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Research paper

Genome-wide identification of shaker K^+ channel gene family in sugar beet (*Beta vulgaris* L.) and function of *BvSKOR* in response to salt and drought stresses

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ABSTRACT

Potassium (K^+) is the most abundant cation in plants, which is absorbed by roots and distributed throughout the plants and within plant cells, and is involved in various cellular processes. Shaker K^+ channel plays crucial roles in the absorption and distribution of K^+ and in the response to abiotic stress in plants. Herein, a total of six shaker K^+ channel genes, *BvKAT1*, *BvKAT3*, *BvAKT1*, *BvAKT2*, *BvAKT5*, and *BvSKOR*, were identified in the genome of sugar beet (*Beta vulgaris* L.). The coding domain sequences (CDS) of these genes ranged from 2232 to 2739 bp, and protein lengths were varied from 743 to 912 aa. The shaker K^+ channel genes contained hormone-related and light responsiveness *cis*-acting regulatory elements. The phylogenetic analysis showed that BvSKOR was highly conserved and contained six transmembrane structures. The expression patterns of *BvSKOR* under salt and osmotic stress were analyzed by qRT-PCR, and found that the expression level of *BvSKOR* under low concentration salt and osmotic stress at short period of treatment were significantly higher than that of the control group. The function of *BvSKOR* was further verified in tobacco (*Nicotiana tabacum*), and the results showed that under salt and osmotic stress, the roots of transgenic plants were significantly stronger than those of wild type (WT) plants, and the relative water content (RWC), chlorophyll, proline, soluble sugar, soluble proteins contents and antioxidant enzyme activity were significantly higher than those of WT plants. These results indicated that overexpression of *BvSKOR* can significantly enhance the salt and drought tolerance in transgenic tobacco plants. This study could provide theoretical support and genetic resources for genetic improvement of crops stress resistance.

1. Introduction

Salt stress is one of the major environmental factors that limits the growth and development of plants worldwide. In addition to large areas of native saline soil, the amount of secondary salinized land is increasing due to irrational irrigation, fertilization or climate change (Abdel Latef et al., 2019; van Zelm et al., 2020). When exposed to high salt conditions, plants can be hindered their growth, development and often subjected to osmotic stress. The combined effects of the two stresses could hinder the photosynthesis and metabolism of plants, thereby inhibiting their growth and even leading to death (Gong, 2021). Firstly, the increased salt concentrations in the soil can generate osmotic stress, resulting in water loss, leaf stomata closure, and photosynthesis inhibition, which affects the normal growth and metabolism of plants. The second effect is Na^+ toxicity, which can compete with K^+ for the main binding sites of key metabolic processes in the cytoplasm, such as enzyme catalysis, protein synthesis, and the function of ribosome (Anschütz et al., 2014; Kumar et al., 2020). Na^+ can also limit the ability of plants to obtain and metabolize other essential nutrients, resulting in serious interference to cell metabolism. Finally, the content of reactive oxygen species (ROS) in plant tissues increased significantly due to salt and drought stresses (Baxter et al., 2014; Petrov et al., 2015), which damages the integrity of cell structures, and ROS can affect a series of cation permeation channels and substantially interfere with cellular ion homeostasis (Demidchik and Maathuis, 2007).

To cope with Na^+ toxicity caused by high salt, some halophytes or

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Table 1
The sequences of primers used for qRT-PCR.

Genes	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
BvACTIN	ACTGGTATTGTGCTTGACTC	ATGAGATAATCAGTGAGATC
BvSKOR	ATGTCGAGGATTAGAGAGGAAGGAG	CCTTCTCTGCTAAACTGTCTCCG
NtActin	ACTGGTATTGTGCTTGACTC	ATGAGATAATCAGTGAGATC

Table 2
Gene identification and protein characterization of shaker-type K^+ channel family in sugar beet.

Gene name	Gene ID	Chr	Chair	CDS (bp)	Exon	Molecular formula	Protein length (aa)	*p*I	MW (Da)	Instability index	GRAVY
BvKAT1	Bv3_066600_wzxk	3	-	2604	11	$C_{4408}H_{6924}N_{1178}O_{1266}S_{36}$	867	6.57	97770.36	37.63	−0.051
BvKAT3	Bv8_202040_fkxj	8	+	2232	11	$C_{3854}H_{5970}N_{1022}O_{1101}S_{36}$	743	6.36	85392.14	48.61	−0.108
BvAKT1	Bv2_043240_aada	2	+	2604	10	$C_{4408}H_{6924}N_{1178}O_{1266}S_{36}$	867	6.57	97832.76	37.63	−0.051
BvAKT2	Bv6_143700_ptey	6	-	2433	9	$C_{4174}H_{6545}N_{1109}O_{1188}S_{32}$	810	8.35	92297.52	30.31	0.013
BvAKT5	Bv8_195260_ywgq	8	-	2739	10	$C_{4629}H_{7242}N_{1288}O_{1336}S_{36}$	912	7.00	103468.41	41.47	−0.170
BvSKOR	Bv2_047040_fjjf	2	-	2487	13	$C_{4277}H_{6706}N_{1140}O_{1229}S_{32}$	828	7.20	94787.11	40.24	−0.166

Chr: Chromosome; CDS: Coding sequence; *p*I: isoelectric point; MW: Molecular weight; GRAVY: Grand average of hydropathicity.

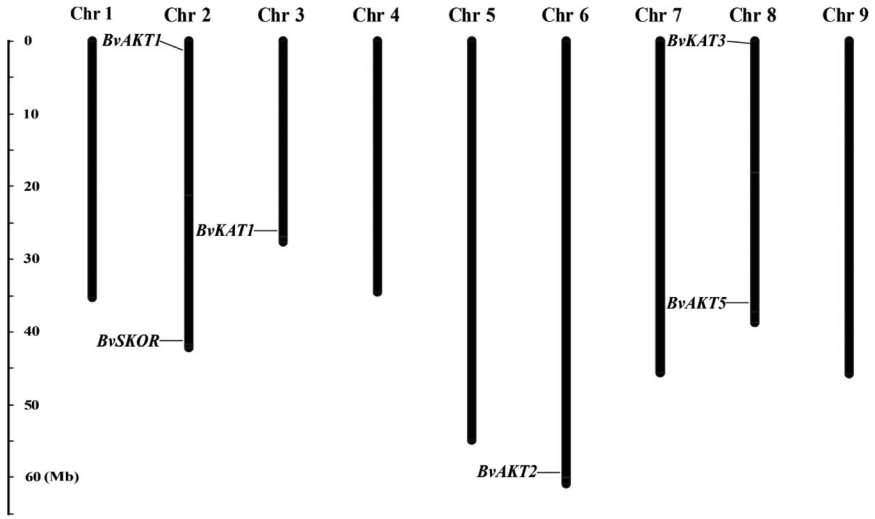

Fig. 1. The location of shaker-type K^+ channel genes on nine chromosomes of sugar beet. The number of chromosomes is shown on the top, and the length of each chromosome is shown on the left.

halophilic crops have formed a series of complex defense mechanisms to enhance their survival probability (Sun and Zhou, 2018; van Zelm et al., 2020). Plants can improve their tolerance to salt and osmotic stresses through osmotic regulation (Eida et al., 2019). Plant cell absorbed osmoregulators, such as K^+, sugar, and proline, to reduce intracellular water potential, indicating that maintaining K^+ homeostasis is one of the critical strategies for plants to deal with these stresses. K^+ is absorbed by the root systems and then distributed to different tissues in plants (Luan et al., 2016). K^+ transporters and channels that have key functions in K^+ maintenance and ion homeostasis under high salt conditions include inward-rectifier shaker K^+ channel AKT1, K^+ channel in *Arabidopsis thaliana* KAT1, high-affinity K^+ transporter HAK5, shaker K^+ outward rectifying channel SKOR, and high-affinity K^+ transporter HKT1;5 (Schmidt et al., 2013; Ahanger et al., 2017; Sustr et al., 2019). The increased channel activity and up-regulation of related gene expression level under salt stress resulted in improving capacity of K^+ transport, maintaining high K^+/Na^+ ratio, and enhancing salt tolerance in plants (Assaha et al., 2017). The first shaker K^+ outward rectifying channel gene *SKOR* was identified in *A. thaliana*, which was preferentially expressed in the stele tissue of roots (Gaymard et al., 1998). Subsequently, the *SKOR* gene has been identified in other species such as rice (*Oryza sativa*) (Kim et al., 2015), *Zygophyllum xanthoxylum* (Hu et al., 2016), *Lycium barbarum* (Zhang et al., 2017), and melon (*Cucumis melo*) (Huang et al., 2018). *SKOR* is preferentially expressed in the cells of pericycle and xylem parenchyma to promote K^+ loading into xylem sap, and then transport K^+ to the shoots by the sap under transpiration pull (Gaymard et al., 1998). The K^+ concentration in xylem sap and K^+ content in shoots of *atskor* mutant were about 50 % lower than those of wild-type (WT) plants, the net uptake rate of K^+ was significantly lower than that of WT plants, and the expression levels of *AtHAK5* and *AtAKT1* in roots were significantly reduced (Nieves-Cordones et al., 2019a), indicating that the transport of K^+ to the shoots was reduced, thereby reducing the K^+ uptake capacity of roots. These results suggested that SKOR plays crucial role in the long-distance transport and distribution of K^+ in both roots and shoots, and is involved in the regulation of abiotic stress response and tolerance mechanism in plants.

Sugar beet (*Beta vulgaris*) is a biennial herbaceous plant which belongs to Chenopodiaceae family, and it was native to southern Europe, eastern Europe and north Africa, mainly cultivated in arid and semi-arid areas in Northern China. Sugar beet is the second largest source of sugar, accounting for about 30–40 % of global sugar production (Hussein et al., 2019; Yolcu et al., 2021). This species is also a typical halophilic crop, its growth is promoted by salt in a range of concentrations, and can tolerate salt concentration of up to 150 mM during the vegetative phase (Wu et al., 2013; 2015,). Additionally, the sequence data of sugar beet genome had been published by Dohm et al. (2014), which provides a

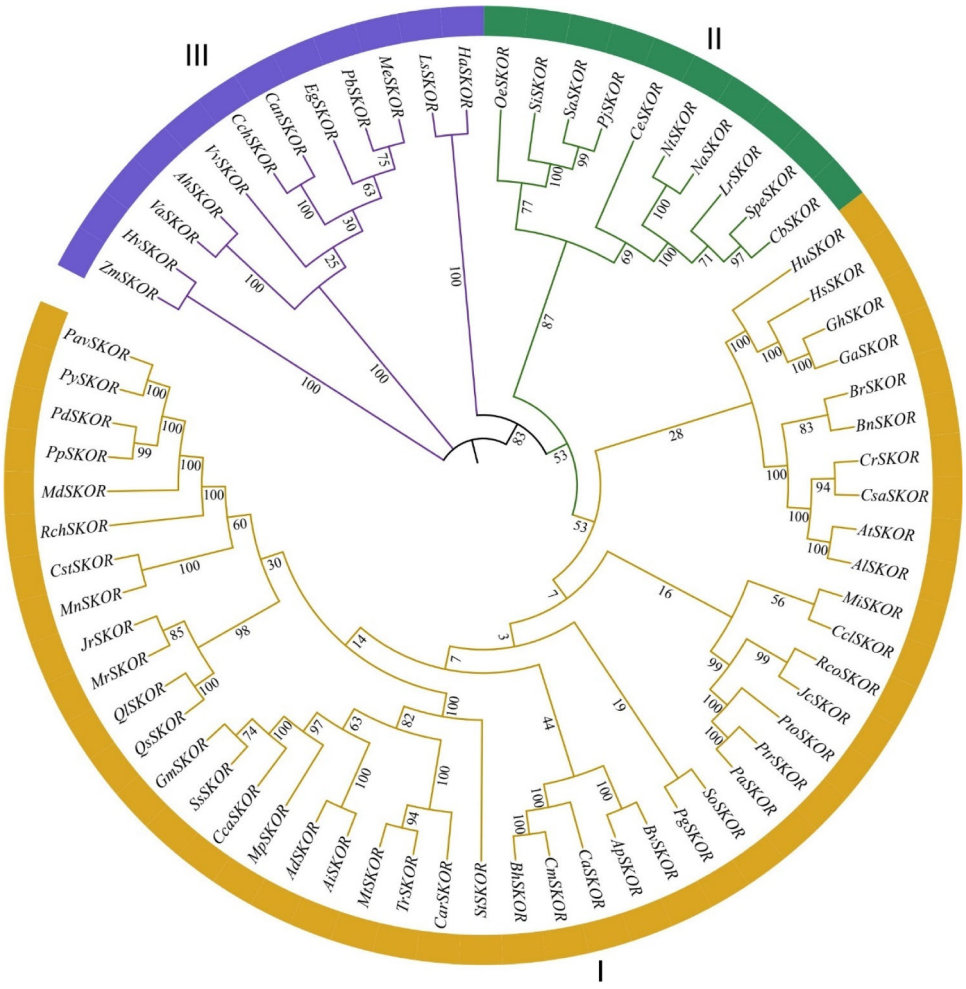

Fig. 2. Phylogenetic analysis of the *SKOR* genes from 69 species. The phylogenetic tree was constructed using the MEGA v11.0 by the neighbor-joining method with 1000 bootstrap replicates. The accession number and protein sequences of all *SKOR* genes used in the present study are listed in Supplementary Data S2.

research basis for the functional analysis of stress resistance mechanism.

Therefore, exploring the molecular mechanism of adaptation to salt and drought stresses and mining genes related to abiotic stress-tolerance in sugar beet has important theoretical significance and practical value for the genetic improvement of crop stress tolerance. However, the shaker K^+ channel family in sugar beet, especially SKOR, a member of shaker family K^+_{out}, has never been identified. To reveal the role of shaker K^+ outward rectification channel, in this study, all genes of shaker K^+ channel family were firstly identified in sugar beet; Secondly, the expression pattern of the shaker K^+ outward rectifying channel gene *BvSKOR* was analyzed under salt and osmotic stress; Thirdly, *BvSKOR* was cloned and overexpression vector was constructed, and the overexpressed *BvSKOR* transgenic tobacco (*Nicotiana tabacum*) infected by *Agrobacterium rhizogenes* were obtained; Finally, the effects of salt and drought stress on growth and physiological parameters in transgenic plants was investigated. The results from this study could provide theoretical support and genetic resources for genetic improvement of crop stress resistance.

2. Materials and methods

2.1. Identification of shaker K^+ channel genes in sugar beet

To identify shaker K^+ channel genes in the genome of sugar beet, the sequences of *AtKAT1*, *AtKAT2*, *AtAKT1*, *AKT2*, *AtKC1*, *AtSKOR*, and *AtGORK* were obtained from The *Arabidopsis* Information Resource (TAIR) (https://www.arabidopsis.org/) and then were blasted the sequence with the BLASTP tool using The *Beta vulgaris* Resource (TBR) (https://bvseq.boku. ac.at/). All putative sequences were examined using the NCBI database (https://www.ncbi), where the sequences can blast out the shaker K^+ channel genes of other species determined as sugar beet shaker K^+ channel family members and named them according to the channel type with their maximum similarity (Supplementary Data S1). The coding domain sequences (CDS) of shaker K^+ channel genes were translated into amino acid sequences with MEGA v11.0 software. The molecular formula, isoelectric point (*p*I), molecular weight (MW), instability factor, and the grand average of hydropathicity (GRAVY) of predicted shaker K^+ channel proteins were computed using the online tool ProtParam (https://web.expasy.org/protparam/).

2.2. Chromosomal location of shaker K^+ channel genes in sugar beet

The chromosomal location of the identified sugar beet shaker K^+ channel genes was retrieved from TBR (https://bvseq.boku. ac.at/) and the physical map of the chromosomal position of these genes were constructed using MapInspect v1.0 software.

2.3. Cis-acting regulatory element prediction of shaker K^+ channels

To analyze the *cis*-acting regulatory elements in the promoter of sugar beet shaker K^+ channel proteins, 2 kb upstream sequence of the transcriptional initiation site (TIS) of the identified genes were estimated using PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/ html/).

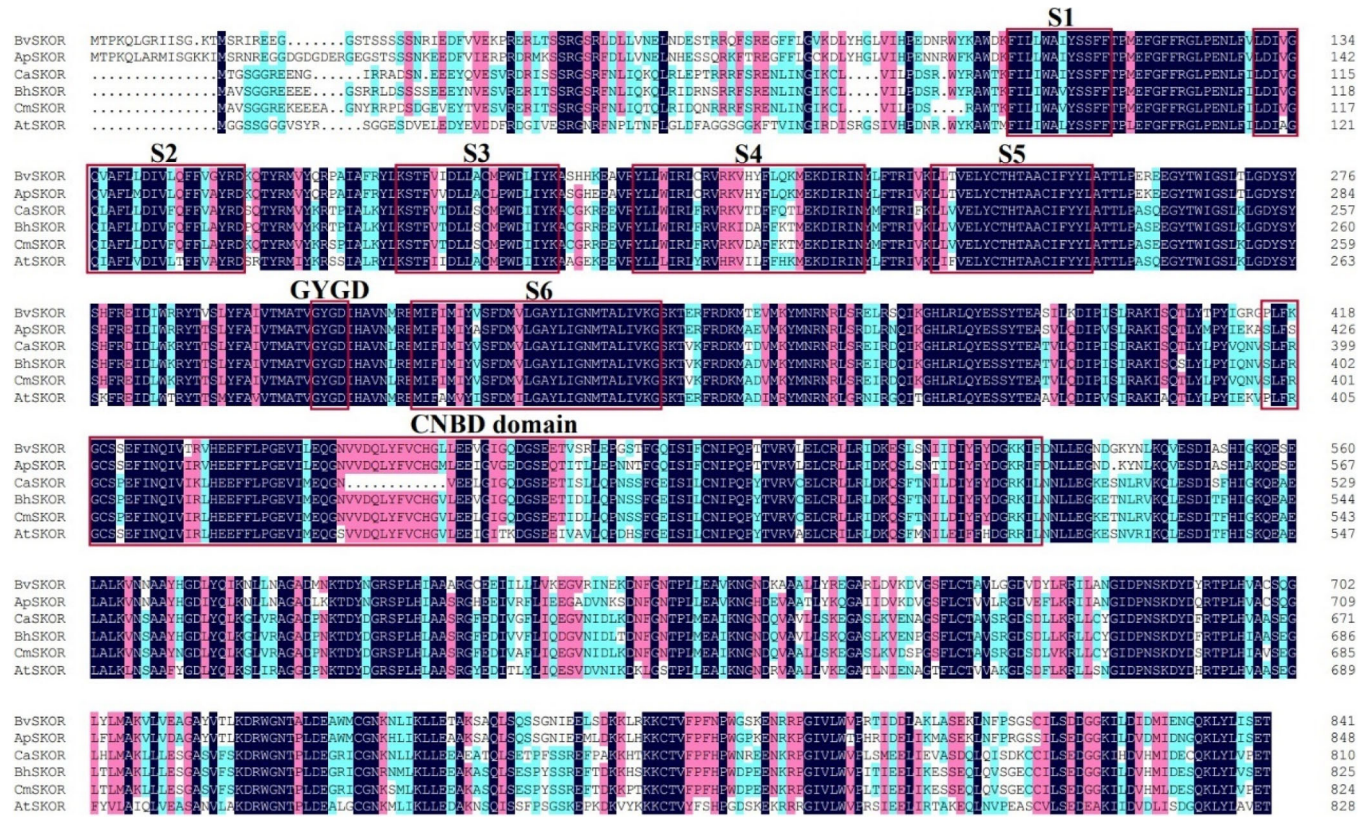

Fig. 3. Multiple sequences alignments of six SKORs from *B. vulgaris* (Bv), *Alternanthera philoxeroides* (Ap), *Cucurbita argyrosperma* (Ca), *Benincasa hispida* (Bh), *Cucumis melo* (Cm) and *Arabidopsis thaliana* (At). The red boxes indicate the six transmembrane domains, the cyclic nucleotide binding domain of the C-terminal, and the conserved GYGD amino acid sequence of SKOR.

2.4. Phylogenetic analysis of shaker K^+ outward rectifying channels

To investigate the phylogenetic relationship between sugar beet shaker K^+ outward rectifying channel *BvSKOR* and other plant species *SKORs*, the protein sequences of *SKOR* for 69 species were downloaded from the NCBI database, and the accession number and protein sequences were shown in Supplementary Data S2, and MEGA v11.0 software was used to construct an unrooted phylogenetic tree based on the conserved domains with 1000 bootstrap replicates.

2.5. Gene structure and protein motif analysis of BvSKOR

The intron/exon structure of *BvSKOR* was predicted using online tool GSDS v2.0 (https://gsds.cbi.pku.edu.cn/). To analyze the conserved motif of BvSKOR with other species SKOR, the amino acid sequence of BvSKOR and other species SKOR were respectively submitted to MEME program (https://meme-suite.org/tools/meme/), with the motif widths ranging from 6 to 50 residues and the maximum number of motifs set at 12.

2.6. Prediction of transmembrane, secondary and three-dimensional structure of BvSKOR

The transmembrane, secondary, and three-dimensional structures of BvSKOR were predicted using TMHMM v2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/), NPSA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa%20_sopma.html), and SWISS-MODEL program (https://beta.swissmodel.expasy.org/), respectively.

2.7. Protein-protein interaction (PPI) prediction of BvSKOR

The functional interacting network of BvSKOR was predicted using the STRING v11.0 database (https://string-db.org/cgi/input.pl).

2.8. Plant materials, growth conditions, and treatments

Seeds of sugar beet (*B. vulgaris* L. cv. "Gantang No. 7") were firstly surface sterilized with 75 % ethanol and rinsed 3 times with distilled water, soaked in distilled water overnight and then sowed in plastic containers filled with vermiculite and germinated in the dark for 3 d. Seedlings were grown in the same growth room where the temperature was set at 25℃/20℃ (day/night), the relative humidity was regulated between 65 % and 75 %, the daily photoperiod was 16 h/8 h (day/night), and light density was 550–600 $mmol{\cdot}m^{-2}{\cdot}s^{-1}$ during the photoperiod. The seedlings were irrigated with the modified Hoagland nutrient solution containing 2.5 mM KNO_3, 1 mM $NH_4H_2PO_4$, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 60 μM Fe-Citrate, 92 μM H_3BO_3, 0.7 μM $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$, 18 μM $MnCl_2{\cdot}4\ H_2O$, 1.6 μM $ZnSO_4{\cdot}7\ H_2O$, and 0.6 μM $CuSO_4{\cdot}5\ H_2O$, once every 3 days. After 4 weeks, for the salt treatments, the healthy seedlings with consistent size were subjected with the modified Hoagland nutrient solution supplemented with additional 50, 100, 150, 200, and 250 mM NaCl, and samples were taken at the 3, 6, 12, 24, 72, and 120 h after treatment, respectively. For drought stress, the seedlings were treated with 5 %, 10 %, 15 %, and 20 % PEG, and samples were taken at 1, 3, 6, 12, and 24 h after treatment, respectively. The unstressed seedlings grown in modified Hoagland nutrient solution at the same time were taken as control. All tissue samples of shoots and roots were immediately frozen in liquid nitrogen and stored at −80℃ until RNA extraction.

2.9. Expression analysis of BvSKOR under salt and drought conditions

To validate functions of *BvSKOR* in response to salt and drought stress, its expression pattern was analyzed by qRT-PCR. Total RNA was

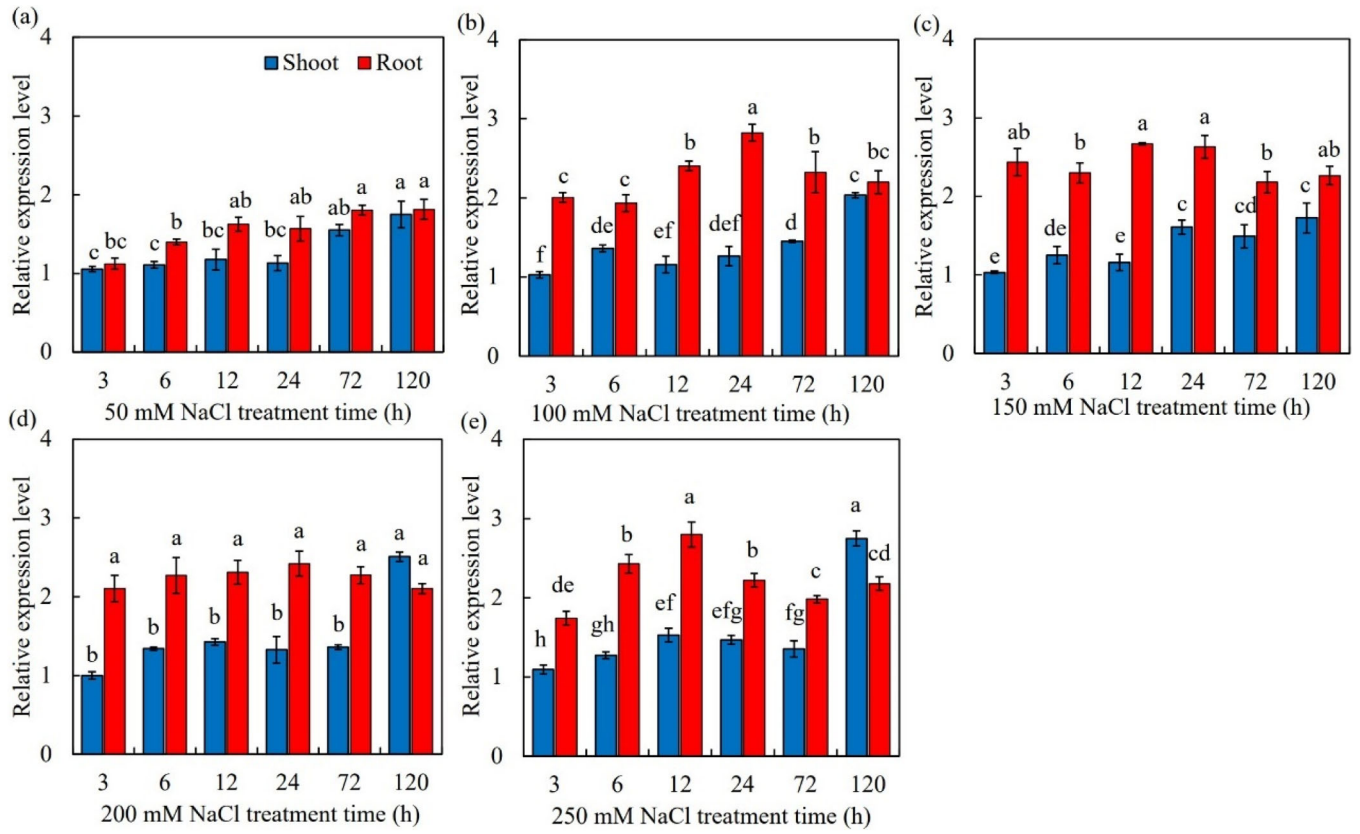

Fig. 4. Effects of salt treatment on the relative expression level of *BvSKOR* in the shoots and roots of sugar beet. The vertical bar represents the standard error (SE) (n=3). Different lowercase letters on the column indicate significant differences at $P < 0.05$.

isolated from samples of roots or shoots using Trizol Total RNA Isolation Kit (Sangon, Shanghai, China) and its concentration and purity was determined using spectrophotometer. First strand cDNAs were synthesized from total RNAs using the PrimeScript™ RT Master Mix Kit (Takara, Dalian, China) according to the manufacturer's instruction. The specific primers of *BvSKOR* and *BvACTIN* were listed in Table 1. The qRT-PCR was conducted on MA-6000 RT-PCR System (Molarray, Suzhou, China) with TB Green Premix Ex Taq™ (Takara, Dalian, China). The conditions followed for the experiment are 95 ℃ for 30 s, and 40 cycles of 95 ℃ for 5 s and 60 ℃ for 60 s. *BvACTIN* was used as the internal reference gene. The relative expression level of *BvSKOR* was calculated by $2^{-\Delta\Delta Ct}$ method with three biological repeats at least.

2.10. Overexpression vector construction and transformation in tobacco

The PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) was used to synthesize cDNAs. The CDS of *BvSKOR* was amplified with specific primers *BvSKOR*-F-*Xho* I and *BvSKOR*-R-*Sma* I (Supplementary Table S1), then inserted into the pEarleyGate 100 vector, and the overexpression vector of pEG-*BvSKOR* was constructed, in which *BvSKOR* was driven by the CaMV 35S promoter. The pEG-*BvSKOR* plasmid was introduced into the *A. tumefaciens* strain GV3101 by the freeze-thaw method. Tobacco (*N. tabacum* cv. "Benshi") was transformed by the leaf disc method. The infected leaves were inoculated on MS medium containing Cefotaxime sodium (Cef), Kanamycin (Kan), Carbenicillin (Cb), 6-Benzylaminopurine (6-BA), and α-Naphthalene acetic acid (NAA) for selective culture. Resistant buds growing on calluses were cut off and then transferred to MS medium until whole plants are formed. Seedlings were transplanted into the matrix which contained soil and vermiculite (3:1), cultured with 16 h/8 h light/dark, and sprayed with 60 mg/L Basta for resistance screening.

Genomic DNA from T_0 generation transgenic tobacco plants was extracted and the *BvSKOR* gene was identified by PCR using primers *BvSKOR*-F/R (Supplementary Table S1), the PCR products were detected by 1.2 % agarose gel electrophoresis to confirm the insertion of *BvSKOR* into the transgenic plants. The relative expression level of *BvSKOR* in T_1 transgenic plants was analyzed by qRT-PCR. *NtActin* was used as the reference gene. The screened T_1 generation lines with higher expression levels of *BvSKOR* were selected to study abiotic stress tolerance.

2.11. Stress treatments of transgenic tobacco plants

Transgenic T_1 generation tobacco seeds overexpressing *BvSKOR* were germinated on 1/2 MS medium containing 30 mg/L Basta for resistance screening. The WT and Basta-positive transgenic seedlings were cultured onto MS medium for 7 d and then transferred to MS medium containing 50, 100, 150 and 200 mM NaCl, and 50, 100 and 150 mM mannitol to simulate salt and drought treatments, respectively. Their root lengths were measured and recorded after 10 d.

To identify the salt and drought tolerance of transgenic tobacco, 10-day-old WT and Basta-positive transgenic seedlings were transferred to pots contained nutritional soil/vermiculite (3/1) and irrigated with the modified Hoagland nutrient solution once every 3 d. After about 30 d of growth, tobacco seedlings of uniform size and growth status were selected for NaCl or mannitol treatment. WT and transgenic plants were respectively treated with 200 mM NaCl and 100 mM mannitol to simulate salt and osmotic stress for 10 d, which grown in normal Hoagland nutrient solution were used as controls.

2.12. Measurement of relative water content (RWC), and the amounts of chlorophyll, proline, soluble sugars and soluble proteins

The 1–2 fresh leaves of control and stress groups were harvested and immediately weighed as fresh weight (FW). After soaking in distilled

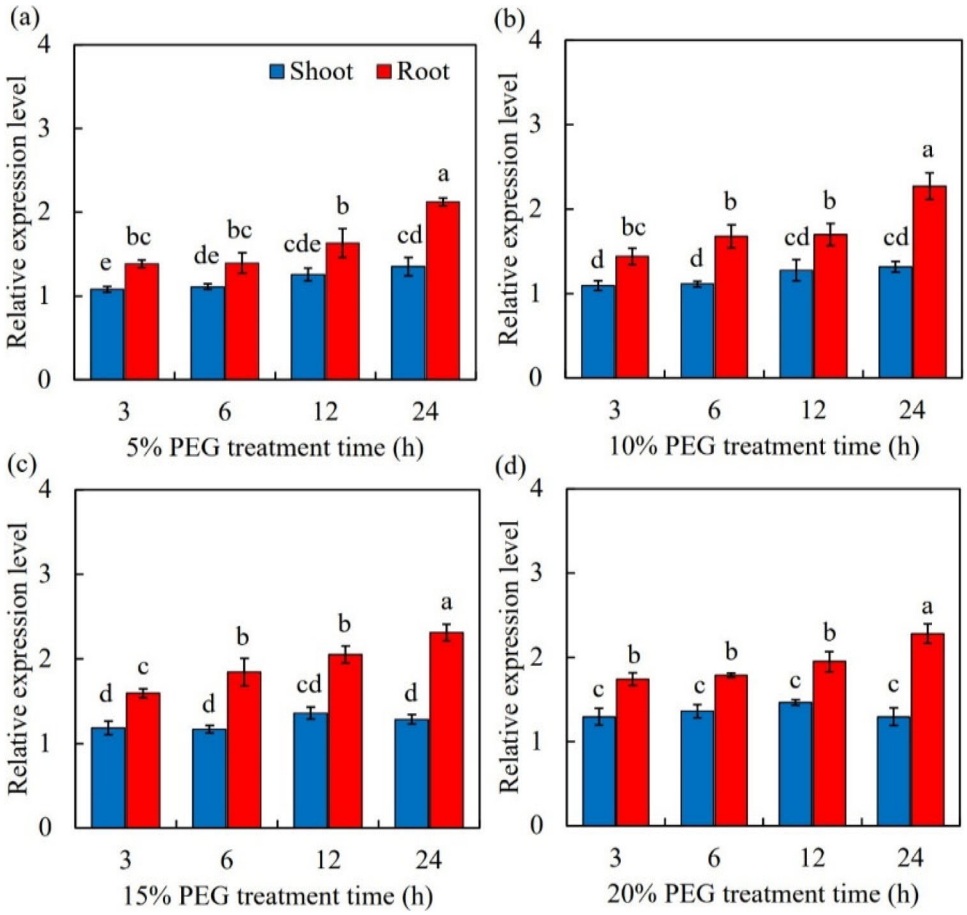

Fig. 5. Effects of PEG treatment on the relative expression level of *BvSKOR* in the shoots and roots of sugar beet. The vertical bar represents the standard error (SE) (n=3). Different lowercase letters on the column indicate significant differences at $P < 0.05$.

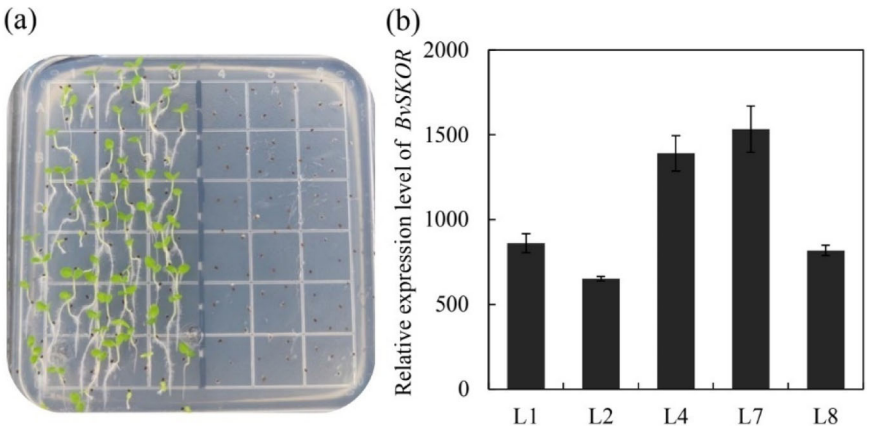

Fig. 6. Screening of T_1 transgenic tobacco and expression level analysis of *BvSKOR*. (a) Basta resistant plate screening transgenic tobacco seeds. The left is the T_1 transgenic tobacco, and the right is the WT tobacco. (b) Relative expression level of *BvSKOR* among transgenic tobacco lines. The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method, while the expression of WT tobacco leaves was calculated as 1.

water for 12 h, the weight of these leaves was determined as the saturated fresh weight (SFW), and then dry weight (DW) was measured after drying all leaves in the oven at 80°C for 48 h. RWC was calculated following the formula: RWC (%) =[(FW-DW)/(SFW-DW)] ×100. The chlorophyll contents were determined according to the method described by Witham et al. (1971). Briefly, leaf samples (0.1 g) of WT, L4, and L7 were washed with distilled water and cut into pieces. Then $CaCO_3$ and distilled water were added into the homogenate and ground until the tissue blanched. Subsequently, samples were soaked for more than 3 h in the dark until the residue was completely white, which used distilled water and acetone as the extraction solution. The absorbance was measured at 663 nm and 645 nm, and chlorophyll content was calculated as: chlorophyll a content (mg/g FW) = 0.1 × (12.7 × A663 - 2.69 × A645), chlorophyll *b* content (mg/g FW) = 0.1 × (22.9 × A645 - 4.68 × A663), and total chlorophyll content (mg/g FW) =0.1× (20.21 × A645 - 8.02 × A663). The contents of proline, soluble sugars, and soluble proteins were measured according to the instructions of Proline Content Determination Kit, Soluble Sugar Content Determination Kit,

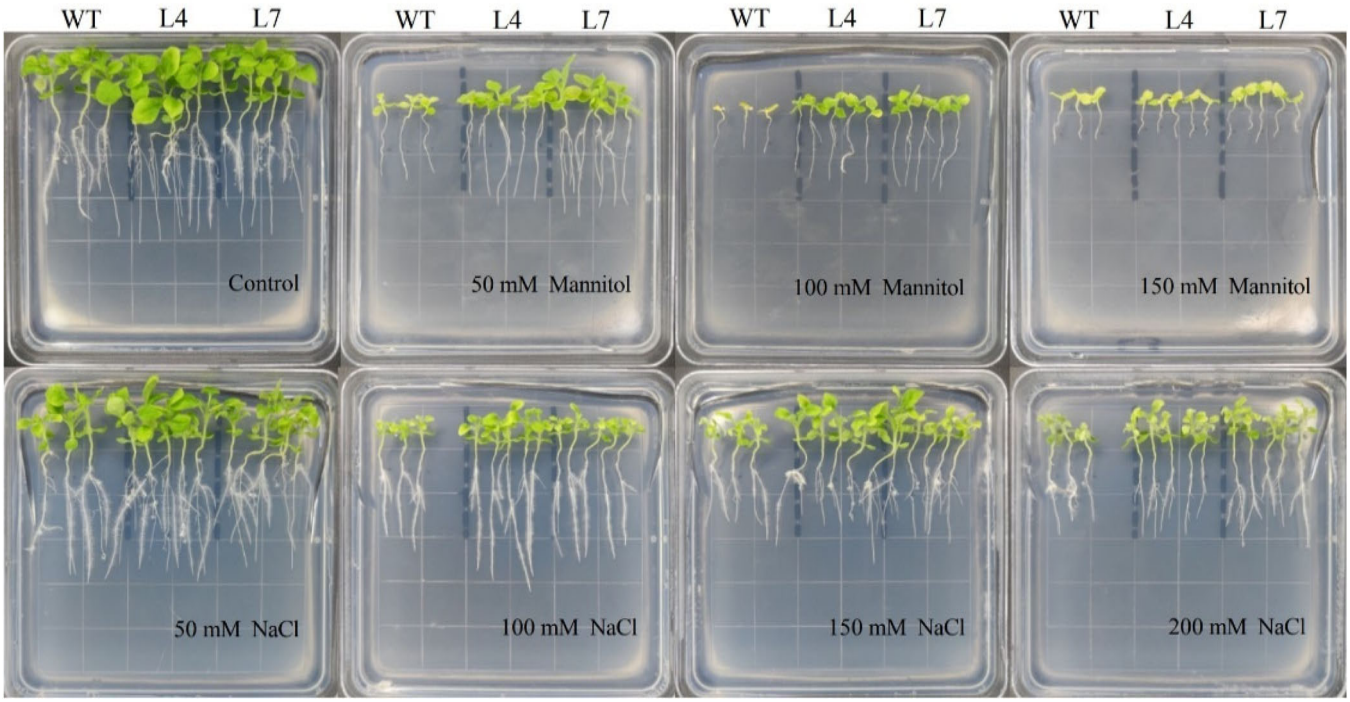

Fig. 7. Effects of different degrees of salt stress and osmotic stress on the shoots and roots of WT and transgenic tobacco. The stress concentration is shown in the lower right corner of the plate; The same control was used for salt stress and osmotic stress; The left, center, and right sides of each plate are WT, L4, and L7, respectively.

and Coomassie Blue Staining Kit for Protein Content from Suzhou Comin Co., Ltd., respectively. The substance contents per gram of plants were calculated according to the standard curve provided in the instructions.

2.13. Determination of activity of antioxidant enzymes

The activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were determined according to the instructions of SOD, POD, and CAT Activity Assay Kits (Comin, Suzhou, China), and enzyme activity per gram of plants were calculated according to the standard curve provided in the instructions.

2.14. Statistical analysis

All the data were presented as means with standard error (SE). Mean ± SE ($n = 3$) values were calculated for each treatment with Microsoft Excel software, the error bars in the figures indicate the SE. Data analysis was examined by one-way analysis of variance (ANOVA) using statistical software (SPSS v19.0, Chicago, USA), Duncan's multiple range tests were used to detect the differences among means at a significance level of $P < 0.05$.

3. Results

3.1. Identification of the shaker K^+ channel genes in sugar beet

Using the sequence of *AtKAT1*, *AtKAT2*, *AtAKT1*, *AKT2*, *AtKC1*, *AtSKOR*, and *AtGORK* from *Arabidopsis* as queries, a total of six genes encoding the shaker-type K^+ channels were identified in the genome of sugar beet, and named as *BvKAT1*, *BvKAT3*, *BvAKT1*, *BvAKT2*, *BvAKT5* and *BvSKOR* (Table 2), according to their sequence similarity. The CDS lengths of these genes ranged from 2232 bp (*BvKAT3*) to 2739 bp (*BvAKT5*), and protein lengths were varied from 743 to 912 aa. Particularly, *BvSKOR* was located on chromosome 2, and length of CDS and protein was 2487 bp and 828 aa, respectively. BvSKOR is a hydrophilic protein and *p*I is 7.20, which is less stable than BvKAT1, BvAKT1, and BvAKT2 (Table 2).

3.2. Chromosomal location of shaker K^+ channel genes in sugar beet

To determine the genome distribution of each shaker K^+ channel genes, physical map of the chromosomal position of these genes were constructed. As shown in Fig. 1, six shaker-type K^+ channels genes were mapped onto four chromosomes (Chr). *BvAKT1* and *BvSKOR* were putative located on Chr 2, and *BvKAT1* and *BvAKT2* were respectively found on Chr 3 and Chr 6, while *BvKAT3* and *BvAKT5* were distributed on Chr 8.

3.3. Cis-acting regulatory elements prediction of promoter regions in shaker K^+ channel gene

To determine the *cis*-acting regulatory elements of shaker K^+ channels in sugar beet, the online tool PlantCARE was used to analyze the 2 kb upstream sequence from transcription initiation site and predict their potential functions. As shown in the Supplementary Table S2, four hormone-related *cis*-acting regulatory elements were retrieved. ABRE was found in the promoter region of *BvAKT1*, *BvAKT2*, and *BvAKT5*, while salicylic acid response elements were found only in *BvAKT2*. Auxin-responsive elements (TGA-elements) were found in *BvSKOR*, and MeJA-responsive elements (TGACG-motif) were found in four members of the shaker K^+ channel family except *BvAKT5*. Additionally, there are *cis*-acting regulatory elements that respond to environment, such as light responsiveness elements (G-box) mainly exist in *BvAKT1*, *BvAKT5*, and *BvSKOR*. The low-temperature responsiveness element (LTR) was identified in *BvKAT1*, *BvAKT1*, and *BvSKOR*. Gibberellin-responsive element (P-box) and wound-responsive element (WUN-motif) were observed in *BvKAT1* and *BvAKT1*.

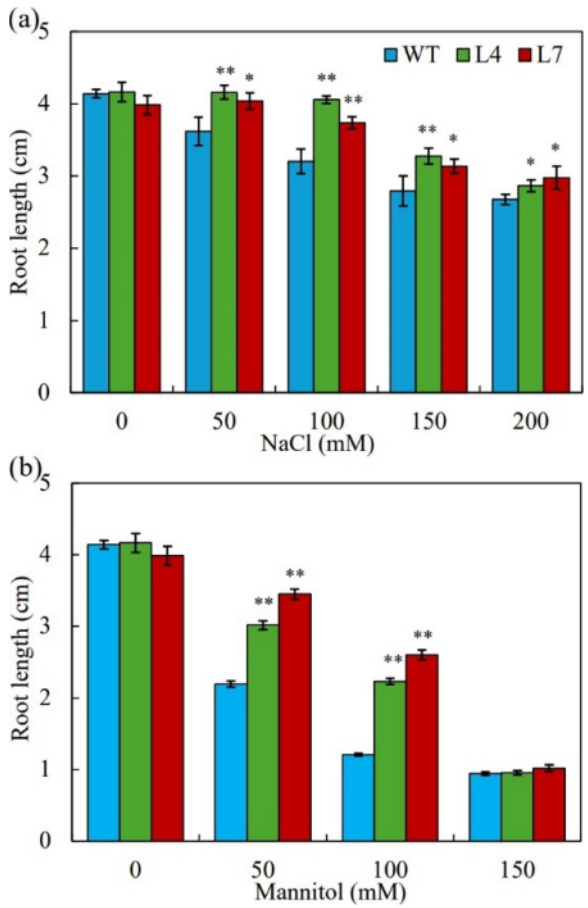

Fig. 8. Effects of different concentrations of NaCl (a) and mannitol (b) treatment on root length of WT and transgenic tobacco. Vertical bars indicate standard error (SE) (n=3), * indicates significant differences at the $P < 0.05$, and ** indicates significant differences at $P < 0.01$.

3.4. Multiple sequence alignments and phylogenetic analysis of shaker K^+ channel genes

To analyze the evolutionary relationship of the *SKOR* genes among different plant species, a phylogenetic tree was constructed through multiple alignments of protein sequences from sugar beet, *Arabidopsis* and other 67 species with MEGA v11.0. The results showed that the 69 *SKOR* genes were divided into three distinct groups according to the similarity of amino acid sequence (Fig. 2). Cluster I was comprised 47 members which included *BvSKOR*, Cluster II contained 10 members, while Cluster III included 12 members. It can be seen from the phylogenetic tree that *BvSKOR* and *ApSKOR* of *Amaranthaceae* family have the highest amino acid similarity, and their evolutionary relationship are the closest.

To further explore the conserved structural domain of SKORs, the protein sequences of *BvSKOR*, *AtSKOR*, *ApSKOR*, *CaSKOR*, *BhSKOR*, and *CmSKOR* were aligned with DNAMAN v10.0. The results indicated that the six transmembrane domains and the C-terminal ring nucleotide binding domain of SKOR are relatively conserved among these genes from different species (Fig. 3).

3.5. Gene structure and protein motif analysis of BvSKOR

To analyze the structural characteristic of *BvSKOR*, the file of gene structure was submitted to the online Gene Structure Display Server 2.0, and the analysis results showed that *BvSKOR* had 13 exons and 14 introns. To better understand the conservation of amino acid sequences of SKOR in different plant species, the motifs of SKOR from six species, such as sugar beet, maize (*Zea mays*) and *Arabidopsis*, were analyzed and visualized by online program MEME. As shown in Supplementary Figure S1, twelve highly conserved motifs, named as Motif 1- Motif 12, were identified in SKOR from six different species, which is consistent with the result of multiple sequence alignment (Fig. 3). All 12 motifs constituted six highly conserved transmembrane domains and the C-terminal ring nucleotide binding domain. Moreover, Motif 2 contained TxGYGD sequence, which is the key amino acid sequence determining ion selectivity.

3.6. Prediction of transmembrane, secondary, and three-dimensional structure in BvSKOR

To investigate the structure of BvSKOR, TMHMM v2.0 was used to analyze the transmembrane domain of BvSKOR. The results showed that the first 400 aa encoded by *BvSKOR* comprised six transmembrane structures (Supplementary Figure S2). This was consistent with the results of other shaker K^+ channel proteins, which were composed of six transmembrane α-helixes and a long C-terminal. As shown in Supplementary Figure S3, the number of α-helixes in BvSKOR is 48.69 %, the extended strand is 14.49 %, while the β-turn and random coil account for 6.77 % and 30.05 %, respectively. Specifically, the α-helix is mainly distributed in the transmembrane region. The second proportion of structure is random coil, which plays the role of interacting with other structures. The extended strand is mainly distributed in the C-terminal domain.

To further explore the structure of BvSKOR, SWISS-MODEL was used to predict the three-dimensional structure of protein. As shown in Supplementary Figure S4, α-helixes occupies most of the transmembrane structure, which is consistent with the predicted structure of the secondary structure. In the functional tetramer, the transmembrane part of the α-helixes formed the core of K^+ transmembrane transport, and the intracellular structure formed the regulatory region of the channel. Additionally, the GMQE of the predicted BvSKOR model is 0.81, which is close to 1, indicating that the three-dimensional structure of BvSKOR is constructed with high accuracy.

3.7. Protein-protein interaction prediction of BvSKOR

The STRING database was used to construct a network of possible interactions between SKOR proteins and with other proteins. As shown in Supplementary Figure S5, BvSKOR interacted with other ion transporters, such as high-affinity K^+ transporter HKT1, Na^+/H^+ antiporter NHX2 and NHX7, K^+ transporter POT1/5/6/7, which belonged to the HAK/KUP family. Meanwhile, K^+-selective plasma membrane ion channel TPK4, K^+ inward rectifier (Kir)-like channel 3 (KCO3) and NRT1/ PTR family 7.3 (NPF7.3) are also predicted to interact with SKOR.

3.8. Expression patterns of BvSKOR under salt and drought treatments

To determine the potential function of *BvSKOR* in response to salt stress, the changes in transcription levels of *BvSKOR* gene were examined in the short- and long- period of salt treatment. When sugar beet was subjected to mild salt stress (50 mM NaCl), the relative expression level of *BvSKOR* in roots and shoots gradually increased with the increase of treatment time, and reached peak value at 120 h after treatment (Fig. 4a). Under moderate salt stress (100 and 150 mM NaCl), the relative expression level of *BvSKOR* in roots increased rapidly at 3 and 6 h after treatments, reached the highest values (reaching 2.5–2.7 times of the control level) at 24 h, and then decreased slightly (Fig. 4b, c). However, the expression level of *BvSKOR* in shoots remained unchanged before 72 h of salt stress. Under severe salt stress (200 and 250 mM), the relative expression level of *BvSKOR* in roots significantly increased by 1.7–2 times at 3 h of treatment, and then reached peak values at 24 h (Fig. 4d, e). Interestingly, the relative expression levels of *BvSKOR* in

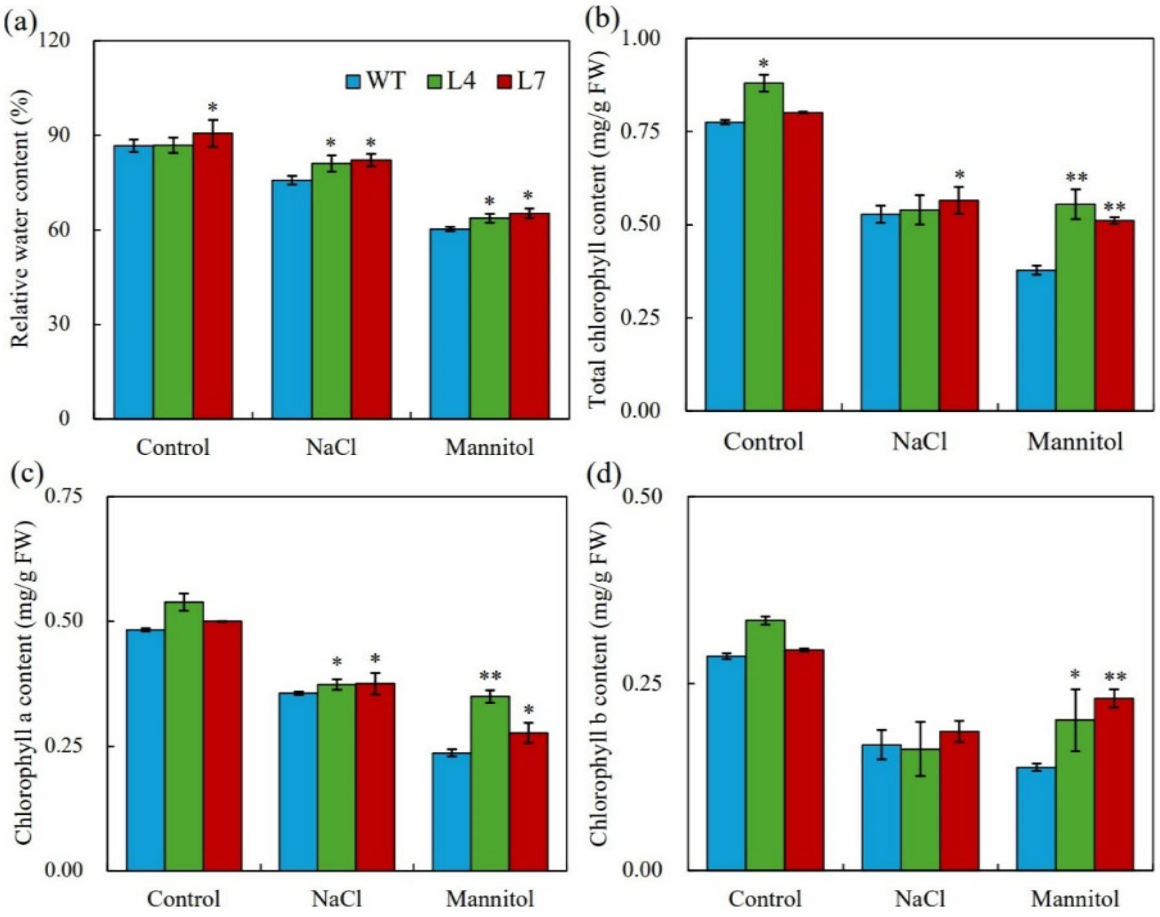

Fig. 9. Effects of different concentrations of NaCl and mannitol treatment on content of relative water (a), total chlorophyll (b), chlorophyll a (c), and chlorophyll *b* (d) in WT and transgenic tobacco. Vertical bars indicate standard error (SE) (n=3), * indicates significant differences at $P < 0.05$, and ** indicates significant differences at $P < 0.01$.

shoots were significantly lower than those in roots at 3–72 h after salt stress, while expression levels in shoots were remarkably higher than those in roots at 120 h salt treatment (Fig. 4d, e). These results suggested that *BvSKOR* plays important roles in the response to salt.

To explore the expression pattern of *BvSKOR* under drought stress, qRT-PCR was used to detect the transcript abundance of *BvSKOR* when plants were treated with PEG for 3, 6, 12, and 24 h, respectively. Under all concentrations of PEG, the expression level of *BvSKOR* in the shoots of sugar beet significantly increased in different treatment times, whereas the expression level in the roots remained unchanged with the increase of PEG concentration (Fig. 5). Under PEG treatments, *BvSKOR* had the higher transcript abundance in roots than in shoots, which directly lead to the plant quickly withered when the PEG processing exceeded 24 h (Fig. 5). These results suggested that *BvSKOR* functions in the response of sugar beet to drought stress.

3.9. Genetic transformation and molecular confirmation of tobacco

To determine the effect of *BvSKOR* on stress tolerance, recombinant vector pEG100-*BvSKOR* was transferred into tobacco plants by *Agrobacterium*-mediated leaf disk transformation. After one month screening in vitro regeneration of the aseptic tissue culture seedlings, a small number of Basta-resistant buds were regenerated from leaf disks on the culture medium containing Basta. Nine regenerated plants were finally obtained in this study. These putative transgenic plants were cultured in soil and sprayed with Basta, while six of them (L1, L2, L3, L4, L7 and L8) survived ultimately. The results of genomic PCR detection with specific primers of *BvSKOR* showed that bands of 2500 bp were amplified in all transgenic lines except WT and L3 (Supplementary Figure S6), indicating that *BvSKOR* was integrated in the genome of tobacco. Seeds of T_0 generation were screened with Basta, and individuals without recombinant vector were eliminated (Fig. 6a), and tobacco seedlings that successfully grew to 4–6 leaves were transferred to soil culture. The qRT-PCR analysis showed that *BvSKOR* was expressed in transgenic plants, while no expression was detected in WT (Fig. 6b). The expression level of *BvSKOR* in the leaves of two transgenic lines (L4 and L7) was higher than that of other transgenic lines (L1, L2, and L8) ($P < 0.05$). Therefore, L4 and L7 were selected to produce T_1 generation seeds for further abiotic stress tolerance research.

3.10. Overexpression of BvSKOR significantly enhanced the stress tolerance of transgenic tobacco

After 10 days of growth on MS medium supplemented with NaCl or mannitol, the growth of all plants was inhibited. The root length of all seedlings was shorter, the plant had fewer and less healthy leaves as the concentration of NaCl and mannitol increased (Fig. 7). However, the WT plants showed more severe growth inhibition than the transgenic tobacco plants. Under 50 and 100 mM mannitol treatments, the leaves of WT were less and the length of roots was significantly shorter than that of transgenic tobacco plants. Lateral roots of WT disappeared, while transgenic tobacco had fewer lateral roots and developed slowly. Under 150 mM mannitol treatment, both WT and transgenic tobacco plants stopped growing. Compared with the control, 50 mM NaCl significantly affected the leaves of tobacco while the length of roots remained unchanged (Fig. 7). Under 100, 150, and 200 mM NaCl, the leaves of WT

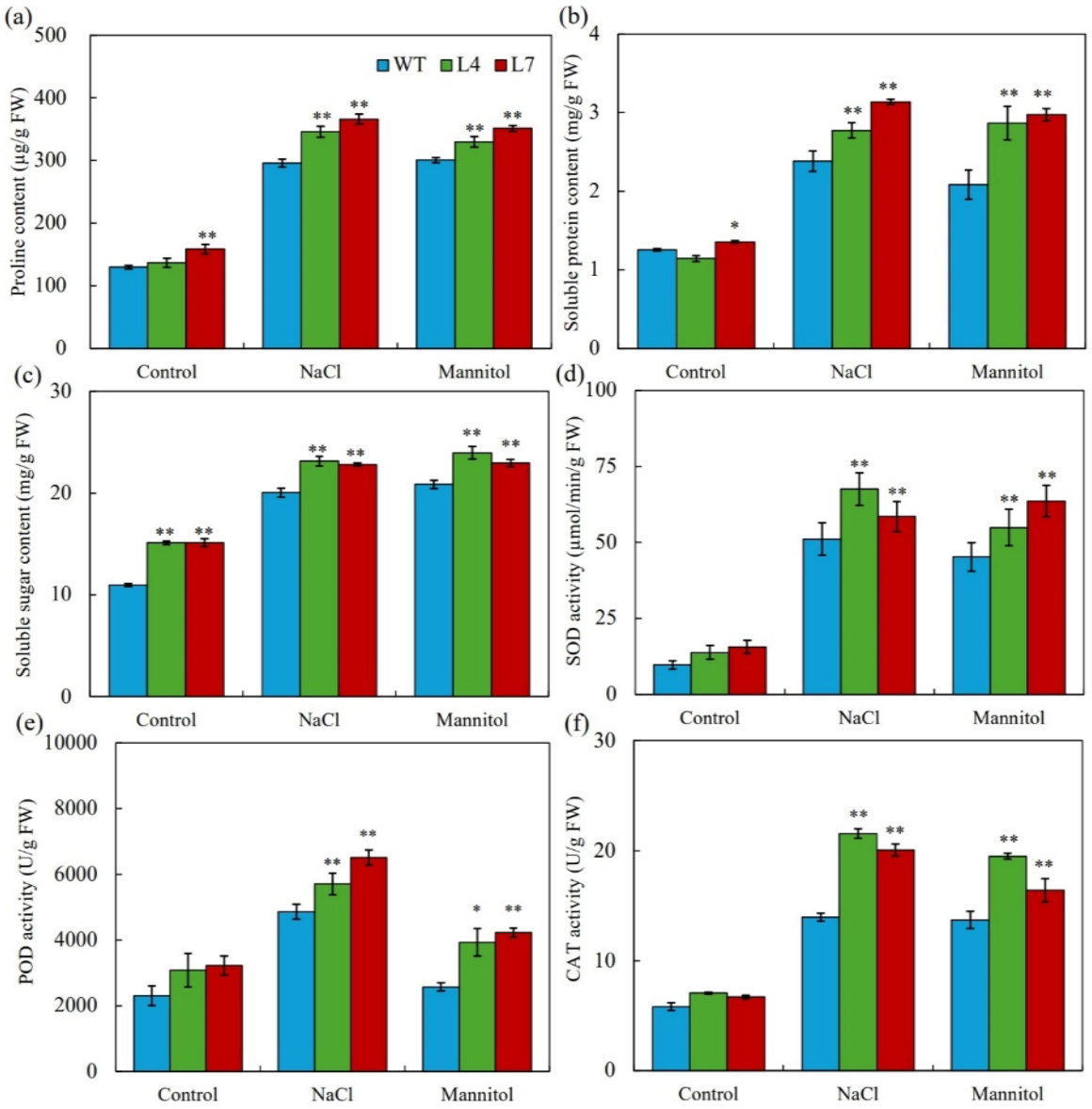

Fig. 10. Effects of different concentrations of NaCl and mannitol treatment on contents of proline (a), soluble protein (b), soluble sugar (c), SOD (d), POD (e), and CAT (f) in WT and transgenic tobacco. Vertical bars indicate standard error (SE) (n=3), * indicates significant differences at $P < 0.05$, and ** indicates significant differences at $P < 0.01$.

and transgenic tobacco plants were further reduced, and the number and length of lateral roots were greatly inhibited. Similarly, roots of WT are shorter than that of transgenic lines (Fig. 7).

There was no significant difference of primary root elongation between WT and transgenic tobacco plants under normal conditions, and the root length of all lines decreased with the increase of NaCl and mannitol concentrations, but the transgenic lines was significantly longer than that of WT (Fig. 8a). Under 50 and 100 mM NaCl treatments, the root length of WT was significantly shorter than that of the control, but the root length of all transgenic plants did not change. Under 150 and 200 mM NaCl, the root length of transgenic lines was significantly reduced compared with control (0 mM NaCl), but significantly longer than WT plants (Fig. 8a). Furthermore, mannitol of 50 and 100 mM significantly reduced the root length of WT and transgenic lines, but the root length of transgenic plants was significantly longer than that of WT plants. However, both WT and transgenic tobacco stopped growing when exposed to 150 mM mannitol (Fig. 8b).

RWC is an important indicator of plant resistance to stress. As shown in Fig. 9a, compared with WT plants, RWC of transgenic plants is equal to or slightly higher under control conditions. However, under salt or mannitol treatments, RWC of both WT and transgenic tobacco was significantly decreased compared to control, whereas the transgenic tobacco was significantly higher than WT (Fig. 9a). These results showed that overexpression of *BvSKOR* increased relative water contents and enhanced salt and drought tolerance in transgenic tobacco plants.

Chlorophyll content is an important factor to increase biomass and yield of plants. Under control condition, the total chlorophyll contents of transgenic L4 were significantly higher than those of WT plants (Fig. 9b). Under salt stress, the chlorophyll contents of WT and two transgenic lines were significantly decreased, but contents of chlorophyll a in transgenic L4 and L7 was significantly higher than those of WT after 10 days of stress treatment (Fig. 9c). Under osmotic stress, the contents of chlorophyll a, chlorophyll *b*, and total chlorophyll in transgenic L4 and L7 were also significantly higher than those of WT (Fig. 9b, c, d).

3.11. Overexpression of BvSKOR significantly increased the ability of osmotic adjustment and activity of antioxidant enzymes in tobacco

When treated with NaCl and mannitol, a series of physiological indicators of WT and transgenic tobacco were tested. As shown in Fig. 10, two stresses significantly increased the content of proline and soluble protein in tobacco leaves, and their contents in WT and transgenic tobacco leaves was 2–3 times higher than those of the control (Fig. 10a, b). The contents of soluble sugars in transgenic lines were also significantly higher than those of WT under either salt or osmotic stress (Fig. 10c). The activities of SOD, POD, and CAT in both transgenic lines and WT were significantly enhanced by NaCl and mannitol, and their activities in transgenic tobacco were significantly higher than those of the WT plants (Fig. 10d, e, f). These results suggested that *BvSKOR* conferred salt and drought tolerance in transgenic tobacco plants through increasing ability of osmotic adjustment and activity of antioxidant enzymes.

4. Discussion

K^+ is one of the most abundant cations in plant cells, accounting for about 10 % dry weight of plants (Kumar et al., 2020). K^+ not only plays important roles in plant cell elongation, enzyme activation, osmoregulation, and photosynthesis, but also enhances plant tolerance to biotic and abiotic stresses (Huang et al., 2017; Kumar et al., 2020; Pathak et al., 2020). The shaker K^+ channel family is a key channel involved in the transport and distribution of K^+ in plants (Sustr et al., 2019; Villette et al., 2019). As a shaker K^+ outward rectifying channel, SKOR plays important roles in the response to abiotic stress. Due to up-regulation of its expression under salt stress, *SKOR* has been speculated as a positive regulatory gene that maintains K^+/Na^+ ratio, avoids K^+ deficiency caused by the competition with high Na^+ concentration, and then enhances salt tolerance in plants (Hu et al., 2016). In previous studies, alkaline salt stress induced the upregulation of the *SKOR* transcription level in roots compared with the control in roots, and H_2S alleviated the alkaline salt-induced increases in *SKOR* expression, indicating H_2S maintained K^+/Na^+ homeostasis by restraining K^+ efflux, which was a result of reduced expression level of *SKOR* to enhance alkaline salt tolerance (Li et al., 2020). Furthermore, Huang et al. (2018) found that after overexpression of *CmSKOR* from *C. melo* in *Arabidopsis*, transgenic plants had stronger salt tolerance by enhancing the flow of K^+ from the roots to the shoots. All the shaker K^+ channel family genes have been identified in some species, such as nine members in *Arabidopsis*, eight members in rice (Musavizadeh et al., 2021; Tian et al., 2021), and nine members in grapes (Cuéllar et al., 2013, 2010; Villette et al., 2019; Dreyer et al., 2019; Nieves-Cordones et al., 2019b; Pratelli et al., 2002). In the current study, members of the shaker K^+ channel family were identified in the genome of sugar beet, the expression patterns of *BvSKOR* under salt and osmotic stress were analyzed, and its function in stress tolerance of transgenic tobacco was verified by observing the phenotypes, measuring and comparing physiological indexes of WT and overexpressed plants.

4.1. Bioinformatics analysis of the BvSKOR gene

The shaker K^+ channel family is one of the most studied K^+ selective channels. According to the genome database of sugar beet, a total of six genes encoding shaker K^+ channels were identified, five inward rectifier channels *BvKAT1*, *BvKAT3*, *BvAKT1*, *BvAKT2*, and *BvAKT5*, and one outward rectifier channel *BvSKOR* (Table 2), which were located on Chr 2, 3, 6, and 8 (Fig. 1), respectively. Promoter regions of these genes contained hormone-related *cis*-acting regulatory elements and light responsiveness elements, indicating that the channel openness may be regulated by these hormones or sensitive to some changes in the external environments (Supplementary Table S2). In this study, *SKOR* from 69 different species were divided into three distinct clusters according to their amino acid sequence similarity (Fig. 2). *BvSKOR* from sugar beet belonged to Cluster I and had the closest evolutionary relation to *ApSKOR* from *A. philoxeroides* (Fig. 2). In SKOR proteins, the six transmembrane domains were highly conserved, and the C-terminal ring nucleotide binding domains were consistent (Fig. 3 and Supplementary Figure S1). Additionally, BvSKOR was found to interact with other ion transporters, such HKT1, HAK/KUP, and NHXs (Supplementary Figure S5).

4.2. The expression pattern of BvSKOR was significantly affected by salt and osmotic stress

There are evidences that *BvSKOR* was expressed in both roots and shoots. As shown in Figs. 4 and 5, *BvSKOR* may be involved in the response of salt and drought stresses, and the up-regulation of expression can be detected within a short term (3 and 6 h) after the stress treatment. The relative expression level of *BvSKOR* in roots and shoots significantly increased with the increase of treatment time when plants was subjected to mild and moderate salt stress (Fig. 4). These results were consistent with previous reports that *EeSKOR*, *MhSKOR*, *PbSKOR*, *VrSKOR*, and *NtSKOR* from *Elytrigia elongata*, *Malus Hupehensi*, *Pyrus betulaefolia*, *Vigna radiat*, and tobacco were significantly up-regulated by low concentrations of NaCl at a short period of time (Azeem et al., 2021; Jin et al., 2021; Li et al., 2021; Zhou et al., 2020; Zhuo et al., 2018). When plants were subjected with high concentrations of NaCl, the expression level of *BvSKOR* in roots increased rapidly and doubled at 3 h, then reached the peak values at 12 and 24 h after treatments (Fig. 4d, e). A similar tendency was also found in *CmSKOR* and *EeSKOR* (Huang et al., 2018; Zhou et al., 2020), which may reflect an adaptation of plants to high salt stress and no longer require sustained high expression. SKOR mainly functions in mediating the transport of K^+ from the roots to shoots of plants, and up-regulation of *BvSKOR* can accelerate the upward transport of K^+ from the root cells, which plays a crucial role in maintenance of ion hemostasis. These results indicated that *BvSKOR* was involved in the response to salt stress in sugar beet. Moreover, at all concentrations of PEG, the expression level of *BvSKOR* significantly increased in roots of sugar beet at different times of treatments (Fig. 5). This is similar to the results observed after PEG treatment in *Vigna radiata* (Azeem et al., 2021) and tobacco (Zhuo et al., 2018). These results proposed that SKOR might be involved in the response of drought in sugar beet.

4.3. Overexpression of BvSKOR significantly enhanced the stress tolerance of transgenic tobacco

Under salt and drought stress, the growth of roots and shoots in both WT and transgenic tobacco plants was inhibited, while the growth of transgenic plants was better than that of WT (Fig. 7). Root length of transgenic tobacco plants was significantly longer than that of WT plants when exposed to NaCl (50–200 mM) and Mannitol (50 and 100 mM) (Fig. 8). These results suggested that overexpression of *BvSKOR* remarkably enhanced salt and drought tolerance in transgenic tobacco plants. The chlorophyll level reflected the ability of plants to photosynthesis under abiotic stress (Li et al., 2022a). In the present study, the chlorophyll contents of transgenic tobacco plants were higher than those of the WT plants under either salt or osmatic stress (Fig. 9b, c, d). The damage to the photosynthesis of transgenic tobacco caused by salt and osmotic stress was less than that of the WT, indicating that tobacco overexpressing *BvSKOR* had stronger tolerance to these stresses. The contents of proline, soluble sugar, and soluble protein in shoots of transgenic tobacco were significantly higher than those in WT (Fig. 10a, b, c). It has been shown that the accumulation of these substances helps to enhance regulatory ability of cells osmotic pressure (Ozturk et al., 2021). In the current study, activities of SOD, POD, and CAT in transgenic tobacco plants were also significantly higher than those in WT under salt and osmotic stress (Fig. 10d, e, f). Higher activities of antioxidant enzymes can help to resist the damage resulting from ROS

produced by abiotic stresses, thus the cells of transgenic plants may encounter less damaged and their survival ability is enhanced under abiotic stresses. These results suggested that *BvSKOR* may functions in the tolerance of transgenic tobacco to abiotic stresses by increasing accumulation of osmoregulators and activity of antioxidant enzymes.

As a shaker K^+ outward rectifying channel, SKOR plays crucial role in maintaining K^+ accumulation and homeostasis in plant cells. The expression level of *SKOR* was significantly up-regulated by reactive oxygen species, and the transport of K^+ from xylem parenchyma cells of roots to shoots was accelerated (Demidchik, 2018; Li et al., 2022b), which accumulated higher levels of K^+ in shoots in response to oxidative stress. There is evidence that plants overexpressed *AtSKOR* accumulated more K^+ and less Na^+ in both roots and shoots compared with WT (Hiya et al., 2024). Similarly, K^+ absorption activity was enhanced when overexpressed *SKOR* in rice, which may lead to an increase in the K^+/Na^+ ratio (Wang et al., 2021). Furthermore, the K^+/Na^+ ratio of *osk5.2* mutant was significantly lower than that of WT when treated with salt (Zhou et al., 2022). These results indicated that SKOR is involved in transport and distribution of K^+ in plants. However, precise functions and regulatory mechanisms of BvSKOR in maintenance of K^+/Na^+ homeostasis need to be addressed in the future research.

5. Conclusion

In the present study, six members of the shaker K^+ channel family, *BvKAT1*, *BvKAT3*, *BvAKT1*, *BvAKT2*, *BvAKT5*, and *BvSKOR*, were identified in the genome of sugar beet, and located on four chromosomes. *BvSKOR* was most closely related to *ApSKOR* from *A. philoxeroides*. Furthermore, the expression levels of *BvSKOR* were significantly up-regulated under salt stress and drought stress. Overexpression of *BvSKOR* significantly enhanced the tolerance of transgenic tobacco to salt and drought stress, and RWC of transgenic plants was significantly higher than that of WT. Transgenic plants accumulated more chlorophyll, soluble sugars and proteins to regulate osmotic pressure balance, and had higher activity of antioxidant enzymes to reduce oxidative damage. These results suggested that *BvSKOR* has research potential in improving the genetic improvement of crop stress resistance by genetic engineering.

CRediT authorship contribution statement

Ya-Dan Hu: Writing – original draft, Investigation, Formal analysis. **Pan-Pan Ren:** Writing – original draft, Investigation, Formal analysis. **Ming Wei:** Formal analysis. **Henri Batoko:** Writing – review & editing. **Guo-Qiang Wu:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2024.106034.

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