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Genomic exploration of *HAK/KUP/KT potassium transporter genes* in *Citrus sinensis* (L.) Osbeck: A comprehensive bioinformatics approach

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Abstract – *Citrus sinensis* (L.) Osbeck, a member of the Rutaceae family, holds significant economic importance. Potassium (K), an essential macronutrient, is vital in diverse physiological processes, such as photosynthesis, osmoregulation, stress tolerance, and disease resistance. The *high-affinity K ion transporters* (*HAK*), *K ion uptake permeases* (*KUP*), and *K transporters* (*KT*) gene family represents the largest group of K transporters. This study aims to comprehensively analyze *HAK/KUP/KT* genes in *C. sinensis* (*Cs*). Phylogenetic analysis, chromosome distribution, gene structure and conserved protein motif analysis, protein interaction, homology modeling, cis-acting element analysis, functional gene ontology, miRNA analysis, and primer search were performed using *CsHAK* sequences. Through bioinformatics tools, 25 *CsHAK* genes were identified and categorized into three distinct groups based on the results of phylogenetic analysis. Furthermore, it has been determined that *CsHAK* genes play a role in K transport, localizing in organelles and plasma membranes. They are found on the first, second, fifth, seventh, and eighth chromosomes. Furthermore, cis-acting elements associated with stress response and miRNAs have been identified. This study provides a robust foundation for future functional genomics research, offering insights into the genetic landscape of K transporters in *C. sinensis*. The findings contribute valuable information for crop improvement strategies and enhance our understanding of plant responses to environmental challenges.

1. Introduction

Potassium is an essential macronutrient in various physiological and metabolic processes in plants, such as photosynthesis, stomatal movement, co-transport of sugars, osmoregulation, regulation of membrane potential, respiration, and enzyme activation [1]. K transporters are categorized into four distinct families: *HAK/KUP/KT, KEA (K^+ efflux antiporter), Trk (transport of K^+)/ HKT (high-affinity K^+/ Na^+ Transporter),* and *CHX (cation/H^+ exchanger)* [2]. *HAK* represents high-affinity K ion transporters, *KUP* stands for K ion uptake permeases, and *KT* is an abbreviation for K transporters [3]. The *HAK/KUP/KT* family represents the largest group of K^+ transporters found in plants, and its presence has also been observed in fungi and bacteria [4]. It is estimated that *HAK/KUP/KT* transporters are divided into five clusters, which are believed to have a common ancestor [5]. *HAK/KUP/KT* genes are expressed in various tissues such as roots, leaves, fruits, and seeds, and their expression is influenced by abiotic stress conditions such as salinity and drought, as well as phytohormones like abscisic acid, ethylene, and cytokinin [6]. Members of the *HAK/KUP/KT* family transporters play a significant role in various physiological processes of plants, including the uptake of K^+ ions, root hair growth, cell stretching, auxin distribution, and the formation of a protective response to osmotic stress, contributing to the regulation of growth and development, salt resistance, as well as the control of osmotic potential [7]. For instance, it has been indicated that *AtHAK5* plays a role in K^+ uptake in roots, while

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AtKUP4 is involved in cell expansion in root hairs in *Arabidopsis*. Similarly, in rice, it has been noted that the genes *OsHAK1* and *OsHAK5* regulate K^+ uptake and K^+ ion translocation [8]. *HAK/KUP/KT* transporters are localized in the plasma membrane, chloroplast thylakoid membranes, vacuolar membranes, and various endomembranes in species, such as *Arabidopsis*, *Oryza sativa* L., and *Zea mays* L. [9].

Citrus species belong to the Rutaceae family, are widely cultivated worldwide, and are thought to originate from Southeast Asia. They are valuable both as a source of nutrition and for human health [10]. *Citrus* fruits are a plentiful source of flavonoids, minerals, carotenoids, limonoids, and vitamins (A, B, and E) [11]. *Citrus* L. is a diverse genus comprising numerous significant cultivated species, including *C. sinensis* (L.) Osbeck (sweet orange), *C. limon* (L.) Osbeck (lemon), *C. reticulate* Blanco (tangerine and mandarin), *C. paradisi* Macfad. (grapefruit) and *C. grandis* Osbeck (pummelo) [12]. *C. sinensis*, a valuable species representing the largest citrus group cultivated worldwide, is evergreen and contains important compounds such as flavonoids, steroids, fatty acids, and carotenoids with high medicinal value [13]. *C. sinensis* is effective in treating various conditions such as the common cold, bronchitis, obesity, hypertension, anxiety, menstrual irregularities, constipation, and diarrhea, and it also possesses immune-boosting properties [14].

In improving crop productivity, nutrients' uptake, transport, assimilation, and biological interactions are crucial, and it is essential to identify and characterize the genes encoding proteins involved in these processes. Plants depend on macro, micro, and trace elements for their growth and development, and K is one of the macro elements essential for this process [15]. K is essential for plant growth and development and plays a role in the plant's resistance to abiotic and biotic stress conditions [16]. Additionally, K plays crucial roles in sugar and starch metabolism in citrus production, and it also has effects on fruit size, color, juiciness, and juice flavor characteristics [17]. Advancements in molecular biology and genomics have significantly contributed to understanding the molecular mechanisms of nutrient uptake and utilization in plants, enabling targeted approaches to enhance plant nutritional value. No study currently provides a detailed bioinformatic analysis of *HAK/KUP/KT* genes in *C. sinensis*. The study aims to conduct a genome-wide analysis of *HAK/KUP/KT* genes in *C. sinensis*, focusing on their physicochemical properties, chromosomal distributions, gene ontology, subcellular localizations, cis-acting elements, miRNA targeting, protein structures, and interactions.

2. Materials and Methods

2.1. Identification of *HAK/KUP/KT Potassium Transporter Genes* in *C. sinensis*

The HAK/KUP/KT protein sequences were extracted from National Center for Biotechnology Information (NCBI) database and subjected to analysis using the BLASTP tool within QIAGEN CLC Genomics Workbench 22.0.1. These sequences were juxtaposed with protein sequences from *C. sinensis*. The conserved regions within the HAK/KUP/KT proteins were also examined using the PFAM 35.0 database, facilitated by QIAGEN CLC Genomics Workbench 22.0.1. (Pfam ID: PF02705). Once the comparative analysis was concluded, repetitive sequences were eliminated, successfully identifying potentially promising HAK/KUP/KT proteins. Subsequently, the identified proteins' physicochemical parameters were calculated using the Expasy ProtParam program [18].

2.2. Phylogenetic Analysis

The amino acid sequences were transferred to the MEGA 11 program [19], and the MUSCLE algorithm was utilized to align multiple sequences. Using the aligned file, a phylogenetic tree was generated through the Maximum Likelihood Tree technique, incorporating bootstrap analysis involving 1000 replicates. The phylogeny was reconstructed using the Jones–Taylor–Thornton (JTT) substitution model. After completing this process, the data were exported in Newick format and submitted to the Interactive Tree of Life (iTOL) v6.7.3 [20]. This web-based software was employed to visually represent the generated phylogenetic tree.

2.3. Chromosome Distribution

The NCBI database searched for genomic sequences of the HAK/KUP/KT proteins to determine the chromosomal positions of the corresponding genes. The MapGene2Chromosome (MG2C) v2.1 software, developed by [21], created visual representations of the chromosomal locations.

2.4. Gene Structure and Conserved Protein Motif Analysis

The Gene Structure Display Server (GSDS 2.0) [22] was utilized to identify exon-intron regions through the comparison of genomic sequences with predicted coding sequences (CDS). HAK/KUP/KT protein sequences were examined using the online program Multiple Em for Motif Elicitation (MEME) Suite version 5.5.1 [23]. Subsequently, detailed information on the motifs was accessed using Motif Alignment & Search Tool (MAST). The classical mode was chosen during the analysis, setting the maximum number of motifs to 10 and defining the optimal width range as 6 to 50.

2.5. Protein Network Analysis and Homology Modelling of *HAK/KUP/KT*

Homology modeling for HAK/KUP/KT proteins was conducted using the intensive mode within the Phyre2 (Protein Homology/Analog Recognition Engine V 2.0) program [24]. This software utilizes advanced remote homology detection methods to construct 3D models of proteins and is also involved in predicting ligand binding sites. To create a 3D model of a protein sequence, the following stages are carried out: gathering homologous sequences, fold library scanning, loop modeling, and side-chain placement. Additionally, protein interaction networks were predicted using STRING 11.5 [25].

2.6. Cis-Acting Elements Analysis

To assess cis-regulatory elements in *HAK/KUP/KT* genes, promoter sequences (2Kb sequences upstream of the start codons) were retrieved from the NCBI database. The promoter sequences were analyzed using the PlantCARE database [26]. Further, the cis-acting elements were quantified using the Microsoft Excel program.

2.7. Functional Gene Ontology and Component Analysis

Gene ontology and localization analysis were performed using STRING 11.5 software [25], which aggregates and scores protein-protein interaction data from various public sources and enhances this information with computational predictions. A comprehensive list of *CsHAK* genes was formatted according to STRING 11.5's input requirements, involving the verification of gene identifiers for consistency with the database's standards. The software's GO enrichment feature was utilized to categorize these genes based on their biological processes, molecular functions, and cellular components, providing insights into their functional roles within cellular systems. STRING 11.5 also mapped each *CsHAK* gene to corresponding Gene Ontology (GO) components, identifying their localization in cellular structures such as plant-type vacuoles, intracellular regions, cytoplasm, vacuole membranes, integral membrane components, organelle membranes, and other cellular anatomical entities. The localization results were then compared across different CsHAK proteins to identify common and unique cellular components.

2.8. miRNA Analysis

The psRNATarget database [27] identified potential target miRNAs using the coding sequences (CDS) of *HAK/KUP/KT*s. The acquired outcomes were imported into Microsoft Excel, and the miRNAs were identified using the microRNA database (miRBase) [28]. Subsequently, plant-specific miRNAs were chosen and incorporated into the article in Excel format.

2.9. In Silico PCR Primer Search

Polymerase chain reaction (PCR) is a technique used in nucleic acid amplification, involving stages such as denaturation of double-stranded DNA into single-stranded DNA, annealing of primers to the single-stranded DNA, and primer elongation facilitated by a thermostable DNA polymerase [29]. In PCR, it is preferred that the primers have balanced melting temperatures (Tm) (e.g., within a range of 1-6 °C difference), and there is generally a desire for minimal variation in the annealing temperatures (Ta) of primer pairs [30]. In silico primers were searched for using the FastPCR Professional 6.8.04 program developed by [29].

3. Results

3.1. Identification of *HAK/KUP/KT Potassium Transporter Genes* in *C. sinensis*

Through comprehensive analyses using *C. sinensis* protein sequences, 25 *HAK/KUP/KT* genes were identified. The *HAK/KUP/KT* genes of the *C. sinensis* were renamed based on their chromosomal distribution. The *C. sinensis (*Cs) initials were used during the naming process, followed by "HAK," and then a numerical identifier was added. Based on the obtained data, the protein lengths of *HAK/KUP/KT* genes range from 613 to 845 amino acids, and their molecular weights range from 67997.91 to 93995.32 Da. Additionally, it has been observed that the theoretical isoelectric points (pIs) of these proteins range from 5.37 to 9.53, with the majority of these proteins being basic (pI > 7). Furthermore, it has been noted that the instability index of HAK/KUP/KT proteins ranges from 27.99 to 47.18 (Supplementary File 1).

3.2. Phylogenetic Analysis

After the phylogenetic analysis, a phylogenetic tree was constructed. As seen in Figure 1, *CsHAK* genes have been divided into three groups. The highest number of members is observed in Group III, which consists of 12 genes (*CsHAK3, CsHAK4, CsHAK12, CsHAK13, CsHAK14, CsHAK15, CsHAK16, CsHAK19, CsHAK20, CsHAK23, CsHAK24,* and *CsHAK25*). The fewest members have been identified in Group I. In this group, there are a total of 6 genes (*CsHAK1, CsHAK2, CsHAK17, CsHAK18, CsHAK 21,* and *CsHAK22*). Based on the data obtained, an analysis within the Group III category reveals that Group IIIB2 comprises 7 genes (*CsHAK3, CsHAK4, CsHAK12, CsHAK14, CsHAK15, CsHAK16,* and *CsHAK24*). In Group IIIA, only the *CsHAK25* gene is present.

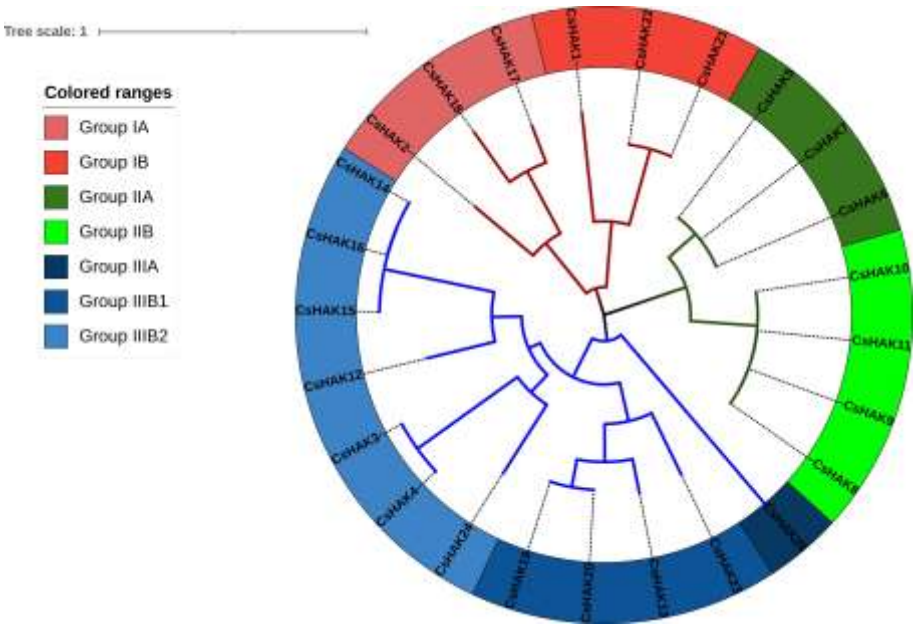

Figure 1. Phylogenetic tree of *CsHAK* genes

3.3. Chromosome Distribution

C. sinensis has nine chromosomes, and the *HAK/KUP/KT* genes have been identified on the first, second, fifth, seventh, and eighth chromosomes. The highest number of genes (*CsHAK3*, *CsHAK4*, *CsHAK5*, *CsHAK6*, *CsHAK7*, *CsHAK8*, *CsHAK9*, *CsHAK10*, *CsHAK11*, *CsHAK12*, and *CsHAK13*) has been observed on chromosome 2. In contrast, only *CsHAK24* has been identified on chromosome 8. On chromosome 1, only *CsHAK1* and *CsHAK2* are found. In addition, the location of the *CsHAK25* gene on any chromosome has not yet been determined (Figure 2). When examining Figure 2, the reason *CsHAK9* and *CsHAK14* locations are not observed is due to *CsHAK9* and *CsHAK10*, as well as *CsHAK14* and *CsHAK15*, having identical gene start and end positions, resulting in the program displaying only one of each gene pair.

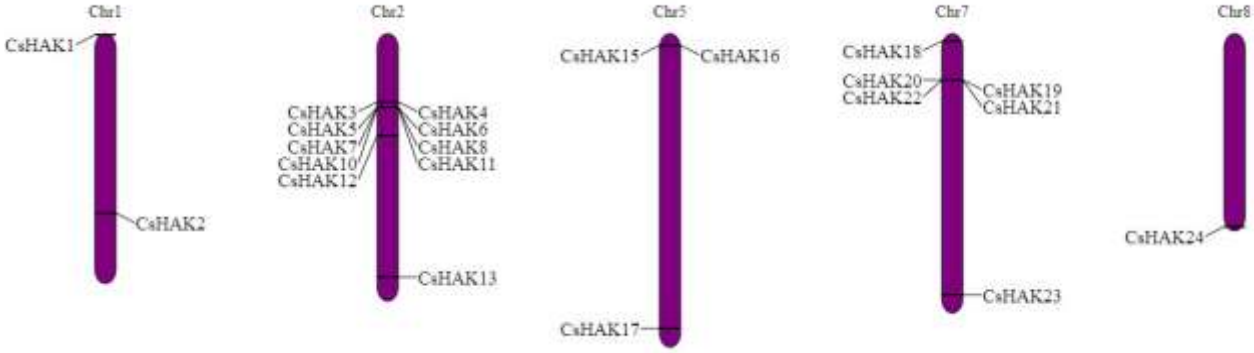

Figure 2. Distribution of *CsHAK* genes

3.4. Gene Structure and Conserved Protein Motif Analysis

The result of gene structure analysis indicates that all genes have exon and intron regions in addition to 5'-UTR and 3'-UTR. The genes with the highest number of exons and introns are *CsHAK1*, *CsHAK3*, *CsHAK8*, *CsHAK9*, *CsHAK10*, *CsHAK11*, *CsHAK15*, and *CsHAK23*, while the gene with the fewest exons and introns is *CsHAK5*. According to the obtained data, the gene with the shortest length is *CsHAK2*, while the longest is *CsHAK6*. The results obtained from phylogenetic analysis are similar and supportive of the gene structure analysis results (Figure 3). According to the results of conserved protein motif analysis, it has been determined that all proteins except CsHAK5, CsHAK8, CsHAK15, and CsHAK22 contain all motifs. In *CsHAK5*, eight out of these motifs have been identified, and this protein lacks the second and ninth motifs.

Similarly, CsHAK22 also has the same number of motifs, but in this protein, the fifth and sixth motifs have not been observed. Moreover, both CsHAK8 and CsHAK15 contain nine motifs, but CsHAK8 lacks the fifth motif, while CsHAK15 lacks the second motif. Additionally, in CsHAK12, there are two copies of the seventh motif, while in CsHAK14, CsHAK15, and CsHAK16, two copies of the first motif have been observed. SQAIISATFSIIKQCVALGCFPRVKVVHTSKKIHGQIYIPEINWILMILC, while the seventh motif is SIQIAFTTVVYPCLJLAYMGQAAYLMKHP (Figure 4).

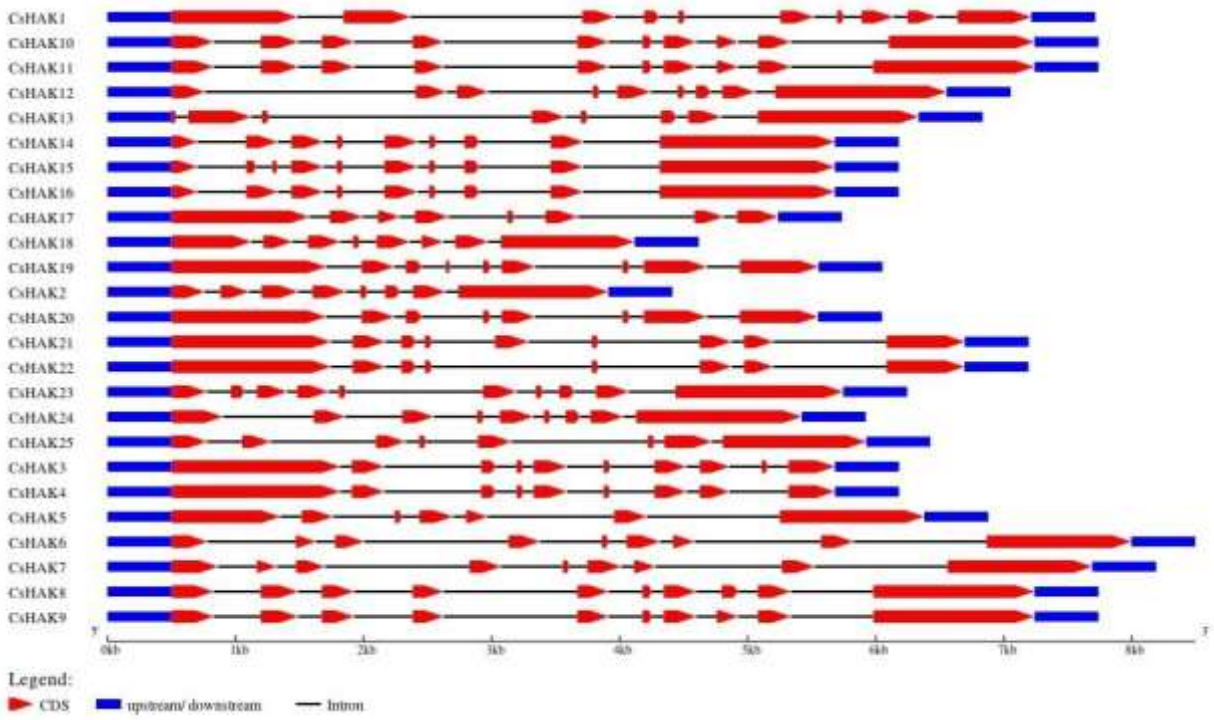

Figure 3. The arrangement of intron and exon in *CsHAK* genes

Figure 4. Distribution of conserved motifs in CsHAK proteins

3.5. Protein Network Analysis and Homology Modeling of HAK/KUP/KT

The similarity rate for homology modeling was determined by selecting the intensive mode from the Phyre2 database, with a confidence level set at 90%. The confidence percentage for all proteins was observed to be above 90%. The confidence percentage for the proteins CsHAK2, CsHAK8, CsHAK9, CsHAK10, CsHAK14, CsHAK15, CsHAK16, CsHAK17, CsHAK23, and CsHAK25 has been determined as 100%. Upon examining the data obtained from the analysis, it was observed that the helix-loop-helix structure is predominant. While only α helix and long loops structures were identified in CsHAK1 and CsHAK22, in CsHAK8, only α helix, β turn, antiparallel β sheets, and long loops were detected. Additionally, α helix, β turn, and long loop structures have been observed in CsHAK2, CsHAK9, CsHAK10, CsHAK14, CsHAK15, CsHAK16, CsHAK17,

CsHAK23, and CsHAK25 proteins. Furthermore, CsHAK3, CsHAK4, CsHAK6, CsHAK7, CsHAK18, CsHAK19, and CsHAK24 exhibit α helix, parallel β sheets, antiparallel β sheets, and long loops structures, the presence of α helix, parallel β sheets, and long loops structures was identified in CsHAK5, CsHAK11, CsHAK12, CsHAK13, CsHAK20, and CsHAK21 (Figure 5). When analyzed in terms of protein interactions, it has been concluded that XP_006474646.1, XP_006474312.1, and XP_006471022.1 proteins are central, and there are interactions between these proteins and CsHAK1, CsHAK2, CsHAK7, CsHAK11, CsHAK12, CsHAK13, CsHAK16, CsHAK17, CsHAK18, CsHAK20, CsHAK22, CsHAK23, CsHAK24, and CsHAK25 (Figure 6).

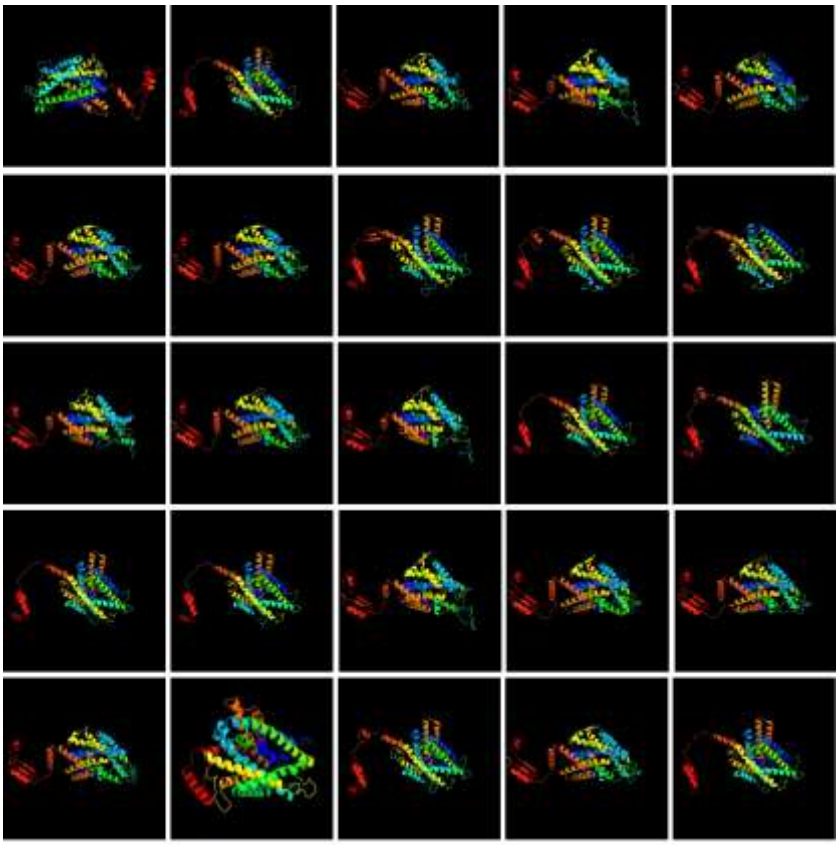

Figure 5. Predicted three-dimensional structures of proteins corresponding to *CsHAK* genes, sorted by gene number

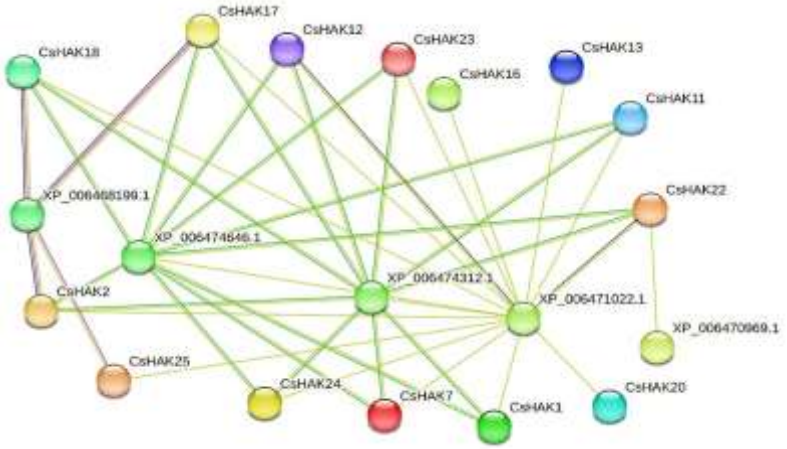

Figure 6. Protein interaction network of CsHAK and some proteins (XP_006468199.1: CBL-interacting serine/threonine-protein kinase 20; XP_006474646.1: cation transporter HKT1;3-like; XP_006474312.1: probable cation transporter HKT6-like; XP_006471022.1: K channel AKT2/3; XP_006470969.1: uncharacterized protein LOC102617546)

3.6. Cis-Acting Elements Analysis

According to the data obtained from cis-acting element analysis, the gene with the highest number of cis-acting elements is determined to be *CsHAK21* (142). *CsHAK7* (136) and *CsHAK12* (132) were identified. The gene *CsHAK19* exhibited the least number of cis-acting elements, with a total of 17 motifs observed. Furthermore, the functions of the identified 54 motifs are detailed in Supplementary File 2, presented in tabular form as a result of this analysis. The analysis results revealed the presence of numerous motifs associated with light response. These include the 3-AF1 binding site, ACE, AE-box, ATC-motif, ATCT-motif, Box II, Box 4, chs-CMA1a, chs-CMA2a, G-box, G-Box, GA-motif, Gap-box, GATA-motif, GT1-motif, I-box, LAMP-element, L-box, MRE, Sp1, TCCC-motif, and TCT-motif. Stress response is a crucial physiological event in plants. *CsHAK* genes contain motifs associated with stress, such as GC-motif (enhancer-like element involved in anoxic specific inducibility), LTR (cis-acting element involved in low-temperature responsiveness), MBS (MYB binding site involved in drought-inducibility), TC-rich repeats (cis-acting element involved in defense and stress responsiveness), and WUN-motif (wound-responsive element). The GC motif has been detected only in *CsHAK17*. LTR has been observed in *CsHAK3, CsHAK4, CsHAK7, CsHAK11, CsHAK12, CsHAK13, CsHAK18,* and *CsHAK24*. MBS has been identified in *CsHAK1, CsHAK6, CsHAK7, CsHAK8, CsHAK12, CsHAK13, CsHAK14, CsHAK15, CsHAK16, CsHAK17, CsHAK18, CsHAK24,* and *CsHAK25*. In addition, TC-rich repeats have been detected in all genes except *CsHAK1, CsHAK3, CsHAK4, CsHAK9, CsHAK10, CsHAK11, CsHAK18, CsHAK19, CsHAK20, CsHAK24,* and *CsHAK25*. WUN-motif has been analyzed to be present in *CsHAK1, CsHAK2, CsHAK5, CsHAK6, CsHAK7, CsHAK8, CsHAK11, CsHAK12, CsHAK17, CsHAK18, CsHAK21, CsHAK22, CsHAK24,* and *CsHAK25* (Supplementary File 2). Gibberellin, auxin, cytokinin, abscisic acid, etc., have important functions in the growth and development of plants. The analysis results revealed the presence of gibberellin-related motifs such as GARE-motif, P-box, and TATC-box; auxin-related motifs including AuxRR-core, TGA-element; abscisic acid-related motif ABRE; methyl jasmonate-related motifs CGTCA-motif and TGACG-motif; and salicylic acid-related TCA-element. The detailed information about the presence of these motifs in specific genes is provided in Supplementary File 2. *CsHAK* genes also encompass motifs with significant functions, such as AACA_motif (involved in an endosperm-specific negative expression), ARE (cis-acting regulatory element essential for the anaerobic induction), CAT-box (cis-acting regulatory element related to meristem expression), circadian (cis-acting regulatory element involved in circadian control), GCN4_motif (cis-regulatory element involved in endosperm expression), HD-Zip 1 (element involved in differentiation of the palisade mesophyll cells), motif I (cis-acting regulatory element root specific), MBSI (MYB binding site involved in flavonoid biosynthetic genes regulation), MSA-like (cis-acting element involved in cell cycle regulation) and RY-element (cis-acting regulatory element involved in seed-specific regulation). AACA_motif is observed only in *CsHAK18,* ARE is present in all genes except *CsHAK12, CsHAK19, CsHAK20,* and *CsHAK24,* CAT-box is found in *CsHAK3, CsHAK4, CsHAK8, CsHAK9, CsHAK10, CsHAK11, CsHAK13, CsHAK16, CsHAK17, CsHAK18, CsHAK23,* and *CsHAK24,* GCN4_motif is only in *CsHAK5* and *CsHAK6,* HD-Zip 1 is in *CsHAK2, CsHAK11,* and *CsHAK24,* motif I is in *CsHAK8, CsHAK9,* and *CsHAK10,* MBSI is only in *CsHAK2* and *CsHAK7,* MSA-like is only in *CsHAK3* and *CsHAK4,* while RY-element is observed in *CsHAK14, CsHAK15,* and *CsHAK25* (Supplementary File 2).

3.7. Functional Gene Ontology and Component Analysis

The analysis results indicate that CsHAK1, CsHAK2, CsHAK4, CsHAK7, CsHAK11, CsHAK12, CsHAK13, CsHAK16, CsHAK17, CsHAK18, CsHAK20, CsHAK22, CsHAK23, CsHAK24, and CsHAK25 are involved in transporter activity, specifically in cation, ion, monovalent inorganic cation, K ion, inorganic molecular entity, and metal ion transmembrane transporter activities. On the other hand, CsHAK3, CsHAK5, CsHAK6, CsHAK8, CsHAK9, CsHAK10, CsHAK14, CsHAK15, CsHAK19, and CsHAK21 have been determined to be involved in every mentioned function except monovalent inorganic cation transmembrane transporter activity. When examined in terms of Gene Ontology (GO) components, CsHAK1 has been analyzed to be

present in the following locations: Plant-type vacuole, intracellular, cytoplasm, vacuole membrane, integral component of membrane, organelle membrane, intrinsic component of membrane, organelle, membrane-bounded organelle, intracellular organelle, intracellular membrane-bounded organelle, whole membrane, cellular anatomical entity. CsHAK16 is found in all mentioned locations; however, unlike CsHAK1, it is also present in the cell periphery. The locations where the majority of proteins (CsHAK2, CsHAK3, CsHAK4, CsHAK5, CsHAK6, CsHAK7, CsHAK8, CsHAK9, CsHAK10, CsHAK11, CsHAK12, CsHAK14, CsHAK15, CsHAK17, CsHAK18, CsHAK19, CsHAK21, CsHAK22, and CsHAK25) are found have been determined to be a membrane, integral component of membrane, intrinsic component of membrane, and cellular anatomical entity. The locations where CsHAK13, CsHAK20, CsHAK23, and CsHAK24 are found include plasma membrane, membrane, an integral component of membrane, intrinsic component of membrane, cell periphery, and cellular anatomical entity.

3.8. miRNA Analysis

miRNAs of the following species target the genes of *CsHAK*: *Acacia auriculiformis* A.Cunn. ex Benth., *Arachis hypogaea* L., *Arabidopsis lyrata* (L.) O'Kane & Al-Shehbaz, *Aquilegia coerulea* E.James, *Aegilops tauschii* Coss., *Arabidopsis thaliana* (L.) Heynh., *Bruguiera cylindrica* (Linnaeus) Blume, *Brachypodium distachyon* (L.) P.Beauv., *Bruguiera gymnorhiza* (L.) Lam., *Brassica napus* L., *B. oleracea* L., *B. rapa* L., *Cynara cardunculus* L., *Cucumis melo* L., *Carica papaya* L., *Citrus sinensis*, *C. trifoliata* L., *Digitalis purpurea* L., *Elaeis guineensis* Jacq., *Festuca arundinacea* Schreb., *Gossypium hirsutum* L., *G. raimondii* Ulbr., *Glycine soja* Siebold & Zucc., *G. max* (L.) Merr., *Helianthus annuus* L., *H. argophyllus* Torr. & A.Gray, *H. ciliaris* DC., *H. paradoxus* Heiser, *H. tuberosus* L., *Hevea brasiliensis* Müll.Arg., *Hordeum vulgare* L., *Lotus japonicus* (Regel) K. Larsen, *Malus domestica* Borkh., *Manihot esculenta* Crantz, *Medicago truncatula* Gaertn., *Nicotiana tabacum* L., *Oryza sativa* L., *Picea abies* (L.) H. Karst., *Pinus densata* Mast., P. *taeda* L., *Populus euphratica* Oliv., *P. trichocarpa* Torr. & A.Gray ex. Hook., *Phaseolus vulgaris* L., *Ricinus communis* L., *Rehmannia glutinosa* (Gaertn.) Steud, *Sorghum bicolor* (L.) Moench, *Solanum lycopersicum* L., *S. tuberosum* L., *Saccharum officinarum* L., *Salvia sclarea* L., *Saccharum* sp., *Triticum aestivum* L., *Theobroma cacao* L., *Vigna unguiculata* (L.) Walp., *Vitis vinifera* L., *Zea mays* L. The result of miRNA analysis has determined that *CsHAK6* is the least targeted gene. On the contrary, it has been detected that *CsHAK21* is the most targeted gene by miRNAs from different species. In the second place, the most targeted gene is *CsHAK22*, while in the third place, *CsHAK23* is situated (Supplementary File 3).

The specific miRNAs and their targets in *C. sinensis* are as follows: csi-miR171b, csi-miR3951, and csi-miR3954 target *CsHAK1*; csi-miR172a-3p targets *CsHAK2*; csi-miR3951 targets *CsHAK7*; csi-miR535 targets *CsHAK8, CsHAK9, CsHAK10*, and *CsHAK11*; csi-miR1515 and csi-miR396c target *CsHAK12*; csi-miR3952 targets *CsHAK18*; csi-miR171b and csi-miR3946 target *CsHAK19* and *CsHAK20*; csi-miR156, csi-miR3946, csi-miR3949, csi-miR3951, and csi-miR535 target *CsHAK21* and *CsHAK22*; csi-miR3946, csi-miR477c, and csi-miR857 target *CsHAK23*; csi-miR396c and csi-miR535 target *CsHAK24*; and csi-miR3946 targets *CsHAK25* (Supplementary File 3).

3.9. In Silico PCR Primer Search

PCR is a widely used method in molecular biology studies. PCR primers can also be designed using FastPCR software. After conducting primer research using this software, the primers considered most suitable for PCR have been selected. These primers were chosen based on the characteristics of a good primer pair as described in the materials and methods section and their high PCR efficiency ratio. It has been determined that the *CsHAK8, CsHAK9, CsHAK10, CsHAK11, CsHAK19, CsHAK20*, and *CsHAK24* genes could be the most suitable for PCR (Table 1).

Table 1. Selected primers for PCR and some of their key characteristics

Primers	Features
>CsHAK8: f_572-593 5'-tgtgcttcgaaactattagcat-3'	Length=22 A=6.0 G=4.0 T=8.0 C=4.0 CG=36.4% Linguistic complexity = 95% Primer's PCR efficiency = 95% Tm = 57.8°C Amplicon size: 826bp Ta=64°C
>CsHAK8: r_1378-1397 5'-gatagtctaacttgacgcca-3'	Length=20 A=6.0 G=4.0 T=5.0 C=5.0 CG=45.0% Linguistic complexity = 97% Primer's PCR efficiency = 97% Tm = 57.8°C Amplicon size: 826bp Ta=64°C
>CsHAK9: f_572-593 5'-tgtgcttcgaaactattagcat-3'	Length=22 A=6.0 G=4.0 T=8.0 C=4.0 CG=36.4% Linguistic complexity = 95% Primer's PCR efficiency = 95% Tm = 57.8°C Amplicon size: 826bp Ta=64°C
>CsHAK9: r_1378-1397 5'-gatagtctaacttgacgcca-3'	Length=20 A=6.0 G=4.0 T=5.0 C=5.0 CG=45.0% Linguistic complexity = 97% Primer's PCR efficiency = 97% Tm = 57.8°C Amplicon size: 826bp Ta=64°C
>CsHAK10: f_572-593 5'-tgtgcttcgaaactattagcat-3'	Length=22 A=6.0 G=4.0 T=8.0 C=4.0 CG=36.4% Linguistic complexity = 95% Primer's PCR efficiency = 95% Tm = 57.8°C Amplicon size: 826bp Ta=64°C
>CsHAK10: r_1378-1397 5'-gatagtctaacttgacgcca-3'	Length=20 A=6.0 G=4.0 T=5.0 C=5.0 CG=45.0% Linguistic complexity = 97% Primer's PCR efficiency = 97% Tm = 57.8°C Amplicon size: 826bp Ta=64°C
>CsHAK11: f_572-593 5'-tgtgcttcgaaactattagcat-3'	Length=22 A=6.0 G=4.0 T=8.0 C=4.0 CG=36.4% Linguistic complexity = 95% Primer's PCR efficiency = 95% Tm = 57.8°C Amplicon size: 811bp Ta=64°C
>CsHAK11: r_1363-1382 5'-gatagtctaacttgacgcca-3'	Length=20 A=6.0 G=4.0 T=5.0 C=5.0 CG=45,0% Linguistic complexity = 97% Primer's PCR efficiency = 97% Tm = 57.8°C Amplicon size: 811bp Ta=64°C
>CsHAK19: f_663-684 5'-gttgtctacggagatttaagca-3'	Length=22 A=6.0 G=6.0 T=7.0 C=3.0 CG=40,9% Linguistic complexity = 98% Primer's PCR efficiency = 98% Tm = 57.2°C Amplicon size: 1837bp Ta=65°C
>CsHAK19: r_2481-2499 5'-caacacttcctgagcgtat-3'	Length=19 A=5.0 G=3.0 T=5.0 C=6.0 CG=47.4% Linguistic complexity = 97% Primer's PCR efficiency = 97% Tm = 57.6°C Amplicon size: 1837bp Ta=65°C
>CsHAK20: f_660-681 5'-gttgtctacggagatttaagca-3'	Length=22 A=6.0 G=6.0 T=7.0 C=3.0 CG=40.9% Linguistic complexity = 98% Primer's PCR efficiency = 98% Tm = 57.2°C Amplicon size: 1804bp Ta=64°C
>CsHAK20: r_2445-2463 5'-caacacttcctgagcgtat-3'	Length=19 A=5.0 G=3.0 T=5.0 C=6.0 CG=47.4% Linguistic complexity = 97% Primer's PCR efficiency = 97% Tm = 57.6°C Amplicon size: 1804bp Ta=64°C
>CsHAK24: f_1577-1596 5'-ttagctgtgacaatcggatt-3'	Length=20 A=5.0 G=5.0 T=7.0 C=3.0 CG=40.0% Linguistic complexity = 97% Primer's PCR efficiency = 97% Tm = 56.5°C Amplicon size: 212bp Ta=62°C
>CsHAK24: r_1768-1788 5'-tcaggaatcttgtaaacgcat-3'	Length=21 A=7.0 G=4.0 T=6.0 C=4.0 CG=38.1% Linguistic complexity = 97% Primer's PCR efficiency = 97% Tm = 56.9°C Amplicon size: 212bp Ta=62°C

4. Discussion

Potassium is one of the crucial plant nutrient elements, playing critical roles in essential functions such as photosynthesis, respiration, enzyme activation, stomatal movement, and osmoregulation in plants [31]. The *HAK/KUP/KT* genes are among the genes responsible for the transport and uptake of K in plants [32]. In a study [33] related to the *HAK/KUP/KT* gene family, the numbers of these genes in 15 species are provided as follows: *Saccharum hybrid cultivar R570* (24), *Saccharum spontaneum* L. (30), *Sorghum bicolor* (29), *Zea mays* (27), *Setaria viridis* (L.) P.Beauv. (28), *Setaria italica* (L.) P. Beauvois (28), *Oryza sativa* (27), *Brachypodium distachyon* (25), *Ananas comosus* (L.) Merr. (12), *Arabidopsis thaliana* (13), *Carica papaya* (8), *Vitis vinifera* (13), *Solanum lycopersicum* (8), *Amborella trichopoda* Baill. (6), and *Chlamydomonas reinhardtii* (1). In other studies, related to these genes [34-36], 27 *HAK/KUP/KT* genes have been identified in *Hordeum vulgare*, 22 *HAK* genes in *Medicago truncatula* and *Medicago sativa* L., and 34 *HAK* genes in *Malus × domestica 'Golden Delicious'*. Another study has reported the presence of 40 *HAK* genes in the *Brassica napus* [37]. In a separate investigation involving the *Casuarina equisetifolia* L., the presence of 25 *HAK* genes has been documented [38]. The current article indicates the identification of 25 *HAK* genes in *Citrus sinensis*. Upon comparison with other mentioned studies, it has been concluded that *C. sinensis* contains fewer *HAK* genes according to some and more *HAK* genes according to others. The phylogenetic analysis results have categorized *HAK* genes into three groups. In the first group, six genes were identified, seven in the second group, and twelve in the third group. When the data obtained from the article were examined, it was observed that the results of phylogenetic, gene structure, and conserved protein motif analyses supported each other.

K accumulates in the cytosol, vacuole, nucleus, and mitochondria. However, the storage of K in the vacuole plays a crucial role in maintaining a specific level of K concentration [39]. The presence of CsHAK proteins on the plasma membrane has been detected in the current article. In addition, it has been determined that CsHAK1 and CsHAK16 are also found in vacuole and organelle membranes. Furthermore, it has been identified that CsHAK proteins play roles in the K ion transmembrane transporter activity. Therefore, the presence of CsHAK proteins in the membrane is essential for facilitating the transport of K and establishing internal balance. *HAK* genes play a role in developing the plant's response under biotic and abiotic stress conditions [40]. In plants, it is believed that *HAK/KUP/KT* genes may play a crucial role in enhancing plant tolerance to adverse conditions such as K deficiency, drought, salt, and heavy metal stress [31]. In a study conducted on the *Gossypium raimondii*, it has been indicated that the *GrHAK/KUP/KT8, GrAKT2.1*, and *GrAKT1.1* genes developed responses to salinity and cold stress [3]. In another study, it has been reported that some of the *VrKUP/HAK/KT* genes in *Vigna radiata* L. undergo significant changes in gene expression under abiotic stress [41]. The current article identifies cis-acting elements associated with stress response in the *HAK/KUP/KT* genes of *Citrus sinensis* (Supplementary File 2). With the FastPCR software, the most suitable genes for PCR were determined as *CsHAK8, CsHAK9, CsHAK10, CsHAK11, CsHAK19, CsHAK20*, and *CsHAK24*. The ABRE associated with abscisic acid response has been identified in the mentioned genes, *CsHAK8, CsHAK9, CsHAK10,* and *CsHAK24*. Additionally, the CGTCA-motif and TGACG-motif, associated with the methyl jasmonate response, have been detected in all genes identified as suitable for PCR. Apart from these, among the cis-acting elements related to the gibberellin response, the GARE-motif was observed in *CsHAK9, CsHAK10,* and *CsHAK11*, while the P-box was only observed in *CsHAK11*. The LTR associated with the low-temperature response has been found in *CsHAK11* and *CsHAK24*. MBS is a motif associated with drought, and it has been identified in *CsHAK8* and *CsHAK24*. In addition, the TCA element related to salicylic acid response has been found in *CsHAK9, CsHAK10,* and *CsHAK11*. Only *CsHAK8* has been identified among these genes with TC-rich repeats associated with defense and stress response. The TGA element (auxin response) has been detected in *CsHAK8, CsHAK9,* and *CsHAK10*, while the WUN-motif (wound response) has been identified in *CsHAK8, CsHAK11,* and *CsHAK24*. Suppose studies related to stress response in Citrus sinensis are to be conducted. In that case, the genes associated with that specific stress factor

can be selected, and their expression profiles can be investigated. The target for research involving *CsHAK8*, *CsHAK9*, *CsHAK10*, and *CsHAK11* should be Chromosome 2. For *CsHAK19* and *CsHAK20*, Chromosome 7 should be examined, and *CsHAK24* should focus on Chromosome 8. miRNAs can play a significant role in conferring tolerance to abiotic stress conditions in plants. miR156, miR159, miR160, miR162, miR165, miR166, miR167, miR168, miR169, miR170, miR171, miR172, miR319, miR390, miR393, miR394, miR395, miR396, miR397, miR398, miR408, miR474, miR528, miR529, miR845, miR851, miR854, miR896, miR901, miR903, miR1030, miR1035, miR1050, miR1088, miR1125, and miR1126 are associated with the response to abiotic stress conditions [42-46]. All mentioned miRNAs except miR162, miR165, miR170, miR390, miR474, miR896, miR901, miR903, miR1030, miR1035, miR1050, miR1088, miR1125, and miR1126 have been identified in *CsHAK* genes (Supplementary File 3). The presence of stress-responsive cis-acting elements and miRNA in the *HAK/KUP/KT* genes of *C. sinensis* suggests the potential role of these genes in developing tolerance to adverse conditions.

5. Conclusion

This study comprehensively analyzes the *HAK/KUP/KT* gene family in *C. sinensis*, elucidating key molecular characteristics and functional roles. A total of 25 *CsHAK* genes were identified and categorized into three phylogenetic groups: six in Group 1, seven in Group 2, and twelve in Group 3. Phylogenetic analysis, gene structure examination, and conserved protein motif studies revealed a high degree of consistency, highlighting the evolutionary conservation of this gene family. The localization of CsHAK proteins on the plasma membrane, as well as on vacuole and organelle membranes (e.g., CsHAK1 and CsHAK16), was determined, emphasizing their essential role in potassium ion transmembrane transport and intracellular balance. Cis-acting elements associated with abiotic and biotic stress responses, such as ABRE, MBS, and LTR, were identified in several *CsHAK* genes. Notably, *CsHAK8*, *CsHAK9*, *CsHAK10*, *CsHAK11*, and *CsHAK24* exhibited elements linked to stress tolerance, highlighting their potential for further functional studies. Additionally, stress-responsive miRNAs, including miR156, miR159, miR160, and miR167, were found to be associated with *CsHAK* genes, suggesting their role in enhancing tolerance to adverse conditions. The findings of this study advance our understanding of the genetic factors involved in potassium transport in *C. sinensis* and provide a valuable foundation for future functional genomic studies. The identified *HAK/KUP/KT* genes and their molecular characteristics can be leveraged to develop strategies aimed at improving citrus crop productivity under challenging environmental conditions. Molecular insights could facilitate gene editing or modification to enhance nutrient uptake efficiency and stress tolerance in citrus plants. Incorporating these genes into breeding programs could result in new citrus varieties with improved productivity traits. Furthermore, applying precision agriculture techniques informed by molecular data could optimize nutrient management and improve the use of environmental resources. Lastly, the findings may serve as a basis for comparative genomic studies across different plant species, contributing to the understanding of the evolutionary dynamics of the *HAK/KUP/KT* gene family.

Author Contributions

The author read and approved the final version of the paper.

Conflict of Interest

The author declares no conflict of interest.

Ethical Review and Approval

No approval from the Board of Ethics is required.

Supplementary Material

https://dergipark.org.tr/en/download/journal-file/32901

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