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ORIGINAL PAPER

Overexpression of *BvSnRK2.1*, a novel gene encoding the sucrose non-fermenting 1-related protein kinase 2 from sugar beet (*Beta vulgaris* L.), confers salt tolerance in transgenic tobacco

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Abstract

The sucrose non-fermenting 1-related protein kinase 2 (SnRK2) family of protein kinases plays a crucial role in plant salt stress tolerance. To investigate the function of the *BvSnRK2.1* gene in the response to salt stress in sugar beet (*Beta vulgaris* L.), the full-length coding sequence (CDS) of *BvSnRK2.1* was cloned, and the transgenic tobacco lines overexpressing *BvSnRK2.1* were generated. According to the findings, transgenic tobacco lines with overexpression of *BvSnRK2.1* gene from sugar beet showed improved root system development, increased levels of proline, sucrose, soluble protein, total soluble sugars and chlorophyll, increased activities of antioxidant enzymes (e.g., peroxidase, catalase and superoxide dismutase) and improved photosynthetic efficiency in saline conditions. Additionally, genes related to proline synthesis (*NbP5CR*), ion transport (*NbSOS1*, *NbNHX*, and *NbAKT*), and reactive oxygen species scavenging (e.g., *NbCAT*, *NbSOD*, and *NbPOD*) were more highly expressed in tobacco plants overexpressing *BvSnRK2.1*. In short, *BvSnRK2.1* coordinated several defense mechanisms, such as ion homeostasis, osmoregulation and antioxidant, to greatly improve plant response to salt stress.

Keywords Sugar beet (*Beta vulgaris* L.) · Sugar crop · Sucrose non-fermenting 1-related protein kinase 2 (SnRK2) · Salt tolerance · Transgenic tobacco

Abbreviations

ABA	Abscisic acid
ANOVA	One-way analysis of variance
BSA	Bovine serum albumin
CAT	Catalase
CDPKs	Calcium-dependent protein kinases
CDS	Coding sequence
DAB	Diaminobenzidine
MAPKs	Mitogen-activated protein kinases
NBT	Nitroblue tetrazolium
NJ	Neighbor-joining
pEG100	pEarleyGate100
Pn	Net photosynthetic rate
POD	Peroxidase
qRT-PCR	Quantitative real-time polymerase chain reaction
RLKs	Receptor-like kinases
ROS	Reactive oxygen species
SnRK2	Sucrose non-fermenting 1-related protein kinase 2
SE	Standard error
SOD	Superoxide dismutase
WT	Wild-type

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Introduction

Soil salinization is a highly destructive environmental stressor that severely disrupts the normal metabolic processes and photosynthetic efficiency of crops, thereby

leading to substantial declines in crop yields (Qadir et al. 2014; Van Zelm et al. 2020; Vinocur and Altman 2005). This not only exacerbates agricultural economic losses but also accelerates ecosystem degradation (Gao et al. 2024; Zhang et al. 2024). When crops are exposed to salt stress, a complex signaling network is rapidly activated, in which protein kinases have been determined to play a pivotal role as key regulators of signaling cascades. Protein kinase-mediated protein phosphorylation is a prevalent and crucial regulatory mechanism in plants, govering almost all core aspects of plant cell metabolism and signal transduction (Qi et al. 2017). Currently, a variety of protein kinases, including receptor-like kinases (RLKs), mitogen-activated protein kinases (MAPKs), calcium-dependent protein kinases (CDPKs), and SNF1-related protein kinases (SnRKs), have attracted much attention in the studies of plant responses to salt stress (Ding et al. 2024; Li et al. 2022; Wang et al. 2024a; Yang et al. 2025).

The SnRK family is classified into three subfamilies (SnRK1, SnRK2, and SnRK3) based on structural characteristics, functional distinctions, and sequence homology (Halford and Hardie 1998). Among them, the SnRK2 subfamily plays an important role in the abiotic stress response and abscisic acid (ABA) signaling pathway in plants (Huang et al. 2024; Iglesias-Moya et al. 2024). SnRK2s can be categorized into three groups (I, II, and III) based on phylogenetic analysis, and this classification closely correlates with their activation patterns in response to ABA: group I members show no ABA-induced activation, group II members exhibit weak activation by ABA, while group III members display strong ABA-dependent activation (Wu et al. 2021).

The first SnRK2 gene *PKABA1* (ABA-induced protein kinase) was characterized in wheat (*Triticum aestivum*) (Anderberg and Walker-Simmons 1992). Since then, the *SnRK2* genes have been identified in several species such as *Arabidopsis thaliana*, rice (*Oryza sativa*), soybean (*Glycine max*), and sugar beet (*Beta vulgaris*) (Boudsocq et al. 2004; Wu et al. 2021; Xu et al. 2013; Zhao et al. 2017). There are evidences that SnRK2 plays a significant role in the plant response to salt stress. For instance, the expression levels of 7 members of the *SnRK2* family from *Haynaldia villosa* were up-regulated under salt treatment (Liu et al. 2024). Similarly, *PvSnRK2.4* and *PvSnRK2.9* from *Phaseolus vulgaris* were induced to varying degrees of expression by salt stress (Cai et al. 2023). The expression of *CeqSnRK2.7* from *Casuarina equisetifolia* was also induced by high salt stress (Ai et al. 2022). Additionally, heterologous expression of *SnRK2* genes has been shown to enhance salt tolerance in transgenic plants. The expression of *TaSnRK2.4* and *TaSnRK2.8* in *A. thaliana* significantly improved the salt tolerance of transgenic lines (Zhang et al. 2016). Similarly, the expression of *TaSnRK2.9* not only improved the salt tolerance of the transgenic plants, but also promoted the growth of root elongation in tobacco (Feng et al. 2019). Furthermore, the salt tolerance of *A. thaliana* was enhanced by the heterologous expression of *PtSnRK2.5* and *PtSnRK2.7* from woolly poplar (*Populus trichocarpa*), which also maintained the chlorophyll content, root length, and survival rate under salt conditions (Yu et al. 2017). Collectively, these findings have demonstrated that overexpression of *SnRK2* genes is an effective strategy to enhance plant salt tolerance.

Increasing evidence indicates that the overexpression of *SnRK2* genes regulates the transcription and function of a series of stress-related genes. For example, heterologous expression of the *AsSnRK2D* gene from oat (*Avena sativa*) in tobacco not only enhanced superoxide dismutase (SOD) activity and chlorophyll content in the transgenic plants under salt stress, but also regulated the expression of various stress-responsive genes, including those encoding: (1) dehydration proteins (*NtERD10B*, *NtERD10D*, and *NtLEA5* genes), (2) cell signaling components (*NtPLC3* and *NtCaMK1* genes), (3) antioxidant enzymes (*NtMnSOD* and *NtCAT* genes), and (4) proline biosynthesis enzymes (*NtP5CS* genes) (Xiang et al. 2020a). Similarly, heterologous overexpression of the *TaSRK2C1* gene in tobacco significantly up-regulated the transcription of genes encoding transcription factors (e.g., *RD29A*, *DREB1A*, and *DREB2*) and enhanced the salt tolerance of transgenic plants (Du et al. 2013). This transcriptional regulation mediates multi-dimensional improvements in stress responses, such as alleviating oxidative damage and maintaining osmotic balance.

Sugar beet is widely cultivated in the arid and semi-arid area of North China and is known for its excellent resistance to salinity and soil infertility (Alves De Oliveira et al. 2020; Skorupa et al. 2019; Yolcu et al. 2021). The salt tolerance of sugar beet is thought to have originated from its wild ancestor, *Beta maritima* L., which evolved strategies to cope with salt, including ion homeostasis regulation and increasing water content (Doney et al. 1990). These adaptations were subsequently adopted by sugar beet cultivars in regions such as Western Europe and along the Mediterranean coast (Lv et al. 2019). Salt tolerance in sugar beet relies on some mechanisms such as osmotic regulation, ion homeostasis management, and compartmentalization of toxic ions (Wang et al. 2024b). With the aid of technologies such as transcriptomics and genomics, research on its mechanisms of salt tolerance has advanced in recent years (Geng et al. 2019; Wu et al. 2023). Since the completion of the sugar beet genome sequence, it has emerged as an ideal model for studying salt tolerance mechanisms (Dohm et al. 2014). A recent study identified six putative *BvSnRK2* genes in the sugar beet genome, and these genes were induced to varying degrees in both shoots and roots under salt stress (Wu et al. 2021). However, further research is required to

fully understand the specific function of the *BvSnRK2.1* gene in salt tolerance.

In this study, we aimed to elucidate the effect of *BvSnRK2.1* overexpression on salt tolerance in tobacco. Therefore, we generated transgenic tobacco constitutively overexpressing *BvSnRK2.1*, assessed its physiological and biochemical paramaters in a greenhouse salt stress environment, and investigated the expression patterns of abiotic stresses-related genes.

Materials and methods

Plant materials and treatments

The seeds of sugar beet (*B. vulgaris* L. cv. 'Gantang 7') were sown in vermiculite-filled pots and grown in a greenhouse environment where the temperature was controlled at 20 to 25 ℃, the relative humidity was maintained between 65% and 75%, and the light/dark cycle were 16 h of light and 8 h of darkness. During this period, the plants were irrigated with a modified Hoagland's nutrient solution (Wu et al. 2019). After 4 weeks, well-grown sugar beet plants were selected and their leaf samples were collected. The leaf samples were immediately frozen in liquid nitrogen and stored at -80 ℃ until required for RNA extraction.

To verify and characterize the specific function of the gene, the model plant tobacco (*Nicotiana benthamiana*) was selected for this study. After vernalization, sterilized tobacco seeds were sown on Murashige and Skoog (MS) solid medium (1× MS salts, 3% sucrose, 1% agar, pH 5.8) containing BASTA (15 mg/L). After about 3 weeks, tobacco seedlings were transplanted into pots containing a mixture of vermiculite and soil (1:3) and continued to grow in the greenhouse and irrigated with modified Hoagland nutrient solution. Following one week acclimation period, tobacco plants with similar and uniform conditions were selected and divided equally into two groups for subsequent experiments. One group served as a control, cultivated under normal growth conditions, while the other group was subjected to salt treatment with 200 mM NaCl. Each group of treatments was repeated three times.

Phylogenetic analysis of *SnRK2s*

SnRK2 family protein sequences from five species, including *A. thaliana*, cucumber (*Cucumis sativus*), rice, tobacco (*N. tabacum*), and sugar beet, were used to construct an unrooted phylogenetic tree using the Neighbor-joining (NJ) method with MEGA v11 software. The AtSnRK2s protein sequences of *Arabidopsis* were downloaded from The *Arabidopsis* Information Resource (TAIR, http://www.arabidopsis.org); the CsSnRK2s protein sequences of cucumber were retrieved from the Cucurbit Genomics Database (CuGenDB, http://cucurbitgenomics.org/); the OsSAPK protein sequences of rice were obtained from the Rice Genome Annotation Project (RGAP, https://rice.uga.edu/index.shtml); the NtSnRK2s protein sequences of tobacco were acquired from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/); and the BvSnRK2s protein sequences of sugar beet were downloaded from the *Beta vulgaris* Resource (BVR, https://bvseq.boku.ac.at/index.shtml). Detailed accession numbers and full-length protein sequences of SnRK2 family members for each species are provided in Supplementary Table S1.

Cloning and vector construction of the *BvSnRK2.1* gene

Total RNA was extracted from 0.1 g fresh leaves of 4-week-old sugar beet seedlings using the UNlQ-10 Column Trizol Total RNA Isolation Kit (Sangon, Shanghai, China). Subsequently, first-strand cDNA was synthesized using the PrimeScript™ II 1st Strand cDNA synthesis Kit (Takara, Dalian, China). Specific primers of BvSnRK2.1 were designed using Primer Premier v5.0 (Supplementary Table S2). The procedure for PCR amplification was as follows: initial denaturation at 94 ℃ for 2 min; denaturation at 94 ℃ for 30 s, annealing at 62 ℃ for 45 s, and extension at 72 ℃ for 2 min for 35 cycles; the extension was continued at 72 ℃ for 10 min, and then placed at a constant temperature of 4 ℃. The PCR products were electrophoresed on a 1.0% (w/v) agarose gel, and then visualized under UV-transmitted illumination. The PCR amplification products were recovered by SanPrep Column DNA Gel Extraction Kit (Sangon, Shanghai, China) and subsequently placed at -20 ℃ for storage.

The pEarleyGate 100 (pEG100) vector was double digested using the enzymes SpeI and XhoI (Takara, Dalian, China). The cleaved vector and the amplification product were seamlessly ligated by Seamless cloning Master Mix (Sangon, Shanghai, China) and the product was transformed into the *Escherichia coli* DH5α receptor cells. The positive clones in white colonies were screened by PCR, followed by sequencing for verification. Finally, the recombinant vector pEG100-*BvSnRK2.1* was successfully constructed (Supplementary Fig. S1).

Transformation of tobacco *BvSnRK2.1* and producing Transgenic plants

The recombinant plasmid pEG100-*BvSnRK2.1* was introduced into *Agrobacterium rhizogenes* GV3101 using a freeze-thaw method, involving liquid nitrogen snap-freezing

(5 min) followed by 37 °C water bath (5 min). The transformed strain was then used to infect tobacco callus tissues according to the method of Horsch et al. (1985). Following differentiation, budding and rooting of the healing tissues, complete plants were regenerated and subsequently transplanted. To select transgenic plants, T_0 seeds were germinated on MS solid medium supplemented with 15 mg/L BASTA. The obtained *BvSnRK2.1* transgenic plants were transferred to the greenhouse and cultivated under controlled conditions until T_2 seeds were harvested for subsequent experiments.

The qRT-PCR analysis of *BvSnRK2.1* and stress-related genes in transgenic tobacco

The expression level of *BvSnRK2.1* in T_0 and T_2 generation plants was measured by quantitative real-time polymerase chain reaction (qRT-PCR) to evaluate the genetic stability of the transgenic lines; qRT-PCR was then carried out on T_2 generation plants treated with 200 mM NaCl for 24 h in order to elucidate the mechanism of salt stress response. First-strand cDNA was synthesized from total RNA using a 5 × PrimeScript RT Master Mix (Perfect Real Time) First Strand cDNA Synthesis Kit (Takara, Dalian, China). The qRT-PCR was performed using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara, Dalian, China) on a Bioer Quant Gene 9600 Real-Time PCR System (Bioer Technology, Hangzhou, China) according to the manufacturer's instructions. *NtActin* was used as an internal control. The qRT-PCR primers are listed in Supplementary Table S2. The procedure was as follows: 94 °C for 2 min, 95 °C for 5 s, 60 °C for 60 s for 40 cycles and 72 °C for 30 s. The relative expression abundance of genes was analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Measurement of root length

The surface-sterilized and vernalized seeds of wild-type (WT) and *BvSnRK2.1* transgenic plants were sown into MS basal solid medium and incubated in a light incubator for 7 days. Subsequently, uniform seedlings were selected and transferred to 1/2 MS agar medium containing 0, 50, 100, 150, and 200 mM of NaCl. After 7 days of incubation, the root length of the seedlings in each treatment group was measured and images of root growth were captured.

Determination of physiological indicators in transgenic tobacco lines

Following 14 days of treatment with 200 mM NaCl, three mature leaves from each of three individual plants per genotype (WT and transgenic lines) were pooled to form one biological replicate, and three independent biological replicates were prepared for each treatment. Physiological parameters were analyzed, including the activities of SOD, peroxidase (POD) as well as catalase (CAT), contents of proline, sucrose, soluble protein, total soluble sugars, chlorophyll, net photosynthetic rate (Pn), and the concentrations of Na^+ and K^+ were determined.

Antioxidant enzyme activities were measured using commercial kit produced by Suzhou Comin Biotechnology Co., following the manufacturer's instructions. Total soluble sugars and sucrose contents were determined with reference to the anthrone-sulfuric acid methods described by Liu et al. (2008). Soluble protein content was determined by the methods as described by Bradford (1976) using bovine serum albumin (BSA) as a standard protein. Chlorophyll extraction was done using 80% (*v*/*v*) acetone and pigment concentration was determined spectrophotometrically according to the methods as described by Witham et al. (1971). Net photosynthetic rate was measured using the ZEAL-GFS portable photosynthesis measurement system (Agripheno, Shanghai, China) from 9:00–11:00 a.m. with a light intensity of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Na^+ and K^+ contents were determined by flame atomic absorption spectrometry (Shimadzu-AA6800F/G, Suzhou, China) (Zhang et al. 2023). The K^+/Na^+ ratios were calculated according to the method of Wu et al. (2015). Three biological replicates were analyzed for each experiment.

Analysis of histochemical staining

To induce oxidative stress, seedlings that had been cultivated in MS solid medium for seven days were chosen and transferred to MS medium supplemented with 150 mM NaCl for another 2 days. The distribution of reactive oxygen species (ROS) in transgenic and WT tobacco seedlings was examined using nitroblue tetrazolium (NBT) staining for $O_{2\cdot-}$ and diaminobenzidine (DAB) staining for H_2O_2. Following the guidelines provided by the ROS detection kits (DAB and NBT, Solarbio, G4815 and G4816), the working solutions were prepared. Whole seedlings treated with salt-stress were then submerged in the DAB and NBT staining solutions, respectively, and subjected to vacuum infiltration at -0.1 MPa for 30 min. The samples were then incubated in the dark at room temperature for 12 h. Following staining, they were treated with tissue decolorizing solution at 75 °C for 20 min, cooled and rinsed three times with distilled water, preserved in tissue preservation solution for 10 min, and finally photographed to document the staining results.

Statistical analysis

All data were expressed as the mean ± standard error (SE) of three independent experiments. Following one-way analysis of variance (ANOVA), Duncan's multiple range test was performed using SPSS v17.0, with a significance level set at $P < 0.05$.

Results

Phylogenetic tree analysis of *SnRK2s*

Phylogenetic trees of SnRK2 family protein sequences of five species (*A. thaliana*, cucumber, rice, tobacco, and sugar beet) were constructed using the MEGA v11 software (Fig. 1). All SnRK2 family members were classified into three main clades (group I, group II, and group III) based

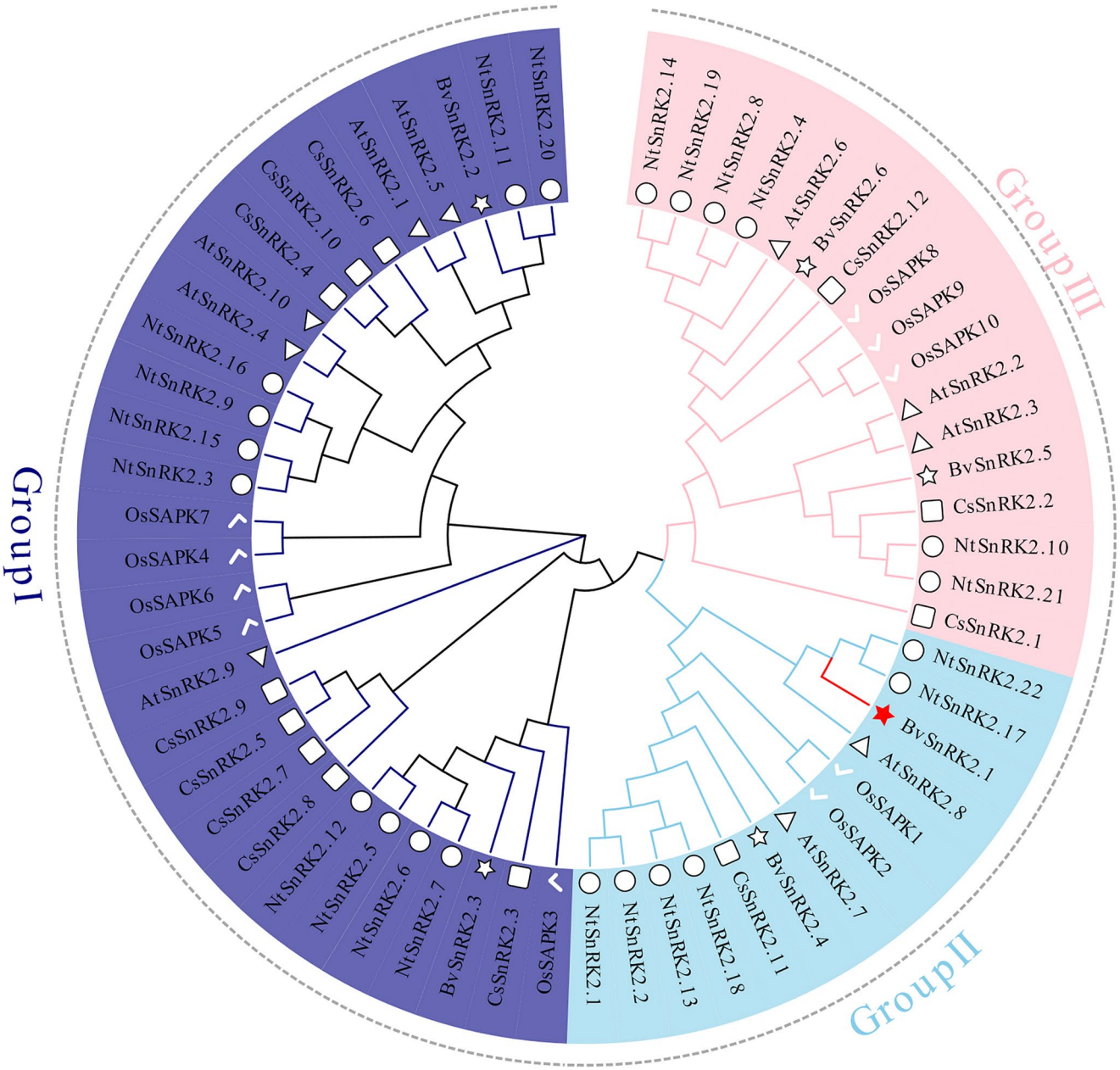


Fig. 1 Phylogenetic analysis of SnRK2 proteins from rice (white check mark, OsSAPK), *Arabidopsis thaliana* (white hollow triangle, AtSnRK2s), cucumber (white hollow rectangle, CsSnRK2s) and sugar beet (pentagram, BvSnRK2s). The unrooted phylogenetic tree was built using MEGA v11 software by the neighbor-joining method. The areas covered by the navy, sky blue, and pink represent Group I, Group II, and Group III, respectively. The accession number and protein sequences of all *SnRK2s* genes used in the present study are listed in Supplementary Table S1

on NJ analysis. BvSnRK2.2 and BvSnRK2.3 were grouped in group I, BvSnRK2.1 and BvSnRK2.4 were grouped in group II, and BvSnRK2.5 and BvSnRK2.6 were grouped in group III.

Genetic transformation of tobacco plants

The recombinant vector pEG100-*BvSnRK2.1* was successfully constructed and subsequently transformed into tobacco plants via *Agrobacterium*-mediated leaf disc transformation (Supplementary Fig. S2). During the 30-day in vitro screening period, discs of transgenic leaf were cultured on MS medium containing 15 mg/L BASTA for selection, followed by regeneration on hormone-supplemented medium. This process induced multiple BASTA-resistant shoots, yielding six positive transgenic lines (L1, L2, L3, L4, L5, and L6) comfired by PCR (Supplementary Fig. S3). qRT-PCR analysis revealed *BvSnRK2.1* expression in lines L2, L3, L4, L5, and L6 lines, with no expression detected in the WT and L1 lines (Fig. 2a). Notably, expression levels of *BvSnRK2.1* in three transgenic lines (L2, L4, and L6) were significantly higher than those in the L3 and L5 ($P<0.05$). T_2 generation plants of the three high expression lines (L2, L4, and L6) were validated to confirm the genetic stability. According to the qRT-PCR results, the *BvSnRK2.1* transcript levels in the three high-expressing lines maintained a consistent expression gradient and did not differ substantially from those of the T_0 generation (Fig. 2b). Based on the molecular validation results of the T_2 generation, the three high-expressing lines were ultimately selected for further functional studies and named as OE1, OE2, and OE3, respectively.

Overexpression of *BvSnRK2.1* promotes root growth and photosynthetic stability in tobacco

To investigate the effect of heterologous expression of the *BvSnRK2.1* gene on salt tolerance and photosynthetic system of tobacco, in vitro culture and soil-based experiments were conducted. In the in vitro culture experiment, OE1, OE2, OE3, and WT seedlings were inoculated onto 1/2 MS solid medium containing different NaCl concentrations for 7 days. The results showed that the growth of all lines was significantly inhibited with the increase of NaCl concentration, as evidenced by progressively shorter root lengths and poorer leaf condition (Fig. 3a). However, the transgenic lines exhibited greater tolerance to salt stress compared with WT. Specifically, under 150 mM NaCl treatment, the root lengths of OE1, OE2, and OE3 were 1.44-, 2.08-, and 2.19-fold longer than those of WT ($P<0.05$) (Fig. 3b). These results suggested that overexpression of *BvSnRK2.1* greatly promoted the growth of the root system of transgenic plants under salt stress. In soil culture, transgenic and WT seedlings were treated with 200 mM NaCl, and the leaves of all lines showed different degrees of wilting after salt stress. Under optimal growth conditions, no significant difference in chlorophyll content was observed between WT and transgenic lines, while Pn in OE1 and OE3 was significantly higher than that in WT. However, chlorophyll contents in OE1, OE2 and OE3 transgentic lines treated with 200 mM NaCl were significantly higher than those in WT plants, as it was increased with 33.94%, 63.25% and 54.02%, respectively (Fig. 3c). Similarly, the Pn of the transgenic lines was significantly higher than that of the WT plants, with the Pn values of OE1, OE2 and OE3 being 2.79-, 2.48- and 2.80-fold higher than those of the WT plants, respectively (Fig. 3d). These results indicated that overexpressing *BvSnRK2.1* enhanced the salt tolerance of transgenic plants by improving the photosynthetic capacity.

Overexpression of *BvSnRK2.1* gene reduces ROS accumulation and increases the activity of antioxidant enzymes in tobacco

To elucidate the role of *BvSnRK2.1* in regulating the antioxidant defense system, ROS (O_2^- and H_2O_2) accumulation patterns and antioxidant enzyme activities (POD, SOD, and

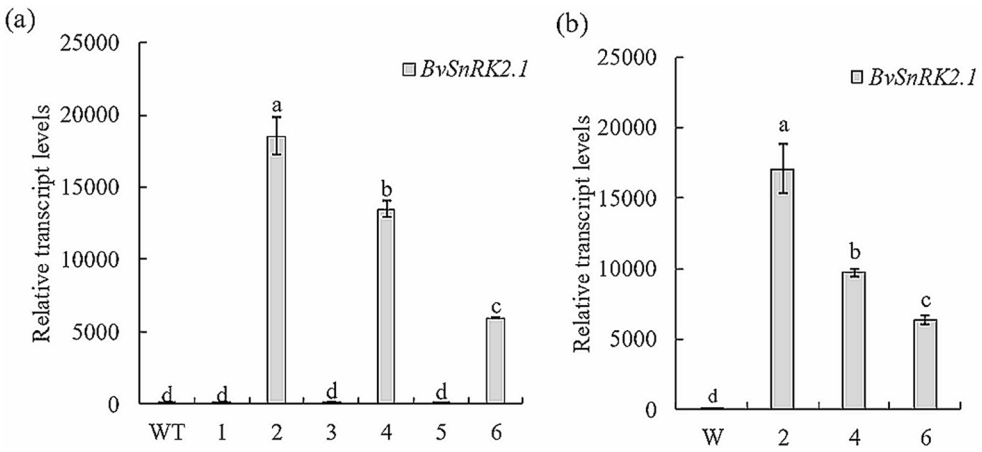


Fig. 2 The expression of *BvSnRK2.1* in T_0**(a)** and T_2**(b)** transgenic tobacco leaves detected by qRT-PCR. Values are means ± standard error (SE), and bars indicate SE ($n=3$). Columns with different letters indicate significant differences, $P<0.05$ (Duncan's test). WT: wild-type; 1–6: six transgenic lines expressing *BvSnRK2.1*

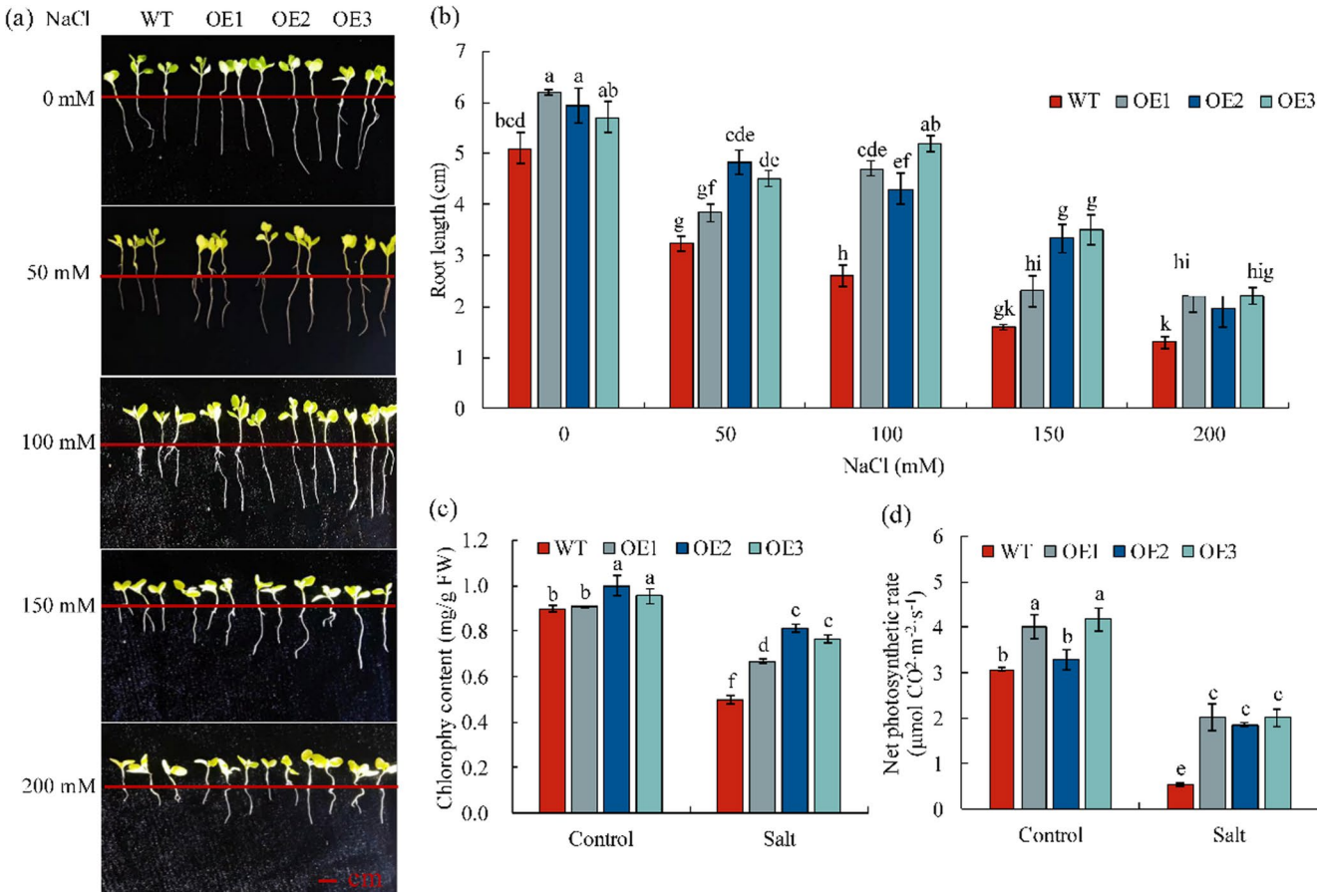


Fig. 3 Effect of salt on growth **(a)**, root length **(b)**, chlorophyll concent **(c)**, and net photosynthetic rate **(d)** in transgenic seedlings and WT. Seedlings were grown on MS basal medium for 7 days, then transferred to 1/2 MS agar medium containing 0-200 mM NaCl for 7 days. **(c)**-(d): Four-week-old soil-grown plants treated with 200 mM NaCl solution for 14 days. Values are means ± standard error (SE), and bars indicate SE ($n=3$). Each replicate contains three seedlings. Columns with different letters indicate significant differences at $P<0.05$ (Duncan's test). WT: wild-type; OE1, OE2, and OE3: three T_2 transgenic lines expressing *BvSnRK2.1*

CAT) were compared between WT and transgenic lines under 150 mM salt stress. DAB and NBT histochemical staining were used to visualize the ROS distribution, and there was a positive correlation between staining intensity and H_2O_2 and $O_{2.-}$ contents. The findings demonstrated that, under optimal growing conditions, there was no discernible difference in staining intensity between WT and transgenic plants. The accumulation of ROS was higher in WT than in overexpression plants, as evidenced by the significantly higher depths of DAB and NBT staining in WT leaf blades following salt stress treatment compared to the leaves of transgenic plants (Fig. 4).

The assay of antioxidant enzymes activities demonstrated that under non-stress conditions, the activities of POD and CAT in the transgenic lines were significantly higher than those in the WT, while there was no significant difference in SOD activity. After salt stress treatment, the activities of all three antioxidant enzymes in transgenic plants were significantly higher than those in the WT plants. Specifically, the POD activities of OE1, OE2 and OE3 were 44.58%, 59.72%, and 39.55% higher than WT; the SOD activities were 23.53%, 31.54%, and 25.81% higher than WT; and the CAT activities were 41.28%, 46.08%, and 32.30% higher than WT, respectively (Fig. 5a-c). In summary, overexpression of the *BvSnRK2.1* gene effectively inhibits the excessive accumulation of ROS under salt stress by enhancing the activity of the antioxidant enzyme system, thereby alleviating the oxidative damage to tobacco plants.

Overexpression of *BvSnRK2.1* enhances osmoregulation in tobacco

To evaluate the impact of *BvSnRK2.1* gene overexpression on the physiological characteristics of tobacco, proline, total soluble sugars, sucrose and soluble protein contents of transgenic lines and WT under salt stress were determined. The results showed that under non-sress conditions, no significant differences were observed between transgenic lines and WT in physiological indices (Fig. 6a-d). However, the accumulation of osmoregulatory substances

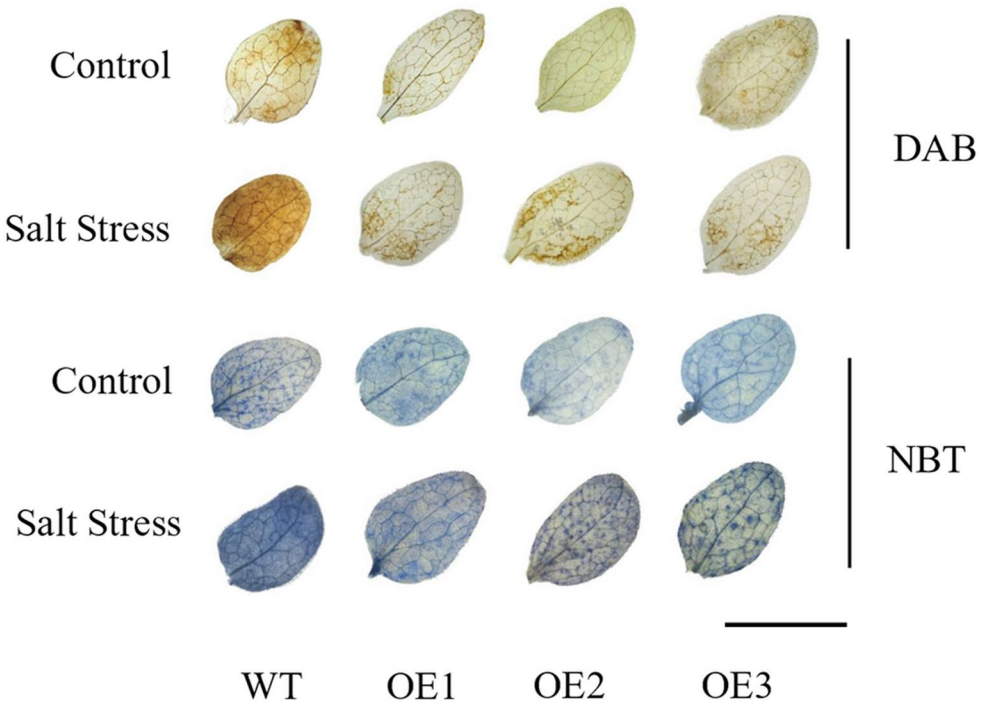


Fig. 4 H_2O_2 and $O_{2.-}$ accumulation in WT, OE1, OE2, and OE3 plants detected by DAB and NBT staining, respectively. Plants were grown on MS solid medium for 7 days, then transferred to medium supplemented with 150 mM NaCl for 2 days. Brown and blue colours represent H_2O_2 and $O_{2.-}$ accumulation, respectively. The scale bar is 0.5 cm

in the transgenic lines was significantly enhanced under salt stress: proline content increased by 37% (OE1), 105% (OE2) and 119% (OE3) compared to WT, respectively (Fig. 6a); the increase in total soluble sugars content ranged from 29.39 to 50.45% compared with that of WT, with the highest increase in OE3 (Fig. 6b); the increase in sucrose content ranged from 32.66 to 47.74% compared with WT, with a significant increase in OE2 (Fig. 6c); soluble protein content increased by 17.01-24.53% compared to WT, with OE2 having the highest increase level (Fig. 6d). These data indicated that *BvSnRK2.1* overexpression significantly enhanced the osmoregulatory ability of transgenic tobacco under salt stress by promoting the accumulation of osmoprotective substances, such as proline and total soluble sugars, thereby maintaining cellular osmotic balance and improving salt tolerance.

Overexpression of *BvSnRK2.1* regulates ion homeostasis in tobacco

The changes in Na^+ and K^+ content in the leaves of WT and transgenic plants under non-sress conditions and NaCl conditions were determined (Fig. 7a-c). Under non-stress conditions, no significant difference in Na^+ and K^+ contents were observed between WT and transgenic plants, with Na^+ contents maintained at low levels in both. However, under salt stress, Na^+ content in both WT and transgenic lines was increased significantly compared with non-stress conditions. Notably, K^+ content in leaves of transgenic lines was significantly higher than that of WT, while Na^+ content was significantly lower than WT, which caused a higher K^+/Na^+ ratio in transgenic plants. These results indicated that overexpression of the *BvSnRK2.1* gene can effectively reduce Na^+ accumulation, increase K^+ content, and maintain a high K^+/Na^+ ratio in leaves, thereby maintaining ion homeostasis under salt stress.

Overexpression of *BvSnRK2.1* regulates antioxidant, osmoregulation and ion transport genes

To elucidate the underlying molecular mechanisms of salt tolerance in transgenic lines, the expression patterns of seven typical abiotic stress-related genes were analyzed, with the aim of ascertaining the function of the *BvSnRK2.1* gene in tobacco. Under non-sress conditions, no significant difference in the expression of the seven abiotic stress-related genes (*NbCAT*, *NbSOD*, *NbPOD*, *NbP5CS*, *NbSOS1*, *NbNHX*, and *NbAKT*) was observed between transgenic lines and WT. However, the expression levels of antioxidant enzyme genes (*NbCAT*, *NbSOD* and *NbPOD*) were significantly upregulated in transgenic lines under salt stress (Fig. 8a-c). Compared to the WT, the expression level of *NbCAT* in the OE2 line increased by 1.28-fold. In addition, the expression levels of *NbSOD* and *NbPOD* in the three transgenic lines ranged from 1.5- to 2.5-fold higher than those of WT. Additionally, the expression level of *NbP5CS*, a pivotal gene encoding proline biosynthetic enzymes, was considerably upregulated under salt stress conditions (Fig. 8f). The expression levels of *NbP5CS* in the transgenic lines were 2.88-, 3.03-, and 2.86-fold higher than those of

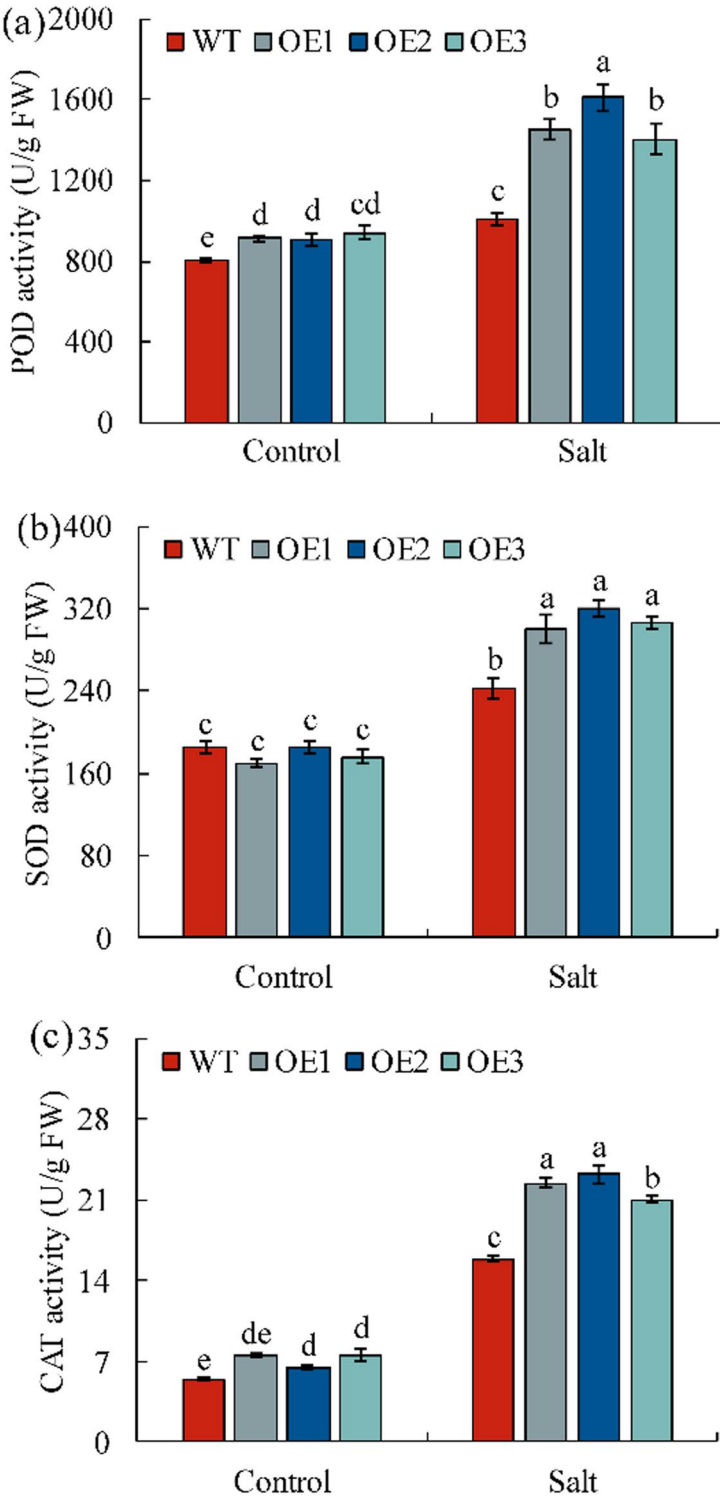


Fig. 5 Effect of *BvSnRK2.1* overexpression on the activities of antioxidant enzymes POD **(a)**, SOD **(b)**, and CAT **(c)** in tobacco under salt sress conditions. Four-week-old soil-grown plants were treated with 200 mM NaCl solution for 14 days. Values are means ± standard error (SE), and bars indicate SE ($n = 3$). Each replicate contains three seedlings. Columns with different letters indicate significant differences at $P < 0.05$ (Duncan's test). WT: wild-type; OE1, OE2, and OE3: three T_2 transgenic lines expressing *BvSnRK2.1*

WT, respectively. Furthermore, the expression levels of ion transport-related genes (*NbSOS1*, *NbNHX*, and *NbAKT*) in transgenic lines were significantly higher than those in WT under salt stress (Fig. 9a-c). Overall, the expression levels of the abiotic stress-related genes were significantly higher in the transgenic lines than in the WT under salt stress.

Discussion

Salt stress imposes major constraints on plant growth and crop yield (Ma et al. 2024). SnRK2 kinases, phylogenetically divided into three groups, play distinct roles in stress responses: group I SnRK2s (e.g., AtSnRK2.4) rapidly

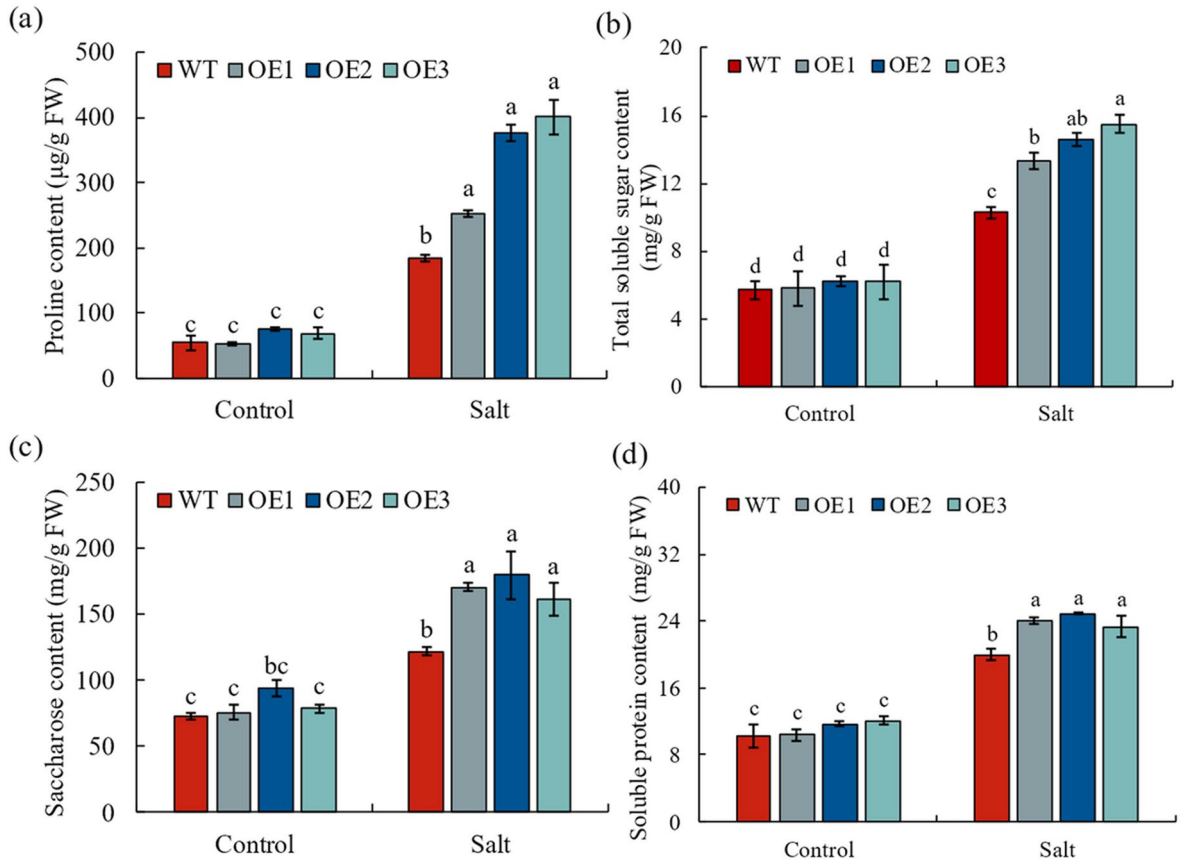


Fig. 6 Effect of *BvSnRK2.1* overexpression on **(a)** proline, **(b)** total soluble sugars, **(c)** sucrose, and **(d)** soluble protein concents in tobacco under salt stress. Four-week-old soil-grown plants were treated with 200 mM NaCl solution for 14 days. Values are means ± standard error (SE), and bars indicate SE ($n = 3$). Each replicate contains three seedlings. Columns with different letters indicate significant differences at $P < 0.05$ (Duncan's test). WT: wild-type; OE1, OE2, and OE3: three T_2 transgenic lines expressing *BvSnRK2.1*

regulate ROS homeostasis in an ABA-independent manner, while group III SnRK2s (e.g., AtSnRK2.6) mediate ABA-dependent stomatal closure (Fujii et al. 2009; Szymańska et al. 2019). In contrast, group II SnRK2s like BvSnRK2.1 remain poorly characterized in salt tolerance mechanisms. Notable examples of salt-responsive genes included *CiPawSnRK2.1* in pecan (*Carya illinoinensis*) (Wang et al. 2024a) and salt-inducible *CoSnRK2* in *Corchorus olitorius*, *CcSnRK2* in *Corchorus capsularis* and *BvSnRK2.1* in sugar beet (Ahmed et al. 2023; Wu et al. 2021). Phylogenetic analysis has revealed that BvSnRK2.1 was clustered within Group II on the evolutionary tree (Fig. 1), a characteristic that clearly distinguishes it from the Group I and III kinases. Given that sugar beet is an important salt-tolerant crop, elucidating the functional roles and molecular mechanisms of the *BvSnRK2.1* under salt stress represents a critical research gap. (I, II, and III)

Role of *BvSnRK2.1* in ROS scavenging and antioxidant system

Under salt stress, osmotic stress, ionic stress, nutrient imbalance and their secondary effects within plants synergistically induce the overproduction of ROS (Hasanuzzaman et al. 2021; Yang et al. 2023). Excessive ROS can disrupt or damage cell membrane through strong oxidative effects, trigger lipid peroxidation, and lead to cellular oxidative damage or even programmed cell death (Ning et al. 2010; Qi et al. 2018), posing a severe threat to plant survival. To cope with this, plants have evolved a sophisticated antioxidant defense system, in which SOD, CAT, and POD play a central roles in collaboratively scavenging ROS to maintain cellular redox homeostasis (Li et al. 2020). Under stress conditions, the expression of genes encoding these enzymes was up-regulated to enhance antioxidant capacity (Nadarajah 2020; Zhao et al. 2021), serving as a key mechanism for plants to adapt stresses. In this study, the results of DAB and NBT histochemical staining revealed that after salt stress treatment, the color development intensity of H_2O_2 and

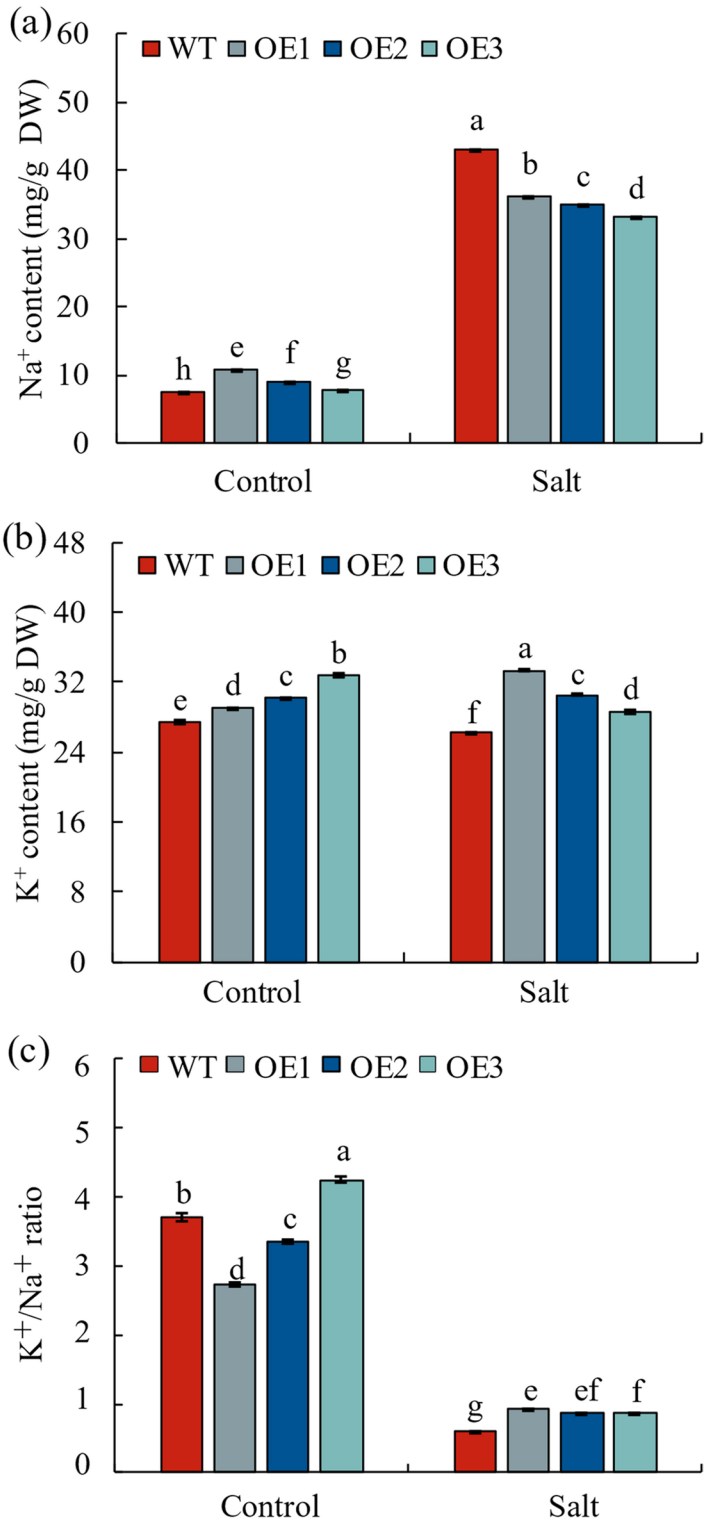


Fig. 7 Effect of *BvSnRK2.1* overexpression on Na^+ content **(a)**, K^+ content **(b)** and Na^+/K^+ ratio **(c)** in tobacco under salt stress. Four-week-old soil-grown plants were treated with 200 mM NaCl solution for 14 days. Values are means ± standard error (SE), and bars indicate SE ($n = 3$). Each replicate contains three seedlings. Columns with different letters indicate significant differences at $P < 0.05$ (Duncan's test). WT: wild-type; OE1, OE2, and OE3: three T_2 transgenic lines expressing *BvSnRK2.1*

$O_2^{\cdot-}$ in the leaves of WT tobacco plants was significantly higher than that in the transgenic lines overexpressing *BvSnRK2.1*. This indicated that the accumulation of ROS in the transgenic plants was significantly lower than that in the WT (Fig. 4). This aligns with the SnRK2 family roles in ROS regulation: *AtSnRK2.4* and *−2.10* (group I) maintain ROS homeostasis through CAT regulation (Szymańska et al. 2019), *TaSnRK2.9* (group III) enhances salt tolerance by increasing SOD, CAT, POD activities and gene expression (e.g., *NtSOD* and *NtCAT*) (Feng et al. 2019). Further analysis demonstrated that overexpression of *BvSnRK2.1* not only significantly enhanced the enzymatic activities of

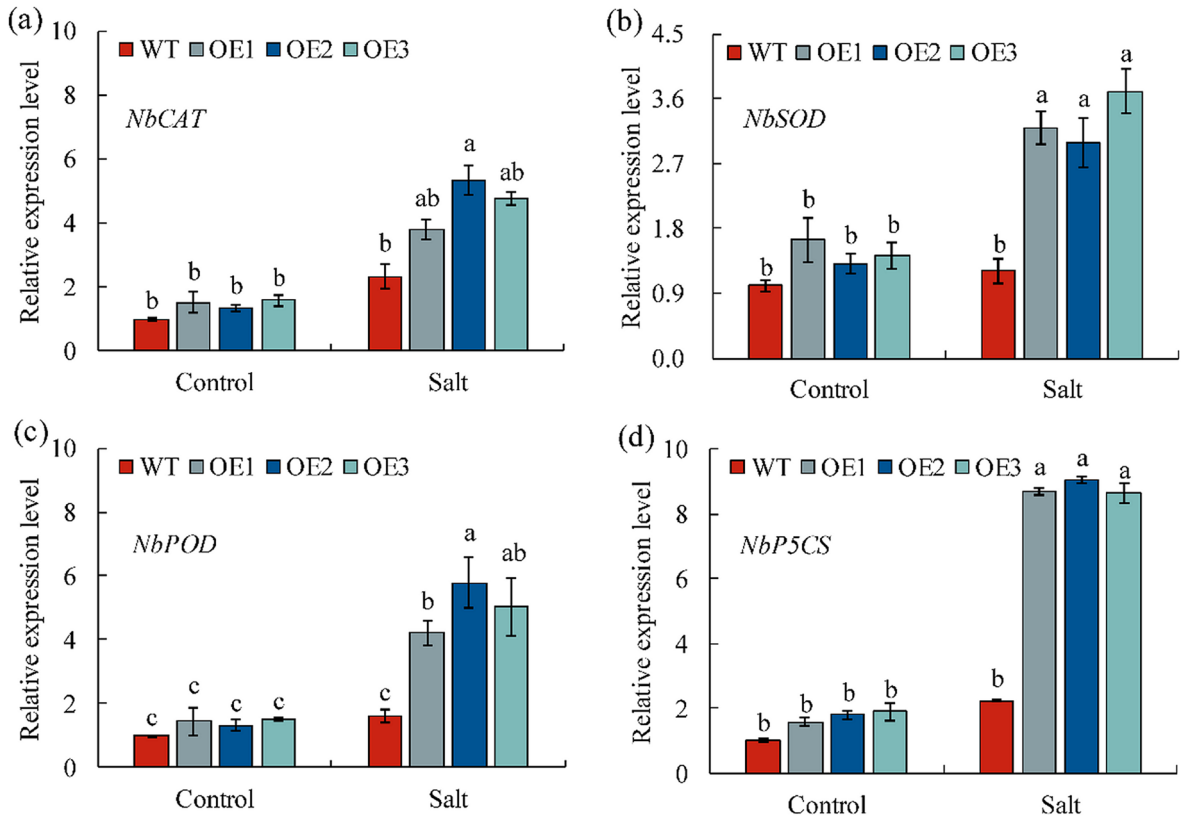


Fig. 8 Transcript levels of ROS and proline synthesis-related genes in WT and transgenic lines under salt stress. Relative expression values of *NbCAT***(a)**, *NbSOD***(b)**, *NbPOD***(c)**, and *NbP5CS***(d)**. Four-week-old soil-grown plants were treated with 200 mM NaCl solution for 24 h. Values are means ± standard error (SE), and bars indicate SE ($n = 3$). Each replicate contains three seedlings. Columns with different letters indicate significant differences at $P < 0.05$ (Duncan's test). WT: wild-type; OE1, OE2, and OE3: three T_2 transgenic lines expressing *BvSnRK2.1*

SOD, CAT and POD under salt stress, but also upregulated the transcript levels of antioxidant-related genes, such as *NbSOD*, *NbCAT* and *NbPOD* (Fig. 8a-c). Consistent with the staining results, the increased enzyme activities and gene expression collectively suppressed the excessive accumulation of ROS, thereby alleviating the oxidative damage induced by salt stress. These results indicated the molecular mechanism by which *BvSnRK2.1* synergistically enhances antioxidative capacity through multiple pathways in plants.

Role of *BvSnRK2.1* in osmoregulation and proline biosynthesis

In response to abiotic stresses, plants initiate a series of complex and delicate physiological regulatory mechanisms (Apostolova 2024; Xu et al. 2024). Among them, dynamic changes in physiological parameters such as proline, total soluble sugars, sucrose, and soluble proteins play a key role in the adaptation of plants to stresses. The essence of these changes is to reduce the cellular osmotic potential and maintain the homeostasis of the intracellular environment through the accumulation of intracellular organic osmoregulatory substances (Zhang et al. 2010; Silva-Ortega et al. 2008; Yamada et al. 2005; Zhu et al. 2022). In the present study, the contents of proline, total soluble sugars, sucrose, and soluble protein were significantly higher in transgenic plants than in the WT plants under salt stress. It was revealed that overexpression of *BvSnRK2.1* induced a series of positive physiological responses in plants, which in turn promoted the synthesis and accumulation of multiple osmoregulatory substances (Fig. 6a-c). Transgenic tobacco overexpressing *BvSnRK2.1* had enhanced resilience due to physiological changes with stronger stress tolerance and can maintain healthy growth in high-salt environments effectively mitigating salt damage.

As key osmoregulatory substances in plants, proline and total soluble sugars are of integral significance in alleviating the negative impacts of salt stress on plants and are widely acknowledged as crucial osmoregulators for enhancing plant salt tolerance (Xiong et al. 2014). In the proline biosynthesis pathway, the enzyme encoded by the *P5CS* gene serves as the main rate-limiting enzyme, exerting a positive regulatory effect on the plant stress response (Verbruggen and Hermans 2008). Under salt stress, cactus

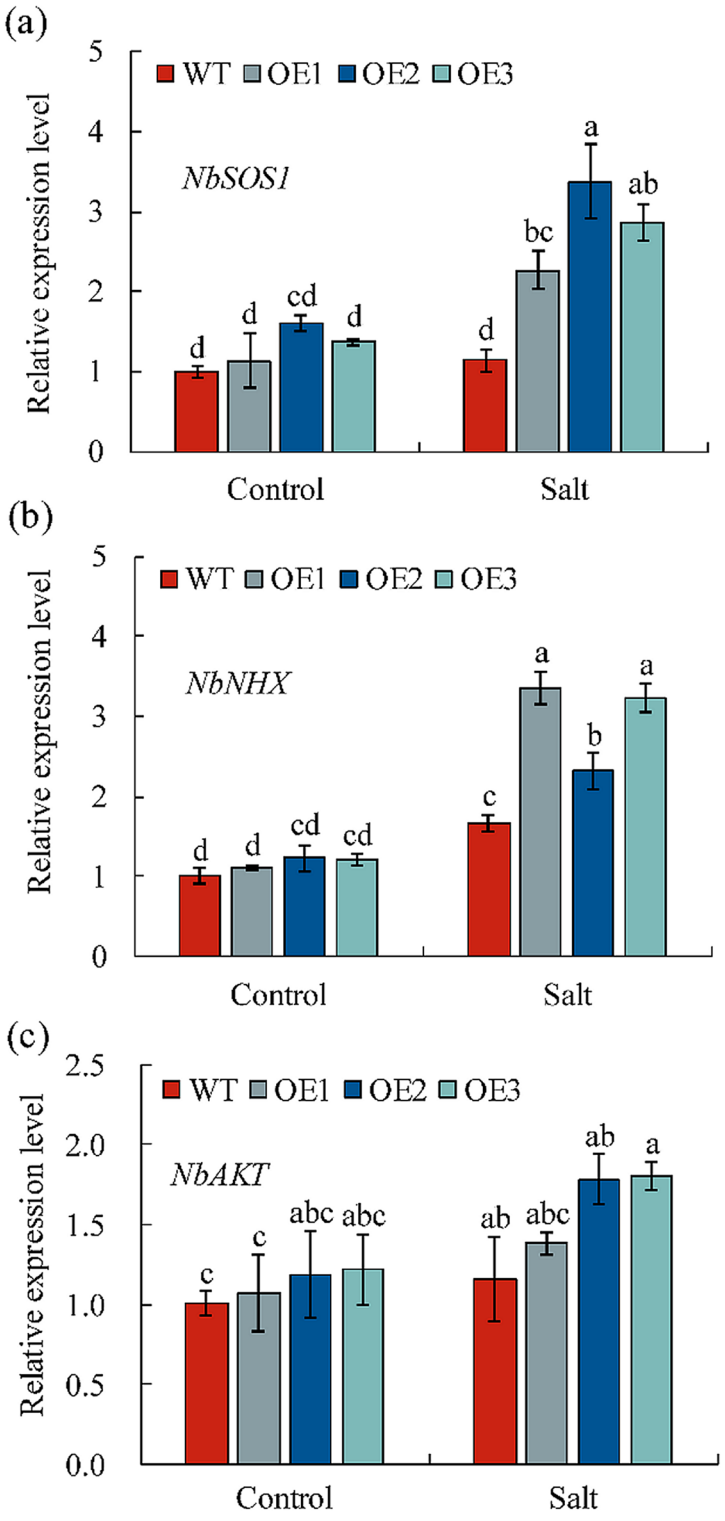


Fig. 9 Transcript levels of ion transport genes in WT and transgenic lines under salt stress. Relative expression values of *NbSOS1***(a)**, *NbNHX***(b)**, and *NbAKT***(c)**. Four-week-old soil-grown plants were treated with 200 mM NaCl solution for 24 h. Values are means ± standard error (SE), and bars indicate SE ($n = 3$). Each replicate contains three seedlings. Columns with different letters indicate significant differences at $P < 0.05$ (Duncan's test). WT: wild-type; OE1, OE2, and OE3: three T_2 transgenic lines expressing *BvSnRK2.1*

pear (*Opuntia streptacantha*) promoted proline accumulation and significantly bolstered salt tolerance by upregulating the expression level of the *P5CS* gene (Silva-Ortega et al. 2008). Similarly, researchers have demonstrated a strong positive correlation between the induced expression of the *P5CS* gene and proline accumulation in *A. thaliana* and rice (Igarashi et al. 1997; Yoshiba et al. 1995). In the current study, transgenic tobacco overexpressing *BvSnRK2.1* significantly up-regulated the expression of the *NbP5CS* gene, in turn, which facilitated a substantial accumulation of proline and was pivotal in mitigating the damage inflicted by salt stress on plants.

Interestingly, the total soluble sugars content of the transgenic lines under 200 mM NaCl was significantly higher than that of WT, with an increase of 29.39-50.45% (Fig. 6b). These results suggested that the *BvSnRK2.1* gene is not only involved in the synthesis of osmotic stress substances, but also in carbohydrate metabolism under salt stress, possibly through the regulation of the expression of sucrose metabolism-related gene. This is similar to the regulatory role of SnRK1 family members in carbon metabolism (Jiang et al. 2013; Xiang et al. 2020b). However, further research is required to fully elucidate the stress-response mechanism of the *SnRK2.1* gene in carbohydrate metabolism and its specific molecular action pathways.

Role of *BvSnRK2.1* in the regulation of ion homeostasis and photosynthesis

As the salinity of the plant growth medium increases, the endogenous Na^+ concentration rises. High concentration of Na^+ have two effects. Firstly, they inhibit enzyme activity. Secondly, they prevent the normal uptake of K^+, which in turn leads to K^+ deficiency in the cytoplasm (Zang et al. 2016). In such a situation, the cell must possess effective mechanisms to transport or segregate Na^+ to keep its level non-toxic. Plants with different salt-tolerant genotypes maintain ion homeostasis in plant cells by adopting diverse strategies to restrict Na^+ entry into the cell (Amin et al. 2021). For instance, the Na^+-specific transporter proteins encoded by the *NHX* family genes are pivotal in regulating Na^+ efflux, compartmentalization, and intercellular transport. Moreover, high-affinity K^+ transporter (HAK) and *Arabidopsis* K^+ transporter (AKT) are mainly responsible for K^+ uptake and transport, which are essential for maintaining intracellular K^+ levels (Chakraborty 2019). Additionally, the K^+/Na^+ ratio in the cytoplasm of plants serves as a key indicator of their salinity tolerance, and higher ratios imply greater salinity tolerance (Alkhateeb et al. 2015; Liu et al. 2021; Mishra et al. 2014). In this study, the expression of *NbAKT*, *NbNHX*, and *NbSOS1* genes was significantly up-regulated in transgenic tobacco following salt treatment (Fig. 9a-c). These findings indicated that the proteins encoded by these genes might contribute significantly to the regulation of K^+/Na^+ homeostasis, as reported in other crops (Bose et al. 2013; Chakraborty 2019; Chakraborty et al. 2016). Overall, *BvSnRK2.1* enhances K^+ retention and reduce Na^+ accumulation by upregaulating *NbAKT*, *NbNHX*, and *NbSOS1* expression (Fig. 9a-c), a regulatory mode distinct from other SnRK2 subgroups. While Group III SnRK2s (e.g., AtSnRK2.6) mediate ABA-dependent ion transport via direct phosphorylation of SOS1 (Fujii et al. 2009), Group I SnRK2s lack established links to ion homeostasis in current reports, with their characterized roles focusing on ROS regulation and mRNA decay (Soma et al. 2017; Szymańska et al. 2019). This demonstrates Group II SnRK2s' unique role in integrating ion homeostasis with salt tolerance.

Under salt stress, the chlorophyll content in leaves can visually reflect the responsiveness of plant photosynthesis (Phan 2016). Higher levels of chlorophyll can protect cells from damage caused by excessive dehydration, thereby maintaining the integrity of intracellular fine membrane structures and enhancing the protective capacity of the cell membrane system (Phan 2016). However, excessive salinity usually leads to a decrease in chlorophyll content in leaves and reduces the effective area for photosynthesis (Mousavi et al. 2022; Navarro et al. 2023). In this study, salt stress reduced chlorophyll levels and photosynthetic efficiency in both transgenic lines and WT plants. Importantly, however, transgenic lines exhibited less pronounced reductions: under 200 mM NaCl, OE1-OE3 chlorophyll levels were 33.94–63.25% higher than WT (Fig. 3c), and Pn values were 2.48–2.80-fold higher (Fig. 3d), indicating *BvSnRK2.1* overexpression alleviated salt-induced reductions in photosynthetic parameters. This aligns with the reports by Song et al. (2016), Wang et al. (2023) and Zhang et al. (2010), showing that *PtSnRK2.7*, *TaSnRK2.8*, and *CsSnRK2.11* overexpressing lines maintained higher chlorophyll levels under stress. Collectively, these results suggest *BvSnRK2.1* actively regulates plant salt stress responses and maintains normal photosynthesis.

BvSnRK2.1 coordinates multi-pathway responses to enhance salt tolerance in plants

Three mechanisms underlie the inhibition of plant growth caused by salt stress, oxidative damage, osmotic imbalance, and Na^+ toxicity (Zhu. 2016). In this study, overexpressing the sugar beet *BvSnRK2.1* gene could greatly increase resistance to salt in tobacco (Fig. 10). Transcription of the Na^+ efflux gene *NbSOS1*, the compartmentalization gene *NbNHX*, and the K^+ uptake gene *NbAKT* was markedly increased in transgenic lines under salt stress (Fig. 9a-c), which resulted in a lower intracellular Na^+/K^+ ratio (Fig. 7c). This regulatory pattern was consistent with the function of *FvSnRK2182* in activating ion transport pathways (Zhang et al. 2022). Furthermore, the activities of antioxidant enzymes such as POD, SOD, and CAT in transgenic plants were enhanced under salt stress (Fig. 5a-c), accompanied by a significant reduction in ROS (H_2O_2 and $O_2^{\cdot-}$) accumulation (Fig. 4), and the induction of antioxidant enzyme-encoding genes (*NbCAT*, *NbSOD* and *NbPOD*) (Fig. 8a-c). These results suggested that *BvSnRK2.1* may enhance ROS scavenging capacity through transcriptional or post-transcriptional regulation. Moreover, chlorophyll content (Fig. 3c) and Pn (Fig. 3d) were significantly increased in

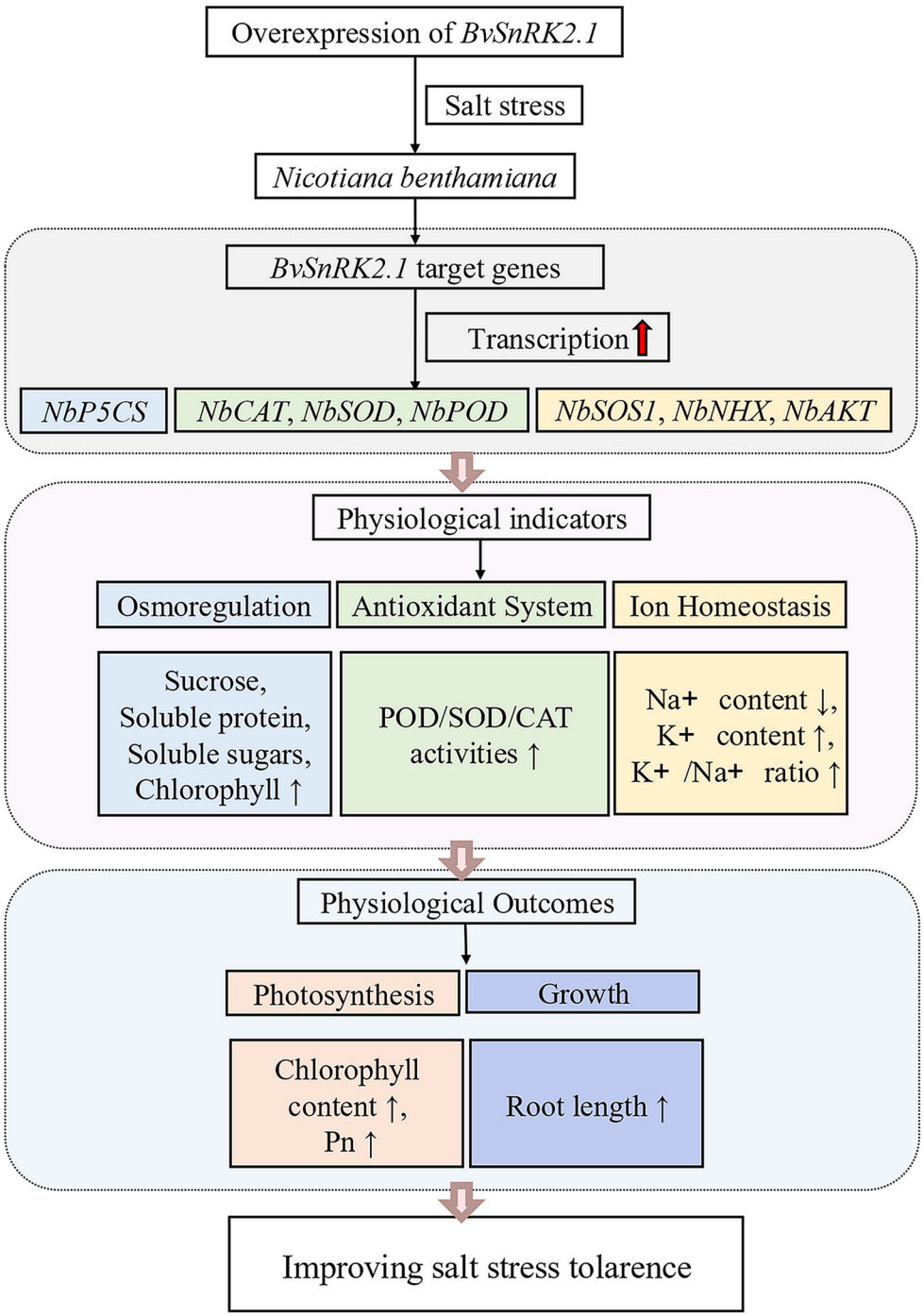


Fig. 1 The working model of the mechanism whereby *BvSnRK2.1* increases salt tolerance. Salt treatment of WT and tobacco overexpressing *BvSnRK2.1* suggested that the overexpression of *BvSnRK2.1* in tobacco triggered genes linked to stress and activated the physiological processes connected with these genes. This helps to improve tolerance to salt stress by maintaining ROS homeostasis, maintaining Na^+ and K^+ homeostasis, and increasing the accumulation of organic osmoregulatory chemicals. The direct targets of *BvSnRK2.1* and upstream signaling pathways require further validation

transgenic plants under salt stress, likely due to enhanced activities of antioxidant enzymes that protect chloroplasts from ROS damage (Mazur et al. 2021). Concurrently, the stress-induced accumulation of proline (Fig. 6a) coincides with the upregulation of osmotic adjustment gene *NbP5CS* expression (Fig. 8d), indicating that it may buffer the osmotic shock of salt stress by enhancing the synthesis of osmoprotective substances.

Notably, BvSnRK2.1 is classified in the phylogenetic tree as a member of SnRK2 group II (Fig. 1), and its enhancement of ion homeostasis, ROS scavenging, and photosynthetic efficiency observed in transgenic plants were consistent with a salt-specific adaptation mechanism (Kaur et al. 2022; Mazur et al. 2021). The upregulation of ion transporter genes highlighted their role in the maintenance of Na^+/K^+ homeostasis, while enhanced antioxidant enzyme activities and increased chlorophyll content respond directly

to Na^+-induced oxidative stress, in contrast to the osmotic stress associated with water deficit (Kaur et al. 2022). Studies from AtSnRK2.10 and TaSnRK2 suggested that these findings are consistent with the function of BvSnRK2.1 kinase in salt stress (Feng et al. 2019; Szymańska et al. 2019). Although the mechanism of transcriptional regulation of salt stress-responsive genes has not been fully elucidated, there is a possibility that SnRK2 kinase may directly phosphorylate downstream proteins without the mediation of transcription factors (Soma et al. 2020) or activate stress-responsive genes through an undefined signalling cascade, and the direct targets and modes of action need to be clarified in the future by using phosphoproteomics, chromatin immunoprecipitation, and promoter binding assays.

Conclusions

In this study, the *BvSnRK2.1* gene from sugar beet was cloned and transfected into tobacco, and overexpression plants were obtained. The results showed that overexpression of the *BvSnRK2.1* gene significantly improved the salt tolerance of transgenic tobacco plants. Under salt stress, transgenic tobacco effectively scavenged ROS and reduced oxidative damage by upregulating the expression of antioxidant-related genes and enhancing their enzymatic activities. Moreover, the *BvSnRK2.1* gene maintained a high K^+/Na^+ ratio by regulating the expression of ion transport-related genes, which safeguarded intracellular ion homeostasis. Additionally, the *BvSnRK2.1* gene significantly increased the chlorophyll content and photosynthetic rate of transgenic tobacco under salt stress, maintaining normal photosynthesis. In summary, the *BvSnRK2.1* gene plays a key role in the plant's response to salt stress.

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Author contributions X.-M. Zhang conducted the research, analyzed the data, and wrote the article. G.-Q. Wu designed the research and revised the article. J.-M. Ren, M. Wei, and B. Cheng analyzed the data and revised the article. All authors read and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare that they have no competing interests.

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