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Identification and characterization of potassium (K^+) transporters in potato (*Solanum tuberosum* L.)

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ABSTRACT: Potassium (K^+) transporter genes have important roles in K^+ acquisition, allocation and signal transduction. The purpose of this study was to analyze the characteristics of the K^+ transporter genes/proteins and to study their expression profiles under K^+ deficiency condition in potato (*Solanum tuberosum*). Strict homology searches were used to find 33 K^+ transporter genes located on potato chromosomes 1 to 12. Gene features, protein features and subcellular Localization were analyzed, and 10 segment duplications were identified from these 33 genes. The functions of K^+ transporter genes were predicted by phylogenetic analysis and analysis of promoter sequences. After Potassium starvation, 12, 13 and 18 K^+ transporter genes were up regulated in roots, stems and leaves, respectively. In addition, the expression of *StHAK5*, *StHAK11* and *StKCO2* were up-regulated in potato roots, stems and leaves under phosphate deficiency. Our findings provide a comprehensive view of members of the K^+ transporter family involved in the response to K^+ starvation growth.

KEY WORDS: K^+ starvation, Potassium transporters, Promoter site, *Solanum tuberosum*.

INTRODUCTION

Potassium (K^+) is the most abundant cation in plants, and it is also one of the three major mineral nutrients that are necessary for plant growth, accounting for 2%–10% of the plant dry weight (Leigh and Jones, 1984). Potassium plays a series of important roles throughout the plant's entire growth and development period. For example, K^+ plays a key role in maintaining the balance between anions and cations, regulating osmotic pressure, promoting photosynthesis efficiency, acting as an activator of enzymes, regulating cell membrane polarization and other metabolic processes in various physiological and biochemical processes of plants (Amtmann *et al*., 2006; Anschütz *et al*., 2014; Schachtman and Shin, 2007). In addition, K^+ can also enhance plant resistance to biotic and abiotic stresses (Anschütz *et al*., 2014; Wang *et al*., 2013).

Potassium transporters can be divided into five families: Shaker potassium channel family, KCO outward potassium channel family, Trk/HKT potassium transporter family, KUP/HAK/KT potassium transporter family and K^+/H^+ antiporter family (Mäser *et al*., 2001). The Shaker potassium channel family includes both inward transporters and outward transporters and is responsible for many physiological activities such as potassium uptake, transport and stomatal movement. In *Arabidopsis*, *AKT1* is the first cloned plant inward rectifying K^+ channel gene, which is strongly expressed in roots and plays a critical role in the regulation of K^+ uptake by plant roots (Dennison *et al*., 2001), the outward rectifying potassium channel gene *GORK* regulates K^+ outward output when stomata are closed (Hosy *et al*., 2003). While *SKOR* and *AKT2* play a major role in potassium transport between roots and crowns. (Gajdanowicz *et al*., 2011; Gaymard *et al*., 1998; Lacombe *et al*., 2000).

The KUP/HAK/KT K^+ transporter family members have high similarity and are very diverse in evolutionary relationship with other transporters. Studies have shown that the K^+ transporter KUP/HAK/KT family is a high-affinity K^+ transporter (Gierth and Mäser, 2007; Grabov, 2007), which has an important relationship with maintaining K^+ balance and osmotic regulation in plants. For example, *AtKUP6* is regulated by SRK2E of the ABA signaling pathway, it can regulate osmotic regulation by regulating potassium balance in cells, and it is a key regulator of cell growth and response to drought in *Arabidopsis* (Osakabe *et al*., 2013). In tomato (*Solanum lycopersicum*), the expression of *LeHAK5* is affected by changes of the K^+ concentration and cell membrane potential, and the gene can promote K^+ uptake by plants at low K^+ concentrations (Nieves-Cordones *et al*., 2008).

The KCO outward potassium channel family is primarily responsible for the outward transport of potassium. The *KCO1* contributes to the outward rectification slow bubble ion current, while has no effect on the outward rectification fast bubble ion current in *Arabidopsis* (Schönknecht *et al*., 2002). The K^+/H^+ anti-transporter family which may be driven by vacuolar protons regulates the exchange of K^+ and H^+ and the entrance of K^+ and H^+ to the acidic environment of the vacuole.

In *Arabidopsis*, the Trk/HKT K^+ transporter family just has one member which is named *AtHKT1* and has a transport effect on Na^+ but not on K^+ (Uozumi *et al*.,

2000). The Trk/HKT K^+ transporter family genes are widely involved in plant stress tolerance (Very and Sentenac, 2003; Wang and Wu, 2013; Waters *et al*., 2013). Studies have found that plant salt tolerance is inversely related to Na^+ accumulation in buds, especially in cereal crops such as rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) (Kader and Lindberg, 2005; Tester and Davenport, 2003).

Potassium transporters have been categorized and described across many plant species. There are 35 and 50 K^+ transporter genes in *Arabidopsis thaliana* and rice, respectively (Amrutha *et al*., 2007; Mäser *et al*., 2001). In addition, K^+ transporter genes have also been studied in cotton (*Gossypium spp.*), tomato, poplar (*Populus trichocarpa*) and grape (*Vitis vinifera*) (Davies *et al*., 2006; Katharina *et al*., 2010; Nieves-Cordones *et al*., 2007; Ruan *et al*., 2001). K^+ plays an important role in the growth and development, yield, quality and stress resistance of potato. The analysis and identification of potato K^+ transporters will help to understand the mechanism of potato absorption and utilization of potassium, which is helpful for improving potato yield, quality and stress resistance. In this study, we carried out a research of the potassium (K^+) transporter families in potato, including the phylogenetic relationships, gene structures and protein features. The traits of the K^+ transporters were characterized by comparison with members of this gene family in *Arabidopsis* and rice. In addition, we used qPCR to characterize the expression levels of some members of the K^+ transporter family to well know about these proteins under K^+ deficiency condition. This study reports the identification expression patterns of K^+ transporters for the first time and provides an essential basis for study of the comprehensive functional genome of the K^+ transporter families in potato. The results have important theoretical value and practical significance for breeding a more efficient use of K^+, reducing the effects of K^+ deficiency on potato growth, development, and yield by using modern biotechnology.

MATERIALS AND METHODS

Plant materials and growth conditions

Potato (*Solanum tuberosum* L.) tetraploid cultivar "Diseree" was used in this study. Potato plantlets were cultured in MS medium (Liu *et al*. 2017) with 0.8% agar and 2% sucrose (pH 5.8) and kept under 16 h light/8 h dark at 22 ± 1°C. Then potato plantlets were treated by following conditions:

One-month-old plantlets of uniform size were then transferred into plastic pots (12 cm × 12 cm) filled with vermiculite media (without K^+). Two-hundred mL modified Hoagland's nutrient solution (1 mM NH_4NO_3, 1 mM $NaH_2PO_4 \cdot H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 50 μM H_3BO_3, 50 μM $MnSO_4 \cdot H_2O$, 15 μM $ZnSO_4 \cdot 7H_2O$, 0.05 μM $CuSO_4 \cdot 5H_2O$, 0.05 μM $CoCl_2 \cdot 6H_2O$, 3 μM $Na_2MoO_4 \cdot 2H_2O$, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$ and 50 μM Fe-Na-EDTA, pH 6.5) (Cellier *et al*. 2004; Hoagland and Arnon 1950) with K^+ (1 mM KNO_3) and without K^+ was irrigated to each pot a week for up to 40 days.

We subsequently sampled root, stem and leaf tissues from the same parts of these plants with a weight of 100mg. All samples were flash frozen in liquid nitrogen and stored at -80°C prior to utilization.

Identification of K^+ transporter genes in potato

Two methods were used to identify K^+ transporters in potato: 35 *Arabidopsis* K^+ transporter protein sequences (Mäser *et al*., 2001) were used as queries to perform a protein search against the database of *Solanum tuberosum* proteins with a strict *E* value ($<1e^{-10}$) requirement. All candidate K^+ transporter protein sequences were quite strictly screened by using the Conserved Domain Database (CDD) of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/cdd/) and Multiple Em for Motif Elicitation (MEME; http://meme-suite.org/tools/meme).

Additionally, we used *Arabidopsis* K^+ transporters AtKUP1 (AT2G30070), AtAKT1 (At2g26650), AtKCO1 (At5g55630), AtHKT1 (AT4G1031) and AtKEA1 (At1g01790) as queries to search (E value < $1e^{-10}$) (Amrutha *et al*., 2007). Then, PROSITE (http://www.ebi.ac.uk/InterProScan/) and Pfam databases were used to screened the candidate K^+ transporter proteins. All the proteins with greater than 25% identity, with at least one of the reference proteins used in the searches, only in case of K^+/H^+ antiporter family genes, the identity degree of 10% were accepted due to the conserved functional domains between this protein and the reference proteins.

All *Arabidopsis* K^+ transporter sequences were obtained from TAIR (http://www.arabidopsis.org/). Incorrect and redundant sequences were removed prior to further analysis of the remaining K^+ transporter sequences. In addition, all relevant sequences of identified potato K^+ transporter genes, such as genomic sequences, transcript sequences, and the chromosome location of each gene, were obtained from Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html) and PGSC database (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml).

Sequence analysis of K^+ transporter genes/proteins in potato

Physicochemical properties of the K^+ transporter proteins were calculated with the ProtParam tool (http://web.expasy.org/protparam/). The sub-cellular localization of K^+ transporter genes were predicted using the CELLO server (http://cello.life.nctu.edu.tw/). Exons

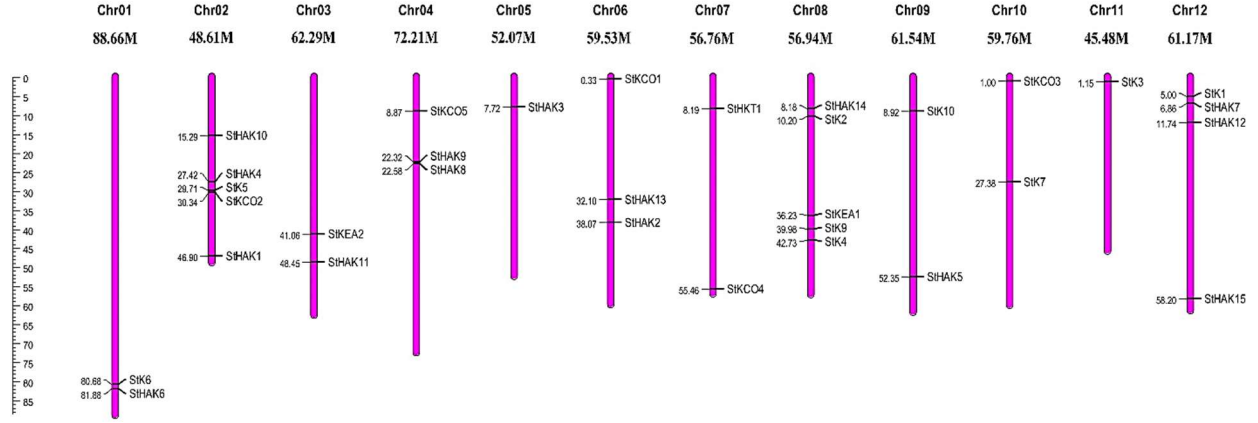

Fig. 1. Chromosomal locations of K^+ transporter family members in potato. Top numbers show the length of chromosomes. M: million.

and introns of all genes were analyzed using the Gene Structure Display Server (GSDS; http://gsds.cbi.pku.edu.cn/index.php). Both multiple amino acid alignments of the sequences and the construction of Neighbor-joining trees were conducted using Molecular Evolutionary Genetics Analysis (MEGA7) with the parameters of the Jones-Taylor-Thornton (JTT) model and 1,000 replicates for bootstrap analysis. The EvolView online tool (http://www.evolgenius.info/evolview/#login) was used to draw and manage the phylogenetic trees. We used MapChart to generate the map showing the position of the K^+ transporter genes in the chromosomes. Duplication events of K^+ transporter genes were analyzed following the methods of Gu *et al*. (2002) and using the Plant Genome Duplication Database (PGDD; http://chibba.agtec.uga.edu/duplication) and visualized using Circos (Gu *et al*., 2002; Krzywinski *et al*., 2009). *Ka* and *Ks* values were calculated by DnaSP software (Librado and Rozas, 2009). Promoter analysis was conducted by extracting 1500 bp upstream regions of K^+ transporter genes from Phytozome database and submitted to the PlantCARE database (Magali *et al*., 2002).

RNA extraction and real-time PCR analysis of K^+ transporter genes

The RNA simple Total RNA Kit (Cat. No. DP419, TIANGEN, Beijing, China) was used to extract RNA. The *ubi3* gene was selected as the reference gene. Specific primers were designed using Primer Premier 5 software. First-strand cDNAs were synthesized from 1μg RNA with the PrimeScript^{TM}RT reagent Kit (Code No. RR047A, TaKaRa, Dalian, China) in 20 μl reaction volume, including gDNA Eraser. Real-time PCR was set up on the basis of 2 × Plus SYBR real-time PCR mixture (Code No. PR7702, BioTeke, Beijing, China), and performed on CFX96^{TM} Real-Time System (BIO-RAD, California, USA) in a 10μl reaction volume. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Liu *et al*., 2016).

RESULTS

Identification, classification and protein features of the K^+ transporter family in potato

We identified and named 33 K^+ transporter genes with 51 transcripts (only primary transcripts were analyzed in this study) (Table 1). Analysis of subcellular localization indicated that 33 genes were all placed in the plasma membrane. These genes were divided into five types based on the gene structure, conserved domains and similarity to *A. thaliana*: KUP/HAK/KT transporter family (15 genes), KCO outward rectifier family (5 genes), Shaker K^+ channel family (10 genes), K^+/H^+ antiporter family (2 genes) and Trk/HKT transporter family (1 gene). With the exception of Shaker K^+ channel family in which the GRAVY value was negative (but the GRAVY value of StK10 was above 0), the members of other four families all had positive GRAVY values.

The members of KUP/HAK/KT transporter family included 8–10 exons with protein length of 745–849 amino acid residues (AAR) and about 83.24–94.55kDa protein molecular weight (PMW) (Table 1). KCO outward rectifier genes contained 2 exons with the lengths of 349–424 AAR and 39.04–46.98kDa PMW. Shaker K^+ channel genes consisted of 611–874 AAR and 69.74–98.50 kDa PMW. The majority of these genes contained 10–13 exons, but StK6 just had one. In addition, K^+/H^+ antiporter family was composed of 20 exons, included 577–599 AAR and 62.96–64.77 kDa PMW. While Trk/HKT transporter family only contained one member, which had three exons with 501 AAR and 57.03 PMW.

Chromosomal location and gene duplications of K^+ transporter genes

To better understand the genomic distribution of K^+ transporter genes, their approximate positions on each chromosome were marked. As shown in Figure 1, *S. tuberosum* K^+ transporters were distributed on chromosomes 01–12. Chromosome 08 had the maximum

Table 1. The gene and protein features of putative K^+ transporter family members in potato.

Gene features							Protein features				
Putative gene Name	Phytozome gene ID	Phytozome transcript ID	Family	Exon no.	Intron no.	Chr. location	Protein length (AA)	MW (kDa)	*pI*	GRAVY	Subcellular Localization
StHAK1	PGSC0003DMG400000501	PGSC0003DMT400001329*	KUP/HAK/KT	8	7	Chr02	772	87.02	8.03	0.374	Plasma Membrane
		PGSC0003DMT400001330									
StHAK2	PGSC0003DMG400002354	PGSC0003DMT400006056*	KUP/HAK/KT	4	3	Chr06	356	39.47	8.43	0.340	Plasma Membrane
StHAK3	PGSC0003DMG400004113	PGSC0003DMT400010525*	KUP/HAK/KT	8	7	Chr05	779	87.10	8.56	0.360	Plasma Membrane
StHAK4	PGSC0003DMG400010431	PGSC0003DMT400027038*	KUP/HAK/KT	8	7	Chr02	792	89.08	8.36	0.304	Plasma Membrane
		PGSC0003DMT400027042									
StHAK5	PGSC0003DMG400011388	PGSC0003DMT400029616*	KUP/HAK/KT	8	7	Chr09	745	83.24	8.66	0.426	Plasma Membrane
StHAK6	PGSC0003DMG400012585	PGSC0003DMT400032781*	KUP/HAK/KT	10	9	Chr01	849	94.55	5.92	0.309	Plasma Membrane
StHAK7	PGSC0003DMG400013739	PGSC0003DMT400035722*	KUP/HAK/KT	8	7	Chr12	784	88.24	8.86	0.177	Plasma Membrane
StHAK8	PGSC0003DMG400013819	PGSC0003DMT400035889*	KUP/HAK/KT	4	3	Chr04	494	55.58	8.80	0.318	Plasma Membrane
		PGSC0003DMT400035888									
StHAK9	PGSC0003DMG400013821	PGSC0003DMT400035898*	KUP/HAK/KT	9	8	Chr04	848	93.98	6.59	0.329	Plasma Membrane
		PGSC0003DMT400035897									
		PGSC0003DMT400035899									
StHAK10	PGSC0003DMG400017862	PGSC0003DMT400046034*	KUP/HAK/KT	8	7	Chr02	784	88.15	7.29	0.283	Plasma Membrane
		PGSC0003DMT400046031									
		PGSC0003DMT400046032									
		PGSC0003DMT400046033									
		PGSC0003DMT400046035									
StHAK11	PGSC0003DMG400019859	PGSC0003DMT400051127*	KUP/HAK/KT	9	8	Chr03	763	85.43	9.09	0.356	Plasma Membrane
StHAK12	PGSC0003DMG400020386	PGSC0003DMT400052541*	KUP/HAK/KT	9	8	Chr12	792	87.61	9.01	0.432	Plasma Membrane
StHAK13	PGSC0003DMG400024311	PGSC0003DMT400062466*	KUP/HAK/KT	9	8	Chr06	790	87.90	7.34	0.396	Plasma Membrane
StHAK14	PGSC0003DMG400025230	PGSC0003DMT400064965*	KUP/HAK/KT	9	8	Chr08	817	91.02	9.05	0.378	Plasma Membrane
StHAK15	PGSC0003DMG400029362	PGSC0003DMT400075514*	KUP/HAK/KT	9	8	Chr12	818	90.79	8.54	0.344	Plasma Membrane
		PGSC0003DMT400075513									
StK1	PGSC0003DMG400001066	PGSC0003DMT400002746*	Shaker	10	9	Chr12	874	98.50	7.06	-0.088	Plasma Membrane
		PGSC0003DMT400002748									
		PGSC0003DMT400002749									
StK2	PGSC0003DMG400009648	PGSC0003DMT400024968*	Shaker	11	10	Chr08	689	79.53	5.99	-0.153	Plasma Membrane
StK3	PGSC0003DMG400013243	PGSC0003DMT400034446*	Shaker	13	12	Chr11	828	94.66	6.45	-0.137	Plasma Membrane
StK4	PGSC0003DMG400014505	PGSC0003DMT400037608*	Shaker	13	12	Chr08	623	70.94	8.94	-0.052	Plasma Membrane
StK5	PGSC0003DMG400021091	PGSC0003DMT400054339*	Shaker	11	10	Chr02	688	78.86	7.00	-0.082	Plasma Membrane
		PGSC0003DMT400054340									
StK6	PGSC0003DMG400023769	PGSC0003DMT400061090*	Shaker	11	10	Chr01	824	94.26	6.54	-0.128	Plasma Membrane
		PGSC0003DMT400061091									
StK7	PGSC0003DMG400024168	PGSC0003DMT400062100*	Shaker	11	10	Chr10	842	96.74	6.21	-0.202	Plasma Membrane
		PGSC0003DMT400062099									
StK8	PGSC0003DMG400025678	PGSC0003DMT400065964*	Shaker	13	12	Chr00#	631	72.41	8.99	-0.009	Plasma Membrane
StK9	PGSC0003DMG400029083	PGSC0003DMT400074787*	Shaker	12	11	Chr08	861	96.73	7.11	-0.056	Plasma Membrane
StK10	PGSC0003DMG400040591	PGSC0003DMT400091020*	Shaker	1	0	Chr09	611	69.47	6.47	0.063	Plasma Membrane
StKCO1	PGSC0003DMG400007275	PGSC0003DMT400018768*	KCO	2	1	Chr06	379	42.40	6.51	0.131	Plasma Membrane
StKCO2	PGSC0003DMG400009702	PGSC0003DMT400025112*	KCO	2	1	Chr02	424	46.98	6.67	0.172	Plasma Membrane
StKCO3	PGSC0003DMG400014411	PGSC0003DMT400037347*	KCO	2	1	Chr10	349	39.13	5.64	0.192	Plasma Membrane
StKCO4	PGSC0003DMG400022284	PGSC0003DMT400057396*	KCO	2	1	Chr07	349	39.04	8.50	0.221	Plasma Membrane
StKCO5	PGSC0003DMG400023600	PGSC0003DMT400060672*	KCO	2	1	Chr04	353	39.61	5.73	0.178	Plasma Membrane
StHKT1	PGSC0003DMG400029966	PGSC0003DMT400077052*	Trk/HKT	3	2	Chr07	501	57.03	9.20	0.322	Plasma Membrane
		PGSC0003DMT400077053									
StKEA1	PGSC0003DMG400029945	PGSC0003DMT400076994*	K^+/H^+ antiporter	20	19	Chr08	599	64.77	7.60	0.541	Plasma Membrane
StKEA2	PGSC0003DMG400031029	PGSC0003DMT400079671*	K^+/H^+ antiporter	20	19	Chr03	577	62.96	7.14	0.652	Plasma Membrane
		PGSC0003DMT400079670									

The ID marked with "*" is the representative transcript of corresponding Gene. #: "Chr00" indicates that the gene is still not mapped in any chromosome in potato.

Table 2. Segmental and tandem duplication events of K^+ transporter paralogous pairs in potato.

Putative gene name	Phytozome transcript ID	Chromosome location	K^+ transporter family	Duplication type	*Ka*	*Ks*
StHAK3	PGSC0003DMT400034446*	5	KUP/HAK/KT	Segmental	0.1587	0.1607
StHAK8	PGSC0003DMT400065964*	4	KUP/HAK/KT			
StHAK4	PGSC0003DMT400037608*	2	KUP/HAK/KT	Segmental	0.1236	0.1115
StHAK10	PGSC0003DMT400091020	2	KUP/HAK/KT			
StHAK5	PGSC0003DMT400054339	9	KUP/HAK/KT	Segmental	0.5481	0.7066
StHAK7	PGSC0003DMT400062100	12	KUP/HAK/KT			
StHAK6	PGSC0003DMT400061090	1	KUP/HAK/KT	Segmental	0.5359	0.5081
StHAK9	PGSC0003DMT400074787	4	KUP/HAK/KT			
StHAK12	PGSC0003DMT400052541	12	KUP/HAK/KT	Segmental	0.1651	0.1587
StHAK14	PGSC0003DMT400064965	8	KUP/HAK/KT			
StK2	PGSC0003DMT400024968	8	Shaker	Segmental	0.2681	0.2182
StK5	PGSC0003DMT400054339	2	Shaker			
StK4	PGSC0003DMT400037608	8	Shaker	Segmental	0.2277	0.3057
StK8	PGSC0003DMT400065964	0	Shaker			
StK6	PGSC0003DMT400061090	1	Shaker	Segmental	0.1970	0.1618
StK7	PGSC0003DMT400062100	10	Shaker			
StKCO1	PGSC0003DMT400018768	6	KCO	Segmental	0.3300	0.9871
StKCO2	PGSC0003DMT400025112	2	KCO			
StKEA1	PGSC0003DMT400076994	8	K^+/H^+ antiporter	Segmental	0.3498	0.6598
StKEA2	PGSC0003DMT400079671	3	K^+/H^+ antiporter			

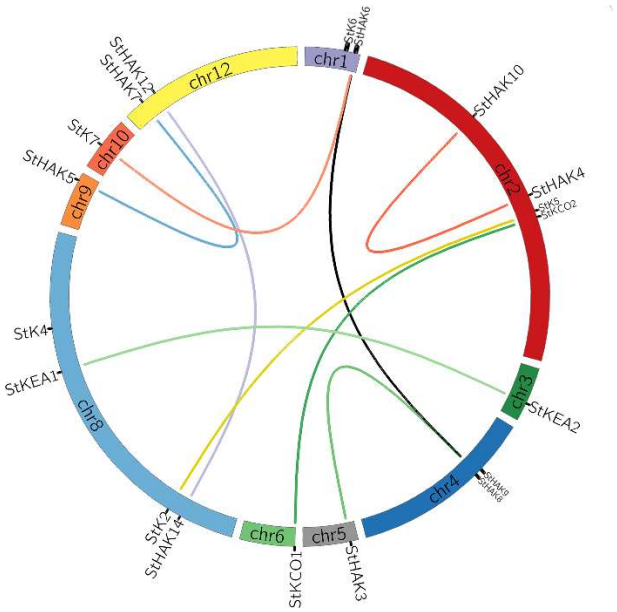

Fig. 2. Segmentally duplicated gene pairs in potato. A total of 33 K^+ transporter genes were unevenly located in 12 chromosomes. There were 10 segmentally duplicated gene pairs identified in the ***S. tuberosum*** genome. This gene pair, *StK4-StK8*, is not shown in the picture because *StK8* is still not mapped in any chromosome.

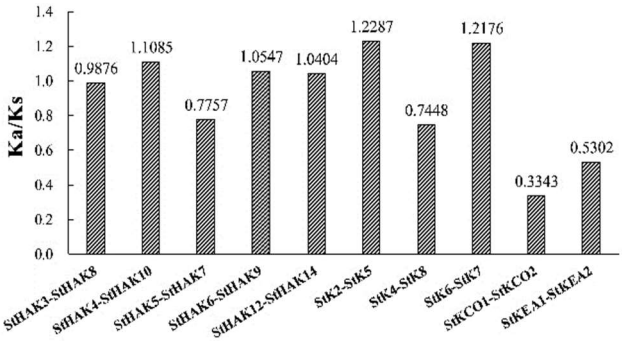

Fig 3. Divergence levels of K+ transporter genes in ***S. tuberosum***.

number, with five genes. Chromosomes 05 and 11 just contained one gene, respectively. Chromosome 02 and 12 included four genes. Chromosome 04 and 06 had three genes. And the other five chromosomes respectively included two K^+ transporter genes. It is worth noting that, *StK8* is still not mapped in any chromosome in potato, thus the gene was not shown in Figure 1.

Furthermore, to investigate the gene duplication events in potato, based on the phylogenetic tree (Figure S1) and the closely related paralogous pairs of K^+ transporter genes, ten gene duplication events were identified (Table 2, Fig. 2) (Liu *et al*., 2017). And then, we calculated the *Ka*, *Ks* and *Ka*/*Ks* of each gene pair to determine whether there was selective pressure on the gene pair. As shown in Figure 3, the result showed that half of the 10 gene pairs had *Ka*/*Ks* values greater than 1, and the other half had *Ka*/*Ks* values less than 1.

Phylogenetic analysis K^+ transporters in potato

We studied the K^+ transporter families in potato by comparative analysis of the phylogenetic distribution of K^+ transporter proteins in potato, rice and *A. thaliana*. This is an established method for examination of the structure and function of a protein family (Sze *et al*., 2014). Therefore, a phylogenetic tree was constructed by utilization of MEGA7 software with the 33 K^+ transporter proteins identified in potato, 35 proteins previously described in *A. thaliana* and 50 proteins of rice (*OsHKT1;2* was not shown since it is a pseudogene)

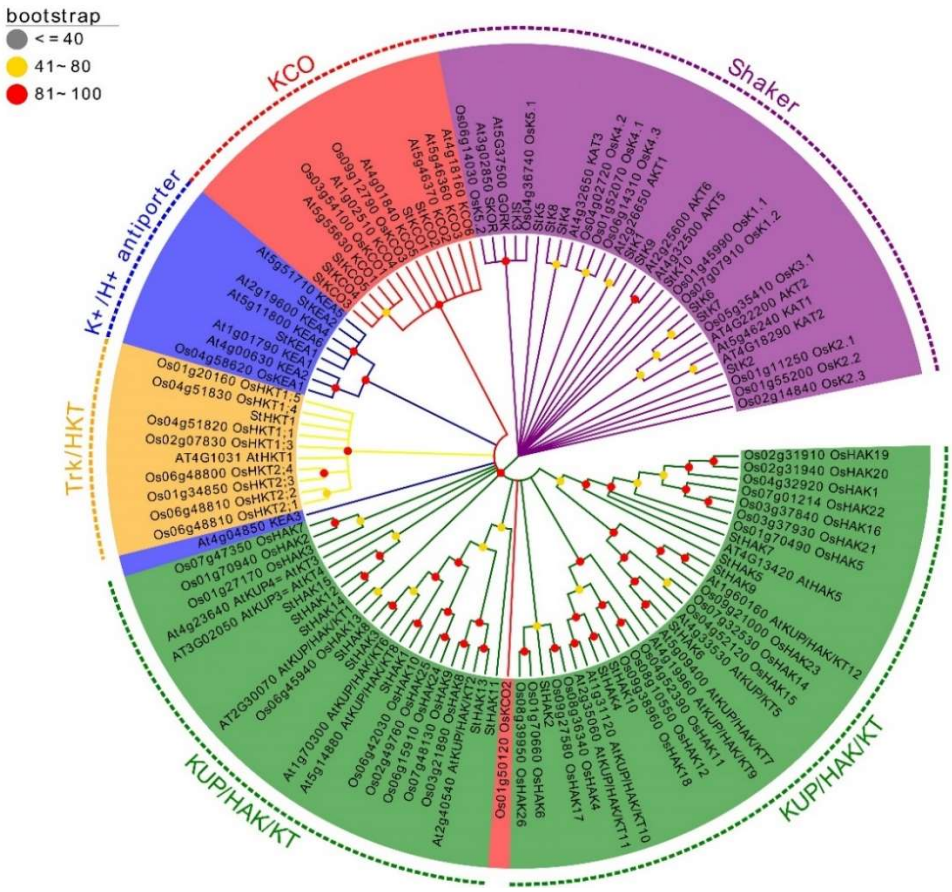

Fig. 4. Neighbor-joining tree for the K^+ transporter family in potato based on characterized K+ transporter genes in Arabidopsis and rice. Bootstrap values were calculated in 1500 replications by using MEGA 7. Analyzed K^+ transporter genes were distributed in four main groups, including Shaker potassium channel family (Shaker), KCO outward potassium channel family (KCO), Trk/HKT potassium transporter family (Trk/HKT), KUP/HAK/KT potassium transporter family (KUP/HAK/KT), and K^+/H^+ antiporter family (K^+/H^+ antiporter), which were marked with different colors. Prefix "St" indicates ***Solanum tuberosum*** (potato), "Os" indicates ***Oryza sativa*** (rice), and "At/AT" indicates ***Arabidopsis thaliana***. At the nodes, gray dots indicated that the bootstrap values were less than or equal to 40; yellow dots showed the bootstrap values were between 41 and 80; and red dots represented bootstrap values that were above 80 but not more than 100.

which were obtained from RiceData (http://www.ricedata.cn/gene/) (Fig. 4) (Amrutha *et al*., 2007). This phylogenetic analysis revealed that these sequences were distributed into five primary groups that were termed KUP/HAK/KT, KCO, Shaker, K^+/H^+ antiporter and Trk/HKT.

In addition, *KEA3* (At4g04850) and *OsKCO2* (Os01g50120) were not distributed in the corresponding clusters. *KEA3* belonged to K^+/H^+ antiporter family but was distributed between the KUP/HAK/KT cluster and the Trk-HKT cluster; *OsKCO2* belonged to KCO outward rectifier family but was distributed in the KUP/HAK/KT cluster. This suggests that although *KEA3* and *OsKCO2* belonged to K^+/H^+ antiporter and KCO outward rectifier families, respectively, they may be functionally closer to the members of KUP/HAK/KT family, especially *KEA3*, and may be also similar to the functions of Trk-HKT family members. However, the specific functions of *KEA3* should be study and determine by more experiment and data.

Promoter cis-elements analyses of K^+ transporter genes in potato

To understand the regulation of the identified K^+ transporter genes at transcriptional level, it is necessary to have insights on promoter region of genes in front of transcription start site (TSS). PlantCARE database was used to analyze the types of cis regulatory elements in K^+ transporter genes within a region 1500 bp upstream close to TSS. In this study, excluding unknown motifs, a total of 59 different cis elements were identified in upstream regions of 33 K^+ transporter genes. To understand these cis regulatory elements, a heatmap was constructed (Fig. 5).

As shown in Figure 5, these cis regulatory elements can be divided into four types: light-responsive elements, stress-responsive elements, hormone-responsive elements and the elements which related to other functions. Regulatory motifs such as CAAT-box and TATA-box were observed in all K^+ transporter genes, while some cis regulatory elements just existed in one K^+

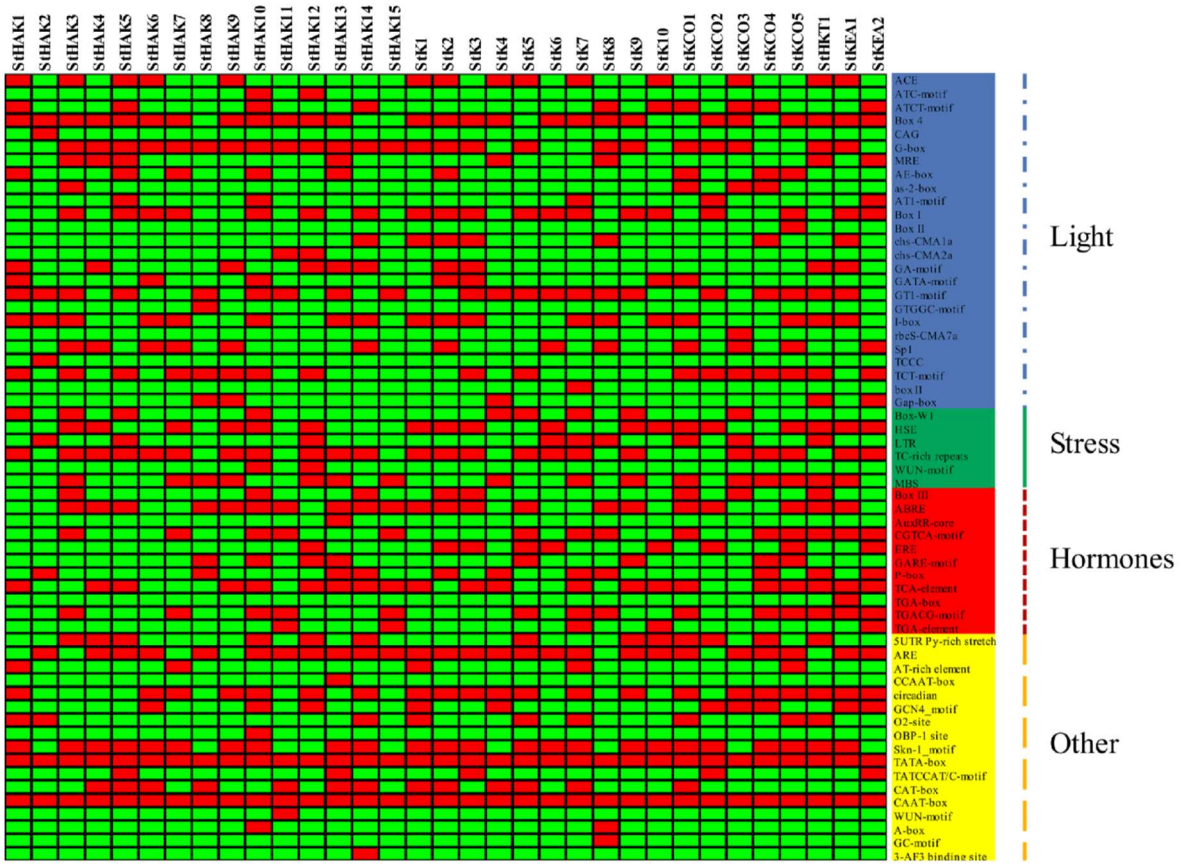

Fig. 5. Heatmap of cis-regulatory elements corresponding to the region 1500 bp upstream of 33 StPHT genes. Motifs were identified in the PlantCARE database. Red color shows the presence of cis regulatory motifs for a designated gene while green indicates the absence of specified motif for that particular gene.

transporter genes. For example, Box II just existed in StK7, WUN-motif just existed in StHAK11.

K^+ transporter expression levels under K^+ deficiency

To better understand the functions of K^+ transporter genes in relation to potassium uptake, the transcription patterns of 33 K^+ transporter genes from different families in the leaves, stems and roots of potato were examined in K^+-medium and K^+-free conditions. The details of specific primers are shown in Table S1.

The expression of 12 genes (*StHAK3*, *StHAK4*, *StHAK5*, *StHAK6*, *StHAK10*, *StHAK11*, *StHAK12*, *StKCO2*, *StK1*, *StK2*, *StK3* and *StK8*) were up-regulated in roots. The expression of 13 genes (*StHAK5*, *StHAK7*, *StHAK8*, *StHAK11*, *StHAK12*, *StHAK13*, *StHAK14*, *StKCO2*, *StKCO4*, *StK5*, *StK8*, *StK10* and *StHKT1*) were up-regulated in stems. The expression levels of 18 genes (*StHAK1*, *StHAK2*, *StHAK4*, *StHAK5*, *StHAK6*, *StHAK8*, *StHAK9*, *StHAK11*, *StHAK13*, *StHAK14*, *StKCO1*, *StKCO2*, *StKCO5*, *StK1*, *StK3*, *StK5*, *StK10* and *StHKT1*) were up-regulated in leaves (Fig. 6). In addition, the expression of three genes (*StHAK5*, *StHAK11* and *StKCO2*) were increased in potato roots, stems and leaves under phosphate starvation. These results suggest that these genes are likely to be involved in the K^+ uptake process which is important to the growth and development of potato.

DISCUSSION

Actually, 51 K^+ transporter transcripts were identified, but some transcripts were not the representative transcripts from corresponding genes. By sorting and analyzing, we finally identified and named 33 K^+ transporter genes with 51 transcripts. Then, a GRAVY value above zero indicates the protein is hydrophobic, while a GRAVY value below zero means the protein is hydrophilic (Drews *et al*., 2004), which suggests most Shaker K^+ channel genes were hydrophilic, but other family genes and StK10 were hydrophobic.

The largest gene family of K^+ transporters that KUP/HAK/KT transporter family was originally described in Bacteria (Schleyer and Bakker, 1993). The characteristic feature of KUP/HAK/KT transporters is the presence of consensus motif GVVYGDLGTSPLY (Rodríguez-Navarro, 2000). In addition, K^+/H^+ antiporters were first described from gram-negative bacteria (Munro *et al*., 2010). In this study, we identified two K^+/H^+ antiporter genes, which

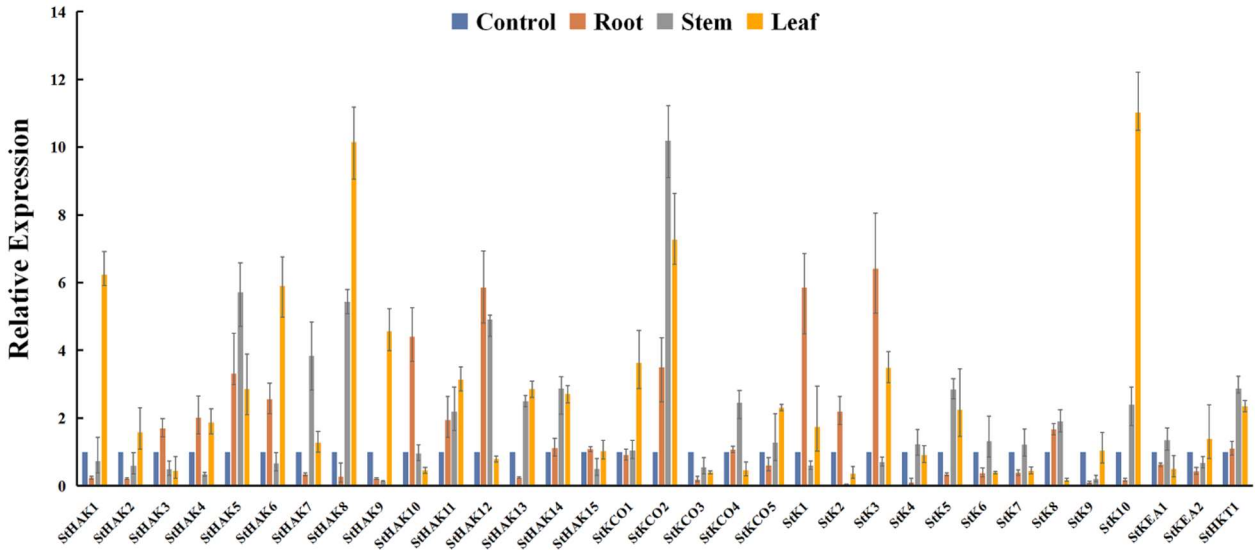

Fig. 6. Expression of 33 K+ transporter genes in potato leaves, stems, and roots at K+ starvation condition. X-axes are representative K^+ transporter genes and the y-axes are scales of relative expression levels. *ubi3* was used as the reference transcript. Root, stem and leaf tissues were sampled from the same parts of control and experimental plants. The control value is "1", so the control value of the roots, leaves and stems is represented by one column in the figure. The quantitative data were detected by taking three biological replicates and two technical replicates, and the relative expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method. The error bars represented the fluctuation range of the experimental values, and the shorter the error bars, the more reliable the values were.

were named as *StKEA1* and *StKEA2*. Trk1 from *S. cerviciae* was the first gene cloned from Trk/HKT family. However, *HKT1* from wheat was the first plant K^+ transporter cloned and identified by functional complementation studies using yeast (Gaber *et al*., 1988). Potassium channels are a structurally diverse group of proteins that facilitate the flow of K^+ ions across cell membranes. They are ubiquitous, being present virtually in all cell types. Plant K^+ channels also play a key role in K^+ uptake, translocation and osmotic regulation too (Maathuis *et al*., 1997; Schroeder *et al*., 1994). K^+ channel families can be categorized by the number of P-loops and presence of transmembrane (TM) domains per monomer. Typical examples of these channels are Shaker-type 1P/6TM channels, the 1P/2TM channels, the ORK like 2P/4TM KCO channels and the TOK like 2P/8TM channels (Goldstein *et al*., 1996; Ketchum *et al*., 1995; Suzuki *et al*., 1994; Tempel *et al*., 1987). The K^+ channel signature sequence comprises TXXTXGYGD motif (Heginbotham *et al*., 1992; Hille, 1992), which is very conserved. KCOs are classified as 2P/4TMS or 1P/2TMS channels and possess K^+ channel signature sequence which is a hallmark for all K^+ channel proteins. In the present analysis, we identified a total of 10 genes corresponding to Shaker-type K^+ channel proteins and five genes to 2P/4TM KCO family.

Genomic duplications are the essential contributes for the origin and evolution of species. However, across evolutionary time, most of these duplications have disappeared and been silenced, with the remaining few playing a strong role in positive or purifying selection (Lynch and Conery, 2000). Of these 10 gene duplication events, nine duplications occurred on different chromosomes, and the other one was on the same chromosome (StHAK4 and StHAK10), but the location of StHAK4 and StHAK10 were not tightly linked on the chromosome 02 (Fig. 1), so the 10 gene duplication events are all considered segment duplication events (Jiang *et al*., 2013).

The substitution rate, known as *Ka*/*Ks* (non-synonymous/synonymous) is an indicator of positive selection pressure (Zhang *et al*., 2006) and is commonly used to investigate the evolutionary direction and selective strength in a coding sequence (Li *et al*., 2009). *Ka*/*Ks* values above 1.0, equal to 1.0 and below 1.0 signify positive, neutral and purifying selection, respectively (Lynch and Conery, 2000). The *Ka*, *Ks* and *Ka*/*Ks* values of each gene pair were calculated (Table 2, Fig. 3), and the results demonstrated that the values of *Ka*/*Ks* of three segmental duplications *StHAK3* and *StHAK8*, *StHAK5* and *StHAK7*, *StK4* and *StK8*, *StKCO1* and *StKCO2* and *StKEA1* and *StKEA2* were lower than 1.0, which indicated that these K^+ transporter genes were under purifying selection. However, the values of *Ka*/*Ks* of the other duplications events were higher than 1.0, which meant that these genes were all under positive selection (Yang *et al*., 2006).

As can be seen from Figure 4, the KUP/HAK/KT group was the largest cluster. This family plays a key role in the normal growth and development of plants, such as cell elongation in roots and shoots (Elumalai *et al*., 2002; Osakabe *et al*., 2013; Rigas *et al*., 2001; Rigas *et al*., 2013; Vicente-Agullo *et al*., 2004). The first K^+ transporter *HvHAK1* in plants was cloned from barley (*Hordeum*

vulgare L) (Santamaría *et al*., 1997), and AtKUPl/KTl was the first K^+ transporter cloned in *Arabidopsis* (Fu and Luan, 1998; Kim *et al*., 1998; Quintero and Blatt, 1997). Members of the HAK/KUP/KT family, such as *AtHAK5* in *Arabidopsis* and *OsHAK1*, *OsHAK5* and *OsHAK22* in rice, can increase plant roots growth to absorb and transfer K^+ in a low external K^+ concentration (Bañuelos *et al*., 2002; Chen *et al*., 2016; Rubio *et al*., 2000; Shen *et al*., 2016; Yang *et al*., 2004).

In *Arabidopsis*, Shaker family could be divided into three types without *KAT3* and *AKT5*: inward rectifier (*AKT1*, *AKT6*, *KAT1* and *KAT2*), outward rectifier (*SKOR* and *GORK*) and weak inward rectifier (*AKT2*) (Reintanz *et al*., 2002; Wang and Wu, 2009). Inward rectification K^+ channels are sensitive to K^+ concentration, dependent on voltage, and have low affinity for K^+. Inward rectifying K^+ channels are mainly expressed on the plasma membrane of cells. *KAT1* is a plant inward rectifying K^+ channel gene screened from an *Arabidopsis* cDNA library simultaneously with *AKT1*. *KAT1*, with a highly selectivity for K^+, is mainly expressed in guard cells and has a low expression level in root and stem vascular tissues, and it is related to the regulation of stomatal movement by outward rectifying K^+ channel gene *GORK*. Outward rectifying K^+ channels are found in various cell types of plants and have a high selectivity for K^+. Studies have shown that SKOR and GORK can physically interact to form functional heterogeneous outward rectifying channels (Dreyer *et al*., 2004). The weak inward rectifying K^+ channel gene AKT2 was only cloned from *Arabidopsis* cDNA library using AKT1 as a probe so far. Also, in the Shaker K^+ channel family, AKT2 is the only gene that can mediate K^+ influx and regulate K^+ efflux (Chérel *et al*., 2002).

In KCO family, *KCO1* is the first gene cloned in the family, which is expressed in all parts of the plant and localized on the cytosol (Czempinski *et al*., 2014; Schönknecht *et al*., 2002). *AtKCO3* is the only putative voltage-independent K^+ channel gene of *Arabidopsis thaliana*. Studies have shown that the lack of *KCO3* under various conditions does not cause significant changes in plant growth, but root growth of the KCO3-1 null allele line will be reduced only under osmotic stress (Rocchetti *et al*., 2012).

For K^+/H^+ antiporter family, Genechip analysis shows that *AtKEA1* is mainly expressed in cotyledons, leaves, petioles and stems and is rarely expressed in roots (Sze *et al*., 2004). By expressing *AtKEA2* lacking the N-terminal domain, indicating that *AtKEA2* may be a cation/H^+ antiporter that confers greater tolerance to Na^+ or K^+ stress in yeast (Aranda-Sicilia *et al*., 2012). The expression levels of AtKEAs are affected by K^+ deficiency and NaCl or osmotic stress, and are also regulated by 2,4-dichlorophenoxyacetic acid, benzyl adenine and sucrose (Han *et al*., 2015). *AtKEA1*, *AtKEA2*, and *AtKEA3* are all located in the chloroplast and are thought to play a fundamental role in chloroplast osmotic regulation, integrity, ion and pH homeostasis (Aranda-Sicilia *et al*., 2016; Kunz *et al*., 2014).

In addition, studies show that Trk-HKT family members play a major role in maintaining intracellular Na^+ and Na^+/K^+ homeostasis, regulating the salt tolerance of plant in *Arabidopsis* and rice (Horie *et al*., 2014; Mäser *et al*., 2002; Rus *et al*., 2004; Sunarpi *et al*., 2010). Trk/HKT family members are widely involved in plant stress resistance (Véry and Sentenac, 2003; Wang and Wu, 2013; Waters *et al*., 2013). For example, *OsHKT2;1* loss-of-function mutation cause rice to grow slowly under low K^+ conditions (Horie *et al*., 2014). Exogenous NaCl treatment can induce *GmHKT1* expression in soybean (*Glycine max*) roots and leaves. And in transgenic tobacco (*Nicotiana tabacum*) overexpressing *GmHKT1*, Na^+ accumulation in roots and shoots is decreased, while K^+ content is increased, and salt tolerance is improved (Chen *et al*., 2011).

The functional relationship could be understood by the phylogenetic distribution, and as a result, the phylogenetic distribution of K^+ transporter genes obtained from *A. thaliana* and rice implied that these K^+ transporters in potato might have a similar function or effect on the development and the responses to some stresses and the stimulation of potato as in their *A. thaliana* counterparts. Therefore, we can speculate on the function of the corresponding K^+ transporter gene in potato based on the existing results that can provide a clear idea and direction for the research.

Through the analyses to the promoter region of K^+ transporter genes, we can further understand the regulation of the identified K^+ transporter genes at transcriptional level. Light is a predominant factor which controls the circadian rhythm of various life processes such as growth, development, nitrate uptake and stress responses in plants. Studies have shown that the GATA motif is required for light to regulate plant growth. For example, in petunia, chlorophyll a/b binding protein gene (Cab22) contains three GATA-motif repeats in the promoter region (Lam and Chua, 1989). Many light-regulated genes present GT1-motif in consensus GT-1 binding sites such as PHYA from rice and oat (Terzaghi and Cashmore, 1995). G-Box is reported to be involved in the light-responsive processes and its binding factors are usually demonstrated to be members of bHLH, bZIP, and NAC families (Liu *et al*., 2016).

Meanwhile, the promoter regions of most K^+ transporter genes also harbored most of the important cis regulatory elements. For example, in the elements that associated with stress response, we identified Box-W1, HSE, LTR, TC-rich repeats, WUN-motif and MBS. Among them, Box-W1 is associated with pathogen stress responsiveness; HSE and LTR are involved in heat stress responsiveness; TC-rich repeats are involved in defense and stress responsiveness; MBS is involved in drought

stress responsiveness. And some elements are associated with hormone response. For example, TGACG-motif and CGTCA-motif are involved in MeJA responsiveness; ABRE is involved in ABA responsiveness; TGA-element is involved in auxin-responsive (Amrutha *et al*., 2007; Liu *et al*., 2017).

In addition, we sought annotated regulatory motifs involved also in other functions. For example, O2-site is zein metabolism regulation elements. Skn-1 and GCN4 motif are required for high levels of endosperm expression; WUN-motif is responsive to wounding; Circadian is involved in circadian control (Sarkar and Maitra, 2008). These results suggest that K^+ transporter genes expression could be modulated by various developmental and/or environmental changes.

Potato was highly affected by K^+ starvation, displaying a low root and shoot growth rate and leading to a visible symptom of small leaves (Fig. S2).

When plants are facing potassium deficiency, the roots are able to sense externally low concentration of K^+ and trigger a range of physiological responses (Schachtman and Shin, 2007; Wang and Wu, 2010). In *Arabidopsis thaliana*, it has been noted that the transcript abundance of *ATKEA* in roots was not strongly affected by K^+ deficiency, While in the shoots, *AtKEA1* is strongly induced by K^+ starvation (Han *et al*., 2015). But in this study, in potato, we found that the expression levels of *StKEA1* and *StKEA2* was hardly affected in stems and roots under K^+ deficiency condition. It was found that the expression of *AtK5* in *Arabidopsis* was up-regulated under K^+ starvation (Ahn *et al*., 2004), so according to the phylogeny, *StHAK11* should be up-regulated. In fact, the expression levels of *StHAK11* was significantly increased in roots, stems and leaves in this study, which was consistent with the predicted results. In addition, the expression of *AtAKT2* is regulated by K^+ concentration (Reintanz *et al*., 2002; Wang and Wu, 2009). *StK1*, which was close to *AtAKT2* on the phylogenetic tree, the expression was increased in roots and leaves, but was decreased in stems under K^+ deficiency. These results suggest that it is feasible to speculate on the function of K^+ transporters in potato based on the phylogenetic distribution.

CONCLUSIONS

In this study, a total of 33 K^+ transporter genes were identified and annotated as KUP/HAK/KT transporter family (15 genes), KCO outward rectifier family (5 genes), Shaker K^+ channel family (10 genes), K^+/H^+ antiporter family (two genes) and Trk/HKT transporter family (one gene), and were analyzed to describe their features, including exons/introns, protein molecular weight, hydrophilicity or hydrophobicity, and subcellular localization. In these genes, we described 10 pairs of duplication events that evolved under the influence of purification, although there were no tandem duplications. These duplication events played an important role in the evolution and development of the potato. In addition, there were differences in the expression levels of 33 K^+ transporter genes at K^+ deficiency, and these genes were up-regulated or down-regulated in leaf, root and stem tissues, suggesting that these genes responded to K^+ starvation. These results provide a basis for studying the function of the K^+ transporter family in the genome. However, the specific functional properties of each individual K^+ transporter gene still need to be identified by further study at the physiological and molecular levels.

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