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Genome-wide identification of potassium transporters and channels in *Malus domestica* genome

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Potassium (K^+) is an essential nutrient for plants. It contributes to most physiological and biochemical pathways for plant metabolism, growth, and development. It is the most available plant nutrient, comprising 10–15% of plant weight. Plants have a sophisticated system of K^+ transporters and channels for distribution in plant body. Apple is one of the most consumed fruits in the world. Its fruit quality and yield are positively affected by K^+. However, limited information is available about K^+ transport systems in Apple. In this study, 47 candidate genes (26 K^+ transporters and 21 K^+ channels) have been identified in Apple (*Malus domestica*) genome. The phylogenetic comparisons with other plants (*Glycine max*, *Arabidopsis thaliana*, and *Oryza sativa*) indicated that the K^+ transport system is much conserved among different plants. The analysis of Gene structure showed the presence of specific introns and exon patterns for these gene families. Transcriptomic data analysis and RT-qPCR demonstrated significant variations in the transcript abundance of these genes in response to abiotic stresses. The current project represents the first report about the K^+ transport system in Apple. Therefore, it may act as a starting point for further functional characterizations.

Keywords K^+ channels, Genomic insights, Auxin, Indole acetic acid, RNA-seq

Potassium (K^+) is the most available and essential nutrient for plants. The dry weight of plant may comprise 10–15% of K^+ with a concentration ranging between 60 and 100 mM in cytosol. Its deficiency may cause metabolic changes and serious malfunctions in the normal growth of plants[1]. Regarding several plant processes, K^+ is also very important for regulating water balance, photosynthesis, and biosynthesis of various bioactives. Plant cells use K^+ in several important biological functions including stomatal movement, regulation of osmotic pressure, and the cell elongation process[2]. The K^+ plays a key role in the normalization of the overall pH of the cytoplasmic cytosol. Plants have evolved a complex but organized K^+ transport system comprising K^+ channels and transporters[3,4]. The K^+ Transport System (PTS) ensures the uptake and translocation of K^+ to the diverse regions (from soil to the tip of leaves) of the plant body. The *Arabidopsis thaliana* has 35 genes comprising PTS as 20 transporter genes and 15 channel genes[5,6].

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The K^+ channels mediate K^+ transport to and from the cell membranes to maintain cell turgidity, hormonal secretions, cell osmotic level, and cellular contents under several conditions[7]. K^+ channel genes have been classified for their functions, i.e., K^+ movement and channel gate opening and closing. Some K^+ channels are ligand-gated, where the opening of channel gate is dependent on an ion that pushes up (opens) the channel gate energetically[8]. Another type of K^+ channel is the voltage-gated transport channel in which the opening of the channel gate is linked to a particle-mediated charge (i.e., K^+, Ca^{+2})[9]. Up till now, the K^+ channels have been found in all life forms including animals and plants[10]. Moreover, their amino acid sequences were found highly conserved[11]. K^+ channels are expressed as multimeric proteins (having pore domains and Transmembrane (TM) domain segments) that are very specific regarding their number of pore and TM domains[1]. K^+ selective conduction is mediated by the association of four pore domains with a multimeric protein. These pore domains possess highly conserved signature sequences (BGYGD/E)[12]. It is observed that according to the nature of their topology, a total of fifteen K^+ ion channels of *A. thaliana* have been categorized into five tandem pore K^+ transport genes (TPK), one K^+ inward rectifier gene (Kir), and nine voltage-gated channel genes[13].

The K^+ transporters are grouped into three subfamilies regarding their nature and structure: K^+ efflux anti-porters (KEA), K^+ uptake permeases (KUP/HAK/KT), and K^+ transporters (Trk/HKT). In *A. thaliana*, a single gene has been reported for high-affinity Trk/HKT, 13 KUP/HAK/KT genes, and 6 KEA genes[5,11]. High-affinity potassium transporters (HKTs) belong to Trk family and contain multiple MPM signature motifs[4,11]. The KT/HAK/KUP family is similar to its close relative members (KUP) from bacteria and fungi (HAKs)[14,15]. KEA (K^+ efflux anti-porters) helps to maintain K^+ balance across membrane as it does not disturb the charges across the membrane by moving it from a region of lower concentration to a higher concentration and vice versa. It also plays an important role in cytosolic pH maintenance[16].

Apple (*Malus domestica)* is among the widely consumable fruits in the world including Pakistan. It has a huge marketplace both nationally and internationally. It is mainly cultivated in temperate regions of the world and exported to other regions with higher prices[17,18]. Apple's genome comprises 17 chromosomes. The average size of proteins ranges between 881 and 1052 amino acids[19]. Phylogenetic analysis of the family pyreae and its genus Malus has revealed the Apple as a special case of hexaploid[20]. There exists an association between gene families' expansion and the development of fruits, including the formation of pome fruit in pyreae tribe[21,22]. A genomic sequence of a diploid apple cultivar called 'Golden Delicious' is used in this work to study potassium transport-related genes[23,24].

In this research, the identification of K^+ transport system in *M. domestica* was carried out. Its characterization, i.e., genomic information (domains, transmembrane domains, gene structures) was also investigated using NCBI server. Furthermore, the phylogenetic analysis, *cis*-regulatory elements analysis, and substitution analysis were also performed to get greater insights into the evolutionary history of genes. Indole acetic acid (IAA) is a phytohormone, and it is critical for plant growth and developmental processes, such as cell division, elongation, and differentiation. This hormone has been shown to promote root growth, lateral shoot formation, and fruit development. This research work will not only develop a deeper insight into potassium regulation for stress resistance but will also help us to improve the production and growth of apples[25].

Materials and methods

Identification of K^+ transporters and channels

Candidate K^+ transport proteins were identified from *M. domestica* proteome using previously known sequences of K^+ transport proteins from *O. sativa* and *A. thaliana*[26]. These sequences were used as a query of BlastP tool, a genomic database of Rosacea (GDR) (https://www.rosaceae.org/ and Genebank (https://www.ncbi.nlm.nih.gov/)[27–29]. Sequences were manually curated to remove incorrect predictions and to reduce redundancy. These sequences were subjected to another screening for the confirmation of transporter or channel-specific motifs. Furthermore, to identify K^+ selective motif (G-Y-G) motif, tBLASTx from NCBI was used to analyze sequences in all six reading frames. The selected genes were also confirmed by a method devised by Gomez and Porras[30]. After verifications, the transporting genes were further searched in other databases such as Pfam (http://pfam.janelia.org/)[31], SMART (http://smart.embl-heidelberg.de/), and CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)[32,33] to check their validity and to confirm their phylogeny and gene structure.

Domain prediction and physicochemical properties

Highly conserved K^+ transporting genes from *M. domestica* were further analyzed to predict TM-Domains. A web-based server TMHMM(http://www.cbs.dtu.dk/services/TMHMM/)" was used to predict TM-domains, as this server uses helical sequences to predict *De novo* based domain prediction[34]. Gene databases (i.e., NCBI's Genbank and Gene) were also searched to locate these genes and find their genomic positions. The physical positions of selected genes on chromosomes and the exons per gene were also identified[35]. Candidate transporters/channel proteins were used to analyze physicochemical properties of identified domains using ProtParam[36].

Motif identification and gene structure analysis

Motifs for all these K^+ transport-related proteins were identified using default parameters of 'MEME' (http://meme.sdsc.edu/meme/meme.html)[37]. The names of genes were selected based on their homology to genes in *A. thaliana*. To identify intron/exon arrangement and gene structures, GSDS (http://gsds.cbi.pku.edu.cn/) was utilized[38].

Sequence alignments and phylogenetics

The alignment of protein sequences from different species was performed using ClustalW, which is a web-based Multiple sequence alignment (MSA) special tool (https://www.genome.jp/tools-bin/clustalw)[39]. Alignment

was expressed as sequence logos using WebLogo3 (http://weblogo.threeplusone.com/), which analyzed highly conserved amino acids[40]. MEGA7 was used to draw phylogenetic trees using neighbor-joining and the maximum likelihood methods[41].

Chromosomal mapping, *Cis*-elements detection, and evolutionary analysis

Complete chromosomal localization for potassium transport-related genes was predicted by the Map Chart tool, which graphically describes the locus positions[42]. Afterward, all the *Cis*-regulatory elements for promoters from all K^+ transport-related genes were detected using an online available tool Plant-Care[43]. DNAsp, an offline tool, was used to find out the gene flow and gene duplications, synonymous and non-synonymous substitutions were calculated, and a timeline was drawn to find out the evolutionary pathway[44].

Transcriptome-based profiling of K^+ channels and transporters in *M. domestica*

To further investigate the transcript abundance of K^+ transport-related genes, NCBI-SRA publicly available RNA-seq BioProject# PRJNA728501 was studied to retrieve transcript abundance during various fruit development stages. Furthermore, another available BioProject, PRJNA645374, was explored to analyze the expression of K^+ transporters and channels in salt stress response. The differential expression was estimated by comparing the reads from treated samples with non-treated. All clean reads (paired-end) were mapped to the reference genome and the reads were counted using Bowtie2 and Cufflinks, respectively. The data were used to generate the heatmap using Tbtools[45–53].

Plant stress treatments, RNA extraction, and RT-qPCR

The plant material for developing plantlets was obtained from Hill Fruit Research Station, Murree, Pakistan (33.9164° N, 73.3968° E). The plantlets of *Malus domestica* were grown in soil at 20 ± 1 °C and 16 h of light and 8 h of dark cycle in a growth chamber for eight weeks under controlled conditions at Government College University, Faisalabad, Pakistan. The Hoagland solution, a mixture enriched with essential elements required for plant development, was used as an initial treatment for the plants' drought stress. Uniformly developed plants were then collected for drought treatment. For drought stress treatment, plants were kept in drought for 14 days. After two weeks of regular development, no water or Hoagland solution was given for the following 14 days for severe drought stress. Samples were taken after 0, 3, 7, and 14 days after drought treatment. These samples were immediately frozen in liquid nitrogen and stored at -80 °C. The total RNA was extracted from *M. domestica* leaves. The Trizol reagent method was employed to extract RNA, and Nanodrop-2000 (Thermo Fisher Scientific, USA) was used to estimate RNA concentration. Reverse transcription was performed using 1 µg of RNA with the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). RT-qPCR was conducted using the iTaq Universal SYBR Green Super-Mix on the CFX96 TouchTM RT-PCR Detection System (BioRad, USA). Oligo Calculator (http://mcb.berkeley.edu/labs/krantz/tools/oligocalc.html/) was used to design gene-s pecific primers, and NCBI-primer BLAST algorithm (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to confirm the specificity of these primers (Supplementary File 1 and Supplementary File 2)). Relative gene expression was calculated using the 2^−ΔΔCt method, where ΔCt = Ct_target − Ct_reference, and ΔΔCt = ΔCt_treatment − ΔCt_control. For each gene, three biological replicates were analyzed, each consisting of tissue pooled from three independent plants. For each biological replicate, three technical replicates were included to ensure consistency and reproducibility. Statistical significance was assessed using one-way ANOVA followed by Student's t-test (for pairwise comparisons), depending on the experimental setup. All statistical analyses were performed using R, and a p-value < 0.05 and $p < 0.001$ was considered statistically significant. The *MdEF1α* (LOC103443462) was employed as the internal control for normalization of expression values.

Results

Characterization of K^+ transport system (PTS) in *M. domestica*

The identity of PTS genes was confirmed by characterizing their domains, motifs, phylogeny, and gene structures. By analyzing the apple genome, it was found that 47 genes, including 21 K^+ channels and 26 K^+ transporters, represented PTS. Domains for all these 47 genes were also predicted using a conserved domain database and TMHMM. It was observed that MdHAK6.2 had a maximum (14) number of TM domains and MdKEA2.2 had a minimum (zero) number of TM domains. Similarly, MdKAT3.2 had a maximum isoelectric point (PI) value of 9.54, and MdKEA3.2 had a minimum PI value of 4.78 (Table 1). Both of these families were then named according to the nomenclature proposed by Very and Sentenac[54,55].

Among the K^+ transporter genes, one high-affinity K^+ transporter, 7 K^+ efflux anti-porters (KEAs), and 18 K^+ uptake permeases (KUP/HAK/KTs) genes were identified in *M. domestica*[4,14]. Members of all three families of K^+ transporters were observed and characterized with reference to *A. thaliana* (Fig. 1A).

After the identification of HAK (higher affinity K^+ transporters), KT (K^+ transporters), and KUP (K^+ uptake premises), this family was named as KT/HAK/KUP due to a very strong phylogenetic relationship between these three subfamilies[56]. Eighteen KT/HAK/KUP genes in *M. domestica* were predicted, which were known to be involved in the K^+ transport system. It was similar to the gene number in *A. thaliana*, a model plant. By further processing of gene similarity evaluation, it was confirmed that these genes code for proteins with an average length between 915-to-1020 amino acids (AAs) (Table 1). The genomic structure analysis of apple showed that the exon number ranges between 9 and 13. An average of 12–14 transmembrane domains were observed in all members of KT/HAK/KUP gene family. By analyzing its domain structures, it was confirmed that this family was closely related to the members present in *A. thaliana*. These results were further verified by the identification of a greater similarity of the other motifs from the same family in different plants (Fig. 1A)[57].

MdHKT1.1 was found as the only member from this gene family in *M. domestica*. It has a protein of length 554 AAs with 9 TM-domains and has a similar structure to AtHKT1. The observed HKT transporter was

Sr.#	Gene name	Protein ID	TM DOMAIN	DOMAINS	Length	Chr.#	Exon	MW (g/mol)	PI	Gravy
1	*MdHKT1.1*	XP_017184880.1	9	TrkH; Cation transport protein	448	15	4	50506.28	8.96	0.301
2	*MdHAK1.1*	XP_017190968.1	12	PotE; K^+_trans;	773	11	9	86098.02	8.57	0.453
3	*MdHAK6.1*	XP_008389622.1	12	PotE; K_trans;	798	13	9	8710.27	8.46	0.287
4	*MdHAK6.2*	XP_008340010.1	14	K_trans; K^+ transporter	776	16	10	86895.52	8.61	0.271
5	*MdHAK2.1*	XP_008345318.1	12	K_trans; K^+ transporter	794	4	10	88997.15	6.81	0.
6	*MdHAK10.1*	XP_008383296.1	11	PotE; K_trans;	805	10	8	90049.23	8.54	0.272
7	*MdHAK12.1*	XP_008389625.1	12	K^+ transporter; Provisional	847	13	9	94048.93	7.18	0.261
8	*MdHAK7.1*	XP_008382037.1	10	PotE; Amino acid transporter	849	10	10	94361.28	5.11	0.191
9	*MdHAK7.2*	XP_008373192.1	12	PLN00151; K^+ transporter;	852	5	10	94914.53	5.24	0.263
10	*MdHAK8.1*	XP_008362145.1	11	K^+ transporter;	775	17	8	86891.01	8.33	0.273
11	*MdHAK4.1*	XP_008341206.1	12	K^+ transporter;	789	17	9	87835.52	8.28	0.230
12	*MdHAK8.2*	XP_017190042.1	11	PotE; Amino acid transporter	781	9	9	87785.76	8.43	0.301
13	*MdHAK5.5*	XP_008369600.1	11	K_trans; K^+ transporter	789	4	9	88555.36	8.58	0.281
14	*MdHAK5.6*	XP_008390229.2	11	K_trans; K^+ transporter	789	13	9	88126.25	8.76	0.264
15	*MdHAK5.1*	XP_008366560.1	12	PotE; K_trans;	799	11	8	89742.59	7.65	0.336
16	*MdHAK5.2*	XP_008350111.1	9	K_trans; K^+ transporter	577	11	5	64904.66	8.32	0.286
17	*MdHAK5.3*	XP_070664292.1	12	PLN00151; K^+ transporter;	798	11	8	89903.56	6.22	0.251
18	*MdHAK1.2*	XP_008346085.2	12	K_trans; K^+ transporter	750	5	10	83839.80	8.72	0.2
19	*MdHAK5.4*	XP_008367107.1	12	K_trans; K^+ transporter	728	13	8	81440.59	8.15	0.301
20	*MdKEA2.1*	XP_008354171.1	10	K_trans; K^+ transporter	789	1	9	132405.5	5.11	0.273
21	*MdKEA2. 2*	XP_008339862.1	0	ERM; Ezrin/radixin/moesin; MttA_Hc f106	790	1	7	87592.29	5.10	0.276
22	*MdKEA6.1*	XP_008377516.1	10	Na_H_Exchanger; Sodium/hydrogen exchanger family	596	8	20	62527.20	5.63	0.221
23	*MdKEA4.1*	XP_008337684.1	11	Na_H_Exchanger; Sodium/hydrogen exchanger family	488	15	20	63938.01	5.49	0.261
24	*MdKEA3.1*	XP_008373236.1	0	Glutathione-regulated K^+efflux system	811	5	19	87830.58	5.57	0.256
25	*MdKEA3.2*	XP_008382240.1	0	Protein KefB; KefB; Kef-type K^+ transport system, TrkA_ N	634	10	19	68131.69	4.78	0.220
26	*MdKEA5.1*	XP_017189304.1	12	Tubulin; Tubulin/FtsZ family, GTPase domain	576	1	20	62070.12	6.33	0.332
27	*MdTPK4.1*	XP_008342353.3	5	Ion_trans_2	397	17	2	44031.66	8.57	0.263
28	*MdTPK4.2*	XP_008380206.1	5	Ion channel	400	9	2	44623.96	8.56	0.273
29	*MdTPK6.2*	XP_008371571.2	5	EF-hand_7; calcium binding motif	429	5	3	47869.23	8.56	0.230
30	*MdTPK1.1*	XP_008345013.1	5	Ion_trans_2	352	3	5	38615.98	7.48	0.301
31	*MdTPK1.2*	XP_008363812.1	5	EF-hand_7	353	3	4	38814.28	7.48	0.281
32	*MdTPK5.1*	XP_008343215.3	5	EF-hand_7	405	17	2	45383.63	8.61	0.264
33	*MdTPK6.1*	XP_008383509.1	5	EFh; EF-hand,	430	10	2	47899.66	8.92	0.336
34	*MdTPK1.3*	XP_008370978.1	5	Voltage-dependent K^+ channel	369	5	4	41336.37	5.48	0.286
35	*MdSKOR1.1*	XP_070660809.1	5	CAP_ED; Voltage-dependent K^+ channel	773	9	13	88290.70	6.84	0.251
36	*MdSKOR1.2*	XP_008343075.1	5	ANK; ANK repeat; CAP_ED	841	17	14	96118.35	6.23	0.236
37	*MdAKT1.2*	XP_008352270.1	5	CAP_ED; Ion_trans_2	874	15	11	97680.30	7.08	0.301
38	*MdAKT6.2*	XP_008394035.1	3	Crp; cAMP-binding domain	891	15	12	100692.6	6.96	0.301
39	*MdAKT6.1*	XP_008392136.1	3	cNMP; Cyclic nucleotide- monophosphate	890	14	12	100778.5	7.86	0.453
40	*MdKAT3.1*	XP_008393996.1	6	Crp; CAP_ED; CAP_ED; CooA, a heme containing CO	620	15	13	71907.09	8.71	0.287
41	*MdKAT3.2*	XP_008365179.1	6	CAP_ED; ANK	443	8	8	51568.93	9.57	0.271
42	*MdAKT1.1*	XP_008362916.1	6	Ion_trans; Ion channel	597	2	10	67789.21	9.00	0.250
43	*MdAKT2.1*	XP_017189417.1	6	Voltage-dependent K^+ channel;	836	8	10	95072.10	8.84	0.272
44	*MdKAT2.3*	XP_008381130.1	5	FNR (fumarate and nitrate reduction)	761	9	11	87151.68	6.37	0.261
45	*MdKAT2.1*	XP_008349191.1	4	Crp; CAP_ED	568	10	10	65246.28	5.99	0.361
46	*MdKAT2.2*	XP_008348556.1	4	Kef-type K^+ transport system, TrkA_N	478	9	8	55501.27	8.64	0.119
47	*MdAKT6.3*	XP_008394036.1	3	CAP_ED; Ion_trans_2	880	15	12	99472.23	6.95	0.223

Table 1. K^+ transporters and K^+ channels for genetic information i.e. Domains, exons, length, chromosome number, molecular weight (MW), isoelectric point (PI), and hydropathicity (Gravy).

classified as MdHKT1.1, as it has a conserved motif S-G-GG-G in which serine was characterized in the first position. This motif was specifically present in HKT family (Fig. 1B)[58].

There were seven KEA members of *M. domestica* (Table 1). These transporters were named according to their close relatives from *A. thaliana*. In Apple, this family has proteins with lengths ranging between 574 and 1198

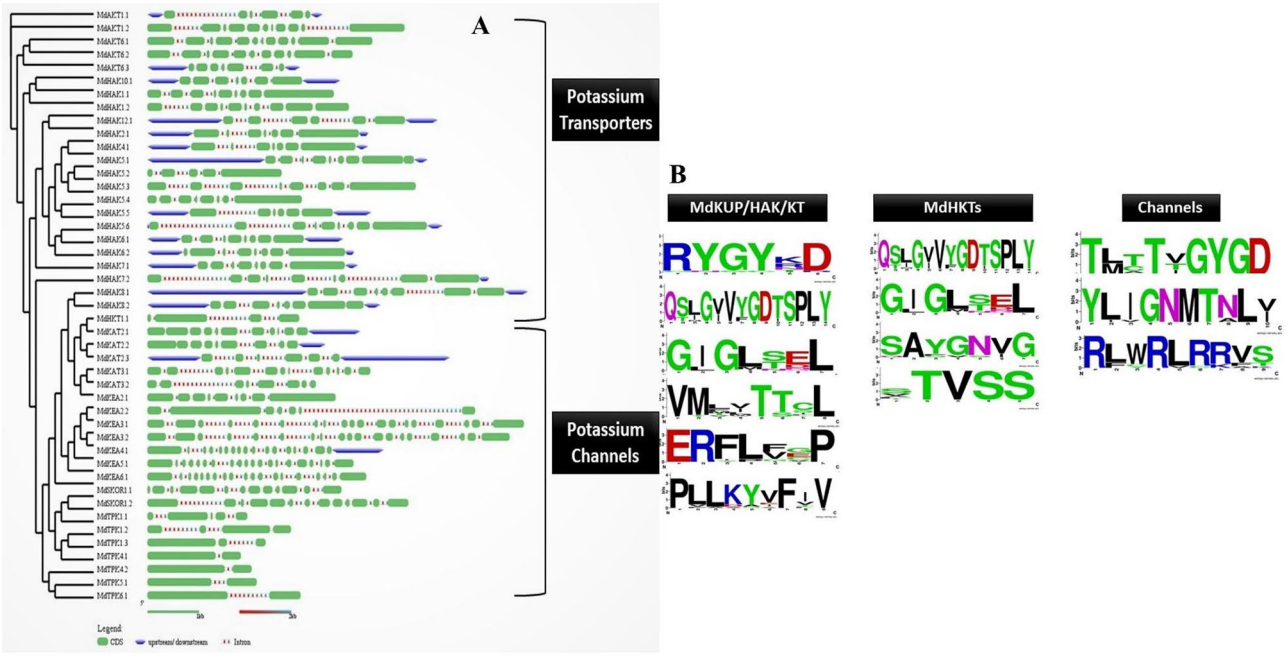

Fig.1. Gene structure and conserved motif analysis of potassium transporters and channels. (**A**) Gene structure analysis. In this figure, a genomic pattern of all transporters & channels of M. domestica has been drawn, which shows the total number of exons and intron parts of all genes relative to their phylogeny relation. (**B**) Sequence logo depicting conserved amino acids in sequences in K+ transport-related proteins.

AAs. Genomic analysis of apples suggested that almost 12–15 exons per gene were present in this gene family (Fig. 1A)[59].

The 21 K^+ channels were characterized in *M. domestica*, out of these, six genes were voltage-gated channels, eight were tandem pore K^+ channels (TPK), five were K^+ inward rectifier channels (Kir), and two were SKOR genes (*MdSKOR1.1* and *MdSKOR1.2*)[60]. At the molecular level, these channels were first studied and reported in *A. thaliana*[61]. Experiments showed that there were six shaker channels in *M. domestica*. Their proteins range between 618 and 892 AAs. The gene structure possessed 9–14 exons (Fig. 1A). Five Kir-like channels and eight TPKs were identified in *M. domestica* genome. Kir-like channel proteins have one hydrophobic core and one domain, while TPKs possess almost 3 transmembrane domains with a single hydrophobic core[8]. Conserved regions/motifs 'RSXpSXpx' were observed. A highly conserved sequence was revealed among all TPKs of *M. domestica*. The protein length of this family ranges between 365 and 401 AAs, and the number of exons is 9 to 11 (Fig. 1A)[4].

Conserved motif analysis by MEME and genomic structure analysis by GSDS

Multiple sequence Alignment was performed by CLUSTAL OMEGA and then represented using WebLogo3. All sequences were aligned, and detailed information about their highly conserved residues was obtained. Conserved motifs from K^+ transporting proteins were identified across the various organisms, i.e., *O. sativa*, *G. max*, *A. thaliana*, *C. arietinum*, and *M. domestica*. These were the most conserved motifs from MdKUP/HAK/KT, MdHKT1.1, and K^+ channels, respectively (Fig. 1B). Exons and introns in all K^+ channels and transporters showed conserved regions among members of subfamilies in different organisms. To find out the overall structural integrity in K^+ transporters and channels in *M. domestica*, the introns and exons configuration was observed[62,63]. In *Malus domestica*, *MdAKT1.1*, *MdAKT1.2*, and *MdAKT1.3* (the Shaker family channel genes) share the same gene structure patterns, including intron and exon patterns and locations. However, *MdAKT1.1* is distinguished by having longer introns and a unique feature not present in the other family members. It could be hypothesized that almost all genes from each family were closely related to their relatives from different organisms, but in the same family as *A. thaliana* and *O. sativa*[64]. It has been predicted that all 47 genes involved in PTS in *M. domestica* have the same features as other genes from different plants' PTS. Reported K^+ transport-related genes exhibit high specificity in their activities, dictated by their locations and structures, yet they primarily serve a singular function: transporting and distributing K^+ ions from roots throughout the entire plant[65]. (Fig. 1A).

Comparative phylogenetic analysis of *M. domestica* with different plants

Potassium transporters and channel protein sequences were utilized to investigate their phylogenetic and evolutionary relationships among *C. arietinum*, *M. truncatula*, *G. max*, *O. sativa*, *A. thaliana*, and *M. domestica*. K^+ channels were divided into both Shaker and TPK subfamilies in agreement with phylogenetic analysis. Shaker channels were categorized into subfamilies called AKT-like, KAT-like, and SKOR-like. In Arabidopsis and rice, AKT (*MdAKT1.1*, *MdAKT1.2*, *MdAKT1.3*, and *MdAKT2.1*) family members with their counterparts showed

a close relationship (Fig. 2). *MdAKT1.1* and *MdAKT1.2* indicated a paralogous relation in *M. domestica*. The *MdAKT1.1* and *MdAKT1.2* genes were duplicated through segmental duplication, indicated by calculations of divergence. In *M. domestica*, KAT family members *MdKAT1.1* and *MdKAT1.2* indicated a neighboring relationship with their counterparts (*MdKAT1.1* and *MdKAT2.1*). Since 52.90 million years ago, the evolutionary study demonstrated the duplication through segmental duplication. Around 40.14 million years ago, segmental duplication resulted in the emergence of two TPK family members, *MdTPK1*.1 and *MdTPK1*.2, within *M. domestica* genome. These duplicates exhibit a paralogous relationship within the apple genome and show co-orthologous and orthologous relationships with *OsTPK1.1/OsTPK2.1* from *O. sativa* and *AtTPK1.1* from *A. thaliana*, respectively. In apples, two members of the SKOR subfamily (*SKOR1.1* and *SKOR1.2*) were also identified, which exhibit a neighboring relationship with respective homologs in *O. sativa* and *A.thaliana* (*SKOR* and *GORK*) (Fig. 3).

Phylogenetic analyses classified K^+ transporters into three subfamilies: KUP/HAK/KTs, KEAs, and HKTs (Fig. 2). In *M. domestica*, only one member of the HKT subgroup, *MdHKT1.1*, was identified. This gene displayed a paralogous relationship within the species, originating from a tandem duplication event approximately 183.67 million years ago. *MdHKT1.1* also showed co-orthologous relationships with *OsHKT1.4/OsHKT1.5* and *AtHKT1.1* with 47.60% and 46.22% identity, respectively, compared to *AtHKT1.1*. Additionally, 18 KUP/HAK/KTs were identified from *M. domestica* genome, revealing a strong relationship with KUP/HAK/KTs from *O.*

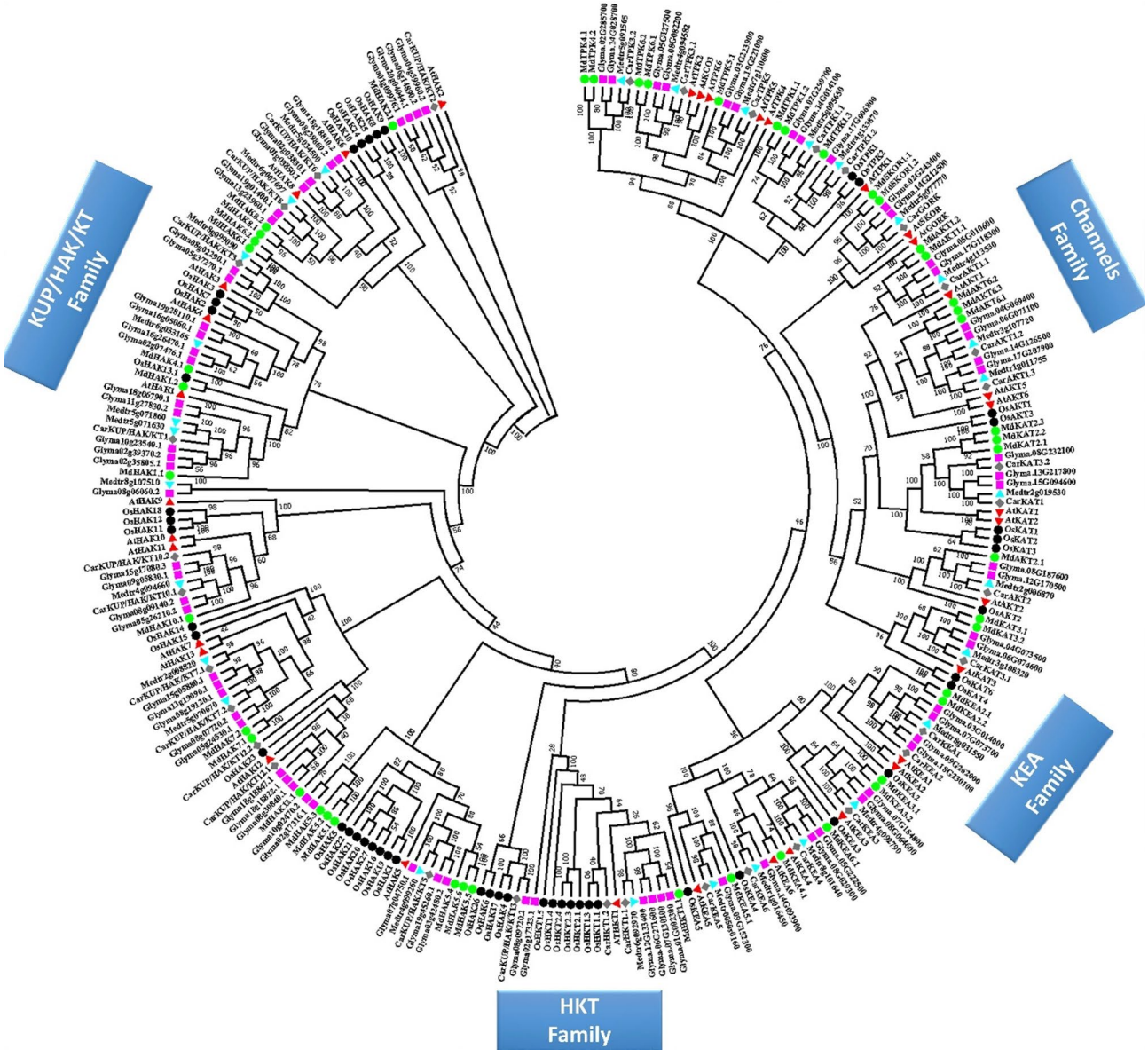

Fig. 2. Phylogenetic tree of Potassium transporters and channels in monocots and dicots. This tree is created by MEGA7, this tree gives us detailed evolutionary information about each member of these 47 K^+ transporting genes in *M. domestica*, all families are distinguished with different shape and color markers to make this tree more understandable (Neighbor-joining analysis) (Kumar et al.[41]).

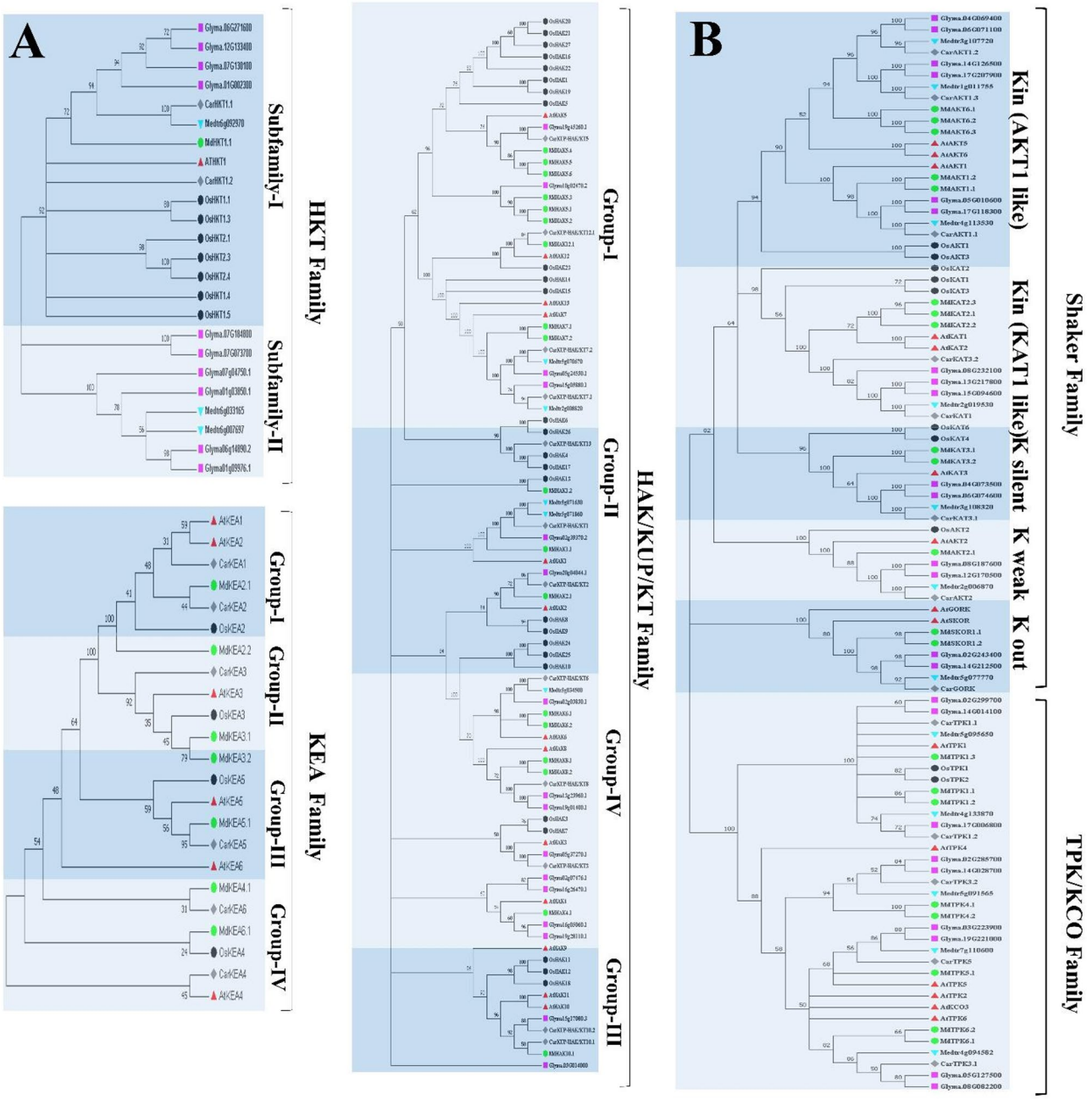

Fig. 3. Phylogenetic evaluation of potassium transporters (**A**) and Channels (**B**) in monocots and dicots. The phylogenetic relationships of K^+ transporters in *G. max*, *O. sativa*, *A. thaliana*, and *M. domestica* were analyzed. Phylogenetic trees were constructed for the HKT, KEA, and HAK/KUP/KT transporter families. These trees were derived using the neighbor-joining method (1000 bootstrap) based on the alignment of protein sequences. Evolutionary analyses were conducted using MEGA7 (Kumar et al.[41]).

sativa and *A. thaliana*. Among them, *MdKUP/HAK/KT8.1* and *MdKUP/HAK/KT6.1* exhibited a paralogous relationship, sharing 44.13% identity within *M. domestica* (Fig. 3). The 163.37 MYA (million years ago), *Md KUP/HAK/KT8.1* and *MdKUP/HAK/KT6.1* were duplicated through tandem duplication. *MdKUP/HAK/KT6.1* demonstrated a 73.16% identity with its ortholog *AtKUP/HAK/KT6.1* in Arabidopsis, the highest identity rate observed among paralogous relationships. Similarly, *MdKUP/HAK/KT8.1* showed the highest identity of 75.12% with its ortholog *AtKUP/HAK/KT8.1* in Arabidopsis. Around 138.5 MYA, *MdKUP/HAK/KT3.1* and *4.1* developed a paralogous relationship due to tandem or segmental duplication. *MdKUP/HAK/KT3.1* shares an orthologous relationship with *AtKUP/HAK/KT3.1*, while *MdKUP/HAK/KT4.1* exhibits a close relationship with its homolog, showing 76.3% identity. *MdKUP/HAK/KT5.1* and *5.3* likely arose from tandem or segmental duplication approximately 178.03 MYA, with *MdKUP/HAK/KT5.1* also showing an orthologous relationship with *AtKUP/HAK/KT5.1*.

Segmental duplication around 67.86 MYA led to a paralogous relationship between *MdKUP/HAK/KT10.1* and *10.2*. Similarly, *AtKUP/HAK/KT9.1*, *10.1*, and *11* share a co-orthologous relationship with their counterparts. *MdKUP/HAK/KT10.1* exhibits a maximum identity of 67.31% with *AtKUP/HAK/KT10*. The *MdKUP/HAK/KT12.1* and *12.2*, resulting from segmental duplication about 30.54 MYA, show a co-orthologous relationship with AtKUP/HAK/KT12. *MdKUP/HAK/KT7.1* and 7.2, originating from segmental duplication around 40.13 MYA, exhibit a close relationship with *AtKUP/HAK/KT7.1*, sharing 70.19% identity.

In *M. domestica*, there are 7 putative members (*MdKEA1.1* to *MdKEA6.1*) of the KEA family, which show a neighboring relationship with their counterparts in *O. sativa* and *A. thaliana*. The *MdKEA1.1* and *2.1* exhibit a paralogous relationship, evolving 32.68 MYA through segmental duplication. *MdKEA1.1* shows a high identity rate of 74.4% and a co-orthologous relationship with *AtKEA1.1* and *2*. The *MdKEA2.1* indicates an orthologous relationship with *OsKEA2*. Similarly, *MdKEA6.1* and *MdKEA4.1* show 60.23% identity with each other, considered paralogous, while *MdKEA4*.1 exhibits an orthologous relationship with *AtKEA4*.1 and *MdKEA6*.1 with *AtKEA6*, showing 70.2% and 71.9% identity, respectively. Evolutionary analysis indicates close relationships among K^+ transporters and channels in *M. domestica*, *A. thaliana*, and *O. sativa.*

Consequently, the K^+ transporters and channels evolved as a result of segmental duplication revealed by the analysis of duplication events. Analysis of the whole-genome sequence also revealed that a notable 71% of annotated apple genes underwent duplication following the split from legumes, *A. thaliana*, and *V. vinifera*, supporting our hypothesis of gene duplication in *M. domestica*. This tree gave the information about the evolutionary pattern of the same transporters and channels in different organisms and a conserved pattern of phylogeny was observed in different organisms (Fig. 2).

Mutation analysis and chromosomal mapping by map-chart

The synonymous and non-synonymous mutations in all 47 genes of (protein or nucleotide) sequences from *M. domestica* were evaluated. There were almost (Supplementary File 1). The chromosomal map defined the locations for all genes in detail (Fig. 4A). A significant correlation among mutations was revealed in a graphical map (Fig. 4B).

Cis-regulatory detection by plant-care database

Cis-regulatory elements are the sites in the upstream regions of transcription start sites (TSS) of genes where transcription factors bind and initiate transcription. In this analysis, some shakers and HKTs sequences found putative cis-elements. To gain insights into tissue-specific and functional regulation of K^+ transport-related genes in *M. domestica*, we screened promoter regions (1000 bp upstream ATG) of these genes for *cis*-regulatory elements (CREs). Analysis revealed several potential CREs in the promoter sequences of both K^+ channels and transporters, including ABRE, NAC Core motif, W-box, GT-1 motif, MYB/MYC recognition site, and G-box. These findings provide valuable information about gene regulatory networks associated with potassium transport in *M. domestica*. Furthermore, *cis*-elements associated with plant response to CO_2, light signals, and K^+ were also identified (Table 2).

Whole transcriptome-based expression profiling

Based on the *cis*-regulatory element analysis, there was a need to further explore the molecular mechanism of potassium transport-related genes in *M. domestica.* Therefore, RNA-seq expression profiling of these genes was performed. Two different transcriptomes were chosen to investigate gene expression. Mostly potassium channels in both experiments were known to be up-regulated. *MdKEA2.1* was known to be the most upregulated gene in both experiments. Many potassium transporters including members of the HKT-family and KT-family were observed to be downregulated in both experiments (Fig. 5).

Gene expression analysis

From *M. domestica* leaf tissues, RT-qPCR was used to estimate the transcript abundance of all 12 chosen potassium-transporting genes. Apple plant when exposed to drought, these stimuli affected the potassium transporting gene family's expression, and alterations in expression resulted in stress tolerance. In response to drought, 12 genes, including *MdAKT6.2*, *MdKEA3.2*, *MdSKOR1.1*, *MdTPK1.2*, *MdTPK4.1*, *MdTPK5.1*, *MdTPK6.2*, *MdHAK1.2*, *MdHAK10.1*, *MdHAK2.1*, *MdHAK5.3 and MdHAK5.6*, were chosen for RT-qPCR-based quantification. Differential regulation of *MdKEA3.2* was seen during drought stress. In reaction to drought stress, the expression of *MdSKOR1.1* was shown to be strongly elevated (up to 3-fold change), but it was found to be decreased under drought stress. Interestingly, *MdHAK5.3* was found to be upregulated under salinity stress conditions, whereas during drought, it is downregulated. It was shown that *MdHAK1.2* was significantly downregulated under salt stress and increased in response to drought (up to a 2-fold change). It was discovered that *MdTPK5.1* was significantly increased in drought conditions (Fig. 6). When compared to the control, *MdTPK6.2* was shown to be downregulated under drought (Fig. 6).

Discussion

As the role of K^+-channels/transporters is well documented in plants, i.e., plants need K^+ in a sufficient quantity for its role in photosynthesis and membrane potential stabilization[64]. K^+ also has a role in plant turgidity level maintenance and cytosol pH level normalization. So, there must be a proper system that involves the K^+ uptake from the soil and its distribution among plant organs[30]. Current study describes the K^+ transport system in Apple and analyzed the K^+ distribution in *M. domestica* plant and the genes involved in this process. A genome-wide analysis was performed for structural and functional characterization of these genes. It has been observed that there is a well-developed system for K^+ transport in *M. domestica* which comprises total 47 genes (26 transporters: 18 HAK/KUP/KTs, 7 KEAs, and 1 HKT) and 21 K^+ channels (2 SKOR, 5 AKT, 6

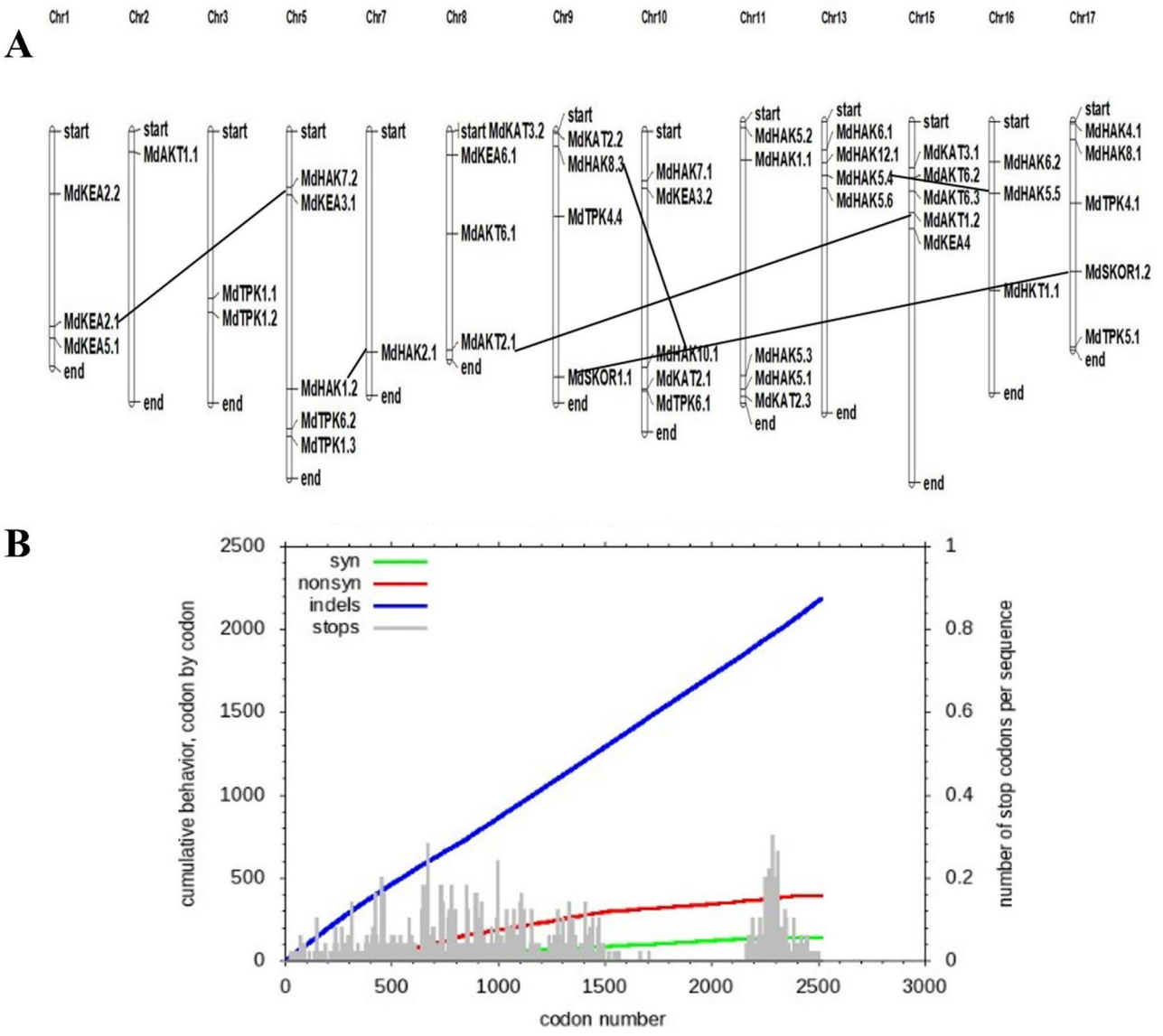

Fig. 4. Map chart of genes involved in Potassium transport system in *M. domestica* (**A**). This diagram represents all gene locations for transporting genes from *M. domestica*. This map is drawn by Map Chart. Mutation analysis of *M. domestica* potassium transport system (**B**). This graph showing us the graphical representation of the above-mentioned table in which the blue line indicating insertion-deletion substitutions, green for synonymous and red for non-synonymous mutations, and gray bars displaying stop codon diversity.

Shakers and 8 TPKs)). The study revealed that orthologous genes exhibit a highly conserved intron and exon position across significant evolutionary periods, whereas paralogous genes show comparatively lesser but still discernible conservation in their intron/exon structures. In exploring the structural diversity of K^+ transporters and channels in *M. domestica*, we analyzed exon and intron organization. This analysis unveiled substantial diversity in the number of exons observed across these genes, ranging from 2 to 24 exons. Interestingly, within specific subfamilies, there was noticeable similarity in gene structure among members, including consistent patterns in intron numbers, exon lengths, and intron phases. For instance, members of the shaker family such as *MdAKT1.1*, *MdAKT1.2*, and *MdAKT1.3* exhibited nearly identical intron/exon patterns, except for *MdAKT1.1*, which featured longer introns compared to the others. This structural conservation within subfamilies suggests functional constraints that may have influenced the evolution of these genes in *M. domestica*.

The comparative study was also performed for the K^+ transport channel genes identified in this study and the previously reported genes from different plants. As *AtGORK* and *AtSKOR* in *A. thaliana*, were closely related to inward rectifier genes of *M. domestica* (*MdSKOR* and *MdSKOR1*). All of the mentioned genes from both plants were K^+ transport channel genes[5,7]. Hence, it could be stated that both *MdSKOR* and *MdSKOR1* were involved in the long-distance transfer of K^+ from roots to shoots on *M. domestica* and also involved in stomata movements[68]. The similar conserved domains of the Shaker family of channels from *M. domestica* and *A. thaliana* were

Regulatory element	Core sequence	MdAKT1.2	MdAKT6.3	MdKAT3.2	MdAKT1.1	MdSKOR1.1	MdKAT2.1	MdAKT6.3	MdKAT3.1	MdHKT1.1	Functions
NAC core sequence	CACG	2	1	1	1	1	2	3	1	1	Response to various stress
	CGTR	2	1	2	4	-	6	-	-	11	
ABRE	ACGTG	1	8	3	2	2	4	2	-	4	Response to ABA signals
MYB recognition site	WAACCA	1	-	32	1	4	2	13	18	10	Response to drought stress and ABA signals
	YAACKG	3	5	3	2	13	1	4	2	1	
MYC recognition site	CANNTG	8	1	10	3	6	10	12	8	2	Response to drought, ABA and cold signals
I-box	GATAA	2	3	6	2	4	6	6	12	2	Response to light signals
G-box	CACGTG	1	1	2	8	18	1	4	2	4	Response to ABA and water deficit
TAAAG motif	TAAAG	1	4	4	6	1	6	4	2	3	Response to K^+ influx channel of guard cells
EEC	GANTTNC	-	1	4	3	-	3	1	10	2	Response to CO_2 signals

Table 2. *Cis*-regulatory elements from K^+ transporters and K + channels are displayed in this table; different core sequences and their abundance is also mentioned.

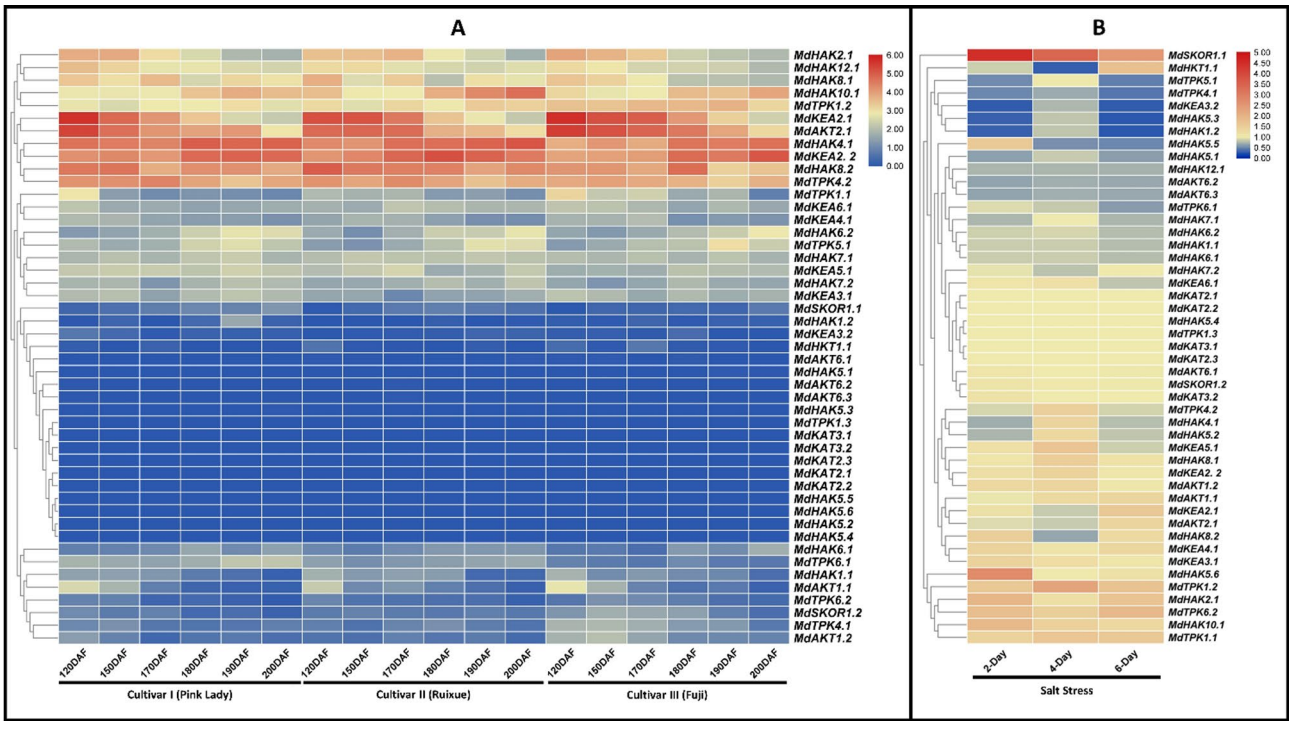

Fig. 5. Transcriptome expression profiling. Representation of all potassium transporting genes in *M. domestica* and their respective RNA-seq expression, (**A**) Different developmental stages of fruits in multiple cultivars, (**B**) Salt stress response in leaf tissues.

revealed. It was further validated by the Phylogenetic tree of all members of PTS from different plants i.e., *G. Max*, *A. thaliana*, and *O. sativa*. These results showed that PTS of *M. domestica* is identical to other plants[4,55,69,70].

Whole-genome analysis of *M. domestica* identified 8 TPK channel transport genes, whereas *A. thaliana* has 5 TPK genes. In *M. domestica*, 6 Kir channels were also discovered and initially classified as a separate group. However, later studies indicated that these Plant Kir-like channels are similar to TPKs in *A. thaliana*. The G-Y-G motif, highly conserved, has been recognized as the signature feature of these channels in *M. domestica*[71]. Genes from the KT/KUP/HAK family have been identified in a wide range of plants beyond the model species *A. thaliana*. In current study, we characterized 18 transporters from this family in *M. domestica*. The sequences of these genes exhibit high similarity to their counterparts in *A. thaliana*, indicating conserved functional roles.

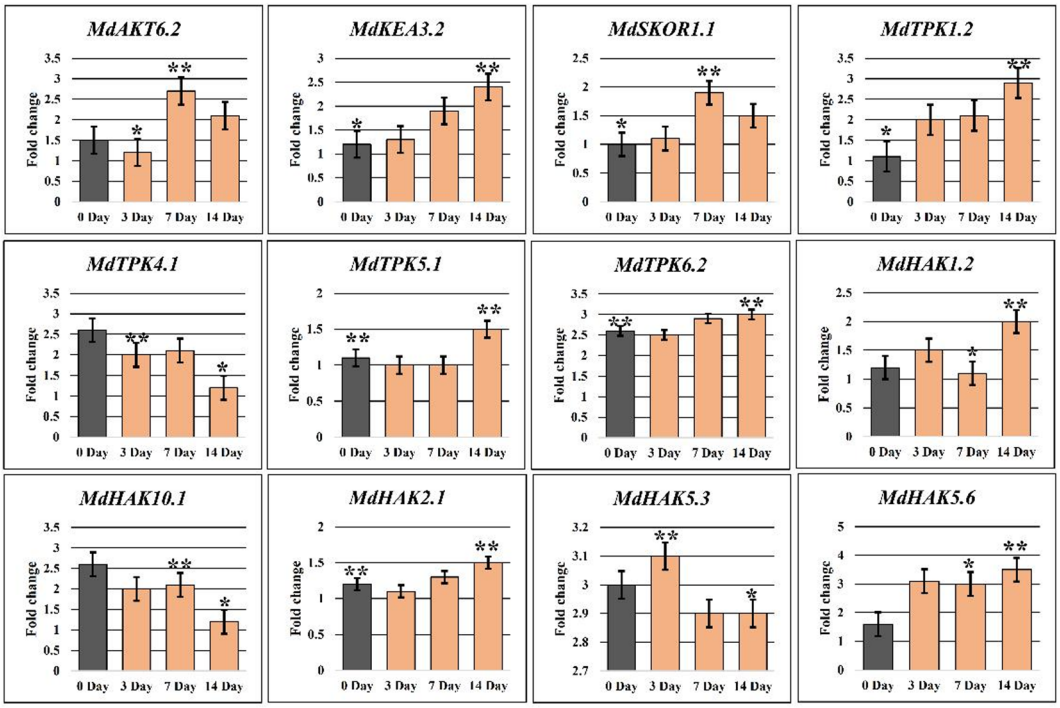

Fig. 6. RT-PCR results. To assess the response of the potassium transporting gene family to drought stress, relative RT-qPCR was conducted. The experiment was performed in triplicate to ensure robustness, and untreated plants served as the baseline with a default expression value set to 1 for each gene. Error bars are included on each column to represent the standard error. Statistical significance (Student's t-test) is denoted by asterisks: * indicates differences with $p < 0.05$, while ** indicates extremely significant differences with $p < 0.001$ for physiochemical measures between environmental stressors and the control.

These genes share identical domain patterns, including the presence of conserved motifs essential for potassium transport. The high degree of sequence and structural conservation suggests that these transporters play a crucial role in maintaining potassium homeostasis across different plant species[72]. Nonetheless, in *M. domestica*, they have 14 domains along with an extra domain for K^+ transportation, which plays a crucial role in its PTS. The MdKUP family containing K^+ transportation domain has been reported to play a significant role in K^+ transport[73]. Through phylogenetic analysis, the KT/KUP/HAK gene family was classified into four subfamilies. Within group I, members such as MdHAK5, MdHAK1, and MdHAK2 are known to be involved in K^+ transport in roots. This analysis revealed significant diversity in potassium transport systems across different plant species. For instance, OsHAK4 exhibited a notably low K^+ influx, showing a reduction of 70% in a 0.2 mM K^+ solution. This variation underscores the functional diversity and adaptation of potassium transport mechanisms among different plant species[74]. The *MdHKT1.1* gene observed in *M. domestica* is closely related to the members of the Trk channel family and is also involved in PTS. Multiple sequence alignment of this family showed that the substitution mutation occurred between both members, i.e., BG was replaced by BS, which changed its function from K^+ transfer to Na^+ transfer[75]. Further evidence of their involvement in Na^+ transportation is supported by the phylogenetic relationships of these genes with *OsHKT1.4* and *OsHKT1.5*. Previous studies have demonstrated that glycosylation influences membrane stability and morphology. N-glycosylation is a common feature observed in many plants, particularly in membrane-associated proteins[76]. In high salt concentrations, Na^+ starts to accumulate in roots of plants, which leads to enzymatic inactivity affecting the growth and development of plant. HKT genes help to stabilize this Na^+-K^+ gradient and enhance plant growth. IAA plays a key role in fruit development, sex determination, and cell elongation[77]. An Auxin-responsive element (motif) was identified in MdHKT1.1, which predicted that MdHKT1.1 was an Auxin-responsive gene. The binding of IAA to MdHKT1.1 can enhance the activity of MdHKT1.1 to stabilize the Na^+-K^+ gradient and enhance the growth of plants in high salt concentrations[79,80].

Conclusion

K^+ ion is well known and proven essential cation among plant nutrients. But unfortunately, previously its significance was not well studied, and this nutrient was given the very least attention by researchers as well as cultivators in Apple. The current study characterized the PTS in *M. domestica*. A total of 47 genes were found to be potentially involved in the K^+ transport system. Among them, 26 genes were K^+ transporters, and the remaining 21 genes were K^+ channels. The phylogenetic analysis, evolutionary studies, and the evaluation of the genetic structure of these genes characterized the PTS genes and provided a deeper insight into their evolutionary and regulatory mechanisms in *M. domestica*.

Data availability

All data is presented in the manuscript and supplementary files of the article furthermore, RNA-seq data and expression pattern data are available at BioProject: PRJNA728501 and BioProject: PRJNA645374, respectively.

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Author contributions

MW, HN, RZ and MARR performed analysis. RR, MN, and SL wrote manuscript. FA devised the main idea and supervised the research. AAA, HA, MJ, SF, NBSAS and FZ re-analyzed, literature review, editing and technical expertise to improve the revised the article. AAA, NBSAS, MJ, SF, MARR, HSR, AMR and FA critically revised, proof-read and edited the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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