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ORIGINAL ARTICLE

Overexpression of a tonoplast Na^+/H^+ antiporter from the halophytic shrub *Nitraria sibirica* improved salt tolerance and root development in transgenic poplar

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Abstract

The sodium/proton exchanger (NHX) mediates Na^+ and H^+ countertransport in plants and plays an important role in regulating intracellular pH and maintaining ion and osmotic balance. Previously, an *NHX1* orthologue was isolated from the halophyte *Nitraria sibirica* Pall (referred to as *NsNHX1*), and its role in enhancing salt tolerance of transgenic *Arabidopsis* was confirmed. To further analyse its features and functions, the 1311-bp sequence of the *NsNHX1* promoter was cloned, and histochemical staining showed that β-glucuronidase (GUS) expression driven by the *NsNHX1* promoter was strongly induced by abiotic stress and phytohormones, such as salt, drought, gibberellins, and methyl jasmonate, in turn indicating that *NsNHX1* might participate in the regulation of various signalling pathways. To determine how *NsNHX1* regulates salt tolerance in forestry trees, *NsNHX1* was introduced into 84K poplar, and salt tolerance analysis of transgenic poplars showed that overexpression of *NsNHX1* increased the overall biomass, survival rate, and plant height, and the contents of chlorophyll, proline and water, all of which are consequences of antioxidant enzyme activity under salt stress conditions. This showed that overexpression of *NsNHX1* enhanced the salt tolerance of transgenic poplars as a result of NsNHX1-mediated Na^+ compartmentalisation, more efficient photosynthesis, greater activity of antioxidant enzymes, and improved osmotic adjustment. Moreover, overexpression of *NsNHX1* enhanced the root development of transgenic poplars; this resulted in increased biomass and height under normal and salt stress conditions, likely due to coupling between NsNHX1 and membrane proton pumps. These results provided a theoretical and experimental basis for further understanding the function and regulatory mechanism of *NsNHX1*, as well as its application for genetic improvement of forestry trees.

Keywords *Nitraria sibirica* · *NHX1* · Promoter · GUS · Transgenic poplar · Salt tolerance

Introduction

In coastal and inland areas, soil salinisation is a major abiotic stress factor that severely restricts the yield and planting range of agricultural crops and forestry trees (Liang et al. 2016). Halophytes can survive in high-salinity environments, but this involves complex cellular, physiological, and biochemical processes. Sodium/proton antiporter–mediated Na^+ and H^+ countertransport plays an important role in plant salt tolerance. Under salt stress, plants maintain a low Na^+ concentration in the cytoplasm in two ways: (1) by limiting Na^+ entry and accelerating efflux, and (2) by compartmentalisation of Na^+ in the vacuole, which plays an important role in establishing the intracellular ion balance (Niu et al. 1995, Blumwald et al. 2000, Zhu 2001, Yamaguchi and Blumwald 2005).

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Cytomembrane Na^+/H^+ antiporter proteins (SOS1) can transport cytoplasmic Na^+ to the exocellular domain, and tonoplast Na^+/H^+ antiporter proteins (NHX1) can deliver Na^+ into the vacuoles against the electrochemical gradient. The synergistic effect of NHX1 and SOS1 with other plant salt tolerance mechanisms can reduce the toxicity of Na^+ on the organelles, regulate pH, maintain intracellular ions and osmotic balance, and improve the salt tolerance of plants.

Tonoplast Na^+/H^+ antiporter activity was first identified in the storage tissues of *Beta vulgaris* in 1985 (Blumwald and Poole 1985) and has been actively researched ever since. *NHX1* is present in almost all plant species, ranging from algae to flowering plants (Chanroj et al. 2012). Shi and Zhu found that the transcription level of *AtNHX1* in *Arabidopsis* was induced by NaCl, KCl, and ABA; and GUS reporter gene driven by the *AtNHX1* promoter expressed in all tissues except the root tip, and strong GUS staining was observed in guard cells, suggesting that *AtNHX1* may play a role in regulating pH and maintaining ion balance in specific cells (Shi and Zhu 2002). *McNHX1* activity in *Mesembryanthemun crystallinum* was detected under normal conditions, but the level of transcription increased when plants were treated with NaCl (Barkla et al. 1995). No expression of *HaNHX1* in *Helianthus annuus* was detected under normal conditions, but high transcription levels were detected under salt stress conditions, indicating that *HaNHX1* is an inducible gene whose expression could be induced by NaCl (Ballesterous et al. 2010). *NHX1* genes were also found in some halophytes, such as *Suaeda salsa*, *Thellungiella halophila*, *Halostachys caspica*, *Salicornia brachiata*, *Nitraria sibirica*, and *Reaumuria trigyna* (Ma et al. 2004, Wu et al. 2009, Guan et al. 2011, Jha et al. 2011, Wang et al. 2016, Li et al. 2017), showing that NHX1 plays an important role in the adaption to salt stress environments of halophytes.

It was previously shown that *NHX1* is important for genetically improving agricultural crops. Overexpression of *AtNHX1* in soybeans enhanced the salt tolerance of transgenic soybeans, as confirmed over six generations (Li et al. 2010). A transgenic tomato overexpressing *AtNHX1* was able to complete a full life cycle in a high-salt environment (200 mM NaCl); moreover, Na^+ did not accumulate in the fruit of the transgenic tomato (Zhang and Blumwald 2001). Transgenic *Brassica napus* overexpressing *AtNHX1* was able to grow normally under 200 mM NaCl stress and complete its life cycle; moreover, the yield and oil quality of *B. napus* were not affected (Zhang et al. 2001). Overexpression of *AtNHX1* in cotton not only increased biomass and cotton yield but also improved the quality of cotton fibres under salt conditions of 200 mM NaCl, due to more efficient photosynthesis and an increased nitrogen assimilation rate (He et al. 2005). Salt tolerance in plants is a complex trait controlled by multiple factors; however, overexpression of *AtNHX1* improves the salt tolerance of transgenic crops, demonstrating the feasibility of improving the salt tolerance of crops via genetic engineering. Recent studies have found that the NHX1s of halophytes have high Na^+/H^+ transport activity. Overexpression of *RtNHX1* from *R. trigyna* and *NsNHX1* from *N. sibirica* significantly improved the salt tolerance of transgenic *Arabidopsis*, as the transgenic plants had an increased capability to regulate their intracellular ion balance, cell osmotic potential, and antioxidants (Li et al. 2017; Wang et al. 2016).

N. sibirica Pall is a small deciduous shrub of *Nitraria* L. (*Zygophyllceae*) that is primarily found in arid and semi-arid regions, and is the major constituent species of forests in Northwest China. It has high ecological and economic value, producing edible forage and serving as a medicinal shrub plant that is widely distributed across Inner Mongolia, Ningxia, Gansu, Qinghai, and Xinjiang; also, it can grow under saline, alkaline, and drought conditions (Yang et al. 2010a, b). *N. sibirica* benefits from the protective and reparative capabilities of its membrane under salt stress, as well as from efficient intracellular ion partitioning and the ability to make osmotic adjustments in response to the accumulation of various substances (Cheng et al. 2015). For these reasons, studying *N. sibirica* could improve our understanding of salt tolerance mechanisms and reveal genes that may enhance salt tolerance in genetically modified crops, and forage and forestry trees. Previously, a tonoplast Na^+/H^+ antiporter gene (*NsNHX1*) was isolated from *N. sibirica*, and its ability to improve the salt tolerance of transgenic *Arabidopsis* was confirmed. In this study, *NsNHX1* was introduced into poplar, and the transgenic plants were shown to be salt-tolerant. Furthermore, 1311 bp of the *NsNHX1* promoter sequence was isolated, and expression feature and promoter activity were investigated using GUS report gene driven by *NsNHX1* promoter. Our results could provide a theoretical and experimental basis to further understanding of the expression and regulatory mechanisms of *NsNHX1*, potentially allowing for genetic improvement of forestry trees through the use of the salt tolerance genes of *N. sibirica*.

Materials and methods

Plant materials and culture conditions

Nitraria sibirica plantlets regenerated through in vitro culture from nodal segments were grown in 1/2 Murashige and Skoog (MS) medium at 25 ± 2 °C, under a photoperiod of 14-h light/10-h dark and a photon flux density of 36–40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Wang et al. 2016). *Arabidopsis thaliana* seeds (columbia-0; Col-0) were surface-sterilised using a solution containing 1% sodium hypochlorite and 0.5% Tween 20, and cultured on 1/2 MS medium at 22 °C under a photoperiod of 16-h light/8-h dark and a photon flux density of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Ten-day-old *A. thaliana* seedlings were transplanted into pots with peat

soil and grown under the same conditions. *Nicotiana benthamiana* seeds were sown into 1/2 MS medium and cultured under a photoperiod of 16-h light/8-h dark. Seedlings with four true leaves were transplanted into pots with peat soil and grown under the same conditions. 84K poplar (*Populus alba* × *Populus glandulosa*) plantlets regenerated through in vitro culture, provided by professor Yang Haifeng, Forestry College, Inner Mongolia Agricultural University, were cultured in 1/2 MS medium at 24 ± 2 °C under a photoperiod of 16-h light/8-h dark and a photon flux density of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Cloning and sequence analysis of the *NsNHX1* promoter

Genomic DNA was extracted from the younger plantlets of *N. sibirica* according to the instructions in the MiniBEST Plant Genomic DNA Extraction Kit (TaKaRa, Dalian, China). Based on the sequence of the *NsNHX1* promoter cloned in a previous study (Wang et al. 2016), three reverse primers, NHX1-SP1, NHX1-SP2, NHX1-SP3, were designed (Suppl. Table S1) and the promoter sequence of *NsNHX1* was isolated using a Genome Walking kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The primers Pro. NsNHX1-F and Pro. NsNHX1-R (Suppl. Table S1) were designed to isolate the upstream promoter sequence of *NsNHX1* by polymerase chain reaction (PCR) with 50-ng genomic DNA, 2.5 μL 10 × *TransStart TopTaq* Buffer, 2.0 μL 2.5 mM dNTPs, 1.0 μL Pro. NsNHX1-F (10 μM), 1.0 μL Pro. NsNHX1-R (10 μM), 0.5 μL *TransStart TopTaq* DNA polymerase (5 $U \cdot \mu L^{-1}$), and 17.0 μL ddH_2O under the following conditions: 94 °C for 3 min; 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 1 min 20 s; 35 cycles; this was followed by 72 °C for 5 min. The transcription initiation site of the *NsNHX1* promoter was predicted using BDGP (http://www.fruitfly.org/seq_tools/promoter.html), and the transcriptional regulatory elements were analysed using the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) online software.

Construction of expression vectors of GUS driven by *Pro. NsNHX1* and its deletion fragments

The four forward primers Pro. NsNHX1(-1063)-F2, Pro. NsNHX1(-841)-F3, Pro. NsNHX1 (-573)-F4, and Pro. NsNHX1(-329)-F5 (Suppl. Table S1) were designed to amplify the four deletion fragments of the *NsNHX1* promoter, together with Pro. NsNHX1-R as the reverse primer. The *NsNHX1* promoter and its deletion fragments were inserted into the binary vector pORE R1 (Provided by Professor Qi Zhi), and the recombinant plasmids were transformed into GV3101 competent cells of *Agrobacterium tumefaciens* (Biomed, Beijing, China).

Transformation of *Arabidopsis* and tobacco

The recombinant plasmid pORE R1-*Pro. NsNHX1*::*GUS* was transferred into *Arabidopsis* according to the simplified in-plant infiltration method of Clough and Bent (1998). The transgenic lines were screened on 1/2 MS medium containing kanamycin (40 $mg \cdot L^{-1}$) and identified by genomic PCR using the primers pORE-F and pORE-R (Suppl. Table S1). The reaction mixture contained 50 ng DNA, 10.0 μL 2 × *EasyTaq* PCR SuperMix, 1.0 μL pORE-F (10 μM), 1.0 μL pORE-R (10 μM), and 5.2 μL ddH_2O, and was amplified under the following conditions: 94 °C for 3 min; 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min 30 s; 35 cycles; this was followed by 72 °C for 5 min. RNA from transgenic *Arabidopsis* was extracted using the MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China). The first strand of cDNA was synthesised by using a TransScript® First-Strand cDNA Synthesis SuperMix Kit (TaKaRa, Dalian, China). Expression of the *GUS* gene was detected using the primers GusF and GusR (Suppl. Table S1). The reaction mixture contained 100 ng cDNA, 12.5 μL 2 × *EasyTaq* PCR SuperMix, 0.5 μL GusF (10 μM), 0.5 μL GusR (10 μM), and 10.5 μL ddH_2O, and was amplified under the following conditions: 94 °C for 3 min; 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 35 s; 35 cycles; this was followed by 72 °C for 5 min. *Actin2* of *Arabidopsis* was used as an internal reference with the Actin2-F and Actin2-R primers (Suppl. Table S1). The reaction mixture contained 100 ng cDNA, 12.5 μL 2 × *EasyTaq* PCR SuperMix, 0.5 μL Actin2-F (10 μM), 0.5 μL Actin2-R (10 μM), and 10.5 μL ddH_2O, and was amplified under the following conditions: 94 °C for 3 min; 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s; 30 cycles; this was followed by 72 °C for 5 min.

A. tumefaciens GV3101, transformed with different recombinant plasmids containing the *GUS* gene (driven by *Pro. NsNHX1*) and its deletion fragments, was suspended in an infecting solution (10 mM MES, 10 mM $MgCl_2$, 200 μM acetosyringone at pH 5.6) and used to infect tobacco leaves, as described by Poulsen et al. (2016).

Histochemical detection of GUS expression

The seeds of the transgenic *Arabidopsis* were sown onto 1/2 MS medium containing kanamycin (40 $mg \cdot L^{-1}$), and 4-, 8-, 12-, 20-, and 32-day-old transgenic plants were subjected to GUS histochemical staining. To investigate the effects of abiotic stress and plant hormones, 2-, 6-, 10-, and 18-day-old transgenic seedlings were transferred onto 1/2 MS medium supplemented with NaCl (100 $mm \cdot L^{-1}$), mannitol (100 $mm \cdot L^{-1}$), gibberellic acid (GA: 160 $\mu m \cdot L^{-1}$), ethylene (ETH: 100 $\mu m \cdot L^{-1}$), and methyl jasmonate (MeJA: 100 $\mu m \cdot L^{-1}$), cultured at 24 °C for 2 days, and then used for GUS staining. Transient expression of GUS driven by the *NsNHX1* promoter

and its deletion fragments in tobacco leaves was detected after 3 days. Leaf discs (diameter, 0.5 cm) containing the injection sites were used for GUS staining and to determine GUS activity.

GUS staining was performed with a GUS Staining Kit (COMIN, Jiangsu, China) according to the manufacturer's instructions. The plant material was submerged in a GUS staining solution (50 × X-gluc: GUS buffer; 1:50, v/v), incubated at 37 °C overnight and decolourised with absolute ethanol 2–3 times before being observed with an SMZ18 stereo microscope (Nikon, Tokyo, Japan). The GUS activity of tobacco leaves was determined with a plant GUS enzyme-linked immunosorbent assay (ELISA) kit (MEIMIAN, Jiangsu, China) according to the manufacturer's instructions.

Genetic transformation, screening, and identification of transgenic 84K poplar

The binary vector pBI101-*NsNHX1* containing *NsNHX1* ORF driven by the CaMV 35S promoter, which was constructed with a pBI101-35::Gus-Hm vector containing an intron-less version of the GUS gene from pIG121-Hm (Akama et al. 1992; Wang et al. 2016), was introduced into *A. tumefaciens* LB4404 for genetic transformation of 84K poplar. The young leaves of the poplars were cut and transferred to a pre-culture medium (Suppl. Table S2) and maintained at 24 °C in the dark for 4 days; then, they were immersed in LB4404 suspension medium supplemented with 0.01% Silwet L-77 for 10 min. The leaves were transferred onto co-culture medium (Suppl. Table S2) and cultured in the dark at 24 °C for 2–3 days, after which they were transferred to the differentiation medium (Suppl. Table S2). When the adventitious buds reached 1–2 cm in length, they were separated from the leaf explants and transferred to the screening medium (Suppl. Table S2). After 3 weeks, the resistant buds were transferred to the rooting medium (Suppl. Table S2).

The transgenic poplar plants were identified with the primers 35S-F and NHX1-pBI-R (Suppl. Table S1) using the TransDirect Plant Tissue PCR Kit under the following conditions: 94 °C for 5 min; 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min 30 s; 35 cycles; this was followed by a final extension at 72 °C for 5 min. The expression of *NsNHX1* in various transgenic lines was confirmed by reverse transcription (RT)-PCR using the NHX1-pBI-F and NHX1-pBI-R primers (Suppl. Table S1). *Paulownia tomentosa* actin (Actin-Poplar-F and Actin-Poplar-R, Suppl. Table S1) was used as an internal reference.

Tolerance analysis of transgenic 84K poplar

To assess the salt tolerance of the transgenic poplars overexpressing *NsNHX1*, nontransgenic and transgenic plants were transferred to rooting medium containing various concentrations of NaCl (0, 50, 100, 150, and 200 mM), and the survival rate and biomass of the roots were measured after 2 weeks. Additionally, nontransgenic and transgenic plants were transplanted into the soil (peat:vermiculite, 5:1) and irrigated with Hoagland nutrient solution twice daily; plants with similar growth status were used to detect salt tolerance (Suppl. Figure S1).

The nontransgenic and transgenic 84K poplars were transplanted into peat soil and cultured in a greenhouse. Healthy leaves from the same location were punched into discs, 1 cm in diameter, with a perforator and placed in solutions containing NaCl (0, 50, 100, and 150 mM), $NaHCO_3$ (0, 100, 200, and 300 mM), and H_2O_2 (0, 1.0, 1.5, and 2.0%). The chlorophyll content of the leaf discs was measured after 72 h of incubation at 24 °C under a photoperiod of 16-h light/8-h dark using a chlorophyll assay kit (COMIN, Jiangsu, China) according to the manufacturer's instructions.

The nontransgenic and transgenic plants were irrigated with Hoagland nutrient solution, with or without 25 mM NaCl, every 2 days during the first week; thereafter, the concentration of NaCl was increased by 25 mM every 2 days until it reached 150 mM. The plants were then irrigated with Hoagland nutrient solution containing 150 mM NaCl every 2 days, and the morphological and physiological indexes were measured after 1 week. The chlorophyll content was measured using a SPAD-502 portable chlorophyll metre. Leaves from the same position on the plants were selected for fresh weight (FW) measurement and were then soaked in distilled water for 24 h at room temperature to measure the swelling weight (SW). Finally, the samples were dried at 80 °C for 48 h and the dry weight (DW) was measured. The relative water content (RWC) was calculated as follows: RWC = (FW-DW)/(SW-DW) × 100% (Chakraborty et al. 2012). The proline (PRO) and malondialdehyde (MDA) contents, and the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), were measured using kits (COMIN, Jiangsu, China) according to the manufacturer's instructions.

Statistical analysis

The data were obtained from three independent biological experiments, and each experiment was repeated at least three times. Data are expressed as means ± standard deviation (SD). Statistical differences were assessed by one-way analysis of variance (ANOVA) using Fisher's least significant difference (LSD) test. A p value < 0.05 was taken to indicate a significant difference between means. All analyses were conducted

using the SPSS software (ver. 19.0; SPSS Inc., Chicago, IL, USA).

Results

Isolation and prediction of cis-acting elements of the NsNHX1 promoter

The 1311-bp of the *NsNHX1* promoter was cloned using genome walking (Fig. 1A), and the transcriptional regulatory elements were analysed using the PlantCARE database (Suppl. Table S3). This showed that the *Pro. NsNHX1* sequence contained not only core promoter elements such as the TATA and CAAT boxes but also cis-acting elements related to abiotic stress and plant hormones, including the salt-inducible SCaM-4 element (GT1GMSCAM), the MYB binding site involved in drought inducibility (MBS), cis-acting elements involved in defence and stress responsiveness (TC-rich repeats), gibberellins (P-box and GARE-motif), and ethylene (ERE) responsiveness. Additionally, cis-acting regulatory elements involved in anaerobic induction (ARE), the fungal elicitor response (Box-W1), meristem-specific activation (CCGTCC-box), photoperiod regulation (circadian), and the light response (3-AF1 binding site and ATC-motif) were also found in the *NsNHX1* promoter region (Fig. 1A).

Expression of the GUS reporter gene driven by the *NsNHX1* promoter in transgenic *Arabidopsis*

The pORE R1-*Pro. NsNHX1*::*GUS* plasmid was transferred into *Arabidopsis*, and 16 transgenic lines were identified by PCR using the primers pORE-F and pORE-R (Suppl. Table S1; Suppl. Figure S1A, B). Three transgenic lines (L2, L8, and L9) were subjected to RT-PCR to detect the expression of the *GUS* gene driven by the *NsNHX1* promoter (Suppl.

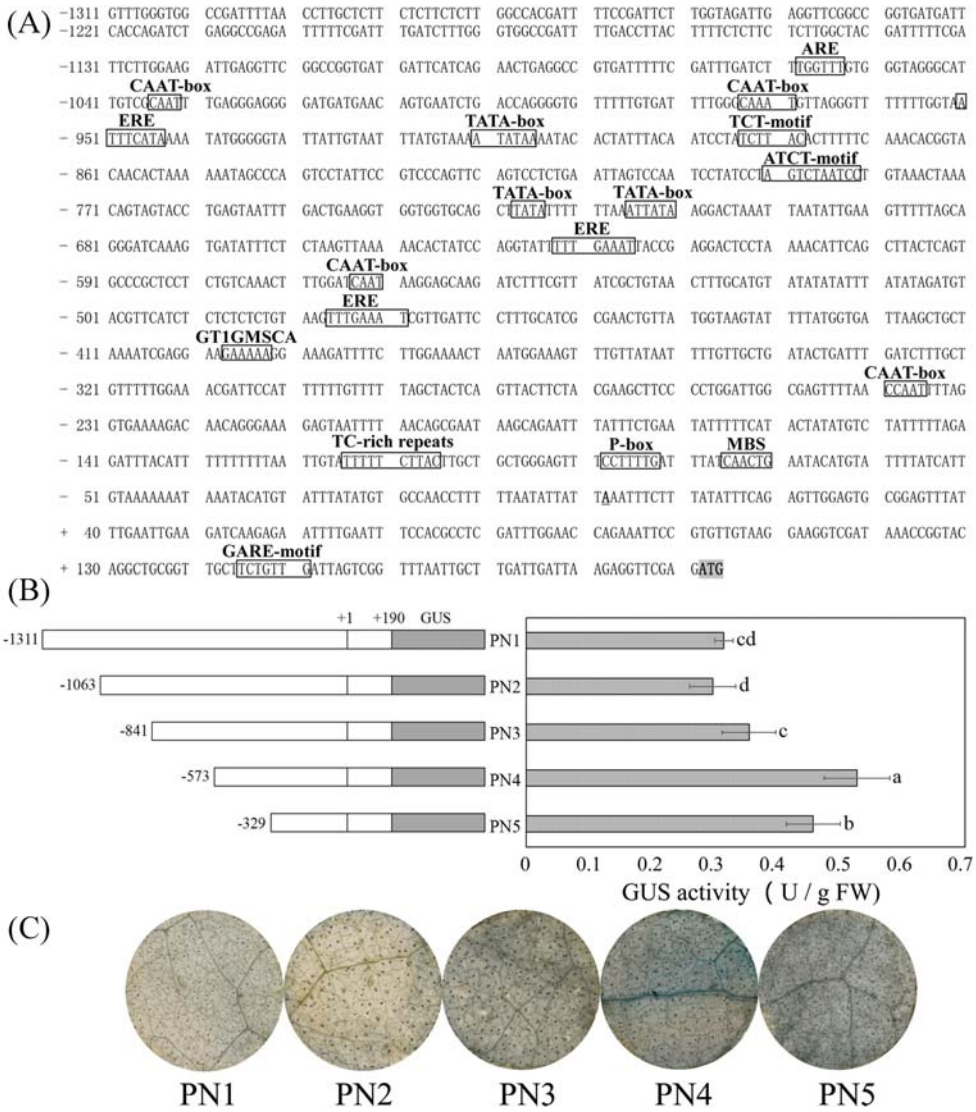

Fig. 1 Sequence of the *Nitraria sibirica* Pall (*NsNHX1*) promoter and β-glucuronidase (GUS) activity driven by different *Pro. NsNHX1* deletion fragments. **A** "A" represents the transcriptional start site, the position of which corresponds to +1. Numbers indicate the positions relative to the transcription start site. "ATG" represents the start codon. **B** *Pro. NsNHX1* deletion fragments (left) and GUS activity driven by different *Pro. NsNHX1* deletion fragments in tobacco (right). **C** GUS staining of a tobacco disc transiently expressing GUS driven by *Pro. NsNHX1* deletion fragments. Values are means ± standard deviation (SD) of three independent biological replicates. The letters indicate p values < 0.05

Figure S4C) with *Arabidopsis actin 2* as an internal reference. This confirmed that the *GUS* gene driven by *Pro. NsNHX1* was stably expressed in the transgenic plants at the transcriptional level. Next, GUS histochemical staining of the transgenic plants, at different developmental stages under different abiotic/hormone-inducible conditions, was performed to determine the expression patterns and regulatory mechanisms of the *NsNHX1* gene. As shown in Fig. 2, no GUS expression was detected in the transgenic *Arabidopsis* at the early developmental stage, and only weak GUS activity was detected in the lateral root apex in 8- and 20-day-old transgenic *Arabidopsis* under normal conditions. However, obvious GUS expression was observed when plants were treated with NaCl, mannitol, GA, and MeJA, which showed different expression patterns under different inducible conditions. GUS signals were detected in the meristem and leaves of transgenic plants when treated with NaCl; a stronger GUS signal was detected as the leaves matured following mannitol treatment, and also at the ambitus of leaves (young and mature) treated with MeJA. A weak GUS signal was detected at the leaf edge following GA and ETH treatment, while no GUS activity was observed in the stems and roots of transgenic plants under the inducible conditions. In the reproductive growth stage, little GUS activity was detected in the rosette leaves of transgenic *Arabidopsis* treated with NaCl and MeJA. However, no GUS signal was detected in the roots and stems of transgenic *Arabidopsis*, even under abiotic- or hormone-inducible conditions (Fig. 2A, B). These results indicated that

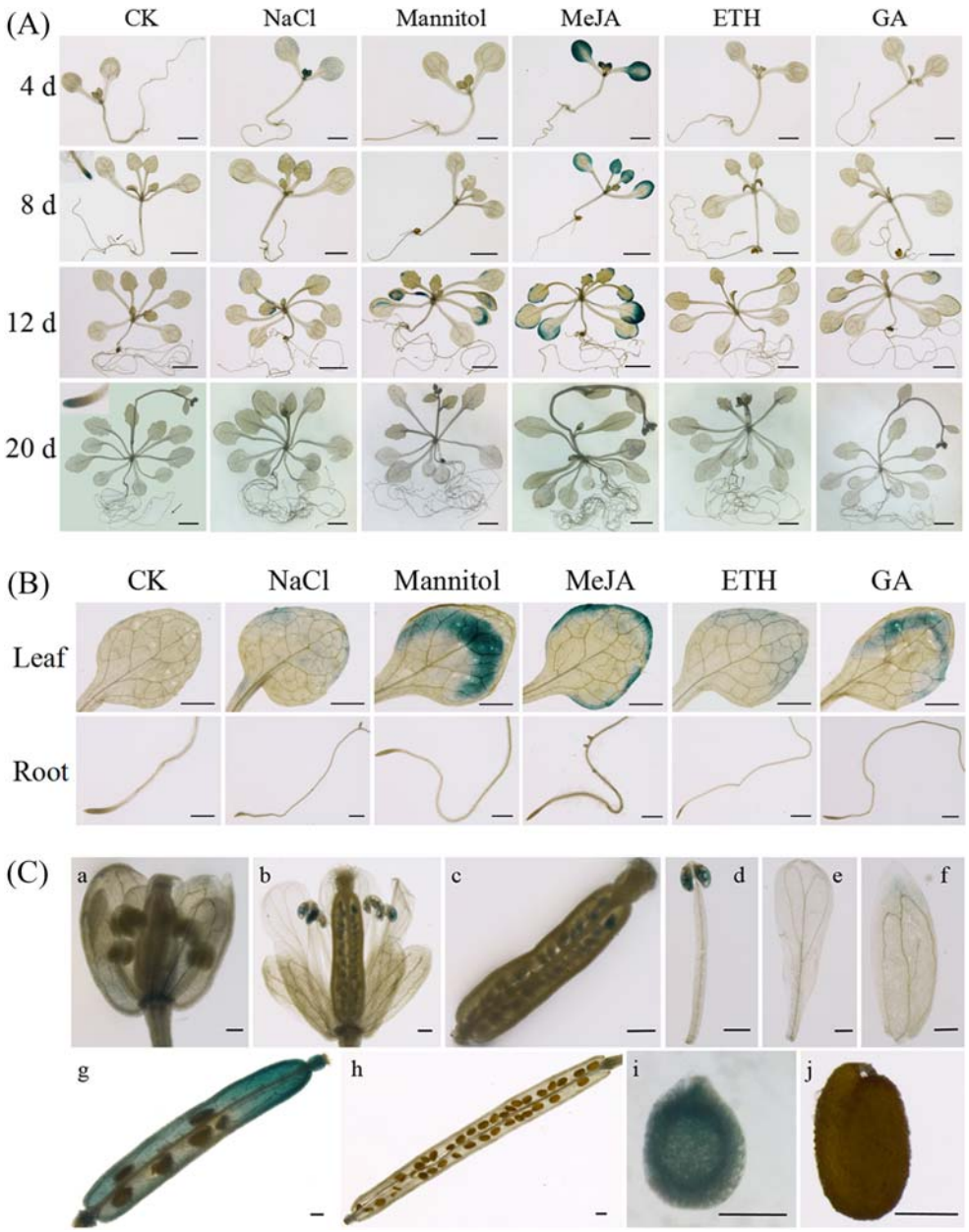

Fig. 2 GUS staining of transgenic *Arabidopsis* expressing the GUS reporter gene driven by the *NsNHX1* promoter. **A** Expression of *Pro. NsNHX1*::GUS in *Arabidopsis* at different growth stages under different induction conditions (scale bar = 3 mm). **B** Expression of *Pro. NsNHX1*::GUS in leaves and roots of *Arabidopsis* (12 days old) under different induction conditions (scale bar = 0.5 mm). **C** Expression of *Pro. NsNHX1*::GUS in the flower and fruits of transgenic *Arabidopsis*. a Alabastrums. b Flowers. c Pistil. d Stamens. e Petals. f Sepals. g Immature pods. h Mature pods. i Immature seeds. j Mature seeds (scale bar = 0.2 mm)

NsNHX1 is transcribed at a low level under normal conditions and that its expression can be induced by abiotic stress and plant hormones, such as salt, drought, GA, and MeJA. After entering the reproductive stage, strong GUS staining was detected in the ovules, the anthers of the stamens, the top of the sepals, and immature pods and seeds, while no GUS activity was detected in the stamen filaments, petals, mature pods, or seeds (at 45 days; Fig. 2C). These results suggested that NsNHX1 might play an important role in regulating the reproductive growth of plants.

Analysis of the functional region of the *NsNHX1* promoter

To analyse the activity of different regions of *Pro. NsNHX1*, five *NsNHX1* promoter fragments of different lengths, i.e. 1311 bp (PN1), 1063 bp (PN2), 841 bp (PN3), 573 bp (PN4), and 329 bp (PN5), were cloned into the pORE R1 vector to construct plasmids expressing the GUS gene driven by the *Pro. NsNHX1* fragments. These plasmids were then transformed into tobacco leaves, and transient expression of the GUS gene was detected (Suppl. Figure S3, Fig. 1B, C). Histochemical staining and GUS activity determination showed no significant difference in GUS activity in the transformants expressing the GUS gene driven by PN1, PN2, and PN3, whereas there was a significant increase in GUS activity in the transformants expressing the GUS gene driven by PN4 and PN5 (Fig. 1B); the GUS activity driven by PN4 was higher than that of PN5. These results provided evidence of negative regulatory elements in the promoter region of -1311 to -573 bp; the upstream sequence of 573 bp plays key roles in the transcriptional activation of the *NsNHX1* gene.

Screening of transgenic 84K poplar

A. tumefaciens–mediated genetic transformation of 84K poplar was carried out using a binary vector of pBI101-*NsNHX1* containing the *NsNHX1* ORF driven by the CaMV 35S promoter (Fig. 3g). Young leaves of the 84K poplar were cut and pre-cultured for 4 days (Fig. 3a), and then immersed in LB4404 suspension for 10 min (Fig. 3b). The leaves were transferred onto co-culture medium (Suppl. Table S2) and cultured in the dark for 2–3 days, after which they were transferred to differentiation medium (Suppl. Table S2). After 2 weeks, adventitious buds were generated from the leaf explants (Fig. 3c). When the adventitious buds reached 1–2 cm in length, they were separated from the leaf explants and transferred into screening medium containing 50 mg/L of kanamycin (Suppl. Table S2, Fig. 3d) for 3 weeks. The kanamycin-resistant buds were transferred to rooting medium (Suppl. Table S2), and transgenic plants were generated after 2 weeks (Fig. 3e). Three transgenic lines, N1–N3, were identified using genomic PCR (Fig. 3h). Stable expression of the *NsNHX1* gene in the transgenic lines was confirmed by RT-PCR using the poplar *Actin* gene as an internal control (Fig. 3i). The expression product of *NsNHX1* was detected in the transgenic plant lines but not in the nontransgenic plants.

Detection of salt tolerance of NsNHX1 transgenic 84K poplars

To determine the role of the *NsNHX1* gene in regulating salt tolerance in transgenic poplars, nontransgenic and *NsNHX1* transgenic plantlets were transferred into 1/2 MS medium containing different concentrations of NaCl (0, 50, 100, 150, and 200 mM), and their growth and phenotypes were investigated. As shown in Fig. 4a and b, under low concentrations of NaCl (0, 50, and 100 mM), no significant difference in growth or phenotype was observed between the nontransgenic and transgenic lines, although growth inhibition was present at 50 and 100 mM of NaCl. At 150 mM of NaCl, obvious damages, such as chlorosis, necrosis, and growth inhibition, were seen in the nontransgenic and transgenic plants; however, the transgenic plants exhibited less damage and growth inhibition and higher survival rates. The survival rates of the three transgenic lines (N1, 83.33%; N2, 50%; N3, 66.67%) were significantly higher than those of the nontransgenic plants (25%) (Fig. 4a, e). Almost all of the nontransgenic and transgenic plants died after 2 weeks of culture in medium containing 200 mM NaCl, indicating that the transgenic poplar could not survive under those conditions (Fig. 4a). Our results showed that the salt tolerance of transgenic 84K poplar could be improved by overexpressing the *NsNHX1* gene, as the transgenic poplars were able to grow at 150 mM NaCl. It was interesting to note that the transgenic poplars had well-developed roots. The root biomass of the three transgenic lines was higher than that of the nontransgenic plants under normal and salt stress conditions, indicating that overexpression of *NsNHX1* improved root growth in the transgenic poplars (Fig. 4b).

The transgenic poplars were transferred to pots with peat soil and cultured in a greenhouse for further analyses. Leaf discs cut from nontransgenic and transgenic plants were used to detect tolerance to salt, alkali, and oxidative stress. Leaf discs from mature leaves of nontransgenic and transgenic poplar were placed in solutions with different concentrations of NaCl (0, 50, 100, and 150 mM), $NaHCO_3$ (0, 100, 200, and 300 mM), and H_2O_2 (0, 1.0, 1.5, and 2.0%) and incubated in a greenhouse for 72 h, after which tolerance was compared according to the extent of damage and chlorophyll content. As shown in Fig. 5A and B, there was no significant difference in the leaf discs between the nontransgenic and *NsNHX1* transgenic poplars, both in the absence of NaCl and with 50 mM NaCl, while the leaf discs from transgenic poplars showed stronger salt tolerance than those of the nontransgenic under

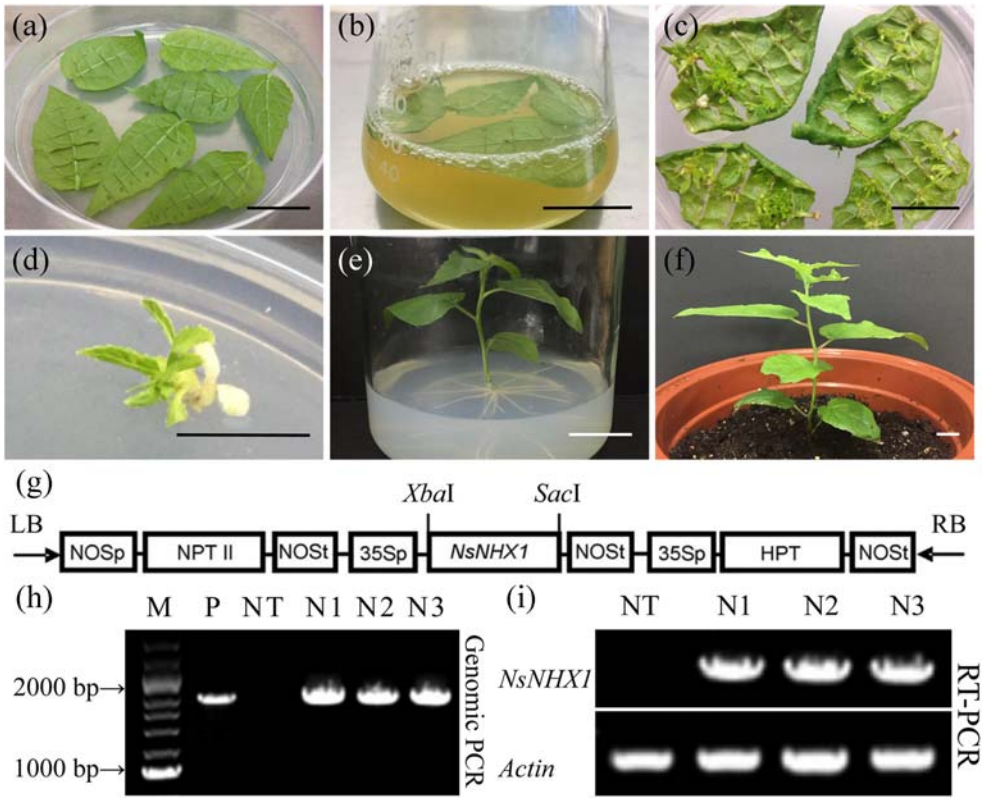

Fig. 3 Genetic transformation and polymerase chain reaction (PCR) identification of transgenic 84K poplar overexpressing *NsNHX1*. **a** Cutting and pre-culture of leaf explants. **b** Dissemination with *A. tumefaciens* LB4404. **c** Differentiated culture. **d** Screening in medium containing kanamycin (40 mg·L^{-1}). **e** Rooting culture. **f** Transplant into the soil. **g** The T-DNA region of pBI101-*NsNHX1*. **h** Genomic PCR identification of transgenic 84K poplar. **i** Reverse transcription (RT)-PCR detection of transgenic 84K poplar. M: 200-bp DNA Ladder. +: Recombinant plasmid of pBI101-*NsNHX1*. NT: nontransgenic 84K poplar. N1–N3: transgenic lines

100 and 150 mM NaCl conditions. Most of the leaf discs were shrunken and had softened due to osmotic stress induced by the high-salt solution. The chlorophyll content of leaf discs from the three *NsNHX1* transgenic lines was higher than that of the nontransgenic under higher concentrations of NaCl (100 and 200 mM) (Fig. 5B). As shown in Fig. 5A and B, obvious damage occurred in the leaf discs of the nontransgenic and transgenic poplar under $NaHCO_3$ (100, 200, and 300 mM), although the leaf discs of the transgenic plants showed stronger alkali tolerance than those of the nontransgenic plants; meanwhile, no significant difference in chlorophyll content between the nontransgenic and transgenic plants was detected. Under 1.0 and 1.5% H_2O_2, the N2 and N3 transgenic lines exhibited stronger antioxidant activity than the nontransgenic and N1, which displayed less disc albinism and higher chlorophyll content. Under 2.0% H_2O_2, no obvious differences in damage or chlorophyll content between leaf discs from the nontransgenic and transgenic poplars were found.

Salt tolerance was also detected in the transgenic plants grown in the greenhouse. As shown in Suppl. Figure S1, nontransgenic and transgenic poplars with similar growth status were irrigated with Hoagland nutrient solution with or without NaCl, and the growth status, biomass, plant height, and relative water and chlorophyll contents of leaves of nontransgenic and transgenic poplars were investigated. Compared to the nontransgenic, all three lines of transgenic plants displayed superior growth status under both salt stress and normal conditions (Fig. 6a), having a higher biomass and plant height than the nontransgenic plants (Fig. 7A, B). Moreover, serious damage, such as chlorosis, necrosis, wilting, and growth inhibition occurred in the nontransgenic plants under salt stress conditions. Obvious differences in the extent of damage and growth inhibition were seen in the seventh leaf from the apex in both the nontransgenic and transgenic plants (Fig. 6b).

The physiological indexes of transgenic poplars, including the activity of antioxidant enzymes, chlorophyll contents, PRO, MDA, and the RWC, were measured. There were no significant differences in chlorophyll contents of the fifth leaf (from the apex) between the nontransgenic and transgenic plants under normal conditions, while the chlorophyll contents of the transgenic plants were significantly higher than those of nontransgenic under salt stress conditions (Fig. 7C). The RWC of the fourth leaf from transgenic poplars was higher than that from the nontransgenic under salt stress conditions (Fig. 7D). The SOD, POD, and CAT activities of the transgenic poplars were significantly higher than those of the nontransgenic (Fig. 7E–G, respectively). Compared to the nontransgenic, the transgenic plants had a higher PRO and

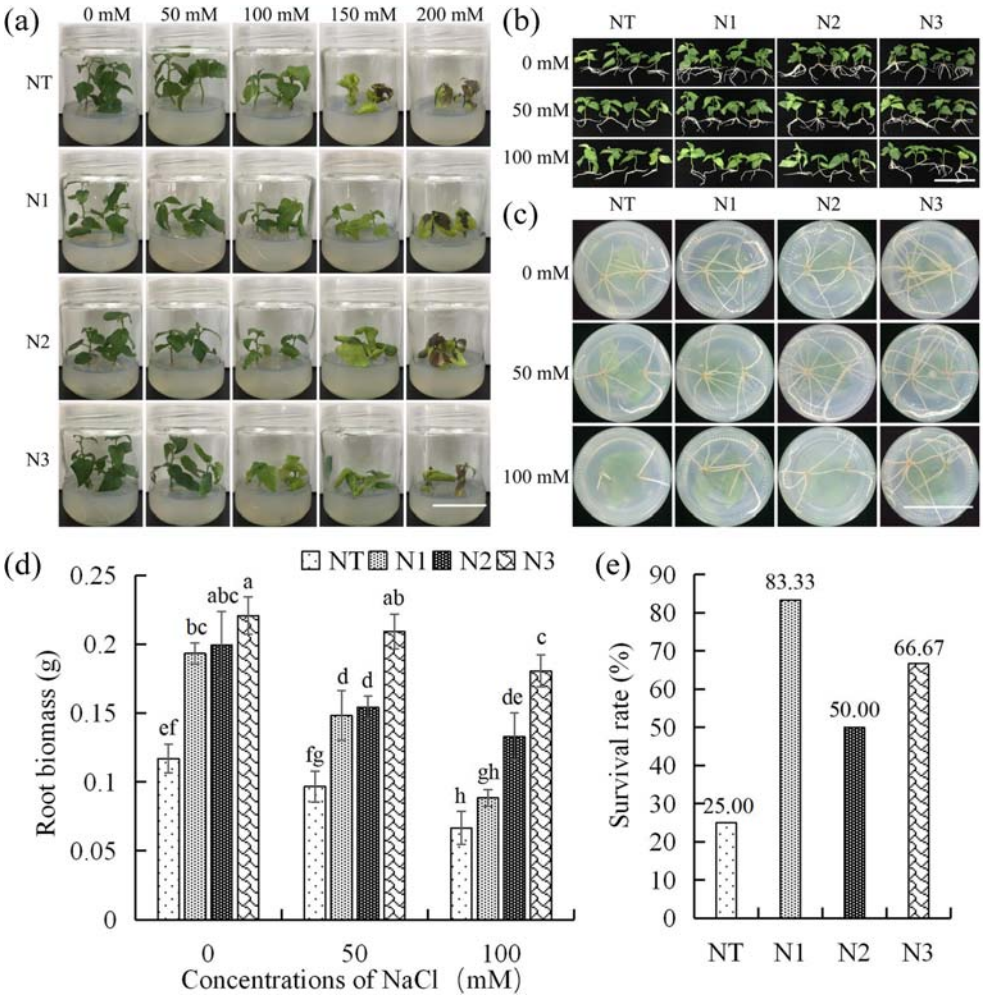

Fig. 4 Detection of salt tolerance in transgenic 84K poplar. **a** NT and transgenic 84K poplar cultured in 1/2 Murashige and Skoog (MS) medium containing different concentrations of NaCl for 2 weeks. **b** Morphological traits of NT and transgenic poplars. **c** The roots of 84K poplar in 1/2 MS medium. **d** Root biomass of NT and transgenic plants. **e** Survival rates of NT and transgenic poplars. NT, nontransgenic 84K poplar. N1–N3, transgenic 84K poplar lines. Scale bar = 10 cm. Values are means ± SD of three independent biological replicates. The letters indicate p values < 0.05

lower MDA contents under the salt stress conditions (Fig. 7H, I). These results indicated that overexpression of *NsNHX1* increased the salt tolerance of the transgenic poplars.

Discussion

The Na^{+}/H^{+} antiporter genes have been isolated from numerous plants, such as *Arabidopsis*, tomato, and soybean, and some *NHX1* genes have been used for the genetic improvement of crops (Wu et al. 1996; Olias et al. 2009; Nie et al. 2015). However, research on the Na^{+}/H^{+} antiporter genes of woody plants, especially in halophyte woody plants, is sparse. Woody plants play important roles in the ecosystem, which makes it essential to understand and use the Na^{+}/H^{+} antiporter genes of woody plants, especially halophyte woody plants, to genetically improve forest trees. In our previous study, an orthologue of *NHX1* was isolated from the halophytic shrub *N. sibirica* Pall, and its function in enhancing the salt tolerance of transgenic *Arabidopsis* was confirmed. Here, we report on the expression features of *NsNHX1* and its function in improving the salt tolerance and root growth of transgenic poplars.

The expression pattern of *NHX1* varies greatly within each plant species; therefore, further analysis of the expression and regulatory mechanisms of *NsNHX1* is very important to aid our understanding of its function, and for its application to improve crops. The promoter of *NsNHX1* contains some cis-elements that respond to biotic and abiotic stress, such as light responsiveness, anaerobic induction, drought, salt and fungal elicitors, and some phytohormone responsive elements, including gibberellin and ETH, indicating that *NsNHX1* may participate in the regulation of various metabolic pathways. Histochemical staining of GUS in transgenic *Arabidopsis* expressing driven by the *NsNHX1* promoter showed different expression patterns under different physiological conditions. Under normal conditions, *Pro*. *NsNHX1*::*GUS* expression was very low in the vegetative organs and was only detected in the root tips of the transgenic plants. In the reproductive stage, GUS activity was detected in the petals, ovules, anthers, sepals, immature pods, and immature seeds of transgenic *Arabidopsis*, indicating that *NsNHX1* may play a key role

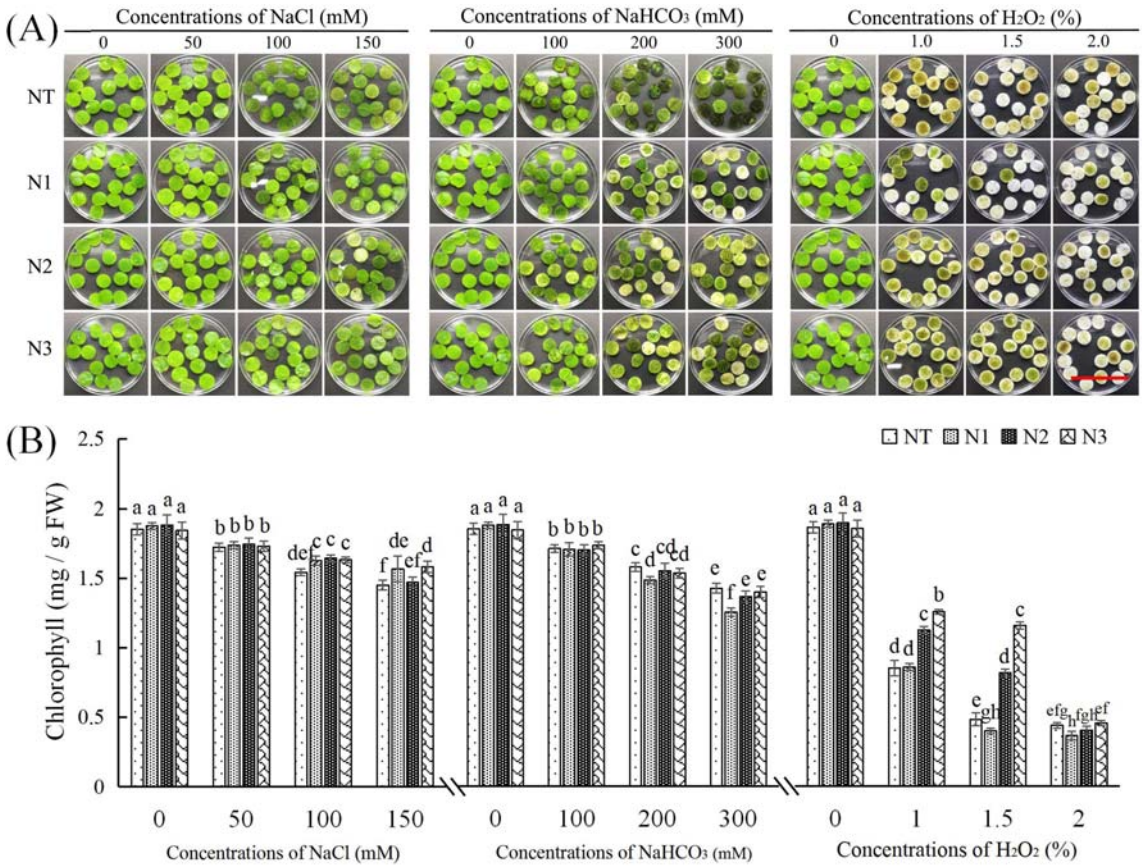

Fig. 5 Detection of resistance of transgenic 84K poplars. **A** Leaf discs treated with NaCl, $NaHCO_3$, and H_2O_2 for 72 h. **B** The chlorophyll contents of leaf discs after 72 h of treatment with NaCl, $NaHCO_3$, and H_2O_2. Values are means ± SD of three independent biological replicates. The letters indicate p values < 0.05. NT, nontransgenic 84K poplar. N1–N3: transgenic lines overexpressing *NsNHX1*

during reproductive stage. *Pro*. *NsNHX1*::*GUS* expression was induced by abiotic stress and phytohormones and showed different expression patterns under the different treatment conditions (NaCl, Mannitol, MeJA, ETH, and GA), indicating that *NsNHX1* expression is regulated by various signalling pathways. Expression of *Pro*. *NsNHX1*::*GUS* was upregulated in leaves and downregulated in roots, indicating that *NsNHX1* mainly accumulated Na^+ in the vacuoles of *N. sibirica* leaves under stress conditions. GUS activity driven by the *NsNHX1* promoter was detected in the leaves of transgenic *Arabidopsis* under salt stress (100 mM NaCl), but not in the roots. These results are consistent with those of the *ZxNHX* gene of *Zygophyllum xanthoxylum*, where *ZxNHX* expression could be detected in the root, stem, and leaf, while the highest expression level was detected in the leaf under stress conditions (Wu et al. 2011). *ThNHX1* of *T. halophila* displayed a different expression pattern, i.e. higher expression in roots than in the stem under salt stress conditions (Wu et al. 2009). The expression of GUS driven by the *OsNHX1* promoter in onion epidermal cells showed that GUS activity was significantly induced by salt, drought, and ABA treatments (Liu et al. 2012), which is consistent with the characteristics of *NsNHX1* expression (Wang et al. 2016).

MeJA is widely distributed in plants and is known to be a hormone and signalling molecule that stimulates the expression of plant defence genes in response to mechanical damage and insect feeding (Elena 2012). TC-rich repeats, the cis-acting element involved in the defence and stress responses of *Pro. NsNHX1*, and GUS expression driven by the *NsNHX1* promoter were strongly induced by MeJA, indicating that *NsNHX1* is involved in MeJA-mediated defence and stress responses.

Stress-inducible promoters should optimise candidate abiotic stress resistance genes to ameliorate negative effects on crop yield. Transient GUS expression driven by *NsNHX1* promoter fragments of different lengths in tobacco leaves showed that the -573 to -329 bp region of the inducible promoter played a key role in transcriptional activation of the *NsNHX1* gene. These results may provide both a theoretical and experimental basis for application of the inducible *NsNHX1* promoter in plant genetic engineering, as well as for analysis of the mechanisms regulating *NsNHX1* expression and its function in stress adaptation.

Overexpression of *NHX1* genes has been shown to improve the growth and development of transgenic plants under salt stress conditions. Overexpression of *AeNHX1* from

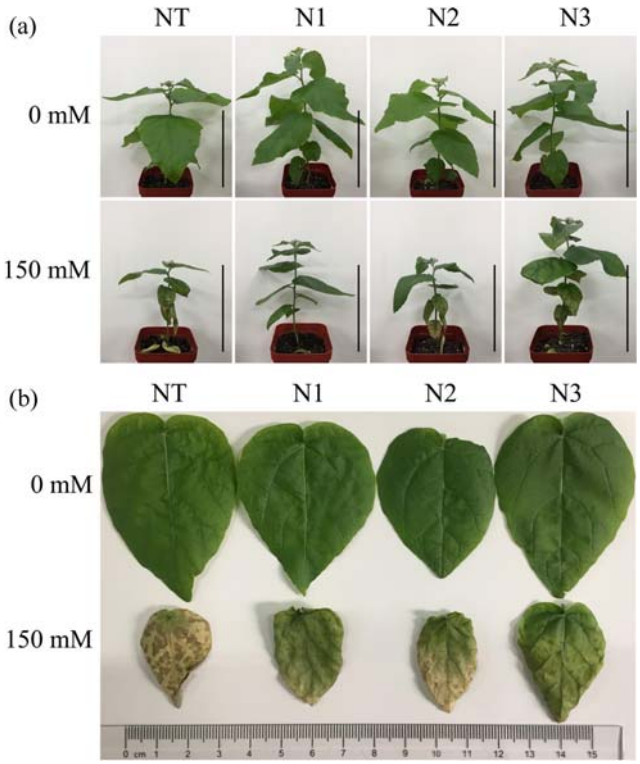

Fig. 6 Phenotypic analysis of nontransgenic and transgenic poplars under NaCl stress conditions. **a** The plants of nontransgenic and transgenic 84K poplars grown in soil under normal (above) and salt stress conditions (below), showing differences in the extent of damage and growth inhibition (scale bar = 10 cm). **b** The seventh leaf from the apex of nontransgenic and transgenic plants. NT, nontransgenic 84K poplar. N1–N3: transgenic 84K poplar

Agropyron elongatum, in both *Arabidopsis* and *Festuca*, showed that the osmotic regulation and photosynthetic efficiency of transgenic plants were improved under salt stress and the seeds of transgenic plants could germinate under high salt concentrations (250 mmol·L^{-1} NaCl) (Qiao et al. 2007). Salt tolerance of transgenic NanLin 895 *Populus* transforming *Arabidopsis AtNHX1* was significantly enhanced, having higher chlorophyll contents and lower MDA content compared to nontransgenic plants (Qiao et al. 2011). Under salt stress conditions, transgenic *Vigna radiata* plants overexpressing *AtNHX1* displayed decreased membrane lipid peroxidation, H_2O_2 and O_2^- accumulation, increased antioxidant enzyme activity, PRO and ascorbic acid accumulation, less damage, and improved growth (Sahoo et al. 2016). Our previous experiments confirmed that overexpression of *NsNHX1* enhanced the salt tolerance of transgenic *Arabidopsis*; the transgenic plants grew better than the nontransgenic under salt stress conditions, with significantly higher germination and survival rates and better root development, especially under 150 and 200 mM NaCl (Wang et al. 2016).

Poplar, an economically and ecologically important tree species due to its fast growth, high yield, and strong adaptability, is widely distributed throughout the world. However, most poplar varieties are extremely sensitive to saline soils (Chen and Polle 2010; Chen et al. 2014; Polle and Chen 2014), which limits their large-scale planting in coastal and other saline soil areas. Due to the complexity of the salt tolerance mechanisms and the relative shortage of genetic information, improvement of salt tolerance in poplar using genetic engineering is difficult (Polle and Chen 2014; Tang et al. 2014; Xu et al. 2018). Here, we attempted to improve the salt tolerance of 84K poplar by overexpressing *NsNHX1* from the halophytic shrub *N. sibirica* Pall, which was confirmed to play an important role in enhancing the salt tolerance of transgenic *Arabidopsis* (Wang et al. 2016). Leaf discs of transgenic plants under salt, alkali, and oxidative stress displayed less damage and higher chlorophyll contents than those of nontransgenic, indicating that the overexpression of *NsNHX1* improved tolerance to salt, alkali, and oxidative stress in the transgenic plants. The salt tolerance of transgenic plants, grown both in medium and in the greenhouse, was confirmed; these plants had a higher biomass, survival rate, height, chlorophyll and PRO contents, and RWC, as well as enhanced activities of SOD, POD, and CAT and lower MDA contents compared to nontransgenic plants. The high salt tolerance of transgenic poplars overexpressing *NsNHX1* could be due to the *NsNHX1*-mediated Na^+ compartmentalisation in the vacuole, which decreases the concentration of Na^+ in the cytoplasm and alleviates the toxicity of Na^+ to the organelles under salt stress. On the other hand, the leaf chlorophyll content of transgenic poplars was higher than that of nontransgenic plants, indicating that photosynthesis in transgenic plants was improved under salt stress. Moreover, the *NsNHX1* transgene may indirectly regulate the activities of antioxidant enzymes and osmotic adjustment mechanisms, thereby increasing the activity of antioxidant enzymes and the PRO content in transgenic plants, and thus alleviating the damage caused by salt stress.

Transgenic poplar plants overexpressing *NsNHX1* had better-developed roots and higher root biomass than nontransgenic plants, indicating that overexpression of *NsNHX1* enhanced growth and development of the roots (Fig. 4). GUS staining showed that the GUS-driven *NsNHX1* promoter was largely expressed in the root tip, revealing its potential role in promoting root growth. After being transferred to the soil, the transgenic plants displayed faster growth and had higher biomass and height, not only under salt stress conditions but also under normal conditions, due to the well-developed roots. Similar results were reported by Verma et al. (2007), where overexpression of *PgNHX1* from *Pennisetum glaucum* not only improved the salt tolerance of the transgenic rice but also promoted root growth and development. However, the specific mechanism underlying the promotion of root development in transgenic plants remains unclear, although it was speculated that

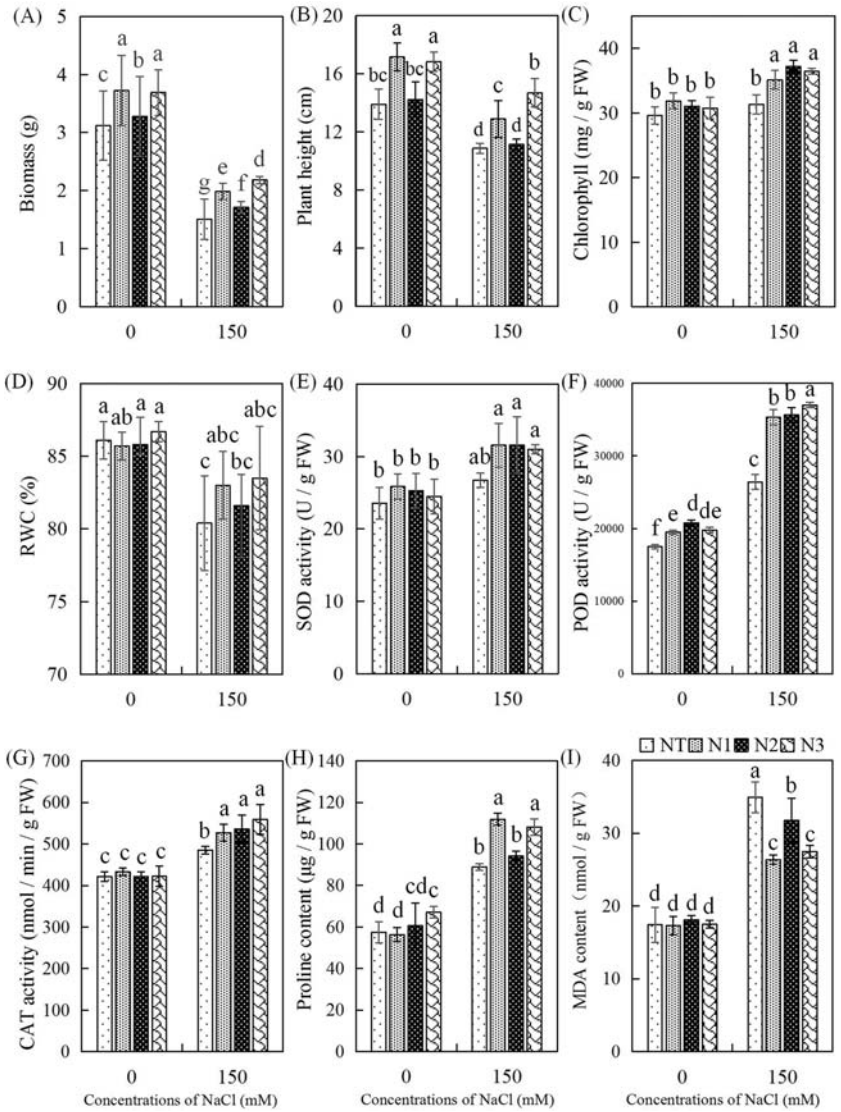

Fig. 7 Detection of physiological indexes of transgenic 84K poplar under normal and salt stress conditions. A Biomass. B Plant height. C Chlorophyll content. D Relative water content (RWC) of leaves. E Superoxide dismutase (SOD) activity. F Peroxidase (POD) activity. G Catalase (CAT) activity. H Proline (PRO) content. I Malondialdehyde (MDA) content. NT, nontransgenic 84K poplar. N1–N3: transgenic 84K poplar. Values are means ± SD of three independent biological replicates. The letters indicate *p*-values < 0.05

coupling between NHX1 and membrane proton pumps may be involved (Verma et al. 2007).

1As a defence mechanism of plants, post-transcriptional gene silencing (PTGS) often occurs in transgenic plants, which can protect the plant genome from the invasion of exogenous genes (Zhang et al. 2016). However, PTGS results in suppression of foreign gene expression, which seriously restricts the application of transgenic plants. Here, we investigated the expression of endogenous *NHX1* (named *PagNHX1*) and foreign *NsNHX1* in 1- to 3-year-old transgenic poplars by qRT-PCR. The results showed that the expression of *PagNHX1* and *NsNHX1* remained stable in 1- to 3-year-old poplars (Suppl. Figure S5), displaying application value of the transgenic poplars in afforestation in saline-alkali soils.

Recent studies have shown that *Arabidopsis* overexpressing *AtSOS1* or *AtNHX1* could not grow in medium with an NaCl concentration > 200 mM, but that *Arabidopsis* overexpressing both *AtSOS1* and *AtNHX1* could grow in medium with 250 mM NaCl (Pehlivan et al. 2016), possibly due to the synergy between the plasmalemma and tonoplast Na^+/H^+ antiporter, and to other genes related to salt tolerance that might also have been activated. We isolated the plasmalemma Na^+/H^+ antiporter gene (*NsSOS1*) from *N. sibirica*, and transgenic 84K poplars transformed with *NsSOS1* showed strong salt tolerance (unpublished data). Therefore, it is expected that transgenic poplars with stronger salt tolerance could be generated by overexpressing both *NsSOS1* and *NsNHX1*.

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Author contributions Conceived and designed the experiments: Xiaofei Lin.

Performed the experiments and analysed the data: Xin Geng, Shouye Chen, Yilan E, Wenbo Zhang, Huiping Mao, Alatan qiqige, Yingchun Wang, Zhi Qi, and Xiaofei Lin.

Wrote the paper: Xin Geng, Xiaofei Lin, and Shouye Chen.

Statement: Xin Geng, Shouye Chen, and Yilan E contributed equally to this paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Data archiving statement *NsNHX1* sequences and information of *Nitraria sibirica* were deposited in the NCBI database (accession number: AB859847, accessible with the following link: https://www.ncbi.nlm.nih.gov/nuccore/820687834).

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