changing environmental conditions while maintaining their conserved domains, allowing them to consistently execute functions in diverse metabolic pathways and physiological processes.

Gene duplication events are known to contribute to the expansion of gene families and evolutionary diversification in plants [40]. In this study, we found 14 pairs of segmental duplications, but no tandem duplications, in peanut, underscoring the significant role of segmental duplications in the expansion of *AhPK* genes. This observation parallels findings in soybean PK genes [14]. Each duplicated gene pair may share close evolutionary relationships and possess similar functions. The K_a/K_s ratio serves as an indicator of the selective pressure faced by duplicated genes during evolution [41]. In our analysis, the K_a/K_s ratios of duplicated gene pairs were less than one, suggesting that PK genes have experienced strong purifying selection during evolution in peanut. Collinearity analyses of homologous genes between different species provide valuable insights into gene functions [42]. We observed that peanut and *Arabidopsis* share more homologous gene pairs than peanut and rice, suggesting greater genetic similarities between peanut and *Arabidopsis*. Future genetic analyses of homologous genes in peanut could elucidate the extent of overlapping functions.

Arabidopsis serves as the reference species, offering insights into gene function across other plants [43]. In this study, we constructed an interaction network for *AhPK* proteins and their target proteins in *Arabidopsis*. The interaction network revealed that several

AhPK proteins can interact with FBA4, APYs, and DLD proteins, which are known to play roles in plant growth, development, or stress responses in Arabidopsis [44–47]. Additionally, PK α members were predicted to interact with PK β members, aligning with findings that PK proteins can interact with each other to form polymeric protein complexes, thereby facilitating their functions in Arabidopsis and rice [13,19,24–26]. These potential interactions will guide future investigations into the biological functions of the *AhPK* gene family.

Cis-elements in the promoter region are critical for controlling gene expression [48]. In this study, the most prevalent elements in *AhPK* genes were those associated with light response, stress, hormones, and development, such as Box 4, G-box, ARE, ABRE, CGTCA-motif and TGACG-motif. These types of *cis*-regulatory elements have also been identified in the promoters of PK genes in rice and soybean [13,14]. In rice, several PK genes have been shown to regulate seed development, which ultimately impacts grain yield [13,24–26]. In this study, *AhPK2*, *AhPK4*, *AhPK5*, *AhPK11*, *AhPK13*, and *AhPK15* were primarily expressed in the shell, while *AhPK6* and *AhPK16* exhibited relatively higher expression levels in the seed, suggesting an overlapping role in crop yield. The rice PK gene *OsPK5*, which regulates seed germination, has been characterized. The transcript levels of this gene gradually increased during seed germination, and its mutation caused a delay in seed germination [27]. In this study, the expressions of *AhPK5*, *AhPK11*, *AhPK15*, *AhPK19* and *AhPK21* significantly increased in embryos after 8 h of seed imbibition, indicating their potential positive role in seed germination. ABRE is recognized as a key *cis*-regulatory element in the ABA signaling pathway [49]. This study found that 19 of the 21 *AhPK* genes contained at least one ABRE element, and most of the selected genes were significantly induced by ABA treatment, implying that PK genes may participate in the ABA signaling pathway in peanut. ABA is well recognized as a crucial component of plant response to drought stress [50]. Interestingly, we found that nearly all tested genes were strongly induced by both PEG and ABA treatments, indicating their critical roles in ABA-dependent drought stress responses. Previous studies have identified PKs involved in drought and ABA responses through multi-omics approaches [51,52]. Our findings support these results, demonstrating conserved roles for PKs in stress adaptation across species. As ABA also mediates responses to salinity and cold stress [53,54], our data suggest broader roles for *AhPKs* in various abiotic stress responses. Future investigations will aim to validate these hypotheses under different stress conditions.

5. Conclusions

In this study, we conducted a comprehensive analysis of the PK gene family in peanut, identifying 21 *AhPK* genes classified phylogenetically into the PKc and PK β subfamilies. Members within the same subfamily exhibited conserved domain patterns and motif compositions. The expansion and evolution of the *AhPK* family were driven by segmental duplication events and purifying selection. Protein–protein interaction network analysis revealed connections between AhPK proteins and other potential interactors. Promoters of *AhPK* genes were found to be enriched with *cis*-elements related to light responses, stress responses, hormones responses, and development. Expression analysis indicated that *AhPK* genes may play important physiological regulatory roles in seed germination and drought stress responses. This study provides the first comprehensive characterization of PK genes in peanut, establishing a foundation for functional studies to enhance drought stress tolerance and seed vigor through genetic engineering or breeding.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae11020200/s1>, Table S1: Primer sequences used for qRT-PCR analysis; Table S2: Physicochemical properties and subcellular localization of the 21 *AhPK* genes and corresponding proteins identified in peanut; Table S3: Estimated Ka/Ks ratios of duplicated *AhPKs*.

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