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Posted Date: 27 March 2026

doi: 10.20944/preprints202603.2200.v1

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Article

Identificaton of the HAK/KUP/KT Potassium Transporter Gene Family and Functional Analysis of IbHAK5A in Sweet Potato

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Abstract

Potassium (K+) is an essential mineral element for plant growth and development. Members of the *HAK/KUP/KT* (*HAK*) gene family play key roles in K+ uptake and homeostasis. Although many of them have been extensively identified in a variety of plant species, sweet potato has not yet undergone systemic characterization. In this work, 22 potential *IbHAK* genes are identified based on phylogenetic analysis, and categorized into four groups (I-IV). We performed comprehensive analysis of *IbHAK* genes, including protein property, chromosome localization, gene structure, collinearity and promoter cis-element investigations for each one. Five IbHAK5 proteins (IbHAK5A-IbHAK5E) were found on the same branch as AtHAK5, OsHAK5, and ZmHAK5, suggesting that small-scale duplication events contributed to the expansion of IbHAK5s in sweet potato. *IbHAK5A*, a gene highly expressed in various tissues and significantly induced under low-K+ (LK) stress, was cloned and functionally characterized in potassium transporter deficient yeast and transgenic Arabidopsis. An AP2/EREBP family transcription factor, IbPTL1, was subsequent identified as having the ability to bind the *IbHAK5A* promoter and playing a role in regulating the K+ signaling pathway. This study provides a foundation for further functional characterization of HAK/KUP /KT transporters in sweet potato and key candidate genes for further functional analysis, which may be useful for breeding sweet potato that utilizes potassium more efficiently in the future.

Keywords: sweet potato; potassium transporter; low potassium stress; transcription factor

1. Introduction

Potassium (K^+) is the most abundant cation in plants and accounts for approximately 2-10% of their dry weight [1]. It plays a crucial role in many physiological processes, such as enzyme activity, osmotic regulation, expansion-driven movement, membrane polarization control, protein biosynthesis, and the transport of assimilates [2,3]. The concentration of potassium ions in plant cells is approximately stable at 80-200 mM [4,5]. Vacuoles are the largest K^+ storage pool, where K^+ concentration ranges from 10 mM to 200 mM [1,6,7], but the concentration of potassium ions in the surfaces of roots is extremely low, typically around 0. 1 to 1 mM [4,8]. Therefore, plants must uptake K^+ from the soil against the concentration gradient. To adapt to this low-K^+ (LK) environment, plants have evolved intricate and complex regulatory mechanisms [9–11], which are mediated by plant K^+ transport components, such as K^+ transporters and K^+ channels.

K^+ transporters are important transporter proteins responsible for potassium absorption and transport in plant root systems, among which the HAK/KUP/KT potassium transporter family members are responsible for high-affinity potassium ion absorption under LK conditions [12–14].These HAK/KUP/KT potassium transporters in plants were first discovered in Arabidopsis and barley [15–17]. Thirty members have been reported in Arabidopsis, among which AtHAK5 is the

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most important high-affinity potassium uptake transporter as it primarily functions under LK conditions, with the *hak5* mutant essentially ceasing growth at potassium levels below 10 mM [5]. Twenty-seven members have been reported in rice, where OsHAK5 has a similar function to Arabidopsis AtHAK5 and participates in potassium ion homeostasis under LK conditions [18]. OsHAK1 has dual affinity and functions under both high and low potassium conditions, with both mediating potassium uptake in the roots and potassium transport from roots to shoots in rice [19]. Additionally, 27 members have been reported in maize, where ZmHAK5 and ZmHAK1 have both been functionally characterized, with ZmHAK5 showing the highest homology to AtHAK5, followed by ZmHAK1, and both are important high-affinity potassium ion transporter proteins responsible for potassium uptake in maize [20]. The HAK/KUP/KT family members are all highly conserved, and through homology and bioinformatics analysis, members of this family have been identified in multiple plants in recent years, such as wheat [21], tea [22], rapeseed [23], and sugarcane [24]. Except for Arabidopsis and rice, the functions and regulatory mechanism of most HAK family members have not yet to be reported.

Sweet potato (*Ipomoea batatas* (L.) Lam.), a member of the *Convolvulaceae* family, is extensively cultivated in tropical and subtropical regions around the world [25]. Among the three primary nutrients nitrogen (N), phosphorus (P), and potassium (K), sweet potato exhibits the highest requirement for potassium, followed by nitrogen and phosphorus [26,27]. K^+ plays a crucial role in promoting the formation and transport of carbohydrates in sweet potato and enhances the activity of the root cambium [2,28,29]. K^+ deficiency impacts root growth, the distribution of photosynthetic products, carbon metabolism, enzyme activity, photosynthetic characteristics, and yield in sweet potato. Conversely, applying potassium fertilizer can enhance sucrose synthesis in sweet potato leaves and promote the conversion of sucrose to starch in storage roots. This process boosts starch accumulation and provides a material foundation for root development and tuber enlargement [28,30,31].

The absorption of potassium ions by plant roots from LK soil mainly relies on high-affinity HAK/KUP/KT family potassium transporters located on root cell plasma membranes [32]. Sweet potato, as a K^+-favorite crop, it is more significant to study its K^+ absorb and use mechanism. However, there is currently limited and insufficiently systematic research into HAK/KUP/KT family members in sweet potato specifically. The key high affinity K^+ transporters and their regulatory mechanism are still poorly understood. The detailed information on structural, evolutionary, and regulatory aspects of the HAK/KUP/KT family, and their tissue expression and response to LK stress have also not yet been reported in sweet potato. Thus, in this study we systematically identified sweet potato HAK/KUP/KT family members using bioinformatics methods, and conducted functional analysis of the *IbHAK5A* gene in transgenic Arabidopsis and potassium transporter deficient yeast systems. A potential transcription factor, IbPTL1, was identified for its ability to bind to the promoters of *IbHAK5A* and the regulate potassium signaling pathway. This study thus provides theoretical support for further investigation into the functions of genes within the sweet potato *HAK/KUP/KT* family.

2. Results

2.1. Genome-Wide Identification and Characterization of HAK/KUP/KT Family Members in Sweet Potato

Through Blast comparison analysis of the sweet potato genome database, a total of 22 members of the HAK/KUP/KT family of potassium transporters were identified (Table 1), and their physicochemical properties of the 22 potassium transporter members were analyzed. The results revealed that the sizes of the IbHAK proteins ranged from 437 amino acids (IbHAK11) to 1,252 amino acids (IbHAK10). Their molecular weights varied from 48618.97 Da (IbHAK11) to 140640.46 Da (IbHAK10), while their isoelectric points ranged from 5.54 (IbHAK14) to 9.31 (IbHAK15). Among them, three proteins were acidic (IbHAK2, IbHAK5A, IbHAK14), and the remaining 19 were basic. The minimum number of transmembrane domains was 6 (IbHAK13), and the maximum was 13

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(IbHAK8). Subcellular localization prediction analysis showed that all HAK/KUP/KT family members were localized to the plasma membrane.

Table 1. The physiological and biochemical properties of 22 deduced IbHAK proteins.

Gene name	Gene ID	Amino acid length	Molecular weight	Isoelectric point	Transmembrane segments	Subcellular location
IbHAK1	g5020.t1	707	78297.95	8.86	11	Plasma membrane
IbHAK2	g25454.t1	795	87886.64	6.71	11	Plasma membrane
IbHAK3	g42476.t1	771	85124.18	9.01	10	Plasma membrane
IbHAK4	g20629.t1	739	82146.06	8.91	12	Plasma membrane
IbHAK5A	g13675.t1	756	84783.58	5.95	11	Plasma membrane
IbHAK5B	g13673.t1	722	80584.73	8.73	10	Plasma membrane
IbHAK5C	g13677.t1	772	87192.37	8.99	10	Plasma membrane
IbHAK5D	g13676.t1	720	81057.02	8.91	9	Plasma membrane
IbHAK5E	g49793.t1	990	110289.62	9.03	12	Plasma membrane
IbHAK6	g12041.t1	706	79171.53	8.66	11	Plasma membrane
IbHAK7	g59493.t1	888	99756.32	8.56	10	Plasma membrane
IbHAK8	g3900.t1	795	88995.95	8.35	13	Plasma membrane
IbHAK9	g1704.t1	769	85930.21	7.15	12	Plasma membrane
IbHAK10	g30149.t1	1252	140640.46	8.58	13	Plasma membrane
IbHAK11	g36794.t1	437	48618.97	8.32	8	Plasma membrane
IbHAK12	g34971.t1	760	84980.12	7.79	11	Plasma membrane
IbHAK13	g17621.t1	594	65776.98	9.26	6	Plasma membrane
IbHAK14	g46647.t1	773	86016.75	5.54	8	Plasma membrane
IbHAK15	g17727.t1	734	81078.81	9.31	9	Plasma membrane
IbHAK16	g40997.t1	783	86884.72	8.55	7	Plasma membrane
IbHAK17	g32726.t1	811	90827.45	8.13	10	Plasma membrane
IbHAK18	g12038.t1	807	88649.68	8.68	12	Plasma membrane

2.2. Phylogenetic Analysis of HAK/KUP/KT Family Members from Different Plant Species

A phylogenetic tree was constructed using the 22 sweet potato HAK/KUP/KT family potassium transporter members along with homologous proteins from Arabidopsis, rice, and maize (Figure 1). The results showed that the 22 HAK/KUP/KT family potassium transporter members of sweet potato were unevenly distributed in four gene clusters (I-IV). The first cluster comprised five *IbHAK* genes (named *IbHAK5A-IbHAK5E*), and notably these five genes group within the same branch as HAK5 from rice, Arabidopsis, and maize. This suggests a close evolutionary relationship and implies that these genes likely play similar roles in potassium uptake and transport. The cluster II contained the largest number of *HAK* genes, including 11 *IbHAK* genes. Cluster III contained 5 *IbHAK* genes, and Cluster Ⅳ has the fewest *IbHAK* genes, with only 1 member. Nearly all *HAK* genes in sweet potato exhibit a closer evolutionary relationship with those in Arabidopsis, as both belong to the dicotyledon group. In contrast, all *HAK* genes in rice and maize show a closer evolutionary relationship to each other, as they both belong to the monocotyledon group.

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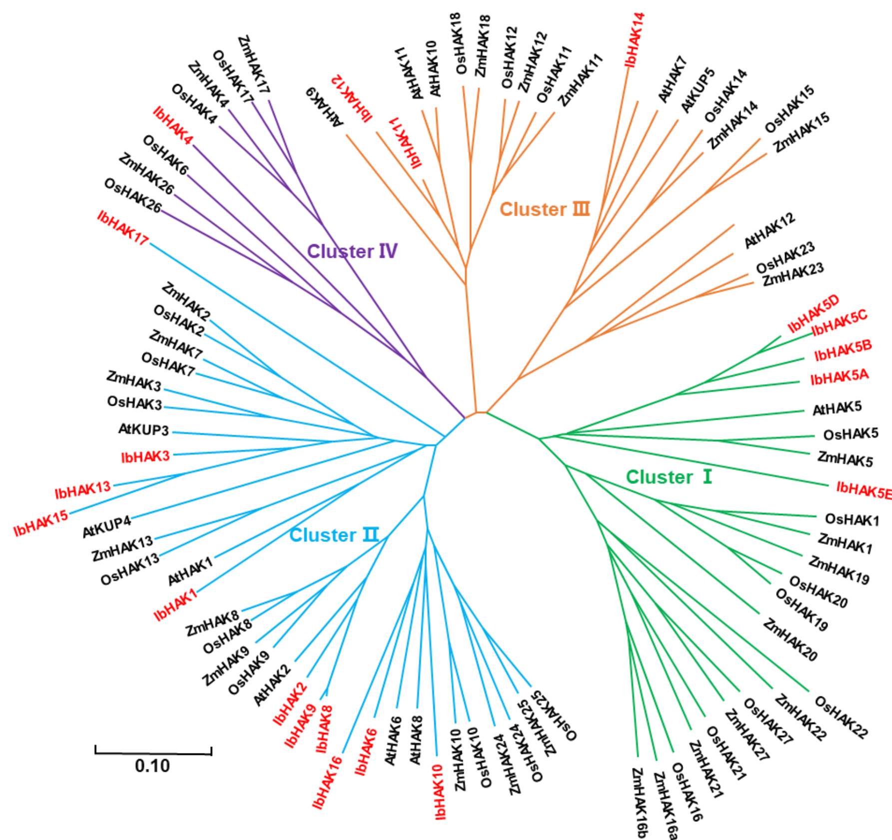

Figure 1. Phylogenetic analysis of HAK/KUP/KT family members in sweet potato, maize, rice, and Arabidopsis. the full length proteion sequences of 13 AtKUPs from Arabidopsis, 27 HAKs from rice, 27 HAKs from maize, and 22 HAKs from sweet potato were aligned using ClustalW. The phylogenetic tree was constructed by the neighbour-joining method in MEGA 11 software with 1000 bootstrap replicates. In total, 22 HAK/KUP/KT family members were divided into four groups with different color (Groups I, II, III and IV filled with green, blue, orange and purple, respectively) consistent with the classification criteria for HAK transporters in maize, rice, and Arabidopsis. all the IbHAKs were marked with red font. All the *IbHAKs* genes are named largely according to their evolutionary relationships with Arabidopsis. Ib: *Ipomoea batatas*, At: *Arabidopsis thaliana*, Zm: *Zea mays*, Os: *Oryza sativa*.

2.3. Chromosome Distribution, Conserved Protein Domains, Gene Structure, and Collinearity Analysis of HAK/KUP/KT Family Genes

An analysis of the chromosomal distribution of the 22 sweet potato HAK/KUP/KT family members showed that they are unevenly distributed on 12 chromosomes (Figure 2a). Among them, *IbHAK5A*-*IbHAK5D* are located on the same chromosome (LG4) and linked at the locus, indicating that these four homologous genes underwent gene duplication during evolution. The analysis of the 15 conserved motifs revealed that motifs 2, 5, and 6 are present in each IbHAK proteins, suggesting that these three motifs are highly conserved. Conserved motif analysis futher showed that only *IbHAK2* and *IbHAK8* included all 15 conserved motifs, implying the two genes were share more similar function and regulation mechanisms. Finally, the analysis of conserved domains revealed that all sweet potato HAK/KUP/KT family members contain a conserved potassium transporter domain (Figure 2b). There are significant differences in gene sequence length and the number of exons among different HAK/KUP/KT family members.

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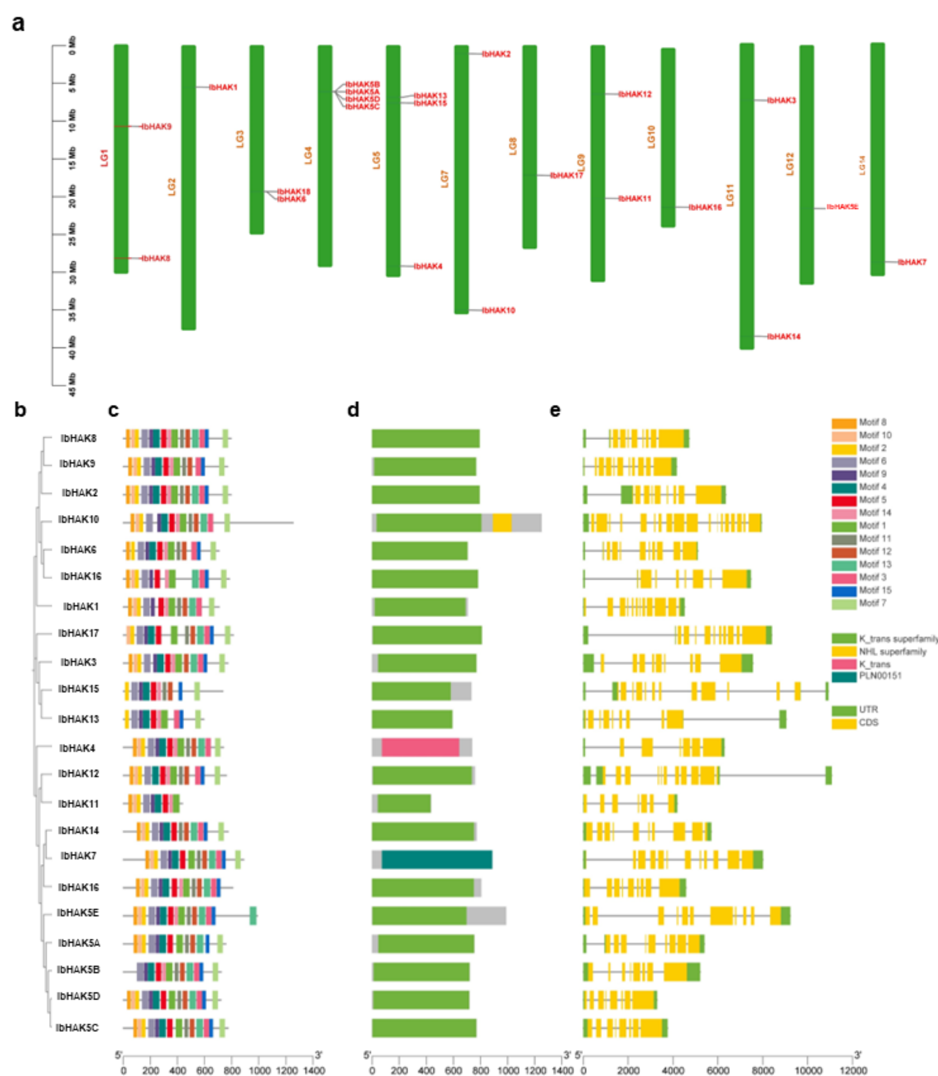

Figure 2. Chromosomal locations, Phylogenetic relationships, conserved motifs, conserved protein domains, and gene structure of *HAK/KUP/KT* family members in sweet potato. (a) Distribution of *HAK/KUP/KT* family genes on the sweet potato chromosomes. The legend on the left indicated the size of the chromosomes. The genes were mapped using the MG2C. LG1–15 represented sweet potato chromosome 1–15; (b) The phylogenetic tree was constructed according to the fulllength sequences of 22 sweet potato HAK/KUP/KT family proteins. (c) The conserved motifs of IbHAK proteins. The different colors on the sequences denoted various motif types. (d) The domain of IbHAK proteins. The protein motifs were predicted using MEME program, the conserved protein domains were searched in NCBI-CDD database, The gray regions indicated areas without specific domains, while the colored sections marked the locations of the K_trans domain. (e) The gene structure of *IbHAK* genes. UTR, untranslated region. the gray horizontal lines represented intron regions, green corresponded to UTRs, and yellow corresponded to CDS regions.

To furtherly explore the potential evolutionary mechanism of the *HAK/KUP/KT* family members in sweet potato, we selected three model plants, including diploid sweet potato *Ipomoea trifida*, *Arabidopsis thaliana*, and *Oryza sativa*, for collinearity comparison analysis with sweet potato (*Ipomoea batatas*) (Figure 3). Here we found 23 collinear genes identified between sweet potato and diploid sweet potato (*Ipomoea trifida*), followed by *Arabidopsis thaliana* with 11 collinear genes, and rice with 4, suggesting that the *IbHAK* genes have the most homologous genes with the diploid sweet potato, followed by *Arabidopsis thaliana*, which is also dicotyledonous, and rice as monocotyledonous. *IbHAK2* and *IbHAK8* exhibited collinearity in all three representative species, indicating that these two genes share more evolutionary conservation.

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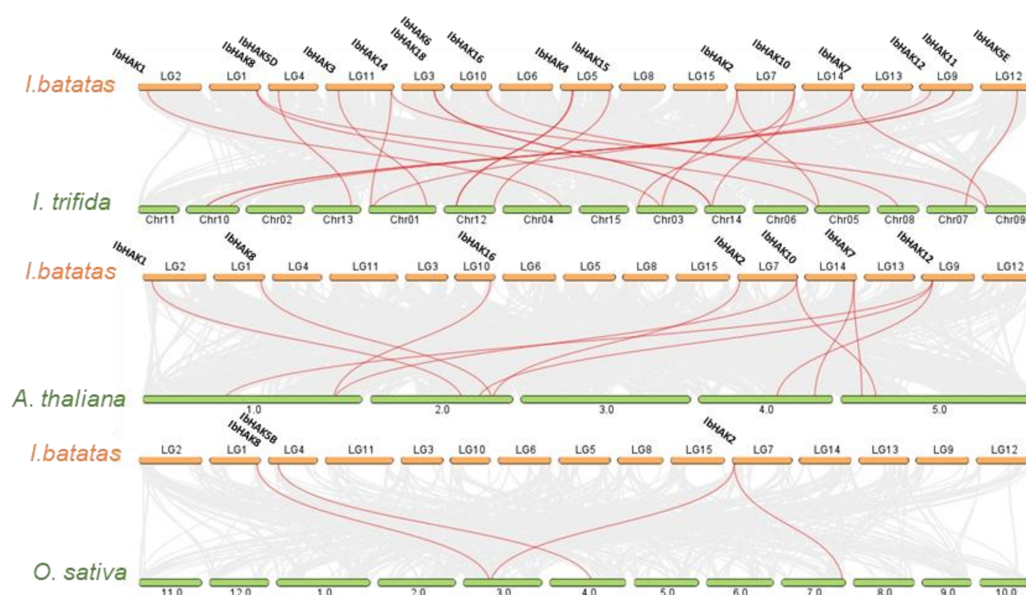

Figure 3. Syntenic analyses between *IbHAK* genes and other three species. Syntenic relationship of *HAK/KUP/KT* family genes shown on chromosome map between *I. batatas* (sweet potato), *I. trilfida* (wild diploid sweet potato), *A. thaliana* and *O. sativa*. Small green rectangles represented sweet potato chromosomes, while small orange rectangles represented chromosomes from three species. Grey lines indicated synteny blocks between sweet potato and other plant genomes, while red lines highlighted syntenic *IbHAK* gene pairs. The number of syntenic gene pairs between sweet potato and other species are as follows: wild diploid sweet potato (23), *Arabidopsis thaliana* (11) and rice (4).

2.4. Cis-Acting Elements of HAK/KUP/KT Family Genes

To further study the transcriptional regulatory mechanisms and potential functions of the *IbHAK* family members in greater detail, a *cis*-acting element analysis was conducted on the 2Kb sequence upstream of the start codon of the *IbHAK* genes (Figure 4a). These results indicated that the promoter regions of the *IbHAK* genes contain numerous abiotic stress and hormone response elements, such as light signals (89), anaerobic signals (35), low temperatures (6), and drought stress (21). The hormone response elements encompass auxin (15), abscisic acid (ABA) (39), methyl jasmonate (MeJA) (50), salicylic acid (SA) (12), and gibberellin (GA) (14). Among abiotic stress response elements, the light signal response elements were the most abundant, followed by anaerobic signals and drought, and in hormone response elements, the MeJA response elements were the most abundant, followed by ABA and Auxin response elements (Figure 4b). Overall, these results suggested that multiple *cis*-acting elements may be involved in the regulation of *IbHAK* genes in response to abiotic stress and hormone induction.

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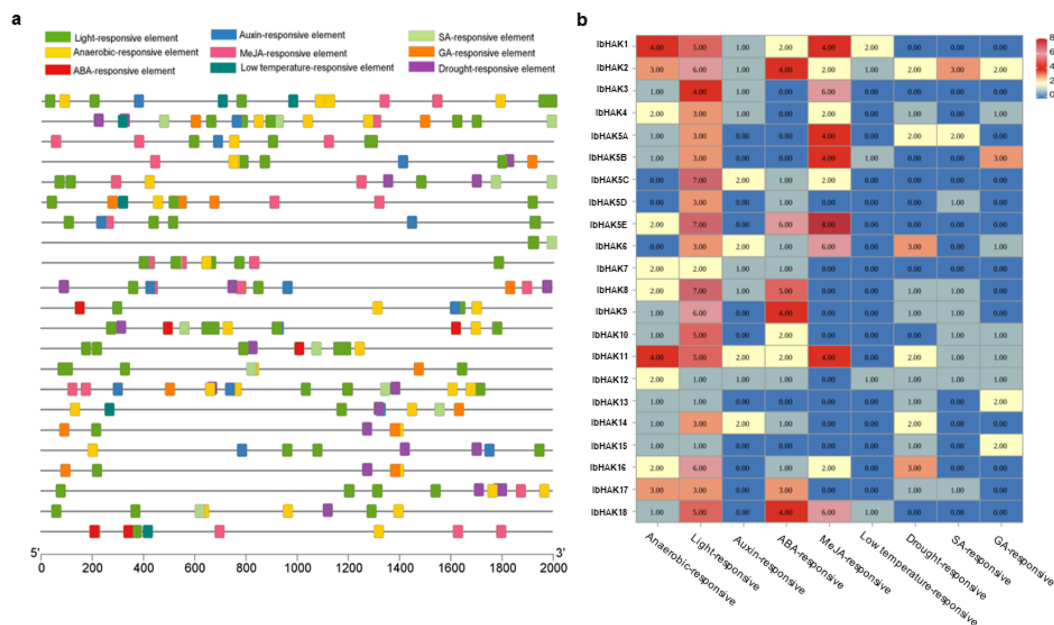

Figure 4. Promoter *cis*-acting elements analysis of *IbHAK* genes. (a) The *cis*-elements in the 2 kb promoter region of *IbHAK* genes were identified using the database PlantCARE and the map was generated by TBtools; The promoter regions of the *IbHAKs* genes were primarily divided into two major categories of elements: hormone response-related elements and abiotic stress-related elements. (b) The number of hormone-related cis-elements and stress-related cis-elements were calculated in *IbHAKs* genes, with a gradient from blue to red signifying an increasing number of elements. The numbers on the color key referred to the number of *cis*-elements.

2.5. Transcriptional Analysis of HAK/KUP/KT Family Genes and the Subcellular Localization of IbHAK5A

To explore the potential biological functions of *IbHAK* family genes, we analyzed the expression of 22 *IbHAK* family potassium transporter members according to the transcriptome data of low potassium stress and different tissue expression (Figure 5a and 5b). These results showed that *IbHAK5A* was obviously induced by LK stress, and mainly expressed in storage and uptake roots, suggesting *IbHAK5A* may play an important role in the potassium absorption function. In addition, *IbHAK3* was highly expressed in each tissue, implying that this gene is responsible for a wider array of functions in sweet potato. To verify the transcriptome data, we carried out RT-qPCR analysis, the results showed that *IbHAK5A* was mainly expressed in storage roots and significantly induced by LK stress (Figure 5c and 5d). This finding was consistent with the transcriptome data. To further reveal the role of IbHAK5A in sweet potato, the subcellular localization was also performed (Figure 5e), and the result showed that IbHAK5A was localized to the plasma membrane.

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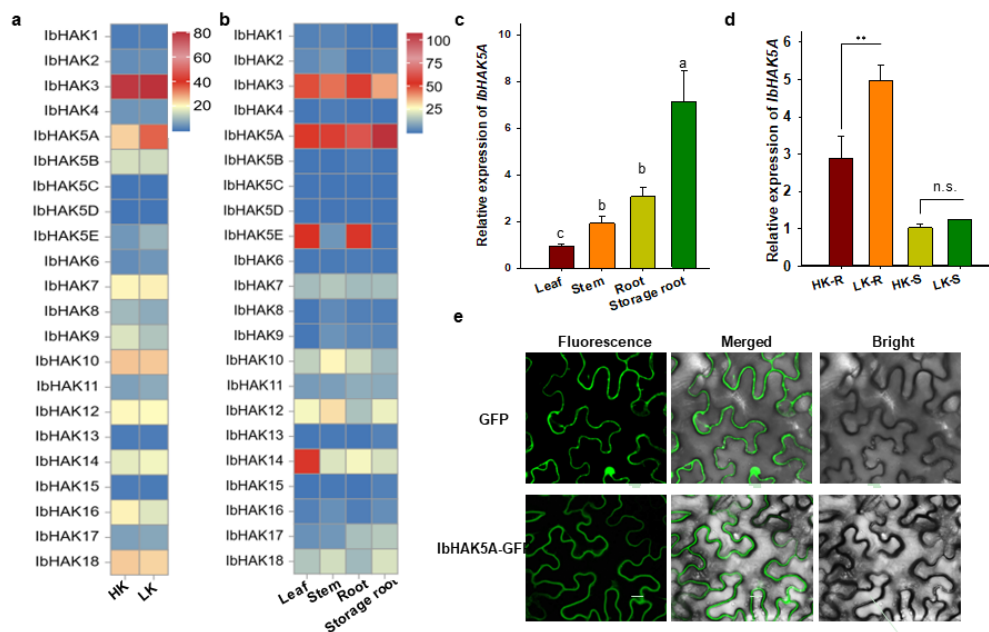

Figure 5. Expression profiles of *HAK/KUP/KT* family members in different tissues and *IbHAK5A* expression analysis. (a) The expression patterns of *HAK/KUP/KT* genes under HK and LK condition; (b) The expression patterns of *IbHAK* genes in leaf, stem, root and storage roots were analyzed; (c) qPCR analysis the expression of *IbHAK5A* in different tissue. Different lowercase letters indicate significant differences among different treatments at the P< 0.05 level. Data represent mean ± SE (n=3); (d) qPCR analysis of *IbHAK5A* expression levels in response to K^+ starvation patterns in roots and shoots of seedling under high (1 mM) and low potassium (0 mM) conditions for 5 days, Data represent mean ± SE (n=3). Student's t-test (**P<0.01) was used to analyze statistical significance. n.s. indicates no significance, HK-R: sufficient K^+ condition root, LK-R: low K^+ condition root, HK-S: sufficient K^+ condition shoot, LK-S: low K^+ condition shoot; (e) Subcellular localization of IbHAK5A in *Nicotiana benthamiana*. The expression data were obtained 1 from RNA-seq data and shown as log_2(FPKM) values. The low and high expression levels are colored with blue and red.

2.6. Functional Characterization of IbHAK5A in Yeast and Arabidopsis

To analyze the potassium transport function of IbHAK5A, we constructed the CDS of *IbHAK5A* into the yeast expression vector *pRS426* and transformed it into the potassium transporter-deficient yeast strain R5421 (Figure 6a). These results showed that IbHAK5A did recover the potassium transport function of the potassium transporter deficient yeast strain, indicating that IbHAK5A is responsible for potassium transport activity. Furthermore, the *hak5/IbHAK5A* transgenic Arabidopsis overexpression material was obtained, and the phenotype, fresh weight and primary root length were analyzed under LK (0.01 mM and 0.02 mM K^+) and HK (1 mM K^+) conditions (Figure 6b–d). The result showed that transgenic *hak5/IbHAK5A* lines complemented the short root phenotype of *hak5*, indicating that IbHAK5A shares a similar function with AtHAK5 and plays an important role in K^+ uptake in sweet potato roots.

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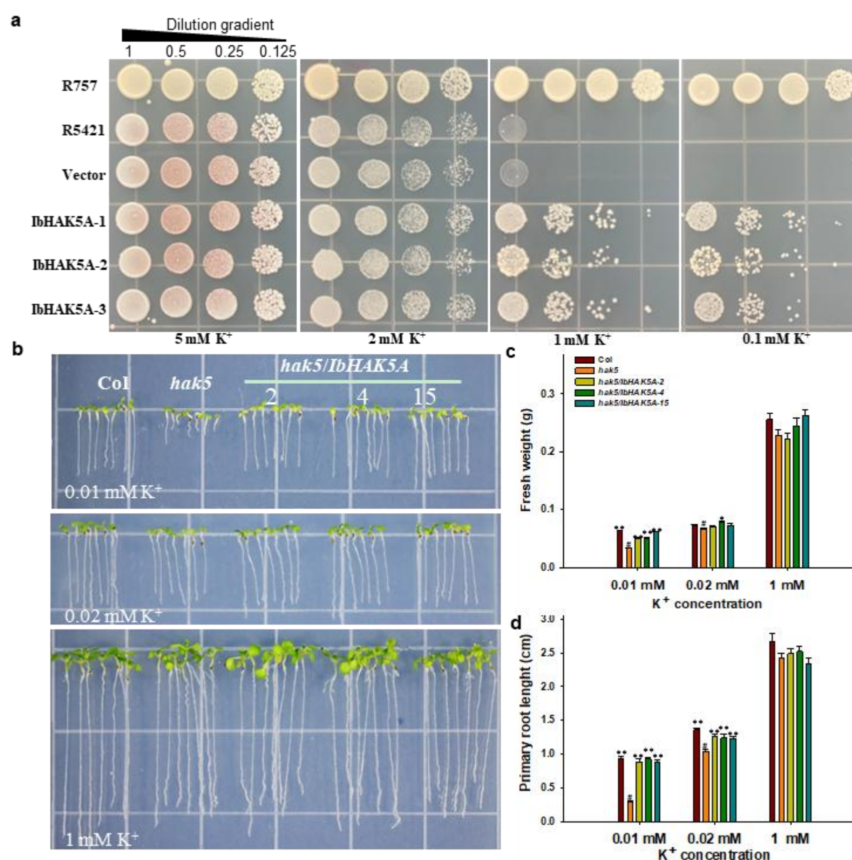

Figure 6. Potassium transport activity of IbHAK5A in yeast mutant and low potassium phenotype of transgenic Arabidopsis. (a) Complementation of *IbHAK5A* in yeast mutant R5421 (*trk1D*, *trk2D*) under different K^+ concentrations. Yeast cell harboring either an empty vector or *IbHAK5A* construct were grown in AP medium to an OD_{600} of 1. Equal volumes of 2-fold serial dilutions were applied to AP medium (pH 5.5) and then incubated at 30°C for 5 d. Three biological replicates were performed for each sample; Different K^+ concentrations phenotype comparison (b), fresh weight (c) and primary root length of various materials (d). Seeds were germinated and grown on low K^+ (0.01 and 0.02 mmol/L) or K^+ sufficient (1 mmol/L) medium for 7 d. Data represent mean ± SE (n=7). Student's t-test (**P<0.01) was used to analyze statistical significance. Asterisks indicate significant differences compared with wildtype plants (WT, $^{\#}$).

2.7. IbPTL1 can Interact with IbHAK5A and Regulate the K^+ Signal Pathway

IbHAK5A was obviously induced under LK conditions, suggesting that there may exist some transcription factors that can positively regulate *IbHAK5A* transcription in sweet potato. Based on previous transcriptome analysis data [38] and yeast-one-hybrid (Y1H) assay, we therefore screened a AP2/EREBP family transcription factor IbPTL1 (Figure 7a). To test whether IbPTL1 can regulates *IbHAK5A* transcription, we performed a dual-luciferase assay (Dual-LUC) in *N. benthamiana* leaves, and a reporter construct in which the expression of the *LUC* reporter gene was driven by the *IbHAK5A* promoter. The LUC activity analysis revealed that IbPTL1 could activate *IbHAK5A* expression, suggesting that IbPTL1 can binds to the *IbHAK5A* promoter (Figure 7b). Furthermore, we constructed the *IbPTL1* transgenic overexpression lines in Arabidopsis, and obtained two different expression lines (Figure 7c). Relative expression level uncovered that *IbPTL1* significantly induced under LK conditions in *IbPTL1*-overexpressing (*IbPTL1OE*) lines (Figure 7d), and the two LK response maker genes, *AtHAK5* and *IbCIPK23*, were also upregulated in *IbPTL1OE* lines (Figure 7e and 7f), suggesting that IbPTL1 can be regulated by K^+ signal. Together, these results suggested that IbPTL1 could combine with *IbHAK5A* promoter and regulate the K^+ signaling pathway.

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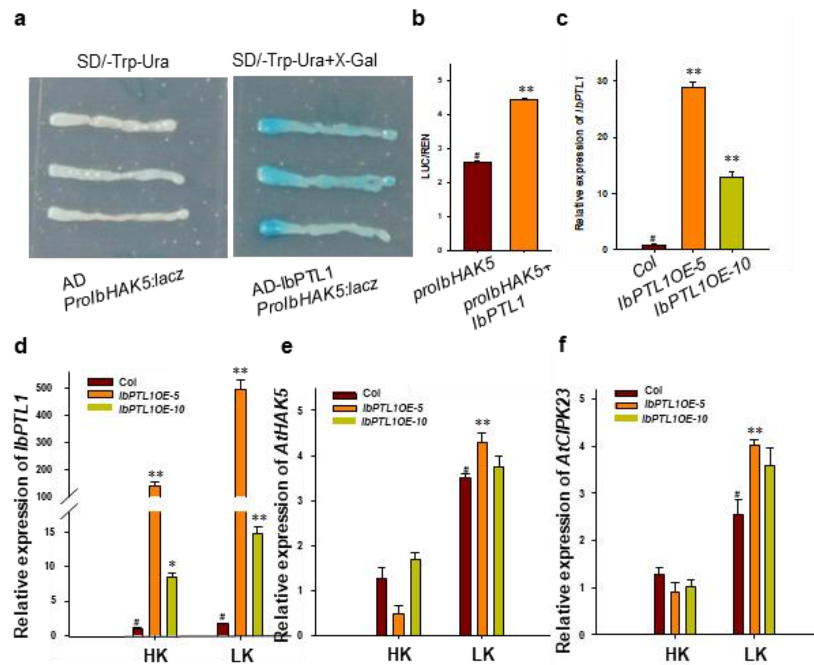

Figure 7. IbPTL1 can bind to the *IbHAK5A* promoter. (A) Yeast-one-hybrid assays showing that IbPTL1 can bind *IbHAK5A* promoter. AD refers to the empty vector expressing the *pB42AD* domain alone. *LacZ* was used as a reporter gene, driven by *IbHAK5A* promoter in yeast; (B) Transient expression assay showing that IbPTL1 can activate the expression of *IbHAK5A*. *IbHAK5A* promoter with the empty effector (*pGreenII 62-SK*) as the control, the *IbHAK5A* promoter with IbPTL1 as the assay; (C) Relative expression level analysis of *IbPTL1* in transgenic Arabidopsis; (D-F) Transcript levels of *IbPTL1*, *AtHAK5*, *AtCIPK23* in WT (Col) and *IbPTL1* overexpression lines under LK (0.01 mM K^+) and HK (1 mM K^+) conditions. Seedlings were germinated and grown on HK and LK medium for 7d. Transcript levels were normalized to *Atactin2/8*. Data represent mean ± SE. Asterisks indicate significant differences compared with control, # indicates control, 0.01<*P<0.05, **P< 0.01.

3. Discussion

Although *HAK* family genes have been reported and the function of the *IbHAK5* gene in sweet potato has been described previously [41,42], these studies remain incomplete and a more comprehensive analysis of the entire genome of the sweet potato has yet to be carried out for the HAK/KUP/KT family. There are 22 KUP/HAK/KT family members in sweet potato (Table 1), and 21 KUP/HAK/KT family members in wild diploid sweet potato were identified in this study (Table S2), suggesting that a large-scale gene duplication event from diploid to hexaploidy evolution did not occur, unlike wheat, which has 56 *HAK/KUP/KT* genes, and happen large-scale gene duplication event in the evolutionary process [21]. Most other plants and crops, such as tea (21) [22], saccharum (30) [24], barley (27) [43], potato (24) [44], mung bean (19) [45], and cassava (21) [46], exhibit similar numbers of *HAK/KUP/KT* family genes, along with transmembrane segments and conserved domains. The suggests that *HAK/KUP/KT* genes family are conserved across different species.

The KUP/ HAK/KT family transporters were identified as candidate high-affinity K^+ uptake transporter; HAK5 are vital for plant growth and development at low K^+ concentrations [5]. In this study, we identified 5 *IbHAK5* members (*IbHAK5A-E*) in sweet potato. Our phylogenetic tree analysis result showed that the five *IbHAK5* genes were in the same branch with Arabidopsis, rice and maize *HAK5* genes (Figure 1). Among the five *IbHAK5* genes, the four genes (*IbHAK5A-D*) were showed closer homology and were arrayed in tight clusters of tandemly duplicated genes (Figure 1 and 2A, Figure S2), while *IbHAK5E* was located on other chromosomes and not occurred duplication, suggesting that I*bHAK5A-5D* were selected for amplification during the evolution of the *IbHAK5* gene but not *IbHAK5E*. We further analyzed the *HAK* family genes in wild diploid sweet potato (Figure S1), and found that there also are 5 *HAK5* genes arrayed in tight clusters of tandemly duplicated

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genes, suggesting that the *HAK5* genes experienced expansion before the diploid evolution to hexaploid sweet potato. The duplicated genes *IbHAK5A-5D* may exhibit functional redundancy, which could enhance the sweet potato's ability to acquire more K^+ for growth and tuber expansion. In addition, transcriptome analysis revealed that the expression levels of *IbHAK5A* under LK stress and across different tissues, indicating its prominence. Therefore, *IbHAK5A* may be a potentially key gene for K^+ acquisition in sweet potato.

Promoting K^+ uptake under high-salt conditions is an important strategy for enhancing plant salt tolerance [19]. To gain a deeper understanding of the *IbHAK* genes family, we obtained salt stress transcriptome data in sweet potato from the NCBI database [39]. The FPKMs were used to calculate gene expression levels and heatmap analysis, which showed that *IbHAK3*, *IbHAK5B*, *IbHAK10*, *IbHAK16*, and *IbHAK18* were obviously unregulated under salt stress, suggesting that these genes may play a more important role in the salt stress signaling pathway (Figure S3). Moreover, we found another highly expressed genes *IbHAK3*, which was also highly expressed in various tissues (Figure 5b) and significantly induced by salt stress. The result showed that *IbHAK3* plays an important role in *IbHAK* genes family, and its function deserves further analysis in the future.

Transcriptional regulation is an important mechanism in a plant's response to LK stress. Under LK conditions, the number of genes regulated at the transcriptional level is limited, including the key potassium transporter *HAK5*. Previous research has shown that the CBLs-CIPK23 and CBLs-CIPK1/9 complex control the high-affinity K^+ uptake mediated by HAK5 [47,48]. AP2/ERF transcription factor RAP2.11 regulates *AtHAK5* expression under LK conditions and also regulates other genes in the LK signaling cascade [49]. In addition, an auxin response factors, ARF2, was reported as a transcriptional repressor to modulate *HAK5* transcription in the response to LK stress [33]. The R2R3-type MYB transcription factor MYB77 has been shown to be able bind to the *HAK5* promoter and activate K^+ uptake [50]. However, these studies were mainly focused on Arabidopsis species; the question of how *HAK5* genes were regulated in crops remains unanswered. In this study, we screened an AP2/EREBP family transcription factor IbPTL1 in sweet potato, which can bind to the promoter of *IbHAK5A*. RT-qPCR showed that the *IbPTL1* was obviously upregulated under LK condition and the genes which response to LK signaling were also upregulated in transgenic Arabidopsis overexpression lines of *IbPTL1* (Figure 7d-f). These results suggest that IbPTL1 may act as an important transcription factor regulating *IbHAK5* under LK conditions. However, further validation using transgenic materials from sweet potato will be necessary to confirm this relationship in future studies.

Genotypes tolerant to LK conditions may be used to breed for improved K utilization efficiency (KUE) as well as for clarifying the genetic basis of sweet potato responses to LK stress [51]. Tolerance LK stress is a complex quantitative trait, with strong interactions between genotypes and the environment [52]. In the present study, we screened eight different genetic backgrounds sweet potato varieties showed different LK tolerant genotypes (Figure S4a). Both vine length and fresh weight were analyzed (Figure S4b and c), which showed that the 'Chuanzishu6', Chuanshu218', 'chuanshu294', and 'Chuanshu217' had the relative LK sensitive phenotype, while 'chuanshu228', 'chuanshu20', 'chuanshu225', and 'Nanshu88' had the LK tolerance phenotype. The different genotype of key potassium efficient use genes needs to be further identified by LK phenotype and whole genome sequencing, and these LK tolerance sweet potato varieties may be utilized for future breeding purposes.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

The wild-type Col and *hak5* mutant of *Arabidopsis thaliana* were generously provided by Professor Yi Wang from China Agricultural University. The overexpression genetic materials of *IbHAK5A* and *IbPTL1* in Arabidopsis were independently constructed in this experiment, with the CDS of *IbHAK5A* and *IbERF* genes cloned into the vector *pSuper1300*, and transformed into the

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Arabidopsis *hak5* mutant and wild-type Col to obtain the transgenic genetic materials *hak5/IbHAK5A* and *Col/IbPTL1*. For the LK germination phenotype experiment, seeds that had undergone vernalization for 3 days were placed on solid media with sufficient potassium (HK, 1mM K^+) and LK (LK, 0.01 mM and 0.02 mM K^+) conditions for light cultivation (22˚C, 16 h light/8 h dark). After 7 days, phenotypic photographs were taken and the primary root lengths of each material were measured using Image-J software [33]. Seven biological replicates were used for Arabidopsis primary root lengths, and three biological replicates were applied for fresh weight count. Student's t-test was used to analyze statistical significance. SigmaPlot v10.0 (Systat Software, https:// systatsoft ware. com/) was used for all graphical analysis and statistical significance.

The experimental materials 'TaiZhong6' and 'NanShu88' were provided by the National Sweet Potato Industry Technology System Resource Library. 'ChuanShu294', 'ChuanZiShu6', 'ChuanShu228', 'ChuanShu20', 'ChuanShu225', 'ChuanShu218', and 'ChuanShu227' are sweet potato varieties independently bred by the Crop Research Institute of Sichuan Academy of Agricultural Sciences and used in the study. For the LK phenotype experiment, consistent sweet potato stem segments (approximately 5 cm) were cut in the field and grown in vermiculite to three-leaf stage, then transferred to Hoagland hydroponic solution for HK (1 mM K^+) and LK (0.01 mM K^+) phenotype experiments, with fresh solution every three days. After 20 days, phenotypic and physiological data were recorded. Three biological replicates were performed for each sample. The conditions in the artificial climate chamber were set to 28 °C, with a light/dark cycle of 16 h/8 h.

4.2. Identification of HAK/CUP/KT Family Members

The sweet potato whole genome sequence and annotation files were download from the sweet potato genome database (public-genomes-ngs.molgen.mpg.de/sweetpotato/). Blast analysis in TBtools was then performed using 13 HAK/KUP/KT family members of Arabidopsis to initially obtain proteins homologous to the Arabidopsis HAK/KUP/KT family. Further analysis of the integrity and reliability of the domains of the HAK/KUP/KT family members using the CD search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SMART (https://smart.embl.de/) online tools (E<0.001). After removing incomplete sequences, and ultimately obtain 22 HAK/KUP/KT family members. Next, TBtools software was used to calculate the number of amino acids, relative molecular weight, isoelectric point, and number of transmembrane domains of the sweet potato HAK/KUP/KT family members. Finally, the subcellular localization of the HAK/KUP/KT family members was predicted using the online website Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/).

4.3. Phylogenetic Analysis of HAK/CUP/KT Family Members

The protein sequences of 22 sweet potato, 13 Arabidopsis, 27 rice, and 27 maize HAK/KUP/KT family members were submitted to MEGA 11 software for multiple sequence alignment using the neighbor-joining method to construct a phylogenetic tree [34], with a bootstrap repeat value of 1000 times and other parameters set to default, and grouped according to the classification method of the Arabidopsis HAK/KUP/KT gene family.

4.4. Gene Locations, Protein Conserved Motifs, and Gene Structure Analysis of HAK/KUP/KT Family Members

The 22 HAK/KUP/KT gene numbers and the sweet potato genome Gff annotation file were submitted together to TBtools software [35], using the Gene Location Visualize from GTF/GFF function for gene chromosome localization and visualization analysis. The HAK/KUP/KT protein sequences were then submitted separately in MEGA 11 software for multiple sequence alignment, using the neighbor-joining method to construct a phylogenetic tree, resulting in a nwk format evolutionary tree. The online software MEME (http://meme-suite.org/tools/meme) was subsequently used to predict the conserved motifs of sweet potato HAK/KUP/KT proteins, with the number of

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motifs set to 15 and the rest as default parameters, saved as an xml file. Last, the conserved domains of sweet potato HAK/KUP/KT proteins were obtained from NCBI's CDD research, and combined with the sweet potato genome GFF annotation file, phylogenetic tree, conserved motif analysis file, and sweet potato genome file, and a final visualization analysis was performed in the Gene Structure View (Advanced) of TBtools.

4.5. Collinear Relationships of HAK/KUP/KT Family Members

MCScan X [36] was used to analyze the tandem and fragment duplication events of the *HAK/KUP/KT* family genes using default parameters and to analyze the collinear relationship of the sweet potato genome (*I. batatas*), the wild diploid sweet potato (*I. trifida*), *Arabidopsis thaliana* (*A. thaliana*), and rice (*O. sativa*). The collinear relationship between the two groups was visualized using TBtools.

4.6. Cis-Regulatory Element of HAK/KUP/KT Family Members

TBtools software was use to extract 2000 bp upstream sequences of the *HAK/KUP/KT* gene family members, which were then submitted to the PlantCARE online website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to predict the *cis*-acting elements in the promoters of the *HAK/KUP/KT* family genes. Excel software was used for statistical analysis of various *cis*-acting elements, while heat maps and TBtools software were employed for visual analysis.

4.7. Subcellular Localization of IbHAK5A and Yeast Complementation Assay

The CDS sequence of *IbHAK5A* was constructed into the *pSuper1300:GFP* vector, and the fused vector *IbHAK5A:GFP* and the empty *pSuper1300:GFP* vector were transformed into *Agrobacterium* GV3101 by electroporation. They were then injected into the lower epidermis of tobacco, and three days later, the expression of *IbHAK5A:GFP* and the control *pSuper1300:GFP* was observed using a confocal laser-scanning microscope (LSM800; Carl Zeiss).

For the potassium transporter-deficient yeast complementation experiment, the CDS of *IbHAK5A* was constructed into the yeast expression vector *pRS426*. The empty vector *pRS426* and *IbHAK5A-pRS426* vector were co-transformed into the potassium transporter-deficient yeast strain R5421 (ura3-52his3Δ200 leu2 Δ1 trp1 Δ1ade2 trk1 Δ::HIS3 trk2 Δ::HIS3), a K^+ uptake-deficient strain of *Saccharomyces cerevisiae* in which the two endogenous K^+ transporter genes (TRK1, 2) were deleted [37]. The WT yeast strain R757 was used as the positive control. The mutant yeast strain R5421 can grow normally in HK conditions, but grow slowly in LK conditions. Three days later, positive clones were selected, and YPDA (supplemented with 0.1 M KCl) was used to culture the yeast to an OD_{600} of 0.8. The strains were washed three times with sterile water, the starting OD_{600} was adjusted to 1, and then diluted in a 2:1 ratio, with different yeast strains spotted on AP medium containing different concentrations of K^+ [20].

4.8. RNA-Seq Analysis and Real-Time PCR Analysis

Based on transcriptome raw data (NCBI SRA accession: PRJNA760652; PRJNA1041734; PRJNA1065644) of sweet potato in different tissues, and under LK and salt stress [38,39], the fragments per kilobase of exon per million fragments mapped (FPKMs) were used to calculate gene expression levels and facilitate heatmap analysis.

For different tissues expression assay, the samples from tissues (stem, leaves, root, storage root) were collected at 80 days (the sweet potato expand stage). For LK stress induced assay, 5 cm stems segments were cut from field condition, and grown in vermiculite to three-leaf stage, then transferred to HK (1 mM K^+) and LK (0 mM K^+) Hoagland hydroponic solution for 5 days, and the roots and shoots were sampled and immediately frozen in liquid nitrogen, then stored at −80 ◦C.

Total RNA was extracted from these samples using a Polysaccharide Polyphenol Plant Total RNA Extraction Kit (TIANGEN; DP441; China) according to the instructions provided by the

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manufacturer. RNA concentration and purity was assessed using a NanoDrop 2000 fluorospectrometer. Sweet potato cultivar 'Taizhong6', whose genome has been sequenced and assembled [40], was used for transcriptome and RT-qPCR analysis in this research. The qRT-PCR experiment for different tissues and LK stress performed with three biological replicates. The qRT-PCR reaction procedure was: 95 °C for 10 s; 95 °C for 15 s, 60 °C for 1 min, for 40 cycles. *IbACTIN* served as the reference gene, and SigmaPlot 10 software was used for graphical analysis. All primers used in this study are listed in Table S1.

4.9. Yeast One-Hybrid (Y1H)

The 2 Kb promoter sequences of *IbHAK5A* were cloned into *pLacZi* vector, and full-length coding sequences of *IbPTL1* were cloned into *pB42AD* vector. The reporter plasmids were then co-transformed into the yeast strain EGY48 as described in the Yeast Protocols Handbook (Coolaber, YH3010), and the transformed yeast was cultured on SD/-Trp/−Ura at 30℃ for 3 days. Positive transformants were grown on proper SD/-Trp-Ura plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 2% galactose, and 1% raffinose for blue color development. Three independent experiments were performed. The respective pairs of primers are shown in Table S1.

4.10. Dual-Luciferase Assays

The *pGreenII0800* vector containing the promoter of *IbHAK5A* and the *pGreen* Ⅱ*-SK-62* vector containing the CDS of *IbPTL1* were respectively transformed into *Agrobacterium tumefaciens* GV3101 and were transiently expressed in *Nicotiana benthamiana* by infiltration. Three days after injection, firefly luciferase and *Renilla* luciferase were detected using a Promega GloMax96 instrument and the Dual-Luciferase Reporter Assay System (Promega).

5. Conclusions

In conclusion, this study identified 22 *IbHAK* family members in sweet potato which were classified into four clusters (I-IV) based on the phylogenetic and structural features analysis. All *IbHAK* genes were unevenly distributed on 12 chromosomes, and there were five *IbHAK5* genes that showed highly homology with Arabidopsis, rice and maize, suggesting the *HAK5* gene had undergone a gene duplication event in sweet potato. One of the highly expressed genes, *IbHAK5A*, was cloned and functionally characterized in potassium-deficient yeast and Arabidopsis, and the results suggested that IbHAK5A functions in K^+ uptake. Moreover, the transcription factor IbPTL1 was found to be able to combine with *IbHAK5A* promoter and to be involved in K^+ homeostasis. Taken together, this study provides important clues to gain insight about the functions of *IbHAK* genes in sweet potato. However, additional research is necessary to uncover the function of key potassium transporters. For example, CRISPR/CAS9 strategy and big data analysis based on genome sequencing of different K^+ uptake efficiency sweet potato materials are necessary to elucidate the precise role of the individual *IbHAK* gene in sweet potato.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Fang Wang conceived and performed the experiments the study. Zhongmei Xie, Songtao Yang, Shuai Qiao, and Wei Song carried out part data analysis. Fang Wang and Wefang Tan prepared the manuscript and performed a critical review of intellectual content. All authors have read, edited, and approved the current version of the manuscript.

Funding: This work was supported by the Natural Science Foundation of Sichuan Province (2024NSFSC1329), the China Agricultural Research System of MOF and MARA (CARS-10), the 1+9 Open Competition Project of Sichuan Academy of Agricultural Sciences (1+9KJGG002).

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Data Availability Statement: The datasets presented in this study can be found in online repositories. The transcriptome expression data are available in the National Center for Biotechnology Information SRA database, accession numbers PRJNA1041734, PRJNA760652 and PRJNA1065644.

Acknowledgments: We thank Prof. Yi Wang (China Agricultural University, China) for kindly providing seeds of the *hak5* mutant and potassium deficiency yeast strain R5421 and WT strain R757. The authors thank AiMi Academic Services (www.aimieditor.com) for English language editing and review services.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

LK	low-K^+
KUE	K utilization efficiency
FPKM	the fragments per kilobase of exon per million fragments mapped
HK	high-K^+
Y1H	yeast one-hybrid
ABA	abscisic acid
MeJA	methyl jasmonate
SA	salicylic acid
GA	gibberellin
Dual-LUC	dual-luciferase assay

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