



Genome-wide analysis of the HAK/KUP/KT gene family in *Spinacia oleracea* L.: Insights into potential stress-related regulatory mechanisms

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Ispanak (*Spinacia oleracea* L.) HAK/KUP/KT gen ailesinin genom çapında analizi: Potansiyel strese bağlı düzenleyici mekanizmalara ilişkin öngörüler

Abstract: Potassium is an essential macronutrient that contributes to ion homeostasis, protein synthesis, and plant responses to biotic and abiotic stresses. The HAK/KUP/KT gene family plays a key role in regulating potassium uptake and transport in plants. Despite the functional importance of these transporters, this gene family has not yet been comprehensively characterized in *Spinacia oleracea*. Therefore, this study conducted a genome-wide analysis of HAK/KUP/KT genes in *S. oleracea*, including chromosomal localization, physicochemical characterization, phylogenetic relationships, gene structure, conserved motifs, secondary and tertiary protein structures, subcellular localization, cis-acting elements, and miRNA target prediction. A total of 32 HAK/KUP/KT genes, designated as SoHAKs, were identified across six chromosomes of *S. oleracea*. Among these genes, ten were located on chromosome 1, indicating an uneven chromosomal distribution. Phylogenetic analysis classified the SoHAK family into three major groups. Physicochemical characterization revealed that most SoHAK proteins were basic in nature and predicted to be unstable. Structural analyses indicated that most SoHAK proteins contained α -helices, β -strands, and loop regions. Subcellular localization prediction showed that most SoHAK proteins were associated with the plasma membrane, supporting their potential roles in potassium transport. Promoter and miRNA target analyses further suggested that several SoHAK genes may be involved in stress-related regulatory mechanisms. Notably, SoHAK2, SoHAK3, SoHAK4, SoHAK10, SoHAK26, SoHAK27, SoHAK29, SoHAK30, and SoHAK32 emerged as candidate genes for future functional studies. Overall, these findings provide a genomic framework for further investigating the potential roles of HAK/KUP/KT genes in potassium transport and stress-related regulatory mechanisms in *S. oleracea*.

Key words: Abiotic stress, HAK/KUP/KT gene family, miRNA, potassium transport, *Spinacia oleracea*

Özet: Potasyum, iyon homeostazının sürdürülmesi, protein sentezi ve bitkilerin biyotik ile abiyotik streslere verdiği yanıtlar açısından temel öneme sahip bir makro besin elementidir. HAK/KUP/KT gen ailesi, bitkilerde potasyum alımı ve taşınmasının düzenlenmesinde kritik rol oynamaktadır. Ancak bu gen ailesi, *Spinacia oleracea*'da şimdiye kadar kapsamlı olarak karakterize edilmemiştir. Bu nedenle, bu çalışmada *S. oleracea*'daki HAK/KUP/KT genleri genom çapında analiz edilerek kromozomal lokalizasyon, fizikokimyasal özellikler, filogenetik ilişkiler, gen yapısı, korunmuş motifler, ikincil ve üçüncül protein yapıları, subselüler lokalizasyon, cis-etkili düzenleyici elementler ve miRNA hedef tahminleri incelenmiştir. Analizler sonucunda, *S. oleracea*'nın altı kromozomu üzerinde yer alan ve SoHAKs olarak adlandırılan toplam 32 HAK/KUP/KT geni belirlenmiştir. Bu genlerin onunun 1. kromozom üzerinde bulunması, kromozomal dağılımın dengesiz olduğunu göstermiştir. Filogenetik analiz, SoHAK gen ailesinin üç ana gruba ayrıldığını ortaya koymuştur. Fizikokimyasal analizler, SoHAK proteinlerinin büyük çoğunluğunun bazik karakterde olduğunu ve kararsız proteinler olarak tahmin edildiğini göstermiştir. Yapısal analizler, çoğu SoHAK proteininin α -heliks, β -tabaka ve döngü bölgelerinden oluştuğunu ortaya koymuştur. Subselüler lokalizasyon tahminleri, SoHAK proteinlerinin büyük ölçüde plazma membranında bulunduğunu ve bunun potasyum taşınmasındaki olası görevlerini desteklediğini göstermiştir. Promotör ve miRNA hedef analizleri ise bazı SoHAK genlerinin strese bağlı düzenleyici mekanizmalarda rol oynayabileceğini ortaya koymuştur. Özellikle SoHAK2, SoHAK3, SoHAK4, SoHAK10, SoHAK26, SoHAK27, SoHAK29, SoHAK30 ve SoHAK32 gelecekte gerçekleştirilecek fonksiyonel çalışmalar için aday genler olarak öne çıkmıştır. Sonuç olarak bu bulgular, *S. oleracea*'da HAK/KUP/KT genlerinin potasyum taşınması ve strese bağlı düzenleyici mekanizmalardaki potansiyel rollerinin daha ayrıntılı olarak araştırılmasına yönelik önemli bir genomik altyapı sağlamaktadır.

Anahtar Kelimeler: Abiyotik stres, HAK/KUP/KT gen ailesi, miRNA, potasyum taşınımı, *Spinacia oleracea*

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1. Introduction

Potassium is an essential macronutrient and the second most abundant element in plants after nitrogen, playing key roles in physiological processes such as membrane potential maintenance, enzyme activation, osmotic regulation, and photosynthesis, while also enhancing plant tolerance to various abiotic and biotic stresses including salinity, cold, drought, and pathogen attacks (Cao et al., 2025). Plants have developed potassium uptake

mechanisms to maintain ionic homeostasis and meet their nutritional requirements, and K⁺ transport proteins are classified into three main families: high-affinity K⁺ transporter/K⁺ uptake permease/K⁺ transporter (HAK/KUP/KT) proteins, cation/proton antiporters (CPAs), and high-affinity K⁺ transporters (HKT) (Liu et al., 2025a). HAK/KUP/KT transporters, first identified as KUP1 in *Escherichia coli* and HAK1 in yeast, constitute a large family of potassium transport proteins in plants that typically contain 10–14 transmembrane domains and play

important roles in potassium uptake, osmotic regulation, K^+/Na^+ homeostasis, and root hair development (Anil Kumar et al., 2024).

Members of the HAK/KUP/KT gene family have been identified and characterized in many plant species (Li et al., 2018; Feng et al., 2020; Yang et al., 2020a; Cai et al., 2021; Jin et al., 2021; Li et al., 2022; Liu et al., 2022; Wang et al., 2022; Chen et al., 2023; Tahir et al., 2023; Liu et al., 2024; Jin et al., 2024; Luo et al., 2025). Expression analyses across different plant species suggest that HAK/KUP/KT genes may perform diverse biological functions (Liu et al., 2022). In particular, several studies have demonstrated that members of the HAK/KUP/KT gene family respond to various abiotic stresses, including salinity, potassium deficiency, and drought, in different plant species (Liu et al., 2024). However, despite the increasing number of studies in various plant species, a comprehensive genome-wide identification and characterization of the HAK/KUP/KT gene family has not yet been reported in *Spinacia oleracea* L.

Spinacia oleracea, a widely cultivated leafy vegetable of the Amaranthaceae family, is valued for its high nutritional content, particularly its richness in vitamins (A, B, and C) and essential minerals such as Ca, Fe, Mn, Mg, and K (Behtash et al., 2025). Therefore, a comprehensive genome-wide analysis of the HAK/KUP/KT gene family in spinach is necessary to better understand their structural and evolutionary characteristics. In this study, the HAK/KUP/KT gene family in *S. oleracea* was systematically identified and characterized using genome-wide bioinformatic approaches, including analyses of chromosomal distribution, phylogenetic relationships, gene structure, conserved motifs, protein structural features, subcellular localization, cis-regulatory elements, and miRNA target prediction. The results may contribute to a better understanding of the potential roles of HAK/KUP/KT genes in potassium transport and plant responses to abiotic stress.

2. Materials and Method

2.1. Chromosomal localization and physicochemical characterization of HAK/KUP/KT genes in *Spinacia oleracea*

First, the protein sequences of HAK/KUP/KT genes belonging to *S. oleracea* were retrieved and analyzed using the BLASTP tool implemented in QIAGEN CLC Genomics Workbench version 24.0.1. Accordingly, the obtained HAK/KUP/KT protein sequences were compared with the non-redundant protein datasets available for *S. oleracea*. To verify the presence of the conserved HAK/KUP/KT domain, the Pfam-A 36.0 database was integrated into QIAGEN CLC Genomics Workbench, and domain searches were performed (Pfam ID: PF02705). The resulting data were exported to Microsoft Excel, and redundant sequences were eliminated to identify putative HAK/KUP/KT genes. Subsequently, the protein, genomic, and coding DNA (CDS) sequences of the identified genes were downloaded from the NCBI database in .fasta format, organized into separate files, and ordered according to their chromosomal positions. Gene nomenclature was assigned based on the combination of the species abbreviation (So; *Spinacia oleracea*), the gene family name, and sequential numbering (e.g., SoHAK1). Finally, the physical positions

of the genes were obtained from NCBI and compiled into a table, while physicochemical properties were calculated using the ExPASy ProtParam tool (Gasteiger et al., 2005) and added to the same table.

The chromosomal distribution of the identified HAK/KUP/KT genes was visualized using the MapGene2Chromosome (MG2C) v2.1 software (Chao et al., 2021).

2.2. Phylogenetic analysis

Phylogenetic analysis was performed using the MEGA12 software (Kumar et al., 2024). Previously downloaded amino acid sequences were imported into MEGA12 and aligned using the MUSCLE algorithm. Based on the aligned sequences, phylogenetic relationships were inferred using the Maximum Likelihood method with 1000 bootstrap replicates under the Jones–Taylor–Thornton (JTT) substitution model. The resulting phylogenetic tree was exported in Newick format and visualized using the Interactive Tree of Life (iTOL) v7.4.2 software (Letunic and Bork, 2024).

2.3. Gene structure and conserved protein motif analysis

To identify exon–intron regions, the CDS and genomic sequences of *S. oleracea* were uploaded to the GSDS 2.0 platform (Hu et al., 2015) in .fasta format. The resulting outputs were saved in .png format and subsequently converted to .jpg format after the necessary adjustments. For conserved protein motif analysis, HAK/KUP/KT protein sequences were submitted to the Multiple Em for Motif Elicitation (MEME) Suite version 5.5.8 (Bailey et al., 2015), and detailed motif information was further evaluated using the Motif Alignment & Search Tool (MAST). The analysis was conducted in classical mode, with the maximum number of motifs limited to 10 and the optimal motif width defined as 6–50.

2.4. Secondary structure prediction and homology modeling of HAK/KUP/KT proteins

The secondary structural features and three-dimensional structures of HAK/KUP/KT proteins were predicted using a homology-based modeling approach, employing the Phyre2 server (Kelley et al., 2015) in Normal mode with the default settings. Structural models were generated based on fold recognition and template-based alignment processes, followed by model refinement steps.

2.5. Subcellular localization prediction of HAK/KUP/KT proteins

The subcellular localization of HAK/KUP/KT proteins was predicted using the WoLF PSORT software (Horton et al., 2007). Protein sequences were analyzed using the plant-specific prediction option with the default settings. Protein sequences were uploaded to the platform, and localization prediction results were obtained. The resulting localization predictions were transferred to Microsoft Excel and subsequently visualized as a heatmap using ClustVis: a web tool for visualizing clustering of multivariate data (BETA) (Metsalu and Vilo, 2015). The generated results were saved as figures.

2.6. In silico analysis of cis-acting elements

Within the scope of promoter region analysis, the 2000 bp upstream promoter sequences of the relevant genes were

retrieved from the NCBI database. The obtained promoter regions were submitted to the PlantCARE database (Lescot et al., 2002) to perform cis-acting element analysis. Cis-acting element results for each gene were recorded separately in Microsoft Excel files. Motifs with annotated functions were identified, and a combined Excel dataset was generated by considering their occurrence frequencies. The distribution and frequency of cis-acting elements were visualized as a heatmap using ClustVis: a web tool for visualizing clustering of multivariate data (BETA) (Metsalu and Vilo, 2015), and the results were saved as figures.

2.7. miRNA target analysis

The CDS of *HAK/KUP/KT* genes were examined using the psRNATarget database (Dai and Zhao, 2011) to identify putative miRNA targets. The predicted miRNAs were exported to Microsoft Excel, and plant-specific miRNAs were selected based on information retrieved from the miRBase database. Among these, miRNAs originating from *Arabidopsis thaliana* (L.) Heynh., a widely used model plant species, were retained for further analysis. A separate table was generated to summarize the *HAK/KUP/KT* genes targeted by the identified miRNAs. In addition, miRNAs previously reported in the literature to be associated with stress-related processes were highlighted (Zhang et al., 2022). The interaction network between miRNA families and their predicted *HAK/KUP/KT* gene targets was constructed and visualized using Cytoscape version 3.10.4 (Shannon et al., 2003).

3. Results

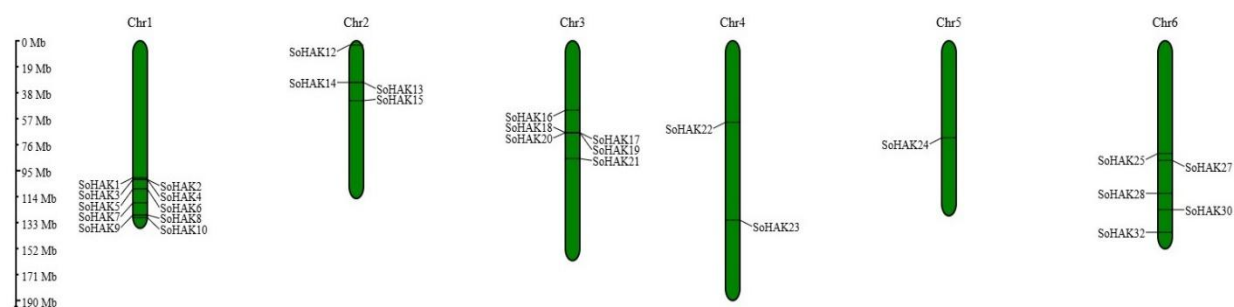


Figure 1. Chromosomal distribution of *SoHAK* genes

3.2. Phylogenetic analysis

Phylogenetic reconstruction classified the *SoHAK* gene family into three distinct evolutionary clades (Group I–III) (Fig. 2). Group II represented the most expanded lineage (15 genes), closely followed by Group I (14 genes), whereas Group III comprised a small, clearly separated cluster of three members. The tree topology demonstrated pronounced interclade divergence, supported by high bootstrap values at major nodes. Members within each clade displayed compact intraclade clustering and reduced branch divergence, indicating strong sequence conservation.

3.3. Gene structure and conserved protein motif analysis

Gene structure analysis demonstrated considerable variation in exon–intron organization among *SoHAK* genes. *SoHAK10* was identified as the longest gene, whereas

3.1. Chromosomal localization and physicochemical characterization of *HAK/KUP/KT* genes in *Spinacia oleracea*

A total of 32 *HAK* genes were identified on six chromosomes in *S. oleracea*. The predicted *SoHAK* proteins ranged from 77 to 846 amino acids (aa) in length. The shortest proteins were *SoHAK15*, *SoHAK21*, *SoHAK24*, *SoHAK25*, and *SoHAK28*, whereas *SoHAK10* was the longest. Consistent with protein length, molecular weights varied between 8,642.13 and 95,220.91 Da. *SoHAK10* exhibited the highest molecular weight, while *SoHAK15*, *SoHAK21*, *SoHAK24*, *SoHAK25*, and *SoHAK28* had the lowest. The predicted isoelectric point (pI) values ranged from 5.05 to 9.33, with 19 basic and 13 acidic proteins identified. The lowest pI values were observed in *SoHAK15*, *SoHAK21*, *SoHAK24*, *SoHAK25*, and *SoHAK28*, whereas the highest pI value was detected in *SoHAK32*. Instability index values ranged from 31.95 to 59.37. A total of 10 proteins were classified as stable, while 22 were predicted to be unstable (Supplementary Table 1).

Chromosomal distribution analysis revealed that the highest number of *SoHAK* genes was located on chromosome 1 (10 genes), whereas only a single gene (*SoHAK24*) was identified on chromosome 5 (Figure 1). Chromosome 6 comprised 8 *SoHAK* genes. Additionally, four gene pairs (*SoHAK11/12*, *SoHAK26/27*, *SoHAK29/30*, and *SoHAK31/32*) shared identical genomic start coordinates and were therefore represented as single loci in Figure 1.

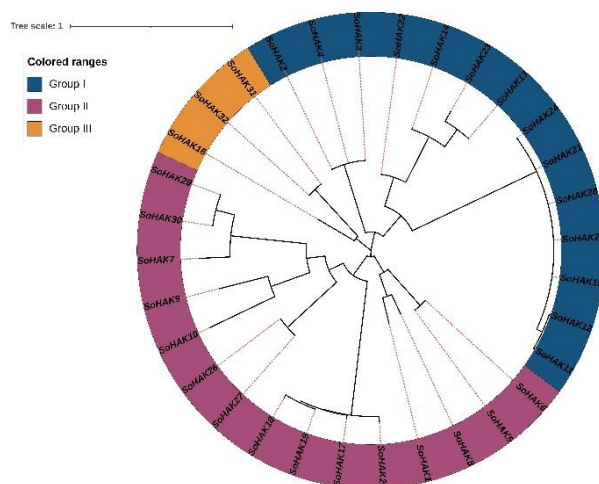


Figure 2. Maximum Likelihood phylogenetic tree of the *SoHAK* proteins in *S. oleracea*

SoHAK28 was the shortest. Four genes (*SoHAK2*, *SoHAK3*, *SoHAK5*, and *SoHAK10*) contained 10 exons and 9 introns, while *SoHAK15* and *SoHAK28* contained only 3 exons and 2 introns. Additionally, relatively long intron regions were observed in several members, including *SoHAK10*, *SoHAK16*, *SoHAK29*, and *SoHAK30* (Fig. 3).

According to the conserved protein motif analysis, SoHAK1, SoHAK2, SoHAK3, SoHAK5, SoHAK7, SoHAK8, SoHAK9, SoHAK10, SoHAK16, SoHAK17, SoHAK18, SoHAK19, SoHAK22, SoHAK26, SoHAK27, SoHAK29, SoHAK30, SoHAK31, and SoHAK32 contained all identified motifs. In contrast, SoHAK13, SoHAK14, and SoHAK23 contained only motif 6. Additionally, SoHAK11, SoHAK12, SoHAK15, SoHAK21, SoHAK24, SoHAK25, and SoHAK28 contained exclusively motifs 2 and 7 (Fig. 4). Furthermore, motif duplication events were detected in certain proteins; for example, motif 8 occurred twice in SoHAK5.

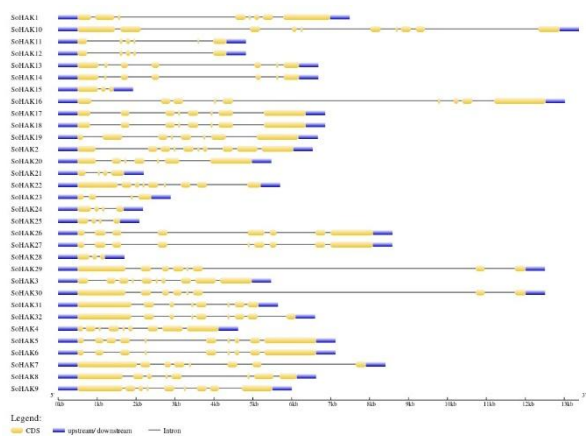


Figure 3. Exon–intron organization of *SoHAK* genes

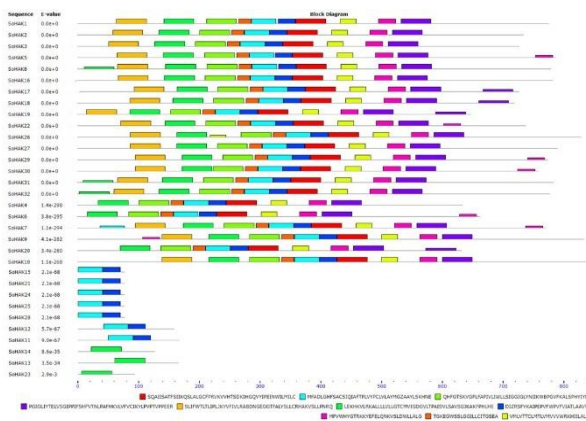


Figure 4. Distribution and organization of conserved motifs in SoHAK proteins. Different colored boxes represent distinct conserved motifs

3.4. Secondary structure prediction and homology modeling of HAK/KUP/KT proteins

The percentages of disordered regions, alpha helices, beta strands, and transmembrane (TM) helices predicted for the SoHAK proteins are presented in Table 1. The highest TM helix proportions were detected in SoHAK15, SoHAK21, SoHAK24, SoHAK25, and SoHAK28, whereas the lowest value was observed in SoHAK23. In terms of alpha-helix content, SoHAK14 showed the highest percentage, while SoHAK12 exhibited the lowest. The highest beta-strand

proportion was identified in SoHAK23, whereas no beta strands were predicted in SoHAK14, SoHAK15, SoHAK21, SoHAK24, SoHAK25, and SoHAK28.

α -helices, β -strands, and long loop structures were observed in SoHAK1, SoHAK2, SoHAK3, SoHAK4, SoHAK5, SoHAK6, SoHAK7, SoHAK8, SoHAK9, SoHAK10, SoHAK17, SoHAK18, SoHAK19, SoHAK20, SoHAK22, SoHAK26, SoHAK27, SoHAK29, SoHAK30, SoHAK31, and SoHAK32. Among these proteins, SoHAK10 exhibited a distinct β -sheet architecture compared with the others, and SoHAK20 also showed a different structural pattern. In contrast, β -strands were not detected in the three-dimensional homology models of SoHAK11, SoHAK12, SoHAK13, SoHAK16, and SoHAK23; however, secondary structure predictions indicated their presence, with the highest β -strand proportion observed in SoHAK23 (21%). Additionally, SoHAK14, SoHAK15, SoHAK21, SoHAK24, SoHAK25, and SoHAK28 were composed only of α -helices and short or long loops (Fig. 5).

Table 1. Percentage distribution of predicted secondary structure elements in SoHAK proteins

Protein	Disordered (%)	Alpha helix (%)	Beta strand (%)	TM helix (%)
SoHAK1	17	47	8	32
SoHAK2	17	50	10	36
SoHAK3	15	50	9	34
SoHAK4	18	50	9	35
SoHAK5	21	48	8	31
SoHAK6	19	48	9	31
SoHAK7	20	48	9	34
SoHAK8	18	47	8	34
SoHAK9	27	46	8	30
SoHAK10	26	45	9	29
SoHAK11	4	41	13	26
SoHAK12	1	39	19	38
SoHAK13	4	52	9	36
SoHAK14	12	68	-	42
SoHAK15	-	61	-	56
SoHAK16	19	47	9	33
SoHAK17	16	50	10	36
SoHAK18	12	51	10	36
SoHAK19	9	53	9	38
SoHAK20	9	51	10	35
SoHAK21	-	61	-	56
SoHAK22	16	46	9	33
SoHAK23	9	48	21	17
SoHAK24	-	61	-	56
SoHAK25	-	61	-	56
SoHAK26	20	47	7	31
SoHAK27	22	46	8	31
SoHAK28	-	61	-	56
SoHAK29	19	49	8	31
SoHAK30	18	46	10	32
SoHAK31	19	47	10	31
SoHAK32	18	48	9	32

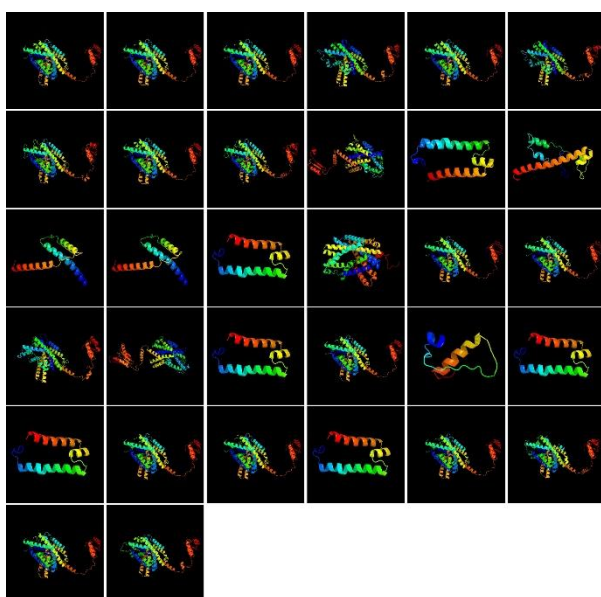


Figure 5. Predicted three-dimensional (3D) structural models of SoHAK proteins in *S. oleracea*, organized by gene number. The models provide insights into the overall folding architecture of the proteins, including α -helices, β -sheets, and loop regions, and may indicate structural variability within the SoHAK protein family

3.5. Subcellular localization prediction of HAK/KUP/KT proteins

Subcellular localization prediction indicated that SoHAK proteins were predominantly localized to the plasma membrane, accounting for approximately 44% of all predictions. This was followed by localization to the cytoplasm (16%), endoplasmic reticulum (9.3%), and extracellular space (8.3%). In contrast, smaller proportions of SoHAK proteins were predicted to localize to chloroplasts (6%), vacuoles (5.9%), mitochondria (4.4%), nuclei (3.4%), and the Golgi apparatus (2.4%), indicating limited nuclear and organelle-associated localization. Notably, SoHAK6 was predicted to localize exclusively to the plasma membrane, whereas SoHAK12 was the only protein predicted to localize to the cytoplasm–nucleus compartment (Fig. 6). Overall, these results suggest that SoHAK proteins are mainly associated with membrane-related cellular components, consistent with their proposed roles in potassium transport.

3.6. In Silico analysis of cis-acting elements

Cis-acting element analysis of the SoHAK genes identified motifs with known functions; these motifs were tabulated, their frequencies were calculated, and the results were visualized as a heatmap (Fig. 7). The highest number of cis-acting elements was detected in SoHAK29 and SoHAK30 (174 each), whereas the lowest was observed in SoHAK21 (62). A total of 52 functionally characterized motifs were identified in SoHAK genes. The highest motif diversity was observed in SoHAK4, SoHAK22, SoHAK26, and SoHAK27 (21 motifs each), followed by SoHAK29, SoHAK30, SoHAK31, and SoHAK32 with 20 motifs each. The lowest motif diversity was detected in SoHAK2 and SoHAK23 (11 motifs each) (Supplementary Table 2).

Moreover, the promoter regions of SoHAK genes were found to contain multiple stress-related cis-regulatory elements, including LTR (low-temperature responsiveness), MBS (drought inducibility), TC-rich

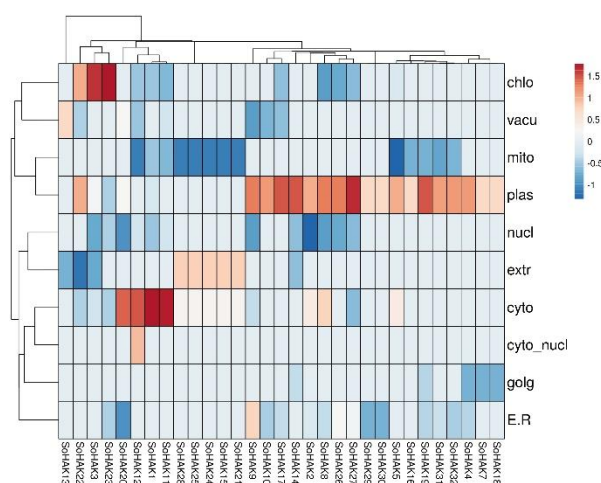


Figure 6. Heatmap of predicted subcellular localization of SoHAK proteins in *S. oleracea*. Abbreviations: chlo, chloroplast; vacu, vacuole; mito, mitochondrion; plas, plasma membrane; nucl, nucleus; extr, extracellular space; cyto, cytoplasm; cyto_nucl, cytoplasm–nucleus; golg, Golgi apparatus; E.R., endoplasmic reticulum

repeats (associated with defense and stress responses), and WUN-motifs (linked to wound-responsive signaling), which may indicate a potential role of these genes in stress adaptation mechanisms. LTR were identified in SoHAK2, SoHAK3, SoHAK4, SoHAK8, SoHAK9, SoHAK11, SoHAK12, SoHAK15, SoHAK16, SoHAK17, SoHAK18, SoHAK21, SoHAK22, SoHAK28, SoHAK29, and SoHAK30, whereas MBS were detected in SoHAK1, SoHAK5, SoHAK6, SoHAK7, SoHAK8, SoHAK10, SoHAK11, SoHAK12, SoHAK13, SoHAK14, SoHAK15, SoHAK22, SoHAK23, SoHAK26, SoHAK27, SoHAK31, and SoHAK32; TC-rich repeats were present in SoHAK1, SoHAK3, SoHAK4, SoHAK9, SoHAK10, SoHAK13, SoHAK14, SoHAK22, SoHAK26, and SoHAK27, while WUN-motifs were identified in SoHAK2, SoHAK3, SoHAK4, SoHAK6, SoHAK7, SoHAK9, SoHAK15, SoHAK17, SoHAK18, SoHAK19, SoHAK20, SoHAK21, SoHAK24, SoHAK25, SoHAK28, SoHAK29, SoHAK30, SoHAK31, and SoHAK32. In addition to these, several light-responsive cis-elements, including the 3-AF1 binding site, AAAC-motif, ACE, AE-box, AT1-motif, ATC-motif, ATCT-motif, Box II, Box 4, chs-CMA1a, chs-CMA2a, G-box, GA-motif, Gap-box, GATA-motif, GT1-motif, GTGGC-motif, I-box, LAMP-element, MRE, Sp1, TCCC-motif, and TCT-motif, were identified. Among these, ACE was exclusively detected in SoHAK7, the ATC-motif in SoHAK23, the Gap-box in SoHAK6, the GTGGC-motif in SoHAK21, and the LAMP-element in SoHAK9, whereas the Sp1 motif was found only in SoHAK26 and SoHAK27 (Supplementary Table 2).

Several hormone-responsive cis-elements were identified in SoHAK genes, including ABRE (abscisic acid responsiveness); AuxRR-core and TGA-element (auxin responsiveness); GARE-motif, P-box, and TATC-box (gibberellin responsiveness); TCA-element (salicylic acid responsiveness); and CGTCA-motif and TGACG-motif (methyl jasmonate responsiveness). Among these elements, AuxRR-core was detected exclusively in SoHAK26 and SoHAK27, whereas the TATC-box was identified in SoHAK11, SoHAK12, SoHAK31, and SoHAK32. Notably, these TATC-box-containing genes lacked other gibberellin-responsive cis-acting elements (Supplementary Table 2).

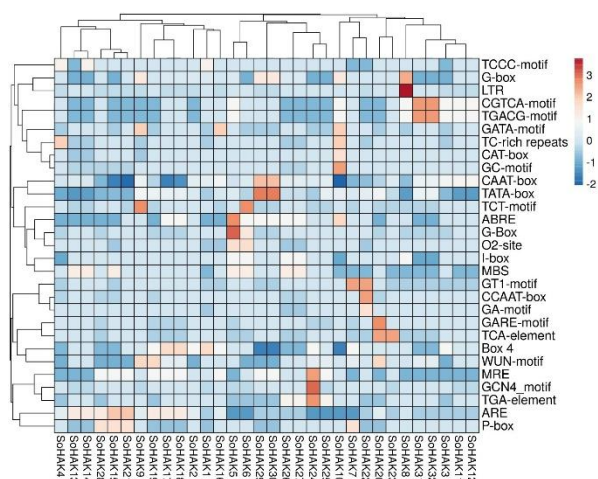


Figure 7. Cis-element distribution heatmap of *SoHAK* gene promoters in *S. oleracea*. Rows represent cis-elements and columns represent *SoHAK* genes; colors indicate their relative abundance

SoHAK genes also contain cis-acting elements associated with important biological processes, including circadian control, differentiation of palisade mesophyll cells, anaerobic induction, anoxic-specific inducibility, regulation of flavonoid biosynthetic genes, and cell cycle regulation. According to the obtained data, the HD-Zip 1 element related to differentiation of the palisade mesophyll cells was detected only in *SoHAK29* and *SoHAK30*, whereas the MSA-like associated with cell cycle regulation was identified exclusively in *SoHAK22* (Supplementary Table 2).

3.7. miRNA target analysis

The miRNA interactions targeting *SoHAK* genes, including miRNAs predicted from different species based on sequence homology, are presented in Supplementary Table 3. From the identified miRNAs, only those originating from *Arabidopsis thaliana* were considered. Subsequently, stress-responsive miRNAs, including ath-miR156, ath-miR157, ath-miR160, ath-miR163, ath-miR164, ath-miR165, ath-miR166, ath-miR167, ath-miR168, ath-miR171, ath-miR172, ath-miR393, ath-miR395, and ath-miR396, were selected. The interactions between these miRNAs and *SoHAK* genes are illustrated in Figure 8.

Among the identified miRNAs, ath-miR165 targeted the highest number of genes, including *SoHAK11*, *SoHAK12*, *SoHAK15*, *SoHAK21*, *SoHAK24*, *SoHAK25*, *SoHAK26*, *SoHAK27*, and *SoHAK28*. In contrast, ath-miR164 and ath-miR395 exclusively targeted *SoHAK10*. Furthermore, ath-miR160 and ath-miR163 targeted only *SoHAK26* and *SoHAK27*, ath-miR166 targeted *SoHAK2* and *SoHAK3*, and ath-miR393 targeted *SoHAK29* and *SoHAK30*. In addition, *SoHAK1* was exclusively targeted by ath-miR172; *SoHAK7* by ath-miR167; *SoHAK9* by ath-miR157; *SoHAK11* and *SoHAK12* by ath-miR165; *SoHAK14* and *SoHAK15* by ath-miR167; *SoHAK16* by ath-miR396; *SoHAK17* and *SoHAK18* by ath-miR156; and *SoHAK21*, *SoHAK24*, *SoHAK25*, and *SoHAK28* were targeted by ath-miR165 (Fig. 8).

4. Discussions

The HAK/KUP/KT gene family is a major gene family comprising numerous members that play a role in

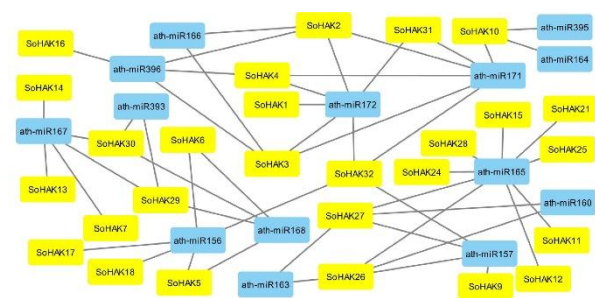


Figure 8. Network of predicted interactions between *Arabidopsis* miRNAs and *SoHAK* genes in *S. oleracea*. Yellow nodes represent *SoHAK* genes, blue nodes represent ath-miRNAs, and the connecting lines indicate predicted regulatory interactions

potassium (K^+) uptake and transport, plant hormone responses, osmotic regulation and abiotic stress responses (Fu et al., 2023). Various studies have demonstrated that the *AtKUP1*, *AtKUP6*, *OsHAK1*, *OsHAK5*, *OsHAK21* and *SlHAK20* genes contribute to stress tolerance (Guo et al., 2024). In a study conducted in *Casuarina equisetifolia* L., the expression levels of *CeqHAK6* and *CeqHAK11* were shown to increase with the duration of salt treatment, and it was concluded that *HAK* genes play an important role in the response to salt stress in *C. equisetifolia* (Wang et al., 2023). A comprehensive analysis of the HAK/KUP/KT gene family in *Spinacia oleracea* has not yet been reported. In this context, the findings were interpreted in light of the relevant literature.

As a result of the analyses carried out in this study, 32 HAK/KUP/KT genes were identified in *S. oleracea*. In other species, the number of members of this gene family was 30 in *Saccharum spontaneum* L. (Feng et al., 2020), 19 in *Vigna radiata* (L.) R. Wilczek (Azeem et al., 2022), 30 in *Chenopodium quinoa* Willd. (Chen et al., 2023), 14 in *Cucumis melo* L. (Fu et al., 2023), 41 in *Phyllostachys edulis* (Carrière) J. Houz. (Guo et al., 2024), in *Gossypium hirsutum* L. 45, in *G. barbadense* L. 44, in *G. raimondii* Ulbr. 24, in *G. arboreum* L. 21 (Yang et al., 2020b), in *Passiflora edulis* Sims 14 (Luo et al., 2025), in *Ipomoea batatas* (L.) Lam. 22 (Jin et al., 2021), *Phaseolus vulgaris* L. 20 (Khan et al., 2023), *Musa acuminata* L. 24, (Liu et al., 2022), *Solanum tuberosum* L. 24 (Liu et al., 2024), *Populus yunnanensis* Dode 32 (Liu et al. 2025b), *Fragaria × ananassa* Duch. 60 (Mercado-Hornos et al., 2025), 17 in *Cajanus cajan* (L.) Huth (Siddique et al., 2021), 20 in *Medicago truncatula* Gaertn. (Zhao et al., 2022b), 20 in *Capsicum annuum* L. (Zhao et al., 2022a) and 40 in *Brassica napus* L. (Zhou et al., 2020). Based on the studies conducted, it is considered that the number of HAK/KUP/KT genes varies between species and that this variation may be related to genomic and evolutionary processes.

In this study, a comprehensive analysis of the HAK/KUP/KT gene family in *S. oleracea* was performed, including chromosomal localization, phylogenetic analysis, gene structure, conserved protein motifs, secondary structure prediction and homology modeling, subcellular localization prediction, and cis-acting element and miRNA target analyses. The integrated evaluation of the phylogenetic, structural, physicochemical, subcellular localization, and regulatory analyses revealed a coherent pattern among certain *SoHAK* members. For example, it was determined that the genes with the shortest protein

lengths were *SoHAK15*, *SoHAK21*, *SoHAK24*, *SoHAK25* and *SoHAK28*, and that these genes had the lowest pI value (5.5). Furthermore, all of these genes were found to be classified in Group I on the phylogenetic tree. These proteins lack β -strands and disordered regions, but have a high number of transmembrane helices. Subcellular localisation analysis suggested that these proteins are localised in the extracellular space, cytoplasm and mitochondria, rather than in the plasma membrane. Conserved protein motif analysis revealed that the *SoHAK21*, *SoHAK24*, *SoHAK25* and *SoHAK28* proteins only contained the 2nd and 7th motifs, while the miRNA analysis showed that the *SoHAK15*, *SoHAK21*, *SoHAK24*, *SoHAK25* and *SoHAK28* genes were solely targeted by ath-miR165. The similarities observed in these genes reveal the existence of an interrelated pattern at the phylogenetic, structural and regulatory levels.

Physicochemical analyses of *S. oleracea* revealed that SoHAK proteins were predominantly basic in nature and unstable. Previous studies on HAK/KUP/KT proteins (Feng et al., 2020; Azeem et al., 2022; Guo et al., 2024; Liu et al., 2025b) have also shown that these proteins tend to be basic, which is consistent with the present findings. Moreover, the high proportion of proteins predicted to be unstable may indicate relatively dynamic structural properties.

An analysis of the chromosomal distribution of *SoHAK* genes revealed that Chromosome 1 contains the highest number of genes, which are located in close proximity to one another. In contrast, the presence of only *SoHAK24* on Chromosome 5 indicates that the gene family is not evenly distributed across the chromosomes. This suggests that the *SoHAK* gene family exhibits a tendency to cluster in specific chromosomal regions within the *S. oleracea* genome. This clustering may be linked to duplication and genomic organisation processes that have played a role in the evolutionary history of the gene family, rather than directly indicating that these chromosomes possess greater functional importance.

A phylogenetic analysis was conducted to determine the evolutionary relationships of *SoHAK* genes, and the results of the analysis revealed that these genes can be divided into three main groups. The close clustering of genes within the same group suggests a common ancestral origin, whereas the separation among different groups may reflect the evolutionary diversification of the HAK/KUP/KT gene family. When the results of the phylogenetic analysis were evaluated in conjunction with analyses of gene structure and conserved protein motifs, it was found that some members belonging to the same group on the phylogenetic tree were consistent with one another in terms of structural features, whereas others did not fully reflect this consistency. For example, *SoHAK17*, *SoHAK18*, *SoHAK19*, and *SoHAK20* clustered within the same sub-branch of Group II in the phylogenetic tree and exhibited similar motif composition and exon–intron organisation. In contrast, although *SoHAK1*, *SoHAK5*, *SoHAK6*, and *SoHAK8* were classified within the same group, their motif composition and gene structures did not exhibit a similar level of concordance with the phylogenetic classification. The fact that some *SoHAK* genes within the same phylogenetic group exhibit similar motif organisation and exon–intron structure supports evolutionary conservation amongst these members. However, the structural

differences observed in some genes suggest that, despite phylogenetic proximity, a certain degree of diversification and possible functional divergence may have occurred within the gene family. This situation demonstrates that the *SoHAK* gene family possesses a dynamic structure characterised by both conserved and divergent features.

Exon–intron organization influences gene responses to environmental conditions; genes with many exons generate diverse protein isoforms via splice diversification, and intron regulation contributes to gene expression (Liu et al., 2024). In the present study, *SoHAK2*, *SoHAK3*, *SoHAK5*, and *SoHAK10* were identified as genes with the highest numbers of exons and introns, and analysis of miRNA indicated that they may be targeted by multiple stress-related miRNAs. For instance, *SoHAK2* and *SoHAK3* were predicted to be targeted by ath-miR166, ath-miR171, ath-miR172, and ath-miR396, which are associated with abiotic stresses such as cold, drought, and salinity. As a result of the analysis, the exon number in *S. oleracea* was found to range from three to ten. In structural analyses of HAK genes performed in *Amborella trichopoda* Baill, *Solanum lycopersicum* L., *Vitis vinifera* L., *Carica papaya* L., *Arabidopsis thaliana*, *Ananas comosus* (L.) Merr., *Brachypodium distachyon* (L.) P.Beauv., *Oryza sativa* L., *Setaria italica* (L.) P. Beauvois, *Setaria viridis* (L.) P.Beauv., *Zea mays* L., *Sorghum bicolor* (L.) Moench, *Saccharum spontaneum*, *Saccharum hybrid* R570, and *Chlamydomonas reinhardtii*, exon numbers were reported to range from two to sixteen, with most HAK genes containing between eight and ten exons (Feng et al., 2020). A similar pattern was observed in the present study, where most *SoHAK* genes also contained eight to ten exons.

Analysis of conserved motifs revealed that the vast majority of *SoHAK* proteins contained all ten identified motifs, whereas a few proteins contained only one or two motifs. This finding suggests that the motif architecture is highly conserved in most members of the *SoHAK* family, while some members exhibit marked structural divergence. The absence of intermediate motif numbers further suggests that this pattern may reflect the coexistence of conserved and divergent members rather than a gradual transition. Furthermore, the limitation of the motif analysis to ten motifs may also have contributed to the detection of fewer motifs in some proteins.

To evaluate the possible intracellular localization patterns and potential functional roles of HAK/KUP/KT proteins, subcellular localization analysis was conducted. The results showed that the majority of *SoHAK* proteins were predicted to localize to the plasma membrane, in agreement with the established roles of HAK/KUP/KT family members in potassium uptake and ion transport across cellular membranes. However, the prediction of several *SoHAK* proteins in other subcellular compartments, including the cytoplasm, endoplasmic reticulum, vacuole, mitochondrion, nucleus, and Golgi apparatus, suggests that members of this gene family may have undergone a certain degree of structural and functional diversification. Consistent with the present findings, previous studies have reported that HAK proteins can be localized not only to the plasma membrane but also to the endoplasmic reticulum, tonoplast, and other endomembrane compartments (Zhao et al., 2022b). Likewise, in *Solanum tuberosum*, although most StHAK proteins were predicted to be associated with the plasma membrane, StHAK4 and StHAK9 were reported

to localize to the cytoplasm, while StHAK8 was associated with the vacuole (Liu et al., 2024).

In addition to their subcellular localization diversity, members of the HAK/KUP/KT family have also been implicated in various abiotic stress responses. Previous studies have shown that *OsHAK5* in rice and *AtHAK5* in *Arabidopsis* are involved in salt stress responses, *IbHAK8* in sweet potato is associated with both drought and salt stress, and *VrKUP/HAK/KT4* and *VrKUP/HAK/KT8.1* in *Vigna radiata* participate in drought and heat stress responses, whereas *VrKUP/HAK/KT4*, *VrKUP/HAK/KT5*, and *VrKUP/HAK/KT8.1* are also implicated in salt stress responses (Azeem et al., 2022; Fu et al., 2023). In *Medicago truncatula*, *MtHAK15*, *MtHAK17*, and *MtHAK18* were strongly upregulated under salt and drought stress conditions, while the promoter regions of *MtHAK* genes were reported to harbour numerous cis-acting elements related to phytohormones and abiotic stress responses (Zhao et al., 2022b). Cis-acting elements are DNA sequences that serve as binding sites for transcription factors, play a role in the regulation of gene expression, and function in plant stress responses (Guo et al., 2024). In the present study, numerous stress-responsive cis-acting elements, including LTR, MBS, TC-rich repeats, and WUN motifs, were identified in the promoter regions of *S. oleracea* *SoHAK* genes. These findings suggest that *SoHAK* genes may be transcriptionally regulated in response to environmental stresses, in agreement with previous studies (Chen et al., 2023; Khan et al., 2023; Liu et al., 2025b). In addition, the presence of hormone-responsive cis-elements, such as ABA- and MeJA-related motifs, in the promoter regions of *HAK* genes suggests that these genes may participate in stress signaling networks (Liu et al., 2025b). Hormone-responsive elements, including ABRE, AuxRR-core, CGTCA-motif, GARE-motif, P-box, TATC-box, TCA-element, TGACG-motif, and TGA-element, were also identified in *SoHAK* genes. Among these elements, the exclusive presence of AuxRR-core in *SoHAK26* and *SoHAK27*, and of TATC-box in *SoHAK11*, *SoHAK12*, *SoHAK31*, and *SoHAK32*, suggests that certain *SoHAK* genes may have roles distinct from those of other family members in the perception and regulation of stress-related hormonal signals.

Abiotic stress conditions such as salinity, high and low temperatures, water deficit, and heavy metal exposure are major factors limiting crop production worldwide, and plants have developed various mechanisms in which miRNAs play a key role in regulating stress tolerance in order to respond and adapt to these conditions (Basso et al., 2019). The identification of miRNA target genes in each plant species is important for elucidating the functional roles of conserved and species-specific miRNAs in plants (Moradi and Khalili, 2018). In *Arabidopsis*, ath-miR157, ath-miR167, ath-miR168, ath-miR171, ath-miR393, ath-miR396, and ath-miR408 have been associated with drought stress responses; ath-miR156, ath-miR158, ath-miR159, ath-miR165, ath-miR167, ath-miR168, ath-miR169, ath-miR171, ath-miR319, ath-miR393, ath-miR394, and ath-miR396 with salt stress responses; and ath-miR165, ath-miR166, ath-miR169, ath-miR172, ath-miR393, ath-miR396, ath-miR397, and ath-miR408 with cold stress responses (Sunkar, 2010). ath-miR156 has been reported to participate in responses to various stress conditions, including drought, heat, cold, gray mold,

pathogen infection, and insect herbivory, while also modulating drought stress tolerance through ABA- and JA-related signaling pathways (Zhang et al., 2026). In the present study, ath-miR156 was predicted to target *SoHAK5*, *SoHAK6*, *SoHAK17*, *SoHAK18*, and *SoHAK32*. Promoter analysis of these genes revealed the presence of stress-related cis-acting elements, including LTR in *SoHAK17* and *SoHAK18*, MBS in *SoHAK6* and *SoHAK32*, and WUN-motif in *SoHAK6*, *SoHAK17*, *SoHAK18*, and *SoHAK32*. In addition, hormone-responsive elements associated with ABA and methyl jasmonate responsiveness, such as ABRE, CGTCA-motif, and TGACG-motif, were identified in their promoter regions, indicating that these genes may be involved in hormone-mediated stress response pathways. Likewise, *SoHAK26*, *SoHAK27*, and *SoHAK32*, which were predicted to be targeted by drought-associated ath-miR157, also contained the drought-responsive MBS element in their promoter regions. The concurrent presence of ABA- and methyl jasmonate-responsive cis-acting elements in these genes further suggests that they may contribute to hormone-mediated drought stress response mechanisms. Furthermore, given the reported role of ath-miR160 in auxin signaling through ARF-targeted regulation (Wang et al., 2017), the prediction that *SoHAK26* and *SoHAK27* are potential targets of ath-miR160, together with the exclusive presence of AuxRR-core in their promoter regions, may suggest their association with auxin-mediated regulatory processes. In the present study, ath-miR164 and ath-miR395 were predicted to target only *SoHAK10*. Since ath-miR164 and ath-miR395 have been reported to be associated with heat and salt stress responses (Kim et al., 2010; Tsai et al., 2023; Bansal et al., 2024; Jiang et al., 2025), this finding suggests that *SoHAK10* may be subject to a specific miRNA-mediated regulatory mechanism under particular stress conditions. Furthermore, the exclusive targeting of *SoHAK10* by these two miRNAs indicates that this gene may differ from other *SoHAK* members in terms of miRNA-mediated regulation. This finding may suggest that *SoHAK10* is regulated through a gene-specific post-transcriptional control mechanism and may have a distinct regulatory role in certain stress responses. ath-miR165 was identified as the miRNA targeting the highest number of *SoHAK* genes, with potential target relationships predicted for nine *SoHAK* members. This finding suggests a possible broad regulatory association between ath-miR165 and the *SoHAK* gene family. It may also indicate that ath-miR165 contributes to the post-transcriptional regulation of multiple *SoHAK* members. However, experimental validation is required to determine whether these predicted interactions are functionally active under stress conditions. Overall, *SoHAK2*, *SoHAK3*, *SoHAK4*, *SoHAK10*, *SoHAK26*, *SoHAK27*, *SoHAK29*, *SoHAK30*, and *SoHAK32* were found to be prominent in both stress-related miRNA target predictions and stress-associated cis-acting element analyses. These findings suggest that the identified *SoHAK* members may be involved in the transcriptional and post-transcriptional regulation of stress responses in *S. oleracea*. Therefore, these genes may be considered priority candidate genes for future functional validation under stress conditions.

Taken together, the present findings provide the first comprehensive genome-wide characterization of the HAK/KUP/KT gene family in *Spinacia oleracea* and offer

new insights into its structural organization, evolutionary diversification, and potential roles in stress-related regulatory mechanisms. The integrated analyses of phylogenetic relationships, gene structure, conserved motifs, protein characteristics, subcellular localization, cis-acting elements, and miRNA target predictions indicate that several *SoHAK* genes, particularly *SoHAK2*, *SoHAK3*, *SoHAK4*, *SoHAK10*, *SoHAK26*, *SoHAK27*, *SoHAK29*, *SoHAK30*, and *SoHAK32*, emerged as promising candidate

genes for future functional validation. These findings establish a valuable genomic framework for investigating HAK/KUP/KT-mediated potassium transport and stress adaptation in *S. oleracea* and may contribute to future molecular breeding strategies aimed at improving abiotic stress tolerance.

Conflict of Interest

The author have declares no conflict of interest.

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